STUDIES ON DEVELOPMENT, OPTIMIZATION AND PRODUCTION OF YEAST CAROTENOID PIGMENTS AND THEIR APPLICATIONS IN FOOD PRODUCTS

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COIMBATORE – 641 003

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STUDIES ON DEVELOPMENT, OPTIMIZATION AND PRODUCTION OF YEAST CAROTENOID PIGMENTS AND THEIR APPLICATIONS IN FOOD PRODUCTS

Thesis submitted in part fulfilment of the requirements for the award of the Degree of

Doctor of Philosophy (Agriculture) in Agricultural Microbiology

to the Tamil Nadu Agricultural University, Coimbatore

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2014

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON DEVELOPMENT, OPTIMIZATION AND PRODUCTION OF YEAST CAROTENOID PIGMENTS AND THEIR APPLICATIONS IN FOOD PRODUCTS"** submitted in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN AGRICULTURAL MICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Mrs. M.RA.MANIMALA** under my supervision and guidance and that no part of the thesis has been submitted for the award of any degree, diploma, fellowship or other similar titles. However, a part of the thesis work has been published in peer reviewed scientific journal of national/ international repute (copy enclosed).

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(M.RA.Manimala)

Abstract

ABSTRACT

STUDIES ON DEVELOPMENT, OPTIMIZATION AND PRODUCTION OF YEAST CAROTENOID PIGMENTS AND THEIR APPLICATIONS IN FOOD PRODUCTS

By

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Degree : Doctor of Philosophy in Agricultural Microbiology

Chairman : Dr. R. MURUGESAN,
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2014

In the present study, a total of 129 samples were collected and analyzed microbiologically for their carotenoid producing colonies. Among thirty eight pigment producing yeast isolates, based on the intensity of carotenoid pigmentation, five yeast isolates *viz*., YP 15, YP 17, YP 19, YP 23 and YP 37 were screened based on their high intensity of carotenoid pigment producing ability. The morphological and biochemical characteristics of yeast isolates were carried out. Antioxidant and free radical scavenging ability of the five yeast carotenoid pigments were assessed using different methods. Carotenoid pigment of YP 17 and YP 37 showed higher percentage of inhibition than other strains *viz*., YP 15, YP 19 and YP 23. Antimicrobial activity of the yeast carotenoid pigment extracts were tested against the selected human pathogens by agar well diffusion assay. Carotenoid pigment extracts of YP 17 and YP 37 were found to exhibit excellent inhibitory effect against selected bacterial human pathogens *viz., E. coli* (3.0 and 2.9 cm), *P. aeruginosa* (2.1 and 2.2 cm), *S. aureus* (2.7 and 2.6 cm), *S. faecalis* (2.0 and 2.2cm), *B. subtilis* (2.0 and 2.3 cm) and *Enterococcus* sp. (2.0 and 1.9 cm) respectively. Based on above studies, it was concluded that two promising carotenoid pigment producing yeast cultures identified as *Rhodotorula mucilaginosa* (YP 17) and *Sporobolomyces* sp. (YP 37), were found to be effective in all aspects and used for further studies.

The Response Surface Methodology (RSM) technique was performed to increase the carotenoid pigments yield of *R. mucilaginosa* and *Sporobolomyces* sp. respectively. The optimal point of physical parameters for *R. mucilaginosa* carotenoid pigment production are, pH 6.0, temperature 30°C and fermentation time 96 h. The optimal chemical concentrations for the five components were calculated as 30 g l^{-1}, 3.5 g l^{-1}, 2 g l^{-1}, 1.25 g l^{-1} and 0.5 g l^{-1} for sucrose, yeast extract, ammonium sulphate, dipotassium hydrogen phosphate and magnesium sulphate respectively, whereas the optimal point of physical parameters for *Sporobolomyces* sp. carotenoid pigment production are, pH 5.0, temperature 30°C and time 120 h. The optimal chemical concentrations for the five components were calculated as 30 g l^{-1}, 3.5 g l^{-1}, 2 g l^{-1}, 1.5 g l^{-1} and 0.5 g l^{-1} for glucose, peptone, ammonium sulphate, dipotassium hydrogen phosphate and ferrous sulphate respectively. The synthetic media yielded CDW and carotenoids about 6.10 g l^{-1} and 14.00-15.00 mg l^{-1} in *R. mucilaginosa* whereas in *Sporobolomyces* sp. (CDW - 5.20 g l^{-1}; carotenoids about 9.50-10.00 mg l^{-1}).

Different agricultural cheap substrates were used to study the submerged fermentation conditions for maximization of carotenoid pigment production. Among the different substrates, cassava bagasse was found to be preferred substrate for *R. mucilaginosa* to produce maximum carotenoid pigment. Response surface methodology (RSM) was used to maximize the pigment production using cassava bagasse with 20 experimental trials for *R. mucilaginosa* pigment. The optimal concentrations for the components were calculated as 30 g (substrate concentration), 4 days (fermentation time), and pH 6 for *R. mucilaginosa.* Casssava bagasse as a cheap substrate *R. mucilaginosa* were yielding 5.60 g l^{-1} (CDW) and carotenoid yield was ranging from 12.00-13.00 mg l^{-1} in *R. mucilaginosa* .

Thin layer chromatography was performed for separation of pigments from *R. mucilaginosa* and *Sporobolomyces* sp. Pinkish red, orange and yellow bands were observed from acetone extracts of *R. mucilaginosa and Sporobolomyces* sp. carotenoid pigments. The bands seperated by TLC were further identified by using reverse phase high performance liquid chromatography (RP-HPLC) which yielded in 3 peaks by employing *R. mucilaginosa* pigment (peak 1–3.35 min; peak 2 – 4.75 min; peak 3-5.93 min) similarly *Sporobolomyces* sp. pigment produced 3 peaks (peak 1- 3.35 min; peak 2- 4.20 min and peak 3- 5.77 min). In both cultures, carotenoid pigment extracts shown a peak at 3.36 min which

was equivalent to the retention time of standard β-carotene. Other two peaks observed at retention time of four and five minutes were denoted as torulene and torularhodin.

The FT-IR spectra of *R. mucilaginosa* carotenoid pigment indicated broad stretching at 2993cm^{-1} due to asymmetrical stretching vibration of aliphatic CH_3 groups. Low intensity bands at 1728 cm^{-1} might be due to >C = O group depict the presence of ester groups. In the FT-IR spectrum stretching frequency observed at 1666 cm^{-1} might be due to C = C due to alkenes which indicates the presence of an olefinic functional group whereas the FT-IR spectrum of *Sporobolomyces* sp. carotenoid pigment showed bands at 2862 cm^{-1} due to asymmetrical stretching vibration of methyl groups. Low intensity bands at 3008 cm^{-1} are related to CH stretching vibration CH = CH and 1705 cm^{-1}. It might be due to C=O stretch ketone group which indicates the presence of an olefinic functional group. Presence of a band at 1226 cm^{-1} might be due to the C – O stretching vibration of the ester. The observed stretching frequencies are however close to olefinic functional groups and ester.

Safety evaluation of both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments were studied in animal system employing male rats at different dose levels. The result demonstrated that there was no changes of biological parameters such as haemoglobin, red blood cells, white blood cells, packed cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential count, mean corpuscular volume, red blood cell distribution width, platelet count and mean platelet volume at different concentrations studied for 28 days trail period. The biochemical parameters like serum glutamate oxaloacetate (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), urea and cholesterol were also studied. There were no significant differences observed in food intake and water intake of animal fed with both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments separately. The pigments were also studied for their stability in different conditions such as pH, heat, light, antioxidants and also in different oils.

Carotenoid pigments from *R. mucilaginosa* and *Sporobolomyces* sp. were incorporated in the preparations of few food products like Indian milk ice cream, almond milk, ice cream, yoghurt and popcorn. On the sensory evaluation of the pigment fortified food products, were found to be acceptable.

CONTENTS

CHAPTER No.	TITLE	PAGE No.
I	INTRODUCTION	1
II	REVIEW OF LITERATURE	5
III	MATERIALS AND METHODS	49
IV	RESULTS	96
V	DISCUSSION	212
VI	SUMMARY	237
	REFERENCES	242
	ANNEXURES	

LIST OF TABLES

Table No.	Title	Page No.
1.	Certified synthetic colours for food usage	8
2.	Studies in India on usage of added colours in various foods	13
3.	Chemical category of naturally derived food colours and their sources	15
4.	Microbial pigments and their properties	18
5.	Structure and applications of important carotenoids	20
6.	Types of reactive oxygen species (ROS)	30
7.	Components of central composite rotatable design of experiments	63
8.	Natural levels, codes and intervals of variation of the independent variables in the design of experiments for *R. mucilaginosa* carotenoid pigment production	64
9.	Experimental design used for optimization of physical parameters on *R. mucilaginosa* carotenoid pigment production	66
10.	Natural levels, codes and intervals of variation of the independent variables in the design of experiments for *Sporobolomyces* sp. carotenoid pigment production	65
11.	Experimental design used for optimization of physical parameters on *Sporobolomyces* sp. carotenoid pigment production	67
12.	Natural levels, codes and intervals of variation of the independent variables in the design of experiments (Chemical parameters) for *R. mucilaginosa* carotenoid pigment production	68
13.	Experimental design used for optimization of chemical parameters on *R. mucilaginosa* carotenoid pigment production	69
14.	Natural levels, codes and intervals of variation of the independent variables in the design of experiments (Chemical parameters) for *Sporobolomyces* sp. carotenoid pigment production	70
15.	Experimental design used for optimization of chemical parameters on *Sporobolomyces* sp. carotenoid pigment production	73

Table No.	Title	Page No.
16.	Natural levels, codes and intervals of variation of the independent variables in the design of experiments for *R. mucilaginosa* carotenoid pigment production by submerged fermentation	81
17.	Experimental design used for optimization of fermentation parameters on *R. mucilaginosa* carotenoid pigment production by submerged fermentation	82
18.	Isolation of pigment producing yeast from various sources	97
19.	Screening of yeast isolates for carotenoid pigment production	102
20.	Morphological and cultural characteristics of intracellular pigment producing yeast	107
21.	Effective intracellular carotenoid pigment producing yeast isolates	109
22.	Growth and pigmentation of yeast on different media	112
23.	Ferric reducing antioxidant power activity of acetone extracts of yeast pigments	114
24.	Inhibition of lipid peroxidation of acetone extracts of yeast pigments	116
25.	DPPH scavenging activity of acetone extracts of yeast pigments	117
26.	Reducing power activity of acetone extracts of yeast pigments	119
27.	Hydroxyl radical scavenging activity of acetone extracts of yeast pigments	121
28.	Nitric oxide anion radical scavenging activity of acetone extracts of yeast pigments	122
29.	Metal chelation activity of acetone extracts of yeast pigments	124
30.	Antimicrobial activity of yeast pigment extracts against human bacterial pathogens	126
31.	Minimum inhibitory concentration of acetone carotenoid pigment extracts of *R. mucilaginosa* and *Sporobolomyces* sp. against bacterial pathogens	129
32.	Effect of pH on cell dry weight and carotenoid pigment production	131

Table No.	Title	Page No.
33.	Effect of temperature on cell dry weight and carotenoid pigment production of yeast isolates	133
34.	Effect of incubation time on cell dry weight and pigment production	136
35.	Effect of agitation on cell dry weight and pigment production	137
36.	Effect of inoculum size on cell dry weight and carotenoid pigment production	138
37.	Effect of different light on cell dry weight and carotenoid pigment production	139
38.	Effect of carbon sources on cell dry weight and carotenoid pigment production	142
39.	Effect of organic nitrogen sources on cell dry weight and carotenoid pigment production	144
40.	Effect of inorganic nitrogen sources on cell dry weight and carotenoid pigment production	145
41.	Effect of trace elements on cell dry weight and carotenoid pigment production	148
42.	Central composite design matrix of physical parameters of independent variables and their corresponding experimental and predicted yield of pigment extracted from *R. mucilaginosa*	149
43.	Analysis of variance (ANOVA) for optimization of physical parameters for pigmentation in *R. mucilaginosa*	152
44.	Regression coefficient for optimization of physical parameters for pigmentation in *R. mucilaginosa*	152
45.	Central composite design matrix of physical parameters of independent variables and their corresponding experimental and predicted yield of pigment extracted from *Sporobolomyces* sp.	150
46.	Analysis of variance (ANOVA) for optimization of physical parameters for pigmentation in *Sporobolomyces* sp.	153
47.	Regression coefficient for optimization of physical parameters for *Sporobolomyces* sp. pigment	153

Table No.	Title	Page No.
48.	Central composite rotatable design matrix of chemical parameters and their independent variables and corresponding experimental yields for intracellular pigmentation in *R. mucilaginosa*	158
49.	Analysis of variance (ANOVA) for optimization of chemical parameters for pigment production in *R. mucilaginosa*	163
50.	Regression coefficient for optimization of chemical parameters for carotenoid pigment production by *R. mucilaginosa*	163
51.	Composite rotatable design matrix of chemical parameters and their independent variables and corresponding experimental yields for carotenoid pigment production by *Sporobolomyces* sp.	160
52.	Analysis of variance (ANOVA) for optimization of chemical parameters for carotenoid pigment from *Sporobolomyces* sp.	162
53.	Regression coefficient for optimization of chemical parameters for *Sporobolomyces* sp. pigment production	162
54.	Effect of agricultural cheap substrates for carotenoid pigment production	170
55.	Chemical composition of cassava bagasse	172
56.	Central composite rotatable design matrix of fermentation parameters on pigmentation in *R. mucilaginosa* by cassava bagasse and their independent variables and corresponding experimental yields	172
57.	Analysis of variance (ANOVA) for optimization of fermentation parameters for pigment production by cassava bagasse in *R. mucilaginosa*	177
58.	Regression coefficient for optimization of fermentation parameters for pigment production by cassava bagasse in *R. mucilaginosa*	177
59.	Effect of different solvents on extraction of yeast carotenoid pigments	179
60.	TLC of acetone extracts of carotenoid pigment from *R. mucilaginosa*	179
61.	TLC of acetone extracts of carotenoid pigment from *Sporobolomyces* sp.	179
62.	Stability of the pigment from *R. mucilaginos* after different time intervals in different pH conditions	186

Table No.	Title	Page No.
63.	Stability of the pigment from *Sporobolomyces* sp. carotenoid pigment after different time intervals in different pH conditions	186
64.	Stability of the pigmentation in *R. mucilaginosa* at different time intervals and temperatures	188
65.	Stability of the pigmentation in *Sporobolomyces* sp. at different time intervals and temperatures	188
66.	Antioxidant stability of the intracellular pigment produced by *R. mucilaginosa* after different time intervals	189
67.	Antioxidant stability of the intracellular pigment produced by *Sporobolomyces* sp. after different time intervals	189
68	Stability of the intracellular pigmentation in *R. mucilaginosa* using different oils under different conditions	190
69	Stability of the intracellular pigmentation in *Sporobolomyces* sp. using different oils under different conditions	194
70	Effect of carotenoid pigment extracted from *R. mucilaginosa* on food intake, water intake and body weight gain by male rats	198
71	Effect of carotenoid pigment extracted from *Sporobolomyces* sp. on food intake, water intake and body weight gain by male rats	199
72	Effect of carotenoid pigment extracted from *R. mucilaginosa* on relative weight of different organs of male rat	200
73	Effect of carotenoid pigment extracted from *Sporobolomyces* sp. on relative weight of different organs of male rats	200
74	Effect of carotenoid pigment extracted from *R. mucilaginosa* on haematological parameters	201
75	Effect of carotenoid pigment extracted from *Sporobolomyces* sp. on haematological parameters	201
76	Effect of feeding carotenoid pigment extracted from *R. mucilaginosa* on serum enzymes and cholesterol of male rats	203
77	Effect of feeding carotenoid pigment extracted from *Sporobolomyces* sp. on serum enzymes and cholesterol of male rats	203

Table No.	Title	Page No.
78	Histopathological examination of rats fed with pigment produced by *R. mucilaginosa* and *Sporobolomyces* sp.	204
79	Organoleptic evaluation of food products coloured with carotenoid pigment extracted from *R. mucilaginosa*	210
80.	Organoleptic evaluation of food products coloured with carotenoid pigment extracted from *Sporobolomyces* sp.	211

LIST OF FIGURES

Figure No.	Title	Page No.
1.	Important physical, chemical and biological properties of carotenoids	25
2.	Isoprenoid pathway of carotenoid biosynthesis	27
3.	Spectrometric scanning of yeast carotenoid pigments	104
4.	Ferric reducing antioxidant power of yeast pigments	115
5.	Lipid peroxidation activity of yeast pigments	115
6.	DPPH scavenging activity of yeast pigments	115
7.	The reducing ability of yeast pigments	120
8.	Hydroxy radical scavenging ability of yeast pigments	120
9.	Nitric oxide scavenging ability of yeast pigments	120
10.	Metal chelation of yeast pigments	125
11.	Response surface curve showing the physical parameters on the pigment production by *R. mucilaginosa*	154
12.	Response surface curve showing the physical parameters on the pigment production by *Sporobolomyces* sp.	155
13.	Response surface curve showing the effect of medium components on the pigment production by *R. mucilaginosa*	166
14.	Response surface curve showing the effect of medium components on the pigment production by *R. mucilaginosa*	167
15.	Response surface curve showing the effect of medium components on the pigment production by *Sporobolomyces* sp.	168
16.	Response surface curve showing the effect of medium components on the pigment production by *Sporobolomyces* sp.	169
17.	Response surface curve showing the effect of cassava bagasse on the pigment production by *R. mucilaginosa*	175
18.	RP-HPLC analysis of standard β-carotene compound	183

Figure No.	Title	Page No.
19.	RP-HPLC analysis of pigment extract from *R. mucilaginosa*	183
20.	RP-HPLC analysis of pigment extract from *Sporobolomyces* sp.	183
21.	FT-IR spectrum of pigment extract from *R. mucilaginosa*	184
22.	FT-IR spectrum of pigment extract from *Sporobolomyces* sp.	184

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LIST OF PLATES

Plate No	Title	Page No.
1.	Growth of efficient carotenoid producing yeast isolates in YM agar media	105
2.	Microscopic view of the yeast isolates	105
3.	Biochemical characters of yeast isolates	108
4.	PCR profile of *R. mucilaginosa* (YP 17) and *Sporobolomyces* sp. (YP 37) yeast isolates	110
5.	Neighbour joining phylogenetic tree showing the relationship of the yeast isolates (YP17 and YP 37) and related species retrieved from GeneBank	107
6.	Antimicrobial activity of carotenoid pigment extract from *R.mucilaginosa* and *Sporobolomyces* sp. against bacterial pathogens	127
7.	Growth and carotenoid pigment production by *R. mucilaginosa* under different pH levels	132
8.	Growth and carotenoid pigment production by *Sporobolomyces* sp. under different pH levels	132
9.	Growth and carotenoid pigment production by *R. mucilaginosa* under different temperatures	134
10.	Growth and carotenoid pigment production by *Sporobolomyces* sp. under different temperatures	134
11.	Growth and pigment production by *R. mucilaginosa* under different light sources	141
12.	Growth and pigment production by *Sporobolomyces* sp. under different light sources	141
13.	Growth and carotenoid pigment production by *R. mucilaginosa* in different carbon sources	143
14.	Growth and carotenoid pigment production by *Sporobolomyces* sp. in different carbon sources	143

Plate No	Title	Page No.
15.	Growth and carotenoid pigment production by *R. mucilaginosa* in different nitrogen sources	146
16.	Growth and carotenoid pigment production by *Sporobolomyces* sp. in different nitrogen sources	146
17.	Optimization of fermentation conditions for *R. mucilaginosa* carotenoid pigment by RSM	157
18.	Optimization of fermentation conditions for *Sporobolomyces* sp. carotenoid pigment by RSM	157
19.	Submerged fermentation on different agricultural cheap substrates	171
20.	Production of yeast carotenoid pigment in fermentor	180
21.	Carotenoid pigment extract from intracellular yeast cells	181
22.	*R. mucilaginosa* carotenoid pigment stored in different oils	191
23	*Sporobolomyces* sp. carotenoid pigment stored in different oils	191
24	Toxicity evaluation of yeast carotenoid pigments on male rats	195
25	Histopathological examination of rats fed with pigment extracted from *R. mucilaginosa*	205
26	Histopathological examination of rats fed withpigment extracted from *Sporobolomyces* sp**.**	206
27	Development of food products with yeast carotenoid pigments	208

LIST OF PUBLICATIONS

Sl. No.	Article	Journal Sl. No.	Journal In. No.	NAAS Rating
1.	Influence of fermentation conditions for carotenoid production on *Rhodotorula mucilaginosa* YP 187	279	B036	3.77
2.	Antimicrobial activity of carotenoid pigment produced from yeast *Rhodotorula mucilaginosa* YP 187	1975	T072	2.74

Abbreviations

LIST OF ABBREVIATIONS

^{o} C	:	Degree Celsius
µ	:	Micron
µg	:	Microgram
G	:	Gram
CDW	:	Cell dry weight
H	:	Hours
Mg	:	Milligram
Min	:	Minutes
Ml	:	Millilitre
Rpm	:	Revolutions per minute
w/v	:	Weight per volume
%	:	Percent
CD	:	Critical difference
Sed	:	Standard error of deviation
pH	:	Negative logarithm of hydrogen ion concentration
NIST	:	National institute of science and technology
IP	:	Inhibition percentage
FRAP	:	Ferrous reducing antioxidant power
DPPH	:	1,1,- Diphenyl-2- picryl hydrazyl
EDTA	:	Ethylene diamine tetra acetic acid
BHA	:	Butylated hydroxyl anisole
RSM	:	Response surface methodology
CCD	:	Central composite design
CAT	:	Catalase
SOD	:	Superoxidase dismutase
RBC	:	Red blood cell
WBC	:	White blood cell
Hb	:	Haemoglobin

GSH	:	Reduced glutathione
GPx	:	Glutathione peroxidase
TLC	:	Thin Layer Chromatography
RP-HPLC	:	Reverse phase High Pressure Liquid Chromatogrphy
FT-IR	:	Fourier transform infra red spectroscopy
ALP	:	Alkaline phosphatase
ALT	:	Alanine Transaminase
AST		Aspartate Transaminase
SGOT		Serum glutamic oxaloacetic transaminase
SGPT		Serum glutamic pyruvic transaminase
MCH	:	Mean corpuscular haemoglobin
PCV	:	Packed cell volume
MCHC	:	Mean corpuscular hemoglobin concentration
DC	:	Differential count
L	:	Lymphocytes
P	:	Polymorph neutrophils
M	:	Monocyte
E	:	Eosinophils
MCV	:	Mean corpuscular volume
RDW	:	Red blood cell distribution width
MPV	:	Mean platelet volume

Introduction

CHAPTER I

INTRODUCTION

Most of the natural pigments are extracted from plants like annatto, grapes, paprika, marigold, beetroot, tomato etc., and microorganisms like yeast of the genera *Phaffia*, *Rhodotorula*, *Cryptococcus* and *Sporobolomyces*, fungi like *Blakeslea trispora*, and algae such as *Dunaliella* and *Haematococcus* and bacteria such as *Flavobacterium* and *Micrococcus* are reported to produce carotenoid pigment (Nelis and De Leenheer, 1991).

Humans acquire carotenoid pigments through diet, from vegetables and fruits as well as from animal products. Carotenoid pigments represent a group of valuable molecules for the pharmaceutical, chemical, food and feed industries, not only because they are vitamin A precursors, but also for their colouring, antioxidant and possible tumor inhibiting activity. Despite the availability of a variety of natural and synthetic carotenoid pigments, there is currently a renewed interest in microbial sources of pigments (Bhosale, 2004). The growing evidence has showed that carotenoid pigments obtained from microbial sources have potential benefits in human and animal health and has led to increased commercial interest in search of alternative natural sources.

β-carotene is probably the most well known of the carotenoids, which are highly pigmented (red, orange, and yellow) fat-soluble compounds naturally present in many fruits, grains, oil and vegetables. β-carotene is considered as a provitamin because they can be converted to active vitamin A. Vitamin A serves several biological functions including involvement in the synthesis of certain glycoproteins, which is a molecule composed of a protein and carbohydrate. Vitamin A deficiency leads to abnormal bone development, disorders of the reproductive system, and ultimately death. β-carotene is also converted to retinol,which is essential for vision. β-carotene might be useful in preventing cancer, and it was found to be an antioxidant. More recently β-carotene has been claimed to prevent a number of diseases, including cystic fibrosis and arthritis, and there is a flourishing trade in vitamin supplements containing carotene (Mathews-Roth, 1990).

Foods rich in β -carotene protect cells from damaging effects of free radicals, provide a source of vitamin A, enhance the functioning of immune system and helps reproductive system function properly. β -carotene has many benefits, such as, it is necessary for growth and repair of body tissue, it helps maintain smooth, soft disease-free skin, it helps protect the mucous membranes on the mouth, nose, throat and lungs, thereby reducing susceptibility to infections, it protects against air pollutants, it counteracts night-blindness and weak eyesight, and it aids in bone and teeth formation. Current medical research shows that food rich in β-carotene will help reduce the risk of lung cancer and certain oral cancers. Some symptoms that may show if body don't have enough β-carotene in diet leads to an increased susceptibility to infections, rough, dry, scaly skin, loss of smell and appetite, lack of tearing, and defective teeth and gum growth (Lawson *et al*., 2007).

Now a days biological sources of carotenoid pigment received major focus because of the stringent rules and regulations applied to chemically synthesized /purified pigments. Compared with the extraction from vegetables or chemical synthesis the microbial production of carotenoid pigment is of paramount interest, mainly because of overcoming the problems of seasonal and geographic variability in the production and marketing of the colourants of plant origin and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources (Frengova and Beshkova, 2009). Moreover, industrial interest is now gradually shifting away from the yellow carotenoids such as β-carotene and lutein towards the considerably more valuable orange-red keto-carotenoids, such as torularhodin and torulene, for which at present no commercially exploitable plant or animal sources exist (Nelis and DeLeenheer, 1991).

Yeasts are more convenient than algae or molds for large scale production in fermenters, due to their unicellular nature and high growth rate (Frengova and Beshkova, 2009). Several yeast species belonging to the genera *Rhodotorula* and *Phaffia* are considered to be as potential pigment sources. Yeasts can synthesize carotenoids when cultivated in commercial medium, containing various refined carbon sources, such as glucose, xylose, cellobiose, sucrose, glycerol and sorbitol nevertheless these type of medium represents high costs. As a result, studies on carotenogenesis have led to find out

low cost substrate as alternative to reduce production costs. Therefore, there have been a growing interest in the use of natural substrates as carbon sources (Gomez *et al*., 2014). By-products from industrial processes are pollutants to the environmental and their treatment represents high costs. In recent years, raw materials and agro-industrial wastes origin have been proposed as low-cost alternative carbohydrate sources.

Humans have traditionally preferred natural sources to add colours to food, clothing, cosmetics and medicines. There has been a relatively brief interlude between the beginning of the new millennium, during which natural pigments were replaced by chemical ones (Canizares *et al.,* 1998). The scrutiny and negative perceptions of synthetic food pigments by the modern consumer have given rise to a strong interest in natural colouring alternatives for human safety and environmental conservation.

Due to the global increase in the manufacture of processed foods and the continued consumers demand for natural food ingredients, the market for natural colourant for food use is estimated to manifold further (Downham and Collins, 2000). Now a days consumers are more nutrition and health oriented and they are developing a growing interest about knowing what is present in the food supply and particularly what is added to it in the way of food additives. Food labeling enhances the consumers' knowledge and interest. Not always understanding the physiological needs of the body for certain chemicals and the body's ability to metabolize other chemical structures, consumers tends to shy away from unfamiliar compounds that comprise food additives, such as antioxidants, preservatives and colours. In search for solution, consumers easily adopt the concept that if the additives are added in natural food it must be safe and good. On the other hand, there is also growing awareness that nature is not infallible, as toxicants are also present in natural foods (Kample and Kaur, 2004).

Although many advances in the development of food colours have been made over the last 25 years, particularly in terms of harmonized legislation and advances in processing and formulation technology, there is still scope for future developments. The overall forecast for colour market is to grow in line with technological and sociological changes that will lead to an overall increase in processed foodstuffs. It is thought that the natural colour market will grow on a global scale at a greater rate than

synthetic colours owing to a continued consumer pressure to ‘go natural’. At present, large quantities of pigments are actually satisfied through synthetic way. However, in food and cosmetics industries, the application of chemically synthetic carotenoid pigment is restricted because of their toxicity. Therefore, commercial production of the carotenoid pigments using yeast has gained more importance owing to its highly efficient and easy manipulation.

In the present study, it was aimed to develop natural carotenoid pigment for ecofriendly and non-toxic colourants, specifically with reference to food, pharmaceutical and cosmetic industries. The research has been formulated with the following objectives,

a. To isolate the pigment producing yeast from diverse potential sources like soil, foliar and floral samples.

b. To determine the antimicrobial and antioxidant potential of yeast carotenoid pigments

c. To optimize the culture conditions for maximization of carotenoid pigment production

d. Production of carotenoid pigments from yeast cultures

e. To identify the pigment components using TLC, RP-HPLC and FT-IR

f. To check the stability of the pigment and

g. To analyze the safety of yeast pigments by employing animal system and application of pigments to food products

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Natural pigments are extracted from fruits, vegetables and microorganisms. Interest in natural pigment such as carotenoids has increased considerably, mainly due to the benefits to human health and also to the growth of certain areas such as agriculture, especially aquaculture and poultry industry, nutritional supplements, food industry where they are used as coloring agents for cooked sausages, soft drinks, baked products and pharmaceutical as additive to cosmetics. According to the study of Britton (1983) 100% of β-carotene can be converted to vitamin A. It has also been used as a nutritional supplement being, the main source of provitamin (Babu and Shenoliker, 1995).

Carotenoids have the ability to act as antioxidants and thus protect cells against photo oxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied (Edge *et al*., 1997). Dietary carotenoids inhibit onset of many diseases in which free radicals are thought to play a role in initiation, such as atherosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and most importantly cancer. Antioxidant properties of carotenoids are exploited also in cosmetics. Cosmetic preparations comprising the carotenoids were reported to be effective in preventing various kinds of damage resulting from oxidation and exposure to UV light. Carotenoids are also popular in food industries as colourants and vitamin A sources. Carotenoid containing preparations are also playing important role as feed additive. Astaxanthin is the major carotenoid used for pigmentation of fishes and salmons (Britton *et al*., 1998). Carotenoids market estimate has resulted in 2010 estimated at nearly $1.2 billion, but the expectations for 2018 are increasing considerably supposing to reach $1.4 billion with a compound annual growth rate of 2.3 (Gomez *et al*., 2014).

Colour is a key organoleptic attribute that provides clues for many food qualities such as flavour, sanity, naturalness or maturity. Based on these clues, consumers make their choices (Mortensen, 2006). Thus, it can be said that colour is a key marketing tool for food. Colour is added to food to enrich or compensate for the loss of colour during storage or processing of food. It may be added to minimize batch-to-batch variations or to

enhance the visual appeal of the otherwise uncoloured food. So, the use of food colorants in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food (Ali, 2011).

2.1. Classification of colourants

For consumers the colourants may appear to be either natural or synthetic, but it is definitely more than that and is worthwhile to take a look at it. Based on chemistry, colourants can be divided into organic or inorganic both of which are mostly synthesized chemically in the lab. The simple examples of which are carotenoids and titanium dioxide respectively. Most of the naturally occurring colourants that are currently in use are typically organic colourants. Furthermore, organic colourants can be classified as pigments and dyes based on their solubility in the media; if they are insoluble and require a fixative to adhere to the surface of the substrates, they are termed as pigments while the dyes are almost soluble in the media to which they are added and do not require any fixatives.

As per the origin and legislation is concerned, food colourants can be categorized into natural, nature-identical and synthetic. Natural colourants are pigments produced, accumulated or excreted by living organisms. They also include colourants made by the modification of materials from living organisms, such as caramel, vegetable carbon and copper-chlorophyllin. Ironically, they are (except for carbon) not found in nature. Nature identical colourants are chemically synthesized man-made pigments which are also found in nature. Examples include, β-carotene, canthaxanthin, and riboflavin. Synthetic colourants are man-made colourants that are not found in nature; these are mostly azo-dyes. Consumers, however, perceive only natural and synthetic classes of colourants. Thus, it can be inferred that natural colours are regarded as safe and have a healthy image for the consumers (Ali, 2011).

2.1.1. Synthetic food colours

The first synthetic organic dye, a purplish lilac colour, was discovered in 1856 by William Henry Perkin and called “mauve.” Over the next 50 years, scores of similar organic aniline dyes, representing every colour and tint of the rainbow, were developed,

and many were used to colour food with little thought or testing regarding their safety. Significant toxicity of many early aniline and coal-tar based colours prompted regulators to examine exactly what was being used to colour food (Pratima and Bhat, 2003).

Of the 80 synthetic food colourants sold in 1907, only 16 were deemed to be "more or less" harmless. By 1907, this list was pared to seven synthetic colourants, which eventually were subjected to "batch certification" to detect and limit toxic impurities (Coulson, 1980; Santhanakrishnan, 1981). The list of "certified" colours had expanded and contracted over the decades to the current group of nine certified colours chemically classified into four chemical families: azo, xanthenes or fluoroscein, triphenylmethane and sulfonated indigoid (Table 1).

2.1.1.1. Health hazards of synthetic food colours

The potential adverse health effects through exposure to synthetic agents in foods is evaluated by risk analysis. Among the synthetic agents, food colours are of major concern in India. In the mid-1980s, there was a growing concern among consumers about the possible adverse effects of additives such as colours, for example, the purported link between tartrazine and hyperactivity in children. A possible link between the ingestion of foods containing synthetic food flavourings and colours and the presence of hyperactive behaviours in children had been suggested (Harley *et al.*, 1978).

Permitted colours like ponceau, tartrazine and sunset yellow have provoked allergic reactions in several individuals even at low levels of intake. The allergic responses vary from urticaria to dermatitis, angioedema and exacerbation of asthmatic patients (WHO, 1991). It has also been reported that the consumption of a particular brand of aniseed having very high levels of ponceau 4R, exhibited symptoms of glossitis of tongue in children (Nin, 1994). Tartrazine was associated with irritability, restlessness and sleep disturbance in atopic or hypertensive children aged between 2 and 14 years. Rowe and Rowe (1994) found that behavioural changes in relation to irritability, restlessness, and sleep disturbance were associated with the ingestion of tartrazine in some children, and reported that those effects were dose-related (1–50 mg per child).

Abdel Aziz *et al.* (1997) revealed the potential adverse effects of erythrosine (ER, FD & C Red No. 3) on the spermatogenesis in adult mice. The sperm count as well as the percentage of motile sperms were significantly reduced by about 50 per cent and

Table 1. Certified synthetic colours for food usage

S. No.	Certified colour name	Common or industrial name (hue)	Uses and restrictions	CFR*
1	FD & C Blue No. 1	Brilliant Blue FCF	Foods generally	74.101
2	FD & C Blue No. 2	Indigotine (royal blue)	Foods generally	74.102
3	FD & C Green No. 3	Fast Green FCF (sea green)	Foods generally	74.203
4	Orange B	-	Casings or surfaces of frankfurters and saus	74.250
5	Citrus Red No. 2	-	Skins of oranges	74.302
6	FD & C Red NO.3	Erythrosine (Cherry red)	Foods generally	74.303
7	FD & C Red No 40	Allura Red (Orange-red)	Foods generally	74.340
8	FD & C Yellow No 5	Tartrazine (Lemon yellow)	Foods generally	74.705
9	FD & C Yellow No 6	Sunset yellow (Orange)	Foods generally	74.706

***CFR - Code of Federal Regulations**

57 per cent respectively. These findings indicated that ER in the used doses had a potential toxic effect on spermatogenesis in mice and in turn, it might affect its testicular function and reproductive performance. Many synthetic colours have been widely used as food additives in various processed foods for economic reasons and for their bright colour and stability (Ozaki *et al.*, 1998). The Prevention of Food Adulteration (PFA) Act in India prescribes a level of 100 ppm of tartrazine, sunset yellow, ponceau 4R, carmoisine, erythrosine, brilliant blue, fast green FCF and indigo carmine to be added to specified food items and a level of 200 ppm in canned foods, jams and jellies.

Tsuda *et al.* (2001) reported the genotoxicity of synthetic red tar dyes (amaranth food red No. 2), allura red (food red No. 40), or acid red (food red No. 106), new coccine (food red No. 18) when orally administered to pregnant and male mice to analyse the DNA damage. The assay was positive in the colon, 3 h after the administration of amaranth and allura red and weakly positive in the lung, 6 h after the administration of amaranth. Acid red did not induce DNA damage in any sample at any sampling time. The 3 dyes induced DNA damages in the colon starting at 10 mg kg^{-1} of new coccine, induced DNA damage in colon, glandular stomach, and bladder. Because, the 3 azo additives examined induced colon DNA damage at a very low dose, more extensive assessment of azo additives was warranted.

Permitted colours usage had also evoked concern because they were being used in excess of the statutory limit (100 ppm) or in foods in which they were not permitted (Bhat and Mathur, 1998; Pratima and Bhat, 2003; Padmaja *et al.*, 2004; Pratima *et al.*, 2004 and 2005). The safety of repeated exposure to the permitted synthetic colours were often questioned. Tanaka *et al*., 2008 studied that tartrazine exerted minimal neuro behavioural effects (only neuromotor development in females) in rats (1.0–2.0 % in the diet).

2.1.1.2. Natural Vs Synthetic colourants

Use of dyes to colour the food stuff have been used historically, however the detrimental effect of such food dyes became more prominent only after the invention of synthetic dyes. For example, it has been reported that synthetic colourant, fuchsine, was used as early as in 1860 in France as a wine additive (Zollinger, 2003). Arsenic acid (toxic) was used as a reagent for its manufacture. Today, consumers are concerned about

the eventual harmful effects of synthetic colourants; Fast Green dye has been shown to be an immunotoxic agent (Ali and Bashier, 2006). Despite the fact that synthetic colourants are relatively more stable, have higher colour strength per mole of a compound and cheaper to produce. Natural colourants are preferred due to their safe and healthy image and the fact that they can be produced by eco-friendly means (Zollinger, 2003). Global sales of natural colours reached an estimated $600 million in 2011, up by almost 29 per cent from 2007 and demonstrating annual growth in excess of 7 per cent. The share of the total food colours market taken by natural sources has increased from 34 per cent in 2007 to nearly 39 per cent in 2011, according to a new report from Mintel and Leatherhead Food Research. Thus, it can be said that naturally derived colorants look set to overtake synthetic alternatives in market value due to the rising demand for the clean label ingredients.

2.2. Legislative issues and food regulations on food colourants

To evaluate the safety of food colours, the Joint FAO/WHO Expert Committee of Food Additives (JECFA) has set an Acceptable Daily Intake (ADI) for each of the permitted colours based on toxicological studies on experimental animals and data from human clinical studies. The ADI has been defined as the amount of a substance that can be consumed everyday throughout the life time of an individual without any appreciable adverse health effects (WHO, 1991). The ADI for permitted colours varies from 0.1 mg kg^{-1} body weight for erythrosine to 25 mg kg^{-1} body weight for fast green FCF. The more toxic the food colour, the lower is the ADI. It becomes extremely important to monitor the total daily intake of all food colours since the ADI level of any food colours should not be exceeded the level prescribed by JECFA, 1996. The International Life Sciences Institute (ILSI) suggested that special ADIs should be established for infants and children as the use of food colours represents a higher risk for infants and children than for adults. The maximum permitted limit in foods is 0.1g per kg of food or one g in 10 kg. In food category, the maximum limit is 0.2 g per kg of food, but even in these, the limit should not exceed 0.1 g per kg at the time of serving or consumption (Pratima and Sudershan, 2008).

In general, children are more susceptible to chemical dyes or colours and the patterns of consumption are different from adults. There are various factors which can influence the intake of food colours, such as the type and quantity of foods consumed and

the socio-economic status of individuals. In India, most of the studies were focussed on the usage of colours in foods while there have been limited studies focused on the exposure of individuals to synthetic food colours. Thus, the safety of repeated exposure to synthetic colours were questioned and there was a need to evaluate total exposure of individuals to colours. The Prevention of Food Adulteration Act (PFA) regulates the usage of colour. At the state level, the implementing authority is the State Food Health Authority and at the local level, municipalities, municipal corporations or Local Health Authorities are responsible (Padmaja *et al.,* 2004).

In most countries, only certain dyes are permitted as food colours, and compound-specific purity standards are governed by strict regulation. The legislation specifies which colourants may be used; the sources of the colourants, the purity of the colourant, to which foods the colourant may be added, and at what level the colourant may be added to a specific food. The European Union (EU) directive, EU Directive 94/36/EC, often referred to as the Colours Directive, is implemented throughout the members state. Here the colours are listed together with conditions for their use. In the United States (US), the use of colours is outlined in Code of Federal Regulations, Title 21 (21CFR). A number of countries also follow the US legislation. The Australian and Japanese legislations on colours in food are also used as the basis for local regulation in a number of other Asian countries. Legislation is often influenced by local, traditional usage of colorants as in case of *Monascus* colourants (pigments of an ascomycetous fungus *Monascus* used as fermentatively produced red rice powder), lac, and gardenia based colourants. None of these are permitted to be used in the EU and the US, partly due to the fact that their sources are too alien for these societies. There are many similarities between the EU and US legislations about the colourants that are allowed, however, there are striking differences when it comes to the sources of the colorants and the foods in which the colorants can be applied. For example, in the USA, sodium copper chlorophyllin may only be made from alfalfa (*Medicago sativa*) and only be used in citrus-based dry beverage mixes, whereas in the EU, allowed sources are alfalfa, grass, nettle, and edible plant material, and a wide range of foods may be coloured (Mortensen, 2006).

2.2.1. Status of permitted food colours in India

The type and extent of colours added to ready-to-eat (RTE) foods prepared in the non-industrial sector of India was investigated. Of the 545 RTE foods analysed, 90 per cent contained permitted colours, 2 per cent contained a combination of permitted and non-permitted colours and 8 per cent contained non-permitted colours (Khanna *et al.,* 1973; Santhanakrishnan, 1981). However in RTE foods with permitted colours, 73 per cent exceeded the 100 ppm, as prescribed by the Prevention of Food Adulteration Act of India and 27 per cent were within the prescribed levels. Among the permitted colours, tartrazine was the most widely used colour followed by sunset yellow. The maximum concentration of colours was detected in sweet meats (18767 ppm), non-alcoholic beverages (9450 ppm), miscellaneous foods (6106 ppm) and hard-boiled sugar confectioneries (3811 ppm). Among the non-permitted colours found, rhodamine was most commonly used. Some of the foods, such as savouries and miscellaneous foods like sugar coated aniseed is not supposed to contain colours as per the Prevention of Food Adulteration Act, but were found to contain colours (Singh, 1997; Pratima and Sudershan, 2008). The studies on permitted and non-permitted colours are presented in Table 2.

2.3. Natural colours

Natural colourants are considered to be safer than synthetic ones, and their applications in foods, cosmetics and pharmaceuticals are growing rapidly (Lauro, 1991). There are a number of natural pigments, but only a few are available in sufficient quantities for industrial production. Production of pigments from microorganisms is advantageous over other sources because microorganisms can grow rapidly which may lead to a high productivity of the product (Kim *et al.,* 1999).

2.3.1. Important chemical classes of natural food colourants

The use of food colourants as additives in the food industry is a significant factor for both food manufacturers and consumers in determining the acceptability of processed food (Pattnaik *et al.,* 1997). Currently, the European Union has authorized approximately 43 colourants as food additives, whereas approximately 30 colour additives are approved

Table 2. Studies in India on usage of added colours in various foods

Food items	Permitted colours	Non-permitted colours	Reference
Milk products (ice cream, khoya, chenna), non-milk products (sweets, namkins), pulses, miscellaneous (confectionery, soft drinks, spices, condiments and tea)	Tartrazine, sunset yellow, carmoisine, ponceau 4R, amaranth, erythrosine, fast red and indigo carmine	Metanil yellow, auramine, rhodamine B, blue VRS, malachite green, Sudan II,III, congo red	Khanna *et al*. (1986)
Sweets, confectionery, Namkins	Tartrazine, sunset yellow, brilliant blue, carmoisine, indigo carmine, erythrosine, ponceau 4R, amaranth	Metanil yellow, orange II, malachite green, rhodamine B, blue VRS, auramine	Dixit *et al.* (1995)
Pulses (*Cajanus cajan, Cicer arietinum, Phaseolus mungo*), powdered spices (turmeric, chillie powder), nonhydrogenated oils	-	Metanil yellow, orange II, sudan II,III, IV and V, auramine, blue VRS, malachite green, rhodamine B	Khanna *et al.* (1987)
Sweetmeats, turmeric, ice candies	-	Metanil yellow, lead Chromate	Kiple and Ornelas (2000)
Edible oil	-	Butter yellow	Chakravarti (1988)
Sweets, confectionery, soft drinks, ice candies, fruit sweetened juices, edible oils, turmeric, pulses, tea and coffee	Tartrazine,sunset yellow, carmoisine, ponceau 4R, brilliant blue	Rhodamine, auramine, orange II	Babu and Shenolikar (1995)
Jilebi	-	Metanil yellow	Toledo *et al.* (1992)
Dried ginger, confectionery, pulses, edible oils, potatoes, pickles, prepared foods *viz.*, Biryani	Tartrazine, ponceau 4R, sunset yellow	Rhodamine, orange II,metanil yellow, ultra marine blue	Sudershan (1994)
Confectionery, sweets, biscuits, beverages, jam, jelly, custard powder	Tartrazine,sunset yellow, carmoisine, ponceau 4R, brilliant blue	Amaranth, orange II, metanil yellow, rhodamine B, congo red, blue VRS, unidentified chocolate brown	Biswas *et al.* (1994)
Flattened rice, fish, fresh vegetables and cut-fruits		Auramine, congo red, malachite green, rhodamine B	Dixit *et al.* (1995)
Sweets, savoury products, powdered spices (turmeric, red chilli)	Tartrazine, sunset yellow, indigo carmine, amaranth, brilliant blue FCF	Metanil yellow, orange II, sudan I, III, IV, rhodamine B, blue VRS, auramine	Dixit *et al.* (1995)

for use in the United States. These legislations do not distinguish between synthetic and natural colour additives; however, in both Europe and US several of the listed colour additives are derived from natural sources by physical and/or chemical extraction.

Although structurally quite diverse and derived from a variety of sources, natural food colourants can be grouped into five important classes (Moss, 2002); tetrapyrrole derivatives, isoprenoid derivatives, benzopyran derivatives, artefacts, and others (Table 3). Chlorophylls belong to the class tetrapyrrols which is a characteristic photosynthetic green plant pigment and is ubiquitous. Other members of photosynthetic apparatus include carotenoids that belong to the pigment class of isoprenoid derivatives. They impart characteristic yellow-orange-red colour to many fruits. Anthocyanins are a group of flavonoids that provide the red-purple shade to many fruits such as strawberries, elderberries, and black currants. They are an example of benzopyran derivatives.

Melanins, melanoidins and caramels are grouped together since they are all complex polymeric molecules, with some similarity in structural units comprising the polymers. The distinction between the melanins and melanoidins and caramels is that melanins occur naturally and are responsible for many of the black, grey and brown colours found in plants and animals, Melanoidins and caramels, however, are formed by non-enzymatic browning reactions, usually during the heat processing of foods, melanoidins are produced as a result of Maillard reactions, essentially a reaction initiated between a reducing sugar and a primary or secondary amine, while caramelization reactions result from the effect of heat on sugars. The pigments of scale insects such as lac and cochineal belong to anthraquinone subclass. For example, carminic acid, which imparts orange-red-purple colour shade; the colour depends on the pH. Beet root is a major source of betalains which comprises of the red-purple betacyanidins and the yellow betaxanthins. Curcumin belongs to the subclass phenalones. The major source of which is the rhizome of the plant *Curcuma longa*. It imparts a characteristic greenish yellow colour. Another less widely used colorant that belongs to the subclass flavin is riboflavin (Wissgott and Bortlik, 1996).

Table 3. Chemical category of naturally derived food colours and their sources

Biochemical origin	Generic name		Example	Source	Colour range	References
Tetrapyrrole	-	-	Chlorophyll	Grass, Lucerne and Nettle	Green to olive	Maoka *et al.* (2002)
Isoprenoid	Carotenoids	Carotene	Lutine	Marigold	Orange	Vernon-Carter *et al.* (1996)
			Lycopene	Tomato	Red	Chen *et al.* (1995)
		Xanthophylls	Canthaxanthin	Paparika, Salmon, Shrimp	Red	Perez - Galvez *et al.* (2003)
			Zeaxanthin	Paparika	Yellow	
	Irodoid		Crocin	Saffron	Red	Tsimidou and Tsatsarani (1993)
N- heterocyclic compounds	Flavin		Riboflavin	*Clostridium acetobutylicum, Ashbya gossypi*	Yellow	Stahmann *et al.* (2000)
	Betalain		Betaine	Beet root	Pink to red	Im *et al.* (1990)
Benzopyran	Anthocyanin		Pelargonidin	Grapes, black carrots	Red	Plamen *et al.*(2007)
			Cyanidin	Apple, Cherry, Fig	Red	Koes *et al.* (1994)
			Delphinidin	Cranberries, grape, Pomegranate	Red	Zwayyed *et al.*(1991)
Quinine			Carmic acid	Female cochineal insect	Orange to red	Ramirez-Crunz *et al.* (2008)

2.3.2. Quality control issues

The extent of quality control depends on the type of colour product, the information available on the certificate of analysis, the expected processing conditions, the finished product and the standard quality control procedures of the food or beverage manufacturer. In some cases the colour strength or colour hue and intensity may be the most important parameter. In others, the microbiological standard and absence of pathogens are also important. For powder formulations to be used in dry blends, the particle size is relevant, whereas density can be a key point if the colour is volumetrically measured by flow meter during the production process (Nielsen and Holst, 2002).

2.3.3. Merits Vs Demerits of natural food colorants currently in use

Since natural colorants are extracted from natural sources, they are in most cases mixtures of varying composition and, therefore, not easy to characterize with respect to purity and contaminants. Anthocyanins and flavonoids form one such an example that is derived from many fruits and vegetable sources. Their use in a pure form as food additives is limited mainly for three reasons:

1. They are expensive to isolate in pure form and large quantities from natural sources.
2. They are difficult to synthesize.
3. The colour is often pH dependent as in case of anthocyanins. An increase of pH enhances blue hues, whereas, under acidic conditions, an orange to red colour is observed (Zollinger, 2003).

The issue of stability of pigments is not to be overlooked. Pigments are highly unsaturated compounds and are prone to light, heat, and oxygen disintegration. However, there exists a large difference in the stability of different classes of existing natural food colorants. Vegetable carbon and caramel are very stable towards heat, light, and oxygen. Carminic acid and carmine are also quite stable but not as stable as vegetable carbon and caramel. On the other hand, turmeric is rapidly bleached by light and beet root pigments turn brownish even under mild heating conditions (Mortensen, 2006). This provides a large incentive to identify alternative ways in which natural colours can be produced in the future.

2.4. Microbial colours

The pigments from microbial sources are good alternatives that could easily be produced in high quantity and capability of producing different coloured pigments. Pigment producing microorganisms and microalgae are quite common in nature. Pigments produced by microorganisms include carotenoids, melanins, flavins, quinones and more specifically monascins, violacein, phycocyanin or indigo. However, there is a long way to go from the Petri dish to the market place as only five productions are operated on an industrial scale (Table 4 and 5).

The microbial colours have the advantage of being climate independent, do not require large area for their growth and can be produced in any quantity in shorter period (Francis, 1987). Microorganisms produce a variety of pigments that includes polyketides, carotenoids, phenazines, acylphenols, pyrones, sclertiorins, anthroquinones but most of these pigments are toxic to humans except for carotenoids and polyketides (Stich *et al.,* 2002). Further, the carotenoids possess nutraceutical properties which gain more importance in food industries (Fontana *et al.,* 1996). Among microalgae, two successful stories yield to efficient production of carotenoids such as β-carotene using *Dunaliella salina* or astaxanthin using *Haematococcus*. The phycobiliproteins or phycocyanin, researchers should first carry out toxicological studies which could allow the use as food colours (Dufosse *et al.,* 2005).

Bacterial sources of food colourants such as zeaxanthin by *Flavobacterium*, canthaxanthin (mostly used in aqua feed) from the photosynthetic bacterium *Bradyrhizobium* spp., and *Halobacterium* spp. have been investigated. Astaxanthin production by *Agrobacterium* and *Paracoccus carotinifaciens* have been investigated. Case of arylcarotenoids such as isorenieratene and hydroxyl derivatives production by *Brevibacterium aurantiacum*; a bacterium associated with red-smear ripened soft cheeses, forms an interesting avenue to be explored further. This is because the pigments produced by this bacterium, thus, have been consumed for a long time. Apart from carotenoids, rubrolone is known to be produced by *Streptomyces echinoruber* (Dufosse, 2006).

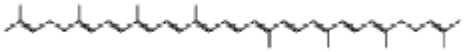
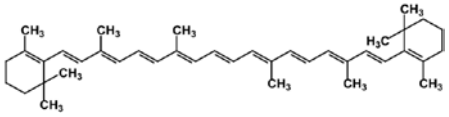
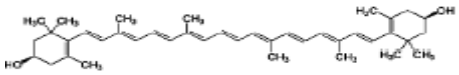
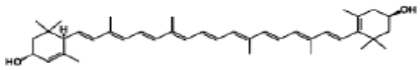
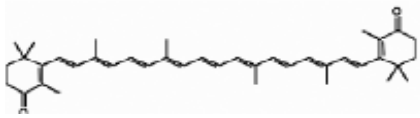
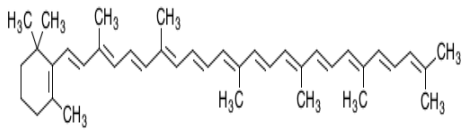
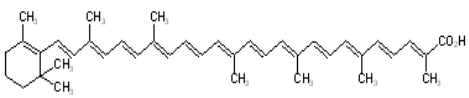
Fungi, particularly ascomycetous and basidiomycetous (mushrooms) fungi, and lichens (symbiotic association of a fungus with a photosynthetic partner usually either a

Table 4. Microbial pigments and their properties

Microbial source	Molecule	Colour	References
Bacteria			
Agrobacterium aurantiacum	Astaxanthin	Pink-red	Yokoyama *et al.* (1994)
Paracoccus carotinifaciens	Astaxanthin	Pink-red	Tsubokura *et al.* (1999)
Paracoccus zeaxanthinifaciens	Zeaxanthin	Yellow	Humbelin *et al.* (2002)
Bradyrhizobium sp.	Canthaxanthin	Dark red	Hannibal *et al.* (2000)
Streptomyces echinoruber	Rubrolone	Red	Iacobucci and Sweeney (1981); Oshima *et al.*, 1981
Flavobacterium sp.	Zeaxanthin	Yellow	Shepherd *et al.* (1976)
Halobacterium sp.	Canthaxanthin	-	Asker and Ohta (1999)
Pseudomonas aeruginosa	Phenazine		Hannibal *et al.* (2000)
Micrococcus sp.	Carotenoid	Yellow	Archer (2000)
Fungi			
Monascus sp.	Ankaflavin Monascorubramin	Yellow	Blanc *et al.* (1994)
Penicillium oxalicum	Anthraquinone	Red	Sardaryan *et al.* (2004)
Fusarium sporotrichioides	Lycopene	Red	Jones *et al.* (2004)
Cordyceps unilateralis	Naohtoquinone	Deep blood-red	Unagul *et al.* (2005)
Ashbya gossypi	Riboflavin	Yellow	Santos *et al.* (2005)
Blakeslea trispora	β -carotene	Yellow-orange	Lampila *et al.* (1985)
Mucor circinelloides	β - carotene	Yellow-orange	Sardaryan *et al.* (2004)
Neurospora crassa	β - carotene	Yellow orange	Hausmann and Sandamann (2000)
Phycomyces blakesleeanus	β - carotene	Yellow orange	Cerda-Olmedo (2001)
Penicillium purpurogenum	Unknown	Red	Watanabe *et al.* (1977)
Paecilomyces sinclairii	Unknown	Red	Cho *et al. (*2002 a)
Fusarium graminearum	Aurofusarin	Red	Miller and MacKenzie (2000)
Fusarium culmorum	Rubrofusarin	orange	Dvorska, (2001)

Microbial source	Molecule	Colour	References
Yeast			
Xanthophyllomyces dendrorhous	Astaxanthin	Pink-red	Ramirez *et al.* (2000)
Rhodotorula sp.	Torularhodin, β-carotene, torulene	Orange, yellow,pink	Sakaki *et al*.(2000)
Sporodiobolus salmonicolor	β-carotene	Orange	Valduga *et al.*(2008)
Sporobolomyces roseus	βcarotene, torulene,torularhodin	Orange,pink	Maldonade *et al.*(2008)
Cryptococcus sp.	B-carotene	yellow	Libkind and Broock, (2006)
Rhodospiridium babjevae	β-carotene	Orange	Sperstad *et al.*(2006)
Algae			
Dunaliella salina	β-carotene	Green	Ben-Amotz (2004)
Haematococcus pluvialis	Astaxanthin	Orange	Wang *et al*.(2004 b)
Dunaliella bardawil	β-carotene	Green	Richmond (2004)
Picochlorum oklahomensis	β-carotene	Red	DelCampo *et al.*(2004)

Table 5. Structure and applications of important carotenoids

Carotenoids	Chemical structure	Major applications	Reference
Lycopene		Against cardiovascular diseases, prostate cancer and cosmetic preparations	Rissanen *et al.* (2002)
β-carotene		Anticancer agent, nutraceutical as provitaminA source,food colorants, photoprotectant and cosmetic preparations, prevention of age related macular degeneration	Pratheeba *et al.* *(*2014) Palozza *et al.* (2004) Wong *et al.*(2011)
Zeaxanthin		Prevention of age-related macular degeneration and cosmetic preparations	Seddon *et al.*(1994)
Lutein		Used in cosmetic preparations and cancer treatment	Kumaresan *et al.* (2008)
Canthaxanthin		Used in animal feed	Krupa *et al.*(2010)
Torulene		Powerful antioxidant to be included in food and drug formulations. Effective in prevention of liver tumour development	Ungureanu *et al.* (2012**)** Sakaki *et al.* (2001)
Torularhodin			

green alga or cyanobacterium) are known to produce extraordinary range of colours that include several chemical classes of pigments. Mushrooms and lichens have a rich history as sources of pigments for textile colouring. Mycelial extracts of some promising mushrooms such as *Chroogomplus vinicolor* gives red tints, *Bankera violascens* gives greens and *Collybia iocephala* gives blues. They have a tremendous potential for dying wool and silk fabrics (Maldonado and Ibarra, 2005). Carotenoids such as β-carotene and lycopene have been known to be produced by fungal cell factories. The successful industrial production of β-carotene by *Blakeslea trispora* is the best example to be given. DSM (The Netherlands) was the first company to produce β-carotene from this fermentative source. Today, there are two other industrial productions of *B. trispora* fungal β-carotene, the first in Russia and Ukraine, the second in Leon (Spain). Another explored fungal sources of β-carotene are *Mucor circinelloides* (zygomycete fungus), and *Phycomyces blakesleeanus*. Widely known vitamin, riboflavin (vitamin B2) is a yellow food colorant that is fermentatively produced by the fungi *Eremothecium ashbyii* and *Ashbya gossypi* (Dufosse, 2006). As far as polyketide class of fungal pigment from *Monascus* have been utilized for making Oriental foods, especially in Southern China, Japan and Southeastern Asia, such as red rice wine, red soya bean cheese and anka (red rice). Anka was traditionally produced by inoculating moistened rice with *Monascus*, and the product contained various pigments linked to proteins, peptides, and amino acids). The new isolate *Penicillium* strain produced violet, red, orange and yellow pigments and these pigments exhibit the monascorubramine components (Ogihara *et al.*, 2000 a,b). Pigments synthesized by micromycetes *Aspergillus* sp. are true melanins. Sanjay *et al.* (2007 b) reported that the pigment obtained from mycelial biomass of *Aspergillus* sp. is a safe food colour.

Among yeasts, the commercial success has been with the case of astaxanthin production by *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*). A major drawback of this production process is that disruption of the cell wall of yeast biomass is required prior to its use as food additive. Another example of production of carotenoids is by the yeast *Rhodotorula glutinis, R. gracilis, R. rubra and R. graminis.* (Dufosse, 2006).

2.5. Carotenoids pigment production by yeast

Carotenogenic yeasts are considered to be ubiquitious due to its world-wide distribution in terrestrial, freshwater and marine habitats and their ability to colonize a large variety of substrates. Carotenoid pigment production by yeasts namely, *Phaffia rhodozyma*, *Rhodotorula* sp., *Sporobolomyces roseus*, *Rhodosporidium* sp., *Sporidiobolus* sp. and *Candida utilis* is documented.

***Phaffia rhodozyma*:** The red yeast *X. dendrorhous* which was formerly known as *P. rhodozyma* was isolated by Herman phaff in the 1960's during his pioneer studies of yeast ecology. Initially, the yeast was isolated from limited geographical region, but isolates were subsequently obtained from Russia, Chile, Finland and United States. The biological diversity of the yeast is more extensive than originally envisioned by Phaff and his collaborators, and atleast two species appear to exist including the anamorph *P. rhodozyma* and the teleomorph *X. dendrorhous*. The Yeast has attracted considerable biotechnological interest because of its ability to synthesize the economically important carotenoid astaxanthin (3, 3'-dihydroxy-beta, beta-carotene-4, 4'dione) as its major pigment. This property has stimulated research on the biology of the yeast as well as development of the yeast as an industrial microorganism for astaxanthin production by fermentation.

Red yeasts can use diverse sources of carbon, including low cost raw materials, such as molasses, whey and agricultural industrial by products, such as crude glycerol. The red yeast is able to grow under a wide range of initial pH conditions from 2.5 to 9.5 and over a wide range of temperatures from 5 to 26°C. It is the only known red yeast that produces astaxanthin. In fact, astaxanthin contributes to 80-90% of its total carotenoids. Carotenoids are produced during late log phase or stationary phase by the mavolanate isoprenoid pathway. The red pigmented fermenting yeast *P. rhodozyma* contains astaxanthin as the principal carotenoid pigment, Echinenone, 3-hydroxyechinenone and phoenicoxanthin were also isolated and identified; isocryptoxanthin and canthaxanthin were absent.

***Rhodotorula* sp:** Yeast in the genus *Rhodotorula* synthesize carotenoid pigments. Feed supplement with a *Rhodotorula* cell mass has been found to be safe and non toxic in

animals. Its use in the nutrition of laying hens has also been documented (Abd El-Rahman *et al*., 2012). Various species of *Rhodotorula* were isolated from plant leaves, flowers, fruits, slime fluxes of deciduous trees, soil, refinery waste water, air and yoghurt. The production of carotenoids by genus *Rhodotorula* is affected by species, medium constituents and environmental conditions. Besides carotenoids, *Rhodotorula* is a rich source of fats and vitamins and can be incorporated in feeds to enhance the nutritional value and to prevent fungal contamination (Joshi *et al*., 2003).

***Sporobolomyces* sp:** In *Sporobolomyces* species, the mainly produced pigment is β-carotene. Production of torulene was not clearly confirmed, nevertheless, some oxidized carotenoid derivatives are produced in *Sporobolomyces* strains. In most of red yeast osmotic and oxidative stress was reported to induce carotenoid production probably as a part of stress response (Marova *et al*., 2012). The genus *Sporobolomyces* contains about 20 species. The most common ones are *S. roseus* and *S. salmonicolor*. *Sporobolomyces* colonies grow rapidly and mature in about 5 days. They produce yeast like cells, pseudohyphae and ballistoconidia. The yeast like cell is the most common type of conidia and oval to elongate in shape. The optimal growth temperature is 25-30ºC. The colonies are smooth, often wrinkled and glistening to dull. *S. roseus* produces other carotenoid pigments such as torulene and torularhodin (Maldonade *et al*., 2007).

***Cryptococcus* sp**: *Cryptococcus neoformans* is the only yeast known that consistently produces a melanin-like pigment from many p-diphenols. Pigment production from diphenols by other members of the genus is very rare. However, pigment production by some *Cryptococcus* species can occur when hydroquinone is present in the substrate. *Cryptococcus* may also produce small amount of pigment from diphenols after prolonged incubation (10 days or more). The strains of *C. neoformans* produced brown cell-associated intracellular pigments while others produced pink extracellular pigments from l- or- dl-tryptophan. Additionally, various other *Cryptococcus* species produced brown or pink pigments when cultured on tryptophan.

2.5.1.1. Functions of carotenoid pigments

Being an important flavonoid compound, β- carotene has powerful antioxidant functions, helps the body scavenge free radicals, thereby limiting the damage to cell membranes, DNA and protein structures in the cell. Research studies suggest that dietary intake of foods high in β-carotene has positive association with decreased risk of cardio-vascular disease as well as oral cavity, and lung cancers. When converted to vitamin A in the intestines it has all the functions of vitamin A such as visual cycle, reproduction (sperm production), maintenance of epithelial functions, growth and development (Davoli *et al*., 2004).

Commercially available β-carotene can come from both natural and nature-identical sources. Though most of the β-carotene used in the food and beverage industries today are produced by synthesis (nature-identical), there are several natural sources available, which are increasingly used to replace the nature-identical variant. Natural β-carotene sources are for example algae, fungi or palm fruit oil (Ekendahl *et al.*, 2003).

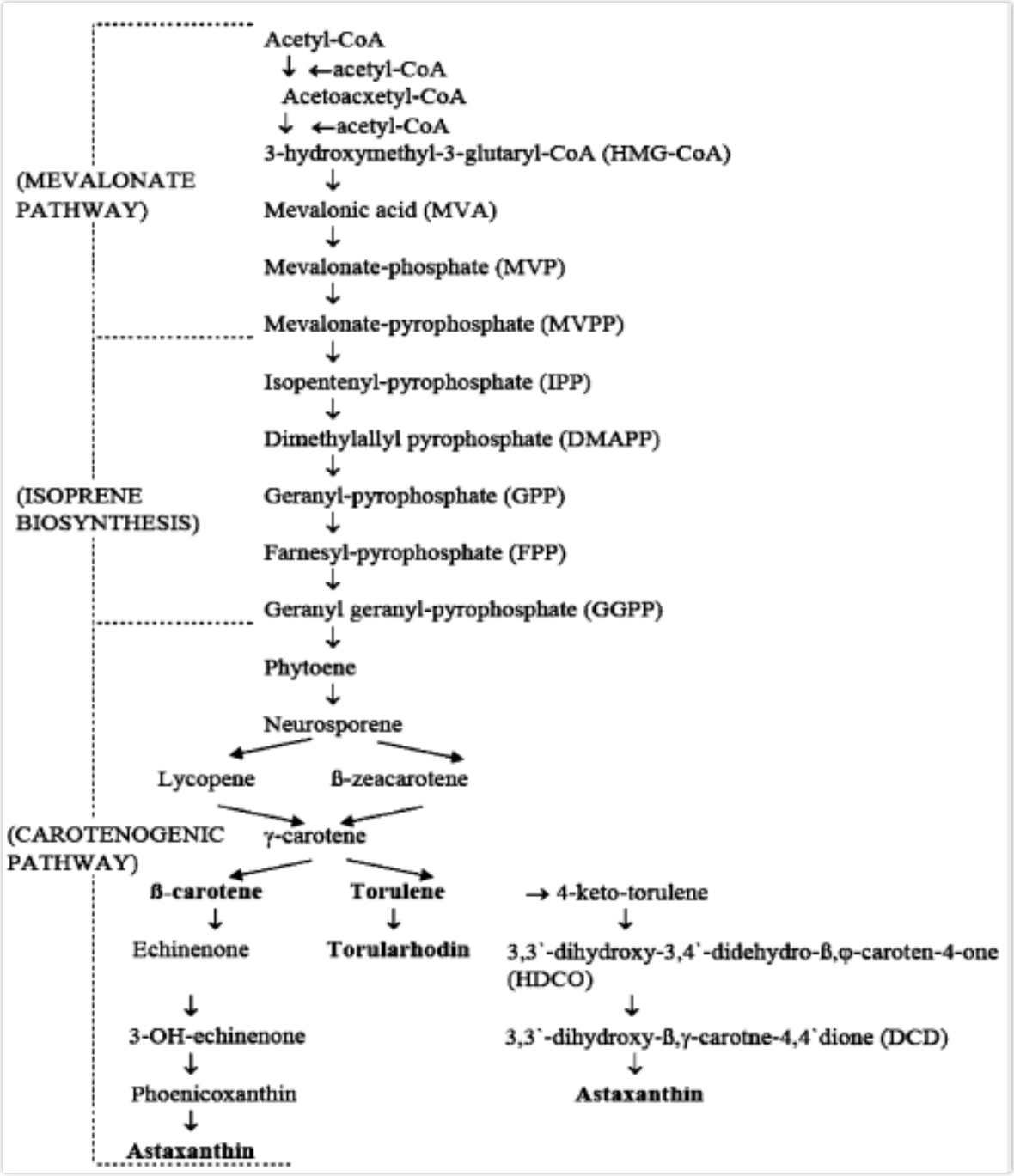
The synthesis of different naturally and commercially important carotenoids (β-carotene, torulene, torularhodin and astaxanthin) by several yeast species, belonging to the genera *Rhodotorula* and *Phaffia,* has led to consider these microorganisms as a potential pigment sources. The red yeast is one of the best potential candidates for biotechnological production (Frengova *et al*., 1994). Perrier *et al.* (1995) reported that *R. glutinis* produce characteristic carotenoids (torulene, torularhodin and β-carotene) in various proportions (Fig.1).

β-carotene: It protect cells from the damaging effects of free radicals, provide a source of vitamin A, enhance the functioning of immune system and helps reproductive system function properly (Pratheeba *et al*., 2014).

Torulene: Torulene is a typical carotenoid found in yeasts. It is a very interesting carotenoid due to its structure, half β-carotene and half lycopene, which is responsible for provitamin A activity and antioxidant property (Maldonade *et al*., 2007).

Torularhodin: It has a very important antoxidant activity and provitamin A. Sakaki *et al*., 2000; Ershov *et al*., 1992; Eugiena *et al*., 1997.

Fig.2. Isoprenoid pathway of carotenoid biosynthesis. (Frengova and Beshkova, 2009).



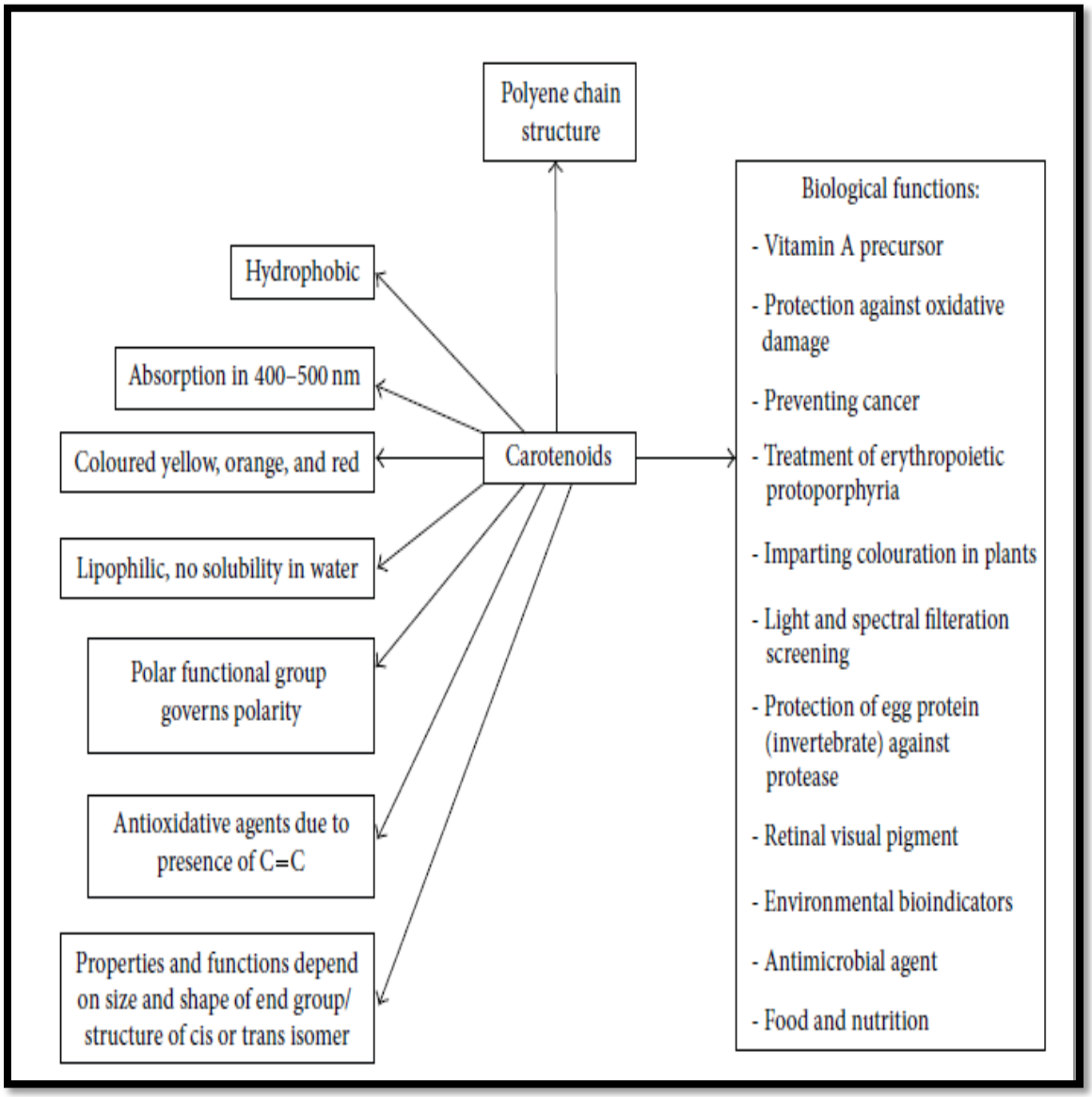
2.5.1. Biosynthesis of carotenoids

Carotenoids are synthesized in nature by plants and many microorganisms. In addition to very few bacterial carotenoids with 30, 45, or 50 carbon atoms, C40-carotenoids represent the majority of the more than 600 known structures. Two groups have been singled out as the most important: the carotenes which are composed of only carbon and hydrogen; and the xanthophylls, which are oxygenated derivatives (Frengova and Beshkova, 2009) (Fig 2).

In the later, oxygen can be present as OH groups, or as oxy-groups or in a combination of both (as in astaxanthin). Hydroxy groups at the ionone ring may be glycosylated or carry a glycoside fatty acid ester moiety. Furthermore, carotenoids with aromatic rings or acyclic structure with different polyene chains and typically 1- methoxy groups can also be found. Typical fungal carotenoids possess 4-keto groups, may be monocyclic, or possess 13 conjugated double bonds. All carotenoids are derived from the isoprenoid or terpenoid pathway. Carotenoids biosynthesis pathway commonly involves three steps: (i) formation of isopentenyl pyrophosphate (IPP), (ii) formation of phytoene and (iii) cyclization and other reactions of lycopene (Armstrong and Hearst, 1996). Before polyprenyl formation begins, one molecule of IPP must be isomerized to DMAPP. Condensation of one molecule of dimethylallyl diphosphate (DMADP) and three molecules of isopentenyl diphosphate (IDP) produces the diterpene geranylgeranyl diphosphate (GGDP) that forms one half of all C40 carotenoids. The head to head condensation of two GGDP molecules results in the first colorless carotenoid, phytoene. Phytoene synthesis is the first committed step in C40-carotenoid biosynthesis Subsequent desaturation reactions lengthen the conjugated double bond system to produce neurosporene or lycopene.

Following desaturation, carotenoid biosynthesis branches into routes for acyclic and cyclic carotenoids. Synthesis of cyclic carotenoids involves cyclization of one or both end groups of lycopene or neurosporene. Typically, beta rings are introduced, but formation of beta rings is common in higher plants and carotenoids with gamma rings are found, for example, in certain fungi. Most cyclic carotenoids contain at least one oxygen

Fig 1: Important physical, chemical, and biological properties of carotenoids (Thakur and Azmi, 2013)



function at one of the ring carbon atoms. Cyclic carotenoids with keto-groups at C4 (C4´) and/or hydroxy groups at C3 (C3´) (e.g. zeaxanthin, astaxanthin, echinenone and lutein) are widespread in microorganisms and plants (Britton *et al*., 1998) (Fig.1).

2.5.2. Screening and identification of potential pigment producing yeast

The molecular and genetic basis for the biosynthesis of any yeast pigment has not yet been examined in detail; thus, genomic screening for pigment producers is not feasible at this premature stage. By contrast, the use of classical tools such as taxonomy, biochemistry and microbial physiology could still be very effective in pre-selecting potential pigment producers.

Prior knowledge of the production of any toxic metabolite by the industrial producer cannot be overlooked. For example, the mycelial food product Quorn™ is produced by *Fusarium venenatum* (Archer, 2000), which also produces a potent cytotoxic metabolite called 4, 15-diacetoxyscirpenol (Miller and Mackenzie, 2000). Such knowledge helps in the hazard analysis and safe design of the production process. In the case of pigments, such knowledge could be used to engineer improved biosynthetic pathways by selectively controlling the production of pigments and uncoupling their formation from the synthesis of toxins, as illustrated in the case of *Monascus ruber*.

Screening for yeast pigments should be based on screening concepts (Maldonade *et al.,* 2007) where a prior knowledge on updated yeast taxonomy and species-specific metabolite profiles can be used to reduce the chances of including pathogenic strains and/or toxin producers from a diverse set of pigment-producing yeast. It would also ensure the correct identification of yeast at the species level to prevent possible mishapes.

2.6. Reactive Oxygen Species (ROS)

Primarily ROS play an important role in the host defense mechanism against microorganisms, but the increased production of ROS is associated with the onset of a variety of diseases including Parkinson's disease (Jenner, 1994), atherosclerosis (Witzum, 1994), pre-mature aging (Orr and Sohal, 1994), inflammation (Sco *et al*., 1995), cancers (Wiseman and Halliwell, 1996) and neurodegeneration (Leboritz *et al*., 1996).

A number of different free radicals and non-radicals are produced during normal aerobic metabolism (Halliwell *et al.,* 1995). A collective term, "reactive oxygen species" (ROS), is used for oxygen-derived species including oxygen bearing free radicals, as well as certain non-radicals. Some non-oxygenated radicals are also generated in biological systems, such as carbon-centered free radicals (e.g. alkyl radical, $R\text{-}H_2C°$) and sulfur-centered radicals (e.g. thiyl radical, R-S°), which are produced by the attack of free radicals on hydrocarbons and the oxidation of glutathione, respectively (Ivanova and Ivanova, 2000). The major ROS have been listed in Table 6.

2.7. Antioxidants

To protect cells and organs from the oxidative stress induced by ROS, living organisms have evolved with an extremely efficient and highly sophisticated protective system, the so-called "antioxidant defensive system". It involves a variety of components, both endogenous and exogenous in origin. These components function interactively and synergistically to neutralize free radicals. A broader definition of an antioxidant is "any substance which, when present at low concentrations compared to those of oxidizable substrates, significantly delays or prevents oxidation of those of substrates". The term oxidizable substrates includes DNA, lipids, proteins and carbohydrates, which are the essential building blocks of a biological system (Halliwell *et al.*, 1995). Oxidative stress occurs as a result of an increase in oxidative metabolism, which produces a number of ROS. To avoid oxidative stress, antioxidants can play an important role conferring beneficial healthy effects (Vaya and Aviram, 2001).

High dietary intake of proven antioxidants can significantly lower the risk of several chronic diseases such as heart diseases, cancers and cataracts. Microbial carotenoids are of considerable interest in nutrition because of their role as antioxidants and potential for preventing or delaying degenerative diseases and for enhancing immune responses in animals and humans (Kirakosyan *et al.*, 2003). Since carotenoids cannot be synthesized by animals, they must be supplied in their diet as colourants, as a source of vitamin A and their potential role as nutraceuticals (Echavarri and Johnson, 2004).

Table 6. Types of reactive oxygen species (ROS)

ROS	Types	Symbol
Radicals	Superoxide	O_2^{*-}
	Hydroxyl	*OH
	Alkoxyl	LO/RO
	Peroxyl	LOO/ROO
	Nitric oxide	NO
	Thiyl radical	R-S
Non-radicals	Hydrogen peroxide	H_2O_2
	Hypochlorous acid	HOCl
	Ozone	O_3
	Singlet oxygen	$^1O_2$
	Peroxyl nitrite	ONOO-
	Lipid peroxide	LOOH

2.7.1. Carotenoids as antioxidants

Epidemiological evidence has suggested that dietary β-carotene and recently lycopene may inhibit certain types of cancer and there are two main theories as to why this might be. The first is its pro-vitamin A activity. The second theory is that carotenoids have the ability to act as chain breaking anti-oxidants and thus protect cells and organisms against photo-oxidation. The ability of carotenoids to quench singlet molecular oxygen is well known (Tinkler *et al*., 1994) and reactions with radical species have also been studied including the prevention of lipid peroxidation (Packer, 1993). Besides cancer prevention, the potential anti-oxidant properties of carotenoids may help to inhibit the onset of other diseases that are believed to be initiated by free radicals. These include atherosclerosis, cataracts, age-related macular degeneration and multiple sclerosis.

Carotenoid pigments, which are abundant in many fruits and vegetables, have been studied lot a number of years because of their diverse roles in photobiology, photochemistry and photomedicine. Singlet oxygen can be generated by electronic energy transfer from the excited state (normally triplet state) of a sensitizer (SENS) to oxygen.

$$SENS \rightarrow {}^1SENS^* \rightarrow * {}^3SENS^*$$

$${}^3SENS^* + {}^3O_2 \rightarrow SENS + {}^1O_2^*$$

In biological systems, sensitisers such as porphyrins, chlorophylls and riboflavin can sensitize ${}^1O_2^*$ production and this can lead to deleterious effects including DNA damage and lipid peroxidation (Piette, 1991). Foote and Denny (1968) first demonstrated that β-carotene could inhibit photo-sensitized oxidation and was therefore an efficient quencher of ${}^1O_2^*$. Farmillo and Wilkinson (1973) showed that electron exchange energy transfer quenching is the principal mechanism of carotenoid photoprotection against ${}^1O_2^*$, although, chemical quenching also occurs leading to destruction of the carotenoid. The carotenoid triplet state (${}^3CAR^*$) is produced via electronic energy transfer.

$${}^1O_2^* + CAR \rightarrow * {}^3O_2 + {}^3CAR^*$$

Once produced ${}^3CAR^*$ can easily return to the ground state dissipating the energy as heat or it can be quenched physically via enhanced intersystem crossing by ${}^3O_2$.

Thus, the carotenoid acts as a catalyst deactivating $^1O_2$*.The ability to quench $^1O_2$* increases with increasing number of conjugated double bonds, reflecting increased exothermicity in the energy transfer (Edge *et al*., 1997). Whilst showing that carotenoids are extremely good quenchers of $^1O_2$* *in vitro,* they are not directly relevant to biological environment and little work has been carried out to test how effectively carotenoids protect cells against $^1O_2$* related damage. Lycopene is the naturally occurring carotenoid that quenches$^1O_2$* most efficiently, but is more susceptible to oxidation than β-carotene and may therefore be used up more quickly.

Unlike the quenching of singlet oxygen which mainly leads to energy dissipation as heat, the reactions of a carotenoid (or any antioxidant) with a free radical will lead to electron transfer or possibly addition reactions. The major fact to note is that the "odd" electron which characterizes a free radical is not lost. Thus for a free radical (R*) reacting with a carotenoid (CAR), reactions such as

$$R^* + CAR\ (H) \rightarrow RH + CAR^*$$

and

$$R^* + CAR \rightarrow R^- + CAR^+$$

are expected, depending on the redox potentials of the species involved. Further more, carbon-centred radicals are known to react readily (by addition), with oxygen, for example $R^* + O_2 \rightarrow RO_2^*$ giving peroxy radicals. Thus, it is expected that antioxidant properties of carotenoids, and other anti-oxidants such as vitamins E and C, may well depend on the oxygen concentrations. The experimental data on chemical parameters are of carotenoid-free radical interactions is limited (Dutta *et al*., 2005). Presently available data have proposed electron transfer, addition reaction and hydrogen abstraction as mechanisms for reactions between carotenoids and a range of free radicals.

2.8. Antimicrobial potential of microbial pigment against human pathogens

Astaxanthin is effective against gram positive and gram negative bacteria when compared with standard chloramphenicol (Ushakumari and Ramanujan, 2013). The antibacterial activity of *Monascus purpureus* was first reported by Wong and Bau (1977). This *Monascus* pigment has antibiotic activities against gram positive

bacteria, but nephrotoxic properties have limited its use as an antibiotic. Pigments and citrinin are secondary metabolites derived from the polyketide pathway (condensation of acetyl-CoA and melonyl-CoA)(Wong and Koehler, 1981a).

Nozaki *et al.* (1991) studied the secondary metabolites of *M. anka,* ankalactone, which they reported as bacteriostatic. Blanc *et al.* (1994) reported the presence of monascidin A in submerged cultures of *M. purpureus* and *M. atiku*, which they identified as citrinin. They also found that addition of crulenine and ethionine could inhibit citrinin and pigment synthesis. Martinkova *et al.* (1999) studied the antibiotic activity of *M. purpureus* extracts containing the orange pigments against various bacteria, fungi and yeast.

Wild strains and mutants of *M. purpureus* were active against *Bacillus*, *Streptococcus* and *Pseudomonas.* Solid-state bioprocessing of soybean by *Monascus* is a biotechnological strategy to produce Monascus-fermented soybeans (MFS) with more beneficial components (Yang *et al.,* 2000). Pyocyanin and pyorubin pigments of *Pseudomonas aeruginosa* showed distinct antibacterial effect against *Citrobacter* sp., a member of the family Enterobacteriace, which causes urinary tract infections, wound infections, and sometimes pneumonia in humans especially in immune compromised persons (Saha *et al*., 2008). Selvameenal *et al*.(2009) reported seven carotenoids, namely, (all-E)-luteoxanthin, (all-E)- neoxanthin, (9Z)-neoxanthin, (all-E)-antheraxanthin, (all- E)-violaxanthin, (9Z)-violaxanthin, and (all-E)-lutein, were isolated from golden delicious apple and showed potent anti-*Helicobacter pylori* activity (CMIC50 = 36 μg/mL). Puspha *et al.* (2009) reported the antimicrobial activity of total red pigment from *M. purpureus* MTCC 410 recorded more antimicrobial activity, which could yield significant information on the scope for *Monascus* pigments substituted or as an adjunct to chemotherapeutic agents. Inhibition of human pathogenic bacteria, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Vibrio cholera*, was observed by endophytic fungal pigment of *Monodictys castaneae* (Visalakchi and Muthumary, 2010).

Neveen (2011) reported that extracellular pigment of *Penicillium purpurogenum* was found to be more significantly effective against all tested microbial species which includes *Epidermophyton floccosum, Microsporum canis*, *Aspergillus niger*, *Candida*

albicans, *E. coli*, *P. aeruginosa*, *S. aureus* and *B. subtilis*. Gunjan and Sanjay kumar (2011) isolated a new red pigment*i.e*.9-(1hydroxyhexyl)-3-(2-hydroxypropyl)-6a-methyl9, 9adihydrofuro[2,3h]isoquinoline 6,8(2H,6aH)-dione from *Monascus purpureus* which showed inhibitory activity against Gram-positive bacteria.

2.9. Optimization of pigment production

Microorganisms accumulate several types of carotenoids as a part of their response to environmental stresses. Aside from the cultural conditions, carotenoid biosynthesis is governed by the level and activity of carotenoid biosynthetic enzymes and the total carbon flux through the synthesizing system. The hyper-production can be achieved by altering either the level or activity of these enzymes or the biosynthetic pathway by using a molecular approach (Sandmann, 2001). To achieve simple and cost effective carotenoid hyper-production, stimulants can be added to carotenogenic microbial culture broth and external cultural conditions can be adjusted correspondingly (Schmidt-Dannern *et al.,* 2006).

Several studies have shown that fermentation broths containing high concentrations of filamentous microorganisms are highly viscous and are characterized by shear-rate-dependent viscosities and by a yield stress (Cho *et al.,* 2002 a).

2.9.1. Factors influencing carotenoid production

Microorganisms accumulate several types of carotenoids as a part of their response to environmental stresses. Aside from the cultural conditions, carotenoid biosynthesis is governed by the level and activity of carotenoid biosynthetic enzymes and the total carbon flux through the synthesizing system. The hyper-production can be achieved by altering either the level or activity of these enzymes, or the biosynthetic pathway by using a molecular approach (Sandmann, 2001). To achieve simple and cost effective carotenoid hyper-production, stimulants can be added to carotenogenic microbial culture broth and external cultural conditions can be adjusted correspondingly (Bhosale, 2004).

2.9.2. Physical factors influencing pigment production

2.9.2.1. Hydrogen ion concentration

Seon-Won *et al.* (1996) reported that enhanced β-carotene production in the strong alkaline condition to be merely due to pellet formation with reduced mass transfer limitations. At extreme pH, delayed lag phase and low specific growth rate were observed. Controlling pH with buffers resulted in several fold increase in cellular carotenoid content and dry cell mass in *P. rhodozyma*. The effect of pH on pigment production by *M. purpureus* 192F using glucose as carbon source was studied in batch fermentor. The highest red pigment was observed at pH 6.5 using glucose-peptone medium (Chen and Johns, 1993). Maximum pigment yields were generally obtained at the initial pH of inoculums at 5.5 to 6.5. *Monascus* pigment production was also negatively affected by the accumulation of pigments and by acidic pH. High pH was also associated with the chemical conversion of the orange pigment to the red pigment.

Cho *et al.* (2002 b) revealed that optimal pH for both mycelia growth and pigment production was 6.0 and it has been reported that many kinds of fungi have more acidic pH optima during submerged culture for pigment production. Mendez *et al.* (2011) evaluated that combined effects of pH and temperature on red pigment production and fungal morphology in a submerged culture of *P. purpurogenum* GH2. The highest production of red pigment (2.46 g l^{-1}) was reached with a pH value of 5.0 and a temperature of 24°C ($p<0.05$).

2.9.2.2. Temperature

Temperature is also important factor affecting the performance of cells and product formation. The effect of temperature depends on the species specificity of the microorganism and often manifests itself in quantity variations of synthesized carotenoid pigment. Temperature changes the carotenogenesis but changing the enzyme concentration (Hayman *et al.,* 1974). *Dunaliella* sp. showed a 7-fold increase in cellular levels of α-carotene when temperature was decreased from 34°C to 17°C. But volumetric level was optimum between 24°C and 29°C. Carotenoid producing thermophilic microbes have rarely been reported in literature. Low temperature was reported to include physiological changes in the unicellular marine cyanobacteria *Synechoccus* sp. (Nakayama *et al*., 1954).

The optimum temperatures for pigment production of *Monascus* were reported as 30°C (Su and Husang, 1977) and 35°C (Border and Kohler, 1980). Martin *et al.* (1993) *Rhodotorula* spp. can be cultivated in wide range of temperatures from 5 to 26°C wherever optimum temperature for *R. rubra* has been reported to be 22°C.

2.9.2.3. Effect of agitation

Carotenogenesis is an aerobic process. The effect of aeration is dependent on the species of the microorganism. The reported optimal values of air flow rate and agitation are in range 0.5 - 1.9 l/min and 180 – 900 rpm for carotenogenesis in yeast *Rhodotorula.* Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product and oxygen (Davoli *et al.,* 2004). If the microorganism requires oxygen, aerating the growth medium is very important for the successful progress of the fermentation. Aeration, by agitation at 200 rpm, significantly reduced cultivation time and enhanced the yield of naphthoquinones (Unagul *et al.,* 2005). Biomass production rapidly increased and reached the stationary phase within 7 days, while it took 21 days when the cultures were grown under static conditions.

2.9.2.4. Effect of light

Carotenogenesis in many organisms is regulated by light. However, the intensity and protocol of illumination varies with the microorganism. The cultivation of red yeast in the dark and under sunlight conditions showed that light was not a limiting factor for growth and pigmentation of *R. glutinis* DFR-PDY (Latha *et al*., 2005). In contrast light stimulated carotenogenesis in many other microorganism such as *P. rhodozyma* where culture exposed to light stimulate more astaxanthin with a redder hue (Johnson and Lewis, 1979). Light frequently stimulates carotenogenesis in fungi which normally form reasonably impressive amounts in dark like *Penicillium oxysporum, Epicoccum nigrum* (Gribanovski and Foppen, 1969), *Neurospora crassa* (Davies, 1976). The aerobic mycelium of wild-type dimorphic zygomycete *Mucor rouxii* accumulated about ten times more β-carotene when grown continuously in the presence of light than corresponding cultures grown in the dark (Mosqueda-Cano and Corona, 1995).

Considerable stimulation is achieved by photoinduction which involves a short simultaneous exposure to oxygen and light followed by a return to darkness. Red light

can induce photoinduction in the presence of redox dyes like methylene blue and toludiene blue. The hydrogen peroxide (10^{-1}-10^{-2}M) and mercuribenzoate (5×10^{-5} M) stimulates photoinduction of carotenogenesis in that they induce synthesis in dark.

Virtually for all organisms light, temperature, etc., are crucial environmental signals for regulating developmental and physiological processes. In fungi, some important secondary metabolites are known to be regulated by blue light. Blue light inhibits mycotoxin production in *Aspergillus flavus*, *A. parasiticus* and *Alternaria alternata* were studied by Lauter (1996). *Monascus purpureus* and *Emericella nidulans* were studied for the production of soluble red pigment and *P. purpurogenum* was studied for the production of yellow pigment (Babitha *et al*., 2008).

Velmurugan *et al.* (2010) investigated the effect of darkness and different color light quality on biomass, extracellular and intracellular pigment yield of five potent pigment producers *Monascus purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides* and *P. purpurogenum*, with different color shades such as red, pink, reddish brown and yellow. Extracellular red pigment produced by *M. purpureus* resulted the maximum production in darkness 36.75 ± 2.1 OD and minimum in open unscreened light condition 5.90 ± 1.1 OD.

2.9.2.5. Incubation time

The sclerotial growth and carotenoid production of *Penicillium* sp.PT95, was studied by Han (1998) and reported the role of incubation time in carotenoid accumulation with the fungus. A positive correlation was observed with the increase in carotenoid content of sclerotia up to 14th day and thereafter a negative impact. Bhosale and Gadre (2001) determined that pigment production in yeast was growth associated. The maximum cell mass concentration (10.2 g/L) obtained on 7th day accompanied by complete utilization of glucose wherever maximum carotenoid yield (3.1 mg /L of culture broth) obtained on 12th day of modified czapek dox broth. Bhosale and Bernstein (2004) reported that the amount of total carotenoid produced by *Flavobacterium multivorum* remained independent of incubation time.

2.9.3. Chemical factors

2.9.3.1. Effect of carbon source

Assimilation of different carbon sources by the red yeast for its growth and pigmentation. During the microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but also as an important energy source (Dunn, 1985). Glucose is an excellent carbon source for growth, which usually interferes with the biosynthesis of many secondary metabolites (Demain, 1986). The production of *Monascus* pigments as food colouring agent by both submerged and agar surface cultures have been studied in the past 10 years (Lin and Demain, 1991). Glucose was reported to be a better carbon source for carotenoid pigment production and accumulation in earlier studies done with *R. glutinis* (Nam and Rhee, 1991) and *P. rhodozyma*. Glucose yielded proportionately higher percentage of β-carotene, whereas the torulene content was considerably higher when fructose was used. Although cellular accumulation of carotenoids (mg/g) in *Rhodotorula* was high with arabinose, rhamnose and xylose, their use in the medium resulted in less growth and relatively low β-carotene proportion. Sucrose yielded a comparable cell mass (g/l), volumetric production (mg/l) and cellular carotenoid accumulation (mg/g), but torulene was the major carotenoid (Bhosale and Gadre, 2001).

Latha *et al*. (2005) investigated the suitable carbon source for pigment production; *R. glutinis* was cultivated in the basal medium containing various carbon sources (2%). Of the 8 carbon sources examined, galactose, sucrose, glucose and xylose were relatively favourable to the cell growth of *R. glutinis* and the pigment production was high in fructose, sucrose and glucose. Aksu and Eren (2007) observed that in molasses, sucrose containing medium carotenoids formation was faster and higher than that of glucose.

Padmapriya (2013) evaluated the suitable carbon source for the red pigment production. *P. purpurogenum* was cultivated in the basal medium containing various carbon sources (2%). Of the 10 carbon sources screened, glucose, fructose, starch and sucrose were relatively favourable to the mycelial growth of *P. purpurogenum* although the pigment production was high. The maximum mycelial growth (3.10 g l^{-1}) and pigment production (670 mg l^{-1}) was achieved in fructose medium.

2.9.3.2. Effect of nitrogen source

The chemical composition and concentration of nitrogen source in medium might also be means of physiological control and regulation of pigment metabolism in microorganisms. Several inorganic and organic nitrogen sources as well as flour extracts and protein hydrolysates have been studied for improvement of carotenoid production. However, it seems that variation in carotene content in yeasts with regard to N-source used in a medium and the rate of pigment production is influenced by the products of catabolism of the nitrogen source rather than being the results of direct stimulation by the nitrogen compound itself (Somashekar and Joseph, 2000).

Han (1998) used six different nitrogen sources to study the effect of carotenoid yield and dry sclerotial weight of *Penicillium* sp. in basal medium. Among sodium nitrate, ammonium nitrate, ammonium chloride, ammonium sulphate, corn meal and urea; urea gave the highest sclerotia growth, but $NaNO_3$ gave the highest pigment content and peak yield of 4120 μg/100g of substrate, which was 1.92 times higher than that of control. Bhosale (2001) reported C: N ratio of 10 enhances β-carotene production and accumulation (mg/l, mg/g) in *Rhodotorula* sp. Yeast extract medium reported maximum carotenogenesis in *R. glutinis* mutant.

Organic nitrogen sources gave rise to higher cell growth compared with inorganic nitrogen sources. Meat peptone, casein peptone, peptone–yeast extract mixture and corn steep powder had a positive effect on pigment production, whereas soy peptone and malt extract strongly inhibited red pigment synthesis. Of all the nitrogen sources tested, meat peptone gave the highest yield for red pigment production (Chen and Johns, 1993). It has been reported that various types of peptone supported greater pigment production in many kinds of pigment-producing fungi (Fang and Cheng, 1993; Kang *et al.,* 1996). A chemically defined medium supporting rapid and intensive red pigment production, using glutamate as nitrogen source was developed and shifted the site of pigment from predominantly cell bound to extracellular.

The effect of nitrogen source on cell growth and pigment production was studied in flask cultures with various nitrogen sources, since nitrogen sources have been found to have a great effect on the quality and quantity of *Monascus* pigments produced.

The optimum nitrogen source was determined to be 1.5 gl^{-1} of monosodium glutamate (MSG) (Lee *et al*., 2001). A stimulatory effect of nitrogen source on red pigment formation from *P. sinclairii* has been reported by Cho *et al*. (2002 b).

Latha *et al*. (2005) reported that among the seven nitrogen sources they selected sodium nitratre gave better carotenoid pigment production which was followed by casein acid hydrolysate, urea and yeast extract gave good results. Some studies showed that *P. rhodozyma* can utilize many kinds of nitrogen sources including ammonium, nitrate and organic nitrogen sources (Du *et al*., 2005). Neveen (2011) reported that peptone had a positive effect on growth and pigment production for all tested species (*Aspergillus nidulans, P. purpurogenum, Phoma herbarum* and *Fusarium moniliforme*). The highest significant extra and intracellular pigments (90% and 77%, respectively) was showed with peptone in *P. purpurogenum*.

2.9.3.3. Effect of trace metals

Bio-elements are one of the important factors affecting pigment production in several microorganisms. Bau and Wong (1979) reported that Mg^{2+} had no influence on the growth of the strain or the yield of the pigment. The pigment would never be produced while Cu^{2+} was added to the broth (An *et al.,* 2001). The presence of Cu^{2+} may inhibit some enzymes which were important to the production of the pigment. Moreover, a critical finding revealed that there was an interaction between pigment and metal ions Fogarty and Tobin (1996).

Lee *et al*. (2001) reported that Fe^{2+} showed the strongest stimulatory effect on red pigment production and some stimulatory effect was also found in Mn^{2+}. The addition of metal ions could influence the yield of quercetin (Liu *et al.,* 2005). The addition of the Zn^{2+} positively increase the yield of quercetin and shorten the time for the appearance of pigment. Danuri (2008) reported that addition of $MgSO_4 \cdot 7H_2O$ (12 mM) increased angkak pigment production by *Monascus purpureus* AKII. Addition of the mineral individually gave significant increase on angkak pigment production by AKII, whereas the addition of minerals mixture give increased pigment production.

2.10. Response surface methodology

Response surface methodology (RSM) is a powerful mathematical model with a collection of statistical techniques wherein, interactions between multiple process variables can be identified with fewer experimental trials (Box and Wilson, 1951). It is widely used to examine and optimize the operational variables for experimental designing, model developing factors and optimization of conditions (Buchanan and Philips, 1990). RSM is a collection of experimental strategies, mathematical methods and statistical inferences increasingly used for optimization steps. The practice of varying one variable at a time does not allow for the evaluation of the combined effects of all the factors involved in the process and demands a time consuming practical oriented processes. These restrictions can be overcome by the simple use of statistical experimental design combined with RSM. This technique evaluates the mutual effects of several factors at different levels and determines a wide region in which the results are valid (Montgomery, 1997). The statistical experiment designs most widely used in the optimization experiments are termed as “response surface designs”. The Central Composite Design (CCD) is the most popular of the many classes of RSM designs due to the following properties:

a. CCD can be run sequentially. It can be naturally partitioned into two subsets of points; the first subset estimates linear and two factor interaction effects while the second subset estimates curvature effects.

b. CCDs are very efficient, providing much information on experimental variable effects and overall experimental error in a minimum number of required runs.

c. CCDs are very flexible. The availability of several varieties of CCDs (Face-centered, rotatable, inscribed) enables their use under different experimental regions of interest and operatability.

Thus, RSM experimental design is an efficient approach to deal with a large number of variables and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation such as enzymes (Hounsa *et al.,* 1996), biomass (Lhomme and Roux, 1991), spores (Yu *et al.,* 1997) and pigment production (Sanjay *et al.,* 2007 a).

Buzzini (2000) optimized carotenoids production from *R. glutinis* 3853 DBVPG yeast in grape juice as carbon source. By this way, the carotenoids yield reached the highest value of 6.9 mg/l and the amount of β-carotene was 1100 μg/l. Sqina *et al*. (2002) optimized carotenoids from *R. glutinis* in a different culturing medium. The medium was supplemented with sugarcane juice as a carbon source in order to gain the highest amount of total carotenoids of 497.2 μg/g dry cell biomass.

Sarada *et al*. (2002) studied the effects of various concentrations of sodium chloride and sodium acetate and the culture age during the period of encystment and astaxanthin accumulation in *Haematococcus* employing RSM. The response functions correlated well ($r > 0.98$, $p < 0.01$) with these variables by second order polynomials consisting of linear, quadratic and interaction terms. The effect of culture age dominated over the sodium acetate and sodium chloride concentrations. Optimum conditions for achieving high astaxanthin content (>1.4 % w/w) and astaxanthin production (13 mg l^{-1}) were found to be with 31- 50 mM sodium acetate and 0.55-0.63 per cent (w/v) of sodium chloride.

There is a need to optimize the fermentation media for efficient utilization of the fermentation technology. Medium optimization by one-factor-at-a-time method involves changing one variable (nutrients, pH, temperature, etc.) while fixing the others at a certain arbitrary levels (Xu *et al.*, 2003). The conventional ‘‘one-factor-at-a-time’’ approach is laborious and time consuming, especially for large number of variables. Moreover, it seldom guarantees the determination of optimal conditions. These limitations of a single factor optimization process can be overcome by using statistical methods. In statistical based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization (Dutta *et al.*, 2004; Xiong *et al.*, 2004).

Plackett-Burman experimental design was used to screen important nutrient parameters influencing the production of red pigments by *Monascus purpureus* MTCC 369 under submerged fermentation. From standard Plackett-Burman data analysis, it was confirmed that, NH_4Cl, NaCl, KH_2PO_4, $MgSO_4.7H_2O$ and $MnSO_4.H_2O$ had contributed to a large extent. Dextrose, $CaCl_2.2H_2O$ and $FeSO_4.7H_2O$ had little impact, while, $(NH_4)_2.SO_4$ contributes moderately for red pigment production by *M. purpureus* MTCC 369 under submerged fermentation (Ahmad *et al*., 2009).

Zhou *et al.* (2009) used RSM to optimize the yellow pigment production by *M. anka* mutant. It was found that all nutrient variables were positively significant factors and the proposed model equation illustrates the interaction between two factors. *M. anka* mutant MYM produced 88.14 OD units of yellow pigment in optimized fermentation media, which is much higher than that of yellow pigment produced by *Monascus* sp.

Venil and Lakshmanaperumalsamy (2009) were applied combination of Plackett – Burman design (PBD) and Box – Behnken design (BBD) for optimization of different factors for prodigiosin production by *Serratia marcescens* SWML08. The media formulations were optimized having the factors such as incubation temperature 30°C, $(NH_4)_2PO_4$ 6 g l^{-1} and trace salts 0.6 g l^{-1}. The maximum experimental response for prodigiosin production was 1.397 g l^{-1} whereas the predicted value was 1.394 g l^{-1}. The high correlation between the predicted and observed values indicated the validity of the statistical design.

Subhagar *et al.* (2010) used RSM for optimization of anticholesterolemic drug lovastatin production by the red mold *M. purpureus*. In this study, the statistical analysis of Plackett-Burman experimental results showed that the medium components glucose, peptone, $MnSO_4 \cdot H_2O$, NaCl and NH_4Cl as the significant components influencing the lovastatin production. The most significant medium components, glucose, peptone and $MnSO_4 \cdot H_2O$ which have confidence level of more than 95 per cent were further optimized using a full factorial central composite design of the response surface methodology.

Ferdes *et al*. (2012) tested *Rhodotorula* strain and determined the highest pigments yield in the case of fructose medium; The total carotenoid pigments was 690 μg/L and the torularhodin concentration was 407μg/L, followed by the concentrations accumulated when glucose and sucrose were used as the carbon source in the medium. Hailei *et al*. (2011) evaluated the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis* by employing factorial design and response surface methodology. The results indicated that glucose and ratio of *C. tropicalis* to strain HSD07B (w/w) in the inoculum were important factors influencing pigment production.

2.11. Identification of compounds

2.11.1. Separation of pigments through chromatography

Teng and Feldheim (1998) revealed that two dimensional TLC could be used to separate the pigments and was found that the pigment patterns in these solvent extracts were different. The solvents, such as with methanol and acetone two yellow and two orange pigments were extracted but with n-hexane or petroleum benzene more than two orange pigments was extracted than the yellow pigments. The methanolic extract of *Penicillium* sp. was separated in silica gel TLC plate and five pigments (orange, violet, red, yellow and red brown) were collected by Ogihara *et al.* (2000a).Vidyalakshmi *et al.* (2009a) produced the MFR (*Monascus* fermented rice) and analysed for its pigment composition, using HP-TLC and colour bands were distinguished based on their Rf values. Red (Rf 0.2), orange (Rf 0.7) and yellow (Rf 0.8) bands were found visible. The study revealed the presence of orange and yellow pigments to be maximum than red pigments. The acetone extract of *R. glutinis* DFR-PDY was separated in silica gel TLC plate and three fractions of crude pigment (orange, red and yellow) were collected by Latha and Jeevaratnam (2010).

2.12. Purification of carotenoid pigment

2.12.1. High-Performance liquid chromatography (HPLC) studies

Bhosale (2001) reported HPLC analysis of the main carotenoids from *Rhodotorula* strains *viz.* torularhodin, torulene and β-carotene, eluted at 2.29, 3.2 and 3.9 min, the absorption maxima were found as 492, 485, 457 nm respectively. The mobile phase comprising acetonitrile: propanol: ethyl acetate (40:40:20) is used in HPLC separation of carotenoids of *Rhodotorula* and the triplicate chromatography runs gave excellent reproducibility.

HPLC analysis of carotenoid is a rather interesting case since elution systems seem to be numerous though not so dramatically different to each other. This is due to the fact that natural extracts usually contain a wide array of carotenoids differing in polarity. For this reason, the systems found in literature most of the times tackle specific needs, *i.e.* complete characterization of the carotenoids found in a test mixture (Echvarri and Johnson, 2004).

An RP-HPLC method has been developed for the analysis of hydroxylated pulvinic acid derivatives which are responsible for the pigmentation of fruit-bodies belonging to the order Boletales. Reversed-phase Variegatic, xerocomic and atromentic acid as well as variegatorubin were detected and separated in methanolic extracts of *Boletus permagnificus* and *Xerocomus parasiticus* and the pigment profile of these species were compared. The identity of the pigments was confirmed by means of LC–atmospheric pressure chemical ionization (APCI) mass spectrometry (Davoli and Weber, 2002). The *Epicoccum nigrum* also produced the oxopolene compound.

Mantzouridou and Tsimidou (2007) gave evidences of a satisfactory separation of three peaks at 453nm in an attempt to locate carotenoids of *Blakeslea trispora* in RP-HPLC with a UV-VIS diode array multiple-wave-length detector. Retention times for peaks are less than 10 minutes. Peak identification was done by comparing retention time to that of standards, confirmed by spiking and comparison of spectral data.

2.13. Toxicological aspects of microbial pigments

Consumer awareness towards many natural products leads to explore new and viable alternatives to the synthetics. All natural colours are not suitable for consumption for human beings. Many of them are certified as unsuitable for consumption because they are carcinogenic in nature.

Safety assessment of β-carotene derived from *B. trispora* has hitherto been limited for genotoxicity and sub acute (4-week) toxicity study (JECFA, 1996). Since it is a potential substitute for synthetic β-carotene, consumers in general prefer natural products; the sub chronic (90-day) toxicity study was performed with administration in the diet to both sexes of 44 rats to allow comparison with the results for synthetic β-carotene. The results showed no adverse effects in the biological system (Woutersen *et al.*, 1999).

The toxicological evaluation of *R. gracilis* CFR-1, in freeze-dried form has been studied to assess its safety as a source of natural colourant and nutritional additive in experimental animals. Acute and sub acute studies on freeze-dried *R. gracilis* were conducted on both sexes of albino rats. Acute doses of *R. gracilis* at 0.5–6.0 g kg^{-1} of body weight (w/w) did not show any symptoms of toxicity for mortality of the adult rats. Feeding of low concentrations of *R. gracilis* at 0.1–2.0 per cent level (w/w) for 14 weeks

did not induce any significant change in food intake and gain in body weight of the experimental rats compared to control animals. There were no significant differences in the absolute and relative weights of vital organs, hematological parameters, macroscopic and microscopic changes in vital organs and serum enzyme levels between the experimental and control groups. The fecal matter of *R. gracilis*-fed rats did not show any viable cells. The results clearly showed that acute and sub acute oral feeding of freeze-dried whole cells of *R. gracilis* for 14 weeks did not produce any toxic effects on male and female rats (Naidu *et al.,* 1999 a,b).

The safety of the natural red colourant extracted from edible algae *Dunaliella* was evaluated in animal model systems. The pigment did not induce any adverse effects in albino rats. The terminal autopsy, vital organs of treated rat groups did not show any biochemical and morphological alteration as compared to untreated animals. On the other hand, *Dunaliella* extract has been shown to have important health benefits as cholesterol, triglycerides and total lipids lowering effect and other additional physiological benefits for human health (Abd El-Baky *et al.,* 2004).

Sanjay *et al.* (2007 b) evaluated the safety of pigment containing *Aspergillus carbonarious* biomass by conducting acute and subacute toxicity studies in both sexes of albino rats. This study revealed the fact that the feeding of fungal biomass did not show any mortality of rats and there is no significant differences in food intake, body weight, hematological parameters (WBC, RBC, Hb etc.), organ weight and serum enzymes (LDH, ALP, ASAT, ALAT and cholesterol).

Mohan Kumari *et al*. (2009) have assessed the safety of *M. purpureus* MTCC 410-fermented rice (Red Mold Rice (RMR)) by conducting toxicological studies on both sexes of albino rats. Dietary feeding of RMR at 2.0 per cent, 4.0 per cent, 8.0 per cent and 12.0 per cent level (w/w) for 14 weeks did not produce any significant changes in food intake or gain in body weight of the experimental rats compared to control rats. The results showed that toxicity studies with RMR of *M. purpureus* did not cause any toxic effects in albino rats.

Poorniammal *et al*. (2011) evaluated the toxicity of yellow pigment from *Thermomyces* sp. in albino mice. Oral feeding of yellow pigment from *Thermomyces* sp.

at 0.25 – 2.0 per cent level (w/w) for 28 days did not produce any significant changes in body weight gain of experimental mice compared to control mice. There was no significant differences in relative weight of vital organs, hematological parameters and histopathological analysis between experimental and control groups.

Latha and Jeevratnam (2012) reported that sub acute oral feeding of red yeast pigment of *R. glutinis* DFR-PDY for 13 weeks did not produce any toxic effect in male and female rats. Further carotenoid pigment from *R. glutinis* DFR-PDY as a colouring substance may have beneficial effects.

Padmapriya (2013) evaluated the toxicity of red and reddish brown pigment from *P. purpurogenum* and *Chetomium aureum* in albino mice. Oral feeding of these two pigments at 2.0 – 4.0 per cent level (w/w) for 28 days did not produce any significant changes in body weight gain of experimental mice compared to control mice. There was no significant differences in relative weight of vital organs, hematological parameters and histopathological analysis between experimental and control groups

2.14. Application of carotenoid pigment

Food industry: Yeast carotenoid pigments are one of the leading food colourants in the world. Carotenoids has been applied to a range of food and beverage products to improve their appearance to customers, including items such as margarine, cheese, fruit juices, baked goods , dairy products, canned goods, confectionary and health condiments. Carotene is used in various pet foods as both a colourant and a precursor to viamin A. Carotenoid pigment can be applied to an array of animal foods designed for pets, including dogs, cats, fish and birds. The antioxidant and precursory vitamin A properties increase the appeal and application of carotenoid pigment in pet foods.

Due to their lipophilic character and poor solubility characteristics special formulations had to be developed for the use of carotenoids as food colourants. Twenty to thirty per cent oily suspensions of microcrystals, particularly of β-carotene, are used for the colouring of margarine, fats and oils, shortenings and other fat containing food, galenic and cosmetic products. Water-dispersable forms, which contain 10 per cent of the pure carotenoid, serve for the colouring of water-base foods such as beverages, particularly carbonated orange drinks, candies, ice-cream, jellies, etc. Similar

preparations of apocarotenoids and of canthaxanthin find more and more use as feed additives for improving and standardizing the colour of egg yolk and poultry meat (Pratheeba *et al*., 2014). The colour range of aqueous solutions obtainable with carotenoids varies from a greenish yellow to a deep bluish red. The colour of the oily solutions is in most cases identical with the one obtained in organic solvents while the corresponding aqueous solutions can differ considerably from it (Gordon and Bauernfeind, 2000).

Milk and yogurt were coloured with *Monascus* pigment and it resembled like strawberry milk. The products were stable for more than a month (Li *et al.*, 2000). Carotenoids, mainly β-carotene, astaxanthin, α-carotene, fucoxanthin, halocynthaxanthin and peridinin, are used as food colorants owing to their antioxidant nature and provitamin A activity. Carotenoids are usually fortified in the food preparations (mainly in baby and infant food) to improve their vitamin content. Applicability of microbial pigment extracted from *R. glutinis* DFR-PDY for colouring foodstuff was studied by adding to puffed products like popcorn, biscuit creams, cake icing and ice creams (Latha and Jeevaratnam, 2010).

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 General

3.1.1. Location

The laboratory experiments were conducted at the laboratories of Dept. of Agrl. Microbiology, Centre of Advanced Studies in Agricultural Microbiology, Agriculture College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India 641003.

3.1.2. Glasswares

The glasswares of "Borosil" grade was used for all the experiments. They were cleaned by soaking in chromic acid solution (100 g potassium dichromate dissolved in one l of water with 500 ml of concentrated sulphuric acid) for 2 h and finally washed with water. After thorough washing the glasswares were rinsed with distilled water and sterilized before use.

3.1.3. Chemicals used

All the chemicals used were of Analytical Reagent (AR) and HPLC grade obtained from M/s Himedia, M/s Merck and M/s Sigma, India and other leading scientific companies.

3.1.4. Sterilization of glass wares

All the glass wares were sterilized in hot air oven at 180ºC for three hours. All the growth media, broth and water blanks were sterilized in an autoclave at 15 lbs pressure for 20 min. Vitamin solutions, growth factors and antibiotics were sterilized using 0.22 µm nitrocellulose membrane filter. Isolation, purification, inoculation and other microbiological works were carried out in laminar airflow chamber (M/s Klenzoids, India).

3.1.5. Composition of media, buffer and solutions

The composition of different culture media and reagents used in this study were presented in Appendix I and II.

3.2. Sample Collection

Soil samples for the isolation of pigmented microorganisms were collected from different locations *viz.,* Western Ghats and Shervaroy hills ranges such as Manjolai forest, Kodai hills, Marudhamalai, Ooty, Yercaud, Mettupalayam and Parambikulam, Tiger Reserve.

Vegetables and fruits *viz.,* Tomato, carrot, sapota, grapes, pomegranate and sugarcane juice and other substances like curd and rice flour were purchased from nearby shops located in Coimbatore. Leaf samples from sugarcane, maize, rice, cabbage and carrot were also collected from Coimbatore and Ooty. The samples thus collected were packed in sterile high density polythene bags and stored under 4ºC refrigerated conditions until further use.

3.2.1. Isolation and screening of carotenoid pigment producing yeast

Pigment producing microbes particularly yeast from flowers, fruits and vegetables were isolated. The samples were washed with 100 ml sterile water prior to isolation of microorganisms. One gram of the samples from each source was inoculated into 5 ml sterile Yeast Extract Malt Extract (YM) broth (Appendix I) in test tubes. The tubes were incubated at 28±1ºC for 48 h (Gupta *et al*., 1994).

After incubation, yeast cultures were isolated from the YM broth by dilution plate technique (Pelczar and Reid, 1958). One ml of culture grown in YM broth was taken and serially diluted upto 10^{-6} dilution in sterile water and one ml of 10^{-5} diluted suspension was spread over YM (Appendix I) agar plates and incubated at 28±1ºC until pigmented yeast colonies appears. Single colonies of yeast isolates were inoculated in 250 ml flask containing 100 ml of the YM growth medium. Screening of isolates were based on the presence of carotenoid pigment production and quantification of total carotenoid yield in yeast cultures.

3.2.2. Purification of yeast isolates

Morphologically distinct, single, well-separated colonies growing on the plates were picked and purified by cross streaking on the YM agar plates and incubated at 28±1ºC for 48 h (Gupta *et al*., 1994). Purification was repeated until to get morphologically identical distinct colonies.

3.2.3. Maintenance of yeast isolates

A purified single colony was selected and streaked on YM agar slants and incubated at 28±1ºC for 48 h. After the incubation the slants were stored inside the refrigerator (M/s. LG, India) at 4ºC and sub cultured at regular intervals in order to maintain the viability.

3.3. Characterization of isolated yeast cultures

The isolated yeast cultures were identified based on their cell morphology, colony characters and biochemical characters (Kreger, 1984; Mesa *et al*., 1999). The yeast isolates were activated by inoculating each strain in a tube containing 25 ml of YM broth (Appendix I) and incubated at 28±1ºC for 24 h. After 24 h, physiologically active cells were used as inoculum for morphological and biochemical characterization.

3.3.1. Reference strains

The isolated yeast cultures were compared with strandard strain *Rhodotorula mucilaginosa* MTCC 9316 obtained from MTCC, Chandigarh.

3.3.2. Morphological characteristics of the isolated yeast culture

The yeast isolates were identified based on the colony characters *viz*., colony shape, appearance, budding pattern and pigmentation.

3.3.2.1. Cell Morphology

To study the morphological characters, 24 h old yeast cultures were inoculated at one per cent level in 100 ml of sterile glucose YM broth in 250 ml conical flask and incubated at 28±1ºC for 48 h.

3.3.2.2. Wet mounts

Direct mounts were made in glass slides order to study the yeast morphology using light microscope (M/s. Novex, Holland) and to determine the purity of the isolates.

3.3.2.3. Ascospore formation

Ascospore formation was studied using restricted growth medium (Appendix I; Herman, 1971). All the isolates were first brought to a state of active growth using

pre-sporulation medium (Appendix I). The sporulation medium was inoculated with actively growing yeast isolates and incubated at 28±1ºC for 72 h and examined under microscope to examine the sporulation in the yeast cultures. In the absence of the sporulation the cultures were incubated further for a known period and sporulation re-examined. Ascospore formation was verified by spore staining (Kreger, 1984). Matured ascospores appeared as bluish green colour when stained, wherever the vegetative cells appeared red.

3.4. Biochemical characterization of the yeast isolates

After the determination of the morphological characteristics, experiments were carried out to verify the biochemical characters.

3.4.1. Fermentation of carbohydrates

The fermentation of five carbohydrates (D-glucose, sucrose, maltose, D-galactose and lactose) by the yeast isolates were studied using yeast nitrogen broth (Appendix I). All sugar solutions were filter sterilized and 0.5 ml of sugar solutions was added to 5 ml of yeast nitrogen broth in test tubes and inoculated with 0.5 ml of 48 h old active yeast cultures. Yeast inoculated, yeast nitrogen broth without carbon source served as control. The tubes were incubated at 28ºC for 72 h and regularly shaken and observed for the accumulation of gas inside the Durham's tube. Depending on the time required for the development of visible amounts of gas and detectable turbidity, the fermentation was rated as positive and negative (Bowman and Ahearan, 1975).

3.4.2. Acid production from glucose

The production of acid by the yeast cultures was documented by inoculating the cultures in the media containing glucose-chalk ($CaCO_3$) agar (Appendix I). Incubation was carried out at 28ºC for seven days. The tubes were observed for the colour change in the glucose chalk agar from red to yellow due to the production of acids (Rij, 1984).

3.4.3. Urease test

Urease test was performed for the production of ammonia from urea on Christensen's Urea Agar (Appendix I) containing the pH indicator (phenol red). The

cultures were inoculated and incubated at 28±1ºC for 24 h. The reaction was recorded as positive with the appearance of deep pink colour (Christensen, 1946).

3.4.4. Tolerance to one per cent of acetic acid

The medium was sterilized and cooled down in room temperature (Sand and Pitt, 1974). Before pouring the medium, into plates 1 ml of glacial acetic acid was added. The plates were inoculated with 1 ml of diluted cell suspensions (2×10^6 cells ml^{-1}). Incubation was at 28±1ºC for 3 to 6 days. The plates were observed for visible growth of cultures.

3.5. Molecular identification of yeast isolates

The efficient carotenoid producing yeast strains were further identified by 18S rRNA sequencing. The selected yeast strains were grown in 5 ml YM broth for 24 h in a shaker incubator (M/s. Lark, India) at 120 rpm. Yeast genomic DNA was extracted from the culture by DNA extraction kit. Polymerase Chain Reaction (PCR) amplification of the Internal Transcribed Spacer (ITS) region was carried out in 50 µl reaction mixtures containing 20 ng DNA, 100 ng of each primer (Forward primer ITS 1, 5' TCCGTAGGTGAACCTGCGG -3' and Reverse primer ITS 4, 5' TCCTCCGCTTATTGATATGC -3') (Kurtzman and Robnett, 1998, 2003), 5 µl of 10× Taq buffer A, 1µl of dNTP mixture (each dNTP at 2.5 mM), 3 U Taq DNA polymerase (Yaazh xenomics, India) and deionized water to make up the volume to 50 µl. PCR amplification was performed in thermocycler (Eppendorf Master Cycler, German). The thermal cycling programme consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR amplicon (3 µl) were subjected to electrophoresis on 1.5 % (w/v) agarose gels along with StepUpTM 500bp DNA ladder (M/s. Fermentas, USA). Gels were stained with ethidium bromide and visualized under UV light. Amplified products were purified and sequences were determined using the sequencer. Phylogenetic analysis of the ITS rDNA sequences was carried out to identify the nearest phylogenetic neighbours, each partial sequences of 18S rRNA ITS genes from yeast strains YP 17 and YP 37 was compared to sequences in the NCBI GenBank database using blast homology search tool, (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997). To produce phylogenetic trees, sequences were aligned using

CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994). Phylogenetic tree was constructed by Neighbour joining method using MEGA software (Version 5.1 http://www.megasoftware.net) .

3.6. Extraction of intracellular carotenoid pigment

The yeast cultures were inoculated onto yeast malt extract broth and incubated at 28±1ºC for 5 days. A known amount (500mg) of freeze-dried red yeast was hydrolyzed with 1 ml of 1N hydrochloric acid in water bath at 70°C for one and half hours. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone until the entire colour was leached out from the cells. Acetone extracts were transferred to light petroleum (20ml) at (40- 60°C) in a separating funnel and washed thrice with distilled water. The absorbance of the light petroleum phase was documented at 474 nm (λ_{max}) and the concentration of carotenoids was determined using the absorption coefficient as $A^{1\%}_{1\ cm} = 1600$. Carotenoid yield is reported on the basis of cell mass (µg g^{-1} dried cell weight) (Latha *et al*., 2005). The total carotenoid was calculated by using the formula

Total carotenoid (µg g^{-1} of yeast) = (ml of petroleum ether) (A474) (100) / (16) (yeast dry weight)

3.6.1. Estimation of yeast dry weight

Yeast biomass was separated from the liquid medium by centrifuged at 6000 rpm for 15 min at 28°C and rinsed twice with double distilled water, and then dried at 80°C overnight to obtain a constant dry weight (Simova *et al.,* 2003).

3.7. Effect of different medium on carotenoid pigment production by yeast

The yeast cultures were grown in different media *viz.*, czapek dox, malt extract, sabouraud dextrose, glucose yeast peptone, czapek yeast, potato dextrose, yeast malt extract and yeast extract dextrose broths for the determination of carotenoid pigment production. One ml of the 24 h old inoculum was aseptically transferred to 100 ml of broth (pre sterilized at 15 lb for 20 min in autoclave). Production of pigment was quantified by following the same procedure as the extraction of pigment yield.

3.8. Antioxidant activity of yeast pigments

3.8.1. Preparation of extracts

Five carotenoid pigment producing yeast isolates along with standard strain were selected for the antioxidant assays. The acetone pigment extracts used for analyses of antioxidant components to a concentration of 1mg ml^{-1} and were then diluted to 20, 60, 80 and 100 µg ml^{-1} for further uses.

3.8.1.1. Ferric Reducing Antioxidant Power (FRAP)

Total antioxidant activity was measured by FRAP assay of Benzie and Strain (1996). FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stiochiometric excess.

FRAP assay was performed as detailed below using a spectrophotometer (M/s Varian, India). Freshly prepared FRAP reagent (redox indicator) 300 µl was warmed to 37°C to which 40 µl distilled water was added and served as blank. In another cuvette, same amount of FRAP reagent was taken and added with 10 µl sample extract and 30 µl of water. Hence, the final dilution of the sample in the reaction mixture was 1/34. An absorbance reading at 593 nm was taken from one minute till completion of reaction *i.e.*, upto 8 min (Benzie and Strain, 1996). The change in absorbance between initial and final readings were noted for each sample and compared with ascorbic acid standard.

3.8.1.2. Lipid peroxidation activity

Antioxidant activity was determined by the conjugated diene method (Lingnert *et al.*, 1979). Each extract (0.5- 10 mg ml^{-1} 100 µl), in distilled water was mixed with 2 ml of 10 mM^{-1} linoleic acid emulsion (pH 6.6) in test tubes and placed in darkness at 37°C to accelerate oxidation. After incubation for 0 or 15 h, 0.1 ml of each tube was mixed with 7 ml of 800 ml l^{-1} acetone in deionised water and the absorbance of the mixture was measured at 234 nm, against a blank in a spectrophotometer (M/s Varian, India). A value of 100 per cent indicates the strongest antioxidant activity.

Inhibition percentage (%): $\left(\frac{C - S}{S}\right)$ x 100

C – control consists of solvent and the reagent solution

S - pigment extract and the reagent solution

3.8.1.3. Determination of diphenyl-2-picrylhydrazyl (DPPH) scavenging activity

Free radical scavenging activities of the extracts were assayed using stable diphenyl-2-picrylhydrazyl (DPPH), following a standard method (Blois, 1958). The hydrogen atom or electron donating ability of the extracts were measured from bleaching of purple coloured (EtOH) solution of DPPH. One ml of pigment extract aliquot and 3 ml of 0.1mM DPPH solution were mixed. After 30 min incubation at room temperature the absorbance was read against a blank at 517 nm in a spectrophotometer (M/s Varian, India).

Inhibition percentage (%): $\left(\frac{C - S}{S}\right)$ x 100

3.8.1.4. Reducing power assay

The reducing power was determined according to the method described by Oyaizu (1986). The extracts (0.5–10 µg ml^{-1}) in acetone (2.5 ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 10 mg ml^{-1} potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. After incubation 2.5 ml of 100 mg ml^{-1} trichloroacetic acid was added and the mixture was centrifuged at 2000 rpm for10 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1ml of 1 mg ml^{-1} ferric chloride and the absorbance was measured at 700 nm against a blank in a spectrophotometer (Ms Varian, India). Higher absorbance value indicates higher reducing power. BHA was used for comparison.

3.8.1.5. Hydroxyl radical scavenging activity

The assay was performed according to the method described by Lloyd *et al.* (1997). Various concentrations of extracts 600 µl in distilled water were taken in different test tubes. Iron-EDTA solution (0.8 ml of 0.021 per cent ferrous ammonium sulphate and 0.03 per cent ethylene diamine tetraacetic acid and 0.8 ml of 0.88 per cent dimethyl sulphoxide (DMSO), 0.1 mol l^{-1} phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.4 ml of 0.26 per cent ascorbic acid. Test tubes were capped tightly and heated in a water bath at 80–90ºC for 15 min. The reaction was terminated by the addition of 1 mL of 17.5 per cent (w/v) ice-cold trichloroacetic acid.

Three ml of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and volume was made upto 1000 ml using distilled water) was added to all of the tubes and left at room temperature for 15 min for colour development. The intensity of the yellow colour was measured spectrophotometrically at 412 nm against reagent blank.

Inhibition percentage (%): $\left(\frac{C - S}{S}\right)$ x 100

3.8.1.6. Nitric oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions measured by the Griess reaction (Green *et al.*, 1982; Marcocci *et al.*, 1994).Two ml of each extract was added in to 3 ml of 10 mM sodium nitroprusside in phosphate buffer and the reference compound in different concentrations (20, 60, 80 and 100 µg ml^{-1}). The solutions were then incubated at 25ºC for 60 min. The similar procedure was repeated with acetone as a blank which served as control. To 5 ml of the incubated sample, 5 ml Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured using spectrophotometer (M/s Varian, India) at 546 nm. All tests were performed in triplicate. Per cent inhibition of the nitric oxide generated was measured by comparing the absorbance values of control and test preparations.

Inhibition percentage (%): $\left(\frac{C - S}{S}\right)$ x 100

3.8.1.7. Chelating ability on ferrous ions

Chelating ability was determined according to the method of Shimada *et al.* (1992). To 2 ml of the mixture consisting of 30 mM hexamine, 30 mM potassium chloride and 9 mM ferrous sulphate were added each extract (0.5–10 mg ml^{-1}) in acetone (2 ml) and 200 µl of 1 mM tetramethyl murexide. After 3 min at room temperature, the absorbance of the mixture was determined at 485 nm against a blank.

Inhibition percentage (%): $\left(\frac{C - S}{S}\right)$ x 100

3.9. Antimicrobial activity of fungal pigments against human pathogens

3.9.1. Culture collection

3.9.1.1. Human pathogens

The human pathogens (bacteria) used in the study were obtained from the Microbiological Laboratory and KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India. The following six bacterial pathogens (four Gram positive bacteria and two Gram negative bacteria) were used in this study.

Bacterial pathogens

Gram positive bacteria

- *Enterococcus* sp. (Urinary tract infections, bacteremia *etc*.,)
- *Staphylococcus aureus* (Skin infections, pneumonia, sepsis, toxic shock syndrome *etc*.,)
- *Streptococcus faecalis* (Meningitis, bacterial pneumonia, endocarditis, erysipelas *etc*.,)
- *Bacillus subtilis* (Food poisoning)

Gram negative bacteria

- *Escherichia coli* (Gastrointestinal infection, urinary tract infection, neonatal meningitis)
- *Pseudomonas aeruginosa* (Inflammation, sepsis, organ failure)

3.9.2. Maintenance of the human pathogens

The human pathogens used for the study were maintained in respective slants (Appendix I) under refrigerated condition (4°C). Extra precautionary measures were taken to preserve these cultures and to avoid cross contaminations. The cultures were frequently sub cultured in fresh slants and stored for further studies.

3.9.3. Preparation of test controls

3.9.3.1. Negative control

Hundred per cent acetone without the test compound was used as the negative control.

3.9.3.2. Positive control

The antibacterial agent (chloramphenicol) at a concentration of 1 mg ml^{-1} was used as the positive control.

3.9.4. Determination of antibacterial activity

Bacterial strains were grown in nutrient broth at 37°C for 18-24 h with a load of 10^8- 10^9 CFU ml^{-1}. Culture (100µl) was spread on nutrient agar plate by spread plate method. Using sterile cork borer, a well was formed and impregnated with 100 µl of acetone extract of crude pigment. The plates were incubated at 37ºC for 24 h. At the end of the incubation period, the susceptibility of the test organism was determined by measuring the zone of inhibition around the well (Iqbal *et al.*, 1998).

3.9.5. Determination of minimum inhibitory concentration (MIC)

The MIC was performed to test the antimicrobial activity of the acetone extracts of carotenoid pigments using tube dilution method (Claeys *et al*., 1988). The MIC was defined as the lowest concentration of extracts at which tested pathogens did not show any growth. This test was performed at four concentrations of the pigment extract (10.0, 1.0, 0.1 and 0.01 mg ml^{-1}).

Twenty-four hour old log phase bacterial culture of each organism was used for the study. Serial dilution was done up to 10^{-4} dilution of each organism. Four number of sterilized micro centrifuge tubes (Eppendorf) were taken to which 900 µl of 10^{-4} diluted pathogenic test organism was added. To the first tube 100µl of culture extract was added and serially diluted. The 4 tubes corresponding to 4 concentrations of the culture extract was obtained (10 mg ml^{-1}, 1 mg ml^{-1}, 0.1 mg ml^{-1} and 0.01 mg ml^{-1}). Likewise, a set of micro centrifuge tubes were prepared for each organism for each test sample. Simultaneously positive and negative controls were also kept for the experiment. Similar to the agar well diffusion method, the petriplates were divided into 4 equal quadrats. After incubation of the micro centrifuge tubes for an hour, 50 µl from each of the tubes were spotted on the petri plates. The plates were then covered and incubated for 24 h. The growth of the organism for each dilution was observed and thus the MIC of the carotenoid pigment extract was calculated.

3.10. Optimization of physical parameters using one-factor-at-a-time related to cell dry weight and carotenoid pigment production

The respective media for carotenoid pigment was already standardized. Yeast extract dextrose broth was used to optimize the various process parameters.

3.10.1. Effect of pH

The effect of pH on the growth and pigment production of the yeast was studied. The respective broth for each pigment was prepared and the pH was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 by 0.5 N HCl or 0.5 N NaOH. Hundred ml of the medium was dispensed in 250 ml Erlenmeyer flasks and sterilized by autoclaving. The broth was inoculated with respective pigment producing yeast cultures. The flasks were incubated for 6 days at 25°C at rotary shaker. After the incubation, the pigment yield was measured as mentioned in section 3.5. Three replicates were maintained for each pH.

3.10.2. Effect of temperature

The effect of temperature on the growth and pigment production of the yeast was studied. In the respective broth for each pigment, pH of the medium was adjusted to 5.0 for YP 37 and 6.0 for YP 17 and standard strain, respectively. Hundred ml of the medium was dispensed in 250 ml Erlenmeyer flasks and autoclaved. The flasks were inoculated with yeast cultures and incubated at different temperatures *viz*., 15, 20, 25, 30, 35 and 40ºC for 6 days under shaking condition for 150 rpm. After incubation, the pigment yield was measured as mentioned in section 3.5. Three numbers of replications were maintained for each temperature.

3.10.3. Effect of incubation time

In order to study the impact of the period of incubation on yeast growth and pigment production, 100 ml of the medium was dispensed in 250 ml Erlenmeyer flasks and steam sterilized. The flasks were inoculated with pigment producing cultures and incubated for 48, 72, 96, 120, 144 and 168 h. After incubation, the pigment yield was measured as mentioned in section 3.5. Triplicates were maintained for each incubation time.

3.10.4. Effect of agitation of culture

Hundred ml of the respective broth for each pigmented cultures were dispensed in 250 ml Erlenmeyer flasks, the pH adjusted to the standardized pH of the respective pigment producing yeast and steam sterilized. The flasks were inoculated with yeast cultures of pigment producers. The flasks were kept over a shaker and the shaking speed was adjusted to 50, 100, 150, 200 and 250 rpm. After the incubation period at 25°C, the pigment yield was measured as mentioned in section 3.5. Three replications were maintained for each rotation per min.

3.10.5. Effect of inoculum size

One 100 ml of the respective broth for each pigment was dispensed in 250 ml Erlenmeyer flasks and steam sterilized. The fermentation medium was supplemented from 0.5 % to 5 per cent (v/v) of inoculum ranging from 10^4 to 10^8 cells ml^{-1} for both pigment producers and incubated at 25°C for respective days for YP17, standard strain and YP 37 under shaking condition. After incubation, the pigment yield was measured as mentioned in section 3.5. Three numbers of replications were maintained for each inoculum size.

3.10.6. Effect of light

The influence of light on the growth and pigment production of the selected yeast was investigated. Hundred ml of respective broth for yeast cultures with standardized pH were taken in 250 ml Erlenmeyer flasks and autoclaved. The flasks were incubated under different light wavelength such as red (640-740 nm), yellow (570-600 nm), green (520-570 nm), blue (450-520 nm), unscreened white light (350-740 nm) and dark for 6 days. Parameters like cell dry weight and carotenoid pigment production in the medium were determined at the end of the experimental duration.

3.11. Optimization of chemical parameters using one-factor-at-a-time

3.11.1. Effect of different carbon sources

Nine carbon sources *viz*., maltose, fructose, xylose, sucrose, lactose, glucose, galactose, ribose and glycerol were used. Culture medium (100 ml) in 250 ml Erlenmeyer flask amended with different carbon sources were steam sterilized. The pH of the medium

was adjusted with diluted NaOH. The flasks were inoculated with 3 per cent inoculum and incubated at 25°C**.** Three replications were maintained with appropriate controls for each carbon source.

3.11.2. Effect of various nitrogen sources

The effect of various nitrogen sources on the growth and carotenoid production of pigment producers were studied. The nitrogen source in the respective broth was replaced with either organic or inorganic nitrogen sources. The organic nitrogen sources such as peptone, beef extract, tryptone, malt extract and yeast extract were tried. The inorganic nitrogen sources screened were sodium nitrite, casein, ammonium nitrate, ammonium chloride and ammonium sulphate.

Hundred ml of culture medium amended with different nitrogen sources were taken in 250 ml Erlenmeyer flasks and sterilized by autoclaving. Yeast cultures were inoculated and the flasks were incubated at 25°C for respective days for YP 17, standard strain and YP 37. After incubation, the pigment yield was measured as mentioned in section 3.5. Three numbers of replications was maintained with appropriate controls for each nitrogen source.

3.11.3. Effect of trace metals

The influence of heavy metal like copper, ferrous, zinc, magnesium and manganese on the growth and pigment production was investigated. Hundred ml of the respective broth for each pigment were taken in 250 ml Erlenmeyer flask and steam sterilized. Filter sterilized metal solution was added to the medium at a concentration of 0.5 g l^{-1}. The flasks were inoculated with 3 per cent of yeast pigment producing yeast cultures. The flasks were incubated at respective days yeast cultures. Triplicates were maintained.

3.12. Optimization by Response Surface Methodology (RSM)

A Central Composite Rotatable Design (CCRD) of experiments (Box and Draper, 1987; Myers and Montgomery, 1995; Montgomery, 1997) under Response Surface Methodology (RSM) was chosen for the experiments for optimization of physical and chemical parameters on pigment production. RSM is a statistical technique that can be used to systematically determine the effects of multiple variables on response variables

while minimizing the number of evaluations that must be conducted. Unlike conventional empirical optimization, the statistical optimization method can take into account the interaction of variables in generating the process response. A combination of factors responsible for a certain optimum response can be identified through the factorial design and the use of Response Surface Methodology (RSM).

Central composite design contains an embedded factorial or fractional factorial design with center points that is augmented with a group of 'star points' that allow estimation of curvature and number of base level points 'n_0' to estimate the experimental error (Table 7). A central composite design always contains twice as many star points as there are factors in the design. The star points represent new extreme values (low and high) for each factor in the design.

The distance from the center of the design space to a factorial point is ± 1 unit for each factor and the distance from the center of the design space to a star point is $\pm \alpha$ with $| \alpha | > 1$. The precise value of 'α' depends on certain properties desired for the design and on the number of factors involved. In order to maintain rotatability, the value of 'α' depends on the number of experimental runs in the factorial portion of the central composite design.

Table 7. Components of central composite rotatable design of experiments

No. of factors	**No. of points**			**Total**	**Scale value for α $[(2^k)^{1/4}]$**
	Factorial (2^k)	**Star (2k)**	**Base level n_0**		
2	4	4	5	13	1.414
3	**8**	**6**	**6**	**20**	**1.682**
4	16	8	7	31	2.000
5	**32**	**10**	**10**	**52**	**2.378**

(Source: Montgomery, 1997)

$$\alpha = [\text{number of factorial runs}]^{1/4} \quad \text{..... (3.1)}$$

If the factorial portion is a full factorial, then

$$\alpha = [(2^k)^{1/4}] \quad \text{..... (3.2)}$$

The number of base level points 'n_0' to be carried out depends on the number of factors 'k'.

Total number of experiments in the first order design $(n_0) = 2^k + n_0$ ..... (3.3)

Total number of experiments in the second order design $(n_s) = 2^k + 2k + n_0$ ..... (3.4)

3.12.1. Optimization of physical parameters by RSM

The natural, coded levels and interval of variation of the independent variables in the experimental plan for the optimization of physical parameters on YP 17 and YP 37 yeast carotenoid pigments production are given in the Table 8 and Table 10 respectively.

Table 8. Natural levels, codes and intervals of variation of the independent variables in the design of experiments for YP 17 pigment production

Factors	Codes	Levels					Interval of variation
		-1.682	**-1**	**0**	**+1**	**+1.682**	
pH	*P*	4.318	4	5	6	7.681	1
Temperature (ºC)	*T*	16.591	20	25	30	33.409	5
Incubation Time (h)	*I*	55.637	72	96	120	136.363	24

where,

$$p = \left(\frac{P - 5}{4}\right) \quad \text{..... (3.5)}$$

$$t = \left(\frac{T - 25}{20}\right) \quad \text{..... (3.6)}$$

$$i = \left(\frac{I - 96}{72}\right) \quad \text{..... (3.7)}$$

The experimental design based on RSM for the optimization of physical parameters on YP 17 carotenoid pigment production is given in detail in the Table 9.

Table 10. Natural levels, codes and intervals of variation of the independent variables in the design of experiments for YP 37 pigment production

Factors	Codes	Levels					Interval of variation
		-1.681	**-1**	**0**	**+1**	**+1.681**	
pH	*P*	3.318	5	6	7	6.681	1
Temperature (ºC)	*T*	16.591	20	25	30	33.409	5
Incubation Time (h)	*I*	79.637	72	96	120	160.363	24

where,

$$p = \left(\frac{P - 6.0}{5.0}\right) \quad \text{..... (3.8)}$$

$$t = \left(\frac{T - 25}{20}\right) \quad \text{..... (3.9)}$$

$$i = \left(\frac{I - 96}{72}\right) \quad \text{..... (3.10)}$$

The experimental design based on response surface methodology for the optimization of physical parameters on YP 37 carotenoid pigments production is given in the Table 11. According to central composite rotatable design for three independent factors, the total experiments to be conducted were found to be twenty (Table 9 and Table 11). Twenty experiments were performed for three variables with five levels of each variable which are shown in Table 9 and Table 11. Fourteen experiments starting from A to O were formulated for optimization of physical parameters on pigment production. The base level treatment I was replicated six times to calculate the reproducibility of the method and the other treatments were replicated thrice. The maximum and minimum variable levels were selected on the basis of preliminary trials. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experiments were

Table 9. Experimental design used for optimization of physical parameters on *R. mucilaginosa* pigment production

Experiment No.	Independent Variable Levels						
	Natural Values			Coded Values			
	P	**T**	**I**	***P***	***t***	***I***	
1 (A)	5.00	20	72	-1	-1	-1	2^k type factorial experiments
2 (B)	7.00	20	72	+1	-1	-1	
3 (C)	5.00	30	72	-1	+1	-1	
4 (D)	7.00	30	72	+1	+1	-1	
5 (E)	5.00	20	120	-1	-1	+1	
6 (F)	7.00	20	120	+1	-1	+1	
7 (G)	5.00	30	120	-1	+1	+1	
8 (H)	7.00	30	120	+1	+1	+1	
9 (J)	4.31	25	96	- α	0	0	2k extra experiments with star points
10 (K)	7.68	25	96	+α	0	0	
11 (L)	6.00	16.59	96	0	- α	0	
12 (M)	6.00.	33.40	96	0	+α	0	
13 (N)	6.00	25	55.63	0	0	- α	
14 (O)	6.00	25	136.36	0	0	+α	
15 (I_1)	6.00	25	96	0	0	0	n_0 base level experiments
16 (I_2)	6.00	25	96	0	0	0	
17 (I_3)	6.00	25	96	0	0	0	
18 (I_4)	6.00	25	96	0	0	0	
19 (I_5)	6.00	25	96	0	0	0	
20 (I_6)	6.00	25	96	0	0	0	

Table 11. Experimental design used for optimization of physical parameters on *Sporobolomyces* sp. pigment production

Experiment No.	Independent Variable Levels						
	Natural Values			Coded Values			
	P	**T**	**I**	***P***	***T***	***I***	
1 (A)	4	20	96	-1	-1	-1	2^k type factorial experiments
2 (B)	6	20	96	+1	-1	-1	
3 (C)	4	30	96	-1	+1	-1	
4 (D)	6	30	96	+1	+1	-1	
5 (E)	4	20	144	-1	-1	+1	
6 (F)	6	20	144	+1	-1	+1	
7 (G)	4	30	144	-1	+1	+1	
8 (H)	6	30	144	+1	+1	+1	
9 (J)	3.31	25	120	- α	0	0	2k extra experiments with star points
10 (K)	6.68	25	120	+α	0	0	
11 (L)	5	16.59	120	0	- α	0	
12 (M)	5	33.40	120	0	+α	0	
13 (N)	5	25	79.63	0	0	- α	
14 (O)	5	25	160.36	0	0	+α	
15 (I_1)	5	25	120	0	0	0	n_0 base level experiments
16 (I_2)	5	25	120	0	0	0	
17 (I_3)	5	25	120	0	0	0	
18 (I_4)	5	25	120	0	0	0	
19 (I_5)	5	25	120	0	0	0	
20 (I_6)	5	25	120	0	0	0	

conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

3.12.2. Optimization of chemical parameters by response surface methodology

Once the critical variables were screened, RSM was performed to optimize the screened medium components for enhanced carotenoid pigments production using the central composite design-uniform precision (CCD-uniform precision). A full factorial central composite factorial design of 2^5 = 32 plus 8 centre points and (2 x 5 = 10) star points leading to a total of 50 experiments were performed. For statistical calculations, the relation between the coded values and actual values for carotenoid pigments were described in Table 13 and Table 15 respectively.

Table 12. Natural levels, codes and intervals of variation of the independent variables in the design of experiments (Chemical parameters) for YP 17 pigment production

Factors	**Coded symbols**	**Coded levels**					**Interval of variation**
		- 2.378	**-1**	**0**	**+1**	**+ 2.378**	
Sucrose	*S*	18.10	25	30	35	41.892	5
Yeast extract	*Y*	0.06	3	4	5	7.06	1
Ammonium sulphate	*A*	0.81	1.5	2.0	2.5	3.18	0.5
Dipotassium hydrogen phosphate	*D*	0.53	0.5	1.0	1.5	3.03	0.5
Magnesium sulphate	*M*	0.02	0.3	0.5	0.7	0.97	0.2

where,

$$s = \left(\frac{S - 30}{25}\right) \qquad \text{..... (3.1a)}$$

$$y = \left(\frac{Y - 4}{3}\right) \qquad \text{..... (3.2a)}$$

$$a = \left(\frac{A - 2}{1.5}\right) \quad \text{..... (3.3a)}$$

$$d = \left(\frac{D - 1}{0.5}\right) \quad \text{..... (3.4a)}$$

$$m = \left(\frac{M - 0.5}{0.3}\right) \quad \text{.....(3.5a)}$$

Table 13. Natural levels, codes and intervals of variation of the independent variables in the design of experiments (Chemical parameters) for YP 37 pigment production

Factors	Coded symbols	Coded levels					Interval of variation
		-α	**-1**	**0**	**+1**	**A**	
Glucose	*G*	18.10	25	30	35	41.89	5
Peptone	*P*	3.81	4.5	5.0	5.5	6.18	0.5
Ammonium sulphate	*A*	0.81	1.5	2.0	2.5	3.189	0.5
Dipotassium hydrogen phosphate	*P*	0.310	1.0	1.5	2.0	2.689	0.5
Ferrous sulphate	F	0.024	0.3	0.5	0.7	0.975	0.2

$$g = \left(\frac{G - 30}{25}\right) \quad \text{..... (3.6a)}$$

$$p = \left(\frac{P - 5.0}{4.5}\right) \quad \text{..... (3.7a)}$$

$$a = \left(\frac{A - 2.0}{1.5}\right) \quad \text{..... (3.8a)}$$

$$p = \left(\frac{P - 1.5}{1.0}\right) \quad \text{..... (3.9a)}$$

$$f = \left(\frac{F - 0.5}{0.3}\right) \quad \text{..... (3.10a)}$$

According to central composite rotatable design for five independent factors, the total experiments to be conducted were found to be fifty. 50 experiments were performed for five variables with five levels of each variable which are shown in Tables 14 and 15. Fifty experiments starting from 1 to 50 were formulated for optimization of chemical parameters on carotenoid pigment production. The base level treatment from 43 to 50 was replicated eight times to calculate the reproducibility of the method and the other treatments were replicated thrice. The maximum and minimum variable levels were selected on the basis of preliminary trials. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experiments were conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

3.12.3. Statistical modeling of responses

Empirical statistical modeling was used to develop an appropriate approximating model between the response (y) and independent variables $(X_1, X_2, X_3, \ldots\ldots X_k)$.

In general the relationship is

$$Y = f(X_1, X_2, X_3, \ldots\ldots X_k) + \varepsilon \qquad \ldots\ldots (3.11b)$$

The variables $X_1, X_2, X_3, \ldots\ldots X_k$ in the Eqn. 3.11b are usually called the natural variables, because they are expressed in µg/g units in RSM it is convenient to transform the natural variables to coded variables $x_1, x_2, x_3, \ldots\ldots x_k$, which are dimensionless having mean zero and variance equal to 1. In terms of the coded variables, the response function (Eqn. 3.12b) will be written as

$$y = f(x_1, x_2, x_3, \ldots\ldots x_k) + \varepsilon \qquad \ldots\ldots (3.12b)$$

The function 'f' is called the response surface. The form of the function 'f' is unknown. The term 'ε' represents other sources of variability not accounted for in 'f' and usually it is treated as statistical error. As the form of the response function 'f' is unknown, it must be approximated. Polynomials are often chosen because they usually

Table 14. Experimental design used for optimization of chemical parameters on *R.mucilaginosa* carotenoid pigment production

Ex. No	Independent Variable Levels										
	Natural Values					Coded values					
	S	Y	A	K	M	s	y	a	k	m	
1	25	2	1.5	0.5	0.3	-1	-1	-1	-1	-1	2^k type factorial experiments
2	35	2	1.5	0.5	0.3	+1	-1	-1	-1	-1	
3	25	5	1.5	0.5	0.3	-1	+1	-1	-1	-1	
4	35	5	1.5	0.5	0.3	+1	+1	-1	-1	-1	
5	25	2	2.5	0.5	0.3	-1	-1	+1	-1	-1	
6	35	2	2.5	0.5	0.3	+1	-1	+1	-1	-1	
7	25	5	2.5	0.5	0.3	-1	+1	+1	-1	-1	
8	35	5	2.5	0.5	0.3	+1	+1	+1	-1	-1	
9	25	2	1.5	2	0.3	-1	-1	-1	+1	-1	
10	35	2	1.5	2	0.3	+1	-1	-1	+1	-1	
11	25	5	1.5	2	0.3	-1	+1	-1	+1	-1	
12	35	5	1.5	2	0.3	1	+1	-1	+1	-1	
13	25	2	2.5	2	0.3	-1	-1	+1	+1	-1	
14	35	2	2.5	2	0.3	+1	-1	+1	+1	-1	
15	25	5	2.5	2	0.3	-1	+1	+1	+1	-1	
16	35	5	2.5	2	0.3	+1	+1	+1	+1	-1	
17	25	2	1.5	0.5	0.7	-1	-1	-1	-1	+1	
18	35	2	1.5	0.5	0.7	+1	-1	-1	-1	+1	
19	25	5	1.5	0.5	0.7	-1	+1	-1	-1	+1	
20	35	5	1.5	0.5	0.7	+1	+1	-1	-1	+1	
21	25	2	2.5	0.5	0.7	-1	-1	+1	-1	+1	
22	35	2	2.5	0.5	0.7	+1	-1	+1	-1	+1	
23	25	5	2.5	0.5	0.7	-1	+1	+1	-1	+1	
24	35	5	2.5	0.5	0.7	+1	+1	+1	-1	+1	
25	25	2	1.5	2	0.7	-1	-1	-1	+1	+1	

Ex. No	Independent Variable Levels										
	Natural Values					Coded values					
	S	Y	A	K	M	s	y	a	k	M	
26	35	2	1.5	2	0.7	+1	-1	-1	+1	+1	2^k type factorial experiments
27	25	5	1.5	2	0.7	-1	+1	-1	+1	1+	
28	35	5	1.5	2	0.7	+1	+1	-1	+1	+1	
29	25	2	2.5	2	0.7	-1	-1	+1	+1	+1	
30	35	2	2.5	2	0.7	+1	-1	+1	+1	+1	
31	25	5	2.5	2	0.7	-1	+1	+1	+1	+1	
32	35	5	2.5	2	0.7	+1	+1	+1	+1	+1	
33	18.19	3.5	2	1.25	0.5	- α	0	0	0	0	2k extra experiments with star points
34	41.89	3.5	2	1.25	0.5	+α	0	0	0	0	
35	30	0.06	2	1.25	0.5	0	- α	0	0	0	
36	30	7.06	2	1.25	0.5	0	+α	0	0	0	
37	30	3.5	0.81	1.25	0.5	0	0	- α	0	0	
38	30	3.5	3.18	1.25	0.5	0	0	+α	0	0	
39	30	3.5	2	-0.53	0.5	0	0	0	- α	0	
40	30	3.5	2	3.03	0.5	0	0	0	+α	0	
41	30	3.5	2	1.25	0.02	0	0	0	0	- α	
42	30	3.5	2	1.25	0.9	0	0	0	0	+α	
43	30	3.5	2	1.25	0.5	0	0	0	0	0	n_0 base level experiments
44	30	3.5	2	1.25	0.5	0	0	0	0	0	
45	30	3.5	2	1.25	0.5	0	0	0	0	0	
46	30	3.5	2	1.25	0.5	0	0	0	0	0	
47	30	3.5	2	1.25	0.5	0	0	0	0	0	
48	30	3.5	2	1.25	0.5	0	0	0	0	0	
49	30	3.5	2	1.25	0.5	0	0	0	0	0	
50	30	3.5	2	1.25	0.5	0	0	0	0	0	

Table 15. Experimental design used for optimization of chemical parameters on *Sporobolomyces* sp. carotenoid pigment production

Ex. No	Independent Variable Levels										
	Natural Values					Coded values					
	S	Y	A	K	M	s	y	a	k	M	
1	25	2	1.5	0.5	0.3	-1	-1	-1	-1	-1	
2	35	2	1.5	0.5	0.3	+1	-1	-1	-1	-1	
3	25	5	1.5	0.5	0.3	-1	+1	-1	-1	-1	
4	35	5	1.5	0.5	0.3	+1	+1	-1	-1	-1	
5	25	2	2.5	0.5	0.3	-1	-1	+1	-1	-1	
6	35	2	2.5	0.5	0.3	+1	-1	+1	-1	-1	
7	25	5	2.5	0.5	0.3	-1	+1	+1	-1	-1	
8	35	5	2.5	0.5	0.3	+1	+1	+1	-1	-1	
9	25	2	1.5	2	0.3	-1	-1	-1	+1	-1	
10	35	2	1.5	2	0.3	+1	-1	-1	+1	-1	
11	25	5	1.5	2	0.3	-1	+1	-1	+1	-1	
12	35	5	1.5	2	0.3	+1	+1	-1	+1	-1	
13	25	2	2.5	2	0.3	-1	-1	+1	+1	-1	
14	35	2	2.5	2	0.3	+1	-1	+1	+1	-1	
15	25	5	2.5	2	0.3	-1	+1	+1	+1	-1	
16	35	5	2.5	2	0.3	+1	+1	+1	+1	-1	
17	25	2	1.5	0.5	0.7	-1	-1	-1	-1	+1	
18	35	2	1.5	0.5	0.7	+1	-1	-1	-1	+1	
19	25	5	1.5	0.5	0.7	-1	+1	-1	-1	+1	
20	35	5	1.5	0.5	0.7	+1	+1	-1	-1	+1	
21	25	2	2.5	0.5	0.7	-1	-1	+1	-1	+1	
22	35	2	2.5	0.5	0.7	+1	-1	+1	-1	+1	
23	25	5	2.5	0.5	0.7	-1	+1	+1	-1	+1	
24	35	5	2.5	0.5	0.7	+1	+1	+1	-1	+1	
25	25	2	1.5	2	0.7	-1	-1	-1	+1	+1	

2^k type factorial experiments

Ex. No	Independent Variable Levels										
	Natural Values					Coded values					
	S	Y	A	K	M	s	y	a	k	M	
26	35	2	1.5	2	0.7	+1	-1	-1	+1	+1	2^k type factorial experiments
27	25	5	1.5	2	0.7	-1	+1	-1	+1	+1	
28	35	5	1.5	2	0.7	+1	+1	-1	+1	+1	
29	25	2	2.5	2	0.7	-1	-1	+1	+1	+1	
30	35	2	2.5	2	0.7	+1	-1	+1	+1	+1	
31	25	5	2.5	2	0.7	-1	+1	+1	+1	+1.	
32	35	5	2.5	2	0.7	+1	+1	+1	+1	+1	
33	18.10	3.5	2	1.25	0.5	- α	0	0	0	0	2k extra experiments with star points
34	41.89	3.5	2	1.25	0.5	+α	0	0	0	0	
35	30	-0.06	2	1.25	0.5	0	- α	0	0	0	
36	30	7.06	2	1.25	0.5	0	+α	0	0	0	
37	30	3.5	0.81	1.25	0.5	0	0	- α	0	0	
38	30	3.5	3.18	1.25	0.5	0	0	+α	0	0	
39	30	3.5	2	-0.53	0.5	0	0	0	- α	0	
40	30	3.5	2	3.03	0.5	0	0	0	+α	0	
41	30	3.5	2	1.25	0.02	0	0	0	0	- α	
42	30	3.5	2	1.25	0.97	0	0	0	0	+α	
43	30	3.5	2	1.25	0.5	0	0	0	0	0	n_0 base level experiments
44	30	3.5	2	1.25	0.5	0	0	0	0	0	
45	30	3.5	2	1.25	0.5	0	0	0	0	0	
46	30	3.5	2	1.25	0.5	0	0	0	0	0	
47	30	3.5	2	1.25	0.5	0	0	0	0	0	
48	30	3.5	2	1.25	0.5	0	0	0	0	0	
49	30	3.5	2	1.25	0.5	0	0	0	0	0	
50	30	3.5	2	1.25	0.5	0	0	0	0	0	

offer an adequate approximation of the true response surface. In many cases, either a first order or a second order model as shown below is used.

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \varepsilon \qquad \text{….. (3.13b)}$$

$$y = b_0 x_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} b_{ij} x_i x_j + \varepsilon \qquad \text{….. (3.14b)}$$

The first model (Eqn. 3.13b) is likely to be appropriate when the experimenter is interested in approximating the true response surface over a relatively small region of the independent variables space in a location where there is no curvature in '*f*'. If there is a curvature in the system, then a polynomial of higher degree such as second order model (Eqn. 3.14b) must be used.

To find out the effect of the independent variables on the dependent variables, the first order linear equation (Eqn. 3.15b) was fitted between '*x*' and '*y*'. For optimization of the independent variables and to check the sufficiency of the experimental design, the second order non-linear regression equation (Eqn. 3.16b) was fitted between dependent and independent variables.

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 \qquad \text{….. (3.15b)}$$

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \qquad \text{….. (3.16b)}$$

where,

y is the response variable

b_0, b_1, b_2 and b_3 are regression coefficients of linear terms

b_{11}, b_{22}, and b_{33} are regression coefficients of quadratic terms

b_{12}, b_{13} and b_{23} are regression coefficients of cross-product terms

x_1, x_2, and x_3 are the coded values of the independent variables *X*, *viz.*, pH (X_1), temperature (X_2), and incubation time (X_3) respectively. The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-test. The significance of the regression coefficient was

determined by p-value. The coefficients of the equation were determined by employing Design Expert software Version 8.0.7.1. Analysis of variance (ANOVA) for the final predictive equation was done using Design Expert software. The response surface analysis was made keeping one independent variable at middle level while changing the other two. The response surface equation was used to optimize the independent variables for the response variables such as pigment yield.

3.13. Carotenoid pigment production using various agricultural cheap sources by submerged fermentation

The promising varied pigment producing yeast cultures were cultivated using potential cheap natural substrates to minimize the production cost of pigments. The tropical agro-industrial residues such as rice bran, wheat bran, coconut oil cake, sesame oil cake, groundnut oil cake, cassava bagasse, sugarcane bagasse and rice flour were selected as substrates for screening the pigment production.

3.13.1. Carotenoid production by YP 17 under submerged fermentation

Carotenoid production by YP 17 using optimized conditions under submerged fermentation was carried out with 2 per cent of cassava bagasse as substrate (carbon source). The cassava bagasse substrate was milled and sieved to a particle size of 200 µ.

3.13.2. Physio - chemical properties of cassava bagasse

The physio-chemical characters *viz.*, carbohydrates, protein, total soluble sugars (TSS), crude fibre, ash, cellulose, starch and moisture content of cassava bagasse were analyzed as per the standard protocols.

3.13.3. Estimation of carbohydrates

The amount of total carbohydrates was estimated by anthrone method (Hedge and Hotreiter, 1962).

Principle

Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxyl methyl furfural. This

compound forms a green coloured product with anthrone at an absorption maximum at 630 nm.

Procedure

About 100 mg of cassava bagasse was taken into a boiling tube. The samples were hydrolysed by keeping it in a water bath for 3 h with 5.0 ml of 2.5 N HCl and cooled to room temperature. The volume was made up with distilled water to 100 ml and centrifuged. Aliquots of (0.5 ml) each sample was pipetted out in test tubes and simultaneously 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard was pipetted out in test tubes. Volume of samples and standards were made up to 1 ml with distilled water. To 1 ml of distilled water blank, 4 ml of anthrone reagent was added to each tube. The tubes were kept in a boiling water bath for eight minutes. After cooling the tubes, the absorbance was recorded at 630 nm using spectrophotometer and the values were plotted in a graph against concentration. Graph was drawn and the amounts of reducing sugars present in the sample were calculated.

3.13.4. Estimation of protein content

The protein content of substrate was estimated as following Lowry *et al*. (1951).

Procedure

Sample aliquot of (0.1 ml) was pipetted out in test tubes and simultaneously 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard was pipetted out into test tubes. Volume of samples and standards was made up to 2 ml with distilled water. To one ml of distilled water blank, one ml of Lowry's stock reagent was added. The tubes were incubated at room temperature for 30 min and the tubes were then added with 2 N folin's reagent. The volume was finally made up to 10 ml with distilled water to 10 ml and left for 10 min. The absorbance was recorded at 620 nm using spectrophotometer and the values were plotted in a graph against concentration. Protein content in the sample was calculated and expressed in terms of percentage.

3.13.5. Estimation of crude fibre content

Crude fibre content of the substrate was estimated according to the method proposed by Prosky *et al*. (1988).

Hundred mg of dried substrate was suspended in 100 ml of distilled water and incubated for 90 min at 37ºC to solubilize sugars. The water soluble fibre components in the substrates were then precipitated by sequentially washing with 78 per cent of ethanol and 95 per cent of acetone, then the substrates was dried at 105ºC and weighed. The total crude fibre content was calculated after deducing the protein and ash content of the substrate.

3.13.6. Estimation of cellulose

The cellulose content in substrate was estimated as following method described by Updegroff (1969).

Principle

Cellulose undergoes acetolysis with acetic or nitric acid reagent forming acetylated cellodextrins, which get dissolved and hydrolyzed to form glucose molecules on treatment with 67 per cent H_2SO_4. This glucose molecule is dehydrated to form hydroxymethyl furfural, which forms green coloured product with anthrone and the colour intensity was measured at 630 nm using spectrophotometer.

Procedure

Ten ml of nitric acid was added to 0.5 g of the sample in a test tube and mixed over vortex mixture. The tube was heated in water bath at 100ºC for 30 min. The supernatant was discarded by centrifugation at 5000 rpm for 5 min. After washing, the sample with distilled water, 10 ml of 67 per cent sulphuric acid was added and allowed to stand for 1 h. One ml of the solution was diluted to 100 ml. To 1 ml of the diluted solution, 4 ml distilled water and 10 ml of anthrone reagent were added and mixed well. Again the tube was kept in boiling water bath for 16 min, cooled and the colour intensity was measured at 620 nm. The quantity of cellulose was calculated from the standard curve and cellulose content was expressed in per cent of dry matter.

3.13.7. Estimation of starch content

The amount of starch content was estimated by anthrone reagent (Hedge and Hofreiter, 1962).

Principle

The sample is treated with 80 per cent alcohol to remove sugars and then starch is extracted with perchloric acid. In hot acidic medium, starch is hydrolysed to glucose and dehydrated to hydroxymethyl furfural. This compound forms with a green coloured product with anthrone.

Procedure

One g of each sample was homogenized in hot 80 per cent ethanol to remove sugars. After centrifuge, the residue was washed repeatedly with hot 80 per cent ethanol. Then, the residue was dried well over a water bath. Five ml of distilled water and 6.5 ml of 52 per cent perchloric acid was added to the residue. Aliquot (0.1 ml) of sample and 0.2, 0.4, 0.6, 0.8 and 1 ml of working standards were pipetted out into test tubes. Volume of sample and standards was made up to 1 ml with distilled water.

3.13.8. Estimation of ash content

Ash content was determined based on the gravimetric loss by heating the substrate to 150ºC for a period of 24 h (Reuter *et al*., 1986).

3.13.9. Estimation of TSS

Standard brix hydrometer (M/S Abbe, India) was used to determine the brix value of cassava bagasse and suitable temperature corrections were made to obtain the corrected brix in degrees (Dogett, 1981).

3.13.10. Estimation of moisture content

Five gram of cassava bagasse was taken in moisture bottle, weighed to record initial weight and dried at 103 $\pm$ 2°C in a hot air oven for 16 h. Then the samples were cooled in a desiccator for 30 min and the dry weight was recorded. The moisture content was calculated and the mean expressed in per cent by using the following formula.

$$\text{Moisture content (per cent)} = \frac{M_2 - M_3}{M_2 - M_1} \times 100$$

where,

M_1 - weight of the moisture bottle (g)

M_2 - weight of the moisture bottle + weight of the sample before drying (g)

M_3 - weight of the moisture bottle + weight of the sample after drying (g)

3.14. Optimization of fermentation parameters for YP 17 carotenoid pigment production by RSM using cassava bagasse

The natural, coded levels and interval of variation of the independent variables in the experimental plan for the optimization of parameters on carotenoid pigment production is given in the Table 16.

Table 16. Natural levels, codes and intervals of variation of the independent variables in the design of experiments for YP 17 carotenoid pigment production

Factors	Codes	Levels					Interval of variation
		-1.681	**-1**	**0**	**+1**	**+1.681**	
Substrate concentration (g)	*S*	21.59	1.5	2.0	2.5	38.40	0.5
Fermentation time (hrs)	*F*	55.63	72	96	120	136.36	24
Initial pH	*I*	4.31	5.0	6.0	7.0	7.68	1.0

where,

$$s = \left(\frac{S - 2.0}{1.5}\right) \qquad \text{..... (3.1c)}$$

$$f = \left(\frac{F - 96}{72}\right) \qquad \text{..... (3.2c)}$$

$$i = \left(\frac{I - 6.0}{5.0}\right) \qquad \text{..... (3.3c)}$$

The experimental design based on response surface methodology for the optimization of parameters on YP 17 pigment production is given in detail in the Table 17.

Table 17. Experimental design used for optimization of fermentation parameters on *R.mucilaginosa* carotenoid pigment production by cassava bagasse

Experiment No.	Independent Variable Levels						
	Natural Values			Coded Values			
	I	**F**	**S**	***I***	***F***	***S***	
1 (A)	5	72	25	-1	-1	-1	2^k type factorial experiments
2 (B)	7	72	25	+1	-1	-1	
3 (C)	5	120	25	-1	+1	-1	
4 (D)	7	120	25	+1	+1	-1	
5 (E)	5	72	35	-1	-1	+1	
6 (F)	7	72	35	+1	-1	+1	
7 (G)	5	120	35	-1	+1	+1	
8 (H)	7	120	35	+1	+1	+1	
9 (J)	4.31	96	30	- α	0	0	2k extra experiments with star points
10 (K)	7.68	96	30	+α	0	0	
11 (L)	6	55.63	30	0	- α	0	
12 (M)	6	136.36	30	0	+α	0	
13 (N)	6	96	21.59	0	0	- α	
14 (O)	6	96	38.40	0	0	+α	
15 (I_1)	6	96	30	0	0	0	n_0 base level experiments
16 (I_2)	6	96	30	0	0	0	
17 (I_3)	6	96	30	0	0	0	
18 (I_4)	6	96	30	0	0	0	
19 (I_5)	6	96	30	0	0	0	
20 (I_6)	6	96	30	0	0	0	

According to central composite rotatable design for three independent factors, the total experiments to be conducted were found to be twenty. Twenty experiments were performed for three variables with five levels of each variable which are shown in Table 17. Fourteen experiments starting from A to O were formulated for optimization of various parameters on YP 17 pigment production. The base level treatment I was replicated six times to calculate the reproducibility of the method and the other treatments were replicated thrice. The maximum and minimum variable levels were selected on the basis of preliminary trials. The experiments were randomized in order to minimize the effects of unexplained variability in the observed responses due to extraneous factors. The experiments were conducted with second order design so that both the first and second order models can be postulated according to the adequacy of fit. For each combination of the independent variables in the experimental design, the dependent parameters were found out.

3.14.1. Statistical Modeling of Responses

Empirical statistical modeling for optimization parameters of yeast carotenoid production was already mentioned in section 3.12.3.

3.15. Production of carotenoid pigments

Mass cultivation was carried out in 3l fermentor to achieve high cell densities for mass production of the crude pigment needed for downstream processing such as separation, purification and characterization of fractions (colourants) for their structure elucidation and to study its stability, toxicity and applicability as food colourant. The RSM optimized media for YP 17 and YP 37 pigments was prepared. Yeast was maintained on YM agar slant at 4°C and subcultured monthly. The inoculum of yeast cultures were grown in 250 ml Erlenmeyer flask containing 100 ml of culture medium composed of the following (g l^{-1}): glucose - 10; malt extract -5; peptone - 5; yeast extract - 3; on a rotary shaker at 200 rpm for 48 h and added at 3 per cent (v/v) to the fermenter containing 1.5 l of following respective medium composition for each culture. Temperature and agitation were maintained at 25-30°C and 200 rpm wherever pH was maintained at 6.0 and 5.0 for YP 17 and YP 37 respectively.

3.16. Comparisons of effectiveness of organic solvents on pigment extracting activity

3.16.1. Procedures for extracting carotenoids using different solvents

Carotenoid pigments were extracted as per the method of Latha *et al.* (2005). Polar and non-polar solvents were used for extraction of the yeast pigments. Methyl alcohol, ethyl alcohol, acetone, ethyl acetate, diethyl ether, benzene, acetonitrile, hexane, chloroform, toluene, petroleum ether and propanol were used as solvents.

3.17. Characterization of yeast carotenoid pigments

3.17.1. Thin layer chromatography (TLC) of the carotenoid pigments

Different solvent systems (ranging from less polar to highly polar) were tested for the effective separation of carotenoid pigments from acetone extracts. Based on the separation of carotenoid compounds suitable mobile phase was standardized. TLC plate spotted with samples was immersed (0.5 cm height solvent column) in the mobile phase filled in TLC tank. The tank was closed with a glass lid in order to maintain the chamber space with solvent volatiles. The plates were kept in the tank till the solvent front reaches the top of TLC plate. Then, the plate was removed from the tank and dried to room temperature for solvent evaporation (Blanc *et al.*, 1994).

The TLC run plates were observed under bright light and the separated spots were marked. The Relative front values were calculated by using the given formula

$$\text{Rf value} = \frac{\text{Distance moved by the solute from the origin}}{\text{Distance moved by the solvent from the origin}}$$

3.17.2. Identification of compounds using HPLC

HPLC analysis (Mantzouridou and Tsimidou, 2007)

The analysis was carried out in HPLC – Hitachi model L 6200 equipped with JASCO PU-1580 pump, JASCO UV-1575 UV/Visible detector using a reverse phase-C18 column to detect the possible compounds present in the active fractions of the extracts of pigment producers.

3.17.2.1. Chromatographic conditions

The carotenoids pigments were analyzed by reverse phase HPLC with detection at 485nm. The mobile phase (HPLC grade solvents were used) and samples were filtered through 0.25µm membrane filter, C18 column consists of acetronitrile, isopropanol and ethyl acetate (40:40:20, v\v\v) with flow rate at 1 ml min^{-1}.

3.17.3. FT-IR

To confirm the structure of yeast carotenoid pigments, FT-IR spectrometer (Impact 400D, Nicolet, Madison, WI) was used to measure the infrared spectra of extract solution in the wave number of 400-4000 cm^{-1} at room temperature. For each IR spectrometer samples 32 scans at 4 cm^{-1} resolution was collected in the transmittance mode.

3.18. Stability of carotenoid pigments to various physical and chemical conditions

3.18.1. pH stability

Five ml of the carotenoid pigment extracts was diluted to 50 ml using deionised water. Adjusting pH from 5 to 7, using 0.1N NaOH or dil HCl (Cesar *et al*., 2005). The carotenoid pigments were measured at an optical density of 360 nm.

3.18.2. Heat stability

For determining the heat stability of carotenoid pigment solutions, extracts were incubated at temperatures ranging from 70 to 100ºC for 20 min and optical density of the pigment was measured at 360 nm.

3.18.3. Antioxidant treatments

Ten mg of antioxidants *viz*., ascorbic acid and butylated hydroxyl anisole were added to the 5 ml of the raw pigment extracts and incubated at different conditions i.e.,4°C, room temperature and 60°C for a period of 60 days in triplicate**.** Optical density of the pigment was measured at 360 nm.

3.18.4. Different types of oils

Stability of the yeast carotenoid pigment was also studied in nine different types of oils namely Sunflower oil, Ground nut oil, Coconut oil, Sesame oil, rice bran oil, corn

oil, mustard oil, safflower oil and palm oil. A known amount of carotenoid extract (0.2 per cent of w/w of oil) was mixed with 20 g of various oil samples were placed in 50 ml beaker and incubated at different temperatures i.e., 4 °C, room temperature and 60°C both in presence and absence of light for a period of 3rd, 45th and 90th days in triplicate. (Latha and Jeevaratnam, 2010).Optical density of the pigment was measured at 474 nm.

3.19. Safety analysis of carotenoid pigments in animal model

3.19.1. Experimental animals and housing conditions

Young male mice (approximately 17-25 g) were used for the acute toxicity studies. The animals were obtained from the KMCH College and Pharmacy, Coimbatore and maintained in their Animal House Facility. The Institute was recognized for animal studies by the ethical committee. The animals were acclimatized for approximately one week and assigned to five groups, all consisting of 6 animals per group. The animals were housed in polycarbonate cages (six mice / cage) on soft chip bedding, which was changed twice per week. Tap water was used as drinking water source for animal. The cages were housed in a room maintained at 25 ± 2ºC with a relative humidity of 60–70 per cent and exposed to a light and dark cycle of 12 h duration.

Animal experiments were carried out based on the ethical guidelines laid down by the committee for the purpose of control and supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment (Appendix III).

3.19.1.1. Diet

Mice were fed with commercial diet (Ms / Amrut – laboratory animal feed, Pranav agro industrial Ltd) obtained from Bangalore. The carotenoid pigments with different concentration were orally administered at levels based on body weight for acute and sub-acute toxicity studies. Control group was fed with sunflower oil through gavages daily. The animals were observed daily for signs of adverse effects and were weighed at the start and on 4 days intervals upto 28 days.

3.19.2. Acute toxicity study

Thirty male mice were randomly divided into five groups with six animals in each group. To the overnight fasted mice of different groups, YP 17 and YP 37 pigment was fed through oral doses of 1.0, 2.0, 3.0, 4.0 and 5.0 mg kg^{-1} body weight on the first day of experiment only. The following general behaviors were observed for first 1h and after 24 h of test drug administration *viz.*, (hypoactivity, passivity, muscle relaxation, narcosis, and ataxia), CNS activity (hyperactivity, irritability, stereotypy, tremors, convulsions, straub reaction, and analgesia) and ANS activity (ptosis, exophthalmia, urination, salivation and lacrimation). Mice were observed thoroughly for onset of any immediate toxic signs and also during the observation period of 1 week for any delayed acute effects.

3.19.3. Oral sub acute toxicity study

The male rats were randomly assigned to control and treatment groups. The mice were fed with control, 2 and 4 per cent of YP 17 and YP 37 carotenoid pigments and orally to assess the cumulative effect of low and high doses of pigments. Body weight gain was monitored and all the animals were observed thoroughly for the onset of any toxicity. At the end of the experimental period all the animals were sacrificed under light ether anesthesia.

3.19.4. Organ weight and histopathological studies

The vital organs of each rat such as brain, heart, liver, lungs, spleen, kidney, pancreas and testis were excised and weighed. Histopathological investigation of lungs, liver, brain and kidney of all experimental and control rats were performed. These tissues were separately sliced in pieces, fixed in 10 per cent formaline for 3 days; processed; stained using hematoxylin, eosin reagent and diphenyl xylene mounting fluid; mounted on glass slides and observed under power microscope.

3.19.5. Hematological study

Blood samples were collected from at least 3 rats of each group and were examined for hematological profiles like haemoglobin concentration (Hb), erythrocytes

/ Red Blood Cell (RBC), leucocytes / White Blood Cell (WBC), Packed Cell Volume (PCV), differential count and total platelet count were calculated according to the method of Bharucha *et al.* (1976).

3.19.5.1. Estimation of haemoglobin (Sahli's haematin method)

Principle

The amount of haemoglobin can be estimated by conversion of known volume of blood into acid haematin by the action of HCl and subsequent colorimetric comparison with a suitable standard.

Procedure

0.1 N HCl was added with the help of a dropper in the graduated haemoglobin meter upto its lowest marking (usually 10 %), 20 µl blood was dropped in the Sahli's pipette. The blood column was carefully adjusted without bubbles. The reaction mixtures were mixed and allowed to stand at room temperature for 10 min. The solution was diluted using distilled water by adding few drops of water carefully and by mixing reaction mixture until the colour matches the colour in the standard. The lowest meniscus of the fluid was noted in g 100 ml^{-1}

3.19.5.2. Estimation of total count of WBC

Principle

Since the normal RBC counts runs in millions, the count is made possible by diluting the sample of blood before counting and subsequently multiplying the amount by the dilution factor.

Procedure

The blood was collected from the mice and the blood was sucked up to 0.5 marks in WBC pipette and RBC diluting fluid (Turk's fluid) was taken in adequate amount and the blood was mixed thoroughly by rolling pipette horizontally in the palms, 2-3 drops was discarded, the neubauer's chamber was cleaned and filled with solution without air bubbles and focused under low power microscope, objective and WBC count was made in all corner squares. WBC count in 64 squares were counted.

WBC = Counted cells x blood dilution x chamber depth

3.19.5.3. Estimation of RBC

Principle

A known volume of blood is diluted and changed into a chamber of known dimension and the number of RBC distributed in the blood is calculated.

Procedure

Blood was taken up to 0.5 mark in the RBC pipette and Hayem's diluting fluid upto mark. The mixture was kept for 3 min and 2-3 drops of fluid was discarded from the pipette devoid of any cells.The counting chamber was charged after 3 min. The RBC in the square were marked R in figure under high power.

$$\text{RBC count} = \frac{\text{No of cells counted x dilution factor x depth factor}}{\text{Area counted}}$$

where, dilution was 1 in 200, depth was 1 in 10mm.

3.19.5.4. Packed Cell Volume (PCV)

The blood sample was well mixed and equilibrated to room temperature. Then the capillary tubes were filled with blood for 2-3rd to three quarters of their total length. The unfilled end of tube was sealed by forcing the special sealing compound into it. The filled tubes were centrifuged at 10,000 – 15, 000 rpm for 5 min. The length of the red cell column to the total length of the column of the ratio of the blood was calculated using triangulation-type haemotocrit reader. PCV was expressed in percentage.

3.19.5.5. Differential count

Differential counts were estimated using blood cell counter which include polymorphs, lymphocytes, monocytes, eosinophils, total RBC count, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC), Red cell Distribution Width (RDW) and Mean Platelet Volume (MPV).

3.19.5.6. Total platelet count

The methodology involved is similar to that of RBC counting. The count was calculated by using the formula,

Total count = Cell count x Blood dilution x Chamber depth x Area of chamber counted

3.19.6. Biochemical analysis

Plasma samples were used to estimate the various biochemical parameters such as aspartate transaminase (SGOT), alanine transaminase (SGPT), alkaline phosphatase, cholesterol and urea by using standard protocols.

3.19.6.1. Determination of Aspartate Transaminase (AST) or SGOT activity

Principle

AST catalyzes the transfer of amino group between L-aspartate and α-ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH in the presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD, was measured as a decrease in absorbance which is proportional to AST activity in the sample (Moss and Henderson, 1999).

Procedure

The following reaction components were added to a cuvette that has a 1.0 – cm light path: L-Aspartate (228 mmol l^{-1}) - 2.30 ml, NADH (4.2 mmol l^{-1}) - 0.10 ml, P-5'-P (3.4 mmol l^{-1}) - 0.10 ml, MD (36,000 U l^{-1}) - 0.05 ml, LD 72,000 U l^{-1}) - 0.05 ml and serum – 0.20 ml. Aliquots (0.2 ml) of the specimen serum were then added into the cuvettes, and the contents were mixed. The contents were preincubated in water bath at 37°C to ensure temperature equilibration and to permit side reactions to proceed and complete. The AST enzyme reaction was initiated by adding 0.2 ml of the 225-mmol l^{-1} oxoglutarate solution that has been prewarmed to 37°C. The cuvette contents were mixed rapidly and absorbance was measured at 340 nm for 5 to 8 min against a dichromate blank. The average absorbance change was calculated per min (A min^{-1}).

$$\text{AST activity in U/L } (\mu\text{mol min}^{-1}\ \text{l}^{1}) = \frac{A\triangle}{\text{min}} \times \frac{1}{6.3\text{x}10^{-3}} \times \frac{3.0}{0.20} \times 2381$$

where

3.0 = total volume in cuvette, in ml

0.2 = volume of serum specimen in ml

6.3 x 10^{-3} = micromolar asbsorptivity of NADH at 339 nm

3.19.6.2. Determination of Alanine Transaminase (ALT) or SGPT activity

Principle

ALT catalyzes the transamination from L-alanine to 2-oxoglutarate, forming L-glutamate and pyruvate. The pyruvate formed is reduced to lactate by LD with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time due to the conversion of NADH to NAD is directly proportional to AST activity (Moss and Henderson, 1999).

Procedure

The following components were pipetted into a 10-mm cuvette and equilibrated at 37ºC: L-alanine 610 mmol l^{-1} (2.30 ml), NADH 42 mmol l^{-1} (0.10 ml), P-5'-P. 34 mmol l^{-1} (0.10 ml), LD 72,000 U l^{-1} (0.10 ml), serum (0.20 ml) and made up the volume to 2.80 ml. Then 20 ml of the serum specimen was added. Then the procedure and calculations were identical to those outlined for the AST procedure. With the large quantity of LD added the endogenous (side) reactions were completed within 2 min. The ALT reaction was initiated by adding 0.2 ml of the 225-mmol l^{-1} oxoglutarate solution.

Calculation

$$\text{ALT activity in U } l^{-1} (\mu\text{mol min}^{-1} \text{ U } l^{-1}) = \frac{A\triangle}{\text{Min}} \times \frac{1}{6.3\text{x}10^{-3}} \times \frac{3.0}{0.20}$$

where,

Total volume in cuvette (ml) = 3.0

Volume of serum specimen (ml) = 0.2

Micromolar absorptivity of NADH at 339 nm = 6.3×10^{-3}

3.19.6.3. Estimation of alkaline phosphatase

Principle

The method is similar to that of King and Armstrong in which disodium phenyl phosphate is hydrolysed with liberation of phenol and inorganic phosphate. The liberated phenol is measured at 700 nm with Folin-Ciocalteau reagent (King and Armstrong, 1964).

Procedure

Buffer substrate (4.0 ml) was taken into a test tube and incubated at 37ºC for 5 min. About 0.2 ml of serum and tissue homogenate was added and incubated further for 15 min. Diluted phenol reagent of about 1.8 ml was added immediately. At the same time, a control was set up containing 4.0 ml buffer substrate and 0.2 ml serum to which 1.8 ml phenol reagent was added immediately. The mixture was mixed well and centrifuged. To 4.0 ml of the supernatant, 2.0 ml of sodium carbonate was added. Then, 4.0 ml of working standard solution was taken up and 3.2 ml of water and 0.8 ml of phenol reagent were used for blank. Then 2.0ml of sodium carbonate was added. All the tubes were incubated at 37ºC for 15 min. The colour developed was read at 700 nm. The activity of alkaline phosphatase was expressed as μmol of phenol liberated min^{-1} mg $protein^{-1}$.

3.19.6.4. Estimation of cholesterol

Principle

The extraction and oxidation of cholesterol is done by acid solution of ferric chloride. Addition of H_2SO_4 to the cholesterol and ferric chloride mixture forms coloured complex which is read at 560 nm (Zak and Ressier, 1955).

Procedure

To 0.1 ml of sample, 4.9 ml of ferric chloride precipitating agent was added and mixed well. The mixture was centrifuged and 2.5 ml of clear supernatant was collected into a dry test tube. 2.5 ml of ferric chloride diluting agent was added. Then 4 ml of conc. H_2SO_4 was added drop by drop. The blank was also prepared simultaneously by adding 5 ml of diluting reagent and treated as above. After 30 min, the intensity of the colour was read at 560 nm.

3.19.6.5. Estimation of urea

Principle

Urease breaks down urea into ammonia and carbon dioxide in alkaline medium. Ammonia liberated from the breakdown of urea reacts with hypochlorite and salicylate to form dicarboxyindophenol. This reaction is catalysed by the presence of nitroprusside. The intensity

of the colour produced by the reaction is directly proportional to the concentration of urea present in the sample and it is measured photometrically at 600 nm (Fawcett and Scott, 1960).

Reaction

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{Salicylate} + CIO \xrightarrow[\text{Nitroprusside}]{\text{Sodium}} \text{2.2 dicarboxylindophenol}$$

Procedure

About 1 ml of working reagent-1 was taken in separate test tubes for preparation of blank, standard and samples respectively. Ten µl of standard solution and samples were added separately in their respective tubes. The mixture was mixed well and incubated at 37°C for 5 min. Then, 1 ml of working reagent-2 was added in all the tubes and incubated at 37°C for 5 min. The absorbance of standard and sample were measured against the reagent blank at 600 nm within 60 min.

3.20. Application of yeast carotenoid pigments in various food items

3.20.1. Ice cream

Milk, sugar and powdered milk were poured in a bowl and heated from low simmer to medium. The mixture was stirred continuously until it was dissolved and kept in warm condition. Egg yolks were separated and whipped until it was thickened using hand mixture. One cup of hot milk mixture was added slowly to the egg yolks until it was blent. Then egg yolk mixture was poured into the bowl of hot milk and increased the heat to medium level. The mixture was stirred continuously with a plastic spoon, until it became solid. Finally the microbial colourants were added. The prepared stuff was refrigerated for 6 h.

3.20.2. Yoghurt

About 1 per cent of selected yeast carotenoid pigments were added in yoghurt and mixed well and stored in refrigerator.

3.20.3. Kulfi (Indian Milk Ice Cream)

Boiled the milk (250 ml) and kept stirring so that it did not boil over. When the milk was boiling, some condensed milk was added slowly. Add some sugar and dissolved in to the milk. Roasted walnut (5 g) and the nutmeg powder (1/2 a teaspoon) was added. Once it was thick, the consistency should be a little less thicker than the condensed milk you had added, took it off the flame. One percent carotenoid pigments were added. Poured into the ice mold and was kept under refrigerate. After half-an-hour, ice-cream mold was opened up and contents were stirred up once more ,so that no icicles formed. Freezed it again.

3.20.4. Almond Milk/Badam milk

Soaked the almonds (100 g) in water overnight. Drained the water from the almonds. Blended the almonds in four cups of fresh water. Add the vanilla extract and continue blending until well mixed. To sweeten the almond milk, honey was added to taste. Using cheesecloth (muslin) poured the almond milk through the filter into a large bowl underneath. The filter was retaining the almond pulp and the liquid went through. Squeeze the remaining pulp to remove the last of the liquid. Before refrigeration add pigment at one per cent. Almond milk could be kept in the refrigerator for 4-7 days. Shaking was needed before serving, to remix the liquid.

3.20.5. Popcorn

Pigment mixed oil was poured at 1.5 per cent to lightly coat the bottom of the pot. Turn on the burner to medium high. Place one or two kernels of popcorn into the pot, then placed the pot onto the burner. Place the lid onto the pot. Wait until you could hear the popcorn pop. This depends on the heat and size of the pan, but it was usually between 1 and 5 min. Once the popcorn has popped, make a light layer of popcorn on the bottom of the pan, not more than one kernel deep. Replace the lid and let the popcorn popped. Listen for the slowing down of popping, being careful not to burn the popcorn.

3.20.6. Evaluation of the products

The products prepared were evaluated for the colour, appearance, flavour, texture and taste of the products.

Experimental Results

CHAPTER IV

RESULTS

4.1. Isolation of pigment producing yeast

Hundred and twenty nine samples were collected from different parts of Tamil Nadu and Kerala and microbial population present in these samples were analyzed. Forest soils of, Kodai hills (24), Yercaud (9), Parambikulam ,Tiger reserve (35), Ooty (8), Mettupalayam (6), Shola forest (17), Marudhamalai (4), Manjolai forest (11) and Shervaroy hills in Eastern Ghats of Tamil Nadu and other sources such as fruits, vegetables phyllosphere of plants etc., (15) were taken for the study. Total microbial load was also enumerated and presented in Table 18. The microbial load presents in the samples show more number of pigmentation. Some soil samples loaded with good number of microbial population and also contain a pigment producing colony.

4.2. Purification and screening of yeast isolates for carotenoid pigment production

Purification of isolates was repeated thrice using repeated streaking on YM agar Petriplate to get individual pure culture. Purified five carotenoid pigment producing isolates were maintained in YM agar slants at 4ºC for further studies.

The yeast isolates were screened based on their carotenoid pigment production, which produced stable pigment even after successive sub culturing was identified. Total numbers of 129 isolates, 38 isolates were screened based on their yeast pigmentation; out of that 5 isolates were found to produce high intensity of carotenoid pigment. Generally, pigment production in the medium started at 2-3 days after inoculation. Hence, the yeast which produced the carotenoid pigments was only selected for further study. The pink and yellow coloured carotenoid pigments were absorbed maximally at 474 -510 nm and 470 nm respectively. The data are presented in Plate 1, Fig.3 and Table 19.

4.3. Characterization of yeast isolates

4.3.1. Characterization of yeast isolates based on cell morphology

Table 18. Isolation of pigment producing yeasts from various sources

S.No.	Sample	Microbial colonies (cfu × 10^4 g^{-1} of soil)	No. of pigment producing yeast	Colour of the pigment
I.	**Kodai hills**			
a.	Kodai hills			
1	Kodai hills	17	-	-
2	Kodai hills	25	-	-
3	Kodai hills	22	14	Yellow
4	Kodai hills	24	-	-
5	Kodai hills	48	-	-
6	Kodai hills	20	-	-
7	Kodai hills	43	7	Yellow
8	Kodai hills	36	-	-
9	Kodai hills	18	-	-
10	Kodai hills	28	23	Yellow
11	Kodai hills	19	-	-
12	Kodai hills	13	-	-
13	Kodai hills	50	41	Yellow
14	Kodai hills	15	-	-
15	Kodai hills	63	16	Yellow
16	Kodai hills	14	-	-
17	Kodai hills	71	-	-
18	Kodai hills	18	-	-
19	Kodai hills	27	-	-
20	Kodai hills	63	5	Brown
21	Kodai hills	19	-	-
22	Kodai hills	26	-	-
23	Kodai hills	40	-	-
24	Kodai hills	39	-	-
b.	**Eastern ghats (Shervaroy hills)**			
25	Yercaud	42	-	-
26	Yercaud	15	-	-
27	Yercaud	53	6	Yellow
28	Yercaud	14	-	-
29	Yercaud	28	-	-

Table 18. Contd..

S.No.	Sample	Microbial colonies (cfu × 10^4 g^{-1} of soil)	No. of pigment producing yeast	Colour of the pigment
30	Yercaud	16	-	-
31	Yercaud	11	-	-
32	Yercaud	19	5	Orange
33	Yercaud	16	-	-
C	**Parambikulam Tiger Reserve**			
34	Kanimara dam	18	-	-
35	Seven members died bend	12	-	-
36	Parambikulam dam site	30	18	Yellow
37	Thunnakadavu dam 1st end	46	-	-
38	Thalavettithadam	19	23	Yellow
39	Thunnakadavu dam	38	-	-
40	Thinnakal grass land	54	-	-
41	63 bit 1 Parambikulam tunnel entry	12	-	-
42	Seven members died bend	27	-	-
43	Thinnakal grass land	71	-	-
44	Deer death place	22	3	Orange
45	Second bridge	20	18	Yellow
46	63 bit 1 Parambikulam tunnel entry	29	-	-
47	63 bit 1 Parambikulam tunnel entry	42	-	-
48	Sungam	54	-	-
49	Belt area	23	-	-
50	Kannimara teak grass land	15	7	Fluorescent yellow
51	Parambikulam dam site	55	-	-
52	Anakkal vayal	37	-	-
53	Anakkal vayal	12	-	-
54	Sungam22	14	-	-
55	Thunnakadavu dam end	56	9	Light pink
56	Thunnakadavu dam end	23	-	-
57	Sungam grass land	40	-	-
58	Thunnakadavu dam end	15	-	-

Table 18. Contd..

S.No.	Sample	Microbial colonies (cfu × 10^4 g^{-1} of soil)	No. of pigment producing yeast	Colour of the pigment
59	Thunnakadavu dam end	37	-	-
60	Deer death place	29	14	Light pink
61	Deer death place	11	-	-
62	Kannimara grass land	42	-	-
63	Kannimara grass land	63	9	Yellow
64	Anakkal vayal	36	22	Pinkish orange
65	4000	62	-	-
66	Peruvaripallam grass land	14	-	-
67	Peruvaripallam grass land	19	-	-
68	4000	29	-	-
D	**Ooty**			
69	Ooty	18	2	Green
70	Ooty	24	-	-
71	Ooty	42	-	-
72	Ooty	17	-	-
73	Ooty	27	-	-
74	Ooty	35	-	-
75	Ooty	13	8	Yellow
76	Ooty	22	-	-
E	**Mettupalayam**			
77	Mettupalayam	31	-	-
78	Mettupalayam	16	9	Pink
79	Mettupalayam	28	2	Brown
80	Mettupalayam	26	-	-
81	Mettupalayam	56	-	-
82	Mettupalayam	15	-	-
83	Shola forest	54	-	-
84	Shola forest	68	-	-
85	Shola forest	23	15	Fluorescent yellow
86	Shola forest	76	24	Bright yellow
87	Shola forest	35	13	Yellow
88	Shola forest	19	-	-
89	Shola forest	31	-	-
90	Shola forest	13	3	Light pink

Table 18. Contd..

S.No.	Sample	Microbial colonies (cfu × 10^4 g^{-1} of soil)	No. of pigment producing yeast	Colour of the pigment
91	Shola forest	25	7	Yellow
92	Shola forest	34	-	-
93	Shola forest	18	-	-
94	Shola forest	29	2	Fluorescent yellow
95	Shola forest	47	-	-
96	Shola forest	43	-	-
97	Shola forest	76	15	Yellow
98	Shola forest	93	-	-
99	Shola forest	48	-	-
F	**Manjsolai forest**			
100	Manjsolai forest	68	13	Pink
101	Manjsolai forest	55	-	-
102	Manjsolai forest	26	-	-
103	Manjsolai forest	17	-	-
104	Manjsolai forest	16	16	Fluorescent yellow
105	Manjsolai forest	65	-	-
106	Manjsolai forest	39	-	-
107	Manjsolai forest	39	-	-
108	Manjsolai forest	48	7	Pink
109	Manjsolai forest	29	-	-
110	Manjsolai forest	56	-	-
g	**Marudhamalai**			
111	Marudhamalai	37	-	-
112	Marudhamalai	24	-	-
113	Marudhamalai	18	-	-
114	Marudhamalai	14	3	Bright yellow
H	**Fruit ,vegetable and other sources**			
115	Grapes	17	-	-
116	Sugarcane juice	36	-	-
117	Pomegranate	53	-	-

Table 18. Contd…

S.No.	Sample	Microbial colonies (cfu × 10^4 g^{-1} of soil)	No. of pigment producing yeast	Colour of the pigment
118	Sapota	12	14	Red
119	Carrot	21	-	-
120	Tomato	6	11	Red
121	Honey	18	-	-
122	Curd	31	-	-
123	Rice flour	7	-	-
124	Cassava bagasse	75	23	Fluorescent yellow
125	Sugarcane leaf	21	8	Yellow
126	Rice leaf	42	13	Pink
127	Maize leaf	20	-	-
128	Cabbage leaf	10	12	Yellow
129	Carrot leaf	36	-	-

Table 19. Screening of yeast isolates for carotenoid pigment production

S. No.	Microbial Isolates	Cell dry weight (g/100 ml)	Carotenoid pigment yield (µg g^{-1})		Colour of the pigment	Incubation time (days)
			470 nm	500 nm		
1	MP1[a]	0.76	171	182	Yellow	1
2	MP2[a]	0.81	123	135	Yellow	2
3	MP3[a]	0.54	189	201	Yellow	1
4	MP4[a]	0.39	111	135	yellow	3
5	MP5[a]	0.65	145	169	Yellow	3
6	MP6[a]	0.40	176	187	brown	1
7	MP7[b]	0.31	123	139	yellow	1
8	MP8[b]	0.71	112	132	Orange	2
9	MP9[c]	0.87	156	178	Yellow	2
10	MP10[c]	0.77	141	165	yellow	2
11	MP11[c]	0.47	189	197	orange	3
12	MP12[c]	0.81	166	171	Yellow	2
13	MP13[c]	0.71	126	141	Fluorescent yellow	3
14	MP14[c]	0.75	111	126	Light pink	3
15	MP15[c]	0.67	203	221	Light pink	2
16	MP16[c]	0.86	132	153	Yellow	3
17	MP17[c]	0.79	272	291	Pinkish orange	2
18	MP18[d]	0.41	126	134	Green	4
19	MP19[d]	0.78	242	254	Yellow	2
20	MP20[e]	0.91	192	176	Pink	2
21	MP21[e]	0.51	175	197	Brown	3
22	MP22[f]	0.32	178	194	Fluorescent yellow	3
23	MP23[f]	0.80	249	263	Bright yellow	2
24	MP24[f]	0.91	121	137	Yellow	2
25	MP25[f]	0.48	164	178	Light pink	3
26	MP26[f]	0.27	94	101	Yellow	1
27	MP27[f]	0.56	29	42	Fluorescent Yellowish	2
28	MP28[f]	0.73	125	145	Yellow	1
29	MP29[g]	0.64	163	187	Pink	3
30	MP30[g]	0.69	171	182	yellow	2

Table 19. Contd…

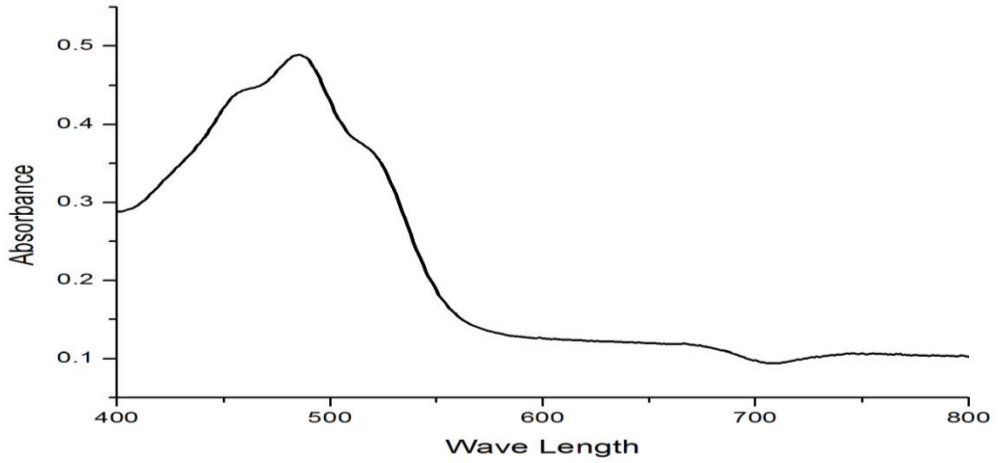
31	MP31[g]	0.84	127	139	Pink	2
32	MP32[g]	0.55	187	208	Bright yellow	3
33	MP33[i]	0.76	147	162	Red	2
34	MP34[i]	0.59	172	195	Red	3
35	MP35[i]	0.41	114	131	Yellow	3
36	MP36[i]	0.69	129	145	Yellow	2
37	MP37[i]	0.78	268	279	Pink	1
38	MP38[i]	0.79	145	167	yellow	3

a – Kodai hills, b – Yercaud, c – Parambikulam Tiger Reserve, d – Ooty, e – Mettupalayam,

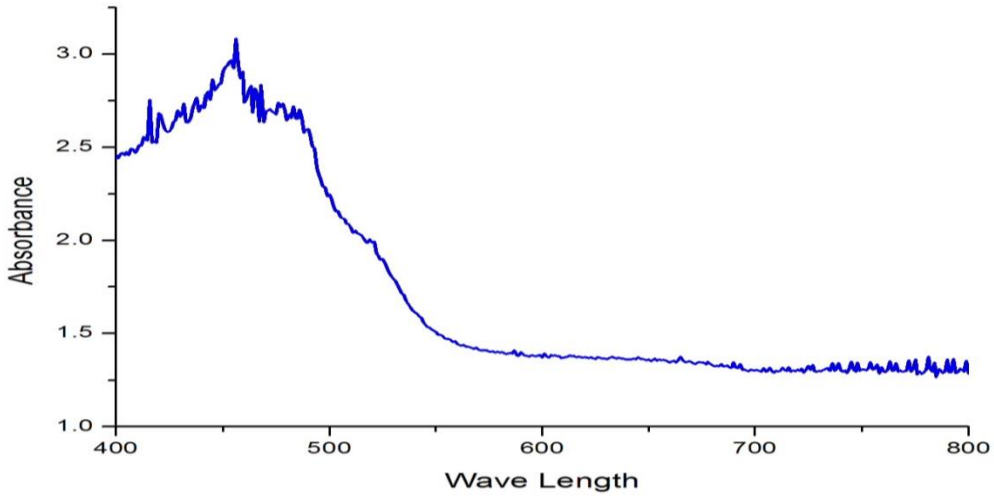
f –shola forest, g – Manjsolai forest, h – Marudhamalai, i – Coimbatore

nd- not determined

Fig 3. Spectrometric scanning of yeast carotenoid pigments

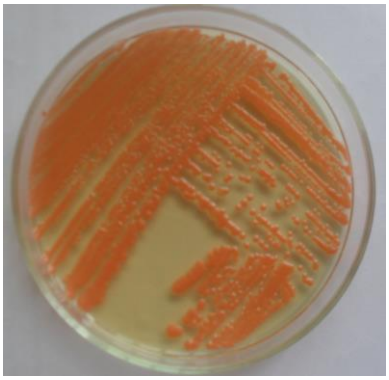


***Sporobolomyces* sp. pigment**



***R. mucilaginosa* pigment**

Plate 1. Growth of efficient carotenoid producing yeast isolates in YM agar media



***R. mucilaginosa* (YP 17)**

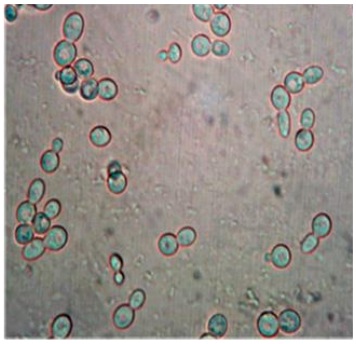


***Sporobolomyces* sp. (YP 37)**

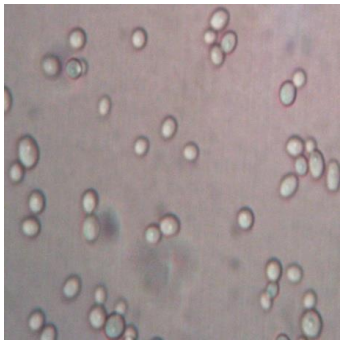


MTCC 9316

Plate 2. Microscopic view of the yeast isolates



***R. mucilaginosa* (YP 17)**



***Sporobolomyces* sp. (YP 37)**



MTCC 9316

Five yeast isolates were identified based on their cell morphological characters *viz*., cell shape, budding pattern, ascospore formation and presence of special structures (pseudohyphae). The results are presented in Table 20 and Plate 2. Yeast isolates (YP15, YP17, YP19, YP23, YP 37 and MTCC 9316) has medium, spherical to oval shaped cells whereas in liquid medium these isolates formed pellicle. The production of spores was not observed in the analyzed cultures. All the isolates formed sediment in liquid medium. The special structure like pseudohyphae was absent in all the yeast isolates except YP 37. Isolates such as YP 17, YP 19 and YP 37 resembled the standard strain MTCC 9316 appeared as glistening while YP 15 and YP 23 appeared as dull.

4.3.2. Biochemical characterization of yeast isolates

Biochemical characters of yeast isolates were assessed using the identification keys (Kergar, 1984). All the yeast isolates were found to grow on glucose, sucrose, maltose and galactose, but none of them utilized lactose. None of the yeast isolates were able to grow in the medium containing $CaCO_3$ agar and were negative for acid production test (Plate 3a). YP 15, YP 17, YP 19, YP 23, YP 37 and standard culture MTCC 9316 produced positive results for urease activity (Plate 3b). All the yeast isolates are able to produce ester compounds and showed positive results for ester production. All the yeast isolates were reported as negative results for tolerance to one per cent acetic acid (Table 20; Plate 3c).

4.4. Yeast identification by 18S rRNA sequence analysis

An almost complete 18S rRNA gene sequence of YP17 and YP 37 was obtained (609 and 1221 bp). The 18S rRNA sequence based phylogenetic analysis of YP 17 and YP 37 revealed that the isolates were more closely related to *Rhodotorula mucilaginosa* and *Sporobolomyces* sp. exhibited 99% and 96% homology with type strain of *Rhodotorula mucilaginosa* EF190221.1 and *Sporobolomyces* sp.N255485.1. The 18S rRNA sequences of *Rhodotorula mucilaginosa* was deposited in the GenBank database under accession no. KF164613. Evolutionary tree based on 18S rRNA with neighbor-joining method, strain YP17 and YP37 formed a stable clade with *Rhodotorula mucilaginosa* EF190221.1 and *Sporobolomyces* sp.N255485.1, which was supported by strong bootstrap value (96% for *R. mucilaginosa* and 95% for *Sporobolomyces* sp.).This neighbour-joining phylogenetic tree

Table 20. Morphological and cultural characteristics of intracellular pigment producing yeast

Morphological, physiological and biochemical character	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316
Shape	Spherical	Ovoid	Spherical	Ovoid	Ovoid	Ovoid
Budding pattern	Monopolar	Multipolar	Monopolar	Monopolor	Monopolor	Multipolar
Ascospore formation	Absent	Absent	Absent	Absent	Absent	Absent
Presence of pseudohyphae	Absent	Absent	Absent	Absent	Present	Absent
Pigmentation	Light pink	Pinkish orange	Yellow	Bright yellow	Pink	Pinkish orange
Appearance	Dull	Glistening	Glistening	Dull	Glistening	Glistening
Growth in liquid medium (Sediment formation)	Significant	Significant	Significant	Significant	Significant	Significant
Growth in liquid medium (Pellicle formation)	+	+	+	-	+	+
Fermentation of carbohydrates a. D- Glucose	+	+	+	+	+	+
b. Lactose	+	-	-	-	-	-
c. Sucrose	+	+	+	+	+	+
d. Maltose	+	+	+	+	+	+
e. D-Galactose	+	+	+	+	+	+
Acid production from glucose	+	+	+	_	_	+
Urease activity	+	+	+	+	+	+
Ester production	_	_	_	_	_	_
Tolerance to 1 per cent acetic acid	_	_	_	_	_	_

(+) positive growth, (-) Negative growth

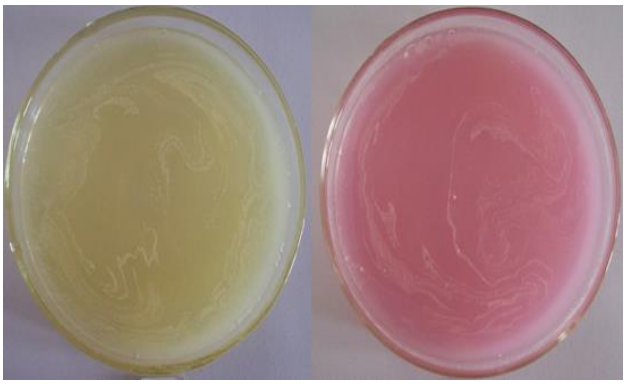
Plate 3. Biochemical characters

3a. Acid production



Control (-)

3b. Urease activity



(-) (+)

3c. Tolerance to 1 per cent acetic acid



(+) (-)

(+) Positive
(-) Negative

indicated that species of *R. mucilaginosa* and *Sporobolomyces* sp. are separated into two groups by *Rhodotorula mucilaginosa* and *Sporobolomyces pararoseus* (Plate 4, 5 and Table 21).

Table 21. Effective intracellular carotenoid pigment producing yeast isolates

S. No	Sample code	Identified yeast isolates
1	YP 17	*Rhodotorula mucilaginosa*
2	YP 37	*Sporobolomyces* sp.

4.5. Growth and intracellular carotenoid pigment production of yeast on different media

The growth and carotenoid pigment production of the screened yeast isolates were studied in eight different media and it was found that yeast extract dextrose medium supported yeast growth and pigment production for all the isolates. In general, the growth and pigment production of all yeast isolates were moderate in other growth media *viz*., czapek yeast agar, glucose yeast peptone and czapek- dox medium. Malt extract medium was not favorable for pigment production and growth of all the cultures. Potato dextrose, sabouraud dextrose and yeast malt extract supported the growth of cultures but pigment production was moderate (Table 22).

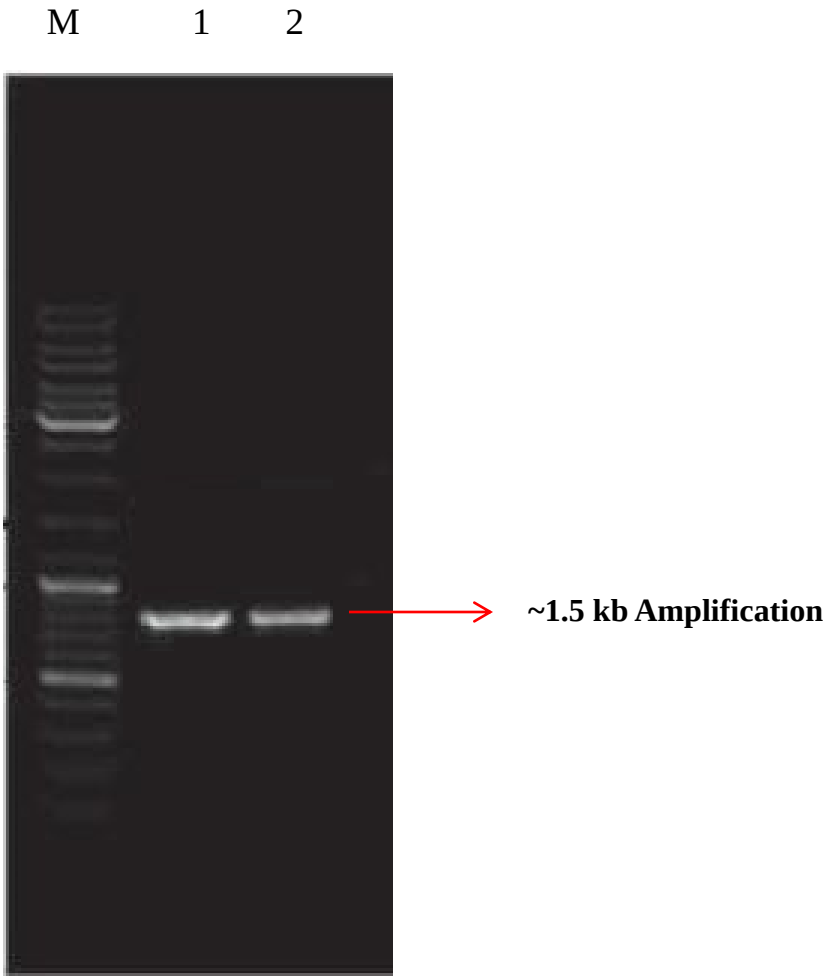
4.6. Antioxidant activity of yeast carotenoid pigments

4.6.1. *In vitro* antioxidant activity

The pigment of five yeast cultures along with standard culture were extracted with acetone and freeze dried. The pigment extract was used for analysis of antioxidant activity to a concentration of 1 mg ml^{-1} and were then diluted to 20, 60, 80 and 100 µg ml^{-1} for further uses.

4.6.1.1. Ferric Reducing Antioxidant Power Activity (FRAP)

Plate 4. PCR profile of *R. mucilaginosa* (YP 17) and *Sporobolomyces* sp. (YP 37) yeast isolates



Lane description:
Lane 1 and 2: PCR Amplification of sample
TM Lane M: StepUp 700bp DNA ladder

Plate 5. Neighbour joining phylogenetic tree showing the relationship of the yeast isolates (YP17 and YP 37) and related species retrieved from GeneBank

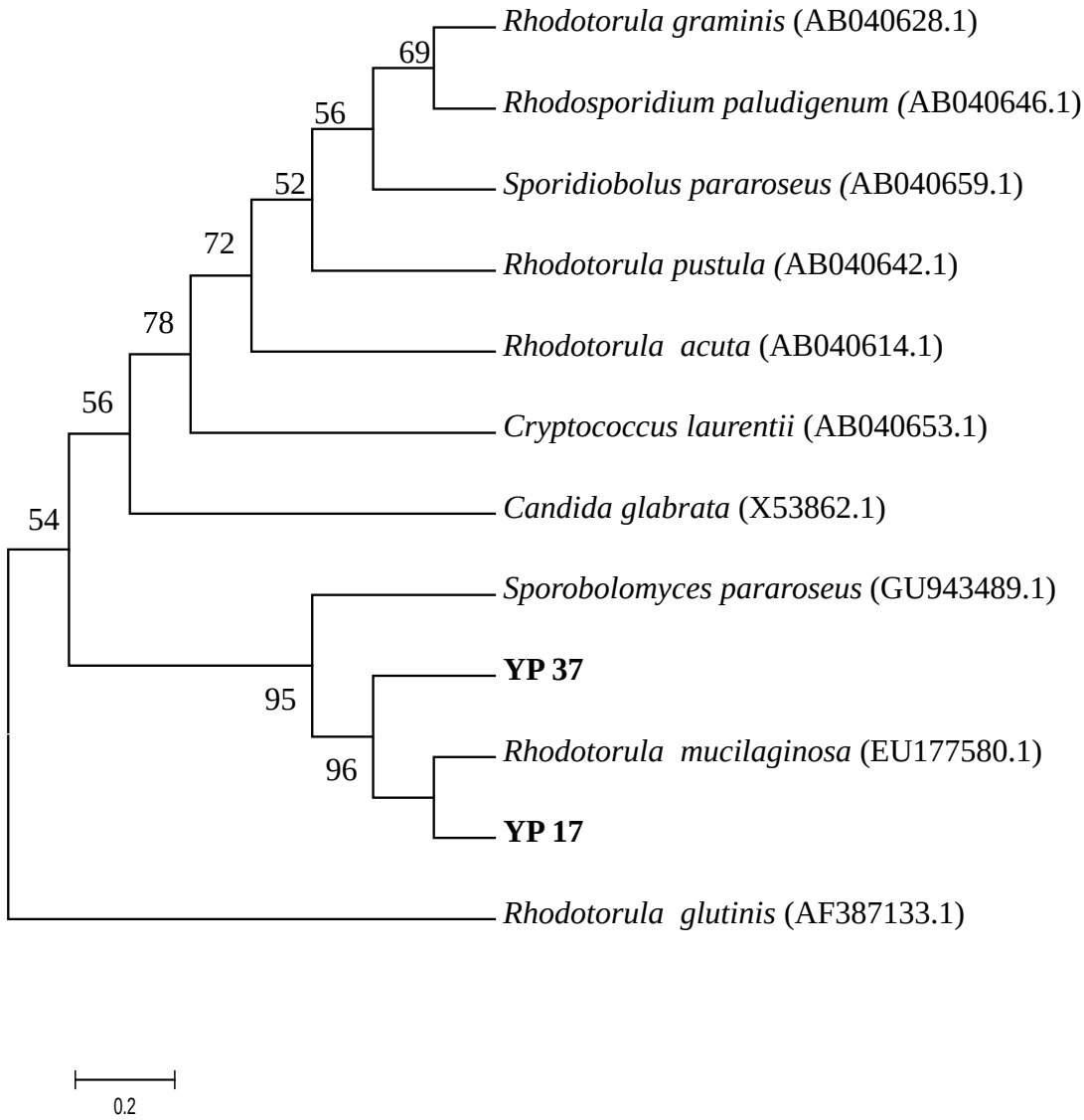


Table 22. Growth and pigmentation of yeast on different media

Different media	YP 15		YP 17		YP 19		YP 23		YP 37		MTCC 9316	
	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 455 nm	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 470 nm	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 455nm	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 470 nm	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 470 nm	CDW (100 g l^{-1})	Pigment (µg g^{-1}) at 470 nm
Yeast malt extract	0.65	202.23	0.79	271.10	0.78	200.20	0.70	222.21	0.74	267.32	0.80	270.44
Potato dextrose	0.60	101.43	0.72	272.30	0.68	112.70	0.61	190.67	0.73	258.14	0.69	273.03
Czapek-dox	0.79	189.66	0.85	280.60	0.80	203.40	0.77	219.60	0.78	275.40	0.81	280.11
Glucose yeast peptone	0.73	224.56	0.81	289.40	0.82	210.60	0.68	253.00	0.82	278.33	0.76	287.10
Sabouraud dextrose	0.80	164.23	0.77	271.80	0.84	158.60	0.74	166.42	0.75	262.87	0.75	270.41
Malt extract	0.22	0.0	0.11	0.0	0.0	0.0	0.0	0.0	0.15	0.0	0.0	0.0
Czapek yeast agar	0.69	100.58	0.71	279.20	0.77	197.47	0.71	222.47	0.77	270.63	0.74	280.14
Yeast extract dextrose	0.70	236.11	0.82	293.12	0.85	238.43	0.78	260.40	0.81	287.42	0.79	288.17

The FRAP assay was selected to be used as the first step in this investigation, because intrinsic antioxidant potential of an extract could be measured, taking antioxidant constituents as reductants in a redox-linked colorimetric reaction. The antioxidant power of extracts was compared with ascorbic acid as a reference standard. The FRAP values of pigment extracts ranged from 0.21 to 10.81 mM μl^{-1} at a concentration of 20, 60, 80 and 100 µg ml^{-1} against ascorbic acid (7.66 to 12.53 mM μl^{-1}).The pigment extracts of YP17, YP 37and MTCC 9316 expressed a significant ability to reduce Fe^{3+} (10.81, 9.99 and 10.02 mM μl^{-1}) at 100 µg ml^{-1} concentration and thus ability to donate electrons. This property suggested that YP17 and YP 37 carotenoid pigment extracts may act as a free radical scavenger, capable of transforming reactive free radical species into stable non-radical products (Table 23 and Fig. 4).

4.6.1.2. Inhibition of lipid peroxidation

This is the most commonly used method for determining antioxidant activity to measure the inhibiton of linoleic acid. The antioxidant activities of the carotenoid pigments increase with increased concentration of the pigment extracts of YP 17, YP 37 and standard strain MTCC 9316 which had exhibited higher antioxidant activities than other yeast carotenoid pigments (Table 24 and Fig. 5). At 20 µg ml^{-1}, YP17, YP 37 and standard strain exhibited 16.53 ,15.01 and 15.71 per cent of antioxidant activity respectively, whereas YP23 showed antioxidant activity of 7.25 per cent at 20 µg ml^{-1}. YP19 and YP15 pigment extracts recorded low antioxidant activities. However, antioxidant activity of standard BHA was 32.37, 44.01, 60.91 and 70.35 per cent at 20, 60, 80 and 100 µg ml^{-1} respectively.

4.6.1.3. DPPH radical scavenging activity

The antioxidant activity has been evaluated in a short time by the ability of pigment extracts to reduce the stable DPPH free radicals. Due to its unpaired electron, DPPH radical gives a strong absorption band at 517 nm (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The DPPH scavenging activities of different concentrations of acetone extracts of carotenoid pigments and BHA results are shown in Table 25 and Fig. 6.

Table 23. Ferric reducing antioxidant power activity of acetone extracts of yeast pigments

Pigment concentration (µg ml^{-1})	FRAP activity (mM µl^{-1})						
	Pigment producing yeast isolates						Ascorbic acid
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	
20	0.39 ± 0.008	4.70 ± 0.095	0.21 ± 0.004	1.33 ± 0.026	4.42 ± 0.089	4.66±0.094	7.66 ± 0.155
60	0.89 ± 0.020	7.72± 0.178	0.44 ± 0.002	1.42 ± 0.032	6.47 ± 0.149	6.59±0.152	10.14± 0.234
80	0.97 ± 0.022	8.79± 0.203	0.72 ± 0.016	1.74 ± 0.040	7.57 ± 0.174	7.01±0.161	11.22± 0.259
100	1.09 ± 0.025	10.81±0.249	0.99 ± 0.022	2.78 ± 0.064	9.99 ± 0.230	10.02±0.225	12.53± 0.289
SEd	0.0286	0.2687	0.0203	0.0614	0.2389	0.2338	0.3390
CD (p = 0.05)	0.0659	0.6196	0.0468	0.1416	0.5509	0.5391	0.7817

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Fig 4. Ferric reducing antioxidant power of yeast pigments

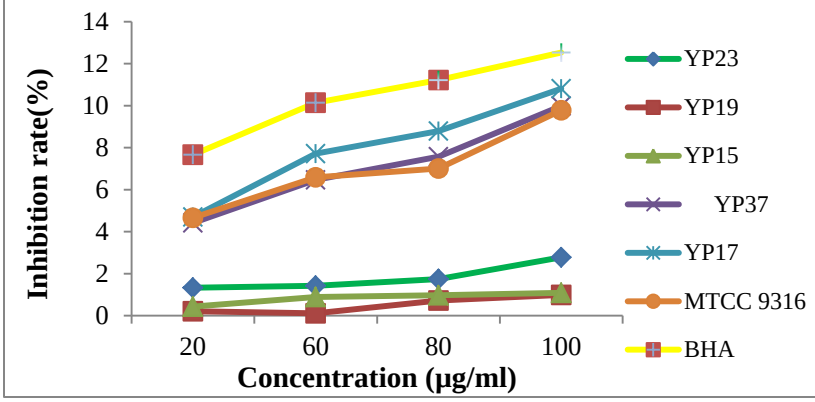


Fig 5. Lipid peroxidation activity of yeast pigments

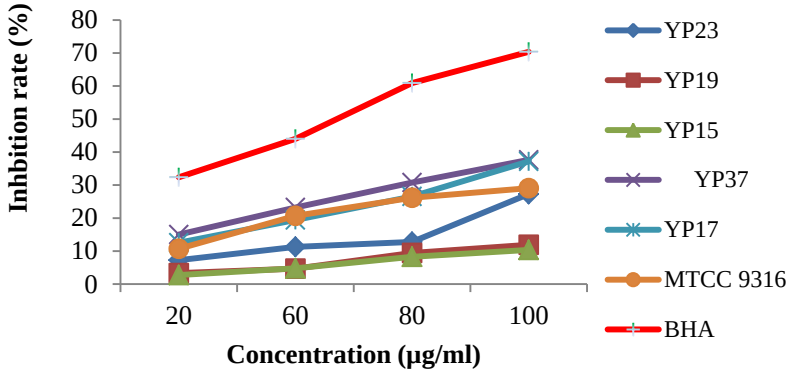


Fig 6.DPPH scavenging activity of yeast pigments

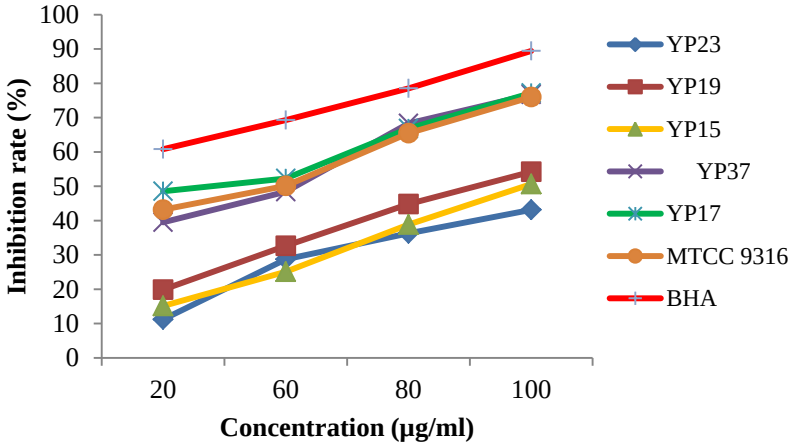


Table 24. Inhibition of lipid peroxidation of acetone extracts of yeast pigments

Pigment concentration (µg ml^{-1})	Inhibition of lipid peroxidation (%)						
	Pigment producing yeast isolates						BHA
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	
20	2.77 ± 0.056	16.53 ± 0.254	3.32 ± 0.067	7.25 ± 0.147	15.01 ± 0.304	15.71±0.217	32.37 ± 0.656
60	4.76 ± 0.109	19.44 ± 0.448	4.71 ± 0.108	11.30 ± 0.260	23.22 ± 0.536	20.66±0.477	44.01 ± 1.01
80	8.29 ± 0.191	26.53 ± 0.612	9.44 ± 0.218	12.77± 0.294	30.76 ± 0.710	26.18±0.604	60.91± 1.406
100	10.38 ± 0.239	37.15 ± 0.857	11.91 ± 0.275	27.25± 0.629	37.66 ± 0.869	36.05±0.670	70.35 ± 1.624
SEd	0.2338	0.8299	0.2641	0.5351	0.9059	0.7384	1.7438
CD (p = 0.05)	0.5392	1.9138	0.6091	1.2340	2.0890	1.7027	4.0214

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 25. DPPH scavenging activity of acetone extracts of yeast pigments

Pigment concentration (µg ml^{-1})	DPPH scavenging activity (% inhibition)						
	Pigment producing yeast isolates						
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	BHA
20	15.12 ± 0.056	48.53 ± 0.983	19.87± 0.402	11.18 ± 0.226	39.51 ± 0.801	43.18±0.875	60.82 ± 1.233
60	25.10 ± 0.109	52.25 ± 1.206	32.62± 0.753	28.72± 0.663	48.39 ± 1.117	50.11±1.157	69.30 ± 1.600
80	38.82± 0. 191	66.80 ± 1.542	44.82± 1.035	36.28± 0.837	68.33 ± 1.578	65.48±1.512	78.51± 1.813
100	50.66 ± 0.239	77.18 ± 1.782	54.24± 1.252	43.14± 0.996	75.04 ± 1.772	76.72±1.755	86.44± 2.065
SEd	1.1407	1.9976	1.2981	1.0454	1.9393	1.9332	2.4120
CD (p = 0.05)	2.6305	4.6066	2.9935	2.4108	4.4720	4.4580	5.5622

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

The carotenoid pigment of YP17, YP 37and MTCC 9316 had significant scavenging effects on the DPPH radical, which increased with increasing concentration from 20 - 100 µg ml^{-1}. The DPPH scavenging activity was more in YP 17 (77.18 %), followed by YP 37 (76.04 %) and MTCC 9316 (75.72 %) and other cultures. The antioxidant activity of YP17 and YP 37 was almost comparable to BHA.

4.6.1.4. Reducing power assay

In the reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (antioxidants) causes the reduction of the Fe^{3+}/ ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+}concentration. Reducing power of the pigment extracts increased in a dose dependent manner. The rate of increase was found to be high for the pigment extract of YP17 and low for the pigment extract of YP 15. At 20 µg ml^{-1} concentration, reducing power of the carotenoid pigment extracts of YP 17 was 1.03. At 100 µg ml^{-1}, the reducing power of pigment was 1.97. However, reducing power of BHA were 1.30 at 20 µg ml^{-1} and as the concentration increases it followed the same pattern as pigment of YP 37 (Table 26 and Fig. 7).

4.6.1.5. Hydroxyl radical scavenging assay

The hydroxyl radical is one of the representative reactive oxygen species generated in the body system. The results of this assay revealed that all the pigment extracts at the concentration of 100 µg ml^{-1} were considerably more active. The carotenoid pigment was found to possess the hydroxyl radical scavenging activity in a dose dependent manner and out of the different culture extracts, YP17 pigment extract was found to be the most active one. It exhibited a maximum of 74.74 per cent activity at 100 µg ml^{-1} followed by MTCC 9316 (74.01%) > YP37 (72.21 %) > YP 23 (66.32 %) > YP 19 (21.56 %)>YP 15 (20.63%) (Table 27 and Fig. 8).

4.6.1.6. Nitric oxide radical scavenging activity

Table 26. Reducing power activity of acetone extracts of yeast pigments

Pigment concentration (µg ml^{-1})	Reducing power activity						
	Pigment producing yeast isolates						BHA
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	
20	0.13 ± 0.002	1.03 ± 0.020	0.49 ± 0.009	0.69 ± 0.013	0.99 ± 0.020	0.97±0.019	1.30± 0.026
60	0.27 ± 0.006	1.61 ± 0.037	0.54 ± 0.012	0.78 ± 0.018	1.56 ± 0.036	1.37±0.031	1.42± 0.032
80	0.45 ± 0.010	1.83 ± 0.042	0.77 ± 0.017	0.84 ± 0.019	1.78 ± 0.041	1.69±0.039	1.59± 0.036
100	0.51 ± 0.011	1.97 ± 0.045	0.89 ± 0.020	1.06 ± 0.024	1.88± 0.043	1.92±0.044	1.84± 0.042
SEd	0.0121	0.0533	0.0223	0.0273	0.0516	0.0494	0.0496
CD (p = 0.05)	0.0279	0.1228	0.0514	0.0631	0.1189	0.1139	0.1144

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Fig 7. The reducing ability of yeast pigments

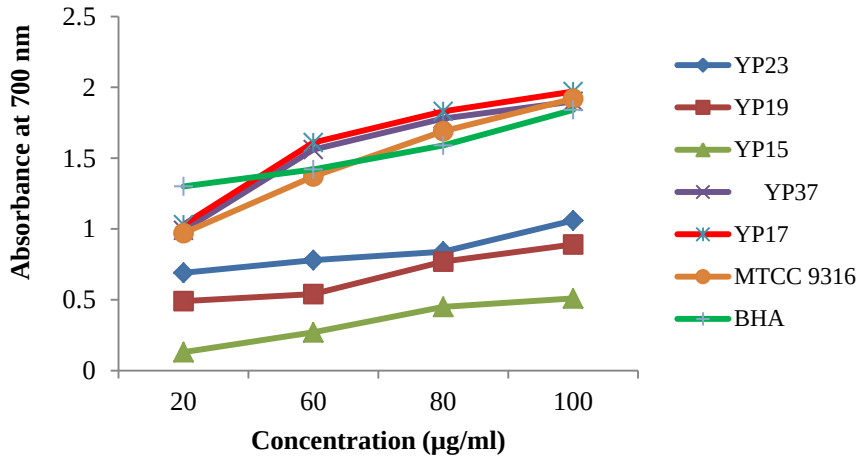


Fig 8. Hydroxy radical scavenging ability of yeast pigments

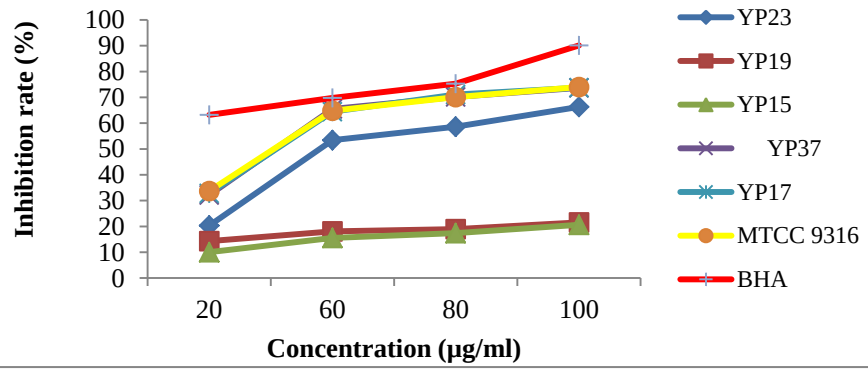


Fig 9. Nitric oxide scavenging ability of yeast pigments

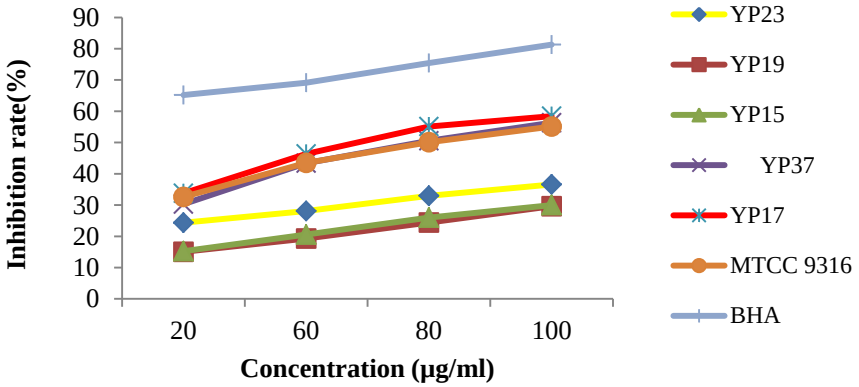


Table 27. Hydroxyl radical scavenging activity of acetone extracts of yeast pigments (% inhibition)

Pigment concentration (µg ml^{-1})	Hydroxyl radical scavenging activity (% inhibition)						
	Pigment producing yeast isolates						
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	BHA
20	10.02± 0.203	33.78 ± 0.664	14.33 ± 0.290	20.30 ±0.411	32.17 ±0.652	33.70±0.683	63.23± 1.282
60	15.53 ± 0.358	64.44 ± 1.488	18.11± 0.418	53.41 ±1.233	65.48 ±1.512	64.82±1.496	69.80± 1.611
80	17.42 ± 0.402	71.11 ± 1.642	18.94± 0.437	58.64 ±1.354	70.16 ±1.620	70.11±1.619	75.28± 1.738
100	20.63 ± 0.476	74.74 ± 1.702	21.56± 0.497	66.32 ±1.539	72.21 ±1.702	74.01±1.709	90.11± 2.081
SEd	0.5286	2.0314	0.5910	1.7133	2.0293	2.0311	2.4078
CD (p = 0.05)	1.2189	4.6845	1.3629	3.9508	4.6796	4.6838	5.5525

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 28. Nitric oxide anion radical scavenging activity of acetone extracts of yeast pigments

Pigment concentration (µg ml^{-1})	Nitric oxide anion radical scavenging activity (% inhibition)						
	Pigment producing yeast isolates						BHA
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	
20	15.27± 0.309	33.78 ± 0.689	15.02 ± 0.304	24.33±0.493	30.30±0.614	32.61±0.661	65.21 ± 1.322
60	20.58± 0.475	46.37 ± 1.070	19.31 ± 0.445	28.11±0.649	43.41 ± 1.00	43.48±1.004	69.11 ± 1.596
80	26.01± 0.600	55.12± 1.272	24.42 ± 0.563	32.94±0.760	50.56 ± 1.16	50.11±1.157	75.42 ± 1.741
100	30.00± 0.692	58.44± 1.349	29.63 ± 0.684	36.56±0.844	55.32±1.300	55.12±1.272	81.33 ± 1.878
SEd	0.7624	1.5902	0.7341	0.9890	1.4896	1.4841	2.3299
CD (p = 0.05)	1.7582	3.6671	1.6929	2.2807	3.4350	3.4224	5.3728

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

The nitric oxide radical scavenging activity of all the five yeast carotenoid pigment along with standard culture were less than that of BHA, which was used as reference for all the assays. The percentage inhibition of nitric oxide generation by carotenoid pigments is shown in Table 28 and Fig. 9. At 100 µg ml^{-1} concentration the pigment of YP 17, YP 37and MTCC 9316 exhibited higher activity of 58.44 per cent, 55.32 per cent and 55.12 per cent inhibition respectively. Whereas BHA standard recorded 81.33 per cent of inhibition.

4.6.1.7. Fe^{2+} chelating ability of yeast carotenoid pigment

At 20 µg ml^{-1}, the chelating abilities of pigment from YP 15, YP 17, YP 19, YP 23, YP 37 and MTCC 9316 were 10.87, 14.79, 15.52, 29.64, 28.21 and 29.11 per cent respectively. The chelating ability of the yeast carotenoid pigment on ferrous ions increases depending on the concentration of the pigments. However, EDTA was as excellent chelating agent for ferrous ions and its chelating ability was 83.86 per cent at 100 µg ml^{-1}. The higher concentration of Fe^{2+} chelation ability was observed in the concentration of 20 µg ml^{-1} (54.32 per cent) (Table 29 and Fig. 10).

4.7. Antimicrobial activities of carotenoid pigments against human pathogens

The antimicrobial activities of the five different yeast carotenoid pigments were tested against bacterial pathogens by agar dilution method. All tested carotenoid pigments exerted different levels of inhibitory effects against pathogenic microbes.

4.7.1. Gram positive bacteria

The bacterial pathogen *Staphylococcos aureus* was found to be highly inhibited by the pigment extracts of YP 17, YP 37 and MTCC 9316 with the inhibition zones of 2.7, 2.6 and 2.6cm respectively, this was comparable to the activity of chloramphenicol (2.9 cm). Whereas the other yeasts pigment extracts possessed less activity against *S. aureus*.

The pigment extracts of YP 17, YP 37 and MTCC 9316 possessed good activity against *Streptococcus faecalis* (2.6, 2.2 and 2.5 cm) and the other carotenoid pigments YP 15, YP 19 and YP 23 with the inhibition zones of 1.3, 1.4 and 1.5 cm. Pathogens like *Enterococcus* sp. and *B. subtilis* were more effectively inhibited by the carotenoid pigment extracts of YP 37 (1.9 and 2.3 cm), YP 17 (2.0 and 2.0 cm), MTCC 9316 (1.8 and 2.0 cm)

Table 29. Metal chelation activity of acetone extracts of yeast pigments (% inhibition)

Pigment concentration (µg ml^{-1})	Iron chelation activity (% inhibition)						
	Pigment producing yeast isolates						EDTA
	YP 15	YP 17	YP 19	YP 23	YP 37	MTCC 9316	
20	10.87 ± 0.220	28.21±0.571	14.79±0.299	15.52 ± 0.314	29.64 ± 0.600	29.11±0.590	54.32±1.101
60	14.99± 0.346	36.56±0.844	15.98±0.369	19.27 ± 0.445	38.21 ± 0.882	38.18±0.881	69.18±1.597
80	18.07± 0.417	48.49±1.119	16.62±0.385	22.10 ± 0.510	50.91 ± 1.175	53.26±1.229	77.54±1.790
100	19.51± 0.450	59.73±1.379	18.23±0.385	28.72 ± 0.663	59.32 ± 1.369	60.77±1.403	83.86±1.936
SEd	0.5223	1.4486	0.5253	0.7062	1.4831	1.5179	2.3155
CD (p = 0.05)	1.2044	3.3405	1.2114	1.6285	3.4200	3.5004	5.3395

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Fig 10. Metal chelation of yeast pigments

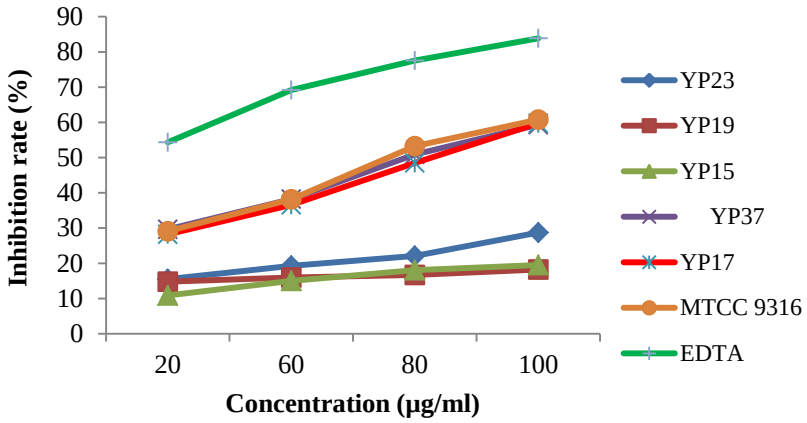


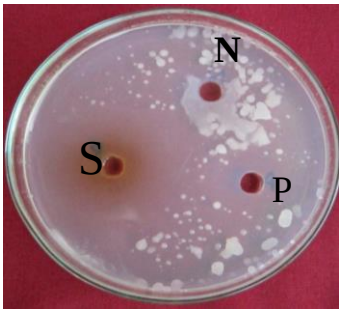
Table 30. Antimicrobial activity of yeast pigment extracts against human bacterial pathogens

Bacterial pathogens	Diameter of the inhibition zone (cm)							
	Pigment producing yeast isolates						Control	
	YP15	YP17	YP19	YP23	YP37	MTCC 9316		
Gram positive							PC	NC
Enterococcus sp.	NI	2.0	1.2	1.5	1.9	1.8	1.8	0.3
S. aureus	0.9	2.7	1.0	2.0	2.6	2.6	2.9	0.0
S. faecalis	1.3	2.6	1.4	1.5	2.2	2.5	2.0	0.2
B. subtilis	1.0	2.0	1.5	1.6	2.3	2.0	2.9	0.1
Gram negative								
E. coli	1.8	3.0	1.9	2.0	2.9	2.8	2.8	0.1
P.aeruginosa	NI	2.1	NI	1.0	2.1	2.0	2.6	0.0

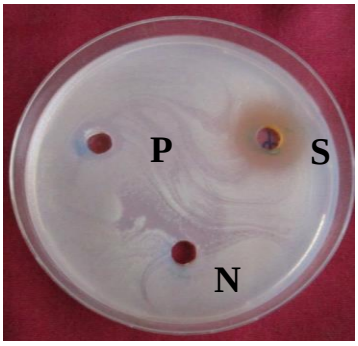
PC = Positive control (Chloramphenicol) ; NC = Negative control (Acetone) ; NI = No inhibition

Plate 6. Antimicrobial activity of carotenoid pigment extract from *R. mucilaginosa* and *Sporobolomyces* sp. against bacterial pathogens

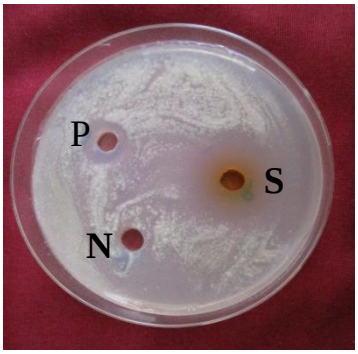
***R. mucilaginosa* pigment**



Staphylococcus aureus



Streptococcus aureus

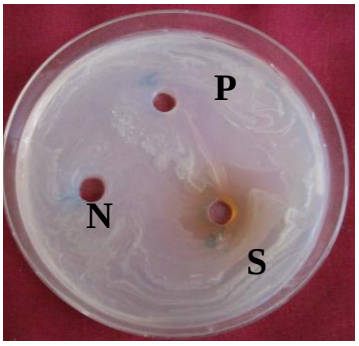


Escherichia coli

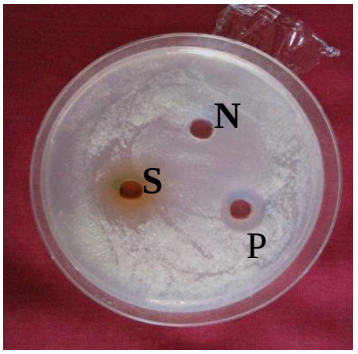
***Sporobolomyces* sp. pigment**



Staphylococcus aureus



Streptococcus aureus



Escherichia coli

S-Sample (Carotenoid pigment)

N-Negative control (100 % acetone)

P-Positive control (Chloramphenicol)

followed by YP 23 (1.5 and 1.6 cm), YP 19 (1.2 and 1.5 cm) and YP 15 (0.0 and 1.0 cm) (Table 30 and Plate 6)

4.7.2. Gram negative bacteria

The carotenoid extracts of YP 17 and MTCC 9316 produced inhibition zones of (3.0 and 2.1 cm) (2.8 and 2.0 cm) against *E. coli* and *P. aeruginosa* respectively and the carotenoid pigment of YP 37 produced the inhibition zone of 2.9 and 2.3 cm respectively. The pigment extracts of YP 23 was also found to inhibit these organisms effectively with inhibition zones of 2.0 and 1.0 cm respectively. The other pigment extracts exhibited lesser activity against the *E.coli* and showed no inhibitory activity against *P. aeruginosa*. The standard antibiotic chloramphenicol produced inhibition zones of 2.8 and 2.6 cm against *E. coli* and *P. aeruginosa* accordingly (Table 30 and Plate 6)

Out of five carotenoid pigment producers, YP 17 and YP 37 exerted higher inhibition zones in both Gram positive and Gram negative organisms. The zones were better than that of positive control. In case of *E. coli* and *S. faecalis*, both cultures outperformed than antibiotic control even after deducting negative acetone control.

4.7.3. Minimum inhibitory concentration (MIC) assay against bacterial pathogens

Minimum inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit any visible microbial growth. The minimum inhibitory concentration for the carotenoid pigment of YP 17 and YP 37 were evaluated against the selected bacterial pathogens. The pigment extracts of YP 17 and YP 37 showed inhibition against all the selected gram positive and gram negative bacteria at a concentration of 10 mg ml^{-1} (dilution 1) and 1 mg ml^{-1} (dilution 2) and this was comparable to that of the standard antibiotic chloramphenicol (Table 31). The pigment extracts of YP 17 and YP 37 had no effect on the growth of the all selected bacterial pathogens to a concentration of 0.1 and 0.01 mg ml^{-1}. The solvent control (acetone) was found to possess negative activity at all the four concentrations against all the organisms.

Among the five carotenoid pigment producers, two isolates *viz*., *Rhodotorula mucilaginosa* (YP17) and *Sporobolomyces* sp. (YP 37) were found to be effective in

Table 31. Minimum inhibitory concentration of acetonic carotenoid pigment extracts of *R.mucilaginosa* and *Sporobolomyces* sp. against bacterial pathogens

Pathogens	Dilutions							
	(10mg ml^{-1})		(1mg ml^{-1})		(0.1mg ml^{-1})		(0.01mg ml^{-1})	
	1	2	1	2	1	2	1	2
Gram positive bacteria								
Enterococcus sp.	I	I	I	I	NI	NI	NI	NI
S. aureus	I	I	I	I	I	NI	NI	NI
B. subtilis	I	I	I	I	NI	NI	NI	NI
S. faecalis	I	I	I	I	NI	NI	NI	NI
Gram negative bacteria								
E. coli	I	I	I	I	I	NI	NI	NI
P. aeruginosa	I	I	I	I	NI	NI	NI	NI
Control								
Solvent control	NI	NI	NI	NI	NI	NI	NI	NI
Chloramphenicol	I	I	I	I	I	I	I	I

1- *R. mucilaginosa*; 2 - *Sporobolomyces* sp.

NI – No inhibition; I – Inhibition

antimicrobial and antioxidant activity. Therefore they were selected for further fermentation,stability, toxicological and application studies.

4.8. Optimization of physical and chemical parameters to increase carotenoid pigment production of *R. mucilaginosa* and *Sporobolomyces* sp.

4.8.1. Optimization of fermentation conditions at one time related to growth and carotenoid pigment production

4.8.1.1. Effect of pH

The data on the influence of pH on the growth medium on pigment production and cell dry weight yield of *R. mucilaginosa, Sporobolomyces* sp. and MTCC 9316 are presented in Table 32, Plate 7 and 8. The pH value of growth medium affects not only biosynthesis activity of culture, but also culture growth rate. In general, pH of the medium set at 6.0 for *R. mucilaginosa* and MTCC 9316 supported higher biomass production but in case of *Sporobolomyces* sp. the better biomass production was found at 5.0. The carotenoid producing yeast was able to grow and form pigments under a wide range of initial pH conditions from 2.5 to 9.5. It may be inferred from this experiments that the optimum pH for better pigment production from *R. mucilaginosa* (299.90 µg g^{-1}), standard strain MTCC 9316 (298.76 µg g^{-1}) and *Sporobolomyces* sp. (281.10 µg g^{-1}) was at pH 6.0 and 5.0 respectively.

4.8.1.2. Effect of temperature

The results of influence of different temperatures on pigment and cell dry weight production are presented in Table 33, Plate 9 and 10. Temperature is an another important parameter affecting the performance of cells and product formation. Among six different temperatures studied (15, 20, 25, 30, 35 and 40°C), 25°C appeared to be optimum for the production of *R. mucilaginosa* ,standard strain MTCC 9316 and *Sporobolomyces* sp. pigments in maximum quantity (384.83, 384.87 and 366.28 µg g^{-1} respectively) but the maximum cell dry weight was observed at 30°C.The yeast cultures was able to grow and produce pigments at all the temperatures examined with the exception of 40°C, at which a negligible growth took place without any pigmentation.

4.8.1.3. Effect of incubation time

Table 32. Effect of pH on cell dry weight and carotenoid pigment production

pH	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
2.0	148.00±3.002	No pigmentation	145.45±2.949	0.59±0.011	No growth	0.54±0.010
3.0	160.17 ± 3.691	56.10± 1.137	158.97±3.671	0.68±0.015	0.61 ± 0.012	0.69±0.015
4.0	268.22 ± 6.190	277.45± 6.407	267.88±6.186	0.71± 0.016	0.78 ± 0.018	0.72±0.016
5.0	283.68± 6.551	296.45± 6.846	284.52±6.570	0.84 ± 0.019	0.82 ± 0.018	0.83±0.019
6.0	299.90± 6.920	281.10± 6.491	298.76±6.901	0.85 ± 0.019	0.81 ± 0.017	0.83±0.019
7.0	276.08± 6.378	249.56± 5.763	276.00±6.377	0.82 ± 0.018	0.78 ± 0.015	0.82±0.018
8.0	263.24± 6.075	231.21± 0.5.339	262.78±6.072	0.81 ± 0.016	0.78 ± 0.014	0.80±0.016
9.0	242.66± 5.609	201.78± 4.659	243.01±5.678	0.66 ± 0.015	0.62 ± 0.015	0.64±0.015
SEd	8.0771	7.3347	8.0542	0.0238	0.0223	0.0242
CD (p =.05)	17.1229	15.5490	17.1217	0.0505	0.0472	0.0502

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Plate 7. Growth and carotenoid pigment production by *R. mucilaginosa* under different pH levels

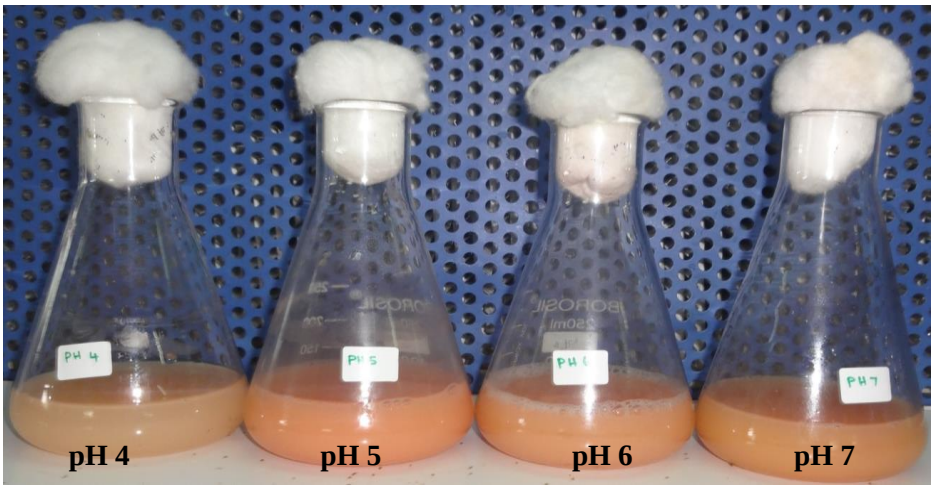


Plate 8. Growth and carotenoid pigment production by *Sporobolomyces* sp. under different pH levels

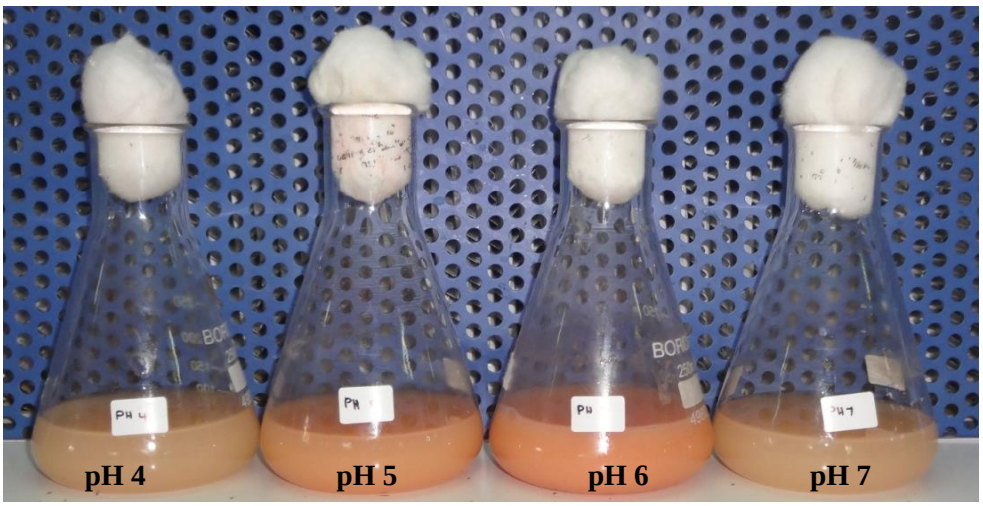


Table 33. Effect of temperature on cell dry weight and carotenoid pigment production of yeast isolates

Temperature (ºC)	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
15	251.88±5.107	No pigmentation	252.26±5.234	0.72±0.014	0.61±0.012	0.70±0.014
20	264.67±6.112	271.76± 06.276	263.56±6.110	0.86± 0.019	0.85 ± 0.019	0.87±0.020
25	384.83± 8.887	366.28 ± 8.458	384.87±8.887	0.81 ± 0.018	0.82 ± 0.018	0.80±0.018
30	379.87 ± 8.772	356.54 ± 8.002	378.98±8.771	0.84 ± 0.018	0.83 ± 0.018	0.84±0.019
35	258.11± 5.960	222.09± 5.128	257.45±5.958	0.72 ± 0.016	0.70 ± 0.014	0.73±0.016
40	No pigmentation	No pigmentation	No pigmentation	No growth	No growth	No growth
SEd	9.2181	8.1914	9.2179	0.0230	0.0218	0.0234
CD (p =0.05)	20.0846	17.8476	20.0845	0.0501	0.0475	0.0502

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Plate 9. Growth and carotenoid pigment production by *R. mucilaginosa* under different temperatures

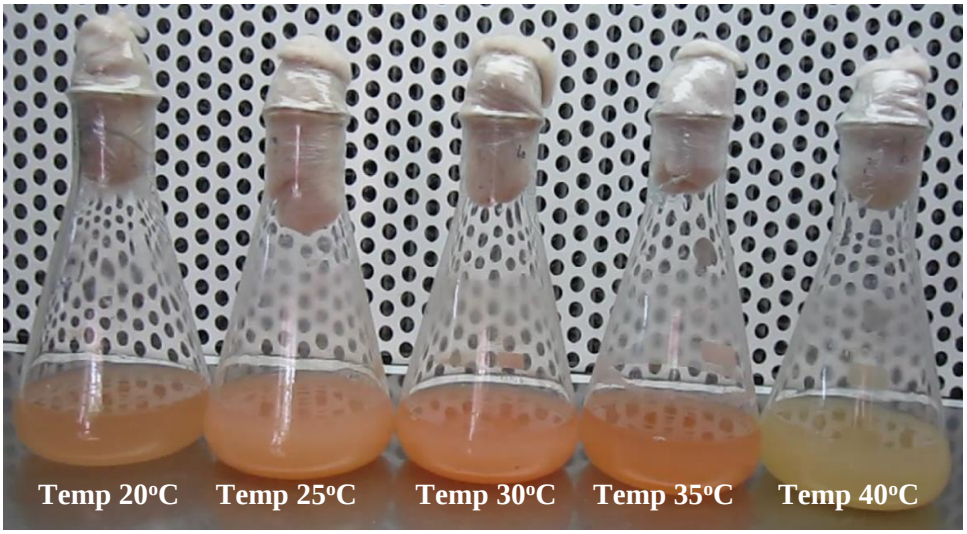
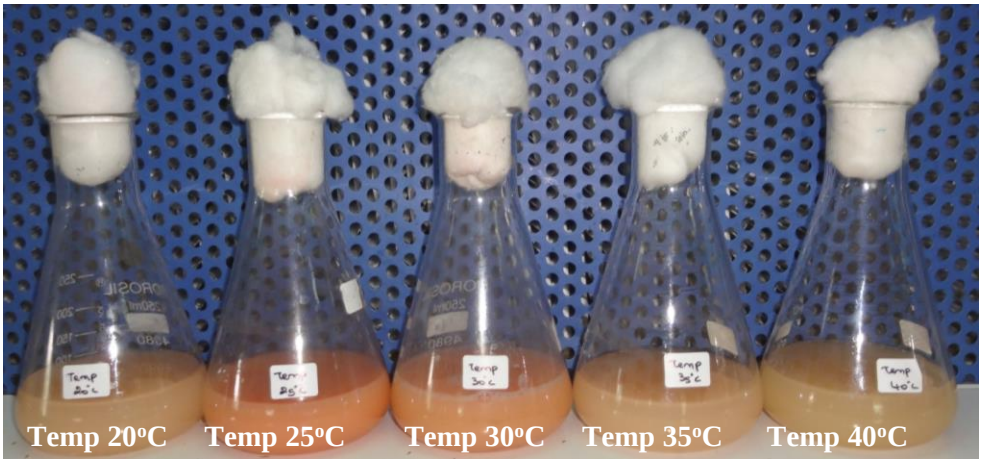


Plate 10. Growth and carotenoid pigment production by *Sporobolomyces* sp. under different temperatures



The maximum production of *R. mucilaginosa* and MTCC 9316 carotenoid pigment (352.25 and 349.21µg g^{-1}) and cell dry weight (0.98 and 0.94g 100 ml $broth^{-1}$) occurred at an incubation time of 96 h (Table 34). Whereas the maximum production of *Sporobolomyces* sp. carotenoid pigment (327.18 µg g^{-1}) observed at an incubation time of 120 h. After 96 h of incubation, a gradual reduction in carotenoid pigment production was observed in *R. mucilaginosa,* MTCC 9316 and whereas 120 h of incubation time results in reduction of pigment production was observed in *Sporobolomyces* sp.

4.8.1.4. Effect of agitation

If the microorganism requires oxygen, aerating the growth medium is very important for the successful progress of the fermentation. As *R. mucilaginosa*, MTCC 9316 and *Sporobolomyces* sp. is an aerobic microorganism, the effect of the aeration rate on the growth and total carotenoids formation of the yeast was also examined and the results obtained were shown in Table 35.The aeration helped yeast to produce more biomass and pigment production as the rate of agitation increased from 100 to 200 rpm. The maximum pigmentation in *R. mucilaginosa,* MTCC 9316 and *Sporobolomyces* sp. (398.85, 399.43 and 389.12 µg g^{-1}) was recorded at 200 rpm.

4.8.1.5. Effect of inoculum size

The effect of inoculum per cent on carotenoid pigment production of *R. mucilaginosa* and *Sporobolomyces* sp. was studied with different levels of inoculums ranging from 0.5 to 6 per cent. An inoculum at 3 per cent v/v was found to be optimum for growth and pigment production of *R. mucilaginosa* (304.83 µg g^{-1}), MTCC 9316 (301.56 µg g^{-1}) and 4 per cent v/v for *Sporobolomyces* sp. (298.56 µg g^{-1}) (Table 36) .

4.8.1.6. Effect of light

It is interesting to note that incubation in total dark condition favored higher production of pigments followed by unscreened white light, blue, red, green and yellow in both yeast isolates and the results are presented in Table 37. In dark, *R. mucilaginosa*, MTCC 9316 and *Sporobolomyces* sp. yielded maximum carotenoid pigment of 296.45, 295.34 and 289.32 µg g^{-1}, followed by white light (*R. mucilaginosa* - 295.01 µg g^{-1}, MTCC 9316 - 290.12 µg g^{-1} and *Sporobolomyces* sp. - 287.10 µg g^{-1}) and blue wave

Table 34. Effect of incubation time on **cell dry weight** and carotenoid pigment production

Time (h)	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
48	253.77±5.145	258.24±5.236	252.67±5.132	0.44±0.008	0.46±0.009	0.45±0.008
72	312.93 ± 7.226	289.21± 6.679	314.87±6.987	0.77 ± 0.017	0.87 ± 0.020	0.76±0.017
96	349.21± 8.064	308.35± 7.121	352.25±8.154	0.98 ± 0.022	0.92 ± 0.021	0.94±0.022
120	301.32± 6.958	327.18± 7.555	305.34±7.127	0.99 ± 0.022	0.89 ± 0.020	0.98±0.022
144	258.40± 5.967	289.34± 6.451	257.78±5.967	0.93 ± 0.021	0.95 ± 0.021	0.96±0.021
168	230.16± 5.315	250.65± 4.864	232.11±5.317	1.00 ± 0.023	0.96 ± 0.022	0.95±0.023
192	150.02± 3.464	221.89± 4.662	156.56±3.654	0.93 ± 0.021	0.82 ± 0.018	0.86±0.020
216	147.89± 3.415	199.08± 4.366	144.78±3.415	0.72 ± 0.016	0.66 ± 0.015	0.73±0.016
SEd	8.3621	8.4545	8.3511	0.0281	0.0271	0.0280
CD(p = 0.05)	17.7270	17.9230	17.7156	0.0596	0.0574	0.0596

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 35. Effect of agitation on cell dry weight and carotenoid pigment production

Agitation (rpm)	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
50	248.11 ± 5.030	254.78 ± 5.165	250.14±5.031	0.80 ± 0.016	0.85 ± 0.017	0.78±0.016
100	282.63± 6.527	288.56± 6.664	281.34±6.520	0.81 ± 0.018	0.81± 0.018	0.80±0.018
150	396.39 ± 9.154	377.66± 8.721	394.87±8.172	0.83 ± 0.019	0.81 ± 0.018	0.82±0.019
200	398.85 ± 9.211	389.12± 8.986	399.43±9.210	0.84 ± 0.019	0.76 ± 0.017	0.78±0.019
250	376.53± 8.695	365.34± 8.437	372.12±8.236	0.78 ± 0.018	0.77 ± 0.017	0.77±0.018
SEd	11.1744	10.9381	11.1467	0.0259	0.0255	0.0252
CD(p=0.05)	24.8982	24.3716	24.8980	0.0578	0.0567	0.0556

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 36. Effect of inoculum size on **cell dry weight** and carotenoid pigment production

Inoculum per cent	**Pigment yield (µg g^{-1})**			**Cell dry weight (g 100ml $broth^{-1}$)**		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
0.5	220.16 ± 4.463	201.04 ± 4.076	217.24±4.108	0.30 ± 0.006	0.33 ± 0.006	0.31±0.006
1	232.55± 5.370	223.72 ± 5.166	229.90±5.223	0.47 ± 0.010	0. 40± 0.009	0.40±0.010
2	285.61± 6.595	278.84± 6.439	282.15±6.435	0.57 ± 0.013	0.64± 0.014	0.52±0.013
3	304.83± 7.039	288.56± 6.894	301.56±6.879	0.56 ± 0.012	0.61± 0.014	0.53±0.012
4	296.71± 6.852	289.53± 6.755	294.12±6.124	0.68 ± 0.015	0.68± 0.017	0.69±0.015
5	271.67± 6.273	270.42± 6.245	265.34±5.786	0.75 ± 0.017	0.76± 0.017	0.73±0.017
6	257.22± 5.940	254.45± 5.876	258.89±5.978	0.82 ± 0.018	0.84± 0.019	0.77±0.018
SEd	8.6757	8.4767	8.6767	0.0200	0.0209	0.0202
CD(p =0.05)	18.6095	18.1826	18.6098	0.0429	0.0448	0.0429

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 37. Effect of different light on cell dry weight and carotenoid **pigment production**

Light source	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
Red (780–622 nm)	269.73 ± 5.469	267.67 ± 5.422	269.56±5.469	0.73 ± 0.014	0.76 ± 0.015	0.72±0.014
Yellow (597–577nm)	180.11 ± 4.159	176.89± 4.085	182.12±4.675	0.74 ± 0.017	0.72 ± 0.016	0.75±0.017
Green (577–492 nm)	268.44± 6.199	264.90± 6.117	265.34±6.213	0.73 ± 0.016	0.72 ± 0.016	0.68±0.016
Blue (492–455 nm)	271.05 ± 6.259	266.86± 6.398	270.89±6.257	0.74 ± 0.017	0.73 ± 0.016	0.71±0.017
White light	295.01 ± 6.842	287.10± 6.954	290.12±76.841	0.70 ± 0.016	0.68 ± 0.015	0.72±0.016
Dark	296.45± 6.846	289.32± 7.051	295.34±6.845	0.69 ± 0.015	0.70 ± 0.016	0.72±0.015
SEd	8.5347	8.6125	8.5225	0.0231	0.0230	0.0231
CD (p = 0.05)	18.5957	18.7652	18.5878	0.0504	0.0500	0.0504

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

length (*R. mucilaginosa* - 271.05µg g^{-1}, MTCC 9316-270.8905µg g^{-1} and *Sporobolomyces* sp. - 266.86 µg g^{-1}). Red, green and yellow light were also found to support the growth and carotenoid pigment production of both the isolates (Plate 11 and 12).

4.8.1.7. Effect of carbon sources

In general, all the carbon sources used in the study encouraged yeast growth and carotenoid pigment production except lactose. Of the 9 carbon sources examined fructose, sucrose, glucose and glycerol were relatively more favourable to the carotenoid pigment production as compared to other sources of carbon. However, sucrose and fructose respectively appears to be the preferred carbon sources for higher yields of *R. mucilaginosa* carotenoid pigment (341.30 µg g^{-1}), MTCC 9316 (338.23 µg g^{-1}) and *Sporobolomyces* sp. carotenoid pigment (302.56 µg g^{-1}). Xylose supported a high rate of growth but only a moderate carotenoid yield, whereas ribose resulted in higher rate of both growth and pigmentation. (Table 38, Plate 13 and 14).

4.8.1.8. Effect of nitrogen sources

Ten nitrogen sources were used in the study. These include nitrate nitrogen, ammonical nitrogen and organic source of nitrogen. As detailed earlier, the data on the cell dry weight on pigment synthesis are set out in Table 39 and 40.

In general, organic sources of nitrogen supported better growth of yeast than inorganic nitrogen sources. Among the organic sources, peptone and yeast extract recorded the highest carotenoid pigment yield. Malt extract and beef extract were also found to be good nitrogen source for growth of isolates (Plate 15 and 16). In this study, among the inorganic nitrogn sources, *viz.*, ammonium sulphate enhanced carotenoid pigment of *R. mucilaginosa*, MTCC 9316 (417.55 and 419.21µg g^{-1}) and sodium nitrate enhanced the *Sporobolomyces* sp. carotenoid pigment (338.70 µg g^{-1}).

4.8.1.9. Effect of trace elements

In order to investigate the effect of trace elements on pigment production and culture growth, *R. mucilaginosa* and *Sporobolomyces* sp. was cultivated in their respective optimized culture medium. Each trace element was added to the culture medium at a concentration of 0.05 per cent. The maximum *R. mucilaginosa* carotenoid

Plate 11. Growth and carotenoid pigment production by *R. mucilaginosa* under different light sources



Plate12. Growth and carotenoid pigment production by *Sporobolomyces* sp. under different light sources



Table 38. Effect of carbon sources on cell dry weight and carotenoid pigment production

Carbon Sources	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
Glucose	277.13 ± 5.619	305.25 ±6.189	275.16±5.602	0.87 ± 0.017	0.78 ± 0.015	0.83±0.017
Fructose	320.21± 7.394	302.56± 6.987	327.12±8.124	0.85 ± 0.019	0.76 ± 0.017	0.86±0.019
Sucrose	341.30± 7.881	284.12± 6.561	338.23±7.992	0.86 ± 0.019	0.79 ± 0.018	0.83±0.019
Maltose	258.51± 5.970	254.77± 5.883	260.56±6.012	0.88 ± 0.020	0.80 ± 0.018	0.84±0.020
Galactose	265.16± 6.123	275.60± 6.364	260.12±6.022	0.79 ± 0.018	0.75 ± 0.017	0.80±0.018
Xylose	249.83± 5.769	248.33± 5.734	251.23±5.897	0.77 ± 0.017	0.84 ± 0.019	0.82±0.018
Ribose	278.55± 6.432	270.70 ± 6.251	277.34±6.321	0.86 ± 0.019	0.81 ± 0.018	0.78±0.017
Lactose	0.00 ± 0.00	0.00 ± 0.00	0.00±0.00	0.12 ± 0.002	0.00 ± 0.000	0.00±0.00
Glycerol	280.01± 6.466	287.91 ± 6.648	281.24	0.78 ± 0.018	0.76 ± 0.017	0.76±0.018
SEd	8.6668	8.4523	8.6557	0.0253	0.0239	0.0254
CD(p=0.05)	18.2085	17.7580	18.2046	0.0531	0.0502	0.0531

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Plate 13. Growth and carotenoid pigment production by *R. mucilaginosa* in different carbon sources



Plate 14. Growth and carotenoid pigment production by *Sporobolomyces* sp. in different carbon sources

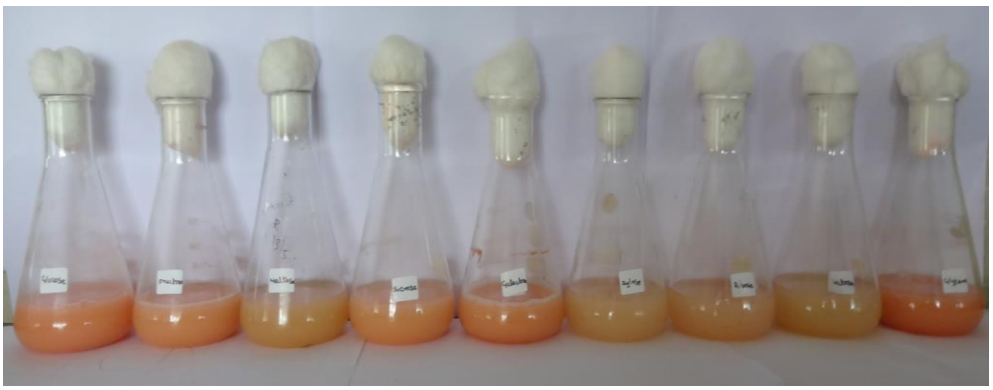


Table 39. Effect of organic nitrogen sources on cell dry weight and carotenoid **pigment production**

Organic nitrogen sources	**Pigment yield (µg g^{-1})**			**Cell dry weight (g 100ml $broth^{-1}$)**		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
Peptone	401.03 ± 8.131	398.92 ± 8.331	400.12±8.012	0.88 ± 0.017	0.84 ± 0.017	0.86±0.017
Beef extract	286.61± 6.618	275.67 ± 6.36	294.25±6.697	0.85 ± 0.019	0.82 ± 0.018	0.84±0.019
Tryptone	271.61± 6.272	288.25± 6.565	270.89±6.272	0.89± 0.020	0.80± 0.018	0.86±0.020
Malt extract	283.54 ± 6.548	294.73± 6.806	284.09±6.540	0.86± 0.019	0.85± 0.019	0.83±0.019
Yeast extract	407.80± 9.417	396.27± 9.151	402.12±9.232	0.97± 0.022	0.80± 0.018	0.89±0.020
SEd	10.5989	10.6647	10.6079	0.0284	0.0262	0.0282
CD(p =0.05)	23.6160	23.7626	23.6156	0.0634	0.0584	0.0633

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Table 40. Effect of inorganic nitrogen sources on cell dry weight and carotenoid **pigment production**

Inorganic nitrogen sources	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
Ammonium nitrate	297.64 ± 6.03	277.43 ± 5.625	299.21±6.67	0.73 ± 0.014	0.86± 0.017	0.72±0.01
Sodium nitrate	399.00± 9.214	338.70± 7.821	397.23±9.123	0.79 ± 0.018	0.74 ± 0.017	0.80±0.018
Casein	382.99± 8.844	318.34± 7.351	384.20±8.971	0.80± 0.018	0.85± 0.019	0.82±0.019
Ammonium chloride	281.90± 6.510	298.12± 6.884	280.54±6.489	0.97 ± 0.022	0.86± 0.019	0.95±0.022
Ammonium sulphate	417.55± 10.335	327.41± 7.561	419.21±10.789	0.81 ± 0.018	0.76 ± 0.017	0.80±0.018
SEd	11.8113	10.0289	11.7112	0.0264	0.0260	0.0264
CD(p =0.05)	26.3174	22.3458	26.3062	0.0589	0.0578	0.0589

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Plate15. Growth and carotenoid pigment production by *R. mucilaginosa* in different nitrogen sources

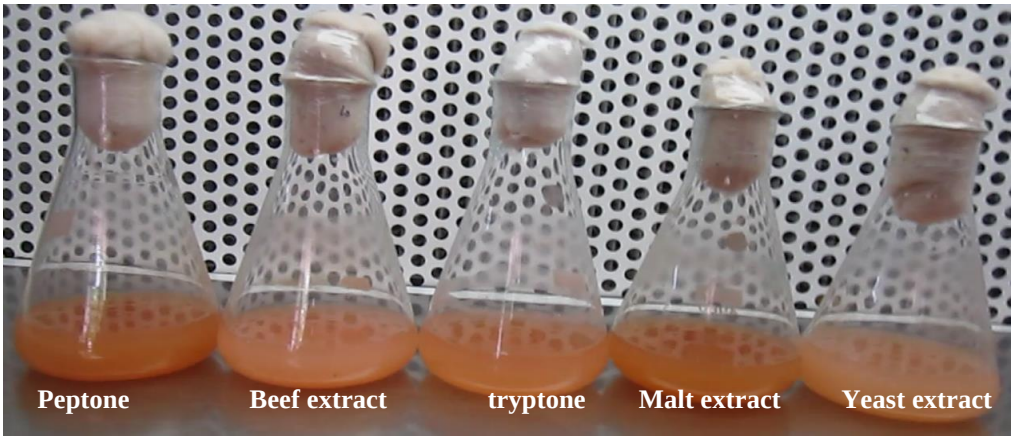


Plate 16. Growth and carotenoid pigment production by *Sporobolomyces* sp. in different nitrogen sources



production of (410.79 µg g^{-1}) was achieved in magnesium sulphate supplemented medium. Whereas Sporobolomyces sp. produced the maximum pigment production (387.66 µg g^{-1}) in ferrous sulphate supplemented medium (Table 41). The other bioelement like copper ions appeared to be detrimental on both culture carotenoid pigment production.

4.8.2. Optimization of various parameters by Response Surface Methodology

4.8.2.1. Physical parameters for maximum carotenoid pigment

The three independent variables (pH, temperature and time) and their concentrations at different coded and actual level of variables employed in the design matrix are shown in Table 8 and Table 10. Three level central composite design matrix and the experimental responses of the dependent variable (pigment yield) are listed in Table 42 and Table 45. The data obtained were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms.

Data from 20 experiments were used for the following equations, where the coded values were obtained from regression analysis for the carotenoid pigment production. The application of RSM gave rise to the following regression equation for pigment yield.

***R.mucilaginosa* carotenoid pigment**

Final equation in terms of coded factors = + 1022.44 -75.38*A – 14.30*B + 20.04*C +2.98*A*B -14.96*A*C -4.26*B*C – 91.12*A^2 – 59.92*B^2 – 57.45*C^2

Final equation in terms of actual factors = - 4490.11500+ 1047.99717* pH + 114.32083* temperature + 24.19511*time - 0.59575*pH*temperature - 0.62318* pH * time - 0.035510* temperature * time - 89.87056* pH^2-2.34692* $temperature^2$ - 0.097569*$time^2$

***Sporobolomyces* sp. carotenoid pigment**

Final equation in terms of coded factors = +989.47 +58.41* A -22.10* B +8.18 *C 19.52* A * B +14.05 * A * C +20.29* B * C -76.68* A^2 -33.49 * B^2 -99.94* C^2

Final equation in terms of actual factors = -4115.50280+852.65512* pH +61.79106* Temperature +34.82877* Incubation time -3.90500* pH * Temperature +0.58521* pH *

Table 41. Effect of trace elements on cell dry weight and carotenoid **pigment production**

Trace element sources	Pigment yield (µg g^{-1})			Cell dry weight (g 100ml $broth^{-1}$)		
	Yeast pigment producers					
	R. mucilaginosa	***Sporobolomyces* sp.**	**MTCC 9316**	***R. mucilaginosa***	***Sporobolomyces* sp.**	**MTCC 9316**
Copper sulphate	0.0 ± 0.000	0.0 ± 0.000	0.0±0.000	0.0 ± 0.000	0.0 ± 0.000	0.0±0.000
Ferrous sulphate	390.83± 9.025	387.66 ± 8.952	391.23±9.034	0.72 ± 0.016	0.70 ± 0.016	0.72±0.016
Zinc sulphate	248.85 ± 5.746	241.26± 5.571	249.45±0.5.765	0.76± 0.017	0.77± 0.017	0.75±0.016
Magnesium sulphate	410.79 ± 9.486	362.11± 8.362	411.39±9.567	0.68± 0.015	0.71± 0.016	0.72±0.016
Manganese sulphate	332.84 ± 7.686	278.67 ± 6.435	330.87±7.634	0.74 ± 0.017	0.79 ± 0.018	0.73±0.017
SEd	10.2679	10.2679	10.2678	0.0212	0.0217	0.0211
CD(p = 0.05)	22.8785	22.8785	22.8785	0.0472	0.0484	0.0472

Data are mean ± SD of three measurements. $P < 0.05$ compared to control

Incubation time +0.16904* Temperature * Incubation time -76.68456 *pH^2 – 1.33943* $Temperature^2$ - 0.17350*$Incubationtime^2$

4.8.2.1.1. Optimization of carotenoid pigment production

Based on the experimental response, the carotenoid pigment produced by *R. mucilaginosa* ranged from 739.99 to 1022.18 µg g^{-1} run 10 and 16 had the minimum and maximum pigment production respectively. While the carotenoid produced by *Sporobolomyces* sp. ranged from 674.33 to 990.65 µg g^{-1} run 9 and 20 had the minimum and maximum pigment production respectively. The ANOVA results of quadratic regression model for pigmentation in *R. mucilaginosa* and *Sporobolomyces* sp. yield is described in Table 43 and Table 46. ANOVA of the regression model for pigment yield demonstrated that the model was significant due to an F- value of 14711.18 and 2514.32 (*R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment respectively) and very low probability value (p models > F- 0.0001). ANOVA for the model explained the response of the depends variable. Table 43 and Table 46 showed that the experimental yields fitted the second order polynomial equation as well as indicated by R^2 value 0.9991 (*R. mucilaginosa* pigment) and 0.9986 (*Sporobolomyces* sp. pigment).

The regression co-efficient along with the corresponding p values for the models of *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments respectively are presented in Table 44 and Table 47. It showed that the regression coefficient of all the quadratic coefficient of P, T, t were significant at 0.05 per cent level.

The 3D response surfaces based on independent variables were obtained using the same software package indicated that a local optimum exists in the area experimentally investigated. Fig. 11 and Fig. 12 show three dimensional (3D response) surface plots of the effect of pH and temperature on the pigment production. As the pH increased from 5 to 6 for *R. mucilaginosa* pigment and 4 to 5 for *Sporobolomyces* sp. pigment, the curve gradually increased *i.e.*, when the temperature was around 25°C, the production of carotenoid pigment increased along with increasing incubation time.

The optimum levels in coded values for pH, temperature and incubation time were 0 and +1. The variable pH and temperature, pH and incubation time indicated that

Table 42. Central composite design matrix of physical parameters of independent variables and their corresponding experimental and predicted yield of pigment extracted from *R. mucilaginosa*

Run No.	Independent variables			Pigment yield ($\mu g\ g^{-1}$)	
	pH	Temperature (°C)	Time (h)	Observed	Predicted
1	5	20	72	871.05	872.10
2	7	20	72	744.17	744.29
3	5	30	72	844.06	845.07
4	7	30	72	732.21	734.18
5	5	20	120	948.03	949.62
6	7	20	120	764.44	762.98
7	5	30	120	907.11	906.54
8	7	30	120	731.32	731.82
9	4.31	25	96	895.08	895.33
10	7.68	25	96	739.99	741.48
11	6	16.59	96	880.34	880.54
12	6	33.40	96	832.01	832.44
13	6	25	55.63	828.69	829.78
14	6	25	136.36	897.14	897.49
15	6	25	96	1022.12	1022.44
16	6	25	96	1022.18	1022.44
17	6	25	96	1021.02	1022.44
18	6	25	96	1020.21	1022.44
19	6	25	96	1021.09	1022.44
20	6	25	96	1022.15	1022.44

Table 45. Central composite design matrix of physical parameters of independent variables and their corresponding experimental and predicted yield of pigment extracted from *Sporobolomyces* sp.

Run No.	Independent variables			Pigment yield (µg g^{-1})	
	pH	Temperature (ºC)	Time (h)	Observed	Predicted
1	4	20	96	748.22	749.68
2	6	20	96	880.35	877.46
3	4	30	96	703.06	705.96
4	6	30	96	753.21	753.64
5	4	20	144	698.01	697.38
6	6	20	144	880.44	881.34
7	4	30	144	730.11	732.80
8	6	30	144	840.32	838.66
9	3.31	25	120	674.34	675.88
10	6.68	25	120	868.99	870.81
11	5	16.59	120	931.44	931.93
12	5	33.40	120	857.04	857.59
13	5	25	79.63	691.90	693.05
14	5	25	160.36	721.44	720.57
15	5	25	120	989.47	991.12
16	5	25	120	980.87	989.47
17	5	25	120	990.87	989.47
18	5	25	120	989.08	989.47
19	5	25	120	987.59	989.47
20	5	25	120	990.65	989.47

mutual interactions between these set of variables had a significant effect on the pigment yield. When the third independent variable fermentation time was kept constant at 96 h (*R. mucilaginosa* pigment) and 120 h (*Sporobolomyces* sp. pigment) (Fig. 11 and 12), the interaction between the two variables (temperature and pH) showed that the pigment yield was sensitive even when pH and temperature were subject to small alterations.

The other pair of the independent variables pH and incubation time showed similar effects while keeping the third independent variable, temperature as a constant at 25°C. The 3D surface response for temperature and fermentation time on the yield of pigment (Fig.11 and Fig 12). The results showed that as the values of process variables increased, the yield also increased but only upto the mid point range of variables and thereafter the yield decreased even though the values of variables increased. The pigment yield was significantly effected by pH, temperature and incubation time where pH produced greater effect. Based on the model, the optimal working conditions were obtained to attain high carotenoid pigment yield.

4.8.2.2. Optimization of chemical parameters

To examine the combined effect of five different medium components (independent variables) on pigment production, a central composite design of 50 experiments were performed (Plate 17 and 18). The five independent variables (sucrose, yeast extract, ammonium sulphate, dipotassium hydrogen phosphate and magnesium sulphate) and their concentrations at different coded and actual levels of the variables employed in the design matrix for pigment production in *R. mucilaginosa* are showed in Table 14. For *Sporobolomyces* sp. pigment production, the five independent variables (glucose, peptone, ammonium sulphate, dipotassium hydrogen phosphate and ferrous sulphate) and their concentrations at different coded and actual levels of the variables used in the design matrix are showed in Table 15. Five level central composite design matrix and the experimental responses of the dependent variables (carotenoid pigment) are listed in Table 48 and Table 51. Second order polynomial equation was used to correlate the independent process variables, with pigment production. The second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert.

Table 43. Analysis of variance (ANOVA) for optimization of physical parameters for pigmentation in *R. mucilaginosa*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance –*F*
Regression	2.734E+005	9	30374.50	14711.18	<0.0001
Residual	20.65	10	2.06	-	-
Cor Total	2.734E+005	19	-	-	-
R^2 = 0.9991, adjusted R^2 = 0.9989					

Table 44. Regression coefficient for optimization of physical parameters for pigmentation in *R. mucilaginosa*

Factor	Regression co efficient	Standard error	P- value
Intercept	1026.10	0.59	< 0.0001
A-P	-75.38	0.39	< 0.0001
B-T	-14.30	0.39	< 0.0001
C-t	20.04	0.39	< 0.0001
AB	2.98	0.51	0.0002
AC	-14.96	0.51	< 0.0001
BC	-4.26	0.51	< 0.0001
A^2	-91.12	0.38	< 0.0001
B^2	-59.92	0.38	< 0.0001
C^2	-57.45	0.38	< 0.0001

Table 46. Analysis of variance (ANOVA) for optimization of physical parameters for pigmentation in *Sporobolomyces* sp.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance –*F*
Regression	2.754E+005	9	30600.94	2514.32	<0.0001
Residual	121.71	10	12.17	-	-
Cor total	2.755E+005	19	-	-	-
R^2 = 0.9986, adjusted R^2 = 0.9982					

Table 47. Regression coefficient for optimization of physical parameters for *Sporobolomyces* sp. pigment

Factor	Regression Co efficient	Standard error	P- value
Intercept	989.47	1.42	< 0.0001
A-P	58.41	0.94	< 0.0001
B-T	-22.10	0.94	< 0.0001
C-t	8.18	0.94	< 0.0001
AB	-19.52	1.23	< 0.0001
AC	14.05	1.23	< 0.0001
BC	20.29	1.23	< 0.0001
A^2	-76.68	0.92	< 0.0001
B^2	-33.49	0.92	< 0.0001
C^2	-99.94	0.92	< 0.0001

Fig 11. Response surface curve showing the physical parameters on the pigment production by *R. mucilaginosa*

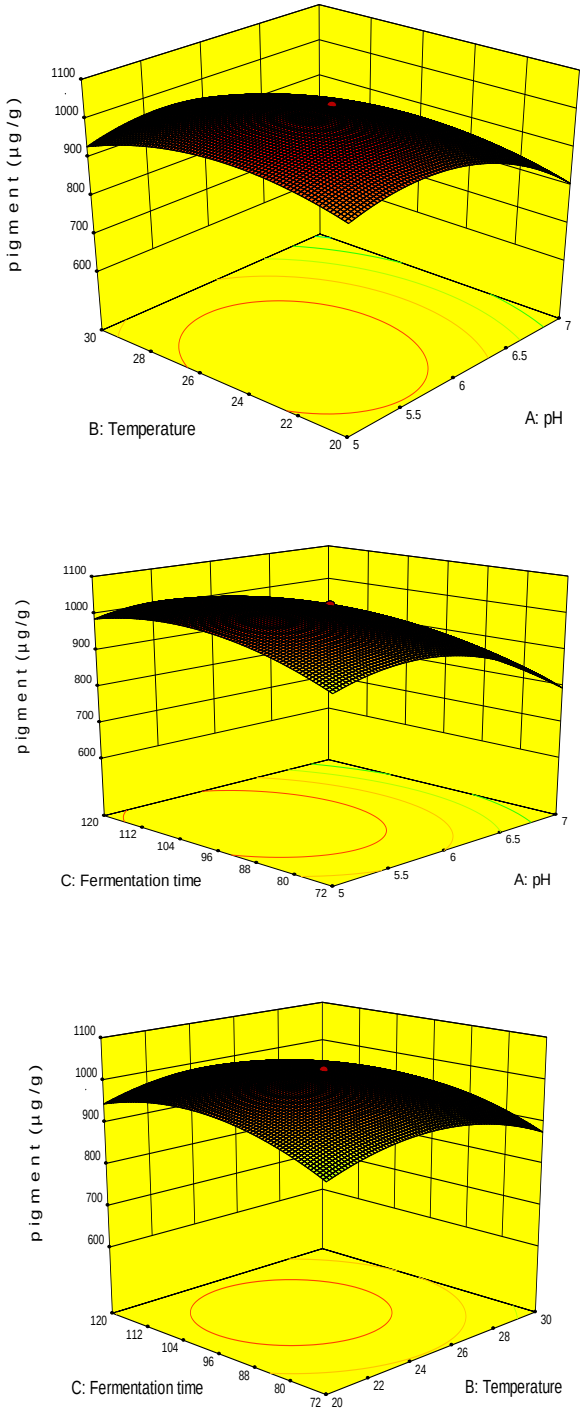
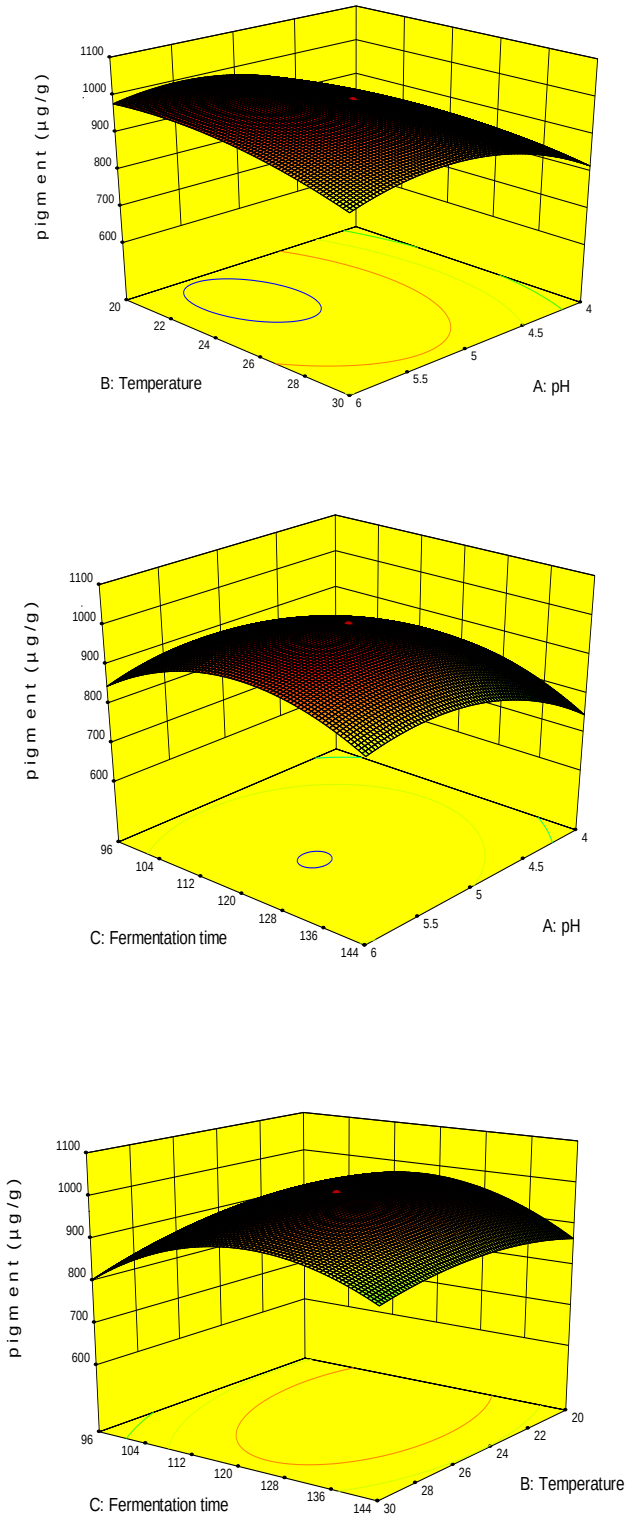


Fig 12. Response surface curve showing the physical parameters on the pigment production by *Sporobolomyces* sp.



The results were analyzed by using ANOVA, *i.e.* analysis of variance suitable for the experimental design. The results for pigmentation in *R. mucilaginosa* and *Sporobolomyces* sp. are shown in Table 49 and 52. The regression co-efficient along with the corresponding p values for the models of *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments respectively are presented in Table 50 and Table 53. The Model F-value of 1674.46 (*R. mucilaginosa* and its pigment) and 172.91 (*Sporobolomyces* sp. pigment) implies that the model is significant. Model F-value is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob > F) is very low (0.0001). The P values were used as a tool to check the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the P, the more significant is the corresponding coefficient. Values of P less than 0.05 indicate model terms are significant.

The coefficient estimates and the corresponding P values suggested that, among the test variables used in the pigment production of *R. mucilaginosa*, A (sucrose), B (yeast extract), C (ammonium sulphate), D (K_2HPO_4), E ($MgSO_4$), A * B (sucrose * yeast extract), A * C (sucrose * ammonium sulphate), A * D (sucrose * K_2HPO_4), A * E (sucrose * $MgSO_4$), B * C (yeast extract * ammonium sulphate), B * E (yeast extract * $MgSO_4$) D *E (K_2HPO_4 * $MgSO_4$), C * E (ammonium sulphate * $MgSO_4$), D * E (K_2HPO_4 * $MgSO_4$) are significant model terms. Sucrose, yeast extract, ammonium sulpahte, K_2HPO_4 and $MgSO_4$ ($P < 0.0001$) had largest effect on *R. mucilaginosa* carotenoid pigment production. The corresponding second-order response model that was found after analysis for the regression was,

***R. mucilaginosa* pigment yield** = +2179.29+ 22.22* A + 38.32 * B + 57.51* C +109.36* D + +31.82*E -18.67 * A * B +44.33* A * C -6.10 * A * D -15.71 * A* E +55.33 * B *C +88.29* B * D –55.51 * B * E +88.21* C * D +31.50* C * E +99.33* D * E -307.75* A^2 -351.46 * B^2 -123.54*

C^2 -128.94* D^2 -110.24* E^2

The coefficient estimates and the corresponding P values suggested that, among the test variables used in the *Sporobolomyces* sp. and its carotenoid pigment production study, A (Glucose), B (peptone), C (ammonium sulphate), D (K_2HPO_4), E ($FeSO_4$), A *

Plate 17.Optimization of fermentation conditions for *R. mucilaginosa* carotenoid pigment by RSM



Plate 18. Optimization of fermentation conditions for *Sporobolomyces* sp. carotenoid pigment by RSM



Table 48. Central composite rotatable design matrix of chemical parameters and their independent variables and corresponding experimental yields for intracellular pigmentation in *R. mucilaginosa*

Ex. No	Independent Variable Levels					Pigment yield (µg g^{-1})	
	Natural Values					Observed	Predicted
	Sucrose	Yeast extract	Ammonium sulphate	Dipotassium hydrogen phosphate	Magnesium sulphate		
1	25	2	1.5	0.5	0.3	1313.87	1320.15
2	35	2	1.5	0.5	0.3	1352.89	1356.92
3	25	5	1.5	0.5	0.3	1034.90	1035.87
4	35	5	1.5	0.5	0.3	998.25	997.94
5	25	2	2.5	0.5	0.3	995.05	996.42
6	35	2	2.5	0.5	0.3	1212.25	1210.49
7	25	5	2.5	0.5	0.3	928.94	933.48
8	35	5	2.5	0.5	0.3	1069.78	1072.85
9	25	2	1.5	2	0.3	996.34	999.41
10	35	2	1.5	2	0.3	1005.27	1011.75
11	25	5	1.5	2	0.3	1056.54	1058.30
12	35	5	1.5	2	0.3	1008.06	1005.95
13	25	2	2.5	2	0.3	1030.45	1028.53
14	35	2	2.5	2	0.3	1193.65	1198.18
15	25	5	2.5	2	0.3	1320.33	1318.76
16	35	5	2.5	2	0.3	1428.36	1429.71
17	25	2	1.5	0.5	0.7	1041.69	1042.54
18	35	2	1.5	0.5	0.7	1014.34	1016.45
19	25	5	1.5	0.5	0.7	977.45	980.31
20	35	5	1.5	0.5	0.7	862.67	869.52
21	25	2	2.5	0.5	0.7	832.05	834.80
22	35	2	2.5	0.5	0.7	976.01	976.32
23	25	5	2.5	0.5	0.7	1005.56	1003.91
24	35	5	2.5	0.5	0.7	1081.58	1080.42
25	25	2	1.5	2	0.7	1106.46	1109.12

Table 48. Contd…

Ex. No	Independent Variable Levels					Pigment yield ($\mu g\ g^{-1}$)	
	Natural Values					Observed	Predicted
	Sucrose	Yeast extract	Ammonium sulphate	Dipotassium hydrogen phosphate	Magnesium sulphate		
26	35	2	1.5	2	0.7	1066.56	1068.61
27	25	5	1.5	2	0.7	1384.54	1386.05
28	35	5	1.5	2	0.7	1279.23	1284.85
29	25	2	2.5	2	0.7	1272.43	1274.23
30	35	2	2.5	2	0.7	1399.76	1401.03
31	25	5	2.5	2	0.7	1785.45	1786.51
32	35	5	2.5	2	0.7	1839.67	1838.61
33	18.10	3.5	2	1.25	0.5	405.96	399.58
34	41.89	3.5	2	1.25	0.5	504.56	494.15
35	30	0.06	2	1.25	0.5	120.87	121.00
36	30	7.06	2	1.25	0.5	286.12	282.30
37	30	3.5	0.81	1.25	0.5	1370.52	1373.68
38	30	3.5	3.18	1.25	0.5	1615.08	1617.23
39	30	3.5	2	-0.53	0.5	1183.19	1189.79
40	30	3.5	2	3.03	0.5	1731.28	1739.99
41	30	3.5	2	1.25	0.024	1480.44	1479.97
42	30	3.5	2	1.25	0.975	1635.56	1631.34
43	30	3.5	2	1.25	0.5	2182.05	2179.29
44	30	3.5	2	1.25	0.5	2175.37	2179.29
45	30	3.5	2	1.25	0.5	2176.35	2179.29
46	30	3.5	2	1.25	0.5	2172.05	2179.29
47	30	3.5	2	1.25	0.5	2179.10	2179.29
48	30	3.5	2	1.25	0.5	2178.02	2179.29
49	30	3.5	2	1.25	0.5	2177.09	2179.29
50	30	3.5	2	1.25	0.5	2177.11	2179.29

Table 51. Central composite rotatable design matrix of chemical parameters and their independent variables and corresponding experimental yields for carotenoid pigment production by *Sporobolomyces* sp.

Ex. No	Independent Variable Levels					Pigment yield (µg g^{-1})	
	Natural Values					Observed	Predicted
	Glucose	Peptone	Ammonium sulphate	Dipotassium hydrogen phosphate	Ferrous sulphate		
1	25	2	1.5	0.5	0.3	812.69	812.70
2	35	2	1.5	0.5	0.3	663.87	660.98
3	25	5	1.5	0.5	0.3	910.11	910.05
4	35	5	1.5	0.5	0.3	1089.22	1093.62
5	25	2	2.5	0.5	0.3	1160.18	1161.07
6	35	2	2.5	0.5	0.3	934.88	934.00
7	25	5	2.5	0.5	0.3	1062.45	1059.58
8	35	5	2.5	0.5	0.3	1167.25	1167.70
9	25	2	1.5	2.0	0.3	884.61	885.28
10	35	2	1.5	2.0	0.3	871.34	870.59
11	25	5	1.5	2.0	0.3	909.11	909.72
12	35	5	1.5	2.0	0.3	1230.23	1229.61
13	25	2	2.5	2.0	0.3	1012.49	1013.18
14	35	2	2.5	2.0	0.3	923.56	924.44
15	25	5	2.5	2.0	0.3	836.44	838.07
16	35	5	2.5	2.0	0.3	1082.86	1084.92
17	25	2	1.5	0.5	0.7	1078.65	1079.44
18	35	2	1.5	0.5	0.7	840.65	840.87
19	25	5	1.5	0.5	0.7	1137.15	1138.58
20	35	5	1.5	0.5	0.7	1237.06	1234.10
21	25	2	2.5	0.5	0.7	1323.8	1322.37
22	35	2	2.5	0.5	0.7	1007.77	1007.84
23	25	5	2.5	0.5	0.7	1180.08	1181.96
24	35	5	2.5	0.5	0.7	1202.03	1203.43
25	25	2	1.5	2.0	0.7	1236.6	1235.65

Table 51. Contd…

Ex. No	Independent Variable Levels					Pigment yield ($\mu g\ g^{-1}$)	
	Natural Values					Observed	Predicted
	Glucose	Peptone	Ammonium sulphate	Dipotassium hydrogen phosphate	Ferrous sulphate		
26	35	2.0	1.5	2.0	0.7	1130.89	1132.90
27	25	5.0	1.5	2.0	0.7	1220.11	1221.18
28	35	5.0	1.5	2.0	0.7	1452.9	1453.02
29	25	2.0	2.5	2.0	0.7	1259.26	1257.40
30	35	2.0	2.5	2.0	0.7	1079.89	1078.61
31	25	5.0	2.5	2.0	0.7	1042.87	1043.38
32	35	5.0	2.5	2.0	0.7	1198.08	1199.18
33	18.10	3.5	2.0	1.25	0.5	705.34	703.99
34	41.89	3.5	2.0	1.25	0.5	707.77	709.66
35	30	-0.06	2.0	1.25	0.5	982.21	985.04
36	30	7.06	2.0	1.25	0.5	1247.32	1249.03
37	30	3.5	0.8	1.25	0.5	581.19	580.02
38	30	3.5	3.1	1.25	0.5	691.56	693.27
39	30	3.5	2.0	-0.53	0.5	1171.05	1172.34
40	30	3.5	2.0	3.03	0.5	1257.56	1257.81
41	30	3.5	2.0	1.25	0.02	1209.34	1213.84
42	30	3.5	2.0	1.25	0.97	1297.29	1297.33
43	30	3.5	2.0	1.25	0.5	1979.39	1978.92
44	30	3.5	2.0	1.25	0.5	1977.06	1978.92
45	30	3.5	2.0	1.25	0.5	1976.23	1978.92
46	30	3.5	2.0	1.25	0.5	1980.01	1978.92
47	30	3.5	2.0	1.25	0.5	1981.45	1984.92
48	30	3.5	2.0	1.25	0.5	1977.09	1978.92
49	30	3.5	2.0	1.25	0.5	1979.35	1979.92
50	30	3.5	2.0	1.25	0.5	1980.45	1978.92

B (Glucose * peptone), A * C (Glucose * ammonium sulphate), A * D (Glucose * K_2HPO_4), A * E (Glucose * $FeSO_4$), B * C (peptone * ammonium sulphate), B * E (Peptone * $FeSO_4$) D *E (K_2HPO_4 * $FeSO_4$), C * E (ammonium sulphate * $FeSO_4$), D * E (K_2HPO_4 * $FeSO_4$) are significant model terms. Glucose, peptone, ammonium sulphate, K_2HPO_4 and $FeSO_4$ ($P < 0.0001$) had largest effect on pigment production of *Sporobolomyces* sp. The corresponding second-order response model that was found after analysis for the regression was,

***Sporobolomyces* sp. pigment** = 1970.09 +0.55* A +0.94* B -0.14* C-0.49* D + 2.61* E -9.062E-003*A * B+0.34* A * C +1.49* A * D +0.79* A * E -1.21* B * C+1.14* B * D +0.43* B * E +1.25* C * D -0.60*C * E + +1.73* D * E -6.14* A^2 -2.10* B^2 -1.38* C^2 -1.31* D^2 -0.98*E^2

The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9991 (*R. mucilaginosa* carotenoid pigment) and 0.9917 (*Sporobolomyces* sp. carotenoid pigment), indicating that 99.10 and 99.17 per cent of t he variability in the response respectively could be explained by the model. The closer the R^2 value is 1, the better the model is fit to experimental data, the less is the distance between the predicted and the observed values. Accordingly, three-dimensional graphs were generated for the pair-wise combination of the five factors, while keeping the other two at their center point levels. Graphs are given here to highlight the roles played by various factors (Fig. 13, 14, 15 and 16). From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. By keeping another variable at its optimal level, three-dimensional plots of two factors versus pigment production were drawn, and the corresponding contour plot was obtained. From the bump of three-dimensional plot or the central point of its respective contour plot; the optimal composition of medium components was identified. The optimal concentrations for the five components as obtained from the maximum point of the model for *R. mucilaginosa* carotenoid pigment were calculated as 30 g l^{-1},3.5 g l^{-1}, 2 g l^{-1}, 1.25 g l^{-1} and 0.5 g $l^{-1}$for sucrose, yeast extract, ammonium sulphate, dipottasium hydrogen phosphate and magnesium sulphate respectively. The optimal concentrations for the five components as obtained from the maximum point of the model for *Sporobolomyces* sp.

Table 49. Analysis of variance (ANOVA) for optimization of chemical parameters for pigment production in *R. mucilaginosa*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Regression	1.293E+007	20	58.54	1674.46	<0.0001
Residual	11200.89	29	386.24		-
Cor Total	1.295E+007	49	-	-	-
R^2 = 0.9910, adjusted R^2 = 0.9985					

Table 50. Regression coefficient for optimization of chemical parameters for carotenoid pigment production by *R. mucilaginosa*

Factor	Regression Co efficient	Standard error	P value
Intercept	2179.29	6.90	< 0.0001
A-A	22.22	2.99	< 0.0001
B-B	38.32	2.99	< 0.0001
C-C	57.51	2.99	< 0.0001
D-D	109.36	2.99	< 0.0001
E-E	31.82	2.99	< 0.0001
AB	44.33	2.99	< 0.0001
AC	-6.10	2.99	< 0.0001
AD	-15.71	2.99	< 0.0001
AE	55.33	2.99	< 0.0001
BC	88.29	2.99	< 0.0001
BE	88.21	2.99	< 0.0001
CD	31.50	2.99	< 0.0001
CE	99.33	2.99	< 0.0001
DE	-307.75	2.99	< 0.0001

Table 52. Analysis of variance (ANOVA) for optimization of chemical parameters for carotenoid pigment from *Sporobolomyces* sp.

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Regression	2906.88	20	145.34	172.91	<0.0001
Residual	24.38	29	0.84	-	-
Total Model	2931.26	49	-	-	-
R^2 = 0.9917, adjusted R^2 = 0.9859					

Table 53. Regression coefficient for optimization of chemical parameters for *Sporobolomyces* sp. pigment production

Intercept	1970.09	0.32	< 0.0001
A-A	0.55	0.14	0.0005
B-B	0.94	0.14	< 0.0001
C-C	-0.14	0.14	0.0037
D-D	-0.49	0.14	0.0016
E-E	2.61	0.14	< 0.0001
AB	0.34	0.14	< 0.0001
AC	1.49	0.14	< 0.0001
AD	0.79	0.14	< 0.0001
AE	-1.21	0.14	< 0.0001
BC	1.14	0.14	< 0.0001
BE	1.25	0.14	< 0.0001
CD	-0.60	0.14	< 0.0001
CE	1.73	0.14	< 0.0001
DE	-6.14	0.14	< 0.0001

Fig 13. Response surface curve showing the effect of medium components on the pigment production by *R. mucilaginosa*

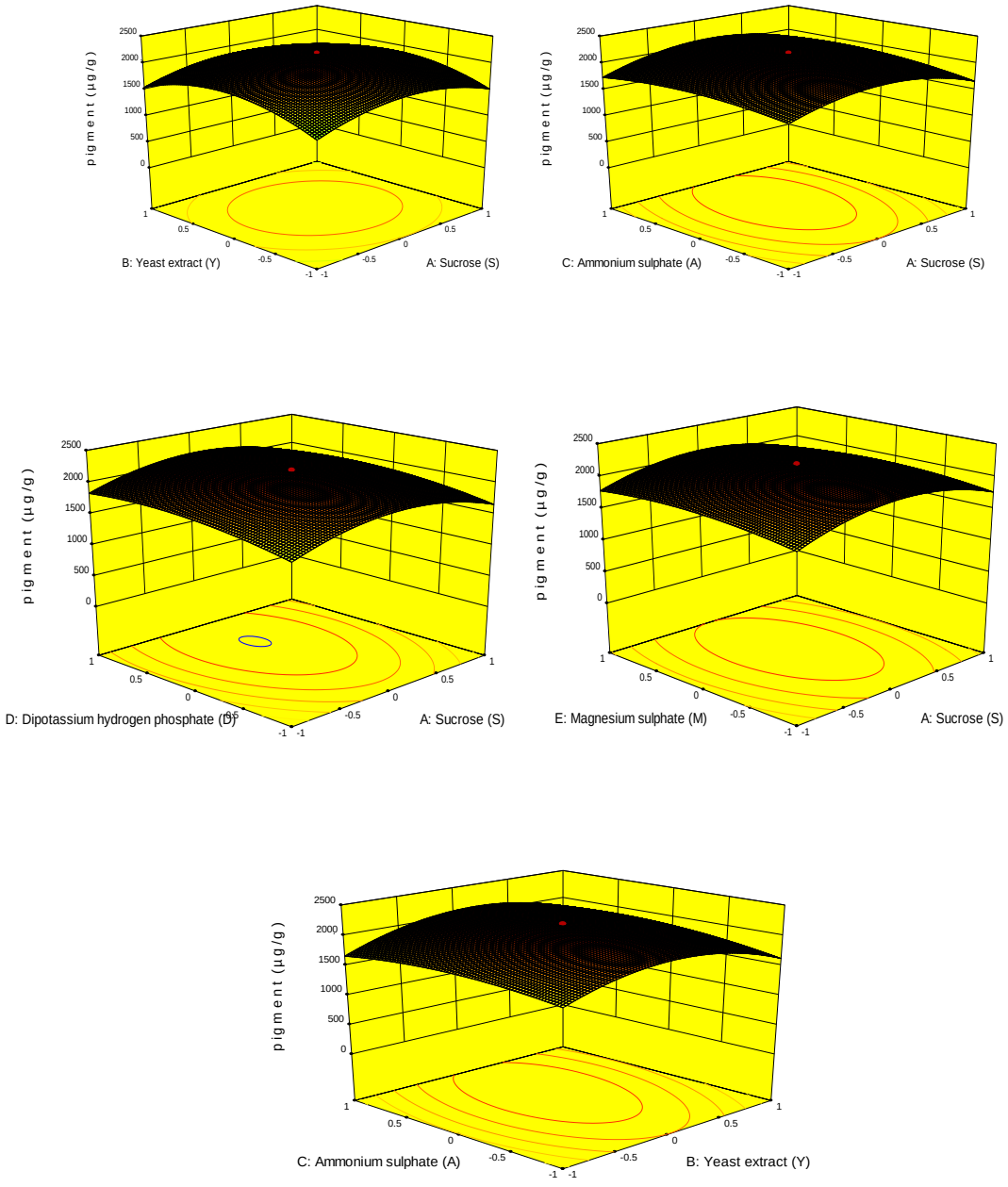


Fig 14. Response surface curve showing the effect of medium components on the pigment production by *R. mucilaginosa*

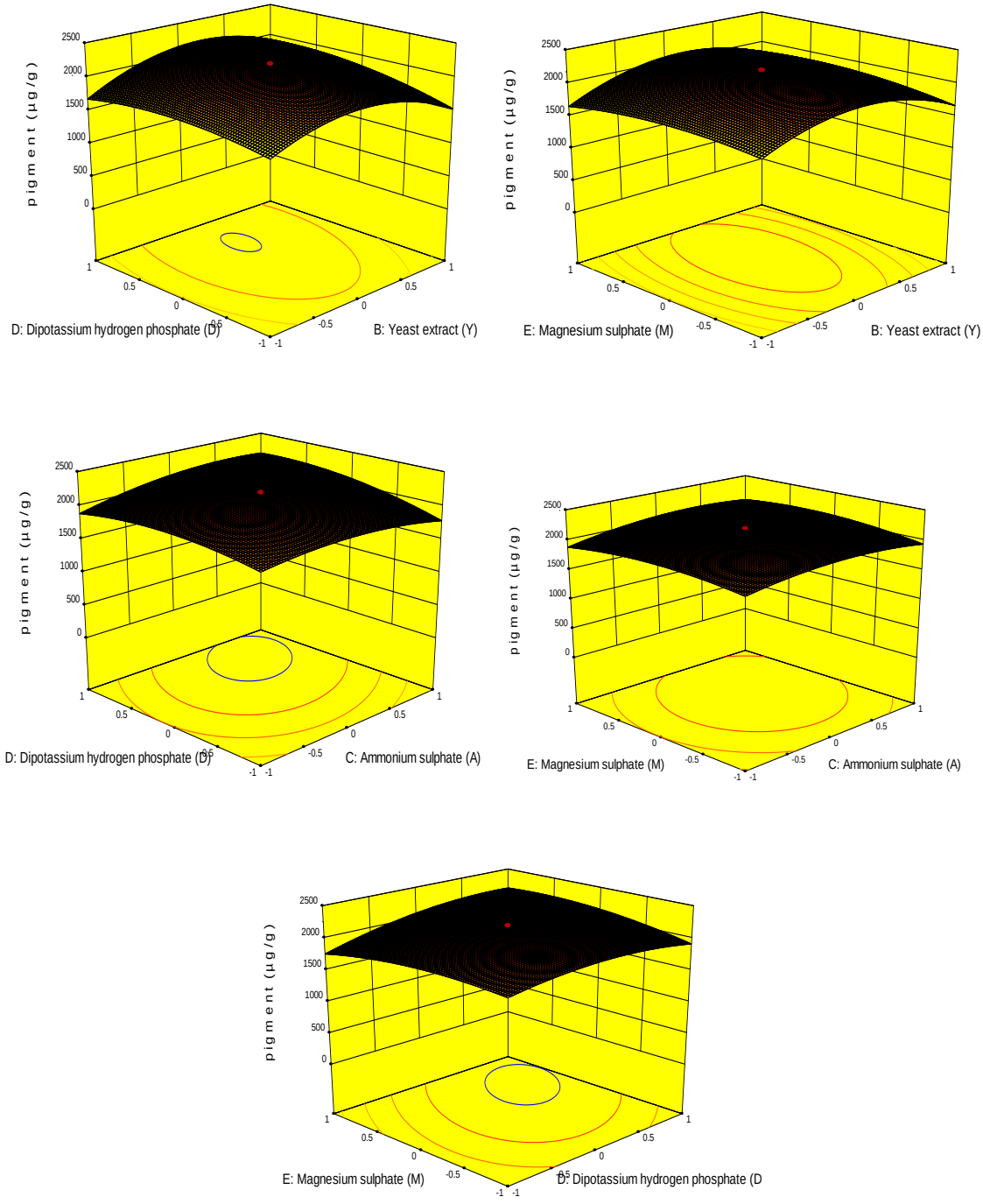


Fig 15. Response surface curve showing the effect of medium components on the pigment production by *Sporobolomyces* sp.

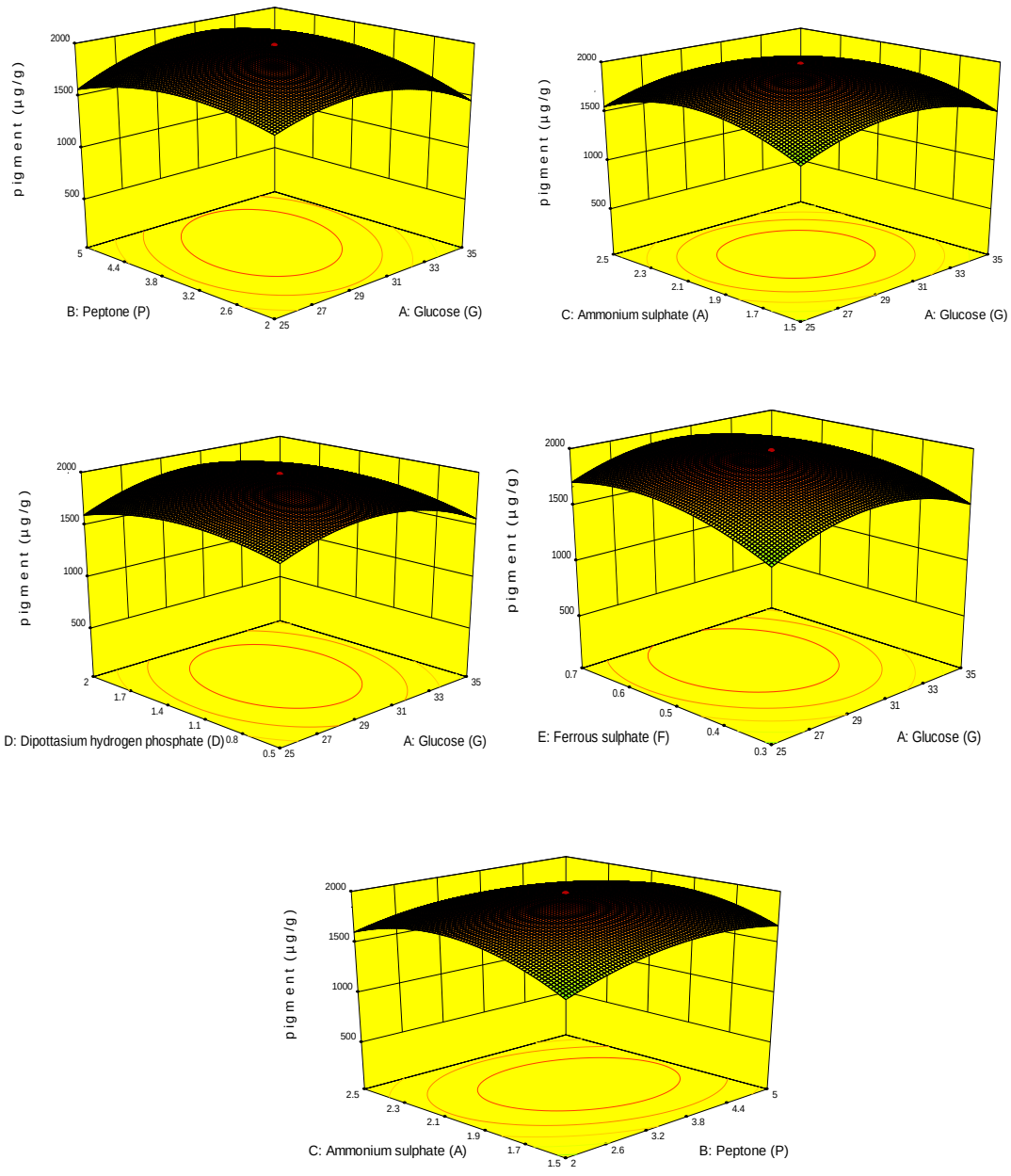
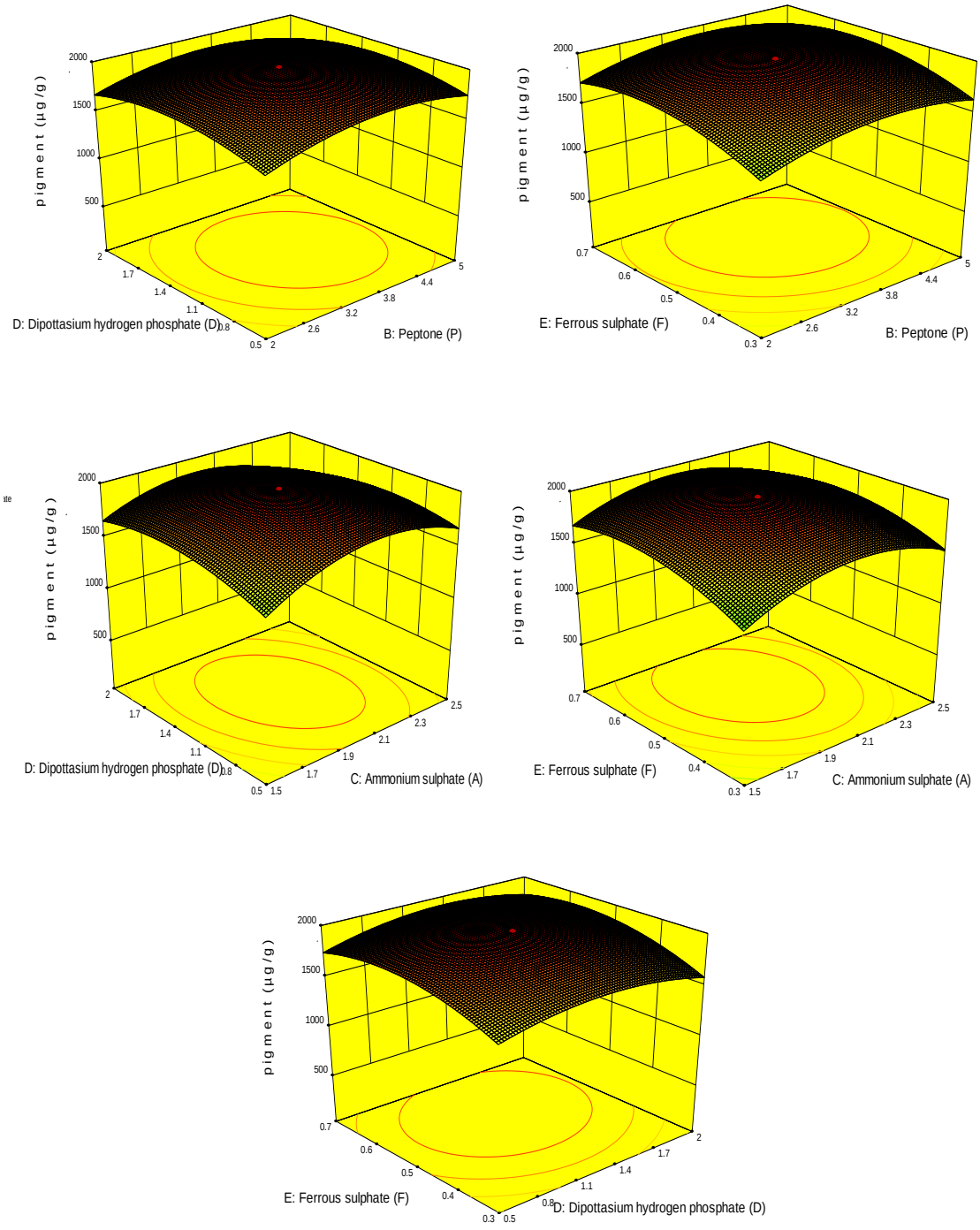


Fig 16. Response surface curve showing the effect of medium components on the pigment production by *Sporobolomyces* sp.



carotenoid pigment were calculated as 30 g l^{-1}, 3.5 g l^{-1}, 2 g l^{-1}, 1.25 g $l^{-1}$and 0.5 g l^{-1} for glucose, peptone, ammonium sulphate, dipottasium hydrogen phosphate and ferrous sulphate respectively. By substituting levels of the factors into the regression equation, the maximum predictable response for both carotenoid pigments production was calculated and was experimentally verified.

Maximum yield

By means of RSM studies pigment yield was enhanced from 6.00 mg l^{-1} to 13.00 mg L^{-1} in *R. mucilaginosa* and in *Sporobolomyces* sp. it increased from 5.00 mg l^{-1} to 10.00 mg L^{-1}.Through optimization studies, synthetic media yielded CDW and carotenoids about 6.10 g L^{-1} and 12.50-13.00 mg L^{-1} in *R. mucilaginosa* whereas in *Sporobolomyces* sp. (CDW - 5.20 g L^{-1}; carotenoids about 9.50-10.00 mg L^{-1}).

4.9. Selection of best substrate for carotenoid production using agricultural cheap sources

Different substrates were evaluated for selecting the best substrates for carotenoid pigment production from yeast cultures but *R. mucilaginosa* only showed growth in few cheap substrates. There was absence of growth in *Sporobolomyces* sp. (Table 54, Plate 19). Highest carotenoid pigment production was achieved with cassava bagasse followed by rice flour and sugarcane bagasse in *R. mucilaginosa* than other substrates which showed poor yields. The substrates such as wheat bran, tamarind seed powder did not support the growth and pigment production. Hence, cassava bagasse was selected and used for subsequent studies.

4.10. Physico - chemical properties of cassava bagasse

The physicochemical properties of cassava bagasse used for pigment production in *R. mucilaginosa* were analyzed and results are presented in Table 55. The components such as starch and cellulose were approximately 56.56 and 24.09 per cent and also contained 10.51 per cent crude fiber, 1.17 per cent protein and 2.12 per cent ash.

Table 54. Effect of agricultural cheap substrates for carotenoid pigment production

S. No.	Substrates	Production of carotenoid pigment
1	Rice bran	-
2	Wheat bran	-
3	Corn cob	+
4	Rice flour	++
6	Cassava bagasse	+++
7	Coconut oil cake	+
8	Sesame oil cake	+
9	Groundnut oil cake	+
10	Tamarind seed powder	-
11	Sugarcane bagasse	++

\+ : Slow growth

++ : Moderate growth

+++ : Better growth

\- : No growth

Plate19. Submerged fermentation with different agricultural cheap substrates (bottom view)



Rice flour



Sugarcane bagasse



Cassava bagasse

Table 55. Chemical composition of cassava bagasse

S. No.	Constituents	Percentage (%)
1	Carbohydrates	78.25 ± 0.90
2	Protein	1.17 ± 0.02
3	Crude fibre	10.51 ± 0.14
4	Cellulose	24.09 ± 0.27
5	Ash	2.12 ± 0.02
6	Moisture	6.20 ± 0.12
7	TSS	42.23±0.51
8	Starch	56.56±0.63

4.11. Submerged fermentation conditions for pigmentation in *R. mucilaginosa* by employing the best agricultural cheap source

4.11.1. RSM optimization conditions for carotenoid pigment production by cassava bagasse

To identify the combined effect for carotenoid pigment production, three factors (substrate concentration, fermentation time, and pH) were chosen for study because of their influence to pigment production. A central composite design of 20 experiments were selected to identify the optimum conditions.

The three independent variables (pH, temperature and fermentation time) and their concentrations at different coded and actual levels of the variables employed in the design matrix are shown in Table 17. Three level central composite design matrix and the experimental responses of the dependent variable (carotenoid pigment yield) are listed in Table 56. The data obtained were used to develop models in which each depended variable was obtained as the sum of the contributions of the independent variables through second order and interaction terms.

Data from 20 experiments were used for the following equations, where the coded values were obtained from regression analysis for the carotenoid pigment production. The application of RSM gave rise to the following regression equation for carotenoid pigment yield.

***R. mucilaginosa* pigment**

Final equation in terms of coded factors = + 2468.62+31.64*A +42.87*B +9.13*C -7.02*A*+47.02*A*C +10.14*B*C -585.45*A^2 -517.93*B^2 -467.75*C^2

Final equation in terms of actual factors = -42381.85387+6803.03928* pH +173.64992* temperature +1059.87524*time -0.29266*pH*temperature +9.40425* pH * time +0.084531* temperature * time -585.45281* pH^2 -0.89919* $temperature^2$ -18.70984*$time^2$

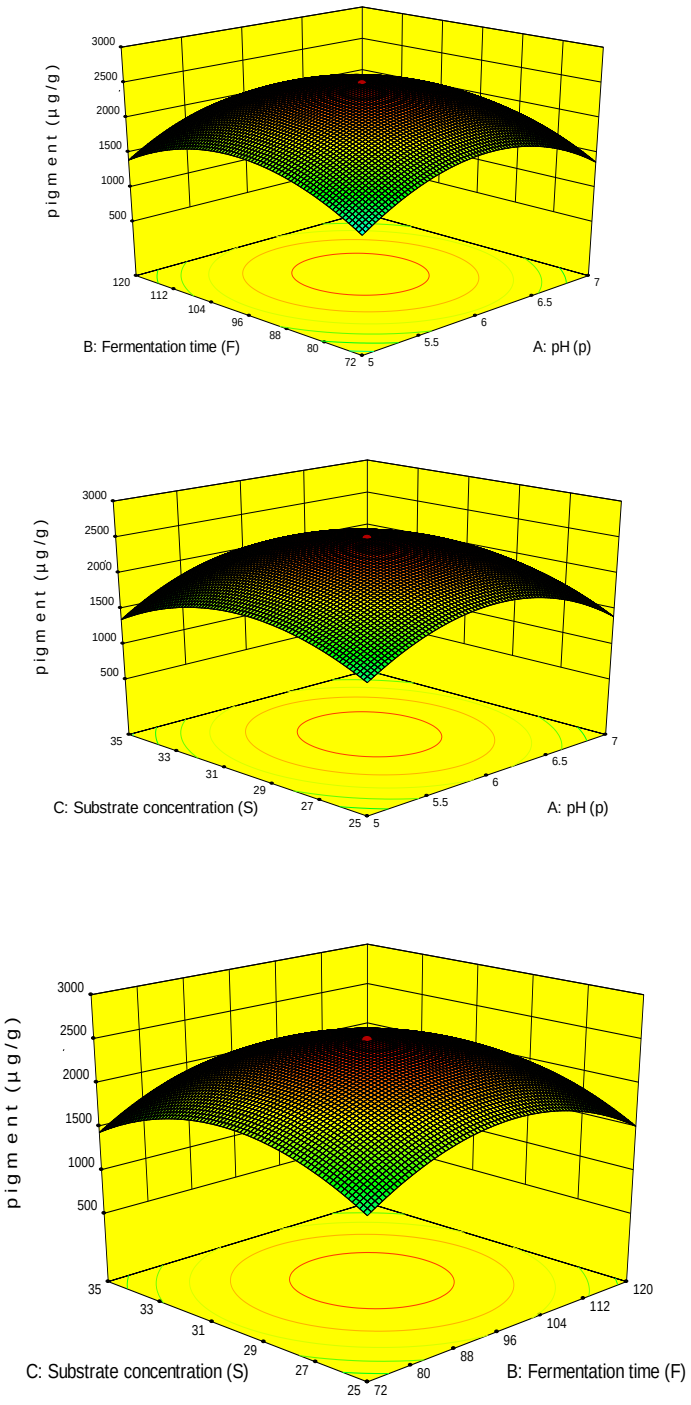
4.11.1.1. Optimization of carotenoid pigment production

Based on the experimental response, pigment produced by *R. mucilaginosa* ranged from 861.97 to 2478.02 run 9 and 15 had the minimum and maximum carotenoid

Table 56. Central composite rotatable design matrix of fermentation parameters on pigmentation in *R. mucilaginosa* by cassava bagasse and their independent variables and corresponding experimental yields

Std	pH	Incubation time (h)	Substrate concentration (g)	Pigment yield (µg g^{-1})	
				Observed	Predicted
1	5	72	25	860.10	864.00
2	7	72	25	850.11	847.28
3	5	120	25	943.02	943.49
4	7	120	25	900.26	898.68
5	5	72	35	963.09	967.92
6	7	72	35	935.91	936.56
7	5	120	35	887.91	887.99
8	7	120	35	1031.01	1031.26
9	4.31	96	30	861.97	863.07
10	7.68	96	30	863.90	865.92
11	6	55.63	30	931.98	933.59
12	6	136.36	30	1059.85	1055.78
13	6	96	21.59	1130.28	1130.78
14	6	96	38.40	1150.94	1150.98
15	6	96	30	2468.02	2468.62
16	6	96	30	2467.78	2468.62
17	6	96	30	2469.43	2468.62
18	6	96	30	2465.21	2468.62
19	6	96	30	2468.03	2468.62
20	6	96	30	2466.18	2468.62

Fig 17. Response surface curve showing the effect of cassava bagasse on the pigment production by *R. mucilaginosa*



pigment production respectively. The ANOVA results of quadratic regression model for *R. mucilaginosa* carotenoid pigment yield is described in Table 57. ANOVA of the regression model for pigment yield demonstrated that the model was significant due to an F- value of 6386.66 (*R. mucilaginosa* pigment) and very low probability value (p models > F- 0.0001). ANOVA for the model explained the response of the depends variable. Table 57 showed that the experimental yields fitted the second order polynomial equation as well as indicated by R^2 value 0.9979 (*R. mucilaginosa* pigment).

The regression co-efficient along with the corresponding p values for the models of pigmentation in *R. mucilaginosa* respectively are presented in Table 58. It showed that the regression coefficient of all the quadratic coefficient of S, T, t were significant at 0.05 per cent level.

The 3D response surfaces based on independent variables were obtained using the same software package indicated that a local optimum exists in the area experimentally investigated. Fig. 17 show three dimensional (3D response) surface plots of the effect of pH and temperature on the carotenoid pigment production. As the pH increased from 5 to 6 for pigmentation in *R. mucilaginosa* the curve gradually increased *i.e.,* when the temperature was around 25°C, the production of carotenoid pigment increased along with increasing incubation time.

The optimum levels in coded values for pH, temperature and incubation time were 0 and +1. The variable pH and temperature, pH and incubation time indicated that mutual interactions between these set of variables had a significant effect on the pigment yield. When the third independent variable fermentation time was kept constant at 96 h (*R. mucilaginosa* pigment) (Fig. 17) the interaction between the two variables (temperature and pH) showed that the pigment yield was sensitive even when pH and temperature were subject to small alterations.

The other pair of the independent variables pH and incubation time showed similar effects while keeping the third independent variable, temperature as a constant at 25°C. The 3D surface response for temperature and fermentation time on the yield of pigment (Fig.17). The results showed that as the values of process variables increased, the yield also increased but only upto the mid point range of variables and thereafter the

Table 57. Analysis of variance (ANOVA) for optimization of fermentation parameters for pigment production by cassava bagasse in *R. mucilaginosa*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Regression	1.006E+007	9	74.51	6386.66	<0.0001
Residual	1750.81	10	0.33	-	-
Cor Total	1.007E+007	19	-	-	-
R^2 = 0.9979, adjusted R^2 = 0.9975					

Table 58. Regression coefficient for optimization of fermentation parameters for pigment production by cassava bagasse in *R. mucilaginosa*

Factor	Regression Co efficient	Standard error	P value
Intercept	2468.62	5.40	< 0.0001
A-p	31.64	3.58	< 0.0001
B-T	42.87	3.58	< 0.0001
C-s	9.13	3.58	0.0289
AB	-7.02	4.68	0.1642
AC	47.02	4.68	< 0.0001
BC	10.14	4.68	0.0553
A^2	-585.45	3.49	< 0.0001
B^2	-517.93	3.49	< 0.0001
C^2	-467.75	3.49	< 0.0001

yield decreased even though the values of variables increased. The pigment yield was significantly effected by pH, temperature and incubation time where pH produced greater effect. Based on the model, the optimal working conditions were obtained to attain high carotenoid pigment yield. Casssava bagasse as a cheap substrate *R. mucilaginosa* were yielding 5.60 g l^{-1} (CDW) and carotenoid yield was ranging from 12.00-12.50 mg l^{-1} in *R. mucilaginosa*.

4.12. Production of intacellular carotenoid pigment

To confirm the aforementioned RSM results, *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments fermentation were conducted respectively in flasks and 3 l fermenter under the optimum conditions of physical and chemical parameters calculated by response surface analysis. The results demonstrated that the good correlation between predicted and measured values of these experiments verified the response of the verified model and the existence of the optimum point (Plate 20).

4.13. Extraction and solubility of intracellular carotenoid pigments using different solvents

Carotenoid pigment extraction was carried out by solvent extraction. Among the tested solvents, acetone and ethanol were more efficient than any of the others to extract intracellular carotenoids from the yeast cells. The carotenoid pigment was also soluble in methanol, benzene, acetonitrile, ethyl acetate, diethyl ether, hexane, petroleum ether, toluene but insoluble in propanol (Table 59 and Plate 21). The pigment extracted with acetone was taken and vacuum dried. The concentrated pigment was used for further studies.

4.14. Thin layer chromatography studies

The different solvent systems ranging from low polar to high polar were tested for effective separation of carotenoid pigment compounds from acetone extracts of *R. mucilaginosa* and *Sporobolomyces* sp. Best separation of the *R. mucilaginosa* pigment compounds achieved with the mobile phase consisting of benzene and petroleum ether (85:15). The carotenoid pigment fractions of *R. mucilaginosa* were separated into 3 bands (pink, orange and yellow). The Rf values of the TLC bands were presented in Table 60. Whereas acetone

Table 59. Effect of different solvents on extraction of yeast carotenoid pigments

S. No.	Solvents	*R. mucilaginosa* pigment	*Sporobolomyces* sp. pigment
1	Methanol	++	++
2	Ethanol	++	++
3	Acetone	+++	+++
4	Ethyl acetate	+	+
5	Diethyl ether	+	+
6	Benzene	+	+
7	Acetonitrile	+	+
8	Hexane	+	+
9	Propanol	-	-
10	Chloroform	+	+
11	Toluene	+	+
12	Petroleum ether	+	+

Table 60. TLC of acetone extracts of carotenoid pigment from *R. mucilaginosa*

Band No.	Colour of the band	Distance travelled (cm)	Rf value (cm)
1	Pink	10.5	0.44
2	Orange	11.5	0.79
3	Yellow	12.6	0.87
4	Yellow (Standard β-carotene)	12.7	0.89

Table 61. TLC of acetone extracts of carotenoid pigment from *Sporobolomyces* sp.

Band No.	Colour of the band	Distance travelled (cm)	Rf value (cm)
1	Pink	9.6	0.41
2	Orange	10.4	0.74
3	Yellow	12.5	0.88
4	Yellow (Standard β-carotene)	12.7	0.89

Plate 20. Production of yeast carotenoid pigment in fermentor



Plate 21. Carotenoid pigment extract from intracellular yeast cells



A . Acetone extract of carotenoid pigment



B. Vaccum dried carotenoid pigment

extract of *Sporobolomyces* sp. pigment compounds were separated using hexane: acetone (70:30) as mobile phase. Three bands (pink, orange and yellow) were separated from the pigment fractions of *Sporobolomyces* sp. The Rf values of the TLC bands were listed in Table 61.

4.15. Purification of pigments by reverse phase high performance of liquid chromatography (RP-HPLC)

RP-HPLC studies were performed for the determination of compounds present in the pigment of *R. mucilaginosa* and *Sporobolomyces* sp. The mobile phase (HPLC grade solvents) and samples were filtered through 0.25µm membrane filter, C18 column consists of acetonitrile, isopropanol and ethyl acetate (40:40:20, v\v\v). The flow rate was maintained at 1 ml min^{-1} with a running time of 30 min and detection wavelength at 485 nm. The standard β-carotene used as reference. Retention time obtained for standard β-carotene was 3.36 minutes, 10µl carotenoid extract injected into the C18 column resulted in 3 peaks for pigmentation in *R. mucilaginosa* (peak 1 – 3.35 min; peak 2 - 4.75 min; peak 3-5.93 min) and for pigmentation in *Sporobolomyces* sp. (peak 1- 3.35 min; peak 2- 4.20 min and peak 3- 5.77 min) (Fig.18,19 and 20). The retention times of β-carotene extracted from *R. mucilaginosa* and *Sporobolomyces* sp. observed were similar to that of standard graph.

4.16. FT-IR

The identification of the major carotenoid fractions extracted from the yeast *R. mucilaginosa* was based on absorption, FT-IR spectra (Fig.21) showed the band at 2993 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH_3 groups respectively and band at 2762 cm^{-1} are due to symmetrical stretching vibration of same groups. The asymmetrical and symmetrical deformation vibrations CH_3 groups are observed at 1350 cm^{-1} respectively. Low intensity band at 1728 cm^{-1} denoted >C = O group. The band at 1666 cm^{-1} indicates C = C due to alkenes .The band at 3657 cm^{-1} indicates the hydroxyl amino group. The band at 3086 cm^{-1} showed that CH stretching vibration .

Whereas the FT-IR spectrum of *Sporobolomyces* sp. carotenoid pigment (Fig.22) showed the band at 2931 $cm^{-1}$are due to symmetrical or asymmetrical stretching vibration

Fig 18. HPLC analysis of standard β-carotene compound

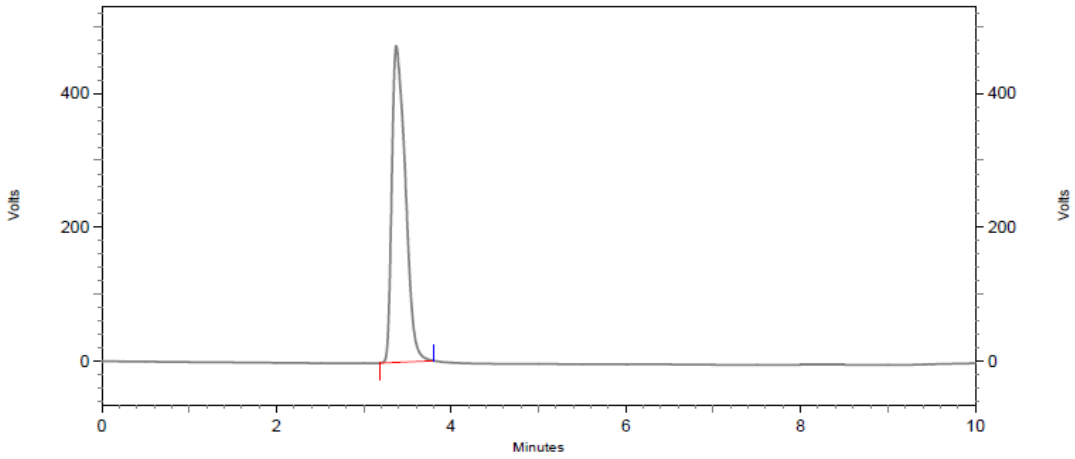


Fig 19. HPLC analysis of carotenoid pigment extract from *R. mucilaginosa*

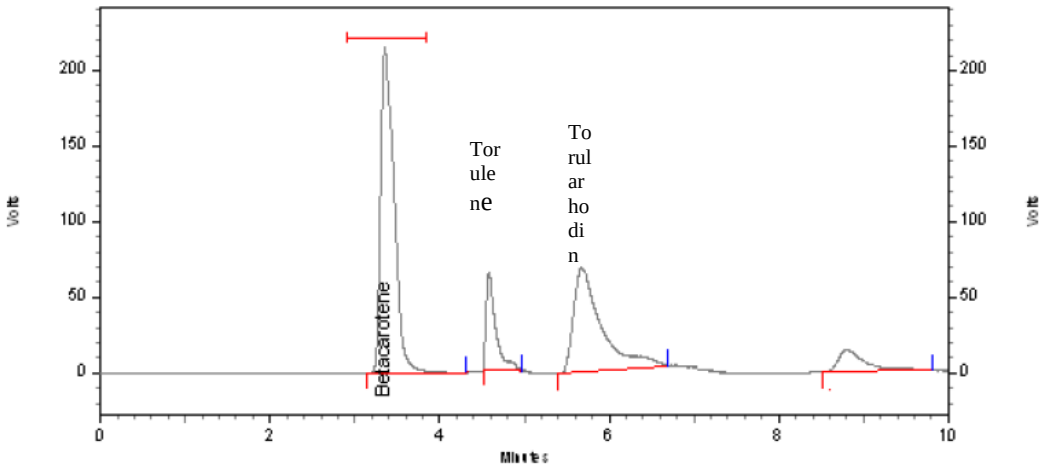


Fig 20. HPLC analysis of carotenoid pigment extract from *Sporobolomyces* sp.

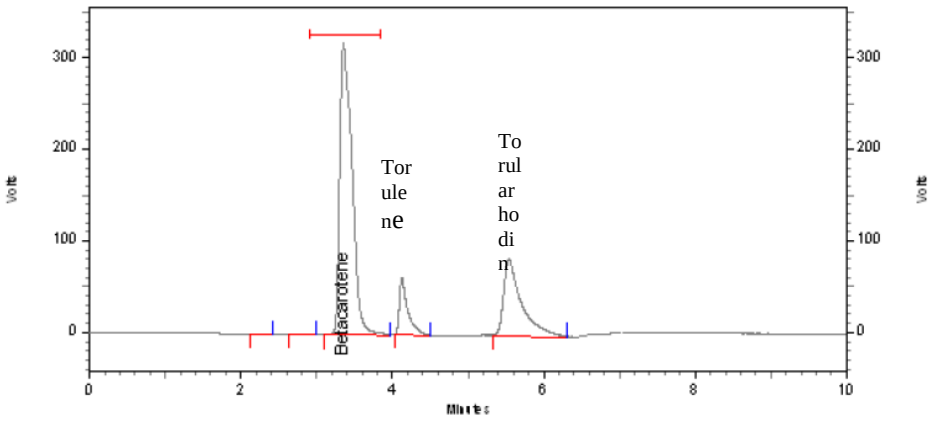


Fig 21. FT-IR spectrum of pigment extracted from *R. mucilaginosa*

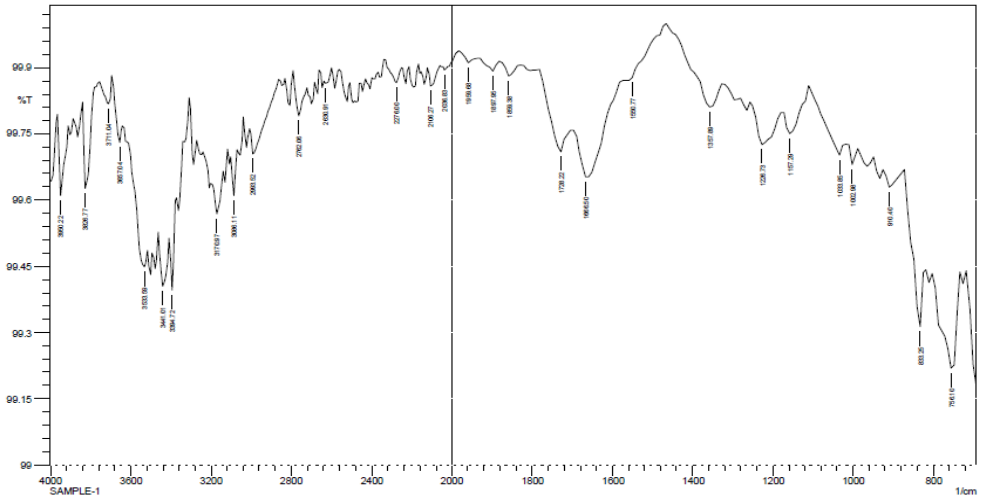
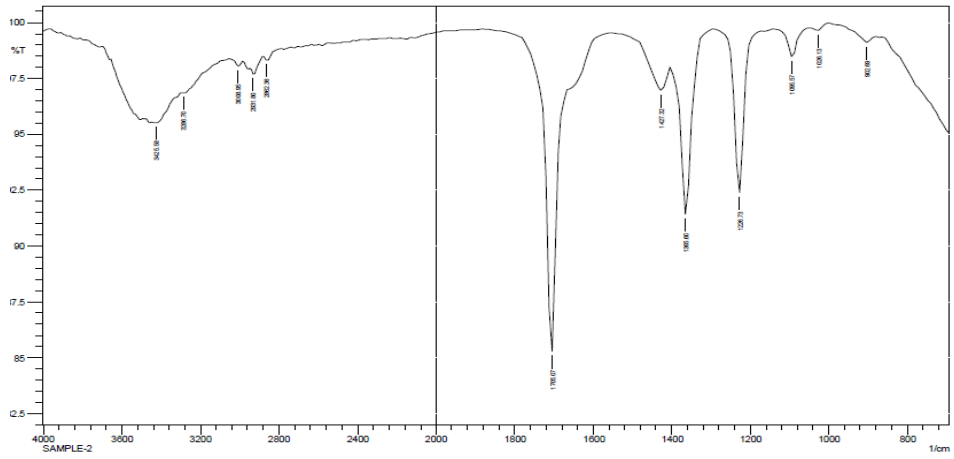


Fig 22. FT-IR spectrum of pigment extracted from *Sporobolomyces* sp.



of aliphatic CH_3 group and bands at 2862 cm^{-1} are due to symmetrical stretching vibration of same groups. The band observed at 1427 cm^{-1} was due to C–C stretch and 1365 cm^{-1} was due to symmetrical stretching vibrations of CH_3 groups. Low intensity band at 3008cm^{-1} is due to CH stretching vibration CH = CH and 1705 cm^{-1} may be due to C=O stretch ketone group. Presence of a band at 1226cm^{-1} due to the C – O stretching vibration of the ester.

4.17. Stability of yeast carotenoid pigments to various physical and chemical conditions

4.17.1. pH stability of intracellular carotenoid pigments

The pH of the pigment extracted from *R. mucilaginosa* and *Sporobolomyces* sp. was adjusted to 5, 6 and 7 using 0.1N NaOH and HCl. Optical density of pigment solution was measured from 0 to 48h of incubation at room temperature and stability of the pigment was measured. Both *R. mucilaginosa* and *Sporobolomyces* sp. pigment solution showed its stability till 1h at pH 5, pH 6 and pH 7. The data presented in Table 62 and Table 63 depicts the stability changes over the time at different pH values. At pH 7, pigmentation in *R. mucilaginosa* and *Sporobolomyces* sp. showed maximum stability with no change in absorbance till 48h as 99per cent residual color remain in the solution. But at pH 5 and 6 absorbance started decreased with increase in incubation time period. Maximum decrease was observed at pH 5 (0.822 to 0.616 OD ml^{-1}) and (0.832 to 0.727 OD ml^{-1}) with 76 per cent and 75 per cent residual color in the solution. At pH 6 (0.830 to 0.732 OD ml^{-1}) and (0.832 to 0.727 OD ml^{-1}) where 88 per cent and 87 per cent residual color was retained by pigment extracted from *R. mucilaginosa* and *Sporobolomyces* sp. From the observations we can conclude that both pigment solutions are having maximum stability at pH 7 than under acidic conditions.

4.17.2. Heat stability of intracellular pigments

The carotenoid pigment extracted from *R. mucilaginosa and Sporobolomyces* sp. was heated from (25-100°C) for 5-20 min and change of optical density was measured by taking OD at 360 nm. It was shown that colour decrease was more rapid in temperature 90°C for 5-20 min (0.753 to 0.362 OD ml^{-1}) for *R. mucilaginosa* pigment and (0.720 to 0.358 OD ml^{-1}) for *Sporobolomyces* sp. pigment and decrease was nearly

Table 62. Stability of the pigment from *R. mucilaginos* after different time intervals in different pH conditions

pH	OD at 360 nm				
	Time intervals (h)				
	Before treatment	0	1	24	48
5.0	0.822±0.018	0.822±0.015	0.822±0.018	0.760±0.017	0.616±0.017
6.0	0.830±0.019	0.830±0.016	0.830±0.018	0.784±0.018	0.732±0.017
7.0	0.864±0.019	0.864±0.016	0.864±0.019	0.860±0.019	0.860±0.018

Table 63. Stability of the pigment from *Sporobolomyces* sp. carotenoid pigment after different time intervals in different pH conditions

5.0	0.810±0.019	0.810±0.016	0.810±0.018	0.730±0.018	0.701±0.017
6.0	0.832±0.018	0.832±0.015	0.832±0.017	0.779±0.017	0.727±0.017
7.0	0.853±0.019	0.853±0.016	0.853±0.018	0.850±0.018	0.849±0.018

Table 64. Stability of the pigmentation in *R. mucilaginosa* at different time intervals and temperatures

Time (min)	Optical density at 360 nm					Before treatment
	Temperature incubation (ºC)					
	25	50	75	90	100	
5	0.910±0.018	0.825±0.018	0.791±0.018	0.753±0.017	0.691±0.015	0.943±0.021
10	0.882±0.018	0.801±0.0181	0.643±0.011	0.517±0.009	0.487±0.006	0.901±0.021
15	0.835±0.016	0.746±0.017	0.612±0.014	0.489±0.011	0.362±0.008	0.856±0.019
20	0.810±0.016	0.676±0.015	0.601±0.009	0.362±0.008	0.301±0.008	0.842±0.019

Table 65. Stability of the pigmentation in *Sporobolomyces* sp. at different time intervals and temperatures

Time (min)	Optical density at 360 nm					
	Temperature incubation (ºC)					
	Before treatment	25	50	75	90	100
5	0.951±0.021	0.931±0.018	0.814±0.018	0.773±0.017	0.720±0.016	0.676±0.014
10	0.913±0.021	0.894±0.018	0.816±0.018	0.717±0.011	0.438±0.009	0.472±0.006
15	0.847±0.019	0.815±0.016	0.770±0.017	0.692±0.011	0.495±0.011	0.306±0.005
20	0.835±0.019	0.931±0.018	0.814±0.018	0.773±0.017	0.720±0.016	0.676±0.014

about 48 and 49 per cent respectively (Table 64 and Table 65). When the temperature was found to be increased to 100°C for 20 min, pigment stability was (0.691 to 0.301 OD ml^{-1}) for *R. mucilaginosa* pigment and (0.676 to 0.289 OD ml^{-1}) for *Sporobolomyces* sp. pigment.

4.17.3. Antioxidant stability of intracellular carotenoid pigments

When an antioxidant such as ascorbic acid and butylated hydroxyl anisole (BHA) (0.3 g l^{-1}) were added to the pigment solution, the stability of *R. mucilaginosa* pigment solution for ascorbic acid stability varied from (0.94 to 0.93 OD ml^{-1}) at 4°C, (0.92 to 0.89 OD ml^{-1}) at room temperature, (0.71 to 0.68 OD ml^{-1}) at 60°C. For BHA stability varied from (0.94 to 0.94 OD ml^{-1}) at 4°C, (0.92 to 0.88 OD ml^{-1}) at room temperature, (0.74 to 0.57 OD ml^{-1}) at 60°C.

With regard to pigmentation in *Sporobolomyces* sp. stability for ascorbic acid stable at 4°C show 0.92 OD ml^{-1}, stability varied slightly at room temperature 0.88 to 0.87 OD ml^{-1} and showed 0.54 to 0.40 OD ml^{-1} at 60°C. For BHA dark condition stability varied from (0.94 to 0.94 OD ml^{-1}) at 4°C, (0.90 to 0.89 OD ml^{-1}) at room temperature, (0.62 to 0.57 OD ml^{-1}) at 60°C (Table 66 and Table 67). Addition of antioxidants to both the pigments was found to increase the stability of the pigment to certain extent.

4.17.4. Stability of intracellular pigments in different oils

Storage stability of the pigment was also studied in different oils at different temperatures (4°C, RT and 60°C) and different conditions (presence and absence of light). *R. mucilaginosa* pigment was found to be more stable in sesame oil in dark condition after 3rd - 90th day stability varied from (0.95 to 0.77 OD ml^{-1}) at 4°C, (0.89 to 0.68 OD ml^{-1}) at room temperature,(0.52 to 0.33 OD ml^{-1}) at 60°C and in light condition stability varied from (0.92 to 0.75 OD ml^{-1}) at 4°C, (0.89 to 0.73 OD ml^{-1}) at room temperature, (0.49 to 0.30 OD ml^{-1}) at 60°C.The sunflower oil also showed more stability in dark condition after (3rd -90th day) stability varied from (0.94 to 0.76 OD ml^{-1}) at 4°C, (0.87 to 0.69 OD ml^{-1}) at room temperature, (0.51 to 0.32 OD ml^{-1}) at 60°C and in light condition stability varied from (0.91 to 0.74 OD ml^{-1}) at 4°C, (0.86 to 0.72 OD ml^{-1}) at room temperature, (0.37 to 0.24 OD ml^{-1}) at 60°C (Table 68 and Plate 22).

Table 66. Antioxidant stability of the intracellular pigment produced by *R. mucilaginosa* after different time intervals

Temperature(ºC)	Antioxidants (0.3 g l^{-1}) (OD at 360 nm)						
	Before treatment	Butylated hydroxyl anisole (BHA) (Days)			Ascorbic acid (Days)		
		3rd day	30th day	60 th day	3rd day	30th day	60 th day
4ºC	0.94±0.021	0.94±0.021	0.94±0.021	0.94±0.021	0.94±0.021	0.94±0.021	0.93±0.021
RT(27±2ºC)	0.90±0.020	0.92±0.020	0.90±0.020	0.88±0.020	0.92±0.020	0.91±0.020	0.89±0.020
60ºC	0.80±0.017	0.74±0.014	0.68±0.013	0.57±0.013	0.71±0.012	0.71±0.011	0.68±0.011

Table 67. Antioxidant stability of the intracellular pigment produced by *Sporobolomyces* sp. after different time intervals

Temperature (ºC)	Antioxidants (0.3 g l^{-1}) (OD at 360 nm)						
	Before treatment	Butylated hydroxyl anisole (BHA) (Days)			Ascorbic acid (Days)		
		3rd day	30th day	60 th day	3rd day	30th day	60 th day
4ºC	0.93±0.021	0.94±0.019	0.94±0.021	0.94±0.021	0.92±0.021	0.92±0.021	0.92±0.021
RT(27±2ºC)	0.90±0.020	0.90±0.021	0.90±0.020	0.89±0.020	0.88±0.018	0.87±0.020	0.87±0.020
60ºC	0.76±0.018	0.62±0.017	0.60±0.015	0.57±0.013	0.54±0.016	0.51±0.016	0.49±0.015

Table 68. Stability of the intracellular pigmentation in *R. mucilaginosa* using different oils under different conditions

Oils	Dark condition(OD at 360 nm)									Light condition(OD at 360 nm)									Before treatment
	3rd day			45th day			90 th day			3rd day			45th day			90 th day			
	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	
Rice bran oil	0.85± 0.01	0.84±0.01	0.48± 0.01	0.81± 0.01	0.82±0.01	0.42± 0.01	0.70± 0.01	0.64±0.01	0.29± 0.00	0.83± 0.01	0.81±0.01	0.46± 0.00	0.81± 0.01	0.79±0.01	0.43± 0.00	0.69± 0.01	0.66±0.01	0.23± 0.00	0.89±0.01
Sesame oil	0.95± 0.02	0.89±0.02	0.52± 0.01	0.89± 0.02	0.86±0.01	0.49± 0.01	0.77± 0.02	0.68±0.01	0.33± 0.00	0.92± 0.02	0.89±0.02	0.49± 0.01	0.86± 0.01	0.85±0.01	0.43± 0.00	0.75± 0.01	0.73±0.01	0.30± 0.00	0.96±0.02
Groundnut oil	0.88± 0.02	0.80±0.01	0.52± 0.01	0.85± 0.01	0.79±0.01	0.49± 0.01	0.72± 0.01	0.66±0.01	0.31± 0.00	0.86± 0.01	0.83±0.01	0.42± 0.00	0.79± 0.01	0.80±0.01	0.39± 0.00	0.63± 0.01	0.62±0.01	0.22± 0.00	0.92±0.02
Sunflower oil	0.94± 0.02	0.87±0.02	0.51± 0.01	0.88± 0.02	0.84±0.01	0.48± 0.01	0.76± 0.01	0.69±0.01	0.32± 0.00	0.91± 0.02	0.86±0.01	0.37± 0.00	0.87± 0.02	0.84±0.01	0.35± 0.00	0.74± 0.01	0.72±0.01	0.24± 0.00	0.97±0.02
Palm oil	0.88± 0.01	0.85±0.01	0.49± 0.01	0.80± 0.01	0.81±0.01	0.38± 0.01	0.69± 0.01	0.60±0.01	0.29± 0.00	0.81± 0.01	0.80±0.01	0.41± 0.00	0.80± 0.01	0.78±0.01	0.39± 0.00	0.70± 0.01	0.64±0.01	0.24± 0.00	0.86±0.01

Table 68. Contd…..

Oils	Dark condition (OD at 360 nm)									Light condition(OD at 360 nm)									Before treatment
	3rd day			45th day			90 th day			3rd day			45th day			90 th day			
	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	
Coconut oil	0.71± 0.01	0.59± 0.01	0.46± 0.01	0.67± 0.01	0.54± 0.01	0.42± 0.01	0.52± 0.01	0.36±0.01	0.21± 0.00	0.69± 0.01	0.64±0.01	0.39± 0.00	0.63± 0.01	0.52± 0.01	0.34± 0.00	0.47± 0.01	0.36±0.01	0.13±0.00	0.76± 0.01
Corn oil	0.90± 0.02	0.89± 0.02	0.50± 0.01	0.84± 0.01	0.82± 0.01	0.43± 0.01	0.71± 0.01	0.70±0.01	0.34± 0.01	0.84± 0.01	0.80±0.01	0.79± 0.01	0.82± 0.01	0.77± 0.01	0.40± 0.00	0.68± 0.01	0.66±0.01	0.23±0.00	0.92± 0.02
Mustard oil	0.80± 0.01	0.78± 0.01	0.44± 0.01	0.76± 0.01	0.76± 0.01	0.40± 0.01	0.59± 0.01	0.63±0.01	0.24± 0.00	0.78± 0.01	0.72±0.01	0.32± 0.00	0.74± 0.01	0.69± 0.01	0.28± 0.00	0.57± 0.01	0.54±0.01	0.13±0.00	0.85± 0.01
Safflower oil	0.82± 0.01	0.80± 0.01	0.49± 0.01	0.79± 0.01	0.77± 0.01	0.47± 0.01	0.66± 0.01	0.64±0.01	0.30± 0.00	0.82± 0.01	0.78±0.01	0.47± 0.01	0.77± 0.01	0.76± 0.01	0.45± 0.01	0.66± 0.01	0.63±0.01	0.20±0.00	0.86± 0.01

Plate 22. *R. mucilaginosa* carotenoid pigment stored in different oils



Plate 23. *Sporobolomyces* sp. carotenoid pigment stored in different oils



With regard to pigmentation in *Sporobolomyces* sp. the stability was more in sunflower and sesame oil. In sunflower oil, pigment showed more stability in dark condition after 3rd-90thday stability varied from (0.95 to 0.82 OD ml^{-1}) at 4°C, (0.89 to 0.74 OD ml^{-1}) at room temperature, (0.51 to 0.34 OD ml^{-1}) at 60°C and in light condition stability varied from (0.89 to 0.73 OD ml^{-1}) at 4°C, (0.88 to 0.74 OD ml^{-1}) at room temperature, (0.36 to 0.22 OD ml^{-1}) at 60°C. In sesame oil, carotenoid pigment stability was observed in dark condition after (3rd-90th day) stability varied from (0.96 to 0.80 OD ml^{-1}) at 4°C, (0.89 to 0.75 OD ml^{-1}) at room temperature, (0.49 to 0.33 OD ml^{-1}) at 60°C and in light condition stability varied from (0.89 to 0.74 OD ml^{-1}) at 4°C, (0.87 to 0.75 OD ml^{-1}) at room temperature, (0.42 to 0.21 OD ml^{-1}) at 60°C (Table 69 and Plate 23).

4.18. Toxicity analysis on the pigment produced by *R. mucilaginosa* and *Sporobolomyces* sp.

The toxicity study for 28 days period conducted for assessing the safety evaluation of carotenoid pigments of *R. mucilaginosa* and *Sporobolomyces* sp. respectively by acute and sub-acute toxicity in rats (Plate 24)

4.18.1. Acute toxicity study

4.18.1.1. Clinical signs and mortality

All the mice were observed at least twice daily with the purpose of recording any symptoms of ill-health or behavioural changes. These observations were also performed on week-ends. The observations included changes in skin and fur, in the eyes and mucous membranes, in the respiratory, circulatory, central nervous and autonomous systems, somatomotor activity and behavior.

All the mice survived until scheduled necropsy and showed normal growth and appeared healthy throughout the study period. Daily general observations, opthalmoscopy and clinical examinations revealed no treatment – related changes (data not shown) occured.

4.18.1.2. Organ weights and histopathology

Relative organ weight of brain, heart, liver, lungs, spleen, kidney (left and right), pancreas and testis of treated groups were comparable to that of controls. No histopathological alterations were observed at any of the dosage levels.

Table 69. Stability of the intracellular pigmentation in *Sporobolomyces* sp. using different oils under different conditions

Oils	Dark condition (OD at 360 nm)									Light condition (OD at 360 nm)									Before treatment
	3rd day			45th day			90 th day			3rd day			45th day			90 th day			
	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	
Rice bran oil	0.91± 0.01	0.87±0.01	0.46± 0.00	0.87± 0.00	0.83±0.01	0.42± 0.01	0.76± 0.01	0.68±0.01	0.24± 0.01	0.82± 0.00	0.78±0.01	0.34± 0.01	0.76± 0.00	0.74±0.01	0.29± 0.00	0.64± 0.01	0.61±0.01	0.18± 0.00	0.94± 0.01
Sesame oil	0.96± 0.02	0.89±0.02	0.49± 0.01	0.93± 0.00	0.86±0.02	0.46± 0.01	0.80± 0.01	0.75±0.01	0.33± 0.01	0.89± 0.00	0.87±0.02	0.42± 0.02	0.86± 0.00	0.87±0.02	0.36± 0.00	0.74± 0.01	0.75±0.01	0.21± 0.00	0.97± 0.02
Groundnut oil	0.95± 0.02	0.89±0.02	0.48± 0.01	0.92± 0.00	0.85±0.02	0.45± 0.01	0.79± 0.01	0.72±0.02	0.31± 0.01	0.87± 0.00	0.85±0.02	0.35± 0.01	0.84± 0.00	0.80±0.01	0.32± 0.00	0.70± 0.01	0.68±0.01	0.19± 0.00	0.97± 0.02
Sunflower oil	0.95± 0.02	0.89±0.02	0.51± 0.01	0.94± 0.00	0.87±0.02	0.45± 0.02	0.82± 0.01	0.74±0.02	0.34± 0.01	0.89± 0.01	0.88±0.02	0.36± 0.02	0.84± 0.00	0.88±0.02	0.34± 0.00	0.73± 0.01	0.74±0.01	0.22± 0.00	0.98± 0.02
Palm oil	0.87± 0.01	0.85±0.01	0.45± 0.00	0.81± 0.00	0.80±0.01	0.39± 0.01	0.77± 0.01	0.67±0.02	0.27± 0.01	0.80± 0.00	0.80±0.01	0.34± 0.01	0.77± 0.00	0.76±0.01	0.33± 0.00	0.62± 0.01	0.58±0.01	0.20± 0.00	0.96± 0.02

Table 69. Contd…

Oils	Dark condition(OD at 360 nm)									Light condition(OD at 360 nm)									Before treatment
	3rd day			45th day			90 th day			3rd day			45th day			90 th day			
	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	4ºC	RT (27-33ºC)	60ºC	
Coconut oil	0.82± 0.01	0.79±0.01	0.36± 0.00	0.78± 0.00	0.77±0.01	0.32± 0.01	0.66± 0.01	0.63±0.01	0.19± 0.01	0.76± 0.00	0.75±0.02	0.39± 0.01	0.73± 0.00	0.69±0.01	0.33± 0.01	0.61± 0.01	0.53± 0.01	0.20± 0.00	0.88± 0.02
Corn oil	0.87± 0.02	0.87±0.02	0.40± 0.00	0.86± 0.07	0.85±0.01	0.37± 0.01	0.72± 0.01	0.70±0.01	0.24± 0.01	0.87± 0.00	0.86±0.01	0.41± 0.01	0.85± 0.00	0.83±0.01	0.37± 0.01	0.72± 0.01	0.69± 0.01	0.22± 0.00	0.90± 0.02
Mustard oil	0.78± 0.01	0.76±0.01	0.31± 0.00	0.76± 0.00	0.72±0.01	0.25± 0.01	0.64± 0.01	0.58±0.01	0.12± 0.01	0.77± 0.00	0.79±0.01	0.28± 0.01	0.76± 0.00	0.74±0.01	0.23± 0.01	0.65± 0.01	0.62± 0.01	0.12± 0.00	0.83± 0.01
Safflower oil	0.87± 0.02	0.82±0.01	0.43± 0.00	0.84± 0.00	0.78±0.01	0.37± 0.01	0.66± 0.01	0.62±0.01	0.12± 0.01	0.86± 0.00	0.83±0.01	0.31± 0.01	0.80± 0.00	0.79±0.01	0.26± 0.01	0.66± 0.01	0.62± 0.01	0.10± 0.00	0.90± 0.02

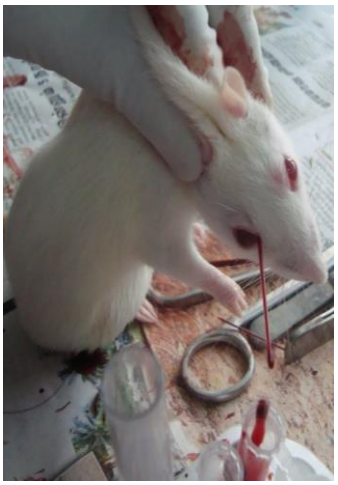
Plate 24.Toxicity evaluation of yeast carotenoid pigments on rats



Treatments



Oral dose



Blood collection



Rat dissection



Vital organs

4.18.2. Sub-acute toxicity study

4.18.2.1. Clinical signs and mortality

Oral dose of carotenoid pigments for 28 days study period did not produce any obvious signs of toxicity or mortality in male rats during the experimental period at any of the dosage levels.

4.18.2.2. Food intake, water intake and body weight

Oral feeding of *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments even at higher dose did not significantly affect food intake and water intake in male rats of treatment groups and were comparable to that of control group. The actual food intake, water intake and mean gain in body weight are presented in Table 70 and Table 71. No difference in food and water intake was observed in treatment groups of male rats during the 28 days experimental period.

The bodyweight of each rat was recorded before the start of the treatment and the changes of body weight recorded every 3-4 days interval. The changes of body weight in both pigment treated male rats were slightly increased. The body weight of male rats exposed to 4 per cent *R. mucilaginosa* and *Sporobolomyces* sp. pigments tend to be higher than the control values.

4.18.2.3. Organ weight

There were no significant difference in the group mean relative weights of various vital organs (brain, heart, liver, lungs, spleen, kidney (left and right), pancreas and testis) in the *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments treated rats compared to that of control rats (Table 72 and Table 73).

4.18.2.4. Hematological profile

Data on the various hematological parameters are presented in Table 74 and Table 75. Haemoglobin, red blood cells, white blood cells, packed cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential count, mean corpuscular volume, red blood cell distribution width, platelet count and mean platelet volume did not show any biologically or statistically significant differences between control and treatment groups.

Table 70. Effect of carotenoid pigment extracted from *R.mucilaginosa* on food intake, water intake and body weight gain by male rats

Duration of feeding (days)	Pigment concentration (%)								
	Control			2			4		
	Food intake ($g^{-1}rat^{-1}day^{-1}$)	Water intake (ml/rat/day)	Body weight (g/rat)	Food intake (g/rat/day)	Water intake (ml/rat/day)	Body weight (g/rat)	Food intake (g/rat/day)	Water intake (ml/rat/day)	Body weight (g/rat)
1	15.50±0.31	7.50±0.15	155.66±3.08	16.08±0.33	9.50±0.19	154.33±3.17	16.80±0.34	7.75±0.16	156.16±3.31
3	17.13±0.40	24.50±0.57	156.83±3.44	18.59±0.43	25.70±0.59	155.33±3.82	18.60±0.42	25.70±0.59	158.00±3.88
7	26.76±0.62	29.10±0.67	157.33±3.63	26.90±0.46	21.75±0.71	159.83±3.92	25.35±0.43	29.15±0.67	159.00±3.97
10	27.32±0.63	35.50±0.82	157.83±3.64	27.15±0.67	36.70±0.92	163.00±4.23	26.70±0.62	36.65±0.89	163.16±4.00
14	32.58±0.75	37.00±0.85	166.00±3.83	33.05±0.76	36.55±0.94	170.33±4.28	32.65±0.75	37.75±0.92	172.66±4.17
17	54.13±1.25	39.75±0.92	172.33±3.98	55.50±1.33	40.50±0.94	180.67±4.33	54.67±1.38	40.60±0.94	181.56±4.35
20	55.53±1.28	40.50±0.94	181.43±3.19	56.40±1.30	41.90±1.01	184.66±4.43	54.89±1.38	40.79±0.94	185.32±4.37
24	59.22±1.37	40.45±0.93	185.76±3.29	60.90±1.41	40.75±1.06	188.34±4.46	58.54±1.35	41.50±1.07	191.76±4.54
28	59.01±1.36	46.15±1.07	198.87±3.55	59.15±1.37	47.50±1.10	198.78±4.59	58.54±1.40	46.55±1.08	199.32±4.56

Table 71. Effect of carotenoid pigment extracted from *Sporobolomyces* sp. on food intake, water intake and body weight gain by male rats

Duration of feeding (days)	Pigment concentration (%)								
	Control			2			4		
	Food intake ($g^{-1}rat^{-1}day^{-1}$)	Water intake ($g^{-1}rat^{-1}day^{-1}$)	Body weight ($g^{-1}rat^{-1}day^{-1}$)	Food intake ($g^{-1}rat^{-1}day^{-1}$)	Water intake ($g^{-1}rat^{-1}day^{-1}$)	Body weight ($g^{-1}rat^{-1}day^{-1}$)	Food intake ($g^{-1}rat^{-1}day^{-1}$)	Water intake ($g^{-1}rat^{-1}day^{-1}$)	Body weight ($g^{-1}rat^{-1}day^{-1}$)
1	13.43±0.22	7.50±0.15	151.96±2.98	13.70±0.30	7.60±0.19	152.08±3.10	14.99±0.30	9.65±0.20	153.21±3.11
3	20.15±0.47	15.50±0.36	152.73±3.46	20.54±0.52	15.75±0.36	156.32±3.68	21.23±0.49	15.50±0.36	158.00±3.65
7	28.30±0.65	15.45±0.36	155.43±3.50	29.77±0.62	16.70±0.39	161.16±3.77	28.43±0.66	18.55±0.43	169.50±3.91
10	34.68±0.80	17.45±0.40	157.81±3.64	36.80±0.85	17.90±0.37	168.83±4.01	34.56±0.80	20.50±0.47	180.33±4.16
14	52.59±1.21	19.50±0.45	162.09±3.74	54.40±1.26	20.65±0.48	173.45±4.24	58.90±1.36	21.75±0.50	183.16±4.23
17	55.63±1.28	21.55±0.50	176.35±4.07	57.65±1.33	23.70±0.55	180.16±4.39	55.00±1.27	25.35±0.59	187.30±4.33
20	61.00±1.41	35.50±0.82	184.33±4.26	60.75±1.40	38.65±1.05	186.00±4.50	62.53±1.44	40.65±0.94	196.75±4.54
24	62.76±1.45	55.40±1.28	195.86±4.52	64.70±1.59	55.70±1.20	194.23±4.51	66.78±1.54	48.90±1.13	194.60±4.49
28	64.21±0.48	56.75±1.31	202.07±4.62	63.75±1.47	57.25±1.39	203.76±4.61	64.80±1.50	58.95±1.36	205.45±4.65

Table 74. Effect of carotenoid pigment extracted from *R. mucilaginosa* on haematological parameters

Concentration of pigment (%)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (g/dl)	DC (%)				MCV (fl)	RDW (%)	Platelet count (10^3/µl)
							L	P	M	E			
Control	14.98± 0.30	8.08 ±0.19	9.67 ±0.22	50.33 ±1.16	21.21 ±0.49	30.33 ±0.70	93.46 ±2.16	2.16 ±0.05	2.68 ±0.06	2.56 ±0.06	222.95 ±1.45	20.11 ±0.46	830.08 ±19.19
2	15.52 ±0.31	9.78 ±0.18	11.30 ±0.21	49.87 ±1.20	24.47 ±0.52	30.76 ±0.71	92.18 ±2.12	3.07 ±0.14	3.42 ±0.08	2.57 ±0.06	244.33 ±1.48	20.01 ±0.46	827.17 ±18.96
4	15.63± 0.32	9.81± 0.19	11.39± 0.22	52.29± 1.21	24.18± 0.56	32.72± 0.71	92.71± 2.13	3.87± 0.14	2.97± 0.07	2.80± 0.05	240.16± 1.39	20.18± 0.47	827.42± 18.46

Table 75. Effect of carotenoid pigment extracted from *Sporobolomyces* sp. on haematological parameters

Concentration of pigment (%)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (g/dl)	DC (%)				MCV (fl)	RDW (%)	Platelet count (10^3/µl)
							L	P	M	E			
Control	15.32 ±0.34	7.89 ± 0.21	9.01 ±0.25	53.66 ± 1.24	21.53 ± 0.50	28.56 ± 0.66	85.41 ±1.97	3.79 ±0.09	6.14 ±0.14	4.09 ±0.09	229.52 ± 1.61	23.40 ±0.54	827.04 ± 19.10
2	16.71 ±0.31	9.02 ±0.17	11.92 ± 0.28	55.77 ± 1.38	22.12 ± 0.51	28.93 ± 0.67	86.18 ±2.11	3.12 ±0.07	6.29 ±0.10	3.19 ±0.07	248.56 ± 1.54	22.51 ±0.47	825.76 ± 18.56
4	16.87 ±0.31	9.7 ± 0.17	11.67 ± 0.22	54.42 ± 1.11	20.19 ± 0.37	28.01 ± 0.59	85.97 ±1.82	3.02 ±0.07	6.76 ±0.07	3.72 ±0.05	242.49 ± 1.31	23.32 ±0.39	824.18 ± 15.67

Hb – hemoglobin, RBC – red blood cells, WBC – white blood cells, PCV – packed cell volume, MCH – mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration, DC – differential count, L – lymphocytes, P – polymorph neutrophils, M – monocyte, E – eosinophils, MCV - mean corpuscular volume, RDW - Red blood cell distribution width,

4.18.2.5. Biochemical parameters

Analyses of various clinical parameters *viz.*, serum enzymes (serum glutamate oxaloacetate (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP), urea and cholesterol are represented in Table 76 and Table 77. No marked alteration in any of the specific activities of enzymes in *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments treated rats was noticed. The record of biochemical parameters in treatment groups of rats were non significantly different in comparison with the control group indicating that *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments have no adverse effect on liver and kidney functioning.

A dose dependent decrease in urea and serum cholesterol level was observed in male rats fed with *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments discretely. The decrease in serum cholesterol levels ranged from 11.67 per cent to 29.74 per cent and 10.44 per cent to 32.33 per cent in *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments fed rats respectively. The results indicate that *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments significantly decreased the urea and serum cholesterol in experimental rats.

4.18.2.6. Histopathological examinations

No abnormality was observed between the control and carotenoid pigments (*R. mucilaginosa* and *Sporobolomyces* sp.) fed rats indicating no treatment related histological changes in various tissues or organs examined. Both of these two pigments have no adverse effect on cellular structure of these organs. Gross examinations of vital organs during autopsy did not reveal any abnormalities that could be attributed to oral dose of carotenoid pigments in male rats. Further on microscopic examinations, no treatment related histopathological alterations were observed in any of the vital organs. Detailed histopathological examinations of each vital organ are shown in Table 78, Plate 25 and 26. Liver was characterized by normal hepatic tissue with distinct nuclei and normal eosinophilic cytoplasm with normal sinusoids. There was no inflammation, fatty change or fibrosis. Kidney displayed normal renal parenchyma with normal glomerules, proximal tubules and collecting ducts. Normal cerebral cortex was observed in brain cells. The cerebellum showed normal purkinjie cells and molecular layer.

Table 76. Effect of feeding carotenoid pigment extracted from *R.mucilaginosa* on serum enzymes and cholesterol of male rats

S. No.	Concentration of pigment (%)	SGOT ($U\ l^{-1}$)	SGPT ($U\ l^{-1}$)	ALP ($U\ l^{-1}$)	Urea ($mg\ di^{-1}$)	Cholesterol ($mg\ di^{-1}$)
1	Control	94.78±2.53	47.60±1.63	91.08±2.10	19.46±0.37	198.33±5.34
2	2	84.50±2.40	49.66±1.26	88.06±1.80	16.21±0.47	175.18±2.89
3	4	77.16±2.25	47.80±1.12	86.93±1.59	15.83±0.39	139.33±2.52

Table 77. Effect of feeding carotenoid pigment extracted from *Sporobolomyces* sp. on serum enzymes and cholesterol of male rats

S. No.	Concentration of pigment (%)	SGOT ($U\ l^{-1}$)	SGPT ($U\ l^{-1}$)	ALP ($U\ l^{-1}$)	Urea ($mg\ di^{-1}$)	Cholesterol ($mg\ di^{-1}$)
1	Control	96.66±2.47	47.23±1.55	90.13±2.08	22.53±0.42	199.83±5.29
2	2	87.00±2.27	47.66±1.10	87.86±2.03	18.21±0.52	178.95±2.98
3	4	75.00±2.05	42.00±0.76	86.25±1.95	17.06±0.49	135.21±2.55

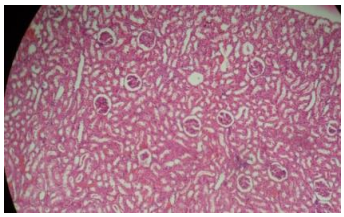
SGOT – Serum glutamic oxaloacetic transaminase, SGPT - Serum glutamic pyruvic transaminase , ALP - Alkaline phosphatase

Table 78. Histopathological examination of rats fed with pigment produced by *R. mucilaginosa* and *Sporobolomyces* sp.

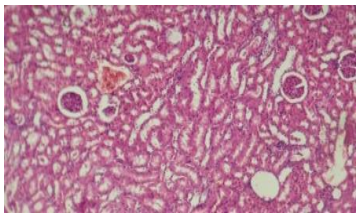
Organ / findings	No. of mice affected / group*					
	***R. mucilaginosa* pigment**			***Sporobolomyces* sp. pigment**		
	Dose (%)					
	0	**2**	**4**	**0**	**2**	**4**
Kidney						
Eosinophilic body, proximal tubule	0	0	0	0	0	0
Tubular Regeneration	0	0	0	0	0	0
Mineralization, cortico – medullary junction	0	0	0	0	0	0
Liver						
Renal parenchyma with glomeregular congestion	0	0	0	0	0	0
Hepatic tissue with dilation	0	0	0	0	0	0
Brain						
Gliosis, edema / degeneration	0	0	0	0	0	0
Lungs						
Inflammation / fibrosis	0	0	0	0	0	0

*Group contains 6 rats

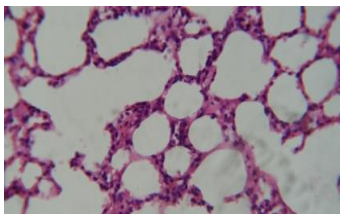
Plate 25. Histopathological examination of rats fed with pigment from *R. mucilaginosa*



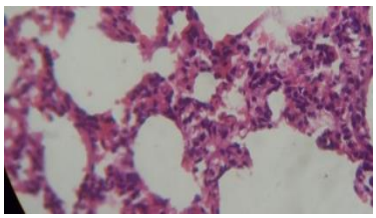
Control brain



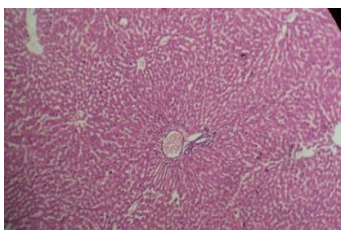
4% dose brain



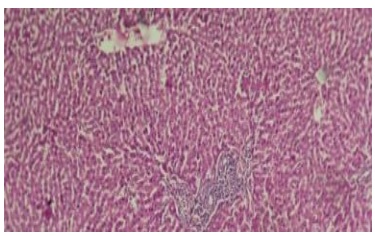
Control lung



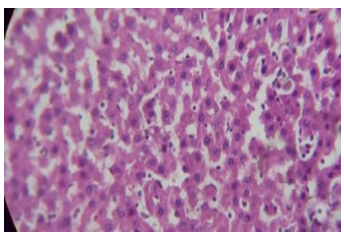
4 % dose lung



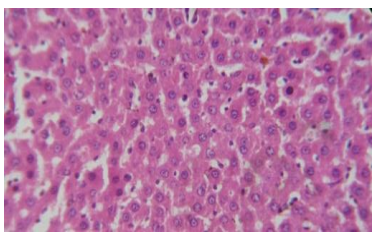
Control liver



4 % dose liver



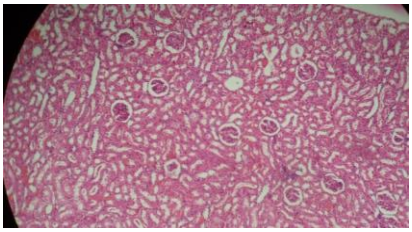
Control kidney



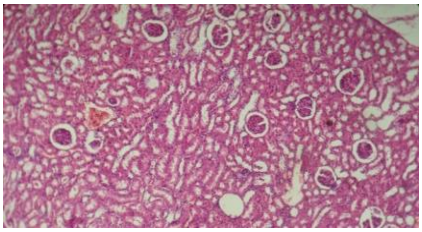
4% dose kidney

Brain	–	normal cerebellum showing unremarkable purkinjie cells and molecular layer
Kidney	–	normal distal convoluted tubule
Liver	–	normal lobular architecture
Lungs	–	normal portal tract and hepatocytes

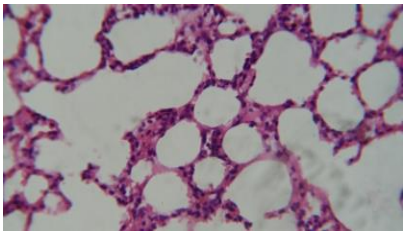
Plate 26. Histopathological examination of rats fed with pigment from *Sporobolomyces* sp.



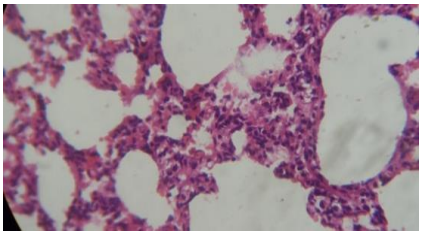
Control brain



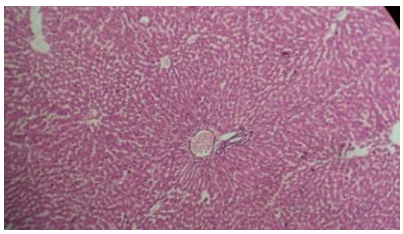
4% dose brain



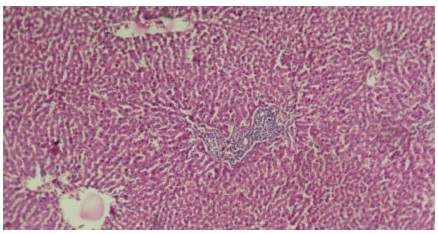
Control lung



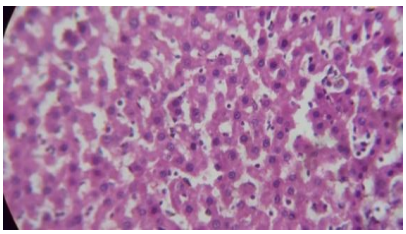
4 % dose lung



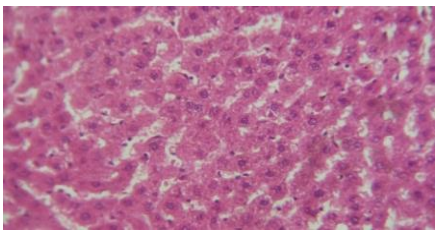
Control liver



4 % dose liver



Control kidney



4% dose kidney

Brain	–	normal cerebellum showing unremarkable purkinjie cells and molecular layer
Kidney	–	normal distal convoluted tubule
Liver	–	normal lobular architecture
Lungs	–	normal portal tract and hepatocytes

The hippocampus displayed normal morphology. Lungs showed normal bronchioles. The alveolar spaces were unremarkable. The interstitium were normal. There were no inflammations or fibrosis.

4.19. Application of *Rhodotorula mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments to food products

An attempt was made to see the applicability of the pigment to different food items. The carotenoid pigments extracted from *R. mucilaginosa* and *Sporobolomyces* sp. respectively were utilized as colour additive for the development of food products. To enhance the appearance and acceptability of foodstuff, carotenoid pigments were added. The pigment was mixed in different concentrations (1.5 per cent to ice cream, Indian milk ice cream, pop corn and 1 per cent to almond milk and yoghurt) the products were seen more appealing (Plate 27) (Table 79 and Table 80).

Plate 27. Development of food products with yeast carotenoid pigments



1 2 3

Popcorn



1 2 3

Ice cream



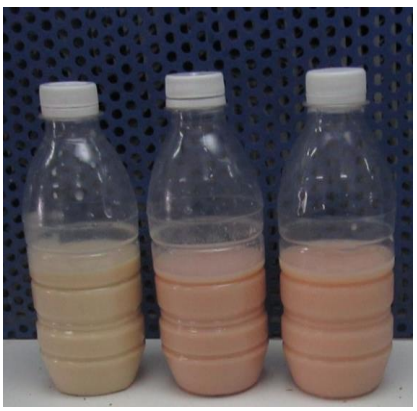
1 2 3

Indian milk ice cream



1 2 3

Yoghurt



1 2 3

Almond milk

1. **Without carotenoid**
2. ***R. mucilaginosa* carotenoid pigment fortified product**
3. ***Sporobolomyces* sp. carotenoid pigment fortified product**

Table 79. Organoleptic evaluation of food products coloured with carotenoid pigment from *R.mucilaginosa*

Colour values	Product scoring*									
	Food products									
	Badam milk/Almond milk		Kulfi/ Indian Milk Ice Cream		Popcorn		Ice cream		Yoghurt	
	Control	Coloured product	Control	Coloured product	Control	Coloured product	Control	Coloured product	Control	Coloured product
Flavour	9.5	5.1	7.8	5.6	9.2	7.5	8.9	6.5	9.2	4.6
Texture	8.7	7.8	8.5	8.1	9.0	9.0	8.5	8.2	8.7	8.6
Taste	9.0	9.0	9.0	8.8	9.3	9.2	8.9	8.7	8.8	8.7
Overall acceptability	9.1	8.9	9.1	8.2	9.4	9.2	8.4	8.2	8.6	7.8
Colour and appearance	9.5	8.4	8.5	8.0	9.3	8.5	8.5	7.9	8.6	7.5

*Scoring are the means of five member judge panel

Table 80. Organoleptic evaluation of food and beverage products coloured with carotenoid pigment from *Sporobolomyces* sp.

Colour values	Product scoring*									
	Food products									
	Badam milk/Almond milk		Kulfi/ Indian Milk Ice Cream		Popcorn		Ice cream		Yoghurt	
	Control	Coloured product	Control	Coloured product	Control	Coloured product	Control	Coloured product	Control	Coloured product
Flavour	9.5	5.1	7.8	5.6	9.2	7.5	8.9	6.5	9.2	4.6
Texture	8.7	7.8	8.5	8.1	9.0	9.0	8.5	8.2	8.7	8.6
Taste	9.0	9.0	9.0	8.8	9.3	9.2	8.9	8.7	8.8	8.7
Overall acceptability	9.1	8.9	9.1	8.2	9.4	9.2	8.4	8.2	8.6	7.8
Colour and appearance	9.5	8.4	8.5	8.0	9.3	8.5	8.5	7.9	8.6	7.5

*Scoring are the means of five member judge panel

Discussion

CHAPTER V

DISCUSSION

Microorganisms have been used for a long time for production of molecules as diverse as antibiotics, pigments, enzymes, vitamins, texturizing agents and so on. The toxicity problems caused by synthetic origin pigments to the environment have created a mounting interest towards natural pigments. Among natural pigments, pigments from microbial sources are potentially good alternative ones to synthetic pigments. There is growing interest in the food industry in the use of natural ingredients. Ingredients, such as colors, are considered natural when derived from biological sources like plants or microorganisms. Further, some natural colorants have commercial potential for use as antioxidants. The industry is now able to produce some microbial pigments for applications in food, cosmetics or textiles. In nature, color rich and pigment producing microorganisms (fungi, yeasts, and bacteria) are quite common (Dufosse, 2009). Microorganisms produce various pigments like melanins, flavins, quinones, prodigiosins and more specifically carotenoids, monascins, violacein or indigo.

Carotenoids such as β-carotene and xanthophylls like astaxanthin play central roles in the metabolism of the eye's macula and retina and in maintaining healthy vision. β-carotene play a role in the prevention of cancer. There is evidence that carotenoid pigments function as chemo-protectives. It may also act as nutraceuticals that prevent carcinogenesis through anti-oxidative, anti-free radical or other mechanisms. The beneficial nutraceutical functions of the carotenes and xanthophylls extend to the prevention of heart attacks and strokes.

The microbial production of carotenoids, when compared with extraction from vegetables or chemical synthesis, seems to be paramount interest mainly because of the problems related to seasonal and geographic variability in the production and marketing of plant origin pigments (De Haan *et al*., 1991). The economic advantages of microbial processes using natural low cost substrates as carbohydrate source (Simova *et al*., 2003). The commercial utilization of microorganisms with biotechnological potential to produce carotenoids is presently limited by the high cost of production. However, the cost of carotenoid production by fermentation can be minimized by optimizing its process, using

highly pigment producing microorganisms cultured in cheap industrial by-products as nutrient sources (Aksu and Eren, 2005). Development of microbial food grade pigments are likely to cut down the high production cost of natural pigments, thus leading to a cheaper source of natural food colourants among the modern consumers.

5.1. Source and isolation of carotenoid pigment producing microbes

Pigmented microorganisms have been reported to be present in the environments such as soil, compost, fruits, vegetables, flowers, leaves, water and air (Maldonade *et al*., 2007). Hence samples were collected from these environments for the isolation of carotenoid pigment producing microorganisms. The highest percentage of pigmented colonies, out of total population was associated with forest soil sample were collected from different parts of Tamil Nadu and also from Tiger Reserve, Parambikulam. The purpose of selecting soil samples was to explore the biodiversity as much as possible. Western Ghats of South India is considered to be rich in biodiversity of microorganisms. The present study revealed that the pigmented microorganism was much higher in the forest soil samples than in cultivated soils due to rich in organic matter content of soil. Though number of pigment producing microbe was low, there existed considerable biodiversity in pigment producing microbes in undisturbed forest soils. Various species of *Rhodotorula* sp. were isolated from plant leaves, flowers, fruits, slime fluxes (or exudates) of deciduous trees, soil, refinery waste water, air and yoghurt (Aksu and Eren, 2007). Almanza *et al*. (2014) also reported that *Rhodotorula* sp. is found in air, soil and phyllosphere surfaces, additionally, are found in milk and cheese products. In this study, pigmented colonies were isolated from forest soil, along with phyllosphere samples of rice, cabbage and other sources such as sapota and tomato.

5.2. Screening of microbes for carotenoid pigment production

Though all the isolates were pigment producing in nature a screening schedule was necessary to recognize the potential carotenoid pigment producer. Isolates were screened to select the producer of carotenoid pigment. This screening yielded five isolates, YP 15, YP 17,YP 19, YP 23 and YP 37.These isolates have very intense colours of light pink, orangish pink, yellow, bright yellow, pink pigments respectively. As these five isolates consistently produced carotenoid pigment, they were selected for detailed studies.

5.3. Characterization of yeast isolates

In the present study, the yeast strains from different sources were observed for their morphological characterization in comparison with the standard strain obtained from IMTECH (*Rhodotorula mucilaginosa* MTCC 9316). Traditionally, yeasts were identified by morphological and physiological criteria which are both laborious and time consuming (Kreger, 1984; Barnett *et al*., 1990; Kurtzman and Fell, 1998).

About five strains were characterized by their cell morphology, colony morphological and biochemical methods. In morphological characterization, yeast cells are medium, spherical to ovoid shaped cells, monopolor and multipolar budding. The morphological characters were also reported in the yeast classification by Bowman and Ahearan (1975). Similar findings were also observed in this study.

The isolates in the present investigation YP 15, YP 19, YP 23 and YP 37 were found to reproduce by monopolar budding and isolate such as YP 17 was reproduce by multipolar budding which was similar to that of standard strain. Carbon assimilation is an important criterion in the taxonomy and identification of yeasts, which depends on organic carbon sources for their energy supply and growth, the carbohydrates being the sources of greater importance. Sugars such as glucose, fructose, mannose etc., are assimilated by all studied species (Barnett *et al*., 1990). In the present investigation, all the isolates were able to utilize glucose, sucrose, maltose and galactose but none of them utilized lactose.

5.4. *In vitro* growth and carotenoid pigment production by the yeast

Most of reports were aimed to enhance the amount of pigment production by cultivation of microbes in rich complex media (Medentsev and Akimenko, 1998; Hestbjerg *et al.,* 2002). The yield of napthoquinone pigments formation in the fungi *F. verticilloides* had dependence on the composition of the medium used. The mineral salt liquid medium with carbon sources, including glucose or sucrose or glycerol was favorably applied and often with the amendment of metals to enhance the metabolite production. The present study indicated that yeast extract dextrose medium supported the growth and carotenoid pigment production of isolates *viz.,* YP15, YP 17, YP 19, YP 23 and YP 37. In general the growth and pigment production of all yeast isolates were better in other growth media

5.5. Antioxidant potential of yeast carotenoid pigments

Carotenoids have the ability to act as antioxidants and thus protect cells against photooxidation. The ability of carotenoids to quench singlet oxygen is well known and reactions with radical species have also been studied (Edge *et al*., 1997). Dietary carotenoids inhibit onset of many diseases in which free radicals are thought to play a role in initiation, such as atherosclerosis, cataracts, age-related macular degeneration, multiple sclerosis and most importantly cancer (Bhosale, 2004). Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart diseases. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins; lipid or DNA to initiate regenerative diseases (Kaur *et al*., 2008). There are number of clinical studies suggesting that the antioxidants in fruits, vegetables, tea and red wine could be the main factors for the observed efficacy of these foods in reducing heart diseases and sore cancer.

The FRAP is versatile and can be readily applied to both aqueous and solvent extracts. In this assay, the antioxidant activity was determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron for all the cultures (Benzie and Strain, 1996). The antioxidant activity ranged from 0.52 to 7.14 µ mol ferrous iron equivalents per µl of sample. The carotenoid pigment extract of YP 17 expressed a significant ability to reduce Fe^{3+} (10.81 mM μl^{-1}) at 100 µg ml^{-1} concentration and thus confirmed its ability to donate electrons. Scavenging of free radicals has been known as an established phenomenon in inhibition of lipid peroxidation which otherwise can be deleterious to cellular function. Lipid peroxidation is free radical mediated propagation for polyunsatuarated fatty acids involving several types of free radicals. Termination occurs through either enzymatic means or by free radical scavenging by antioxidant. In the present study, concentration dependent scavenging activity was observed in all samples, being very high for two yeast carotenoid pigments *viz*., YP 17 (37.15 %) and YP 37 (37.66 %) at a concentration of 100 µg ml^{-1}.

DPPH is a free radical, stable at room temperature and produces a violet solution in ethanol in the presence of antioxidant compounds; the DPPH is reduced and producing a non colour ethanol solution (Hayet *et al*., 2008). It is well known that antioxidants can seize the free radical chain of oxidation and form stable free radicals, which would not initiate further oxidation. In the present study, the scavenging activities of DPPH exerted by YP17 pigment extract at the concentration of 100 µg ml^{-1} exhibited 77.18 per cent inhibition; whereas the standard BHA at the same concentration exhibited 86.44 per cent inhibition.

Likewise in the reducing power assay, antioxidant activity could be increased as extract concentration increased, indicating that the compounds present in yeast cultures were both electron donars and could react with free radicals and convert them into more stable products to terminate radicals chain interaction. In this experiment all concentration of YP 17 showed higher activities followed by YP 37 than control. From this it could be inferred that antioxidant properties were associated with development of reducing power as described by Tanaka *et al.* (1998). The reduction of ferric ions is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action and is strongly correlated with other antioxidant properties. Using the $K_3[Fe(CN)_6]$ reduction method, the reducing capacities of red mould rice (RMRP), red mould dioscorea (RMDP) and DP at a concentration of 10 mg ml^{-1} were determined to be 0.20, 0.53 and 0.16 respectively (Tseng *et al*., 2012).

Hydroxyl radical produced may cause sugar fragmentation, base loss and leakage of DNA strand (Xu *et al.,* 2007). Hydroxyl radicals are the major ROS causing lipid peroxidation and enormous biological damage (Morales and Jimenez-Perez, 2004). All the tested yeast carotenoid pigments scavenge off these free radicals and hence inhibit cellular damage. It is apparent from the present study that the YP 17 and YP 37 pigments not only scavenges off the free radical but also inhibits the generation of free radicals.

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons etc., and involved in the regulation of various physiological processes (Lata and Ahuja, 2003). Excess concentration of NO is associated with several diseases (Ialenti *et al*., 1993). Oxygen reacts with the excess nitric oxide to generate

nitrite and peroxynitrite anions, which act as free radicals (Sainani *et al.,* 1997). In the present study, the carotenoid pigment extracts competes with oxygen to react with NO and thus inhibited generation of the anions. The percentage inhibition of NO generation by carotenoid pigment was well documented.

Metal-chelating activity is an antioxidant mechanism, since it mediates transition metal catalysis during lipid peroxidation. Iron and copper are essential transition metal elements in the human body required for the activity of a large range of enzymes and for some proteins involved in cellular respiration, O_2 transport and redox reactions. Unfortunately, they contain unpaired electrons that enable them to participate in one-electron transfer reactions. Hence, they are powerful catalysts of autoxidation reactions (Lloyd *et al*., 1997). With this assay, YP 17 carotenoid pigment showed strong Fe^{2+}-chelating activity at the concentration of 100 µg ml^{-1}. Its chelating rate was 59.73 per cent. Since ferrous ions are the most effective prooxidants in the food system, high chelating abilities of methanolic extracts from various monascal products would be beneficial (Tseng *et al.,* 2006). Tseng *et al*. (2012) reported that the ferrous ion-chelating effects of red mould rice (RMRP), red mould dioscorea (RMDP) and DP at a concentration of 100 mg ml^{-1} were 20.82, 68.84 and 52.14 per cent respectively.

5.6. Antimicrobial potential of yeast carotenoid pigments

Antibiotic resistant pathogens pose an enormous threat to the treatment of a wide range of serious infections. To prevent this exponential emergence, a periodic replacement of the existing antibiotic is necessary (Llic *et al.,* 2005). Currently**,** the greatest cause of concern is infection caused by methicilin and vancomycin resistant strains of *Staphylococcus aureus,* ESBL strains of *E. coli, Klebisiella* sp., and *Pseudomonas aeruginosa* (Selvameenal *et al.,* 2009). The development of novel drugs against drug resistant pathogen is the need of the hour. The antibiotic activity of astaxanthin pigment has been investigated by Ushakumari and Ramanujam, 2013. The antibacterial activity of *Monascus* pigment was first tested against *Bacillus* sp. Beni-koji was prepared with *M. pilosus* to efficiently control the contaminants (*Micrococcus varians* and *B. subtilis)* during koji making in the open air.

Five carotenoid pigment producing yeasts were screened for the antibacterial potential. The acetone pigment extracts of YP 17, YP 37 and standard strain MTCC 9316 was found to exhibit excellent inhibitory effect against selected bacterial human pathogens *viz., E. coli* (3.0, 2.9 and 2.8 cm), *S. aureus* (2.7, 2.6 and 2.6 cm), *S. faecalis* (2.6, 2.2 and 2.5 cm), *B. subtilis* (2.0, 2.3 and 2.0 cm) and *Enterococcus* sp. (2.0, 1.9 and 1.8 cm) and *P. aeruginosa* (2.1, 2.1 and 2.0 cm). The intensity of inhibition produced by isolates YP 17 and YP 37 was higher when compared to other culture pigments. The pinkish red pigment extracted from *Sclerotinia* sp. was tested against seven bacterial pathogens and all the pathogen were inhibited except *E. coli* (Perumal *et al.,* 2009). Velmurugan *et al.* (2009 b) also confirmed that the fungal pigments have excellent antibacterial activity against *S. aureus, E. coli, S. typhi* and *P. aeruginosa*. Ushakumari and Ramanujam (2013) reported that the astaxanthin pigment was found to be more significantly effective against all tested pathogen species such as *S. typhi* produced 20mm diameter for zone of inhibition, *P.aeroginosa* 24 mm, *B. subtilis* 18 mm and *S. aureus* 16mm. In the present study, it is apparent that the pigment of all tested species were more toxic towards Gram-negative strains, while the maximum inhibition (3.0 cm) was showed in *E. coli* than Gram-positive strains, where the highest inhibition (2.7 cm) which recorded in *S. aureus*. The reason was referred to the difference in the structures of the cell walls (Singh *et al*., 2007). Selective antibacterial activity may be due to several factors, including charge density, structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in Gram-negative and Gram-positive bacteria (Devine and Hancock, 2002). The observed results suggest that the potential therapeutic drug could be developed from yeast carotenoid pigment in future to treat human pathogenic microbes.

5.6.1. Minimum inhibitory concentration of *R. mucilaginosa* and *Sporobolomyces* sp. pigment

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotics or pigment that did not show any growth of tested pathogens at a minimum concentration (Abere *et al.,* 2007). Kono and Himeno (1999) reported that the MIC of *M. pilosus* ethylacetate extract inhibited koji contaminated pathogens including *B. subtilis* and *Micrococcus varians* up to 30 mg ml^{-1} concentration. Velmurugan *et al*. (2009 b) reported that pigments *viz., M. purpureus, Isaria farrinosa, E. nidulans,*

F. verticulloides and *P. purpurogenum* effectively inhibited the human pathogen at 3.5 per cent concentration. In the present study, the MIC of acetone extracts of YP17 and YP 37 was found to be 10 mg ml^{-1} for tested pathogens. The results indicated that the chemistry of the pigments has a significant influence on its antimicrobial activity. These results may suggest that the pigment might be developed as antibiotic drug.

5.7. Optimization of physical and chemical parameters to increase the carotenoid pigment production

5.7.1. Optimization of physical parameters of fermentation conditions at one time

Microbial fermentation are widely explored in industries for the production of a variety of fine and commodity chemicals like alcohols, organic acids, antibiotics, vitamins, amino acids and pharmacologically active substances (Goksungur *et al*., 2002). In optimization process cultural parameters is a prerequisite since they are not defined earlier. Various parameters that affect microbial physiology and metabolite yield are inoculum density, pH, temperature, agitation and media composition (Sanjay *et al.*, 2007 a).

5.7.1.1. Effect of pH

The pH of the culture medium has been reported to play a key role in pigment synthesis. Aksu and Eren (2007) reported that with raising the pH, specific growth and carotenoid production rates increased and reached to a maximum level at pH 6. On the other hand, a further increase of the pH over 6.0 resulted in a reduction of both the rates. Although the optimal pH 6 yielded the highest values of total carotenoids concentration and product yields, maximum biomass concentration was obtained at an initial pH value of 6.5. The yeast cultures *viz*., *R. mucilaginosa* and *Sporobolomyces* sp. was grown at different initial pH levels (2.0-9.0) in their respective media. The results depicted that biomass and pigment production was significantly affected with increase in pH levels. It may be inferred from this experiments that the optimum pH for higher pigment production (299.90 µg g^{-1}) from *R. mucilaginosa* was at pH 6.0, whereas the optimum pH for *Sporobolomyces* sp. pigment production (296.45 µg g^{-1}) was at pH 5.

Voaides and Dima (2011) observed that optimum intial pH was 6.0-6.5 for carotenoid production by *Rhodotorula* sp. Aksu (2005), Maldonade *et al*. (2008) and

Choudhari and Singhal (2008) also showed that as the pH increased, specific growth and carotenoid production rates increased and reached to a maximum level at pH 6-7 with drop off at higher pH levels. Moreover, the optimal pH also yielded the highest values of biomass cells and carotenoid. Yongsmith *et al*. (2000) reported that a lower pH of the culture medium promotes synthesis of yellow pigments, whereas a higher pH results in red pigments. These results suggest that pH can affect the activities of enzymes involved in the biosynthesis of pigments, but the effect depends on the particular microorganism utilized. The medium pH may affect cell membrane function, cell morphology, cell structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis. In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH (Kim *et al*., 2003).

5.7.1.2. Effect of temperature and incubation time

Temperature is an another important parameter affecting the performance of cells and product formation. The temperature of the growth medium had also a considerable effect on both growth and carotenoid production of *R. glutinis* (Latha *et al*., 2005). The specific growth rate of the yeast increased notably with raising the temperature up to 30°C and lessened sharply above 30°C due to the denaturation of the enzyme system of microorganism at higher temperatures (Aksu and Eren, 2007). However, *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment production at all temperature except at 40°C. The highest yield of pigment was detected at 25-30°C. Among six different temperatures studied, 25°C appeared to be optimum for the production of both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments, but the maximum biomass production was observed at 30ºC. The effect of temperature on yeast carotenogenesis is dependent on the species characteristics of the microorganism (Frengova *et al*., 1995). Taskin *et al*. (2011) showed the effect of cultivation time on carotenoid production in extract from waste loquat kernels as substrate. The highest increases for total carotenoid concentration were observed from the beginning of the 96th hour up to the 120th hour.

In order to optimize the production of pigment, the cultures were incubated for different periods and analyzed for the quality of carotenoid pigment production. Among the six incubation period tested, the maximum production of *R. mucilaginosa* carotenoid

pigment (384.83 µg g^{-1}) and cell dry weight (0.81 g 100 ml $broth^{-1}$) occurred at an incubation time of 96 h; Whereas the maximum production of *Sporobolomyces* sp. carotenoid pigment (366.28 µg g^{-1}) and cell dry weight (0.82 g 100 ml $broth^{-1}$) observed at an incubation time of 120 h. The results of this study showed that maximum pigment production was obtained at 96 and 120 h of incubation time for *R. mucilaginosa* and *Sporobolomyces* sp. pigment production, after which there was a decrease in pigment production. Incubation beyond the optimum period causes catabolism of pigments (Han, 1998).

5.7.1.3. Effect of agitation

If the microorganism requires oxygen, aerating the growth medium is very important for the successful progress of the fermentation. Carotenogenesis is an aerobic process. The effect of aeration is dependent on the species of the microorganism. The reported optimal values of air flow rate and agitation are in range 0.5 - 1.9 l min^{-1} and 180- 900 rpm for carotenogenesis in *Rhodotorula* sp. (Davoli *et al*., 2004).The aeration influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids. At higher aeration, the concentration of total carotenoids increased relative to the biomass and fatty acids in *R. glutinis*, but the composition of carotenoid (torulene > *β*-carotene > γ carotene > torularhodin) remains unaltered. In contrast, *S. roseus* responds to enhanced aeration by a shift from the predominant β-carotene to torulene and torularhodin (Davoli *et al*., 2004).

In the present study, yeast such as *R. mucilaginosa* and *Sporobolomyces* sp. are an aerobic microorganisms, the effect of the aeration rate on the growth and total carotenoids formation of the yeast was also examined and aeration helped yeast to produce more biomass and pigment production as the rate of agitation increased from 100 to 200 rpm. There was significant increase in biomass and carotenoid pigment yield when the organism was grown under agitation. It may be concluded that agitation speed from 150 to 200 rpm was ideal rotation for both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment enhancement. Thus it was showed that aeration was important for both growth and pigment production.

5.7.1.4. Effect of light

Carotenogenesis in many organisms is regulated by light. However, the intensity and protocol of illumination varies with the microorganism. The cultivation of the yeast in the dark and under sunlight conditions showed that light was not a limiting factor for growth and pigmentation of *R. glutinis* DFR-PDY (Latha *et al*., 2005). In contrast, light stimulated carotenogenesis in many other microorganisms, such as *Phaffia rhodozyma*, where cultures exposed to light synthesize more astaxanthin with a redder hue (Frengova and Beshkova, 2009). In the present study carotenoid pigment production observed in the white unscreened (295.01and 296.45µg g^{-1}) and dark condition (287.10and 289.32 45µg g^{-1}) found significant result. A slightly low yield of pigments was observed in yellow wavelength. The red, green and blue wavelength observation finds significance as it is against the postulated photo-protective role of biopigments (Blumenstein *et al*.,2005). The results are in agreement with Latha *et al*. (2005) who reported the effect of light on carotenoid pigment production.

5.7.2. Optimization of chemical parameters at one time

5.7.2.1. Effect of carbon and nitrogen sources

Assimilation of different sugars as carbon source by the red yeast for its growth and carotenoid pigmentation. Latha *et al*. (2005) reported that among the carbon sources carotenoid yield was higher in sucrose followed by fructose and glucose. Padma priya (2013) also studied that sucrose to be a best carbon source for reddish brown pigment production. At present study among monosaccharides, in *Sporobolomyces* sp. carotenoid yield was higher on glucose (305.25 µg g^{-1}) followed by fructose and galactose. On disaccharides, maximum growth and pigmentation was observed on sucrose (341.30 µg g^{-1}) in *R. mucilaginosa*. It may be due to the fact that sucrose can be easily assimilated in the metabolic pathway for biosynthesis of pigment production (Cho *et al.,* 2002b). Further, lactose supported no growth and pigmentation to both yeast cultures. It may be noted here that lactose-assimilating yeasts are rarely found in natural conditions and *R. lactis* is the only species that could assimilate lactose among 163 strains of *Rhodotorula* (Frengova *et al*., 1994). D-xylose supported

a high rate of growth but only a moderate carotenoid yield, whereas D-ribose resulted in higher rate of both growth and pigmentation.

A stimulatory effect of nitrogen source on pigment formation has been reported by Hamdi *et al.* (1998). It is well known that utilization of different nitrogen sources in fermentation had effects on growth and pigment production of microorganism (Lin and Demain, 1991). In the present study out of 10 nitrogen sources tested for growth and carotenoid pigment production, inorganic nitrogen source ammonium sulphate (417.55 µg g^{-1}) and organic nitrogen source yeast extract (407.80 µg g^{-1}) were preferred . According to Chen and Johns (1993) ammonium and peptone served as good nitrogen sources that yielded better growth and pigmentation of *Monascus purpureus.* In this study, peptone and sodium nitrate had positive effect for *Sporobolomyces* sp. carotenoid pigment (398.93 and 338.70 µg g^{-1}). Latha *et al.* (2005) found that sodium nitrate was the best nitrogen source for the red yeast pigment production. Tamil selvam (2001) reported that by providing appropriate sources of carbon and nitrogen in the growth medium one could harvest heavy yields of biocolourants. Organic sources of nitrogen were rapidly absorbed without much biochemical transformation.

5.7.2.2. Effect of trace metals

The trace elements have been demonstrated to act as stimulants for growth of red yeasts, which have a stimulatory effect on β-carotene and γ-carotene synthesis. The observed effect of trace elements on the biosynthesis of specific carotenoids in red yeasts may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoids biosynthesis (Manowattana *et al.*, 2012). Bioelements are one of the important factors affecting pigment production in several micro organisms (Kim *et al.,* 1998; An *et al.,* 2001). A critical finding between pigment and metal ions were reported by Fogarty and Tobin (1996). In order to investigate the effect of bioelements on carotenoid pigment production and growth of *R. mucilaginosa* and *Sporobolomyces* sp. was cultivated in their respective optimized culture medium. Each trace element was added to the culture medium at a concentration of 0.05 per cent. The maximum *R. mucilaginosa* carotenoid pigment production of 410.79 µg g^{-1} was

achieved in magnesium sulphate supplemented medium, whereas the *Sporobolomyces* sp. pigment production 387.66 µg g^{-1} was higher in ferrous sulphate supplemented medium. These results are consistent with Lee *et al*. (2001) who reported Fe^{2+} has the strongest stimulatory effect on red pigment production. Some stimulatory effect has also been attributed to $Mn^{2+,}$ because trace metals have important effects on secondary metabolism (Weinberg, 1989). The other bioelement like copper ions appeared to be detrimental on both pigment production and yeast growth. In fungal nutrition magnesium and calcium are noted as macronutrients and manganese, iron, copper and zinc as micronutrient but in case of red pigment production by *Monascus purpureus*, magnesium contribution was higher than calcium, iron and manganese. This may be due to magnesium, acting as cofactor for different enzyme required for pigment biosynthesis (Yu *et al.,* 1997).

5.8. Optimization by RSM (Response Surface Methodology)

Factorial design and response surface techniques are important tools to determine the optimal process conditions. This methodology has been successfully used in many areas of biotechnology, particularly to optimize the production of bioactive molecules (Xu *et al.,* 2003).

5.8.1. Optimization of physical parameters by RSM

In the present study, the independent variables *viz.*, pH, temperature and fermentation time were selected for maximization of carotenoid pigment production. The effect of physical parameters on *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment production were performed with the quadratic model expressed by determination of R^2, which was found to be 0.9991 and 0.9986 respectively. The optimal point of pH 6, temperature 25°C and fermentation time 96 h was found to be best for maximum *R. mucilaginosa* carotenoid pigment production (1022.18 µg g^{-1}), whereas the optimal condition for *Sporobolomyces* sp. pigment (990.87 µg g^{-1}) as follows; pH 5, temperature 25°C and time 120 h. The 3D response surface plots described by the regression model were drawn to illustrate the effects of interaction of each independent variable (pH, temperature and time) on the response variable (Bocchini *et al.,* 2002). The shape of the corresponding contour plots indicated that the mutual interaction between the independent variable were orientation of principle axes of contour plots (Cui *et al.,* 2005).

Sanjay *et al.* (2007a) also studied the same parameters (pH, temperature and time) for maximization yield of xanthin pigment by *Aspergillus carbonarious.* They reported that it could be achieved at the conditions when pH of growth media was 3.3, temperature 29.3°C and fermentation time of 46.7 h. Malisorn and Suntornsuk (2008) studied a face centered central composite design which was applied to optimize a cultivation condition for improved β-carotene production by *R . glutinis* using fermented radish brine as solid substrates. In this study pH, temperature and dissolved oxygen were considered as independent variable. Optimum condition for β-carotene was at 30°C; pH 6 and 80 per cent dissolved oxygen to yield 201µg g^{-1} of β-carotene. Manowattana *et al*. (2012) employed Placket-Burman design and RSM to enhance carotenoid production by *Sporobolomyces pararoseus* using waste glycerol as carbon source. In that study pH, temperature and waste glycerol were considered as independent variables. Optimum condition for carotenoid pigment production were 5.64 pH, 23.90 temperature and 34 g l^{-1} waste glycerol to yield 16.55 mg l^{-1} of carotenoid pigment. (Yu *et al.,* 1997).

5.8.2. Optimization of chemical parameters by RSM

In the present study physical parameters pH, temperature and fermentation time were optimized using three level central composite designs (CCD). The physical parameters greatly influences the carotenoid pigment production. Based on the results, chemical parameters were optimized. The fermentation media was optimized by five factor central composite design. The combined effect of five different medium components (independent variables), on pigment production was performed and fit of the model expressed by the coefficient of determination R^2, which was found to be 0.99 for both *R. mucilaginosa* and *Sporobolomyces* sp. pigments, indicating 99.0 per cent of the responses. The optimal concentrations for the five components for *R. mucilaginosa* carotenoid pigment production (2179.10 µg g^{-1}) as obtained from the maximum point of the model were calculated to be as 30, 3.5, 2, 1.25 and 0.5 g l^{-1} sucrose, yeast extract, ammonium sulphate, dipotassium hydrogen phosphate and magnesium sulphate respectively, whereas the optimal concentrations for the five components for *Sporobolomyces* sp. pigment production (1981.45 µg g^{-1}) as obtained from the maximum point of the model were calculated to be as 30,3.5, 2, 1.25, and 0.5 g l^{-1} glucose, peptone, ammonium sulphate, dipotassium hydrogen phosphate and ferrous sulphate respectively.

Choudhari and Singhal (2008) studied the optimum condition for β-carotene production from *B. trispora.* The study was agreement with our results. The fit of the quadratic model was found to be significant and the yield of 139 ± mg l^{-1} β-carotene was recovered using RSM.

Silveria *et al.* (2008) used grape waste as a substrate for submerged fermentation of *Monascus purpureus* for pigment production employing factorial design. The three independent variables (carbon source, peptone and monosodium glutamate) were evaluated having as response to pigment production. The result clearly showed that peptone concentrations were the most significant variable for pigment production.

Peptone, ammonium nitrate and dipotassium hydrogen phosphate as the most significant variables for pigment production by *Monascus anka* in submerged fermentation (Zhou *et al.*,2009) The RSM optimized these nutrients for maximum yellow pigment production. Designing the medium is an open ended, time-consuming and laborious process involving large number of experiments. The CCD experimental design is the preliminary technique for rapid illustration of the effects of various medium constituents whereas Maldonade *et al*. (2012) evaluated glucose, yeast extract, magnesium sulphate, dipottasium hydrogen phosphate and pH as the most significant variables for growth and carotenoid pigment production by *R. mucilaginosa*. The best condition in the region studied for biomass and carotenoid production was in the range of 15 to 20 gl^{-1} glucose and 15 to 17.07 gl^{-1} yeast extract. Finally 8.0 gl^{-1} biomass yielded maximum total carotenoid production of 745 µg l^{-1}. Poorniammal *et al*. (2013) studied a face centered central composite design which was applied to optimize a cultivation condition for improved yellow pigment production by *Thermomyces* in modified czapek yeast extract medium. In this study pH, temperature and fermentation time were considered as independent variables. Optimum condition for yellow pigment was 30.17°C temperature; pH 8.14 and 135 h fermentation time to yield 781 OD units.

By optimizing the culture conditions for *R. mucilaginosa* and *Sporobolomyces* sp. an almost two fold improvement in carotenoid pigment production was achieved under optimal culture conditions by using 3 l batch fermentor. The cultivation of red yeast in 3 l fermentor, the pigment production was higher and faster when compared to growth in

conical flasks. This may be due to the design of the fermentor leading to better mixing and mass transfer. Currently, the cost of natural colours in most cases is higher than synthetic colours of similar shades but this hurdle can be overcome by mass production of natural colours and by increasing the demand also.

5.9. Utilization of the agricultural cheap materials for cultivation of pigment producing yeast

There has been a growing interest in the use of carotenoid pigment as a functional food and pharmaceutical supplement because of its proven and potent antioxidant activity (Guerin *et al*., 2003). However, the costs in natural carotenoid production were high (U.S. $ 2,500-3,000 kg^{-1}) (Olaizola, 2000). As a consequence, researchers had been studying the use of alternative carbon sources for the production of carotenoids. The raw material and by-product of agro-industrial origin have been proposed as low cost alternative carbohydrate sources for microbial metabolite production (Demain *et al*., 1998), with the view of minimizing environmental and energetic problems related to their disposal. Starch rich raw materials of agro-industrial origin (eg. corn, barley and other cereals, potatoes and cassava) are widely available as feed stock and could be considered as cheap sources of sugars. Several strains of *Rhodotorula* had been studied for carotenoid production on grape juice (Buzzini and Martini, 1999).

Production of carotenoids by yeast can be made economically viable by using cheap industrial byproducts / wastes as nutrient sources. Latha and Jeevaratnam (2010) studied that out of four waste materials tried, waste mango pulp, dry tapioca powder and ethanol showed positive result but no growth was observed in whey. Among the cheap substrates they observed, dry tapioca powder yield high on both growth and pigment. The present study was focused to select the best natural substrate for carotenoid pigment production by *R. mucilaginosa* using various best cheap agricultural products were evaluated. It was found that cassava bagasse, rice flour and sugarcane bagasse would be good substrates, for the production of pigment. Some of the substrates presented a slow culture growth.

The large quantities of cassava waste materials produced each year especially in Tamil Nadu. Pigment production from agro industrial waste, cassava bagasse may offer

an opportunity to develop new avenues on natural colourant production and provide economic diversification in rural areas. Due to the changing in the processing practice in Thailand, a large amount of starch remains in the cassava pulp (up to 50 – 60 %, dry basis) (Grace, 1977; Balagopalan *et al*., 1994); in addition the pulp is also high in moisture (60 - 70 %). These factors combine to create a difficult drying process that is both inefficient and expensive method. Poorly dried or fresh cassava pulp spoils rapidly in the humid warm tropical environment as microorganisms quickly multiply on this substrate high in nutrients. Alternative means of processing the pulp are limited; compacting and heating are difficult due to starch and water interaction at high temperature. The only viable alternative that can help to reduce environmental impact and add value to the cassava pulp is to efficiently recover the starch, either as starch or sugar. Then, the treatment of cassava bagasse for by products utilization including pigment production by using microorganisms can be taken up.

Yang *et al*. (2011) reported that cassava residue as an inexpensive raw material for fermentation and promising substrate for producing *P. rhodozyma* with increased astaxanthin.

5.9.1. Optimization of fermentation parameters by utilizing source cassava bagasse by RSM

In the present study fermentation parameters such as substrate concentration (cassava bagasse, fermentation time and pH were optimized using three level central composite designs (CCD). The fermentation condition was optimized by three factor central composite design. The combined effect of three different fermentation components (independent variables), on carotenoid pigment production was performed and fit of the model expressed by the coefficient of determination R^2, which was found to be 0.99 for *R. mucilaginosa* indicating 99.0 per cent of the responses. The optimal concentrations for the three components for pigment production in *R. mucilaginosa* obtained from the maximum point of the model were calculated to be as 30 g (substrate concentration), 4 days (fermentation time) and pH 6. The pigment yield was obtained in *R. mucilaginosa* (2469.43 $\mu g\ g^{-1}$) and biomass yield (5.60 $g\ l^{-1}$).

Carnecka (2009) studied that carotenoid production in yeast *Rhodotorula glutinis* addition of non-processed lyophilized whey into production medium led to about 3-times increase in b-carotene production. β-carotene formation in media with deproteinated whey was even higher. Deproteinated lyophilized whey added into both, inoculation and production media, appeared to be the most suitable for carotenogenesis in *Rhodotorula glutinis* (33.48 mg of β – carotene l^{-1} culture fluid; 3137.1 μg g^{-1} of CDW).

Yang *et al*. (2011) used Plackett-Burman experimental design to optimize carbon source influencing the production of carotenoid pigments by *P. rhodozyma.* They reported that the optimum growth conditions determined in this work were sugar content 40 g l^{-1}, nitrogen content 8 g l^{-1}, initial pH 4 the time which temperature was changing is at the 36th hour. Manawattana *et al. (*2012) employed RSM design to enhance the carotenoids production by using waste glycerol as carbon source in *S. pararoseus* TISTR5213. The CCD was used to simulate the optimal conditions for high total carotenoids production were; waste glycerol 34.00 g l^{-1}, initial pH at 5.64 and 23.90°C for 5 days. Total carotenoids production obtained from the optimized medium was 16.55 mg l^{-1}.

5.10. Extraction of intracellular yeast carotenoid pigments

Different solvents such as acetone, ethanol and methanol have been used to extract carotenoids from yeast, like *Rhodotorula glutinis, Sporobolomyces roseus, Sporobolomyces coprosmae, Xanthophyllomyces dendrorhous, Dioszegia takashimae* (Roland *et al*., 2007). Namthimp *et al*. (1997) used acetone for extraction and analysis of astaxanthin from *Phaffia rhodozyma.* Carotenoids are readily soluble in organic solvents such as acetone, ethanol, diethyl ether, chloroform. The red pigment, Monascorubramine from *Monascus* was soluble in ether, methyl alcohol, ethyl alcohol, benzene, chloroform, acetic acid and acetone (Su and Huang, 1977). *Monascus* pigments are readily soluble in methanol, ethanol. Hawksworth and Pitt (1983) reported that pigment could be extracted from *Monascus* by methanol, ethanol, chloroform, acetic acid, acetone, benzene and ether. Among the tested solvents, acetone and ethanol were obviously more efficient than any of the others to extract carotenoids from the yeast cells. Similar results were in agreement with the earliest report of Ni *et al*. (2008).

Carotenoid is a lipid soluble substance and can be dissolved in the solvents listed in Table 59 except propanol. The phenomena can be explained from the basic properties of cell membrane. When the cell is wet, the yeast membrane is still double lipid layer structure and capsulated with a water layer. If the solvent is not water-soluble, chances are that it is difficult to permeate the water surface of the double-layer membrane and thus could rarely contact the membrane fixed with carotenoid pigment. Some water-soluble solvents such as acetone, ethanol and methanol can permeate the outer water cover easily and can extract pigment out of the yeast efficiently.

5.11. Thin layer chromatography studies

Bioautography allows localizing pigment composition of an extract on the chromatogram; it supports a quick search for new compounds through bioassay guided isolation. This method avoids the need of previous purification of the substance thus reducing the cost of the initial screening (Scorzoni *et al.*, 2007). Latha *et al*. (2005) analyzed the separation of the crude extract of carotenoid pigments from *R. glutinis* DFR-PDY by thin layer chromatography. They revealed the presence of multiple components. The bands are separated based on the Rf value, yellow (Rf 0.9) and orange (Rf 0.7) bands were visible. In the present study, the acetone extract of *R. mucilaginosa* was subjected to TLC separation, using benzene: petroleum ether (85:15) as per the mobile phase were separated based on the Rf value, yellow (0.87), orange (0.79) and pink (0.44) . Three bands such as pink, orange and yellow colour bands were observed and identified by RP-HPLC. The acetone extract of *Sporobolomyces* sp. carotenoid pigment compounds were separated using hexane: acetone (70:30) as mobile phase. Three bands (pink, orange and yellow) were separated from the pigment fractions of *Sporobolomyces* sp. based on the Rf value yellow (0.88), orange (0.74) and pink (0.41) and used for further identification. By comparing standard β-carotene and yeast cultures β-carotene pigments values are quite similar with standard β-carotene spot from this the carotenoid pigment were identified by Rf values. The results are in accordance with the reports of Latha and Jeevaratnam, 2010;Golkhoo *et al*., 2007;Pratheeba *et al*. (2014).

5.12. RP-HPLC analysis of carotenoid pigment

High performance liquid chromatographic methods for the separation of carotenoids were first reported in 1970 (Craft, 1992). HPLC analysis of carotenoids produced by *B. trispora* indicated the presence of three compounds, Lycopene, γ-carotene and β-carotene (Mantzouridou and Tsimidou, 2007; Mapari *et al.*, 2008). C18 column was used for the HPLC analysis of β-carotene produced by *Blakeslea trispora*. They reported the presence of peaks corresponding to carotenoids on the basis of spectral characteristics, at the retention time of less than 10 min at 453 nm. Bhosale and Gadre (2001) reported that the carotenoids, β-carotene, torulene and torularhodin eluted at 2.39, 3.39 and 3.49 min respectively. In the present study data indicated the presence of three peaks in *R. mucilaginosa* pigment: β-carotene, torulene and torularhodin at retention time of 3.35, 4.55, 5.92 min and in *Sporobolomyces* sp. pigment at retention time of 3.35, 4.20 and 5.77 min respectively. In agreement with the results of Bhosale and Gadre (2001) identified pigment was confirmed as carotenoid compounds. The retention time of β-carotene extracted from both yeasts were similar to the standard β-carotene. Therefore our study demonstrated that the extracted pigment of both red yeast is a carotenoid compound.

5.13. FT - IR

FT-IR spectroscopy has widely been used for the characterization and identification of fungi, bacteria and yeasts which are hydrophilic microorganisms (Fischer *et al.*, 2006). In the present study, the infra red spectra of *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment showed the band at 2993 and 2931 cm^{-1} due to asymmetrical stretching of aliphatic CH_2 groups respectively. The asymmetrical and symmetrical deformation vibrations CH_3 groups are observed at 1350 cm^{-1} for *R. mucilaginosa* pigment and 1365 cm^{-1} for *Sporobolomyces* sp. respectively. Low intensity bands at 1728 cm^{-1} and 1705 cm^{-1} may be due to >C = O group indicate the presence of ester groups for *R. mucilaginosa* and *Sporobolomyces* sp. pigment. This assignment is supported by the presence of a band at 1222 cm^{-1} for both cultures pigment which could be the C – O stretching vibration indicate the presence of ester. Bands at 1666 cm^{-1} for *R. mucilaginosa* pigment and 3008 cm^{-1} for *Sporobolomyces* sp. indicate the presence of an olefinic functional group. Similar results were also reported by Latha and Jeevaratnam

(2010) in *R. glutinis*. Marshell (1998) reported that FT-Infra red spectrum of freshly isolated β-carotene bands around 2930, 1720, 1450 and 1370 cm^{-1} respectively.

5.14. Stability of intracellular yeast carotenoid pigments

Stability of natural colourants towards formulations, different processing conditions, additives, pH, uniformity in distribution in products and availability of raw materials, cost economics of preparation, *etc*., are the major factors affecting the selection and application of natural food colours in processed food products (Rao *et al*., 2002).

The pH of the medium had a major influence on colouration of the pigment solution inducing sometimes a modification in their structure. In the present study pigment extracts from *R. mucilaginosa* and *Sporobolomyces* sp. were introduced into solutions with different pH, greater sensitivity was observed for both the pigments at acidic pH. Carotenoid pigments showed more stability near neutral pH than acidic. These results are in accordance with Kaur *et al*.(2008) who reported that *R. rubra* carotenoid pigment are more stable under neutral pH. Carvalho *et al*. (2005) found that colour of *Monascus* pigment decreases more rapidly in low pH values.

Carotenoids impart yellow to orange colours to foods and are fairly stable to heat and pH changes (Lewis *et al*., 1997). Kaur *et al.* (2008) reported that carotenoids produced by *Rhodotorula* also got chemically denatured on exposure to heat (70-100°C) for 15 min (51% decolouration). Both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment showed stability up to 70°C.

With the help of biotechnology intervention, production of some food grade natural pigments such as β-carotene from *R. glutinis, R. rubra, Blakeslea trispora*, riboflavin from *Ashbya gossyppi*, astaxanthin from *Xathophyllomyces dendrorhous*, Arpink red from *Penicillium oxalicum*, red pigments from *Monascus purpureus, M. ruber* have gained considerable consumer acceptance. Generally, these pigments are produced in cell bound state and have low water solubility. Many natural pigments are also sensitive to heat, pH change and light. Several reports have already indicated that substitution of their free functional groups *viz* carbonyl group in monascorubrine, monascin, ankaflavin, rubropunctatine; free –OH, -COOH, >C=O of Arpink red; free >C=O, -OH of astaxanthin, riboflavin; free –CHO and >C=O group of

Xanthomonascins A and B by amino group of various amino acids, peptides or proteins, could lead to a color change, improve their water solubility and also enhance their stability towards light, high temperature, low pH so that the pigments can easily replace synthetic colorants for food and pharmaceutical applications (Kaur *et al*., 2008).

Latha and Jeevaranam (2010) studied that storage stability of carotenoid pigment using different oils. The present study was carried out to observe the storage stability of pigment in different oils. Both yeast carotenoid pigments was found to be more stable in sunflower oil and sesame oil which was followed by ground nut oil, rice bran oil, corn oil, palm oil, safflower oil and coconut oil. The stability is more at 4°C and also even at room temperature (RT) in some cases and very poor stability was observed at 60°C. The observed result was in agreement with Latha and Jeevaratnam (2010). Addition of antioxidants to the red yeast pigment was found to increase the stability of the pigment to certain extent. The red and yellow pigment obtained from *Monascus ruber* when treated with antioxidants such as ascorbic acid, the discolouration of the red pigments decreased from 70 to 50 per cent after 5 h (Fabre *et al.,* 1993).

Since carotenoids are light sensitive the pigment here is less stable when exposed to light during the storage whereas it is stable when stored in dark. In a recent report, the carotenoids, the main pigment produced by *Rhodotorula* sp. was shown to get denatured on exposure to the light, heat and oxygen (Kaur *et al*., 2009). However, we found that the red yeast pigment is pretty stable when stored in different oils and it can be of use in oil based food products. These characteristics of red yeast pigment give them considerable potential for development to use in the food industry.

5.15. Toxicity evaluation of carotenoid pigment

Oral toxicity evaluation using 28 days toxicity test is an accustomed practice in a sub acute study. Sub acute toxicity has been advocated as a fundamental test for assessing safety and has been previously used in many safety assessment studies (Kitano *et al.,* 2004; Arts *et al.,* 2004; Wang *et al*., 2007). Acute and sub-acute toxicity study was conducted on albino mice of yellow pigment from *Thermomyces* sp. to assess its safety for food application (Poorniammal *et al*., 2011). Toxicological studies carried out on *R. gracilis*, a

carotenoid lipid producing yeast was found to be safe in acute and sub acute toxicity studies in a albino rats (Naidu *et al.*, 1999 a, b).

Safety of carotenoid pigment from *R. mucilaginosa* and *Sporobolomyces* sp. respectively was analyzed for acute and sub acute toxicity which are a prerequisite for food application. The carotenoid pigments from both cultures were fed separately as oral dose to albino mice for toxicity evaluation. In both the studies, no treatment related adverse effects were observed for any parameters in male rats receiving upto 4 per cent level oral dose of pigment. Although the body weights were increased, there were no significant changes of body weights in male rats or between treatment and control groups. The RBC and other hematological parameters did not show any biologically or statistically significant differences between rats fed with both pigments individually or controls. However slight increase or decrease in Hb, RBC, WBC, PCV, MCH, MCHC, DC, MCV, RDW, platelet count and MPV were noted in rats fed with pigment while these data also fell within the earliest report by Sanjay *et al.* (2007b); Kumari *et al.* (2009); Poorniammal *et al*. (2011).

There were no significant changes in histological findings in lungs, liver, brain and kidneys and were comparable in incidence and severity in control and treatment groups. Minor changes observed were common in rats and were not considered to be treatment related. No other significant or treatment related increases in incidence of abnormalities were observed in any of the other tissues examined. Gross examination and microscopy examination also revealed that administration of *R. mucilaginosa* and *Sporobolomyces* sp. pigments could not be lethal.

Incorporation of both cultures carotenoid pigments even at 4 per cent did not significantly affect food and water intake in treatment groups and were comparable to that of control group. Clinical chemistry evaluations in 28 days oral dosing groups did not show any statistical significant difference between treatment and control groups. Feeding *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments significantly decreased the urea and serum cholesterol in experimental rats. The results are similar to those of Kumari *et al.* (2009); Latha and Jeevarathnam,(2012).

Kumari *et al.* (2009) confirmed that the rat fed with red mould rice, fermented with *M. purpureus* proved no adverse effects on the organ weight, histopathology, body weight, food intake and serum enzymes. The organ to body weight ratio is one of the fundamental judgments to diagnose whether organ exposed to injury or not impaired organ often have abnormal tumidity or atrophy. There were no significant changes of organ to weight ratio between treatment groups. Nabae *et al.* (2005) reported that 90 days oral toxicity study of β-carotene from *B. trispora* fed at 5 per cent levels indicated that no treatment related effects with regard to body weight, histopathology and serum enzyme level. Padma priya (2013) studied that 28 days oral toxicity evaluation of *Penicillium purpurogenum* and *Chetomium aureum* proved no adverse effects on the organ weight, histopathology, body weight, food intake and serum enzymes. The present studies are the first step to assess the safety of carotenoid pigments from *R. mucilaginosa* and *Sporobolomyces* sp. as a source of natural food colourant. The results of both the studies clearly concluded that carotenoid pigment extract from *R. mucilaginosa* and *Sporobolomyces* sp. may be used safely in as food colourant for direct consumption by humans with an added benefit of antioxidant activity.

5.16. Development of food products with carotenoid pigments

Food products gain more intense and stable red colour and improved organoleptic characteristics when *M. purpureus* pigment was used (Blanc *et al.,* 1995). Moreover, application of the natural pigment promotes consumers´ health protection by decreasing the intake of salt and allows manufacturing fully natural food without any synthetic additives (Su *et al.,* 2005). In the present study, carotenoid pigments from *R. mucilaginosa* and *Sporobolomyces* sp. were added individually to the common food products such as almond milk, Indian milk ice cream, popcorn, ice cream and yoghurt to impart not only colour and also added antioxidant to these products. Traditionally, carotenoid pigments in colouring various food products such as margarines, beverages, dry soups, canned soups, dairy substitutes, salad dressings, meat products, etc., and its use in nutrition of laying hens have also been documented (Francis, 2000). Vidyalakshmi *et al.* (2009 a) studied incorporation of monascus fermented rice for colouring flavoured milk, which showed appealing colour, appearance and were overall acceptable. The pigment distributed evenly in the food product giving a pleasing

appearance. Vidyalakshmi *et al.* (2009 b) applied *Monascus* red pigment in the preparation of flavoured milk. On the sensory evaluation of the flavoured milk, the application of pigments was found to be acceptable. Latha and Jeevaratnam (2010) tested the applicability of carotenoid pigment extracted from *R. glutinis* DFR-PDY for colouring puffed products like popcorn, biscuit creams, cake icing and ice creams. The present study has also revealed that both cultures carotenoid pigments fortified food preparations received very high acceptability. It is fervently hoped that in the foreseeable future, these yeast carotenoid pigments could receive greater attention.

Application of new technologies in food industry requires untraditional processing of food stuffs with the aim to improve their quality, durability, storage, nutritional value and visual attraction. Moreover application of the carotenoid pigments promotes consumers health protection and allows manufacturing fully natural food without any synthetic additives which cause unexpected undesirable side effects.

Biotechnological industries harness the metabolic activity of compound produced for use in food, pharmaceutical, chemical and health care industries (Parekh *et al*., 2000). In recent years, production of natural food colourants through microbial fermentation is an extensive area of investigation, since they overcome concerns of unfavorable side effects by synthetic colours (Delgado-Vargas *et al*., 2000). Today there is great interest in the microbial production of various compounds that can be used in different areas, such as the pharmaceutical, cosmetic and food industries, to name a few, as these compounds have particular characteristics that increase the benefits of their consumption and it also reduces production costs and take advantage agro industrial wastes to perform this type of bioprocesses. The carotenoid pigment produced by *R. mucilaginosa* and *Sporobolomyces* sp. indicated its importance in food, pharmaceutical and nutraceuticals industries.

Summary

CHAPTER VI

SUMMARY

Many artificial synthetic colorants, which have widely been used in foodstuff, dyestuff, cosmetic and pharmaceutical manufacturing processes, comprise various hazardous effects. To counter the ill effect of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources. The utilization of natural pigments has been increasing in recent years thanks to the apprehension about the harmful effects of synthetic pigments and their industrial byproducts on humans and the environment (Unagul *et al*., 2005). Natural pigments can be obtained from two major sources, plants and microorganisms (Cho *et al*., 2002b). The accessible authorized natural pigments from plants have numerous drawbacks such as instability against light, heat or adverse pH, low water solubility and are often non-availability throughout the year. The latter are of great interest owing to the stability of the pigments produced and the availability of cultivation technology (Parekh *et al*., 2000). The advantages of pigment production from microorganisms include easy and fast growth in the cheap culture medium, independence from weather conditions and colors of different shades. The commercial utilization of microorganisms with biotechnological potential to produce carotenoids is presently limited due to high cost of production. However, the cost of carotenoid production by fermentation can be minimized by optimizing its process, using highly pigment-producing microorganisms cultured in cheap industrial by-products as nutrient sources (Aksu and Eren, 2005). Development of microbial food grade pigments are likely to cut down the high production cost of natural pigments, thus leading to a cheaper source of natural food colourants among the modern consumers. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications. The results of the work done are summarized as below.

- A total of 114 soil samples were collected from various parts of the Tamil Nadu, Tiger Reserve Parambikulam and 15 samples were carried out from various sources like fruits, vegetables, flowers and leaves were analyzed microbiologically for their pigment producing microbial flora.

- Cultural, morphological and biochemical characteristics of yeast isolates were carried out based on carotenoid pigmentation, colony characteristics, etc.
- Among thirty eight pigment producing isolates, based on the intensity of carotenoid pigmentation, five yeast cultures *viz*., YP15, YP 17, YP 19, YP 23 and YP 37 were selected based on their carotenoid pigment producing ability.
- Antioxidant and free radical scavenging ability of the five yeast carotenoid pigments were assessed using different methods *viz.*,Ferric-Thiocyanate method, lipid peroxidation, DPPH radical scavenging, reducing power, hydroxyl radical, nitric oxide and metal chelation ability. The YP 17 and YP 37 pigment showed higher percentage of inhibition than other strains.
- The human pathogens *viz*., gram positive bacteria - *Enterococcus* sp*., S. aureus, S. faecalis, B. subtilis;* gram negative bacteria - *E. coli, P. aeruginosa* were collected from Microbiological Laboratory, Coimbatore and KMCH College of Pharmacy, Coimbatore.
- Antimicrobial activity of the yeast carotenoid pigment extracts were tested against the selected human pathogens by agar well diffusion assay. The maximum inhibition of carotenoid pigment of YP 17and YP 37 was found to inhibit human pathogens *viz., E. coli* (3.0 and 2.9 cm), *P. aeruginosa* (2.1 and 2.2 cm), *S. aureus* (2.7 and 2.6 cm), *S. faecalis* (2.0 and 2.2cm), *B. subtilis* (2.0 and 2.3 cm) and *Enterococcus* sp. (2.0 and 1.9 cm) respectively.The carotenoid pigment of YP 17and YP 37 were found to exhibit MIC of 10 mg against the selected human pathogens.
- Carotenoid pigment of YP 17 and YP 37 showed higher percentage of inhibition than other strains such as YP 15, YP 19 and YP 23. Based on above studies, it was concluded that two promising carotenoid pigment producing yeast cultures were identified as *Rhodotorula mucilaginosa* (YP 17) and *Sporobolomyces* sp. (YP 37) were found to be effective in all aspects and used for further studies.
- For optimizing culture conditions to maximize *R.mucilaginosa* carotenoid pigment production, in one factor at a time method, the selected yeast culture was grown in Yeast extract dextrose broth with different pH, temperature, incubation time, agitation, light, inoculums size, carbon source, nitrogen source and trace metal level for red pigment production and their yield were estimated. Physical

parameters *viz.*, pH 6, temperature 25-30°C, incubation time 96 h, 150-200 rpm of agitation and incubation in dark, 3 per cent inoculum (v/v) and chemical parameters *viz.*, sucrose, yeast extract, ammonium sulphate ,dipotassium hydrogen phosphate and magnesium sulphate were the optimal parameters to harvest higher carotenoid pigment and biomass from the yeast.

- For optimizing culture conditions to maximize carotenoid pigment production, in one factor at a time method, the selected yeast *Sporobolomyces* sp. was grown in yeast extract dextrose broth with different pH, temperature, incubation time, agitation, light, inoculums size, carbon source, nitrogen source and trace metal level for carotenoid pigment production and their yield were estimated. Physical parameters *viz.,* pH 5.0, temperature 25-30°C, incubation time 120 h, 150-200 rpm of agitation and incubation in dark, 4 per cent inoculums (v/v) and chemical parameters *viz.*,glucose, peptone, ammonium sulphate, dipottasium hydrogen phosphate and ferrous sulphate were the optimal parameters to get higher carotenoid pigment and biomass from the yeast.
- Response surface methodology (RSM) with collection of statistical technique where in, interactions between multiple process variables can be identified with fewer experimental trials. The optimal point of physical parameters for *R.mucilaginosa* carotenoid pigment production are, pH 6.0, temperature 30°C and time 96h. The optimal chemical concentrations for the five components were calculated as 30 g l^{-1}, 3.5 g l^{-1}, 2 g l^{-1}, 1.25 g $l^{-1}$and 0.5 g l^{-1} for sucrose, yeast extract, ammonium sulphate, dipotassium hydrogen phosphate and magnesium sulphate respectively, whereas the optimal point of physical parameters for *Sporobolomyces* sp. carotenoid pigment production are, pH 5.0, temperature 30°C and time 120 h. The optimal chemical concentrations for the five components are calculated as 30 g l^{-1}, 3.5 g l^{-1}, 2 g l^{-1}, 1.5 g l^{-1} and 0.5 g l^{-1} for glucose, peptone, ammonium sulphate, dipotassium hydrogen phosphate and ferrous sulphate respectively.
- Different substrates were used to study the fermentation condition for maximization of carotenoid pigment production. Rice bran, wheat bran, rice flour, cassava bagasse, tamarind seed powder, coconut oil cake, groundnut oil cake, sesame oil cake and

sugarcane bagasse were used. Among the different substrates used, cassava bagasse was found to be mostly preferred substrate by *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigment production.

- Response surface methodology (RSM) was used to maximize the both cultures carotenoid pigment production using cassava bagasse with 20 experimental trials. The optimal concentrations for the components were calculated as 30 g (substrate concentration), 4 days (fermentation time), and pH 6 for *R. mucilaginosa* carotenoid pigment. Whereas for *Sporobolomyces* sp. carotenoid pigment optimal concentrations for the components were calculated as 30 g (substrate concentration), 5 days (fermentation time), and pH 5.
- The synthetic media yielded CDW and carotenoids about 6.10 g l^{-1} and 14.00-15.00 mg l^{-1} in *R. mucilaginosa* whereas in *Sporobolomyces* sp. (CDW - 5.20 g l^{-1}; carotenoids about 9.50-10.00 mg l^{-1}).Casssava bagasse as a cheap substrate *R. mucilaginosa* were yielding 5.60 g l^{-1} (CDW) and carotenoid yield was ranging from 12.00-13.00 mg l^{-1} in *R. mucilaginosa* .
- Both *R.mucilaginosa* and *Sporobolomyces* sp. pigment was subjected to TLC seperation using benzene and petroleum ether (85:15) and hexane: acetone (70:30) as mobile phase respectively. The bands seperated by TLC were further identified by using reverse phase high performance liquid chromatography (RP-HPLC which yielded in 3 peaks by employing *R. mucilaginosa* pigment (peak 1–3.35 min; peak 2 – 4.75 min; peak 3-5.93 min) similarly *Sporobolomyc*es sp. pigment produced 3 peaks (peak 1- 3.35 min; peak 2- 4.20 min and peak 3- 5.77 min). In both cultures, carotenoid pigment extracts shown a peak at 3.36 min which was equivalent to the retention time of standard β-carotene. Other two peaks observed at retention time of four and five minutes were denoted as torulene and torularhodin.
- The infra red spectra of *R.mucilaginosa* carotenoid pigment showing broad stretching at 2993 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH_3 groups. Low intensity bands at 1728 cm^{-1} may be due to >C = O group indicate the presence of ester groups. In the IR spectrum stretching frequency were also observed at 1666 cm^{-1} may be due to C = C due to alkenes which

indicate the presence of an olefinic functional group whereas the FT-IR spectrum of *Sporobolomyces* sp. carotenoid pigment showed bands at 2862 cm^{-1} are due to asymmetrical/symmetrical stretching vibration of methyl groups. Low intensity bands at 3008cm^{-1} is due to CH stretching vibration CH = CH and 1705 cm^{-1} may be due to C=O stretch ketone group which indicate the presence of an olefinic functional group. Presence of a band at 1226cm^{-1} due to the C – O stretching vibration of the ester. The observed stretching frequencies are however close to olefinic functional groups and ester.

- Safety evaluation of both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments were studied in animal system employing male rats at different dose levels. The result demonstrated that there was no changes of biological parameters such as haemoglobin, red blood cells, white blood cells, packed cell volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, differential count, mean corpuscular volume, red blood cell distribution width, platelet count and mean platelet volume at different concentrations studied for 28 days trail period. The biochemical parameters like serum glutamate oxaloacetate (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), urea and cholesterol were also studied. There were no significant differences observed in food intake and water intake of animal fed with both *R. mucilaginosa* and *Sporobolomyces* sp. carotenoid pigments separately. The pigments were also studied for their stability in different conditions such as pH, heat, light, antioxidants and also in different oils.
- Carotenoid pigments from *R. mucilaginosa* and *Sporobolomyces* sp. were incorporated in the preparations of few food products like Indian milk ice cream, almond milk, ice cream, yoghurt and popcorn. On the sensory evaluation of the pigment fortified food products, were found to be acceptable.

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Appendices

APPENDIX- I

1. Yeast extract Malt extract agar medium (Adams *et al.*, 1997)

Glucose	-	10.0 g
Malt extract	-	5.0 g
Yeast extract	-	3.0 g
Peptone	-	5.0 g
Agar	-	2.0 g
Demineralised water	-	100 ml
pH	-	6.0

2. Glucose Yeast Peptone Medium (General purpose medium for yeast)

Glucose	-	25.0g
Peptone	-	5.0g
Yeast extract	-	3.0g
Agar	-	20.0g
Distilled water	-	1000 ml
pH	-	6.8±0.2

3. Yeast Extract Peptone Dextrose medium (Adams *et al.*, 1997)

Glucose	-	30.0 g
Yeast extract	-	4.0 g
Peptone	-	4.0 g
Agar	-	20.0 g
Demineralised water	-	1000ml
pH	-	6.0

4. Restricted growth medium (Adams *et al*., 1997)

Glucose	-	20.0 g
Yeast extract	-	10.0 g
Peptone	-	20.0 g
Agar	-	20.0 g
Demineralised water	-	1000 ml

5. Pre-sporulation medium (Fast, 1973)

Yeast extract	-	10.0 g
Peptone	-	20.0 g
Potassium acetate	-	10.0 g
Tetracyclin	-	20.0 mg
Demineralised water	-	1000 ml

6. Yeast nitrogen broth (Adams *et al*., 1997)

Yeast nitrogen base	-	0.67 g
Agar	-	20.0 g
Demineralised water	-	1000 ml

7. Glucose – chalk agar (Kreger-Van Rij, 1984)

Yeast extract	-	0.5 g
Glucose	-	50.0 g
Chalk	-	5.0 g
Agar	-	20.0 g
Demineralised water	-	1000 ml

8. Christensen's urea agar (Christensen, 1946)

Peptone	-	1.0 g
Glucose	-	1.0 g
Sodium chloride	-	5.0 g
Potassium dihydrogen phosphate	-	2.0 g
Phenol	-	0.012 g
Agar	-	20 g
Demineralised water	-	1000 ml

9. Medium (Sand and Pitt, 1974)

Glucose	-	10.0 g
Tryptone	-	1.0 g
Yeast extract	-	0.5 g
Demineralised water	-	100 ml

10. Czapek – Dox agar medium

Sucrose	:	30.0 g
Sodium nitrate	:	2.0 g
Dipotassium hydrogen phosphate	:	1.0 g
Magnesium sulphate	:	0.5 g
Pottasium dhloride	:	0.5 g
Ferrous sulphate	:	0.01g
Distilled water	:	1000 ml
Ph	:	5.5- 6.5
Agar	:	15g

11. Potato dextrose agar (PDA) medium (Ricker and Ricker, 1936)

Potato (peeled)	:	250.0 g
Dextrose	:	20.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	5.5 – 6.8

Peeled potatoes were cut into small pieces, boiled in 500 ml of distilled water, mashed and the extract was strained through muslin cloth. To which, 20 g dextrose was added, dissolved and final volume was made to 1000 ml using distilled water. After adjusting pH, 20 g of agar was added and sterilized at 121°C for 20 min

12. Malt yeast extract medium

Malt extract	:	50.0 g
Distilled water	:	1000 ml
pH	:	5.5 – 6.5
Agar	:	15 G

13. Sabouraud dextrose agar medium

Peptone	:	10.0 g
Dextrose	:	40.0 g
Distilled water	:	1000 ml
pH	:	5.5 -6.5
Agar	:	15 g

14.Czapek Yeast Extract Agar (Atlas, 1997)

Sucrose	:	30.0 g
Agar	:	15.0 g
Yeast extract	:	5.0 g
K_2HPO_4	:	1.0 g
Czapek concentrate	:	10.0 ml
Distilled water	:	1000 ml
Czapek concentrate per litre:		
$NaNO_3$	:	30.0 g
KCl	:	5.0 g
$MgSO_4.7H_2O$	:	5.0 g
$FeSO_4$ $.7H_2O$	:	0.1 g
$ZnSO_4$ $.7H_2O$	:	0.1 g
$CuSO_4$. $5H_2O$	:	0.05 g

15.Nutrient agar medium

Glucose	:	5 g
NaCl	:	5 g
Peptone	:	5 g
Beef extract	:	3 g
Distilled water	:	1000 ml
Agar	:	15 g
pH	:	7.0

16.Enterococci Agar (Atlas, 1997)

Pancreatic digest of casein	:	15.0 g
Agar	:	10.0 g
Papaic digest of soybean meal	:	5.0 g
Yeast extract	:	4.0 g
K_2HPO_4	:	4.0 g
Glucose	:	2.0 g
$NaNo_3$	:	0.4 g
Triphenyl tetrazolium chloride	:	0.1 g
pH	:	7.2 ± 0.2

17.Ampicillin L Broth Medium (Atlas, 1997) (*Escherichia coli*)

Pancreatic digest of casein	:	10.0 g
NaCl	:	5.0 g
Yeast extract	:	5.0 g
Glucose	:	1.0 g
Ampicillin solution	:	10.0 ml (Filter sterilize)
pH	:	7.0 $\pm$ 0.2

18.Beef Extract Agar (Atlas, 1997) (*Pseudomonas aeruginosa*)

Agar	:	25.0 g
Beef extract	:	10.0 g
Peptone	:	10.0 g
NaCl	:	5.0 g

19.CCY Modified Medium (Atlas, 1997) (*Staphylococcus aureus*)

Yeast extract	:	30.0 g
Casamino acid	:	20.0 g
Na_2HPO_4	:	2.48 g
KH_2PO_4	:	0.41 g
$MgSO_4.7H_2O$	:	20.0 mg
$MnSO_4.7H_2O$	:	7.5 mg
Citric acid	:	6.4 mg
$FeSO_4.7H_2O$	:	6.4 mg
Sodium pyruvate solution	:	100.0 ml
pH	:	7.3 $\pm$ 0 .2

Sodium pyruvate solution (Filter sterilize)

Sodium pyruvate	:	23.2 g
Distilled water	:	100 ml

20. Streptococcus Agar (Atlas, 1997) (*Streptococcus faecalis*)

Glucose	20.0 g
Pancreatic digest of casein	20.0 g
Agar	15.0 g
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	1.0 g
pH	6.8 $\pm$ 0.2

APPENDIX -II

1. Reagents for estimation of FRAP assay

Acetate buffer (300 mM, pH 3.6) : Dissolve 3.1 g of sodium acetate in 16 ml of glacial acetic acid, make up to 1 litre with distilled water and stored at 4ºC.

Diluted HCl (40 mM) : make up 1.46 ml concentrated HCl (11 mM) to 1litre using distilled water and stored at 30ºC.

TPTZ (2,4,6 – tri (pyridyl) – s triazine) (10 mM) : add 0.03 g of TPTZ in 10 ml of 40 mM HCl, dissolve at 50ºC in water bath.

Ferric chloride (20 mM) : dissolve 0.054g of ferric chloride in 10 ml distilled water.

The solution should be straw coloured, if its tinged with blue discard and prepare fresh solution after rinsing the plastic bottle thoroughly with deionized water.

2. Reagents for estimation of DPPH assay

0.1 Mm of DPPH solution: 0.039 g of DPPH was dissolved in 1000ml of 100% methanol

3. **Reagents for estimation of reducing power assay**

Sodium phosphate buffer (pH 6.6): Dissolve 27.8 g of NaH_2PO_4 and 53.65 g of $Na_2HPO_4.2H_2O$ in distilled water and make up the volume to one litre.

1% potassium ferricyanide : 1 g of potassium ferricyanide was dissolved in 100 ml of distilled water.

10% TCA: 10 g of trichloroacetic acid was dissolved in 100ml of distilled water.

4. Reagents for estimation of Hydroxyl radical scavenging assay

0.021% FAS: 0.021 g of ferrous ammonium sulphate was dissolved in 100 ml of distilled water.

0.037% EDTA: 0.037 g of ethylene diamine tetraacetic acid was dissolved in 100 ml of distilled water.

0.88% DMSO: 0.88 ml of dimethyl sulphoxide make up to 100 ml of phosphate buffer.

0.264% ascorbic acid: 0.264 g of ascorbic acid in 100 ml of distilled water.

5. Reagents for estimation of Nitric oxide radical scavenging assay

10 mM sodium nitroprusside : 0.2975 g sodium nitroprusside in 100 ml of phosphate buffer

Griess reagent: 1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4 was added.

6. Reagents for estimation of chelating ability

9 mM ferrous sulphate

30 mM potassium chloride

1 mM tetramethyl murexide

Publications

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INFLUENCE OF FERMENTATION CONDITIONS FOR CAROTENOID PRODUCTION ON *RHODOTORULA MUCILAGINOSA* YP 187

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ABSTRACT : Carotenoid pigment producing yeast, *Rhodotorula mucilaginosa* yp187 was isolated from soil and its optimal fermentation conditions were evaluated. Optimum pH and temperature for total carotenoids production were determined as 6.0 and 25- 30°C with an incubation period of 96 h produced the highest quantity of biomass and carotenoids respectively. Agitation speed from 150 to 200 rpm min^{-1} was optimum for carotenoid pigment production. However, light was not a limiting factor for the growth and pigmentation of the yeast. Among the various carbon and nitrogen sources examined, maximum growth and pigmentation was observed sucrose and fructose as carbon sources and yeast extract (organic) and ammonium sulphate (inorganic) as nitrogen source.

Key words : Carotenoid, fermentation, *Rhodotorula mucilaginosa* YP 187.

INTRODUCTION

Carotenoids are important natural pigments, displaying yellow, orange and red colours that are ubiquitous in nature. They are a group of 600 molecules which can be found in plants and microorganisms, including bacteria, algae, molds and yeasts. Several microorganisms, including bacteria, algae, molds and yeasts of the genera *Rhodotorula, Rhodosporidium, Sporobolomyces* and *Phaffia* are able to produce carotenoids naturally (Frengova and Beshkova, 2009). Carotenoid biosynthesis is a specific feature of the *Rhodotorula* or *Sporobolomyces* yeasts and *Phaffia* genera (Razavi and Marc, 2006).

Carotenoids are currently produced for use as food colorants, nutritional supplements, cosmetics or health purposes. In addition to their pigmenting abilities, carotenoids may function as antioxidants by quenching photosentizers, interacting with singlet oxygen, and scavenging peroxy radicals (Frengova *et al*, 1994). Several studies have shown that carotenoids can be used as therapeutic agents against various type of cancer and other diseases due to their antioxidant and/or provitamin A properties (Astorg, 1997).Besides carotenoids, *Rhodotorula* is a rich source of fats and vitamins and can be incorporated in feeds to enhance the nutritional value and prevent fungal contamination (Joshi *et al*, 2003).

Interest in carotenoids has increased considerably, due to the growing evidence of benefits to human health and also to the growth of certain areas of agriculture, especially aquaculture and poultry industry (Nelis and Deleenheer, 1991). Color is an important characteristic of food. The development of foods with an attractive appearance is an important goal in the food industry. Increasingly, food producers are turning to natural food colors, since certain artificial color additives have demonstrated negative health issues following their consumption. Because of the deficiencies of existing natural food colorants, the demand for natural pigments is repeatedly raised by the food industry.

There is growing interest in microbial pigments due to their natural character, medicinal properties and nutritive value; production being independent of season, geographical conditions, controllable and predictable yield and safety to use (Frengova and Beshkova, 2009). Microbial synthesis offers a promising method for production of carotenoids. This explains the increasing interest in production of microbial carotenoids as alternative for synthetic food colourants.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism used in this study was isolated from soil, collected from Tiger reserve Parambikulam, Kerala India. Stock cultures were maintained on yeast malt extract agar slants at 4ºC after being incubated at 25- 30ºC for 4-5 days.

The basal medium for liquid culture contained 10.0 g glucose, 1.0 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 1.0 g $MgSO_4.7H_2O$ and 1.0 g yeast extract (YE) (per litre),

Table 1 : Effect of different temperature on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

Temperature (^{0} C)	Pigment carotenoid yield (mg/L)	Cell dry weight (g/ L)
15	1.81 ±0.02	7.2±0.144
20	2.27 ± 0.04	8.6±0.172
25	3.11 ± 0.06	9.1±0.182
30	3.02 ± 0.06	9.4±0.188
35	1.85 ± 0.03	6.2± 0.124
40	Negligible	Nil

Table 2 : Effect of different incubation time on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

Incubation time (hours)	Pigment carotenoid yield (mg/L)	Cell dry weight (g/ L)
48	1.0 ± 0.02	4.4 ± 0.08
72	2.5 ± 0.05	5.7 ± 0.11
96	3.6 ± 0.07	9.8 ± 0.19
120	2.9 ± 0.05	9.9 ± 0.19
144	2.4 ± 0.04	10.0 ± 0.2
168	1.4 ± 0.02	9.3 ± 0 .18

with the pH adjusted to 5.5 (buffered by 0.1 M potassium hydrogen phosphate). For stock culture, the yeast was sub cultured on agar slop (basal medium + 15 g/l agar) and incubated at 25 - 30p C for 72 h. The continuous sub culture was done in every 3 months. For starter culture, one loop of the yeast was transferred from the culture slop into 100 ml of basal medium contained in 250 ml Erlenmeyer flask. The flask was kept on the rotary shaker at 250 rpm and 25- 30p C for 72 h. To investigate carbon, nitrogen and growth factor sources, the liquid culture was done in 250 ml Erlenmeyer shake flasks, each was filled up with 100 ml medium and incubated on the rotary shaker at 250 rpm, 25- 30p C. Each of the flasks was inoculated with 5 per cent starter culture. Three replication experiments were carried out.

The physiochemical parameters studied were pH, temperature, aeration, incubation period, light sources, carbon sources, nitrogen sources and trace elements. Experiments were conducted in shake flasks, yeast growth and carotenoid pigment production was determined in 5 days interval and all experiments were performed in triplicate.

Estimation of pigment yield

The total carotenoid content of yeast was determined according to An *et al* (1996). One millilitre of yeast culture was harvested and resuspended in distilled water, washed twice and centrifuged to collect cell pellet. One millilitre of DMSO was added together with glass beads to break cells using vortex mixer. Then carotenoid extraction was performed by serially adding of each one millilitre of acetone, petroleum ether and 20% (w/v) NaCl solution, following with vortexing. After the solvent layers settled, the carotenoid in petroleum ether layers was monitored absorbance at wavelength 474 nm (A474) by using Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan). All the samples were carried out in triplicate and the values represented in figures are mean ± SD reported. The total carotenoid composition was calculated by using the 1% extinction coefficient = 2,100 by the formula:

Total carotenoid (µg/g of yeast) = (ml of petroleum ether) (A474) (100) / (21) (yeast dry weight)

Dry weight determination

Yeast biomass was separated from the liquid medium by centrifuging and rinsed twice with double distilled water, and then dried at 105^{0}C overnight to constant weight, yielding the dry weight.

RESULTS AND DISCUSSION

Effect of pH, temperature and incubation time on carotenoid pigment production

The pH value of growth medium affects not only biosynthesis activity of culture, but also culture growth rate. Latha *et al* (2005) reported that pigment producing red yeast was able to grow and form pigments under a wide range of intial pH conditions from 2.5 to 9.5. As seen from Fig. 1, with raising the pH, specific growth and carotenoid production rates increased and reached to a maximum level at pH 6.The results depicted that cell dry weight increased gradually with an increase in the pH of the broth, while the highest carotenoid yield (2.7mg/l) was obtained at pH 6.0 and it can be considered as the optimum pH for pigment production in this yeast.

The effect of temperature on yeast carotenogenesis is dependent on the species specificity and strain characteristics of the microorganism. The results of influence of different temperature on pigment and biomass production are presented in Table 1. In general, the optimum temperature for the growth of *R.mucilaginosa* appears to be 25-30ºC, low temperature (15°C) and high temperature (35ºC) reduced the biomass and pigment production. Among six different temperatures studied (15, 20, 25, 30, 35and 40°C), 25-30°C appeared to be optimum for the production of pigment in maximum quantity (2.8mg/l). The specific growth rate of the yeast increased notably with raising the temperature up to 30°C and lessened sharply above 30°C due to the denaturation of the enzyme system of microorganism at higher temperatures (Aksu and Eren, 2007). The total

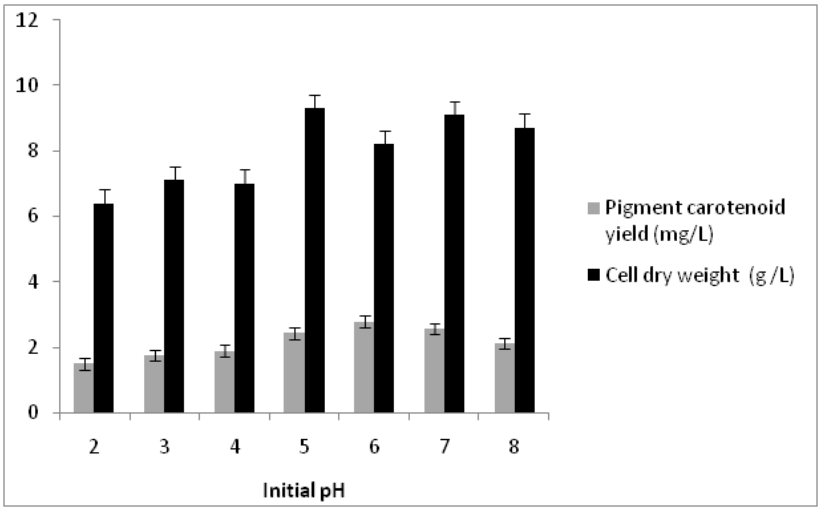

Fig. 1 : Effect of different pH on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

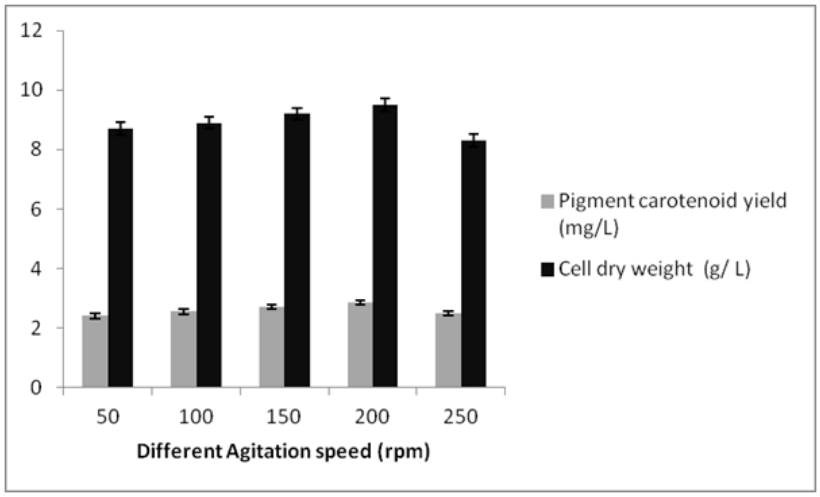

Fig. 2 : Effect of different aeration rate on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

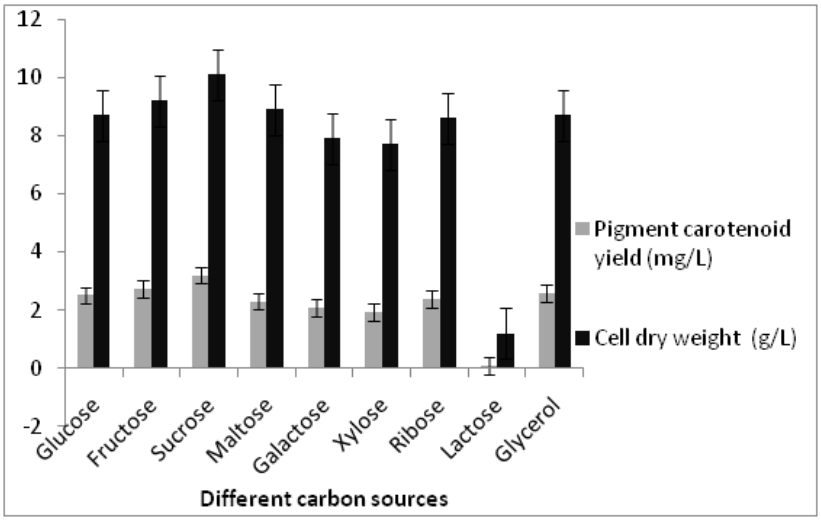

Fig. 3 : Effect of different carbon sources on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

carotenoids formation rate was also affected by the variation of fermentation temperature, enhanced strictly up to 30°C and increased slightly with further increase in temperature.

In order to optimize the production of pigment, the cultures were incubated for different periods and analyzed for the quality of pigment production. Among the six incubation period tested, the maximum production of pigment (3.06 mg/L) and biomass (9.8g/L) occurred at an incubation time of 96 h and lowest growth occurred at 48 h (Table 2). The results of this study showed that maximum pigment production was obtained at 96 h of incubation time for pigment production, after which there was a decrease in pigment production. Incubation beyond the optimum period causes catabolism of pigments.

Effect of light sources and aeration rate on pigment production

The cultivation of red yeast in the dark and under sunlight conditions showed that light was not a limiting factor for growth and pigmentation of *R.glutinis* DFR-PDY (Latha *et al*, 2005). A low yield of all the shades of pigments was observed in green wavelength, whereas the yellow wavelength inhibited the production of pigments. The darkness, red and blue wavelength observation finds significance as it is against the postulated photo-protective role of biopigments. The results are in agreement with Velmurugan *et al* (2009) who reported the effect of light on extra and intracellular pigment production.

If the microorganism requires oxygen, aerating the growth medium is very important for the successful progress of the fermentation. As *R. mucilaginosa* is an aerobic microorganism, the effect of the aeration rate on the growth and total carotenoids formation of the yeast was also examined and the results obtained were shown in Fig. 2. Both the specific growth and total carotenoids formation rates changed significantly with varying the aeration rate. It may be concluded that agitation speed from 150 to 200 rpm min^{-1} was optimum for carotenoid pigment production.

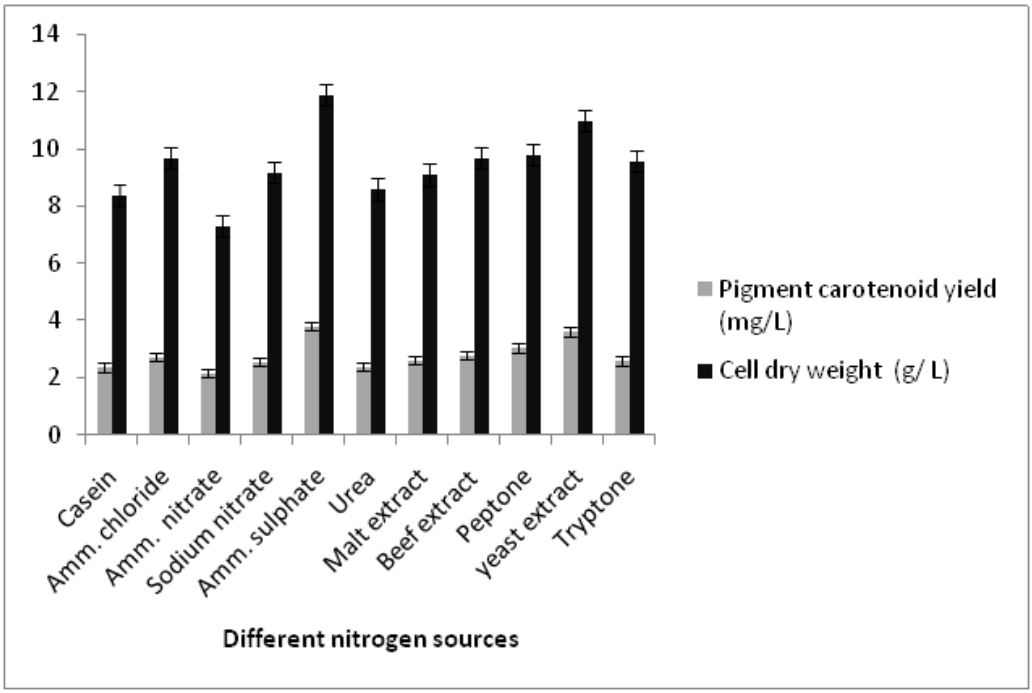

Fig. 4 : Effect of different nitrogen sources on carotenoid pigment production and cell dry weight in *R.mucilaginosa* YP 187.

Effect of carbon sources, nitrogen sources and trace metals on pigment production

During the microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but also as important energy source. Assimilation of different sugars as carbon source by the red yeast for its growth and pigmentation. In general, increase in sugar concentration in the growth medium increased the growth of yeast and carotenoid formation. Among the carbon sources evaluated in this study, fructose and sucrose gave a maximum yield (2.7mg/l) and (3.2mg/l) of carotenoid pigment were shown in Fig 3. As this yeast utilizes sucrose effectively, by-products from the sugarcane as well as fruit and vegetable processing industries may be considered as cheap substrates for its cultivation and production of carotenoids (Bhosale and Gadre, 2001). Glycerol, glucose and ribose supported moderate rate of both growth and pigmentation followed by sucrose and fructose. The ability of *R. mucilaginosa* yeast for growing on a variety of carbon sources, such as glucose, sucrose and fructose is a remarkable advantage. When compared with the results obtained with other yeasts in the literature, the high carotenoid productivity of the yeast also suggests a feasible process.

A stimulatory effect of nitrogen source on pigment formation has been reported by Hamdi *et al* (1998). Nitrogen source is one of the necessary components in the fermentation medium and different components of the nitrogen source could greatly affect the yield of astaxanthin (Du *et al*, 2005).It is well known that utilization of different nitrogen sources in fermentation had effects on microorganism growth and pigment production (Lin and Demain, 1991). In the present study various nitrogen sources were tested for growth and pigment production, among them inorganic nitrogen source ammonium sulphate yield maximum pigment production (3.8mg/l) and organic nitrogen source yeast extract (3.6mg/l) and peptone(3.0mg /L) were preferred by *R. mucilaginosa* yp187. Neveen (2011) reported that peptone had a positive effect on growth and pigment production of *P. purpurogenum.*

The maximum carotenoid pigment production of (2.9 mg/l) was achieved in magnesium sulphate supplemented medium and followed by ferrous sulphate and manganese. These results are consistent with Lee *et al* (2001) who reported Fe^{2+} has the strongest stimulatory effect on red pigment production. Some stimulatory effect has also been attributed to $Mn^{2+,}$ because trace metals have important effects on secondary metabolism (Weinberg, 1989)

CONCLUSIONS

Higher concentrations of sucrose, yeast extract and ammonium sulphate positively enhance both biomass and carotenoid accumulation in *R. mucilaginosa* YP 187. Sucrose had a significant positive impact on biomass accumulation and yeast extract had a significant positive impact on carotenoid production. Our finding therefore presents the optimized medium for higher volumetric production of carotenoid production from *R. mucilaginosa*

YP 187 and has potential for exploitation by biotechnological industries. Thus, the yeast *R. mucilaginosa* will be one of the most promising microorganisms for the commercial production of carotenoids by the use of agricultural wastes as a cheap source.

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Antimicrobial Activity of Carotenoid Pigment Produced from Yeast*rhodotorula Mucilaginosa* YP 187

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ABSTRACT

A carotenoid pigment present in red yeast and they possess a wide range of antimicrobial activity. The aim of the present study was to study about the antibacterial activity of carotenoid, extracted from soil pigmented yeast. Yeast strain *Rhodotorula mucilaginosa* YP 187 was isolated from the soil sample collected from Tiger reserve, Parambikulam, Kerala, India. The antibacterial activity of carotenoid pigment was studied on several organisms like *Bacillus Subtilis, Streptococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus* sp. The pigment showed excellent antibacterial activity than the standard chloramphenicol. Among this, pigmentshowed maximum inhibition against *Bacillus subtilis andStaphylococcus aureus.*

Key words *Carotenoid, Rhodotorula mucilaginosa YP 187, Antibacterial activity, well diffusion assay*

Yeasts can produce many bioactive substances, such as protein, amino acids, vitamins, polysaccharides, fatty acid, phospholipid, polyamine, astaxanthin, carotenoid, trehalose, glutathione, superoxide dismutase, chitinase, amylase, phytase, protease, killer toxin and so on; they have been receiving much attention for many decades. Red yeasts are ubiquitous microorganisms. They occur in soil, fresh and marine water, on plants, are commonly associated with animals and are also found frequently in man-made habitats such as food(Haggag,2013). *Rhodotorula* is pigmented yeast, part of the Basidiomycota phylum, quite easily identifiable by distinctive orange/red colonies. This distinctive colour is the result of pigments that the yeast creates to block out certain wavelengths of light that would otherwise be damaging to the cell. Colony colour can vary from being cream coloured to orange/red/pink or yellow. Members of the genus *Rhodotorula glutinis* have long been known for their potential to reduce the plant disease caused by fungal pathogens and they have gained considerable importance as potential antagonistic microorganisms. Recently, an antagonistic yeast strain of *Rhodotorula glutinis* have been reported as an effective biocontrol agent (*in vivo*) against postharvest decay of fruits and vegetables (Malisorn and Suntornsuk, 2008)

Martinkova, *et al.,* 1999 studied the antibiotic activity of *Monascus purpureus* extracts containing the orange pigments against various bacteria, fungi and yeast. Puspha, *et al.,* 2009 reported the antimicrobial activity of total red pigment from *M.purpureus* MTCC 410 recorded more antimicrobial activity, which could yield significant information on the scope for *Monascus* pigments substituted or as an adjunct to chemotherapeutic agents. Neveen, *et al*., 2011 reported that extracellular pigment of *Penicillium purpurogenum* was found to be more significantly effective against all tested microbial specieswhich includes *Epidermophyton floccosum, Microsporum canis*, *Aspergillus niger, Candida albicans*, *E. coli, P. aeruginosa, S. aureus* and *B. subtilis*. Gunjan and Sanjay Kumar, 2011 isolated a new red pigment *i.e.* 9-(1hydroxyhexyl)-3-(2-hydroxypropyl)-6a-methyl9,9adihydrofuro[2,3-h]isoquinoline 6,8(2H,6aH)-dione from *Monascus purpureus* which showed inhibitory activity against Gram-positive bacteria.

In industry, â-carotene and astaxanthin can be used as the additives of food and feed. â-carotene also can serve as the precursor of vitamin A in mammals. In recent years, many studies on enhanced production of carotenoid by mutants of *Rhodotorula* spp. and optimization of the fermentation processes for cultivation of *Rhodotorula* spp. were undertaken (Bhosale and Gadre, 2001). The aim of the present study is to determine the antimicrobial activity of carotenoid pigment extracted from the yeast culture *Rhodotorula mucilaginosa* YP 187.

MATERIALS AND METHODS

Sample and Culture collection:

The microorganism used in this study was isolated from soil, collected from Tiger reserve Parambikulam, Kerala, India. Stock cultures were maintained on yeast malt extract agar slants at 4ºC after being incubated at 25- 30ºC for 4-5 days.

Human pathogens:

The human pathogens (bacteria) used in the study were obtained from the Microbiological Laboratory, Coimbatore, Tamil Nadu, India and KMCH College of

Table 1. Minimum inhibitory concentration of acetonic pigment extract of *R. mucilaginosa* YP 187 against bacterial pathogens

Pathogens	Dilution 1 (10mg ml^{-1})	Dilution 2 (1mg ml^{-1})	Dilution 3 (0.1mg ml^{-1})	Dilution 4 (0.01mg ml^{-1})
Enterococcus sp.	I	I	NI	NI
S. aureus	I	I	I	NI
B. subtilis	I	I	NI	NI
S. faecalis	I	I	NI	NI
E. coli	I	I	I	NI
P. aeruginosa	I	I	NI	NI
Solvent (Acetone)	NI	NI	NI	NI
Chloramphenicol	I	I	I	I

NI – No inhibition; I – Inhibition

Pharmacy, Coimbatore, Tamil Nadu, India. The following six bacterial pathogens were used in this study *Enterococcus* sp., *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa*.

Analytical methods:

Pigment extraction:

The yeast cultures were inoculated onto yeast malt extract broth and incubated at 28±1ºC for 5 days. A known amount (500mg) of freeze-dried red yeast was hydrolyzed with 1 ml of 1N hydrochloric acid in water bath at 70°C for one and half hours. After removal of excess acid by washing with water, the cells were soaked overnight in acetone: methanol (1:1) solution. The pigment was extracted with acetone until the entire colour was leached out from the cells. Acetone extracts were transferred to light petroleum (20ml) at (40- 60°C) in a separating funnel and washed thrice with distilled water. The absorbance of the light petroleum phase was documented at 474 nm (λmax) and the concentration of carotenoids was determined using the absorption coefficient as A1% 1 cm = 1600. Carotenoid yield is reported on the basis of cell mass (µg g-1 dried cell weight) (Latha et al., 2005). The total carotenoid was calculated by using the formula

Total carotenoid (µg g-1 of yeast) = (ml of petroleum ether) (A474) (100) / (16) (yeast dry weight)

Dry weight determination:

Yeast biomass was separated from the liquid medium by centrifuging and rinsed twice with double distilled water, and then dried at 105°C overnight to constant weight, yielding the dry weight.

Determination of antibacterial activity:

Bacterial strains were grown in nutrient broth at 37°C for 18-24 h with a load of 10^8- 10^9 CFU ml^{-1}. 0.1 ml of culture broth was spread on nutrient agar plate by spread plate method. Using sterile cork borer, a well was formed and impregnated with 100 ìl of acetonic extract and crude extract. The plates were incubated at 37ºC for 24 h. At the end of the incubation period, the susceptibility of the test organism was determined by measuring the zone of inhibition around the well (Iqbal, *et al.,* 1998)

Determination of minimum inhibitory concentration (MIC):

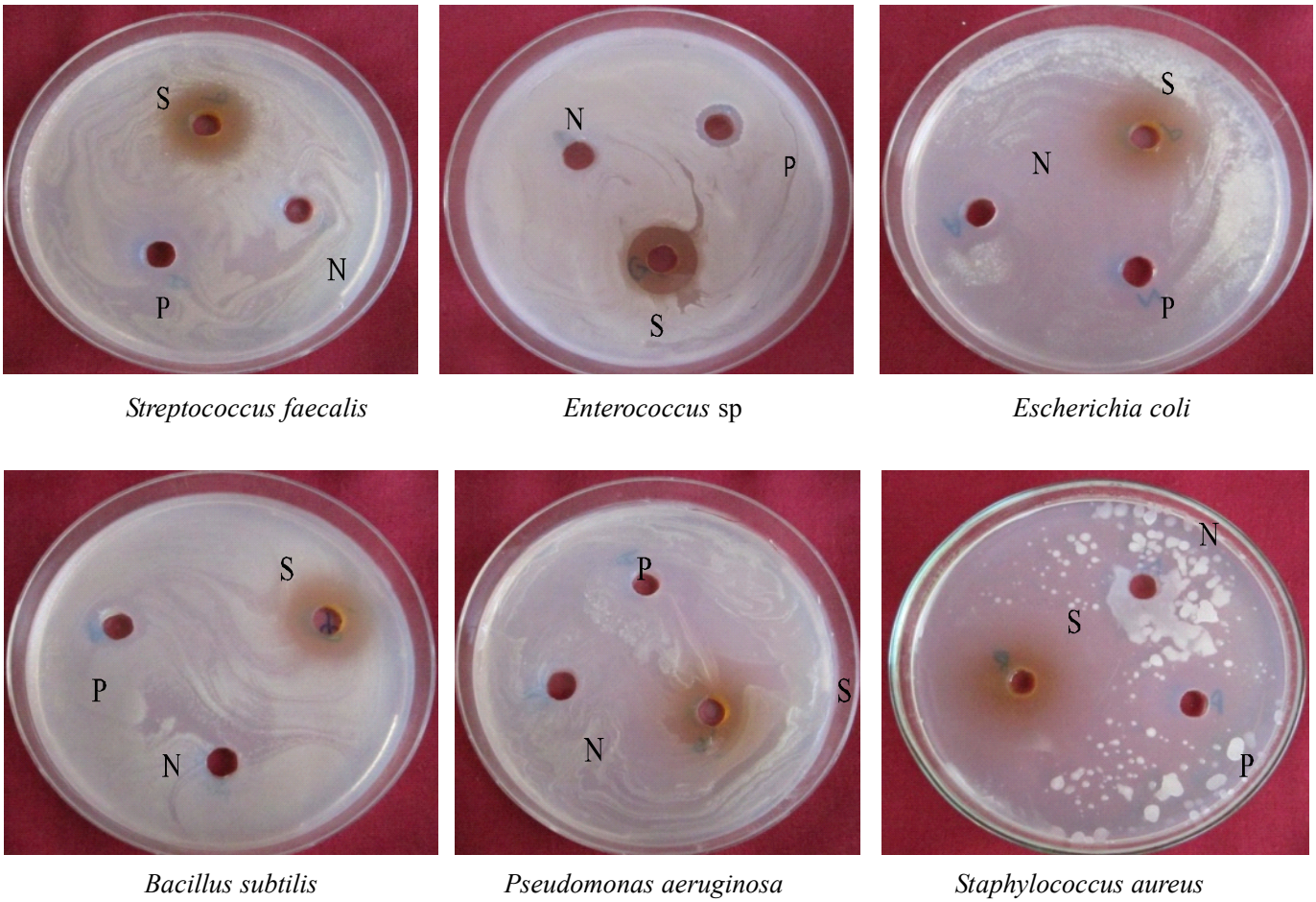
The MIC was performed to test the antimicrobial activity of the acetonic extracts of carotenoid pigment using tube dilution method (Claeys, *et al*., 1988). The MIC was defined as the lowest concentration of extracts at which tested pathogens did not show any growth. This test was performed at four concentrations of the pigment extract (10 mg ml^{-1}, 1 mg ml^{-1}, 0.1 mg ml^{-1} and 0.01mg ml^{-1}). Chloramphenicol (10 mg ml^{-1}) was used as the positive control (standard).Similar to the agar well diffusion method,the petriplates were divided into 3 equal quadrats. After incubation of the eppendorf tubes for an hour, 50 µl from each of the tubes were spotted on the petri plates.The plates were then covered and incubated for 24 h. The growth of the organism for each dilution was observed.

RESULTS AND DISCUSSION

Antimicrobial activities of yeast carotenoid pigment against human pathogens:

The bacterial pathogen *Escherichia coli* was found to be highly inhibited by the pigment extract of *R. mucilaginosa* YP187with the inhibition zoneof3.0 cm respectively; this was comparable to the activity of chloramphenicol (2.8 cm). The carotenoid pigment possessed good activity against *Staphylococcus aureus* (2.7 cm). Pathogens like *Streptococcus faecalis*and *Bacillus subtilis*weremore effectively inhibited by the pigment extract of*R. mucilaginosa* YP187 (2.6and 2.0 cm), followed by *Enterococcus* sp and *Pseudomonas aeruginosa* (2.0 and 2.1cm) (Fig. 1).The standard antibiotic chloramphenicol produced inhibition zones of 2.9, 2.0, 1.8, 2.9, and 2.6 cm against *Staphylococcus aureus*, *Streptococcus faecalis*,*Enterococcus* sp, *Bacillus subtilis*and *P. aeruginosa* accordingly. The reason was referred to the difference in the structures of the cell walls (Singh, *et al*., 2007). Selective antibacterial activity may be due to several factors, including charge density, structure of lipopolysaccharides and lipid composition of the cytoplasmic membrane in Gram-negative and Gram-positive bacteria (Devine and Hancock, 2002).

The observed results suggest that the potential therapeutic drug could be developed from yeast pigment in future to treat human pathogenic microbes.



Streptococcus faecalis *Enterococcus* sp *Escherichia coli*

Bacillus subtilis *Pseudomonas aeruginosa* *Staphylococcus aureus*

Fig. 1. Antimicrobial activity of acetonic extract of carotenoid pigment from *R. mucilaginosa* YP 187

S-Sample (pigment), N-Negative control (100% Acetone), P-Positive control (Chloramphenicol)

Minimum inhibitory concentration (MIC) assay against bacterial pathogens:

Minimum inhibitory concentration (MIC) was defined as the lowest concentration able to inhibit any visible microbial growth. The Minimum inhibitory concentration for the pigment extract of *R. mucilaginosa* YP 187was evaluated against the selected bacterial pathogens. The pigment extractshowed inhibition against all the selected gram positive and gram negative bacteria at a concentration of 10 mg ml^{-1}, this was comparable to that of the standard antibiotic chloramphenicol (Table 1).The carotenoid pigment extract had no effect on the growth of the all selected bacterial pathogensto a concentration of 1.0 mg ml^{-1}, 0.1 mg $ml^{-1}$and 0.01 mg ml^{-1}. The solvent control (acetone) was found to possess negative activity at all the four concentrations against all the organisms. The results indicated that the chemistry of the pigments has a significant influence on its antimicrobial activity

The yeast pigment which exerted higher inhibition zones in both Gram positive and Gram negative organisms better than that of chloramphenicol. The results indicated that carotenoid pigment has broad spectrum of activity against pathogenic cultures.These results may suggest that the pigment might be developed as potential antimicrobial drug.

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