Optimization and Characterization of pigment produced from the bacteria *Serratia nematodiphila*

काशी हिन्दू विश्वविद्यालय BANARAS HINDU UNIVERSITY

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Submitted by

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Dear Sir,

I have great pleasure in forwarding the thesis titled **"Optimization and Characterization of pigment obtained from the bacteria *Serratia nematodiphila*"** submitted by **Ms. Deepika Yadav, I.D. No. FST-15254,** in partial fulfillment of the requirements for the degree of **Master of Science** in **Food Science and Technology,** from Centre of Food Science and Technology, Institute of Agricultural Sciences, BHU Varanasi.

I certify that the entire scheme of investigation, presented here in, was planned and carried solely by the candidate under my guidance. To the best of my knowledge, the data presented in the thesis are genuine and original.

Thanking you.

Yours Faithfully,

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Optimization and characterization of pigment produced from the bacteria *Serratia nematodiphila*

By

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2017

I.D.No.FST-15254 **Enrolment No. 376515**

Thesis submitted in partial fulfillment of the requirements for the award of degree of Master of Science in Food Science and Technology from Centre of Food Science and Technology, Institute of Agricultural Sciences, Banaras Hindu University Varanasi-221005

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(DEEPIKA YADAV)

ABBREVIATIONS USED

α	:	Alpha
β	:	Beta
oC	:	Degree centigrade
%	:	Per cent
&	:	And
bp	:	Base pair
cfu	:	Colony forming units
cm	:	Centimeter
DNA	:	Deoxyribonucleic Acid
Fig.	:	Figure
g	:	Gram
g/l	:	Gram per litre
hrs	:	Hour
i.e.	:	That is
l	:	Litre
M	:	Molar
mg	:	Milligram
min	:	Minutes
ml	:	Millilitre
mm	:	Millimeter
N	:	Normal

nm	:	Nanometer
OD	:	Optical density
ppm	:	Parts Per Million
PCR	:	Polymerase Chain Reaction
RNA	:	Ribonucleic Acid
rDNA	:	Ribosomal DNA
rRNA	:	Ribosomal RNA
rpm	:	Rotations per minute
sp.	:	Species
temp	:	Temperature
UV	:	Ultra violet
v/v	:	volume/volume
viz.	:	Visually
w/v	:	weight/volume

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CHAPTER-1

INTRODUCTION

Color is one of the significant visual properties and is an important attribute of any article. The color determines the acceptance of a product and has paramount influence on human life. Many synthetic colors used in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing pose various hazardous effects like allergies, tumor, cancer and severe damages to the vital organs **(Duran *et al*. 2002)**. Moreover, the effluent of synthetic dyes pose serious threat to the environment. Consequently, many synthetic colors have been banned due to their toxicological problems. With the increasing awareness about the toxic effects of synthetic colors and consumer safety, there is an increasing interest in the development of colors from natural sources **(Babu and Shenolikar, 1995).**

Natural colors are generally extracted from fruits, vegetables, roots and microorganisms which are often called as bio-colors due to their biological origin. The utilization of natural pigments in foodstuff, dyestuff, cosmetics and pharmaceutical manufacturing processes has increased in the recent years due to their nontoxic nature **(Unagal *et al*., 2005)**. Moreover, their eco-friendly, antioxidant, anticancer and antimicrobial activities further add to their positive effects. The significant growth in the naturally derived colors has been attributed to their stability and consumer acceptance. Further the annual growth rate of naturally derived colors has been predicted to be 5-10 per cent in comparison to synthetic colors, with a low growth rate of 3-5 per cent **(Parmar and Phutela, 2015).** Although there are a number of natural pigments, only a few are available in sufficient quantities to be useful for industry because they are usually extracted from plants. In spite of the availability of variety of pigments from fruits and vegetables, there is an ever growing interest in microbial pigments due to several reasons like their natural

character and safety to use, production being independent of seasons and geographical conditions, controllable and predictable yield **(Kim *et al.*, 1998 and Gunasekaran and Poornima, 2008)**. In the last few years scientific research has focussed itself on studying detailed attributes of not only potentially pathogenic microorganisms, but has also thrown light on a whole new approach to studying them. The rapid growth of microbes reduces the production time to a matter of days compared to plant and animal sources, the production is flexible and can easily be controlled **(Joshi *et al.*, 2003)**. The presence of bio pigments has been reported in almost all the classes of microorganisms including bacteria, fungi, yeasts and algae. These microorganisms can produce variety of bio pigments such as carotenoids, melanins, flavones, quinines, prodigiosin, and monascins **(Jiang *et al.* 2005 and Dofosse, 2006)**. Many yeasts like *Rhodotorula* (pink), *Yarrowia lipolytica* (brown), *Cryptococcus* (red) and *Phaffia rhodozyma* (carotenoids) are good source of microbial pigments. The pigment production by molds of *Monascu*s group especially *Monascus purpureus* and *Monascus 'anka'* for use as a good food grade color is well known **(Sharma, 2014)**. The algae which produce pigments are *Chlorococcum, Chlamydomonas, Chlorella*, *Hematococcusi* and *Sporangium*. Another algae namely, *Dunaliella salina* belonging to class chlorophylaceae occur in marine environment and produces β-carotene which can be used as food colorant **(Joshi *et al.*, 2003)**. The pigment produced by algae and fungi may be less accessible for exploitation because of the structural complexity of the pigment bearing tissues and the pigment production at critical points of development within a complex lifecycle. Bacteria are good source of pigments. Bacterial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications **(Venil and Lakshmanaperumalsamy, 2009)**. The advantages of pigment production from bacteria include easy and fast growth in cheap culture medium and faster fermentation for bulk production **(Venil *et al.*, 2013)**. Some pigment producing bacteria are *Staphylococcus aureus* for golden yellow, *Serratia marcesans* for red,

Micrococcus lutes for yellow, *Micrococcus roseus* for pink, *Staphylococcus roseus for* red and *Pseudomonas cynxanatha* for yellow pigment **(Ahmad *et al.*, 2012)**. Extreme environments generally characterized by a typical temperature, pH, salinity, toxicity and radiation level are inhabited by various microorganisms. The hot springs and geothermal vents are found in different parts of the world which contain several prokaryotes, especially adapted to grow in these environments. These microorganisms are often colored due to the presence of photosynthetic and carotenoids pigments. Due to their ability to grow and survive at high temperature of hot springs, microbes are classified as thermophiles. These thermophiles are adapted to survive in extreme conditions with higher reaction rates, higher solubility and stability of most chemicals. The bio colors are used in food, textile and pharmaceutical industries. The use of non-allergic, non-toxic and eco-friendly natural colorants in food has become a matter of significant importance due to increased awareness. Hence, due to harmful effect of chemical colorants on health as well as the environment, a number of countries have issued strict regulations so as to preserve our environment. As a consequence there is a revived interest in the use of natural pigments and dyes, which could be subjected to biodegradation in the environment.

Natural dyes produce very uncommon, soothing and soft shades as compared to synthetic dyes. Although, synthetic colors are widely available at economical price in wider range of colors but these dyes produce skin allergies, less stable and also produce highly toxic wastes that pose a threat. The color stability under extreme temperature, variable pH, and processing conditions is the pre requisite for industrial application. Therefore, microbial diversity has been a great source for exploration for application of bio pigments. Limited research studies have been conducted for exploration of such pigment-producing microorganisms under Indian conditions.

Hence, the present study was focused on pigment producing bacteria for their pigment production. The study also envisaged on the optimization of conditions for

maximum pigment production with the following objectives:

1. To obtain and enumerate colour producing microorganisms.
2. To optimize various physico-chemical conditions for maximum color production.
3. To partially purify and characterize microbial colorants.
4. To carry out solid state fermentation and submerged fermentation.

CHAPTER-2

REVIEW OF LITERATURE

Colors provide attractive appearance to the marketable products such as food, textiles and pharmaceutical products. There are many artificial synthetic colors which have been used widely in several industries like food, cosmetics, textiles and pharmaceuticals. Although these synthetic colors are reliable and economical as compared to natural colors, which are expensive, less stable and possess lower intensity, many of such synthetic colors have also been banned due to their hyper-allergenicity, carcinogenicity and other toxicological problems. These adverse effects of synthetic colors have triggered intense research on natural colors and dyes **(Reyes *et al.*, 1996**). Recent research efforts have been made to replace synthetic pigments with natural pigments from plant, animals and microorganisms.

Microorganism are known as a potential source for bio pigment production due to their advantages over plants in terms of availability, stability, cost efficiency, labor, yield and easy downstream processing **(Joshi *et al.*, 2003).** A limited number of research studies have been conducted on the exploration of microorganism for pigment production which calls for exploring microbial pigments in more detail. More recently, the current trends and future perspective of microbial pigments as natural colorants has been reviewed by **Tuli *et al*. (2015).**

1) Food Colors

Colour plays a special role with the food we eat. For example, when confronted with an unattractive colour, the consumer assumes that the food is poor or spoiled. On the other hand, products with atypical colour – for example, green cheese or blue drink – in most cases are rejected by the consumers. Typically, one associates colours with food items such as cherry with red, lemon with yellow, or orange with

carrot. Therefore, colours can serve as the primary identification of food and are also a protective measure against the consumption of spoiled food. Colours of foods create physiological and psychological expectations and attitudes that are developed by experience, tradition, education and environment: We inevitably eat with our eye.

The controversial topic of synthetic dyes in food has been discussed for many years. The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural colouring alternatives. Some companies decided to colour food with food, using mainly plant extracts or pigments from plants, *e.g.* red from paprika, beetroots, berries or tomato; yellow from saffron or marigold; orange from annatto; green from leafy vegetables, *etc.* Penetration of the fermentation-derived ingredients into the food industry is increasing year after year. Examples could be taken from the following fields: thickening or gelling agents (xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), flavor compounds (gamma-decalactone, diacetyl, methyl ketones), acidulants (lactic acid, citric acid), *etc*. Some fermentation-derived pigments, such as b-carotene from the fungus *Blakeslea trispora* in Europe or pigments from *Monascus* in Asia, were developed later on and are now in use in the food industry. Efforts have been made in order to reduce the production costs of fermentation pigments compared to those of synthetic pigments or pigments extracted from natural sources. Innovations will improve the economy of pigment production by isolating new or creating better microorganisms, by improving the processes. This review focuses on research works related to this field published over the past ten years by private companies or academic laboratories.

3) Isolation and Characterization of Pigment Producing Microorganisms

A large number of species of bacteria, yeast, mold and algae produce pigments but only few of them have been found suitable for pigment production. Most of the microbial pigments are carotenoids in nature. Among different organisms, bacteria are

known to produce a wide range of colors. Some of them are *Chromobactor* sp. (red), *Bacillus* sp.(brown), *Bervibacterium* sp. (orange yellow), *Pseudomonas* sp.(yellow), *Rhodococcus maris* (bluish red) and Sarcina sp. (dark yellow) **(Thirkell and Strange, 1967, Broder and Koehler, 1980 and Careilla *et al*., 2001).** A number of pigment producing microorganisms are enumerated below (Table 1). These pigment producing bacteria have been isolated and studied for morphological, physiological and biochemical characteristics as per Bergey's manual of Determinative Bacteriology **(Holt *et al*., 1994).**

Table 1: Pigment Producing Microorganisms

Organism	Pigment	Color
Serratia marcesans	Prodigiosin	Red
Corynebacterium insidiosum	Indigoidine	Blue
Monascus roseus	Canthaxanthin	Orange, pink
Staphylococcus aureus	Zeaxanthin	Yellow
Pseudomonas aeruginosa	Pyocyanin blue	Green
Dunaliella salina	β carotene	Orange
Hematococcus pluvialis	Astaxanthin	Red
Bradyrhizobium sp.	Canthaxanthin	Orange /dark red
Alteromonas rubra	Prodigiosin	Red
Flavobacterium sp.	Zeaxanthin	Yellow
Asbhya gossypi	Riboflavin	Yellow
Blakeslea trispora	Lycopene	Rred
Rhodotorula sp.	Torularhodin	Orange-red
Penicillium oxalicum	Anthraquinone	Red
Streptoverticillium	Prodigiosin	Red
Streptomyces echinoruber	Rubrolone	Red
Janthinobacterium lividum	Violacein	Purple
Haloferax alexandrinus	Canthaxanthin	Dark Red

Source: (Gupta *et al*. 2011)

Goswami *et al.* (2010) isolated an unidentified yellow pigment producing bacterium from the soil from Durgapur, West Bengal. They reported that the colonies were circular, convex and yellow in color. The isolated strain was Gram positive coccus.

Cardona *et al*. (2010) isolated two blue pigment producing bacterial isolates BPB-W and BSB-SW from soil samples. One isolate was isolated from the south region of Puerto Rico (BPB-W) and other from the south west region of Puerto Rico (BSB-SW). The mineral composition test of the soil sample from where BSB-SW was isolated have shown quartz as common mineral followed by vermiculate. The soil sample containing BSB-SW have shown presence of minerals like anorthite, calcite and vermiculite. They have reported that both blue pigmented isolates showed a circular form, convex elevation and metallic deep blue color and were identified as *Vogesella indigofera*.

Bhatt *et al*. (2013) isolated pigment producing bacteria from the food samples like samosa, bread, soup and juice procured from local market of Srinagar Kashmir, India. Among the colonies of bacteria formed on nutrient agar, two colonies were found to produce pigments. They reported that one colony were found to produce orange color pigment and strain was named as MJ-O and other colony were found to produce yellow pigment and was named as MJ-Y. They have reported that these two pigment producing bacteria were round, smooth, cocci, non-motile and Gram negative. They identified the bacteria by biochemical characterization as per Bergey's manual as *Micrococcus nishinomiyaensis* and *Micrococcus luteus*.

Arulselvi *et al.* (2014) screened 41 soil samples collected from various locations with different environmental conditions such as fertile land, waste land sewage areas and location of Salem district. They reported that 24 out of 41 were found to produce yellow pigmented bacterial colonies. They reported predominant number of yellow pigmented bacterial colonies in soil samples of fertile land and hill

stations where the climate is slightly colder. The strains were further identified by colony morphology and biochemical identification. They reported that all bacterial colonies were circular, convex, gram positive, cocci, catalase positive, oxidase negative and yellow in color and out of twenty four bacterial isolates YCD3b showed highest pigment production with maximum absorbance around 1.40 at 450 nm.

4) Molecular Characterization of Pigment Producing Bacteria

Peix *et al*. (2005) performed 16S rRNA gene sequencing from pigment producing isolates CH01 and PA01. Complete sequences for these two isolates exhibited 1532 nucleotides and 1530 nucleotides. Comparison of sequence 16S rRNA gene sequences showed a maximum similarity of 99.3 per cent between them, and phylogenetic analysis revealed that the strains belong to the genus *Pseudomonas straminea* within the γ-subclass of *Proteobacteria*. The G+C DNA content was 57.5 mol% for CH01 and 58.0mol% for PA01.Amplification of the 16S rDNA was done by PCR with positive amplification of approximately 1500 bp. Sequencing of the whole 16S rDNA was performed. *In-silico* analysis of both blue pigmented bacteria suggested that they were 90 percent similar with each other, but 97 per cent similar with *V. indigofera*. The phylogenetic analysis demonstrated the two isolated bacteria were very similar to *V. indigofera* **(Cardona *et al*., 2010).**

Mukherjee *et al*. (2012) identified the aquatic isolate from Bakreshwar hot spring of West Bengal by 16S rDNA sequencing and phylogenetic study. The DNA was isolated from the culture and its quality was evaluated in 1.2 per cent agarose Gel. The fragments of 16S rDNA were amplified by PCR. The sequence of 1421 bp 16S rDNA of the isolated bacteria showed 100 per cent similarity with 16S rDNA gene sequence of *Pseudomonas aeruginosa* GIM 32 (Gene bank accession no HM067869.1). Phylogenetic tree constructed by neighbor joining method showed that the isolated bacterial culture was closely related to *Pseudomas aeruginosa* strain D2 (Gene bank accession No EU 915713.1) and distantly related to *Bacillus* sp. W4

(Gene bank accession no EU 596423.0.1)

Kurian *et al.* (2014) isolated a melanin producing bacterial strain BTCZ10 from marine sediments. Phylogenetic analysis revealed 100 per cent homology of 12 the 16S rDNA sequence with *Pseudomonas stutzeri* strains in NCBI database. The UV visible spectrum revealed a higher absorption at the UV region which decreased as it reached the visible region. Strain BTCZ10 produced 47.47±0.2 µg/mL of melanin.

Vora *et al.* (2014) also isolated red pigmented bacterial strain from the soil sample of Kharaghoda area of Gujrat. The 16S rRNA gene sequencing have shown the strain JR3 was matched with *Serratia marcesans* CMG 3090.

5) Optimization of Cultural Conditions for Maximum Pigment Production

The microorganisms use different medium, carbon and nitrogen sources for their growth and pigment production. Numerous workers have reported different conditions of temperature, pH and incubation period for pigment production by the microorganisms.

Khanafari *et al.* (2010) optimized the conditions for pigment production by bacterial strain *Halorubrum sodomense* isolated from water samples of solar salt lake in Southwest Iran. They reported maximum pigment production with 30 per cent NaCl concentration. The maximum growth and pigment production was found at pH 7.2-7.5 indicating that acidic or alkaline pH have inhibitory effect on the bacterial growth and pigment production. The optimum temperature for maximum growth and pigment was found to be 25°C indicating that these strains are not psychrophilic or thermophilic microorganisms. They further reported that when sucrose (1%) is added as extra carbon source and NH4 NO3 as extra nitrogen source in the media, the pigment production increased.

Goswami *et al.* (2010) studied the effect of pH and temperature on pigment

production by a yellow pigment producing bacteria isolated from soil in areas around Durgapur, West Bengal. Pure colonies were grown at 1 to 14 pH and temperature between 4 °C and 45 °C. The maximum pigment yield was obtained at pH 7 and at 30 °C. This suggested that pigment production is influenced by physical parameters.

Dikshit and Tallapragada (2011) studied growth pattern, temperature and pH on pigment production and mycelial growth of *Monascus purpureus* in submerged culture. They reported that maximum red pigment was produced on the 16th day of incubation. The favorable temperature was found to be 30 °C with a pH value of 5.5.

Shahitha and Poornima (2012) tested various media for enhanced production of prodiogiosin from *Serratia marcesans*. The highest pigment production was observed in powdered peanut seed broth and powdered seasame broth. Among different oils, peanut oil exhibited high pigment production. They reported that the optimum conditions for pigment production were found to be in nutrient broth at 28 °C and at 7 pH. The maltose was reported as best source substrate for enhancing pigment production in nutrient broth which yielded 2.72 mg/ml prodigiosin.

Chaudhari and Jobanputra (2013) screened 9 nutrient parameters viz., sucrose, yeast extract, peptone, NH_4Cl, $(NH4)_2SO4$, KH_2PO4, NaH_2Po4, MgSO4 and FeSO4 for the production of pigment by *Planococcus maritimus* under shake flask conditions. Among the nine nutrients sucrose, yeast extract, NH4Cl, KH2PO4 and MgSO4 had contributed to large extent for bio pigment production. They also reported that sucrose as carbon source and yeast extract as a nitrogen source was found best for maximum carotenoid pigment production.

Goswami and Bhowal (2014) studied the optimum growth optimum the growth conditions for red pigment production by novel strain of *Bacillus* sp. From soil samples. They reported the appearance of red pigment from bacteria in nutrient broth after 72 hrs of incubation in the culture medium. The maximum pigment production was at 30-37 °C and at pH 7 after 7 days of incubation.

Chandran *et al.* (2014) isolated the bacterial pigments from the four strains namely; *Bacillus subtilis, Enterococcus hirae, Acinetobactor mufti* and *Pseudomonas aeruginosa*. One ml each of the test organisms such as *Bacillus subtilis, Enterococcus hirae, Acinetobacter mufti* and *Pseudomonas aeruginosa*, were inoculated into 20 ml test tube containing 10 ml nutrient broth and brain heart infusion broth separately. The *Pseudomonas aeruginosa* was reported to produce maximum pigment production at optimum temperature of 37 °C at 72 hrs. They stated that the temperature, pH and incubation period usually play an important role in cell synthesis and suitable conditions are needed for culture growth and their pigment productions.

Bhat and Marar (2015) studied various factors affecting the growth and pigment production by *Salinicoccus* sp. MKJ 997975 and found that the growth and pigment production were higher when the *Salinicoccus* sp. MKJ 997975 was grown in nutrient broth (4.5 g/l) than in LB medium (4.10 g/l). Maximum growth and pigment was obtained within 3 days by *Salinicoccus* sp. and it declined after third day i.e., 72 hrs. Microorganism generally exhibit pigment production during log phase or at stationary phase **(Kaur *et al.*, 2009).** The species showed maximum pigment production of 257.26 µg/g at pH 7. They reported maximum growth and pigment production at 30 °C.

6) Extraction and Characterization of Pigment

Water insoluble and lipophilic pigments are usually extracted with water miscible organic solvent such as acetone, methanol, ethanol or mixture of these solvents for better solvent penetration. Dried materials can be extracted with water immiscible solvents. The extract usually contains a substantial amount of water, which can be removed by partition to hexane, petroleum ether, diethyl ether or mixtures of these solvents. The chromatographic behavior and the ultraviolet/visible absorption spectrum provide the first clues for the identification of pigments.

Goswami *et al.* (2010) extracted the yellow color bacterial pigment using methanol solvent. The pigment extract was analyzed using UV-Vis spectrophotometer with absorption maxima at 437 nm and reported it as carotenoid.

Sasidharan *et al.* (2013) extracted yellow pigment from the four isolates RS7, RSS3, RS13 and RS14 using methanol as solvent. They reported that all the yellow pigments gave identical absorption spectra of carotenoid. The spectra were characterized by maximum peaks at 493 and 527 nm with broad shoulder at 467 nm indicating the main carotenoid is bacterioruberin. The HPLC analysis showed the retention time close to 3.3 indicating the carotenoid as Astaxanthin.

Masi *et al.* (2014) extracted the pigment from the isolates *Pseudomonas aeruginosa*, and extract were prepared by chloroform extraction method. The samples were analyzed by using UV spectrophotometer and HPLC. The maximum absorbance of the pigment was obtained at 690 nm and 682 nm. The HPLC chromatogram reported that the pigment belongs to the chlorophyll family and pyocyanin.

Vora *et al.* (2014) used red pigmented bacterial strain for the extraction and characterization study. This halophile, Gram negative bacteria was identified as a *Serratia marcesans* strain by 16S rRNA gene sequencing. The highest extraction of this pigment was yielded in 2:1= 85 per cent, Methanol: Acetone mixture among different concentration of different solvents. The Rf value of the extracted pigment was 0.96, 0.80 0.64 and 0.58 by TLC method. The maximum absorption spectrum was observed at 535 nm by UV-Visible Spectroscopy.

Prasad (2015) reported the pigment extraction from bacterial strains isolated from effluent water samples by solvent extraction using petroleum ether and acetone (1:1). The thin layer chromatography was carried to identify the pigment. They further reported that the pigment produced by *Bervibacterium* sp. and *Arthobactor* sp. as _-carotene with Rf value of 0.95 and the pigment produced by *Serratia marcesans* identified as prodigiosin with Rf value 0.83.

7) Properties of Microbial Pigments

Microorganisms are known to produce a variety of biologically and pharmacologically active compounds. A number of studies have been carried out to find antioxidant, anticancer, antimicrobial activities of microbial pigments. It can be an alternative for synthetic compounds in food and pharmaceutical technology in order to treat various pathological disorders.

Pigment	Properties	Source
Violacein	*Antiprotozoan* Anticancer Antiviral antibacterial Antioxidant activities	**(Martz *et al.* 2004)** **(Kodach *et al.* 2006)** **(Sanchez *et al.* 2006)** **(Mojib *et al.* 2010)**
Prodigiosin produced by *Serratia marcesans*	Antibacterial activity against gram positive bacteria including *Staphlococcus aureus, S. Saprophyticus, B.subtillus*	**Mekhael and Yousuf (2009)**
Green pigment from *Pseudomonas aeruginosa*	Antibacterial activity against Gram negative bacteria such as *E.coli*, *Pseudomonas* sp. And *Rhizobium* sp.	**Mukherjee *et al.* (2012)**
Isolates RS7	DPPH free radical scavenging activity	**Sasidharan *et al.* (2013)**
Pigment from the isolates *Pseudomonas aeruginosa*	Anti-oxidant activity Antibacterial activity	**Masi *et al.* (2014)**
Red pigment of halophile *Serratia marcesans*	Antibacterial activity against *B. Cereus, S. Aureus* and *E.coli* with inhibition zone 12 mm, 07 mm and 6 mm respectively. Antioxidant activity	**Vora *et al.* (2014)**
8 yellow pigmented isolated strains of YCD 3b strain	Higher amount of (78%) DPPH free radical scavenging effect.	**Arulselvi *et al.* (2014)**

Figure 1 Properties of microbial pigments

Thus pigments from bacteria offer the wide range of biologically active properties and continue to provide promising avenues for applied biomedical research. **Prodigiosin – Red Pigment**

Many microorganisms have the unique property of producing a secondary metabolite which is characterized by a unique color. However, most microbial pigments are water insoluble, have poor stability and show different intensities of color at varying pH, temperature and nutrient sources **(Joshi *et al.*, 2003).** Nevertheless, microorganisms have the capability of producing a wide variety of pigments such as carotenoids, melanins, flavins, quinones, prodigiosin, monascins and violacein or indigo. **(Venil and Lakshmanaperumalsamy, 2009).** .

For several decades, *prodigiosin* has been known to be a natural cytotoxic compound of broad range activity as displayed by *Vibrio psychroerythrus, S.marcesans* and *Pseudomonas magnesiorubra* **(Furstner, 2003).**

i. Structure of Prodigiosin:

Secondary metabolites of bacterial origin include various enzymes, pigments, and antibiotics which could be of importance to mankind in many ways **(Giri *et al.*, 2004).**The prodigiosin group of natural products is a family of tripyrrole red pigments that contains a common 4-methoxy, 2-2 bipyrrole ring system. The pigment is a multifaceted secondary metabolite produced by many microorganisms *like Serratia marcesans, Pseudomonas magneslorubra, Vibrio psychroerythrous, S. rubidaea, Vibrio gazogenes, Alteromonas rubra, Rugamonas rubra* and Gram positive actinomycetes, such as *Streptoverticillium rubrireticuli* and *Streptomyces longisporus* ruber form prodigiosin and/or derivatives of this molecule. Each of these organisms produces different derivatives of prodigiosins called as prodiginines **(Khanafari *et al.*, 2006).** Prodigiosin is therefore a typical member of a group of compounds with a common pyrroylpyrro-methene (PPM) skeleton and has a series of close relatives bearing the same PPM core with different alkyl substituents **(Wei and Chen, 2005).**

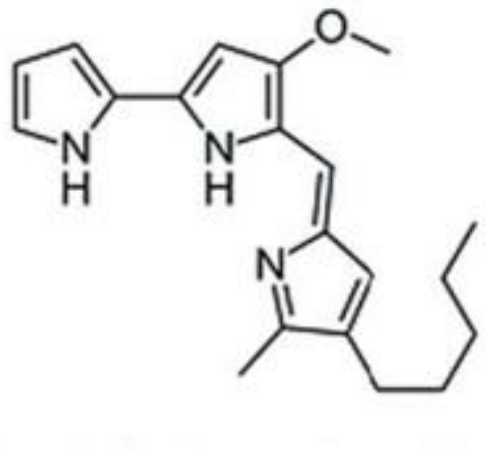

Figure 2 Structure of prodigiosin

ii. Estimation of Prodigiosin:

One of the earlier prodigiosin estimation protocols was established by **Haddix *et al*. (2000)** according to which optical density values at 620 nm (OD620) could be used to measure cell density without interference due to prodigiosin absorbance. According to **Hardjito *et al.* (2002)** cell-bound and extracellular pigment was measured spectrophotometrically at 540 nm, and the total pigment was calculated by adding the cell-bound and extracellular values.

A 5 ml broth was centrifuged at 10,000 rpm for 5 min. The extracellular pigment was determined directly from cell free supernatant at pH 5 by adding hydrochloric acid, while the cell-bound pigment was extracted by adding 5 ml ethanol-chloroform (2:18).

Wei and Chen (2005) proposed a modified method for the estimation of prodigiosin. The culture broth was mixed with an equal volume of 2% (w/v) alum placed in a vial. 4 ml of methanol was added to the vial and the mixture was vigorously vortexed. The solution was then centrifuged at 1200 x g for 10 minutes. A fixed amount (0.8 ml) of the supernatant was further mixed with 0.2 ml of 0.05 N HCl/methanol mixture (4:1 (v/v)). The optical density of the resulting solution was measured at 540 nm. The OD540 was converted to mass concentration via appropriate

calibration using the purified prodigiosin like protein (PLP) product as the standard.

iii. Applications of Prodigiosin:

Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene) is a bacterial metabolite that has anticancer and antimetastatic properties. However, the molecular mechanisms responsible for these abilities are not fully understood. Gene expression profiling of the human breast cancer cell line MCF-7 treated with prodigiosin was analyzed by cDNA array technology. The majority of the significantly modified genes were related to apoptosis, cell cycle, cellular adhesion, or transcription regulation. The dramatic increase of the nonsteroidal anti-inflammatory drug activated gene 1 (NAG-1) made this gene an interesting candidate regarding the possible mechanism by which prodigiosin induces cytotoxicity in MCF-7 cells. **Soto-Cerrato *et al*. (2007)** showed that prodigiosin triggers accumulation of the DNA damage response tumor suppressor protein p53 but that NAG-1 induction was independent of p53 accumulation. Moreover, prodigiosin caused AKT dephosphorylation and glycogen synthase kinase-3B (GSK-3B) activation, which correlated with NAG-1 expression. Prodigiosin-induced apoptosis was recovered by inhibiting GSK-3B, which might be due, at least partly, to the blockade of the GSK- 3B–dependent up-regulation of death receptors 4 and 5 expression. These findings suggest that prodigiosin mediated GSK-3B activation is a key event in regulating the molecular pathways that trigger the apoptosis induced by this anticancer agent.

Park *et al*. (2008) carried out their research on the therapeutic potential of prodigiosins, which further stimulated research into their mechanism of action. Here, a possible relationship between the cytotoxicity of the prodigiosins and their DNA damaging capacity was demonstrated. In the presence of redox-active metal cations, preferably copper^{2+} (Cu (II)), prodigiosins facilitated ss & ds DNA cleavage. These events were thought to have been derived from formation of the π -radical cation at the electron-rich pyrroylpyrro-methene chromophore through interaction with Cu(II)

to yield Cu(I), which fostered reductive-activation of molecular oxygen (O2) to form the superoxide radical anion (O2 •–) and hence hydrogen peroxide (H2O2). The interaction of H2O2 with a Cu-bound prodigiosin species was thought to initiate dsDNA cleavage. Structure- activity relationships demonstrated that replacement of the individual metal-coordinating pyrrole rings by other weaker Cu (II)-ligating arenes resulted in marked loss of nuclease activity and cytotoxicity. Hence their study showed photo-induced cytotoxicity of the natural product prodigiosin along with three structure analogues. The antibacterial activity of prodigiosin (PG) was the result of their ability to pass through the outer membrane and to their capacity for inhibiting target enzymes, such as DNA gyrase and topoisomerase IV, which inhibited the cell growth **(Berlanga and Vinas, 2000)**

CHAPTER-3

MATERIALS AND METHODS

1) Instruments

Table 2 List of instruments used

S.No	Instrument	Manufacturer
1	Analytical Balance	Metller Toledo, JB1603-C/Fact, Switzerland
2	Autoclave	Tony SX-500, UK
3	Bacterial Incubator	Remi, India
4	Centrifuge	Sigma, 3-30K, Germany
5	Hot Air Oven	Perfit, 992/10, India
6	Laminar Air Flow	Labtech LCB 1201V, Daihan Pvt.Lmt,India
7	Micropipette	Tarsons
8	Microscope	Nikon
10	pH Meter	Thermo Scientific, Sn B21899, Singapore
11	Shaking Incubator	Lab tech, germany
12	Spectrophotometer	Shimadzu, Japan
13	Refrigerator	Godrej

2) Media, Chemicals and Glass wares

Table 3 List of chemicals used

S.No.	Chemical	Company
1	Nutrient agar	HiMedia
2	Starch	Fischer scientific
3	Glucose	Fischer scientific
4	Sucrose	Fischer scientific
5	Glycerol	Fischer scientific
7	Urea	HiMedia
8	Peptone	HiMedia
9	Sodium Chloride	Finar reagent
10	Beef Extract	HiMedia
11	Malt Extract	HiMedia
12	Yeast Extract	HiMedia
13	Agar Agar	HiMedia

All the chemicals and reagents were of molecular biology and analytical grade.

All the glassware used was washed properly with detergent, rinsed with distilled water and autoclaved prior to use.

1) Enumeration of Pigment Producing Bacteria in Maintenance Media

The microbial culture used in the study was procured from NCIM, Pune. Nutrient agar was used for the revival and preculture of *Serratia nematodiphila* from a freeze dried stock.

Nutrient agar (NA)

Table 4 Composition of nutrient agar

Component	Amount
Beef extract	3 g
Peptone	5 g
NaCl	5 g
Agar	20 g
Distilled water	1000 ml
pH	6.5

2) Morphological characterization & Biochemical characterization

The selected pigment producing bacterial isolates were marked on the basis of their morphological and biochemical characteristics and the results are presented in table 4. Gram's staining reaction for pigment producing bacterial isolates was performed to check the gram's reaction and shape of bacterial isolates. The gram's reaction of the bacterial isolates was determined by light microscopy after gram staining.

Oxidase Test, Catalase Test, Indole Test, Methyl red Test, Voges Proskaur Test, Citrate Test, Urease Test and H2S Test were done.

3) Optimization of Cultural Conditions for Maximum Pigment Production

i. Optimization of Incubation Period

The screened pigment producing bacterial isolates were grown in nutrient agar medium (NA) for different incubation period viz. 24, 36, 48, 96 and 120h and

incubated at 37±2oC for maximum pigment production.

ii. Optimization Of pH

The pH of the selected media was adjusted ranging from 4 to 8 with the help of pH meter. The autoclaved liquid media was inoculated with the pigmented bacterial isolates and incubated at 37±2oC.

iii. Optimization of Incubation Temperature

In order to determine the optimum temperature for maximum pigment production, the selected pH of the media was adjusted and inoculated with pigmented bacterial isolates. The flasks were then incubated at different temperatures ranging from 25oC to 40oC with the interval of 5oC.

iv. Effect of Carbon Sources

Different carbon sources namely glucose, glycerol, sucrose and starch each at the rate of 1.0 per cent were added in the media and their effect on the growth and pigment production was studied after incubation

v. Effect of Nitrogen Sources

Different nitrogen sources viz., Peptone, ammonium sulfate, ammonium nitrate and ammonium chloride were added at the rate 1.0 per cent in the selected media and their effect was studied on growth and pigment production after incubation

4) Solid State Fermentation

SSF was carried out by using agro industrial waste viz. wheat bran husk, rice bran husk, green gram husk and orange peel powder. 20g of each substrate was mixed with 20ml of trace metal solution, pH maintained at 7 and incubated at 28Oc.

Table 5 Composition of TMS

Metal	Concentration (mM)
Calcium chloride dihydride	60 mM
Ferrous sulphate heptahydride	1.5 mM
Magnesium sulphate heptahydride	2.5 mM
Manganese sulphate monohydride	9 Mm
Copper sulphate pentahydride	1.5 mM

5) Identification and Characterization of Pigment

i. Identification of the Pigment by TLC

Thin layer chromatography is based on the principle of distribution coefficient. The stationary phase is coated as a thin layer on a glass or metal foil plate. The test sample is applied as a spot or band near one end of the plate that is then placed in a reservoir of mobile phase that is allowed to pass over the plate. The analyte rises by capillary action along with the mobile phase.

ii. Quantification of the Pigment by Spectral Analysis

1. Supernatant of a culture broth (0.5 ml) was mixed with an equal volume of 2% (w/v) alum in a vial.
2. Four milliliters of methanol was added to the vial
3. Mixture was vigorously shaken.
4. The solution was then centrifuged at 1200× g for 10 min.
5. The supernatant was filtered through a 0.45 m filter
6. The clear supernatant was used for the identification of extracted pigment by scanning at different wavelength region from 200-1000 nm at 50 nm interval

under UV Visible spectrophotometer.

6) Statistical Analysis

The data obtained was subjected to appropriate statistical analysis as per the requirement of the experiment.

CHAPTER-4

RESULTS AND DISCUSSION

1) Enumeration of Pigment Producing Bacteria in Maintenance Media

The culture of *Serration nematodiphila* obtained from NCIM pune was grown on nutrient agar medium at 28oC. The colonies obtained were red in color and distinct. Biochemical tests were conducted of the culture thus obtained.

The same culture was maintained on nutrient broth at 28oC. A deep red colored broth was obtained after incubation of 2-3 days.

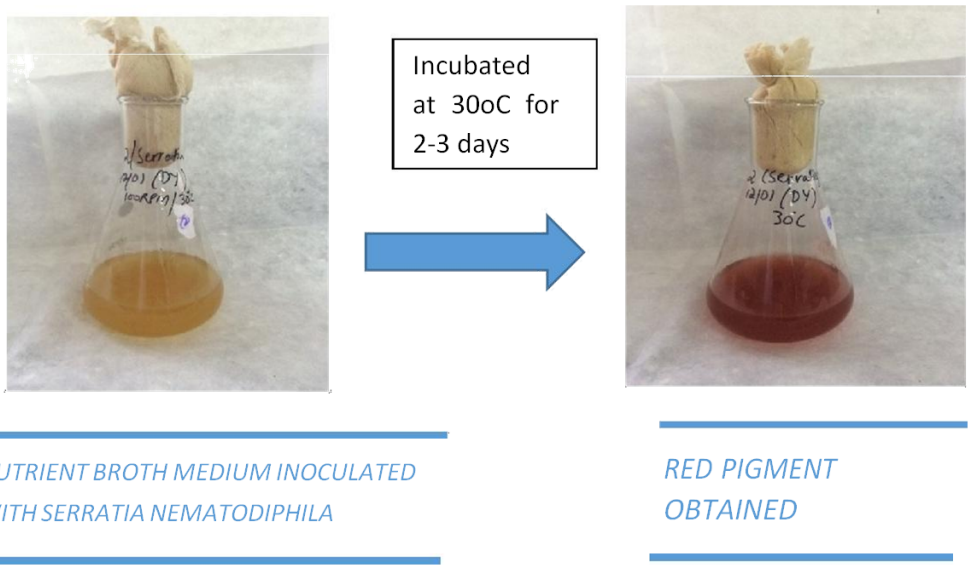

Figure 3 Pigment production in nutrient broth

2) Morphological and Biochemical Characterization

The isolates were identified by studying morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1989). Detailed morphological and biochemical tests of the isolates are given in Tables:

Table 6 : Morphological characteristics of Serratia nematodiphila

Morphological characteristics of Serratia nematodiphila							
Culture isolate	Configuration	Elevation	Surface	Pigment	Opacity	Gram Staining	Cell shape
A	Round	Convex	Smooth	Red	Opaque	Gram positive	Rod

Table 7: Biochemical characteristics of Serratia nematodiphila

Biochemical characteristics of Serratia nematodiphila	
Oxidase Test	Negative
Catalase Test	Positive
Indole Test	Negative
Methyl red Test	Negative
Voges Proskaur Test	Positive
Citrate Test	Positive
Urease Test	Negative
H2S Test	Negative

3) Effect of incubation time on growth and prodigiosin production

The pattern of growth of *Serratia nematidophila* showed a steady increase and the prodigiosin production was also enhanced with increasing incubation time at 28˚C at pH 7.0 grown on nutrient agar media. The isolate were incubated separately at 24, 48, 72 hours respectively. Grew rapidly in the first 48-72 hours. The pigment production was initially negligible since it is a secondary metabolite but increased considerably over the next day.

Table 8 : Effect of Incubation Time

Incubation time at 28˚C at pH 7.0 (hrs)	OD540		
24	0.004	0.004	0.002
48	0.261	0.264	0.262
72	0.423	0.422	0.422
76	0.424	0.423	0.422

4) Effect of incubation temperature

The biosynthesis of a pigment is significantly affected by the physiological parameter, temperature **(Hejazi and Falkiner, 1997).** In order to determine the effect of temperature on the production of prodigiosin by *Serratia*, the cells were incubated at 25, 30, 35, 40 and 45˚C, respectively. The incubation time was kept at the optimum 48-72 hrs for isolate from the above data obtained from the above parameter. Incubation temperature was found to be a critical parameter as it affected the growth as well as pigment production.

5) Effect of pH

The growth and type of pigment produced by an organism is affected largely by the pH of the medium in which the microorganism is grown. Slight changes in pH can also alter the shade of colour produced **(Joshi *et al.*, 2003).** The influence of pH on the production of prodigiosin was studied at different pH values ranging from 4 to 8. Shows that maximum production of prodigiosin occurred at pH 7. However, the acidic and the basic pH showed the lowest synthesis of the pigment.

Figure 4 : Effect of pH on pigment at temperature 30oC

Table 9: Effect of pH at temperature 30oC

At temperature 30oC				
SAMPLE	pH	OD_{540}		
A2	pH 4	0.119	0.118	0.119
A4	pH 5	0.226	0.228	0.227
A3	pH 6	0.301	0.303	0.301
A5	pH 7	0.522	0.524	0.521
B8	pH 8	0.310	0.310	0.311

Figure 5 Effect of pH on pigment at temperature 45oC

Table 10 : Effect of pH at temperature 45-C

At temperature 45oC				
SAMPLE	**pH**	**OD540**		
A6	pH 4	0.002	0.001	0.001
A1	pH 5	0.004	0.002	0.004
B6	pH 6	0.011	0.010	0.009
A7	pH 7	0.020	0.018	0.020
B7	pH 8	0.003	0.001	0.001

6) **Effect of carbon source**

The use of alternate sources of carbon is important for the synthesis of the pigment since it can not only produce a variety of colors, but can also influence the production. Various carbon sources such as glucose, glycerol, sucrose and starch, at 0.5% were evaluated for their contribution towards pigment production by maintaining all the above physical parameters to a constant level.

When glucose was used in the media, the level of prodigiosin produced was relatively reduced when compared to previous levels of production. According to **Haddix and Werner (2000),** glucose reduced pigment production to nearly half when compared to other carbon sources. The same result was obtained in the present study. Glucose addition causes catabolic repression resulting in reduced prodigiosin production **(Khanafari *et al.*, 2006).** The use of glycerol as a source of carbon showed substantial increase production of pigment when compared to glucose. Sucrose and starch produced faint colored pigments.

Figure 6 Effect of carbon source on pigment production

Table 11 Effect of carbon source on pigment production

Carbon sources				
SAMPLE	**SOURCE**	**OD_{540}**		
01	Starch	0.332	0.331	0.332
02	Dextrose	0.241	0.242	0.242
03	Sucrose	0.332	0.332	0.331
04	Glycerol	0.401	0.401	0.403

7) **Effect of nitrogen source**

Four different nitrogen sources were used to study their effect on prodigiosin production. Peptone, ammonium sulfate, ammonium nitrate and ammonium chloride were used as nitrogen sources separately in each trial at concentration of 0.5% in each media preparation.

Ammonium sulfate, ammonium nitrate and ammonium chloride gave low to negligible quantities of prodigiosin. Except for peptone, all the rest are inorganic sources of nitrogen lacking in the rich supplements of vitamins, minerals and other proteinaceous nutrients present in peptone. Highest production of prodigiosin was seen when peptone was used as a nitrogen source.

Figure 7 Effect of nitrogen source on pigment production

Table 12 Effect of nitrogen source on pigment production

Nitrogen sources				
SAMPLE	**SOURCE**	**OD_{540}**		
01	Ammonium sulfate	0.151	0.151	0.152
02	Ammonium nitrate	0.266	0.264	0.265
03	Ammonium chloride	0.154	0.152	0.152
04	Peptone	0.881	0.881	0.882

8) Taguchi

The carbon and nitrogen sources were optimized, establishing that the best results were obtained when glycerol and peptone are taken as carbon and nitrogen sources, respectively. So, taguchi DOE trials were set up, the results of which have been tabulated (Table 13).

According to the results obtained, it can be clearly established that 30oC is the optimum temperature for pigment production, when the glycerol and peptone at 1.5%

and 2%, respectively, are used.

Table 13 Design of experiment of Taguchi

S.No.	Temp	Glycerol	Peptone	OD_{540}	Conc. (Mm)	Amount of pigment (g)
1	25	0.5	0.5	0.301	2.74	0.886
2	30	0.5	1.0	0.403	3.68	1.190
3	35	0.5	1.5	0.152	1.38	0.446
4	40	0.5	2.0	0.106	0.96	0.310
5	30	1.0	0.5	0.5.0	4.58	1.481
6	25	1.0	1.0	0.332	3.03	0.971
7	40	1.0	1.5	0.009	0.08	0.025
8	35	1.0	2.0	0.192	1.75	0.565
9	35	1.5	0.5	0.123	1.12	0..362
10	40	1.5	1.0	0.013	0.11	0.035
11	25	1.5	1.5	0.318	2.90	0.937
12	30	1.5	2.0	0.522	5.04	1.629
13	40	2.0	0.5	0..11	0.10	0.032
14	35	1.5	1.0	0.137	1.25	0.404
15	30	1.5	1.5	0.482	4.40	1.306
16	25	1.5	2.0	0.336	3.07	0.992

9) Solid state fermentation

Deep red color was obtained on growth of bacteria on the green gram husk after 24 hrs of incubation at 28oC. Light red color was also seen on the wheat bran husk. However, no significant color was obtained on the rice bran husk and the orange peel powder.

The production of deep red color after only 24 hrs of incubation can be attributed to the supplementation with the trace metal solution, which clearly enhanced the pigment production.

10) TLC

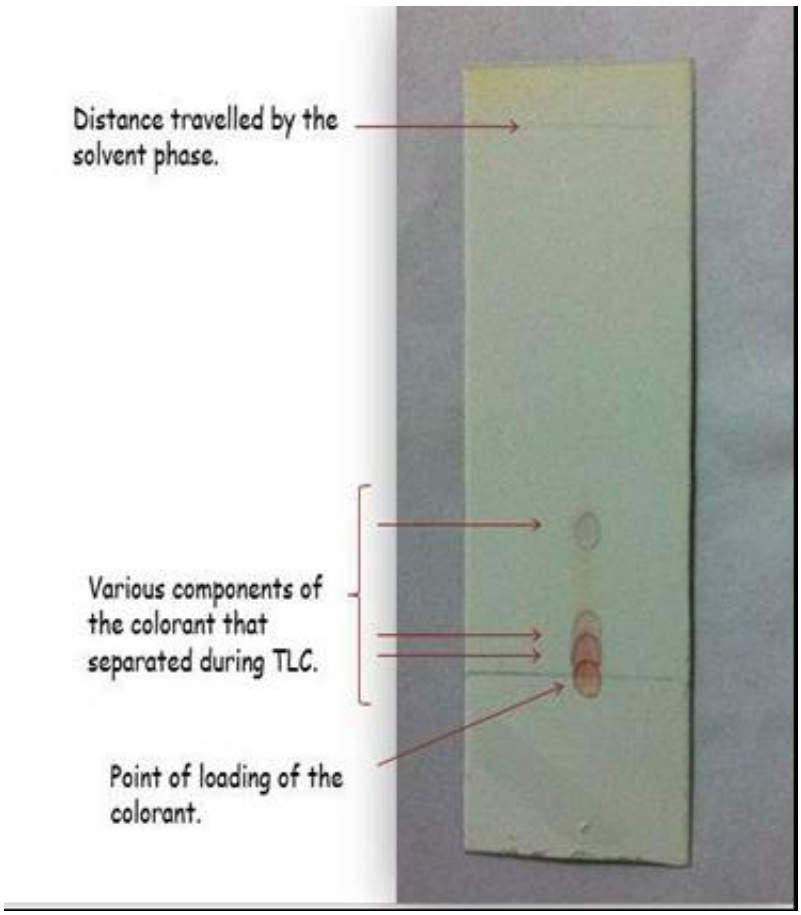

Figure 8 TLC plate of the colorant

Table 14 : Identification of pigment by TLC

Bacterial isolate	Rf value	Rf value per literature	Compound	Reference
A	0.81	0.83	PRODIGIOSIN	Latha and Jeevarthnam, 2010

11) Spectral analysis

The identification of extracted pigment was done by scanning at different wavelength region from 200-700 nm under UV Visible spectrophotometer. Maximum absorption was shown at 536nm, which resembles the absorption maxima of prodigiosin pigment.

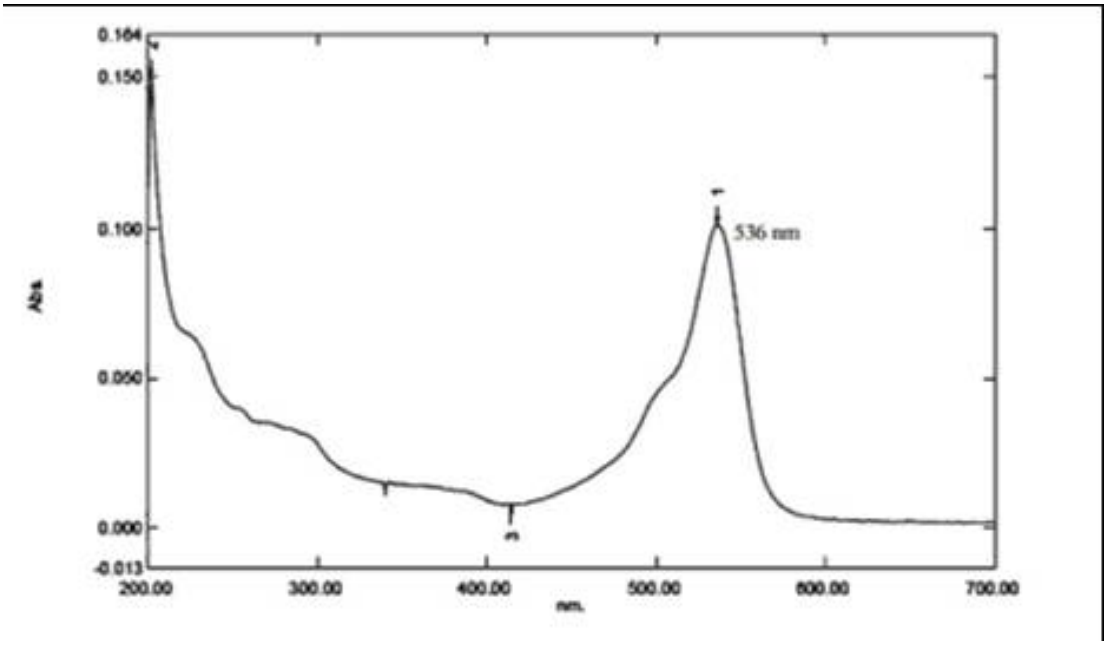

Table 15 Spectral analysis of crude pigment

12) Statistical analysis

i. **Qualitek-4**

The table number 15 to 21 represent qualitek-4, which automatically designs experiments based on user-indicated factors and levels. The program performs the three basic steps in analysis i.e. main effect, analysis of variance and optimum studies.

The interaction severity index indicates the interaction amongst the different variables and their effect on the pigment production. Interaction among glycerol and peptone exert the maximum effect, folled by that between glycerol and temperature. Interaction between peptone and temperature has comparatively less effect.

The most significant factor is shown to be the temperature, followed by the carbon source, glycerol, while the nitrogen source i.e. peptone has the least effect amongst the three factors.

ANOVA determines the significance and adequacy of the model. The analysis of variance in the present study demonstrated that the model is highly significant, with the error being only 0.002%

Table number 20 gives the optimum condition of each factor. For maximum pigment production, glycerol at 2%, peptone at 0.5% and temperature of 250C is to be taken, according to the statistical analysis based on trials and factors.

The variance reduction plot captures a comparative performance status of the system under investigation, that is maximum pigment production. It provides an improved shape of the distribution and the resulting savings expected.

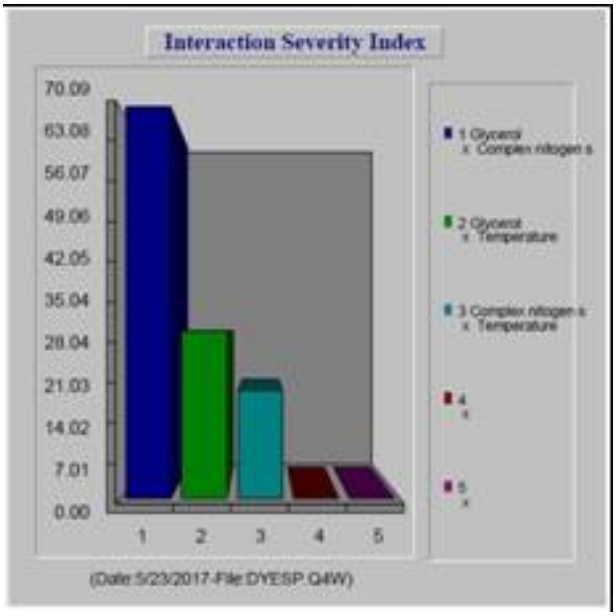

Table 16 Interaction severity index

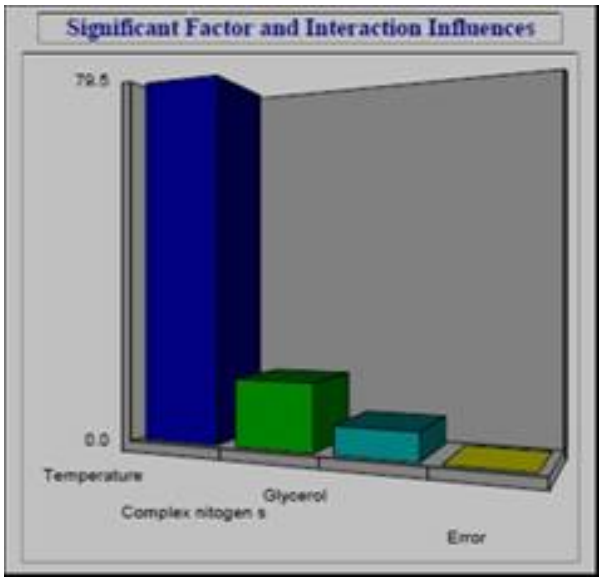

Table 17 Significant factors and interaction influences

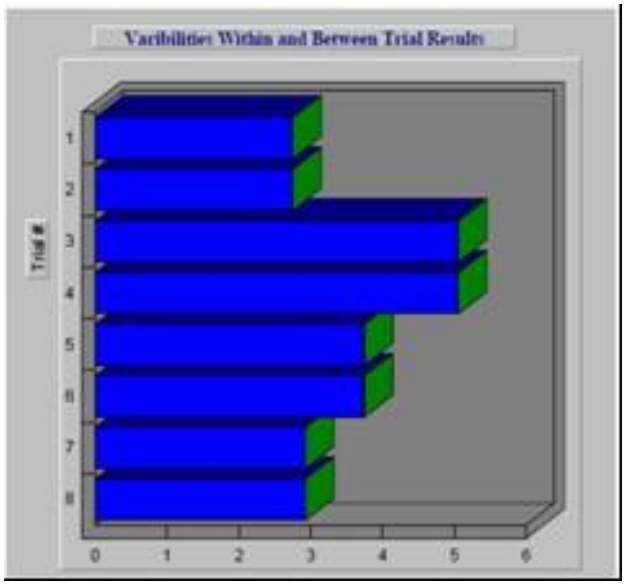

Table 18 Variabilities within and between trials

Analysis Of Variance (ANOVA)

	Factors	DOF	Sums Of Squares	Variance	F-Ratio	Pure Sum	Percent
1	Glycerol	1	2.094	2.094	83,762.715	2.094	6.030
2	Complex nitogen s	1	5.025	5.025	201,013.079	5.025	14.472
3	Temperature	1	27.604	27.6041	104,167.249	27.604	79.496
	Other/Error	4	-0.001	-0.001			0.002
	Total:	7	34.723				100.000%

Table 19 Analysis of variance

Optimum Condition and Performance

	Factors	Level Desc.	Level	Contribution
1	Glycerol	2.0	2	0.511
2	Complex nitogen s	0.5	1	0.792
3	Temperature	25	1	1.857

Total Contribution From All Factors...	3.160
Current Grand Average Of Performance...	-10.905
Expected Result At Optimum Condition...	-7.745

Table 20 Optimum conditions of the factors

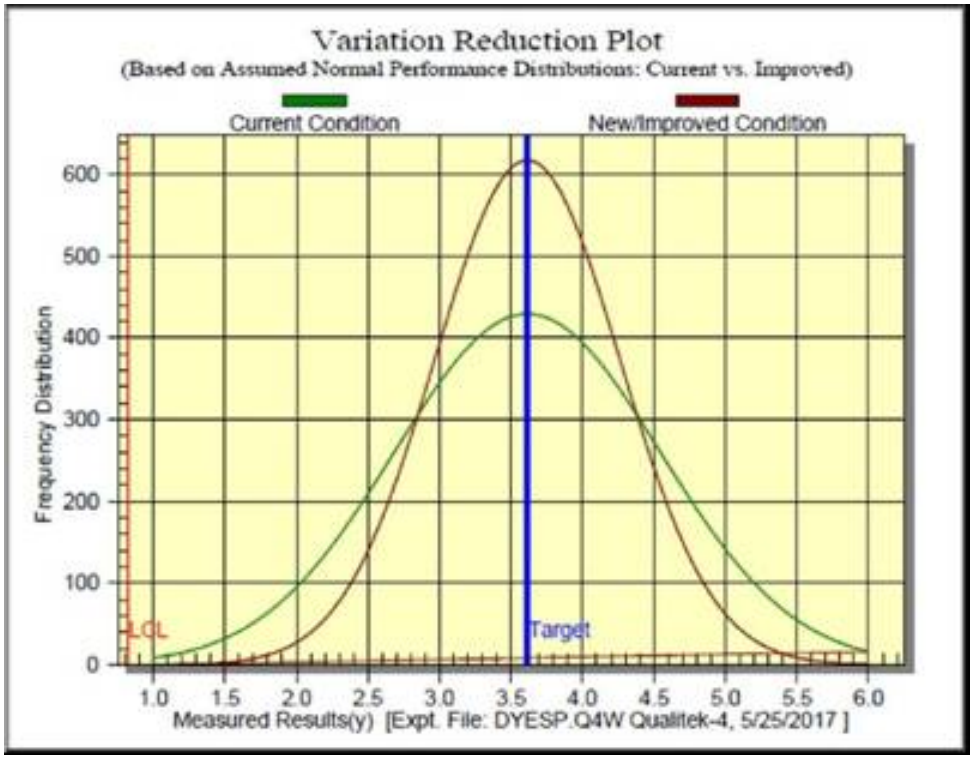

Table 21 variance reduction plot

CHAPTER-5

SUMMARY AND CONCLUSION

Bacterial pigments have economic potential and industrial importance offering opportunities for application in textile, food, pharmaceuticals, cosmetics etc. But their current volume of production still has not attained the optimum level to meet the demand aroused due to the recent awareness for natural products. The current novel strategies like genetic engineering, molecular biology techniques and fermentation technologies are greatly contributing to higher production of bacterial pigments. For cost-competitive and higher production of bacterial pigments, these current processes of screening of new pigmented bacteria should continue in order to support the discovery and application of novel bacterial pigments that possesses high activities and useful properties from less expensive sources.

The pigment yield by *Serratia nematodiphila* was observed to be regulated mainly by unconventional carbon source such as glycerol and sucrose while the most common carbon source i.e. glucose showed little impact on pigment production. Maximum increase in pigment yield was noticed with glycerol supplementation. The nitrogen source also plays an important role in pigment production. Inorganic nitrogen sources had negligible effect while peptone showed maximum pigment production. Incubation temperature is the most prominent physiological factor which regulated the pigmentation yield, with maximum pigment obtained at 30oC. Over all the combination these three factors resulted in improving the pigmentation yield, indicating the imperative role of growth parameters and their concentrations in regulation of metabolically mediated pigment production in this isolate.

SSF was also successful in pigment production, especially while using green gram husk, which gave maximum pigment production. However, submerged fermentation was preferred over solid state fermentation due to greater ease of handling of microorganisms in submerged fermentation as compared to SSF. Also, the extraction and quantification of pigment is easier in submerged fermentation

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