

BIOPROSPECTING OF PECTINASE ENZYMES FROM FUNGI

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CONTENTS

Sl. No.	Chapter Particulars
	CERTIFICATE
	ACKNOWLEDGEMENT
	LIST OF TABLES
	LIST OF FIGURES
	LIST OF PLATES
	LIST OF APPENDICES
	LIST OF ABBREVIATIONS
1.	INTRODUCTION
2.	REVIEW OF LITERATURE
	2.1 Microbial enzyme biotechnology: Tool of the age
	2.2 Isolation, screening and identification of pectinolytic fungi from different sources
	2.3 Purification and characterization of pectinase enzyme
	2.4 Application of fungal pectinase in fruit juice extraction and clarification
3.	MATERIAL AND METHODS
	3.1 Collection of samples of fruits and vegetable wastes
	3.2 Isolation and primary screening of pectinolytic fungi
	3.3 Optimization of culture conditions for maximum enzyme production under submerged condition for the most efficient fungal isolates
	3.4 Screening of agro wastes for maximum enzyme production under solid state fermentation (SSF) condition
	3.5 Application of pectinase in orange and guava fruit juice clarification
	3.6 Statistical analysis
4.	EXPERIMENTAL RESULTS
	4.1 Isolation of pectinolytic fungi
	4.2 Solid State Fermentation (SSF) studies for optimization of culture parameters for maximum pectinase production
	4.3 Ammonium sulfate precipitation of the crude pectinase enzyme
	4.4 Extraction of orange juice using pectinase enzyme
5.	DISCUSSION
	5.1 Isolation, screening and identification of pectinolytic fungi
	5.2 Screening of agro wastes for pectinase production under solid state fermentation (SSF)
	5.3 Purification of pectinolytic enzymes
	5.4 Application of pectinase enzyme
6.	SUMMARY AND CONCLUSIONS
	REFERENCES
	APPENDICES

LIST OF TABLES

Table No.	Title
1.	An extensive classification of pectinolytic enzymes
2.	Biochemical and physicochemical properties of pectinases
3.	Pectin yield and calcium pectate content in different substrates
4.	List of fungi isolated from different fruit and vegetable wastes on pectin agar medium
5.	Pectinase potency index values of the fungal isolates
6.	Pectinolytic profile of the pectinase positive fungal isolates
7.	Tentative identification of the pectinase positive fungal isolates based on colony morphology, growth characters and microscopic observations
8.	ITS rDNA partial sequences of the potent pectinolytic fungal isolates
9.	<i>In silico</i> BLAST analysis of the ITS rDNA sequences of potent pectinolytic fungal isolates for molecular identification
10.	Effect of inoculum size on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under submerged fermentation
11.	Influence of inoculum size on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under submerged fermentation
12.	Effect of inoculum size on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under submerged fermentation
13.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under submerged fermentation as influenced by different pH levels
14.	Effect of pH on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under submerged fermentation
15.	Influence of pH on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under submerged fermentation
16.	Influence of temperature on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under submerged fermentation
17.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under submerged fermentation as influenced by temperature
18.	Influence of temperature on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under submerged fermentation
19.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under submerged fermentation at different substrate concentrations
20.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under submerged fermentation at different substrate concentrations
21.	Pectin esterase production by <i>Aspergillus oryzae</i> RC27 under submerged fermentation at different substrate concentrations

Contd....

Table No.	Title
22.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under submerged fermentation at different incubation periods
23.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under submerged fermentation at different incubation periods
24.	Pectin esterase production by <i>Aspergillus oryzae</i> RC27 under submerged fermentation at different incubation periods
25.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation at different moisture content of substrates
26.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation at different moisture content of substrates
27.	Pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation at different moisture content of substrates
28.	Effect of substrate particle size on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
29.	Effect of substrate particle size on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation
30.	Effect of substrate particle size on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
31.	Influence of inoculum size on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
32.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation as influenced by different inoculum sizes
33.	Effect of inoculum size on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
34.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation during different incubation periods
35.	Pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation during different incubation periods
36.	Pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation during different incubation periods
37.	Influence of pH on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
38.	Effect of pH on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation
39.	Effect of pH on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
40.	Influence of temperature on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
41.	Effect of temperature on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation

Contd....

Table No.	Title
42.	Influence of temperature on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
43.	Effect of nitrogen sources on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
44.	Influence of nitrogen sources on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation
45.	Effect of nitrogen sources on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
46.	Polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation as influenced by different carbon sources
47.	Influence of carbon sources on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation
48.	Effect of carbon sources on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
49.	Effect of mineral salts on polygalacturonase production by <i>Aspergillus niger</i> RBF96 under solid state fermentation
50.	Effect of mineral salts on pectin lyase production by <i>Penicillium citrinum</i> DLP51 under solid state fermentation
51.	Effect of mineral salts on pectin esterase production by <i>Aspergillus oryzae</i> RC27 under solid state fermentation
52.	Protein recovery in the pectinase crude extract of different fungi as influenced by different concentrations of ammonium sulfate
53.	Purification of polygalacturonase produced by <i>Aspergillus niger</i> RBF96
54.	Purification of pectin lyase produced by <i>Penicillium citrinum</i> DLP51
55.	Purification of pectin esterase produced by <i>Aspergillus oryzae</i> RC27
56.	Optimization of temperature for maximum polygalacturonase activity
57.	Optimization of temperature for maximum pectin lyase activity
58.	Optimization of temperature for maximum pectin esterase activity
59.	Optimization of pH for maximum polygalacturonase activity
60.	Optimization of pH for maximum pectin lyase activity
61.	Optimization of pH for maximum pectin esterase activity
62.	Extraction of orange and guava juice using pectinase enzyme
63.	Physico-chemical characteristics of orange and guava juice treated with pectinase enzyme
64.	Sensory attributes of orange and guava juice treated with pectinase enzyme

LIST OF FIGURES

Figure No.	Title
1.	Schematic representation of the conventional (A) and recently proposed alternative (B) structure of pectin
2.	Interaction through insertion of Ca^{2+} ions between the unesterified carboxyl groups of the galacturonosyl residues of two Homogalacturonan (HG) chains
3.	Mode of action of pectinases
4.	Potency index values of different pectinolytic fungal isolates
5.	Polygalacturonase activity of pectinolytic fungal isolates
6.	Pectin lyase activity of pectinolytic fungal isolates
7.	Pectin esterase activity of pectinolytic fungal isolates
8.	A linearized phylogenetic tree showing evolutionary relationships of different closely related fungal taxa
9.	Optimization of temperature for maximum polygalacturonase activity
10.	Optimization of temperature for maximum pectin lyase activity
11.	Optimization of temperature for maximum pectin esterase activity
12.	Optimization of pH for maximum polygalacturonase activity
13.	Optimization of pH for maximum pectin lyase activity
14.	Optimization of pH for maximum pectin esterase activity
15.	Lineweaver-Burk plot for polygalacturonase from <i>Aspergillus niger</i> RBF96 showing Michaelis-Menten kinetics
16.	Lineweaver-Burk plot for pectin lyase from <i>Penicillium citrinum</i> DLP 51 showing Michaelis-Menten kinetics
17.	Lineweaver-Burk plot for pectin esterase from <i>Aspergillus oryzae</i> RC27 showing Michaelis-Menten kinetics
18.	Lineweaver-Burk plot for polygalacturonase from <i>Aspergillus niger</i> MTCC1344 showing Michaelis-Menten kinetics
19.	Lineweaver-Burk plot for pectin lyase from <i>Aspergillus niger</i> MTCC1344 showing Michaelis-Menten kinetics
20.	Lineweaver-Burk plot for pectin esterase from <i>Aspergillus niger</i> MTCC1344 showing Michaelis-Menten kinetics

LIST OF PLATES

Plate No.	Title
1.	Fruit and Vegetable waste samples used for isolation of pectinase fungi by enrichment culture technique
2.	Preparation of agro wastes for solid state fermentation experiment
3.	Solid state fermentation experimental set up
4.	Occurrence of fungus on pectin agar medium from different fruit and vegetable waste samples
5.	Qualitative plate assay of fungal isolates showing the clear zone formation on pectin agar medium
6.	Microphotographs of pectinolytic fungal isolates (40 X)
7.	PCR amplification of DNA from pectinolytic fungal isolates with primers ITS1 and ITS4
8.	Colony characteristics and microscopic features of potent pectinolytic fungal isolates
9.	Various steps in purification of pectinase enzymes
10.	SDS-PAGE for determination of molecular weight of pectinase enzymes
11.	Orange and guava fruit juices extraction and clarification using pectinase enzymes

LIST OF APPENDICES

Appendix No.	Title
I.	Pectin agar medium
II.	Composition of Czapek yeast extract agar
III.	50 mM Iodine-potassium iodide solution
IV.	Polygalacturonase assay
V.	Pectin Lyase Assay
VI.	Pectin esterase activity assay
VII.	Total protein estimation by Lowry's method
VIII.	Lactophenol Aniline Blue stain
IX.	CTAB extraction buffer for fungal DNA isolation
X.	Estimation of pectin as calcium pectate
XI.	Amounts of solid ammonium sulfate required to change the per cent saturation of crude enzyme solution
XII.	Gel filtration chromatography
XIII.	Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS PAGE)
XIV.	$V_{\max}$ and K_m of the Michaelis-Menten equation
XV.	Score card for sensory evaluation of orange and guava juice

LIST OF ABBREVIATIONS

@	- At the rate of
≈	- Approximately
°B	- Degree Brix
°C	- Degree Celsius
ATCC	- American Type Culture Collection
a _w	- Water activity
BLAST	- Basic Local Alignment Search Tool
CaCl ₂ .2H ₂ O	- Calcium chloride dehydrate
CFTRI	- Central food technological research institute
cm	- Centimetre
CO ₂	- Carbon dioxide
CRD	- Complete randomized design
CTAB	- Cetyl trimethyl ammonium bromide
CuSO ₄ .5H ₂ O	- Copper sulphate penta hydrate
DNA	- Deoxyribonucleic acid
e.g.	- Example
EC	- Enzyme Commission
EDTA	- Ethylene diamine trichloro acetate
FeCl ₃ .6H ₂ O	- Ferric Chloride Hexahydrate
FeSO ₄	- Ferrous sulphate
Fig.	- Figure
g	- Gram
gds	- Gram dry substrate
GRAS	- Generally regarded as safe
h	- Hour(s)
HCl	- Hydrochloric acid
HPLC	- High performance liquid chromatography
IMTECH	- Institute of Microbial Technology
ITS	- Internally transcribed spacer
K ₂ HPO ₄	- Dipotassium Hydrogen Phosphate
KDa	- Kilo Dalton
Kg	- Kilograms
Km	- Michaelis constant
L	- Litre
Lb	- Pound
M	- Molar
M.W	- Molecular weight
Mg	- Magnesium
mg	- Milligram
MgSO ₄ .7H ₂ O	- Magnesium Sulphate Heptahydrate
Min	- Minute(s)
mL	- Millilitre
Mm	- Milli metre
mM	- Milli molar
MnCl ₂	- Manganese chloride
MPa	- Mega Pascal
MS	- Mass Spectrometry
MTCC	- Microbial Type Culture Collection
N	- Normality

NaCl	- Sodium chloride
NaOH	- Sodium hydroxide
NCBI	- National Centre for Biotechnology Information
NCIM	- National Collection of Industrial Microorganisms
NH ₄ Cl	- Ammonium chloride
NH ₄ SO ₄	- Ammonium sulphate
nm	- Nano meter
PCR	- Polymerase Chain Reaction
PE	- Pectin esterase
PG	- Polygalacturonase
PGA	- Polygalacturonic acid
pI	- Isoelectric Point
PL	- Pectin lyase
ppm	- Parts per million
rpm	- revolutions per minute
s	- Seconds
SDS-PAGE	- Sodium Dodecyl Sulphate Poly Acrylamide gel electrophoresis
SmF	- Submerged fermentation
Sp.	- Species
SSF	- Solid state fermentation
TSS	- Total soluble Solids
U	- Units (enzyme activity)
U/mL	- Units per millilitre
Ugds ⁻¹	- Units per gram dry substrate
USFDA	- United States Food and Drug Administration
V	- Volt
V/v	- Volume/volume
Viz	- Namely
Vol	- Volume
W/v	- Weight/volume
ZnSO ₄ ·7H ₂ O	- Zinc sulphate Heptahydrate
α	- Alpha
β	- Beta
δ	- Delta
μ	- Micro
μl	- Microlitre(s)
μmole	- Micromole
γ	- Gamma

INTRODUCTION

In nature, microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the years. Pectinases from microorganisms are of great significance with tremendous potential to offer in juice and food industry (Kashyap *et al.*, 2001). Pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances, present mostly in plants. Chemically, pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1→4) linkage. The side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose. Pectin in native form is located in the cell wall and may be interlined with other structural polysaccharides and proteins to form insoluble protopectin. In an unripe fruit, pectin is bound to cellulose microfibrils in the cell wall. Such pectin is insoluble and hence confers rigidity on cell walls. Pectic substances account for about 4 per cent of the weight of fresh material. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity of the pulp particles, whereas other pectin molecules remain bound to cellulose fibrils by means of side chains of hemicellulose and, thus, facilitate water retention (Pedrolli *et al.*, 2009).

It has been reported that microbial pectinases account for 25 per cent of the global food enzymes sales. Most of the commercial preparations of pectinases are produced from fungal sources. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Jayani *et al.*, 2005). Fungi such as *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium expansum*, *Penicillium restrictum*, *Trichoderma viridae*, *Mucor piriformis* and *Yarrowia lipolytica* etc, which are generally regarded as safe (GRAS) by United States Food and Drugs Administration (USFDA) are employed for pectinase production (Meyer, 2008).

Pectinases can be produced by both submerged and solid state fermentation (SSF). Submerged fermentation (SmF) is the cultivation of microorganisms in liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. Solid-state fermentation has been defined as the fermentation process which involves a solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms. SSF incorporates microbial growth and product formation on or within particles of a solid substrate under aerobic conditions, and does not generally require aseptic conditions for enzyme production.

The potential of SSF lies in bringing the cultivated microorganism in close vicinity of the substrate and achieving the highest substrate concentration for the fermentation (Singhania *et al.*, 2009). SSF resembles the natural habitat of microorganisms and is, therefore, a preferred choice for microorganisms to grow and produce useful value added products. SSF reproduces the natural microbiological processes like composting and ensiling. On one hand, by utilizing the low cost agricultural residues, SSF adds on to economic feasibility of the process, and on other hand, it solves the problem of its disposal which otherwise cause's pollution.

The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues, the major components of which are cellulose, starch, pectin, lignin and xylan. These materials have attracted considerable attention as an alternative feedstock and energy source, since they are abundantly available. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches (Pedrolli *et al.*, 2009). Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used are sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, grapevine trimmings, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc. Wheat bran, however, holds the key, and has most commonly been used, in various processes. And, the use of by-products from food industries is of great interest because of its economic profitability, since residues are available in large quantities and may be cheaper (Pandey, 1994).

Pectinolytic enzymes are of prime importance for plants as they help in cell wall extension and softening of some plant tissues during maturation and storage. They also aid in maintaining ecological balance by causing decomposition and recycling of waste plant materials. These macerating enzymes play a key role in food biotechnology and their demand is likely to increase for extraction of juice from an array of fruits and vegetables. In wine industry, pectinases are mainly used for decreasing astringency by solubilizing anthocyanins without leaching out procyanidin polyphenols, and pectinases have also been shown to increase pigmentation by extracting more anthocyanins (Tucker and Woods, 1991). Pectic substances are responsible for the consistency, turbidity and appearance of fruit juices. In fact, the presence of pectic substances in fruit juices that suspend toward insoluble fruit pulp particles leads to problems in the clarification of fruit juices (Grassin and Fauquembergue, 1996). Mechanical crushing of pectin-rich fruit yields a fruit juice with high viscosity, which remains bound to the pulp in the form of a jellified mass. It is difficult to extract this juice by pressing or using other mechanical methods. Pectinases have been used in the extraction and clarification of a number of fruit and vegetable juices (Galante *et al.*, 1998).

Use of macerating enzymes increases both yield and process performance of fruit juices without additional capital investment. The cocktail is generally used in two steps: in the first instance, it is added during crushing to macerate the fruit pulp either to partial or complete liquefaction, which not only increases the juice yield and reduces the processing time, but also improves the extraction of valuable fruit components. Secondly, after the juice extraction, they are used for clarification, thereby lowering the viscosity of fruit juice prior to concentration, thus, increasing the filtration rate and stability of the final product.

Pectinases are an integral part of fruit processing industry and in the ever growing field of biotechnology. Presently available commercial preparations of pectic enzymes are obtained from many filamentous fungi which are not very efficient. Thus, there is a pressing need to explore novel sources of pectinolytic enzymes.

Selection of efficient strains, isolated from the natural environment, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Once an efficient strain is selected, its fermentation condition needs to be optimized. Hence, in order to obtain highly productive strains for exploring their application in fruit juice clarification, the present study was conducted with the following objectives.

1. Isolation, characterization and screening of pectinase producing fungi from different sources.
2. Screening different agro wastes for maximum pectinase production under solid state fermentation condition and optimization of process parameters for selected most potent fungal isolates.
3. Purification and characterization of the pectinase enzymes produced by the selected most potent fungal isolates.
4. Exploring the application of the fungal pectinase from different strains in orange and guava fruit juices extraction and clarification.

REVIEW OF LITERATURE

This thesis focuses on exploring the highly productive fungal strains isolated from the natural environment to produce the commercially important pectinase enzyme using the regional agro-industrial residues under solid state fermentation (SSF) condition. The literature review encompasses about nature and properties of the pectin molecule, classification of pectinase enzyme, biochemical characterization of pectinase enzyme, isolation of fungi producing pectinase, purification and application of pectinase in fruit juice extraction and clarification.

2.1 Microbial enzyme biotechnology: Tool of the age

Biotechnology is finding its applications in four major industrial domains viz. health care, crop production and agriculture, non food (industrial) uses of crops and other products (e.g. biodegradable plastics, vegetable oil, biofuels etc.) and environmental uses. One of the significant applications of agro-industrial wastes is biotechnological production of enzymes such as pectinases for their food applications. There is an increasing tendency among the people to use chemical free foods. The use of enzymes like pectinases in the food processing can meet such public demands (Pandey *et al.*, 2000). Tremendous amounts of agro-based wastes/by-products are generated every year over the globe and their improper management causes environmental pollution. Being a co-partner, food industry is contributing a significant share in producing agro-based biological wastes. The elimination of wide range of pollutants and wastes is an absolute requirement to promote a sustainable and friendly environment. Biotechnology plays a major role in the bioconversion of agro-industrial wastes and is taking advantage of the greater adaptability of microorganisms to degrade/convert such compounds into value added products.

In view of rapidly growing economic demand, it is imperative to exploit all indigenous resources at their maximum to attain the goal. The production of value added products from agro-industrial wastes can reduce, to some extent, the cost of production (Durrani *et al.*, 2002). Advent of biotechnology helped to unlock novel food ingredients through the use of biotechnologically-derived industrial enzymes. Microbes have played pivotal role in this progress, and currently the fermented products contribute an adequate share. Their role in bioconversion of waste commodities into value added products has been highlighted in the recent decades (Ahuja *et al.*, 2004).

The ability of filamentous fungi to grow on rather simple and inexpensive substrates as well as their capacity to produce a wide range of commercially interesting metabolites have attracted considerable interest to exploit them as production organisms in biotechnology. Nowadays, filamentous fungi are used in biotechnology as cell factories for a wide range of products. Diverse compounds ranging from simple organic acids to complex secondary metabolites are produced for the use in various market segments. Due to their exceptional high capacity to express and secrete proteins, filamentous fungi have become indispensable for the production of enzymes of fungal and non-fungal origin (Aro *et al.*, 2005). Filamentous fungi exhibit characteristics that make them good models for industrial applications. Noteworthy, among some of these, are their capacity for fermentation, the production of large quantities of extracellular enzymes (e.g. several grams per liter in strains of *Aspergillus*), the feasibility of cultivation, and the low-cost of production in large bioreactors.

2.1.1 Pectin: The miracle molecule

Pectin, in native form is located in the cell wall and may be interlined with other structural polysaccharides and proteins to form insoluble protopectin. In an unripe fruit, pectin is bound to cellulose microfibrils in the cell wall. Such pectin is insoluble and hence confers rigidity to cell walls. However, during ripening, the structure of pectin is altered by naturally occurring enzymes in the fruits (Kashyap *et al.*, 2001). These alterations involve the breakdown of the pectin chain or of side chains attached to the units, which make up the main chain. In either case, the result is that the pectin becomes more soluble and its grip on the surrounding cell walls is loosened and the plant tissue softens. The pectic substances account for about 0.5 ± 4 per cent of the weight of fresh material. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity and the pulp particles, whereas other pectin molecules remain bound to cellulose fibrils by means of side chains of hemicellulose and, thus, facilitate water retention (Pifferi *et al.*, 1989).

All terrestrial plants contain pectin, which binds with cellulose, and creates protopectin, a substance that gives plants their structure. Given this role, it should not be surprising that pectin is found primarily in the plant cell walls, and in the region between cell walls, called the lamella, where it

assists in the binding of one cell wall to another. In addition to giving plants their structure, pectin has other important roles, such as determining how porous the cell is, and its pH (Voragen, *et al.*, 2009).

2.1.2 Structure of pectin

Chemically, pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1→4) linkage. The side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose. The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by sodium, potassium or ammonium ions. Based on the type of modifications of the backbone chain, pectic substances are classified into protopectin, pectic acid and pectin (Kilara, 1982). Protopectin is a parent pectic substance and upon restricted hydrolysis, yields pectin or pectinic acid. Protopectin is occasionally a term used to describe the water-insoluble pectic substances found in plant tissues and from which soluble pectic substances are produced (Be Miller, 1986).

Pectic acids are the galacturonans that contain negligible amounts of methoxyl groups. Normal or acid salts of pectic acid are called pectates. Pectinic acids are the galacturonans with various amounts of methoxyl groups. Pectinates are normal or acid salts of pectinic acids. Pectinic acid alone has the unique property of forming a gel with sugar and acid or, if suitably low in methyl content, with certain other compounds such as calcium salts. Pectin is not one specific molecule, but a class of polysaccharide carbohydrates that encompasses many different structures with a common saccharide as its subunit. There are three major types of chains in pectin (Willats *et al.*, 2006), but all three types of pectin chain consist primarily of rings of methylated D-galacturonic acid. D-galacturonic acid is the oxidized form of D-galactose, in which the sixth carbon, in the R-group external to the saccharide ring, is converted from an alcohol to a carboxylic acid group through oxidation (Mohnen, 2008; Ovodov, 2009).

Three major pectic polysaccharides groups include:

2.1.3 Homogalacturonan (HG)

HG is a linear polymer formed by D-galacturonic acid which can be acetylated and/or methyl esterified. It can be called smooth regions of pectin. The molecule is classified according to its esterification level: pectin has at least 75 per cent of the carboxyl groups methylated; pectinic acid has less than 75 per cent of the carboxyl groups methylated; pectic acid or polygalacturonic acid has no methyl esterified carboxyl groups (Willats *et al.*, 2006).

2.1.4 Rhamnogalacturonan I (RGI)

RG I is composed of the repeating disaccharide rhamnose- galacturonic acid. The galacturonic residues can be acetylated and both residues can carry side chains of neutral sugars as galactose, arabinose and xylose.

2.1.5 Rhamnogalacturonan II (RGII)

Despite its name, RGII is a homogalacturonan chain with complex side chains attached to the galacturonic residues. Vincken *et al.* (2003) have proposed a pectin molecule structure model in which HG and RGII are long side chains of RGI backbone (Fig. 1). Both RG chains are also called hairy regions of pectin molecule. In unripe fruit, pectin is found as a water insoluble pectic substance, the protopectin, bounded to cellulose micro fibrils conferring rigidity on cell walls.

Pectic substances tend to form a gel structure when portions of HG are cross-linked forming a three dimensional crystalline network in which water and solutes are trapped (Fig. 2). Various factors determine gelling properties including temperature, pectin type, esterification degree, acetylation degree, pH, sugar and other solutes, and mainly the interaction between calcium ions and pectin unesterified carboxyl groups. In high-ester pectins, the junction zones are formed by the cross-linking of HG by hydrogen bridges and hydrophobic forces between methoxyl groups, both promoted by high sugar concentration and low pH (Gummadi and Panda, 2003).

As a plant matures, the protopectin, which is insoluble, is broken down by the enzymes pectinase and Polygalacturonase by hydrolysis. This process, started by the enzyme pectin methylesterase, creates low methyl pectin which is the substrate for polygalacturonase (Mohnen, 2008). The physical manifestation of this process can be seen in such things as ripened fruits and vegetables, and released leaves on deciduous plants. In fact, the sweeter taste of the ripened form of many fruits is due to different polysaccharides, such as pectin, getting broken down into their

monosaccharide subunits. Since more mature plant materials and fruits have had some time to be exposed to these enzymes, the pectin is not highly desirable for extraction, as these will be fewer long chains, and the level of esterification will be harder to determine (Vicens, *et al.*, 2009).

2.1.6 Classification of pectic enzymes

Pectinases are a group of enzymes that attack pectin and depolymerise it by hydrolysis and transelimination as well as by deesterification reactions, which hydrolyse the ester bond between carboxyl and methyl groups of pectin (Ceci and Loranço, 1998). The mode of action of different pectinase enzymes is shown in Fig.3.

Pectinases are classified under three headings according to the following criteria: whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, whether pectinases act by trans-elimination or hydrolysis and whether the cleavage is random (endo-liquefying or depolymerizing enzymes) or endwise (exo- or saccharifying enzymes). The extensive classification of pectinases is given in the Table 1.

2.2 Isolation, screening and identification of pectinolytic fungi from different sources

Isolation is the first stage in the screening of microorganisms for potential industrial application. This involves obtaining either pure or mixed cultures. This is followed by their assessment whether they carry out the desired reaction or produce the desired product. The isolation procedure is to be designed so as to favour the growth of these organisms possessing the industrially important characteristic. The ideal isolation procedure commences with environmental source, which is highly probable to be rich in the desired types of microorganisms and also incorporates a simple test to distinguish them.

Pectin is the substrate for pectinolytic activities. The microorganisms which synthesize the pectic enzymes, are usually found associated with dead and decaying plant materials. Pectic enzymes play decisive role in the microbial spoilage. Many spoilage organisms are usually strongly pectinolytic and characteristically are responsible for the extensive maceration of fruits and vegetables. Pectinolytic microorganisms are widely distributed in soil, spoiled fruits, vegetables, decayed leaves, wood, *etc.*

Forty-six fungal strains, isolated from soil and samples of cacao (*Theobroma cacao* L.) jelly, were screened for pectinase production. All the strains were positive for pectinase activity in a cup-plate assay, as evident from clear hydrolysis halos. The cultivation of the best strain (*Peecilomyces clavisporus* 2A.UMIDA.1) by submerged and semi-solid fermentation resulted in high quantities of endopolygalacturonase enzyme. (Souza *et al.*, 2003).

A strain of *Fusarium moniliforme* was isolated from a tropical mangrove ecosystem near Mumbai, and deposited in the National Collection of Industrial Microorganisms (NCIM) as *F. moniliforme* NCIM 1276 (Niture and Pant, 2004). The organism produced extracellular polygalacturonase (PG I) [EC 3.2.1.15] at pH 5.0 and pectate lyase (PL) [EC 4.2.2.2] at pH 8.0 in liquid medium containing 1 per cent citrus pectin.

A thermophilic fungal strain producing both pectinase and polygalacturonase was isolated after primary screening of 120 different isolates (Phutela *et al.*, 2005). The fungus was identified as *Aspergillus fumigatus* Fres., MTCC 4163. Using solid-state cultivation, the optimum levels of variables for pectinase and polygalacturonase (PG) production were determined. Maximal levels of enzyme activities were achieved upon growing the culture in a medium containing wheat bran, sucrose, yeast extract and ammonium sulfate after 2-3 days of incubation at a temperature of 50 °C. The highest enzyme activities of 1116 Ug⁻¹ for pectinase and 1270 Ug⁻¹ for Polygalacturonase were obtained at pH 4.0 and 5.0, respectively

Aspergillus strains (262 in number), isolated from vineyards for pectinase production, were evaluated in the screening methodology that studied the diameter of the pectin hydrolysis and the size of the colony in an agar culture media containing pectin as the sole carbon source (Taskin *et al.*, 2008). *Aspergillus foetidus* var. *pallidus* Ege-K-730 and *Aspergillus aculeatus* Ege-K-355 presented the highest pectinase production ratio, and *Aspergillus foetidus* var. *pallidus* K-635 and *Aspergillus aculeatus* K-398 presented the highest colony diameter. *Aspergillus carbonarius* CFTRI 1047 was used as the reference strain. In submerged fermentation (SbF), the strains Ege-K-730, Ege-K-355, Ege-K-635 and CFTRI 1047 produced the highest PG (Polygalacturonase) and PMG

(Polymethylgalacturonase) levels; however, Ege-K-730 was the faster producer. In solid state fermentation (SSF), the strains Ege-K-730, Ege-K-635, Ege-K-398 and CFTRI 1047 produced the highest PG and PMG levels. In the experimental conditions, SSF was better than Sbf for pectinase production.

Aspergillus repens isolated from cultivated soils released pectinmethylesterase (PE) into the liquid culture medium during growth (Arotupin *et al.*, 2008). On screening, the strains which were active producers of pectinase were selected among the microscopic fungi under conditions of deep cultivation. The most active producers were selected by screening among the cultures of thermophilic, alkali, acidophilic and halophilic microscopic fungal strains. The cultures have been selected from the following genera *Aspergillus*, *Penicillium*, *Mucor*, *Trichoderma*, *Rhizopus*, *Sporotrichum*, *Chaetomium* and *Stempillium*. Among the collection of pectinase producers, the representatives of the following genera dominated: *Aspergillus* and *Penicillium*. Among active producers of pectinase note worthy were *Penicillium canescens* I-85 (acidotolerant), *Aspergillus niger* T₁-1 (thermotolerant), and *Trichoderma viride* Ts-2 (alkali tolerant). Myco-toxicological studies have shown that the selected strains were neither toxic nor pathogenic. The strain *Penicillium canescens* I-85 revealed the highest pectinase activity at 20°C and pH 4.0; while the strain *Aspergillus niger* T₁-1 at 40°C, pH 6.0 and *Trichoderma viride* Ts-2 at 30°C, pH 7.5. As a result of optimization of the nutrient media, the activity of pectinase was increased by 122, 28 and 98 per cent, respectively (Kutateladze *et al.*, 2009).

Ten moulds isolated from municipal waste soil sample were screened for pectinolytic enzyme production when grown on pectin containing solid media (Banu *et al.*, 2010). *Penicillium chrysogenum* was selected based on clearance zones and pectinase enzyme production was carried out in submerged fermentation.

Pectinase producing microorganisms were isolated from pectin industry waste using selective isolation technique by Patil and Chaudhari (2010). Among them, a potential culture was identified, on morphological basis, as *Penicillium* spp. It was found to produce significant amount of extracellular pectinase under submerged fermentation process. The type of enzyme produced was subsequently identified as polygalacturonase (PG).

Thirty five white and bluish green filamentous fungi, dominant during ripening of Cabrales cheese, were phenotypically and genotypically identified (Florez *et al.* 2007). Morphotypic analysis identified *Penicillium* spp. (12 isolates), *Penicillium roqueforti* (11 isolates), *Geotrichum candidum* (10 isolates), *Acremonium charticola* (1 isolate), and one isolate that could not be identified. Polymerase chain reaction (PCR) amplicons of their ITS1-5.8S-ITS2 region were subjected to restriction fragment length polymorphism (RFLP) analysis with six restriction enzymes. Strains morphotypically identified as *P. roqueforti* showed identical RFLP profiles with all enzymes, as did all the *G. candidum* strains; confirming their phenotypic classification. However, molecular analysis showed the *Penicillium* spp. group to be composed of *P. commune* (5 isolates), *P. chrysogenum* (4 isolates), *P. roqueforti* (2 isolates), and *P. citreonigrum* (1 isolate). The genetic diversity found among the different *P. roqueforti* and *G. candidum* isolates was assessed by Rapid amplified polymeric DNA-polymerase chain reaction (RAPD-PCR) with two primers. Nine *P. roqueforti* strains were defined among the 13 isolates, and every *G. candidum* isolate represented an individual strain.

Restriction digestion analysis of the ITS products was tested as an easy method to identify isolates of filamentous fungi on grapes (Diguta *et al.*, 2011). Endonucleases S₁ul, H₁infl, M₁sel, H₁aell₁ were used. Endonucleases B₁fml, C₁fr9₁, H₁py188₁, M₁aell or P₁spG₁ were used as necessary to complete discrimination. The 43 species studied generated 42 different composite profiles. Only the species *P. thomii* and *P. glabrum* gave the same composite profile. About 96.3 per cent strains tested could be differentiated to the species level with only four enzymes. One hundred and ninety nine strains of filamentous fungi isolated from various vineyards in Burgundy were identified by this method. *Penicillium* (58.5 %) was the genus most frequently isolated and no strains of the genus *Aspergillus* was isolated. *P. spinulosum* was the most isolated species of *Penicillium* (22.70 %). The species *C. cladosporioides*, *B. cinerea*, *E. nigrum*, *A. alternata*, *T. koningiopsis*, *P. diplodiella*, *C. herbarum*, *A. alternatum*, *T. cucumeris* and *F. oxysporum* were also isolated.

Baffi *et al.* (2012) investigated the fungal diversity of fresh olive (*Olea europaea* L.) fruits, olive paste (crushed olives), olive pomace (solid waste) by screening and quantifying enzymatic activities with biotechnological applications. Fungi were randomly isolated from olive cultivars from Castilla La Mancha region (Spain). Identification included comparison of their polymerase chain reaction (PCR) amplicons of the ITS1–5.8S–ITS2 ribosomal DNA region, followed by nucleotide sequence analysis. Fourteen different species with DNA sequences of different similarities were identified, belonging to

seven different genera (*Aspergillus*, *Penicillium*, *Rhizomucor*, *Mucor*, *Rhizopus*, *Lichtheimia* and *Galactomyces*). *Aspergillus fumigatus*, followed by *Galactomyces geotrichum*, *Penicillium commune* and *Rhizomucor variabilis* var. *regularior* were the most frequent species. Specific enzyme screening was performed on agar plates, using cellobiose, carboxymethylcellulose (CMC), polygalacturonic acid and CaCl_2 /Tween 80 as substrates for β -glucosidase, carboxymethylcellulase (CMCase), Polygalacturonase and lipase, respectively.

2.2.1 Different agro-wastes for pectinase production under solid state fermentation (SSF) conditions

Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism (Pandey, 2003). The potential of SSF lies in bringing the cultivated microorganism in close vicinity of substrate and achieving the highest substrate concentration for the fermentation. It resembles the natural habitat of microorganism and, is therefore, preferred choice for microorganisms to grow and produce useful value added products (Kelecom, 2002; Pandey, 2003; Holker *et al.*, 2004). Though historically known since centuries, SSF gained a fresh attention from researchers and industries all over the world since recent few years, mainly due to few advantages it offered over liquid (submerged) fermentation, particularly in areas of solid waste management, biomass energy conservation and its application to produce high value–low volume products such as biologically active secondary metabolites, *etc.*, apart from the production of food, feed, fuel and traditional bulk chemicals (Pandey *et al.*, 2007).

Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce pectinase enzyme. Some of the substrates that have been used included sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, grapevine trimmings dust, saw dust, corn cobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, *etc.* The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus, may involve screening of several agro-industrial residues (Pandey *et al.*, 2007). In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (*e.g.* ligno-cellulose), there by making them more easily accessible for microbial growth.

2.2.2 Challenges and advantages of SSF

There are various important factors that produce immense impact on the success of a particular technology, hence, needed to be considered for the development of any bioprocesses and so is with the SSF. It includes selection of microorganism and substrate, optimum process parameters and also purification of the end product, which has been a challenge for this technology. Fungi and yeast were termed as suitable microorganisms for SSF according to the theoretical concept of water activity. The establishment of the relationships between the physiology of the microorganisms and the physico-chemical factors is the aim for the development of proper models. These factors include temperature, pH, aeration, water activity and moisture, bed properties, nature of solid substrate employed, *etc.* Among several critical factors, moisture and nature of solid substrate employed are the most important factor affecting SSF processes. Selection of moisture depends on microorganism employed and also the nature of substrate. Fungi need lower moisture, 40–60 per cent moisture could be sufficient. High moisture percentage results in low substrate porosity which, in turn, prevents oxygen penetration, whereas low moisture content may lead to poor accessibility of nutrients resulting in hampered microbial growth (Pandey *et al.*, 2000). The consequences of these difficulties for bioreactor performance have been well explored (Mitchell *et al.*, 2000). Heat generation due to metabolic activities of the microorganisms, is often fatal for biotechnological processes because a large part of the enzymes produced during the fermentation can be heat denatured at the end of the process (Muller dos Santos *et al.*, 2004).

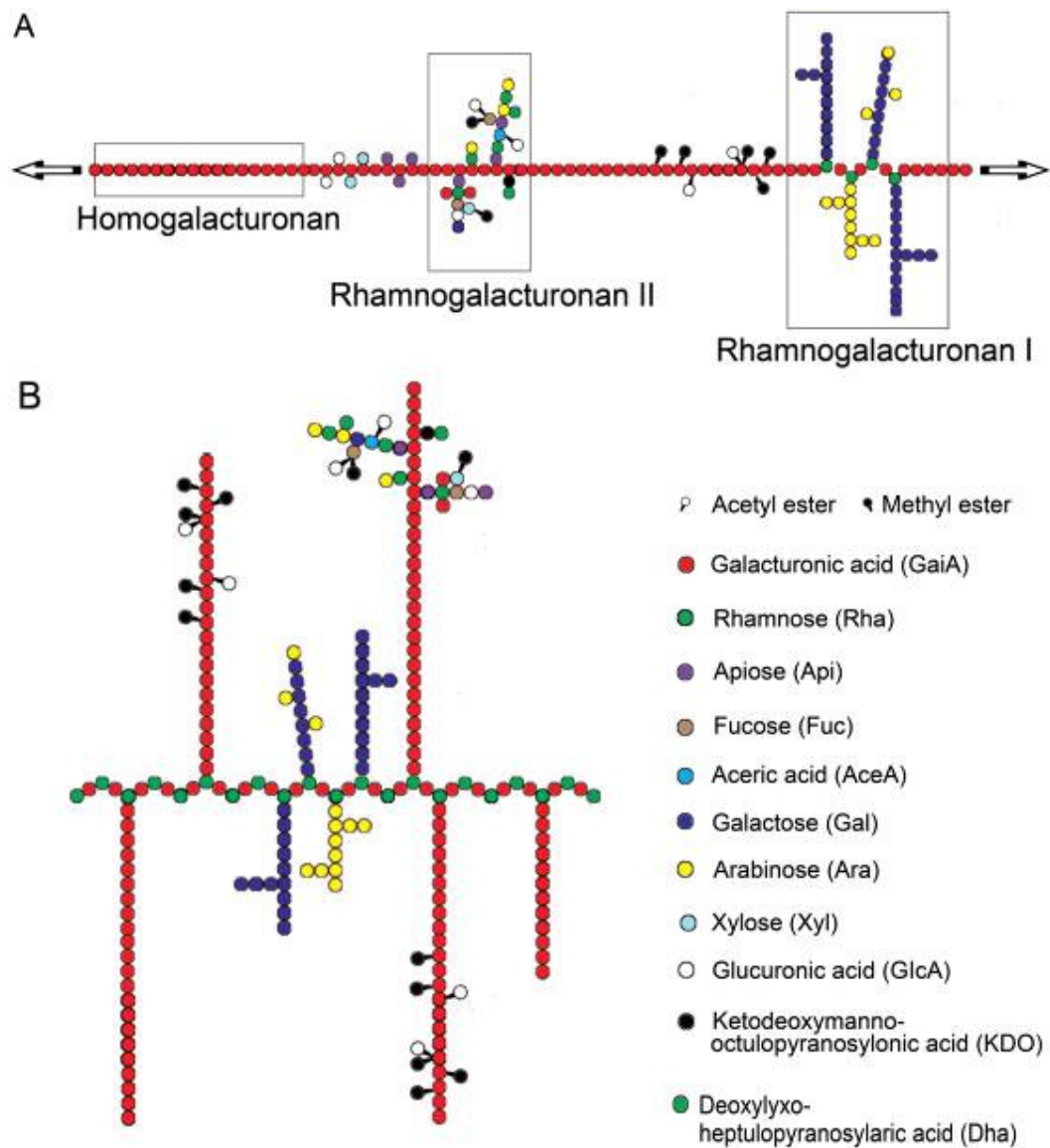


Fig.1: Schematic representation of the conventional (A) and recently proposed alternative (B) structure of pectin

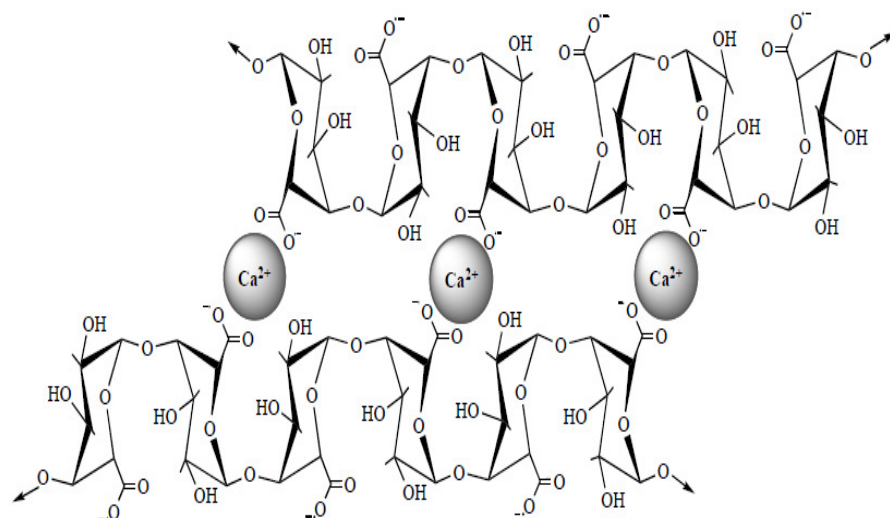


Fig. 2: Interaction through insertion of Ca^{2+} ions between the unesterified carboxyl groups of the galacturonosyl residues of two Homogalacturonan (HG) chains (Adopted from Pedrolli *et al.*, 2009).

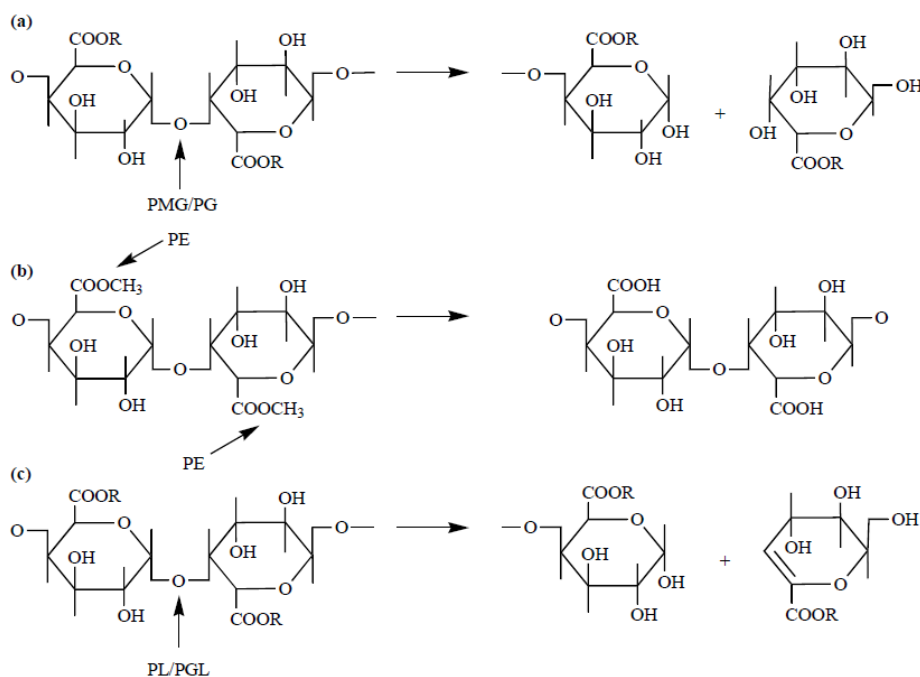


Fig. 3: Mode of action of pectinases: (a) $\text{R} = \text{H}$ for PG and CH_3 for PMG; (b) PE; and (c) $\text{R} = \text{H}$ for PGL and CH_3 for PL. The arrow indicates the place where the pectinase reacts with the pectic substances. PMG, polymethyl galacturonases; PG, polygalacturonases; PE, pectin esterase; PL, pectin lyase (Adopted from Lang and Dornenburg, 2000).

Table 1. An extensive classification of pectinolytic enzymes

Enzyme	E.C. No.	Modified EC systematic name	Action mechanism	Action pattern	Primary substrate	Product
Esterase						
Pectin methyl esterase	3.1.1.11	-	Hydrolysis	Random	Pectin	Pectic acid + methanol
Depolymerizing enzymes: Hydrolases						
1. Protopectinases	-	-	Hydrolysis	Random	Protopectin	Pectin
2. Endopolygalacturonase	3.2.1.15	Poly-(1-4)- α -D-galactosiduronate Glycanohydrolase	Hydrolysis	Random	Pectic acid	Oligogalacturonates
3. Exopolygalacturonase	3.2.1.67	Poly-(1-4)- α -D-galactosiduronate Glycanohydrolase	Hydrolysis	Terminal	Pectic acid	Monogalacturonates
4. Exopolygalacturonan-digalacturono hydrolase	3.2.1.82	Poly-(1-4)- α -D-galactosiduronate Digalacturonohydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase	-	-	Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates
6. D4: 5Unsaturated oligogalacturonate hydrolases	-	-	Hydrolysis	Terminal	D4:5(Galacturonate) _n	Unsaturated monogalacturonates And saturated (n-1)
7. Endopolymethyl-galacturonases	-	-	Hydrolysis	Random	Highly esterified pectin	Oligomethylgalacturonates
8. Endopolymethyl-galacturonases	-	-	Hydrolysis	Terminal	Highly esterified pectin	Oligogalacturonates
Lyases						
1. Endopolygalacturonase lyase	4.2.2.2	Poly-(1-4)- α -D-galactosiduronate Lyase	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	4.2.2.9	Poly-(1-4)- α -D-galactosiduronate Exolyase	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galactosiduronate lyase	4.2.2.6	Oligo-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate Lyase	4.2.2.10	Poly(methyl galactosiduronate) Lyase	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyl oligogalacturonates
Exopolymethyl-D-galactosiduronate Lyase	-	-	Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyl monogalacturonates

(Adopted from Jayani *et al.*, 2005).

Viniegra-Gonzalez *et al.* (2003), have attempted to develop a general approach for the comparison of productivity of enzymes employing SSF and SmF and have tried to explain the reason for higher production in SSF. Higher biomass, high enzyme production and lower protein breakdown, contributes to the better production in SSF. Scale up, purification of end products and biomass estimation are the major challenges that led the researchers to thrive hard to find the solutions. SSF processes offers several biotechnological advantages (Holker *et al.*, 2004), such as higher fermentation productivity, higher end-concentration of products, higher product stability, lower catabolic repression, cultivation of micro-organisms specialized for the water-insoluble substrates or mixed cultivation of various fungi, and last but not least, lower demand on sterility due to the low water activity used in SSF.

Scale up in SSF has been a limiting factor since long but recently with advent of biochemical engineering a number of bioreactors have been designed which could overcome the problems of scale up and to an extent also the on-line monitoring of several parameters, as well as heat and mass transfer. Separation of biomass is a big challenge in SSF, which is essential for the kinetic studies. However, certain indirect methods are available such as, glucosamine estimation, ergosterol estimation, protein estimation, DNA estimation, dry weight changes and CO₂ evolution, but all of them have their own limitations. Recently digital image processing has been developed as a tool for measuring biomass in SSF. The images were acquired by stereomicroscope and a digital camera and processed using KS400 software (Couri *et al.*, 2006). There are major challenges that need to be addressed for the successful implementation of SSF technology, such as scale up in SSF, which is a major bottle neck; providing adequate heat and mass transfer within the substrate bed, to monitor on-line several key processes parameters and also to mix the bed adequately without damaging the microorganisms as well as the substrate particles of bed (Khanahmadia *et al.*, 2006).

Although, product recovery and purification are more expensive processes employing natural supports, minimises the production costs with much higher activities. (Rodriguez-Couto and Sanroman, 2006). Hence, an economical evaluation of the overall process should be done in order to determine its feasibility to a specific purpose. This system is especially suitable for the production of high-value products like enzymes. There are particular applications where concentrated end products with high titers are required rather than degree of purity. In recent times, estimation of oxygen intake and carbon dioxide evolution rate are considered to be the most accurate methods for determination of the growth of the microorganism (Pandey *et al.*, 2007).

2.2.3 Optimization of process variables for pectinase production

Aspergillus carbonarius produces extracellular pectinases when grown on wheat bran in solid-state fermentation (SSF) on a laboratory scale in flasks (Singh *et al.*, 1999). The downstream processing parameters for recovery of the crude pectinases from the fermented bran were optimized. Optimum time and temperature of extraction were determined to be 15 min and 30°C. The enzyme could be concentrated at least 4-fold in the extract by sequential extractions. Extraction using water or acetate buffer (pH 4.3) containing sodium sulfate resulted in the maximum enzyme recovery. Colours and polysaccharides in the enzyme extract could be minimised by treatment with sodium chloride and activated charcoal. Concentration of the enzyme by ultrafiltration resulted in a 50-fold concentration of activity with 91% recovery.

Fruit-processing wastes including apple pomace, cranberry pomace and strawberry pomace were used as substrates for polygalacturonase (PG) production by *Lentinus edodes* through solid-state fermentation (Zheng and Shetty, 2000). Strawberry pomace was the best substrate for maximum PG yield, followed by apple pomace, while cranberry pomace was not a suitable substrate for PG production in this study. The highest PG activity was obtained after 40 days of culture and the yields from strawberry pomace, apple pomace and cranberry pomace were 29.4 U, 20.1 U and 14.0 U per gram of pomace, respectively. The PG activity was increased by addition of polygalacturonic acid in apple pomace and cranberry pomace media, but was not affected in strawberry pomace medium. The PG produced by *L. edodes* from strawberry pomace exhibited a maximal activity at 50°C and at pH 5.

Castilho *et al.* (2000) used soy and wheat bran as raw materials for the production of pectinases by *Aspergillus niger* through solid-state fermentation. The kinetics of enzyme synthesis was investigated in the range from 13 to 96 h with moisture contents varying from 25% to 70% (w/w). A medium moisture content of 40 per cent and a fermentation time of 22 h were selected, as these conditions resulted in high pectolytic activity and enhanced Polygalacturonase productivity. In order to

optimise the recovery step, the best combination of temperature of extraction, contact time and solvent type was investigated. Acetate buffer (pH 4.4), 35°C and 30 min provided the best recovery.

Teixeira *et al.* (2000) studied the effect of different carbon sources on the pectinesterases, endo- and exo-polygalacturonase activities from *Aspergillus japonicus* 586 in liquid medium supplemented with different substrate concentrations. The culture medium was inoculated at the rate of 5×10^6 spores mL^{-1} and maintained under agitation (140 rpm), at 30°C, during 122 h. The enzyme evaluation was carried out 24 h after filtration. The crude extract from *A. japonicus* 586 indicated that the best enzymatic activities were afforded in the presence of 0.5% pectin (pectin esterase), 0.2% pectin and 0.2% glycerol (endo polygalacturonase), and 0.5% pectin associated to 0.5% glucose (exopolygalacturonase). Carbon sources, their concentrations significantly affected the pectin esterase, and endo- and exo polygalacturonase activities.

Pectin lyase and polygalacturonase production by newly isolated *Penicillium viridicatum* strain Rfc3 was carried out by means of solid state fermentation using orange bagasse, corn tegument, wheat bran and mango and banana peels as carbon sources (Silva *et al.*, 2002). The maximal activity value of polygalacturonase (PG) (30 Ug^{-1}) was obtained using wheat bran as carbon source while the maximal pectin lyase (PL) (2000 Ug^{-1}) activity value was obtained in medium composed of orange bagasse. Mixtures of banana or mango peels with sugar cane bagasse resulted in increased PG and PL production as compared to fermentations in which this residue was not used. The mixture of orange bagasse and wheat bran (50 %) increased the production of PG and PL to 55 Ug^{-1} and 3540 Ug^{-1} respectively.

The synthesis of pectolytic enzymes appears to be considerably influenced by the glucose content of the cultivation medium containing mixed carbon sources in a predetermined optimal ratio. Studies conducted to examine the effect of glucose showed that glucose severely reduced the synthesis of polygalacturonase and polymethylgalacturonase at concentrations above 5 g L^{-1} while pectin lyase was unaffected. *In vitro* studies showed that the inhibition of the two pectin hydrolases was reversible and competitive in nature. The results of the study also indicated induction of pectolytic enzymes by corn in *Aspergillus niger* NCIM 548 (Panda *et al.*, 2004). Growth on semi-solid medium containing wheat bran and orange bagasse resulted in a three-fold increase in PG production and a two-fold increase in PL production in comparison with that in liquid medium (Niture and Pant, 2004). The increased production of PG on semi-solid media, as compared to production in liquid media was investigated. The second PG II was a 30.6 kDa enzyme, had an alkaline pH of 8.6, with 0.166 mg mL^{-1} K_m value, $13.33 \text{ mmol min}^{-1} \text{ mg}^{-1}$ of $V_{\max}$ and 403 min^{-1} of Kcat. It had a specific activity of 18.66 Umg^{-1} . The differences between the PGs (PG I and PG II) suggested that the two enzymes were the products of different genes.

Pectinase production studies were carried out in submerged and solid-state conditions from deseeded sunflower head employing *Aspergillus niger* (Patil and Dayanand, 2006). Two potential strains of *A. niger*, DMF 27 for submerged and DMF 45 for solid-state were isolated by multi-step screening technique based on coefficient of pectolysis and capability of pectinase production. Process variables such as size of inoculum, pH, temperature, particle size and moisture content were optimized with an aim to achieve the maximum production of pectinases. The increased level of pectinase production was recorded at pH 5.0 and temperature 34°C in submerged and solid-state conditions. The optimum inoculum size was $1 \times 10^5 \text{ mL}^{-1}$ for submerged and $1 \times 10^7 \text{ g}^{-1}$ for solid state conditions. Five hundred micrometer particle size and 65% moisture content of the substrate were optimum for the maximum production of pectinases in solid-state condition. Under optimum conditions, maximum production of exo-pectinase was 34.2 U mL^{-1} in SSF and endo-pectinase was 12.6 U mL^{-1} in SmF.

The enzyme preparation was partially purified by ammonium sulfate precipitation and dialyzed (Arotupin *et al.*, 2008). The ammonium sulfate-dialysate fraction of the enzyme was separated by molecular exclusion and ion exchange chromatography. The molecular weight of the enzyme was found to be 141,300 Daltons. The optimum temperature for PE activity was 30°C and pH 6.5. The activity of the enzyme was increased with an increase in substrate concentration reaching maximum at 4 mg mL^{-1} .

The pectinase production parameters were first studied at shake flask level. The parameters that were studied included different carbon sources, different strains of *Aspergillus niger* and different pH (Khairnar *et al.*, 2009). During growth studies, the maximum biomass or cell mass was obtained in between 24 h and 48 h. The production of Pectinase from *Aspergillus niger* was greatly influenced by pH of initial culture. The optimum pH for production of pectinase by *A. niger* was 3.8.

Palaniyappan *et al.* (2009) conducted an experiment on different natural substrates such as wheat flour and corn flour in comparison with synthetic pectin for the production of pectinase using *Aspergillus niger* MTCC 281. The work involved optimizing various parameters like substrate concentration, pH, temperature, rpm, time of fermentation for the production of pectinase and the effect of carbon sources on the synthesis of the pectinase enzyme. The experimental studies indicated that maximum synthesis of pectinase (6.1 U mL^{-1}) was obtained with *A. niger* MTCC 281 by using wheat as substrate under the influence of the additional carbon source, starch. The optimal conditions were found to be substrate concentration in wheat (1%), pH 5.5, temperature 30°C , time 72 h, rpm 170, and carbon source starch (0.025 %).

Maciel *et al.* (2011) studied the activity of endo-polygalacturonase (endo-PG), exo-polygalacturonase (exo-PG), pectin lyase (PL) and pectinesterase (PE) produced by *Aspergillus niger* URM4645 in solid-state fermentation (SSF) using forage palm as the substrate. The effect of variable substrate amount, inoculum concentration and temperature on the pectinase production was studied using a full factorial design (2^3). The maximum activity obtained was 66.19 U mL^{-1} for endo-PG, 3.590 U mL^{-1} for exo-PG and $40,615.62 \text{ U mL}^{-1}$ for PL, respectively at 96, 24 and 72 h of fermentation. PE showed no activity. The production of endo-PG and exo-PG was significantly influenced by varying substrate amount, inoculum concentration and temperature, but these variables had no influence on PL production. The best conditions for production of the three enzymes at the same time were obtained with 10.0 g of substrate, 10^7 spores g^{-1} at 28°C . Endo-PG and PL presented optimum activity at pH 5.0 and exo-PG at pH 7.0. The maximal activity of endo-PG and PL was determined at 50°C and exo-PG at 40°C . Endo-PG and exo-PG were stable at a pH range of 3.5 - 11.0 and at 50 and 80°C , respectively. PL showed stability only at pH 5.0 and at 50°C .

Possibility of producing pectinase utilizing fruit wastes of cashew, banana, pineapple, and grape under controlled fermentation with *Aspergillus foetidus* was studied (Venkatesh *et al.*, 2009). Among the different media composition tried, medium containing 5 g fruit waste + 0.05 g urea + 0.25 g ammonium sulfate supported better growth of the microorganism. Enzyme production was maximum in the medium with grape waste. As extractant of the enzyme, distilled water was better than CaCl_2 . The ideal temperature and duration of fermentation were 40°C and 8 days respectively.

Martos *et al.* (2009), using response surface methodology optimized Polygalacturonase (PG) and pectinesterase (PE) production in submerged fermentation by *Aspergillus niger*. Variables studied were: fermentation time (24 to 120 h), pH (3.5 to 6.5) and initial concentration of pectin (5 to 20 g L^{-1}). Maximum PE production was 220 U L^{-1} , after 72 h of culturing in a medium containing 20 g L^{-1} of pectin (pH 6.5). The optimal conditions for PG production were pH: 4.1, 20 g L^{-1} of pectin and 96 h of fermentation with a maximum value of 1032 U L^{-1} .

Banu *et al.* (2010), observed higher enzyme production by *Penicillium chrysogenum* at pH 6.5, temperature of 35°C using sucrose and ammonium per sulfate as carbon and nitrogen sources, respectively. However, the maximal activity of *P. chrysogenum* pectinase was at 50°C , pH 6.5 and was thermostable up to 40°C . MgCl_2 and CaCl_2 had little effect on pectinase activity. K_m and $V_{\max}$ values were 1.0 mg mL^{-1} and 85 U mg^{-1} protein, respectively with an apparent molecular weight of 31 kDa on SDS-PAGE.

On partial optimization, Patil and Chaudhari (2010) reported that the *Penicillium spp.* culture showed maximum enzyme production at 35°C in a medium having pH 6.0 containing 1.5 per cent pectin on 72 h of incubation. The PG produced by the isolate was further purified by ammonium sulfate precipitation, size exclusion and ion exchange chromatography. The molecular weight of purified enzyme was determined to be 35 kDa by SDS-PAGE. Under optimized conditions, purified PG showed 98.66 U mL^{-1} activity which was almost 12 fold higher than crude. In view of cost effective pectinase production, substrate optimization using solid state fermentation was carried out amongst which orange bagasse gave 64.50 U mL^{-1} PG activity which was higher than other natural substrates.

The continuous production of polygalacturonases (PGases) by a strain of *Aspergillus awamori* in surface culture fermentation was investigated (Abbasi *et al.*, 2011). Wheat flour acted as a good substrate for the cultivation of the fungus and production of PGases in surface culture fermentation. Fermentation started in batch mode until mycelia completely occupied the medium following growth of the microorganism, after which it was turned to the continuous mode by the introduction of fresh feed. The process continued for 34 days, and the thickness of the microbial layer on the surface of the liquid medium became almost constant after approximately one week. The production of PGases, however, continued throughout the experiment, and maximum activities of 1.2 U mL^{-1} and 0.014 U

mL⁻¹ were obtained for exo polygalacturonase (exo-PGase) and endo-polygalacturonase (endo-PGase), respectively.

SSF process was described (Ruiz *et al.*, 2012) for the production of pectinase by *Aspergillus niger* Aa-20 and lemon peel pomace (LPP) as both support and carbon source in a solid-state bioreactor. Invasion ability of selected fungal strain was analyzed on four particle sizes of LPP; (3) SSF process was operated in a column-tray bioreactor at 30°C and 70 per cent moisture content with 194 mL min.⁻¹ of air flow rate and substrate particle size (2–0.7 mm) of LPP for 96 h. Results showed, that high levels of pectinase activities were obtained. The maximum pectinase activity obtained was 2181 U L⁻¹.

2.2.4 Scale up studies and techno-economic analysis of pectinase enzyme production

The effect of solid substrates, inoculum and incubation time were studied on the production of polygalacturonase enzyme and spores following response surface methodology (RSM) in solid-state fermentation using *Aspergillus sojae* ATCC 20235 (Ustok *et al.*, 2007). Two stage optimization procedure was applied using D-optimal and face-centered central composite design (CCD). Crushed maize was chosen as the solid substrate, for maximum polygalacturonase enzyme activity based on D-optimal design. Inoculum and incubation time were determined to have significant effect on enzyme activity and total spore ($p < 0.01$) based on the results of CCD. The individual optimum values of inoculum and incubation time for maximum production of the two responses were 2×10^7 total spores and 5–6 days respectively. The predicted enzyme activity (30.55 U mL⁻¹) and spore count (2.23×10^7 spore mL⁻¹) were very close to the actual values obtained experimentally (29.093 U mL⁻¹ and 2.31×10^7 spore mL⁻¹, respectively).

Fontana *et al.* (2009) studied the production of endo and exo-polygalacturonase (PG) by *Aspergillus oryzae* IPT 301 in a stirred tank bioreactor (STR) and an internal circulation airlift bioreactor. Using a factorial experimental design, a soluble culture medium was defined which allowed the production of exo- and endo-PG comparable to that obtained in a medium containing suspended wheat bran. The soluble medium was used in tests to compare the production of these enzymes in the STR and airlift bioreactor. In these tests, after 96 h, maximum values of enzymatic activity were achieved for exo- and endo-PG with 65.2 U mL⁻¹ and 91.3 U mL⁻¹ respectively, in the STR, with similar activity values of 60.6 U mL⁻¹ and 86.2 U mL⁻¹, being achieved in the airlift bioreactor.

Stirring effect in a stirred membrane cell and the type of pumping action in a cross-flow filtration system were assessed by Nakkeeran and Subramanian (2010) from the viewpoint of recovery of polygalacturonase (PG) from *Aspergillus carbonarius* culture broth. PG and protein loss increased with stirring speed (shear stress) as well as processing time under simulated conditions of a stirred membrane cell. When the stirring conditions did not exceed the critical speed of 400 rpm, corresponding to a shear stress of 2.1 Pa, higher recoveries of PG (88%) and protein (96%) were obtained. Processing culture broth using a gear pump increased PG and protein loss with processing time and resulted in 71% PG and 54% protein loss after 3 h of pumping, while the loss of PG and protein was negligible when a peristaltic pump was used.

Nakkeeran *et al.* (2012) took up a techno-economic analysis of submerged (SmF) and solid state fermentation (SSF) processes for *Aspergillus carbonarius* polygalacturonase production to make an appropriate process selection. The downstream processing involved integrated membrane process (IMP) and alginate affinity precipitation (AAP). For a production scale of 30 kL purified polygalacturonase concentrate per year, the total upstream cost of SmF was 14 per cent lower than the SSF process. Downstream processing cost by IMP was 47 per cent lower than AAP. The SmF-IMP process required a total capital investment that was 15–24% lower than the SmF-AAP and SSF-AAP processes. The corresponding unitary product cost was also lowered by 24–36 per cent in SmF-IMP process.

2.3 Purification and characterization of pectinase enzyme

During enzyme production process, in addition to the target enzyme, growth medium may have some undesirable metabolites of the micro-organisms. The purified enzymes exhibit higher activity, lesser risk of harmful substances and, thus, better affectivity for the specific product. Moreover, characterization of enzyme is imperative, to achieve better performance in a particular

application; as it is a prerequisite to get information regarding the suitable conditions of enzyme action.

2.3.1 Removal of microbial cells and other solid matter

Purification of the microbial enzymes face the problems of removing the contaminating substances present in the starting materials and also the separation of the enzyme from other pectinolytic enzymes produced by the microorganisms. Normally, one protein homogenate contains thousands of different proteins and the purification seems to be difficult. However, in practice, only four different steps are needed for the purification of proteins. In some cases a single chromatographic step is enough based on the purpose of the purification. For structural and functional studies, 100% purification is not necessary. Enzymes can be purified several folds but the yield of the enzyme may be very poor. Industrial enzymes are purified as little as possible from impurities. Other products are isolated by using modifications of the flow system (Stanbury *et al.*, 1997).

Microbial cells and other insoluble materials are separated from the harvested broth by filtration or centrifugation. Filtration separates particles simply on the basis of their size. Commonly used filter is diatomaceous earth. Centrifugation separates on the basis of the particle size and density difference between the liquid and solid phases. Ultrafiltration is one of the purification steps whereby water and low molecular weight materials are removed by passage through a membrane under pressure, while the enzyme is being retained. Enzymes can be concentrated by precipitation, and this is generally used as the initial step of purification. Salting out ammonium sulfate is the best known method for concentration and purification of the enzyme. Some enzymes do not survive with the ammonium sulfate precipitation. In such cases, organic solvents such as ethanol, propanol and acetone can be used as the alternative. For further purification, the ammonium sulfate present in the protein precipitate is to be removed; this can be achieved by dialysis, ultrafiltration or using sephadex G-100 column. The biochemical and physiological classification of pectinases is furnished in Table 2.

Purified papaya (*Carica papaya* L. var. *exotica*) pectinesterase (EC 3.1.1.11) was investigated for its activity as influenced by NaCl, pH, temperature, and its kinetic parameters were determined. The activity was linear up to 20 min with an enzyme concentration of up to 6.14 μg . Optimum activity was obtained with 0.25 M NaCl concentration and the optimum pH was found to be 8. The energy of activation of enzyme was $5690 \text{ cal mol}^{-1}$ observed in the temperature range of $30\text{--}50^\circ\text{C}$ and the optimum temperature for the enzyme activity was 65°C . The K_m value for citrus pectin was 0.11 mg mL^{-1} , corresponding to a V_{max} value of $730 \text{ mole min}^{-1} \text{ mg}^{-1}$ of protein (Fayyaz *et al.*, 1995).

The kinetics of commercial pectinase, Pectinex Ultra SP-L, in a batch reactor was studied by Mutlu *et al.* (1999). The change in viscosity of pectin solution was followed by an enzymatic assay to determine pectolytic activity of the enzyme preparation. The pectolytic activity of Pectinex Ultra SP-L was measured at pH 3.5 and 35°C and found to be $0.025 \text{ pectin \% (w/v) sec}^{-1} \text{ enzyme}^{-1} \text{ \% (v/v)}$. The Michaelis-Menten constant (K_m) of enzyme preparation was found to be 1.13 per cent pectin (w/v). The temperature dependence of the reaction rate was obeying the Arrhenius Law. The activation energy of the biochemical reaction catalysed by commercial pectinase was calculated as $9.316 \text{ kcal mol}^{-1}$.

A polygalacturonase with a molecular mass of 74 kDa, an isoelectric point around pH 4.2. pH and temperature optima of 3.9 and 50°C , respectively, was purified from a culture fluid of *Penicillium frequentans*. The enzyme was characterized as an $\text{exo-1, 4-polygalacturonase (exo-PG I)}$. K_m and V_{max} for sodium polypectate hydrolysis were 0.68 g L^{-1} and 596.8 Umg^{-1} , respectively. The enzyme, a glycoprotein with a carbohydrate content of 81%, is probably the main pectinase of *Penicillium frequentans* responsible for cleaving monomer units from the non-reducing end of pectin (Chellegatti *et al.* 2002).

An endo-polygalacturonase, named PGI, was purified to homogeneity from the culture filtrate of *Aspergillus kawachii* IFO 4033 grown in a glucose-tryptone medium (Esquivel and Voget, 2004). The molecular mass of PGI was estimated to be 60 kDa by SDS-PAGE and 40 kDa by gel filtration on Sephacryl S-100. The isoelectric point was 3.55 as determined by isoelectric focusing. The remarkable property of PGI was its high activity in the pH range 2.0-3.0 towards soluble and insoluble substrates, while being inactive at pH 5.0. Enzyme stability at low pH was markedly enhanced by different compounds, such as proteins, polysaccharides, simple sugars and the substrate pectin. PGI was very efficient to extract pectin from lemon protopectin and to macerate carrot tissues at pH 2.0.

Affinity precipitation is a simple, single plate separation process in which the complex of a smart macroaffinity ligand with the target protein (from a crude broth) can be selectively precipitated

by application of a suitable stimulus. Alginate is a copolymer of guluronic acid and mannuronic acid residues and precipitates with Ca^{2+} ions. It was found to bind to pectinase present in a commercial preparation of *Aspergillus niger*, Pectinex Ultra-SPL (Mondal *et al.*, 2004). Microwave pretreatment of alginate at 75°C was found to enhance the selectivity of the affinity precipitation. Using microwave-treated alginate, 83 per cent of the enzyme activity with 20-fold purification could be recovered. SDS-PAGE upon silver staining confirmed the enhanced selectivity of affinity precipitation when microwave-treated alginate was used.

Ortega *et al.* (2004), studied the Kinetic properties and thermal inactivation of polygalacturonase (PG) assayed in commercial preparations (Rapidase C80, Pectinase CCM and Pectinex 3XL). The PG activities in all samples followed Michaelis-Menten kinetics and their catalytic efficiencies were calculated. The optimum pH for enzyme activity was 4.7 for Pectinex 3XL and 4.0 for both Rapidase C80 and Pectinase CCM. The PG optimum temperature lay within the range of 50–55°C. The activation energies of Rapidase C80, Pectinase CCM and Pectinex 3XL PG were 26.5, 45.6 and 4.2 kJ mol⁻¹, respectively. The thermal inactivation curves were not linear in the range of 40–60°C. The biphasic curves fitted to a two-fraction first-order model suggested the presence of two groups of PG isozymes in Rapidase C80 and Pectinase CCM.

Pectinase from *Aspergillus niger* associated with polygalacturonase activity, caused considerable debranching-depolymerization of guar galactomannan. Zymogram analysis further confirmed the action of pectinase on the latter. Optimum activity occurred at pH 5.0 and 50°C and the reaction obeyed Michaelis-Menten kinetics with K_m and V_{max} values of 3.72 mg mL⁻¹ and 1.852 mmoles min⁻¹ mg⁻¹ respectively. Viscometry, GPC and HPLC analyses of the native guar galactomannan showed a molecular weight (Mw) of ≈240 kDa with a galactose:mannose (G:M) ratio of 1:1.6, as revealed by GLC analysis, whereas, after enzymatic catalysis for 60 min, the major resultant product had a (Mw) of ≈70 kDa with a G:M ratio of 1:2.8, respectively. The latter had a solubility of 98% in water at ambient temperature (within 30 min) compared to ≈ 60% for native galactomannan (Shobha *et al.*, 2005).

An extracellular pectin lyase (PL) 4.2.2.10, produced by *Penicillium oxalicum* produced on mandarin peel meal on solid state fermentation was studied by Yadav and Shastri (2005). The enzyme was partially purified by ammonium sulfate precipitation. The partially purified pectinase showed pH optima of 8.0 and stable over pH range of 4.0–8.0. The optimum temperature of the thermolabile enzyme was 40°C. The enzyme, however lost its activity quickly at temperatures over 50°C. The K_m of the enzyme was found to be 2.08 mg mL⁻¹ of citrus pectin, which was substantially lower than the values already reported. Phenolic compounds except tannic acid and several divalent ions and other inhibitors examined had no significant effect on the enzyme activity. An extracellular polygalacturonase (PGII) from *Trichoderma harzianum* was purified to homogeneity by two chromatography steps using DEAE-Sephacryl and Sephacryl S-200. The molecular weight of *T. harzianum* PGII was 31,000 Da by gel filtration and SDS-PAGE. PGII had isoelectric point of 4.5 and optimum pH of 5.0. PGII was very stable at pH 5.0. PGII had very low activity toward non-pectic polysaccharides. The apparent K_m value and K_{cat} value for hydrolyzing polygalacturonic acid (PGA) were 3.4 mg mL⁻¹ and 592 s⁻¹, respectively. PGII was found to have temperature optimum at 40°C and was approximately stable up to 30°C for 60 min of incubation (Mohamed *et al.*, 2006).

An extracellular pectinase was purified to apparent homogeneity from liquid state cultures of the thermophilic fungus *Acrophialophora nainiana* by ultrafiltration and a combination of gel filtration and ion-exchange chromatographic procedures (Celestino *et al.*, 2006). The molecular masses of PECl were 35,500 and 30,749 Daltons, as determined by SDS-PAGE and mass spectrometry, respectively. It was more active at 60°C and pH 8.0 and showed high stability at 50°C with half-life of 7 days.

A comprehensive study on the purification and characterization of pectinolytic enzymes produced by *Aspergillus niger* MIUG 16 on raw materials solid-state fermentation has been reported by Dinu *et al.* (2007). Five pectinolytic enzymes were purified using a combination of chromatographic techniques. The purified enzymes were classified with respect to their biochemical properties and substrate specificity. Among these proteins, one revealed polygalacturonase activity, another appeared to be a pectin methylesterase and three were identified as pectate lyases. The capacity of the fungus *A. niger* to produce pectate lyases with optimum pH in acidic domain was reported for the first time.

An acetylesterase was purified from *Aspergillus aculeatus* on the basis of its ability to deacetylate homogalacturonan. A series of well-characterized substrates were prepared and used to

investigate its specificity, including pectin with various degrees of methylation and acetylation, pectic domains with various structures, and oligomers. It was then compared to a rhamnogalacturonan acetyltransferase previously isolated from the same fungus. Both enzymes were active towards various acetylated pectins, and were able to release acetyl groups from acetylated homogalacturonan, oligogalacturonates as well as rhamnogalacturonan at different extent. It was thus concluded that while the two enzymes differed in their efficiencies, they were both pectin acetyltransferases, able to act in the “smooth” as well as in the “hairy” regions of pectin (Bonnin *et al.*, 2008).

Several isolates of *Aspergillus niger* were screened for ability to produce pectinases by the enzymatic index method. All the 34 isolates screened were producers while isolates JGIm2, JGIm3 and JGIm5 being the best. Optimum enzyme production was found after 48 h of fermentation with medium containing 1.5% pectin. Partial purification of the enzyme was carried out by ethanol precipitation to give two fold purification and 56% yield. The enzyme had pH and temperature optima of 4.0 and 45°C, K_m and V_{max} values of 0.178 g dL⁻¹ and 11.62 IU mg⁻¹ protein, respectively (Rashmi *et al.*, 2008).

A long-term investigation of the enzyme hydrolysis of pectin, using a free enzyme membrane reactor (FEMR) was conducted by Rodriguez-Nogales *et al.* (2008). Production of crude enzymes (Polygalacturonase and pectin lyase), in *Aspergillus niger* CECT 2088 cultures grown on an agricultural sub product (apple pomace) as a low cost inducer, was also studied. Observing the retention of induced pectinases by the ultrafiltration membranes, the best FEMR configuration was achieved with the membrane of 10,000 nominal molecular weight limit (NMWL), since the biocatalyst was retained with no loss of enzyme activity.

An alkaline pectin lyase secreted by *Aspergillus flavus* MTCC 7589 was purified to electrophoretic homogeneity using ammonium sulfate fractionation, anion exchange chromatography on DEAE cellulose and gel filtration chromatography on sephadex G-100 (Yadav *et al.*, 2008). The pH and temperature optima of the enzyme were found to be 8.0 and 50°C. The enzyme was found to be stable for 24 h in the pH range 4-10. The enzyme did not loose activity up to 50°C when exposed for 1 h. Addition of ammonium sulfate in the range of 0.1-2.0 M increased the thermostability of the enzyme, 0.6 and 1.8 M of ammonium sulfate providing complete stability at 60 and 70°C respectively. The values of K_m and k_{cat} of the enzyme were 0.59 mg mL⁻¹ and 52.2 S⁻¹ respectively. The molecular weight was found to be 38±01 kDa.

Rashad *et al.* (2010), studied extracellular polygalacturonase (PG) from *Pleurotus ostreatus* NRRL-0366 using lemon peel (*Citrus limonium*) as a substrate and characterized it. The enzyme was purified to a homogenous form with about 27 fold purification and specific activity of 158 U mg⁻¹ proteins by two chromatographic steps in DEAE cellulose and sephacryl S-300. The extracellular PG was a monomer protein with a molecular weight of 21 kDa determined by SDS gel electrophoresis and 20 kDa determined by gel filtration techniques. The optimal pH and temperature values were 3.5, 30°C respectively. The enzyme was stable at 30°C, pH (3.0-3.5). PG was able to hydrolyse pectin and partially polygalacturonic acid exhibiting K_m of 1.33 mg mL⁻¹ and V_{max} of 28.6 μmol min⁻¹. The effect of different metal cations at 1mM concentration on the enzyme activity was studied.

Purification of polygalacturonase (PG) from the cultures of *Aspergillus carbonarius* obtained by acetate buffer extraction after solid-state fermentation was attempted by integrated membrane process and alginate affinity precipitation (Nakkeeran *et al.*, 2010). The carbohydrates were completely eliminated (98 % - 99 %) with a PG recovery of 72-80 per cent during integrated membrane process, which would otherwise interfere with the purification process and lead to enzyme loss. However, specific activity of PG did not improve (1.19–1.21 fold) due to the presence of other similar molecular mass proteins. Under optimum conditions of affinity precipitation, the specific activity of PG enhanced to 2450 U mg⁻¹ (4 fold) with almost complete elimination of carbohydrates and colour compounds resulting in a PG recovery of 61 per cent. The results suggested suitability of affinity precipitation for purification of PG from solid-state cultures and the potential of UF as a single step process for handling eluted PG.

Aspergillus carbonarius, when grown under submerged and on solid-state fermentation, produced different molecular forms of polygalacturonase (PG; EC 3.2.1.15), among them a 42 kDa PG with a high specific activity of 7000 U mg⁻¹ protein (Nakkeeran *et al.*, 2011). When the enzymes were purified by integrated membrane process (IMP) and alginate affinity precipitation (AAP), the two processes concentrated different forms of the enzyme. The AAP process selectively purified and concentrated the high active PG whereas the IMP yielded different PGs along with amylase and protease.

Table 2. Biochemical and physicochemical properties of pectinases

Source of Pectinase	Nature	Molecular weight (kDa)	Isoelectric point (pI)	Specific activity (U mg ⁻¹)	Michaelis constant (Km)	Optimum temperature (°C)	Optimum pH	Temperature stability (°C)	pH stability	Reference
I. Polygalacturonases										
<i>Penicillium frequentans</i>	Exo	63	–	2571	1.60	50	5.0	–	–	Favey <i>et al.</i> (1992)
	Exo	79	–	185	0.06	50	5.8	–	–	
<i>Mucor flavus</i>	-	40	8.3	–	–	45	3.5–5.5	40	2.5–6.0	Margo <i>et al.</i> (1994)
<i>Fusarium oxysporum</i> f.sp. <i>lycopersci</i>	Exo	38	–	209	–	69	11.0	–	7.0–11.0	Pietro and Roncero (1996)
<i>Aspergillus awamori</i>	Endo	41	6.1	487	–	40	5.0	50	4.0–6.0	Nagai <i>et al.</i> (2000)
<i>Aspergillus niger</i>	Endo (PG I)	61	–	982	0.12	43	3.8–4.3	50	–	Singh and Rao (2002)
	Endo (PG II)	38	–	3750	0.72	45	3.0–4.6	51	–	
<i>Aspergillus japonicus</i>	Endo (PG I)	38	5.6	-	-	30	4.0–5.5	-	-	Hasunuma <i>et al.</i> (2003)
	Endo (PG II)	65	3.3	–	-	30	4.0–5.5	–	–	
II. Pectin lyases										
<i>Penicillium italicum</i>	PMGL	22	8.6	-	3.20	40	6.0–7.0	50	8.0	Alana <i>et al.</i> (1990)
<i>Aspergillus japonicus</i>	PMGL	–	7.7	-	0.16	55	6.0	–	-	Dinnella <i>et al.</i> (1994)
<i>Penicillium adametzii</i>	PMGL	–	–	–	-	60	8.0	40	7.0	Sapunova <i>et al.</i> (1995)
<i>P. citrinum</i>	PMGL	–	–	–	-	45	7.0	40	7.0	
<i>P. janthinellum</i>	PMGL	–	–	–	-	40	6.5	40	7.0	
<i>Fusarium monoliforme</i>	PGL	–	-	-	-	-	8.5	75	-	Dixit <i>et al.</i> (2004)
III. Pectin esterases										
<i>Aspergillus niger</i>	-	–	-	-	1.01	45	5.0	-	-	Maldonado and Saad (1998)
<i>A. japonicus</i>	-	46(PE I)	3.8	-	-	-	4.0–5.5	50	-	Hasunuma <i>et al.</i> , (2003)
		47(PE II)	3.8	-	-	-	4.0–5.5	50	-	

Fusarium asiaticum (strain 7071) from wheat (China) produced pectin methylesterase (PE), Polygalacturonase (PG), and pectin lyase (PL) (Glinka and Liao, 2011). The activity of these pectolytic enzymes was detected by a gel diffusion assay. Three forms of PE were identified in a culture filtrate of *F. asiaticum*. Two forms of PE with molecular weights of 31 kDa and 42.5 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were purified using a combination of chromatographic techniques. These PEs did not bind to Concanavalin A (Con A), which was confirmed by chromatography using a Con A agarose column. The 31 kDa purified PE was thermostable in a temperature range of 25-55°C. The optimal pH for the PE of *F. asiaticum* was 6.5.

An endo-polygalacturonase gene (pg I) was cloned from *Penicillium* spp. CGMCC 1669 and expressed in *Pichia pastoris* (Yuan *et al.*, 2011). The full-length cDNA consisted of 1140 bp and encoded a glycoside hydrolase protein with a calculated molecular mass of 37.5 kDa. The optimal pH and temperature of purified recombinant endo-PG I were 3.5 and 40°C, respectively. At 0°C, endo-PG I still retained 7.3 per cent of the maximal activity. The enzyme had a high affinity and specificity towards polygalacturonic acid. The K_m and V_{max} values were 19.5 mg mL⁻¹ and 909.1 U min⁻¹ mg⁻¹, respectively. Addition of endo-PG I (3.4 U mL⁻¹) reduced the intrinsic viscosity of apple juice by 4.5 per cent, and increased the light transmittance by 71.8 per cent.

Gomes *et al.* (2011) conducted an experiment to evaluate polygalacturonase (PG) production and characterization by *Aspergillus niger* ATCC 9642. The maximum PG activity (51.82 U mL⁻¹) was obtained using pectin, L-asparagine, and, iron sulfate at concentrations of 32 g L⁻¹, 2 g L⁻¹, 0.06 g L⁻¹ and 1.0 g L⁻¹, respectively with 180 rpm of stirring rate, 25°C and an initial pH of 4.0. The kinetic study showed that the highest enzyme activity was achieved at 27 h of fermentation. The evaluation of the optimum pH and temperature permitted to observe that the highest PG activities were achieved at 37°C and pH of 5.5. The enzymatic extract presented higher stability at 55°C and pH of 5.0. The results showed that at low temperatures the enzyme extract kept the initial activity until 40 days of storage.

Al-Najada *et al.* (2012), reported partial purification and characterization of polygalacturonases from fruit spoilage. *Fusarium oxysporum* and *Aspergillus tubingensis* were isolated from banana and peach, respectively. By using diethylaminoethyl (DEAE)-Sephacel column, PGaseI and PGaseII forms of polygalacturonases were separated from *F. oxysporum* (PGase) and *A. tubingensis* respectively. The polygalacturonases examined had higher affinity toward various polygalacturonic acids and pectins. The apparent K_m and V_{max} values were reported for the enzymes. Acidic pH optimum (4.0 to 6.0) was also reported for the enzymes. Optimal temperature and thermal stability of the enzymes showed a range from 40 to 60°C.

Vasu *et al.* (2012) purified a *Carica papaya* pectin methylesterase (CpL-PE; EC 3.1.1.11) from a commercial papain preparation. This CpL-PE was separated from the abundant cysteine endopeptidases activities using sequential hydrophobic interaction and cation-exchange chromatographies and then purified by affinity chromatography using Sepharose-immobilized kiwi PE inhibitor protein to obtain a single electrophoretically homogeneous protein. The enzyme was purified 92-fold with 38% yield, providing a specific activity of 1200 U mg⁻¹, and the molecular weight was 35,135 Daltons as determined by MALDI-TOF-MS in linear mode.

Kant *et al.* (2013) conducted an experiment on the production of polygalacturonase from *Aspergillus niger* (MTCC 3323). The enzyme precipitated with 60 per cent ethanol resulted in 1.68-fold purification. The enzyme was purified to 6.52-fold by Sephacryl S-200 gel-filtration chromatography. On SDS-PAGE analysis, enzyme was found to be a heterodimer of 34 and 69 kDa subunit. Homogeneity of the enzyme was checked by native-PAGE and its molecular weight was found to be 106 kDa. The purified enzyme showed maximum activity in the presence of polygalacturonic acid at temperature of 45°C, pH of 4.8 and a reaction time of 15 min. The enzyme was stable within the pH range of 4.0–5.5 for 1 h. At 4°C it retained 50 per cent activity after 108 h but at room temperature it lost its 50 per cent of activity after 3 h. The K_m and V_{max} values of the enzyme were found to be 0.083 mg mL⁻¹ and 18.21 μ mol mL⁻¹ min⁻¹.

2.4 Application of fungal pectinase in fruit juice extraction and clarification

Pectinases are an integral part of fruit processing industry and in the ever growing field of biotechnology. The fruit juices containing vitamins and minerals play pivotal role in the human health and are considered as important processed, value-added products. The production of fruit and vegetable juices is influenced by extraction methods, their clarification and stabilization. Latest

development in the fruit and vegetables processing industry and difficulties encountered during various steps especially in juice filtration diverted the attention to devise new methods to resolve the allied issues. In addition to pectinases, other enzymes such as cellulases and hemicellulases were used in the production of fruit juices. While there are no restrictions on the use of microbial pectinases, the principles of the Council of European Union, forbids hemicellulases and cellulases in fruit juice directive (Reberio *et al.*, 2010).

Guava is one of the richest sources of vitamin C. Its ascorbic acid content may be improved through enzymatic treatment from the peel (Askar *et al.*, 1992). Pectin for example, affects the flavour release from food commodities by enhancing viscosity and/or binding of the aroma compounds with the food matrix.

The raw press juice is rich in insoluble particles, which are mainly made up of pectic substances. These particles are known as 'cloud particles'. In these particles, a protein nucleus with a positive surface charge is coated by negatively charged pectin molecules (Pilnik and Voragen, 1993). This negative charge causes the pectin molecules to repel one another. Pectinases degrade this pectin and expose part of the positively charged protein beneath, thus reducing electrostatic repulsion between cloud particles which causes these particles to aggregate to larger particles. These larger particles eventually settle out, but to improve the process, flocculating agents (finings) such as gelatin, tannin or bentonite (a type of clay) can be added. Yeasts and other microbes, which may contaminate the juice, are also precipitated by finings. What is left is a transparent but by no means clear juice. A second centrifugation and subsequent filtration is needed to give the clear juice that many consumers prefer.

During extraction of pineapple juice from pineapple pulp, Sreenath *et al.* (1994), observed an increase in the juice recovery after treatment with commercial cellulase, a pectinase or their combination at a concentration of 0.025% at 27-30°C for 30 min. The per cent juice recovery of pineapple after enzyme addition reached up to 81-86% as compared to 72% in case of control possibly due to extraction of more soluble solids from the mash. The enzymatic process is accredited to offer a number of advantages over mechanical-thermal comminution of fruit pulps. The enzyme application facilitates the juice extraction process and improves the quality attributes of the finished product. The enzymes not only help in softening the plant tissue but also lead to the release of cell contents that may be recovered with high yield.

Currently, a combination of pectinases, cellulases and hemicellulases collectively called macerating enzymes is used in the extraction and clarification of fruit and vegetable juices (Grassin and Fauquembergue, 1996; Galante *et al.*, 1998). It is considered that pectic substances are enclosing cellulose in the cell wall, Therefore; pectinase hydrolyzes the pectic polysaccharides and expose cellulose to cellulase degradation.

Research was focused on industrially suitable enzymes from food-grade microorganisms, together with increased knowledge on fruit components (Uhlig, 1998). The clarity and homogeneity of clarified products can be achieved by the complete removal of all suspended solids. These suspended solids are mainly polysaccharides (pectin, cellulose, hemicellulose, lignin and starch), proteins, tannin, metals and microorganisms (Vaillant *et al.*, 2001). For clarified fruit juices, turbidity is undesirable from marketing perspectives. Pectinases are extensively used for extraction and clarification of fruit juices (Kashyap *et al.*, 2001). Therefore, it is desirable to test the efficacy of any potential pectinase for the above application from the view point of commercial utilization. Apple juice is more popular worldwide next only to orange juice and contains high amount of pectin. Accordingly, the efficacy of purified PGs from *Aspergillus carbonarius* was tested for the extraction and clarification of apple juice.

For producing date syrup, Al-Hooti *et al.* (2002) used pectinase and observed increased recovery of total soluble solids (68 %) compared with that of control (35 %). The addition of enzymes, even at the level of 0.5%, increased the extraction yield of soluble solids from 63.10 to 66.58 %. The maximum juice was attained by the treatment of fruit pulps with the enzyme mixture containing xylanases (2.0 U mL⁻¹), pectinase (1.5 U mL⁻¹) and cellulase (0.68 U mL⁻¹), respectively. The use of 1 per cent pectinase and cellulase enzyme preparations was found to be optimal for the maximum extraction yields of soluble solids from date pulps.

Carbohydrates, organic acids and phenolic compounds are the major constituents of fruits determining the quality of juices (Versari *et al.*, 2002). During the formation of aroma compounds, the concentration of volatiles changes both qualitatively and quantitatively. The physico-chemical properties of volatiles along with the chemical nature and structure of the food stuff influence the

amount and perception of volatile compounds (Bezman *et al.*, 2003). The sum of sugars, organic acids and volatile compounds affect the sensory properties of fruits and vegetables (Azondanlou *et al.*, 2003).

Flavour enhancement in apricot by the enzymatic treatment has been reported by Riu-Aumatell *et al.* (2004), who assessed the volatile composition of juices of apricot, peach and pear. Peaches were the poorest in volatile compounds, probably because the high polysaccharide content in these samples could cause a higher retention of volatile compounds. Lee *et al.* (2006) studied banana juice treated with pectinase at various enzyme concentrations (0.01– 0.1%), temperatures (30–50 °C) and time (30–120 min). At 30 °C, the clarity of banana juice was increased rapidly at the beginning but with a slower rate towards the end, with an increase in enzyme concentration. The turbidity decreased markedly as enzyme concentration was elevated irrespective of incubation time and temperature of the enzyme treatment.

The studies on pectinase produced from *Aspergillus niger* on clarification of commercial apple juice revealed that most effective clarification along with a viscosity drop of 35.5% was achieved using an enzyme dose of 15 U mL⁻¹ in the presence of 0.01% gelatine at 45°C for 6 h (Singh and Gupta 2004). Kaur *et al.* (2004), reported increased juice yield while treating apple, banana and grapes with PG from *Sporotrichum thermophile*.

Semenova *et al.* (2006), tested the efficacy of chromatographically purified *Penicillium canescens* for the clarification of non clarified apple juice, and reported that its performance was comparable with commercial pectinase preparations.

Roldan *et al.* (2006) investigated on the effect of enzymatic treatment on juice clarification, fermentation and characteristics of young wine. A positive effect on clarification was observed with commercial preparations (E4: pectinases, cellulases and xylanases and E5: mixture of pectinases and cellulases) added to grapes juice. The preparation E5, comprising CMCase and pectinase showed higher than that of E4. Likewise, application of composite enzymes (pectinase, cellulase, papain and α -amylase) to treat the mashed litchi fruit and to evaluate the effects of individual enzyme on the transmittancy, stability and nutritional profile of resulting juice

Shah (2007) treated Litchi pulp with various levels of pectinase (0-0.133% w/w), cellulase (0-0.266% w/w) and hemicellulase (0-0.20% w/w) for different durations (30-150 min) at 45 °C. The enzymatic treatment exhibited an increase in yield, clarity, TSS and reduction in apparent viscosity.

Oszmianski *et al.* (2009) studied the effect of different commercial pectolytic enzymes on apple mash treatment and reported that it had a positive effect on the production of cloudy apple juices by improving its yield and polyphenolic contents. The influence of enzymatic mash treatment at various temperatures (30-60 °C) on the analytical composition of the apple juice revealed that the highest value of juice yield, colloidal concentration and polyphenol content were obtained at 50°C, which was the optimum temperature for the commercial pectinase used in this study.

The study conducted by Sandri *et al.* (2011) analysed the efficiency of fungal pectinolytic preparations produced in laboratory along with commercial products used for the clarification process of apple, butia palm fruit, blueberry, and grape juices. Two crude enzymatic extracts, produced by *Aspergillus niger* T0005007-2 (TE1) and *Aspergillus oryzae* IPT 301 (TE2), were tested in solid-state and submerged cultures, respectively, in comparison to the commercial preparations Pectinex^R Clear and Pectinex^R BE Colour, used for the clarification of clear (apple, butia palm fruit) and dark (blueberry and grape) juices, respectively. The total activity at 1 U mL⁻¹ of fruit juice, reactions were conducted at 30 and 50°C, for 30 and 60 min. Time increase resulted in an improved clarification, whereas temperature increase could not be clearly linked to a greater clarification. When compared to the commercial products, the use of the crude preparation TE1 resulted in similar clarification of apple and blueberry juices.

Juice clarity is vital for the uniformity of end product to have a pleasing hedonic response. In this context, enzymatic treatment is considered to be the single largest processing aid contributor. By the use of multi-enzyme preparations different technological effects can be achieved, like viscosity reduction, improved processing efficiency, clarification and cloud stability of juices

The enzymatic process is accredited to offer a number of advantages over mechanical-thermal comminution of fruit pulps. The enzyme application facilitates the juice extraction process which improves the quality attributes of the finished product. The enzymes not only help in softening the plant tissue but also lead to the release of cell contents that may be recovered with high yield. The

fruit juices containing vitamins and minerals play a pivotal role in the human health. The production of fruit juices is influenced by extraction methods, clarification and stabilization. Latest development in the fruit processing industry and difficulties encountered during various steps especially in juice filtration diverted the attention to devise new methods to resolve the allied issues. Research should be focused on industrially manageable candidate enzymes from food-grade microorganisms, together with increased knowledge on fruit components.

MATERIAL AND METHODS

A series of experiments were conducted in the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad to isolate, characterize and screen pectinase producing fungi from various sources and to produce pectinase under solid state fermentation condition by optimizing process parameters for the selected efficient fungal isolates. Attempts were also made to purify and characterize the pectinase enzymes produced by the selected efficient fungal isolates; and its application in orange and guava fruit juice extraction and clarification was explored. The details of the materials used and the methods followed during the course of investigation are described in this chapter.

3.1 Collection of samples of fruits and vegetable wastes

Decayed, rotten and fungus infected fruit wastes like decayed jackfruit rind (DJFR), decayed pineapple fruit waste (DPW), rotten guava fruit (RGF), decayed lemon peel (DLP) rotten papaya (RP), decayed pomegranate fruit waste (DPFW), rotten citrus fruit (RCF), decayed sapota fruit (DSF), rotten apple fruit (RAF), rotten banana fruit peel (RBFP), rotten ber fruit (RBF) and vegetable wastes like rotten tomato (RT), rotten beans (RB), rotten green chilli (RGC), rotten carrot (RC), and soil with decomposed fruit and vegetable waste in the market yard (S) were collected from Dharwad city fruit and vegetable market. Partially degraded coffee husk waste (CHW) was also collected from the dumping yard near M/s. Amalgamated Bean Coffee Trading Company Ltd. (ABCTCL), Hassan, Karnataka. All the samples were collected in sterile polypropylene bags and stored at 4°C in a refrigerator until the isolation experiments were completed (Plate 1).

3.2 Isolation and primary screening of pectinolytic fungi

3.2.1 Chemicals, media and standard enzyme

All the chemicals used in the present investigation were of analytical grade (AR grade) and procured from M/s. HiMedia Laboratories Limited, Mumbai and from M/s. Sigma-Aldrich Corp, MO, USA. All the experiments for isolation, screening and pectinolytic enzyme production were conducted under sterile conditions with adequate safety measures.

3.2.2 Reference strain *Aspergillus niger* MTCC 1344

An active culture of *Aspergillus niger* MTCC 1344 was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, and maintained by sub culturing on the Czapek dox yeast extract agar (CYA) medium.

3.2.3 Enrichment

Isolation was carried out in two steps viz. i) enrichment and ii) isolation as given by Patil and chaudhari (2010).

One gram of each sample was ground uniformly using a sterile pestle and mortar and inoculated in to 100 mL pectin broth (5 % w/v yeast extract, 1 mL 0.1% v/v bromothymol blue, 0.6 mL 10% v/v $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH 6.0), supplemented with 0.5 per cent w/v pectin (Hi-Media, Mumbai) and incubated on a rotary shaker (120 rpm) at 30°C for 7 days. One mL of this enriched culture was inoculated in to 100 mL of freshly prepared pectin broth supplemented with 1.0 per cent w/v of pectin and enriched for 7 days. Several such serial transfers were made in pectin broth, each time with increasing pectin concentration up to 3.0 per cent. The sample was finally subjected for isolation of pectinase producing fungi.

3.2.4 Isolation of Pectinolytic fungi

After fifth week of enrichment, dilutions upto 10^{-6} were made and aliquots from 10^{-4} , 10^{-5} and 10^{-6} dilutions were plated on modified pectin agar medium as given by Jayasankar and Graham (1970). Ampicillin antibiotic @ 100 mg mL^{-1} was added to the molten and cooled medium to prevent bacterial growth (Appendix I). The above dilutions were plated in triplicates. The plates were then incubated at 30°C for 7 days. The colonies, thus isolated, were sub-cultured 3 times on Czapek dox yeast extract agar (CYA) till active growth of isolates were obtained and thereafter maintained on the Czapek dox yeast extract agar (CYA) medium slants for further experimental work (Appendix II). The isolates were assigned code numbers based on their sources.



Rotten tomato (RT)



Rotten beans (RB)



Rotten carrot (RC)



Rotten green chilli (RGC)



Decayed Sapota Fruit (DSF)



**Fruit and vegetable
market yard soil (S)**



Rotten guava fruit (RGF)



Rotten citrus fruit (RCF)



Rotten papaya (RP)



Coffee husk waste (CHW)



Decayed pineapple



Decayed lemon peel (DLP)



**Decayed pomegranate fruit
waste (DPFW)**



Rotten ber fruit (RBF)



Rotten apple fruit (RAF)



**Rotten banana fruit
peel (RBFP)**



**Decayed jackfruit rind
(DJFR)**

Plate 1: Fruit and Vegetable waste samples used for isolation of pectinase fungi by enrichment culture technique

3.2.5 Primary screening for pectinase activity

Agar discs of purified fungal isolates were transferred to pectin agar medium containing pectin as the sole carbon source and plates incubated at 30°C for 7 days. At the end of the incubation period, the plates were stained with 50mM iodine solution (Appendix III) to view the clear zones around the colonies (Phutela *et al.*, 2005). Selection was done on the basis of formation of clear zones and the corresponding diameter of zone and colony were measured and recorded.

3.2.6 Quantitative estimation of pectinolytic activity

Quantitative estimation of pectinolytic activity of 48 isolates was done under submerged fermentation condition. One mL of freshly grown broth culture of each isolate was transferred to the sterilized pectin broth medium and the flasks incubated on a shaker incubator at 250 rpm for 8 days at 30°C for carrying out different assays namely: polygalacturonase (PG) assay, pectin lyase (PL) assay, pectin esterase (PE) assay and total protein production.

3.2.7 Polygalacturonase (PG) assay

Polygalacturonase (PG) assay was performed by determining the amount of reducing sugars released (galacturonic acid) using dinitrosalicylic acid (DNSA) method (Miller, 1972). D-galacturonic acid was used as standard (Appendix IV).

One unit of Polygalacturonase (PG) has been defined as the amount of enzyme that released one μ mol of galacturonic acid mL min^{-1} under the assay conditions.

3.2.8 Pectin Lyase Assay

Pectin lyase was assayed by measuring the increase in absorbance at 550 nm according to method described by Albersheim and Killias (1962) (Appendix V).

One unit of activity is the amount of enzyme causing a change in absorbance of 0.01 under the conditions of assay.

3.2.9 Pectin esterase (PE) assay

The PE activity was estimated by the method of Kertesz (1955) (Appendix VI).

The enzyme unit was expressed as the amount of enzyme required to produce carboxylic groups to be titrated by one micro-equivalent of NaOH.

3.2.10 Protein determination

Protein was estimated following the method of Lowry *et al.* (1951) using bovine serum albumin as the standard and absorbance was measured at 600 nm (Appendix VII).

3.2.11 Characterization of the fungal isolates

3.2.11.1 Morphological and cultural characteristics of the fungal isolates

Pectinase producing fungal isolates were identified on the basis of colony morphology, cultural and microscopic features. The isolates were inoculated on to plates containing Czapek dox yeast extract (CYA) agar medium and incubated at different temperature (20°C, 30°C, 40°C, and 50°C) for 7-20 days (Florez *et al.*, 2007). The macroscopic variables analyzed included colony type, obverse and reverse mycelium colour, degree of sporulation, and the presence or absence of exudates. The microscopic characteristics of the mycelia were examined by following the procedure given by Campbell and Stewart (1980) in a lactophenol aniline blue stain (Appendix VIII). A drop of 95 per cent alcohol was applied on the slide and the fragments of the culture of the fungus were placed on the slide. It was teased out gently with needles and the alcohol allowed to evaporate. Lacto phenol aniline blue stain was added and the cover slip applied carefully without allowing air bubbles to form. The excess stain around the coverslip was removed with the edge of a blotting paper. The slide was observed under a compound microscope equipped with an image analyser (Motic Images plus 2.0, Digital microscopy software). The isolates were tentatively identified by referring to Hand Book of Soil Fungi by Nagamani *et al.* (2006).

3.2.12 Molecular characterization

3.2.12.1 DNA extraction by the modified CTAB extraction method (Karthikeyan *et al.*, 2010)

The selected 10 efficient fungal isolates were grown on potato-dextrose agar (PDA) at 30°C for three days and 150 mg of fungal mycelia was scraped, frozen in liquid nitrogen and ground to a fine powder using a sterilized pestle and mortar. The grounded mycelium (50-100 mg) was taken in 1.5 mL microfuge tubes and 500 µl of pre warmed (60°C) TES lysis buffer (Appendix IX) (100 mM Tris pH 8.0; 10 mM pH 8.0; 2% SDS), and 50 µg of proteinase K were added to the grounded material, incubated at 60°C for 60 min. A volume of 140 µl of 5M NaCl and 64 µl of 10% (w/v) of CTAB were added to the suspension and incubated at 65°C for 10 min. DNA was extracted by adding equal volume of chloroform: isoamyl alcohol (24:1), centrifuged at 13,000 rpm for 10 min. Extracted DNA was precipitated by adding 6 volumes of ice cold isopropanol and left undisturbed for 4 min. Then the contents were centrifuged and washed twice with 70 % ethanol and suspended in 100 µl TE buffer (10 mM Tris pH 8.0; 1mM EDTA pH 8.0). The RNA was digested by adding 10 mg mL⁻¹ of RNase-A and incubated at 37°C for 45 min for drying and re-dissolved in T₁₀E₁ and stored at -20°C for further use.

3.2.12.2 Polymerase chain reaction

The ITS region was amplified with the universal primers ITS1 (forward primer 5'-TCCGTTGGTGAACCAGCGG-3') and ITS4 (reverse primer 5'-TCCTCCGCT TATTGATATGC-3') reported by White *et al.* (1990). Amplification was performed in 100 µl of reaction mixture. The reactions were performed on PalmCycler, gradient thermal cycler with a palm top computer interface (Corbett Life Sciences (QIAGEN) Mortlake, Australia) using a program described by Phalip *et al.* (2004). The reaction conditions of thermal cycler were set as follows:

Reaction mix	Quantity per reaction
1. Genomic DNA (40 ng µl ⁻¹)	: 2.5 µl
2. Buffer B	: 10.0 µl
3. MgCl ₂ 15 mM	: 6.0 µl
4. dNTPs 2.5mM	: 2.5 µl
5. Taq polymerase (1 U µl ⁻¹)	: 1.0 µl
6. Primer Forward (100 ng µl ⁻¹)	: 5.0 µl
7. Primer Reverse (100 ng µl ⁻¹)	: 5.0 µl
8. Milli Q water	: 68.0 µl

The PCR conditions

1. Lid temperature	: 105°C
2. Initial denaturation	: 94°C for 2 min.
3. Primer annealing	: 52°C for 30 sec.
4. Primer extension	: 72°C for 90 sec.
5. Final extension	: 72°C for 5 min.
6. Number of cycles	: 30

3.2.12.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.5 per cent agarose in 1x TAE (Tris Acetate EDTA) buffer, 0.5 µg mL⁻¹ of ethidium bromide, and a loading buffer (0.25% bromophenol blue in 40% sucrose) (Appendix IX). Four µl of the loading dye was added to 20 µl of PCR product and loaded to the agarose gel. Electrophoresis was carried at 65 V for 1.5 h. The gel was observed under UV light and documented using gel documentation unit.

3.2.13 DNA Sequencing

3.2.13.1 DNA sequencing and sequence analysis

The PCR products generated using the primers ITS1/ITS4 were used as a template for DNA sequencing. The PCR eluted product was sequenced through outsourcing. Samples were sent to M/s. Chromous Biotech Pvt Ltd, Bangalore, India. The sequences obtained were compared with sequences available in the NCBI database using the Basic Local Alignment Search Tool (ntBLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Based on sequence homology, the identification of pectinolytic fungal isolates were confirmed.

3.2.14 Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics Analysis) version 4 given by Tamura *et al.* (2007). Evolutionary history was inferred using Neighbor-Joining method. The DNA sequences were given in FASTA format for multiple sequence alignment using ClustalW2 software. The alignment was done for sequences of newly identified isolates and highly similar sequences retrieved from GeneBank for comparison. The evolutionary distances were computed using the Maximum Composite Likelihood method, and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset using complete deletion option.

3.3 Optimization of culture conditions for maximum enzyme production under submerged condition for the most efficient fungal isolates

3.3.1 Inoculum preparation

The pectin broth was prepared, adjusted to pH 5.5 and autoclaved for 15 min at 121°C and cooled. A loop-full of culture from sporulation medium of fungi raised on Czapek dox yeast extract agar (CYA) slants, was transferred aseptically in a laminar air flow into the conical flask (250 mL) containing 100 mL sterilized inoculum medium. It was incubated on an orbital shaker (130 rpm) at 35°C. After 72 h, the spore count in the inoculum was adjusted between 1×10^5 to 1×10^7 spores mL⁻¹ with the help of a haemocytometer (Fernandez and Soria, 1996).

3.3.2 Effect of inoculum size

The effect of inoculum size at two different dilutions namely, 1×10^5 and 1×10^7 spores mL⁻¹ were tested on pectinase production by the selected isolates under submerged fermentation conditions.

3.3.3 Effect of pH

The effect of different pH values varying from pH 3.0, 4.0, 5.0, 6.0 and pH 7.0 was studied on pectinase production by the selected isolates.

3.3.4 Effect of temperature

The effect of different temperature ranging from 20°C, 30°C, 35°C and 40°C was evaluated on pectinase production by the selected isolates.

3.3.5 Effect of substrate (pectin) concentration

The effect of pectin concentration in the medium varying from 0.1%, 0.5%, 1.0%, 1.5% and 2.0% was tested on pectinase production by the selected isolates.

3.3.6 Effect of incubation time

The effect of different incubation time such as 2, 4, 6, and 8 days was studied on pectinase production by the selected isolates.

3.3.7 Enzyme production

After incubation, the mycelial mass was separated by filtration and the resulting supernatant centrifuged using a refrigerated centrifuge (Plasto Craft) at 10,000 rpm for 10 min. The supernatant was carefully transferred to a sterile container and the samples used for further studies.

3.4 Screening of agro wastes for maximum enzyme production under solid state fermentation (SSF) condition

3.4.1 Collection of different agro wastes used as substrates for solid state fermentation (SSF)

Citrus peels were collected from different fruit juice centers, located in Hubli and Dharwad city. Pineapple peel and jackfruit rind waste were collected from push cart vendors in Dharwad city market. Sunflower head and banana pseudostem were collected from threshing yard of MARS, UAS, Dharwad. Coffee cherry husk was collected from Amalgamated Bean Coffee Trading Company Ltd (ABCTCL) Hassan, Karnataka. Wheat bran was collected from a local flour mill in Dharwad. The pectin content in these wastes were analysed and results are furnished in Table 3.

3.4.2 Rapid screening of agro wastes for pectinase production by solid state fermentation (Singh *et al.*, 1999).

The agro wastes were washed many a times with clean water to remove all adhering substances. Small pieces of peels were prepared using a knife and dried in a hot air oven at 50°C for 48 h. The dried substrates were powdered using a pilot scale Willey huller mill to a 2.0 mm and 4.0 mm size and stored in polypropylene covers for further use. The wheat bran was dried, ground, treated with 1.5% NaOH and stored in sterile polythene covers for subsequent use in the fermentation medium (Plate 2).

3.4.3 Extraction of pectin from different substrates

Pectin was extracted by the method given by Rao and Maini (1999). Each substrate powder (15 g) was weighed and taken in 150 mL flask, 30 mL of 0.05N Hydrochloric acid added to it and the extraction was done by boiling the above mixture at 100°C for 60 min. The mixture was then separated from the extract after filtration. The extraction was performed twice. The filterates obtained were combined and cooled. Two volumes of absolute alcohol were added to precipitate pectin.

Yield of pectin was calculated by the following formula:

Table 3. Pectin yield and calcium pectate content in different substrates

Sl. No.	Name of agro waste	Crude pectin yield (%)	Calcium pectate (%)
1	Wheat bran (WB)	5.17	2.71
2	Sunflower head (SFH)	12.00	9.73
3	Coffee husk waste (CHW)	5.70	5.00
4	Banana pseudo stem (BSS)	2.73	1.57
5	Citrus peel (CP)	13.93	12.89
6	Jackfruit rind (JFR)	8.27	7.63
7	Pineapple peel (PP)	6.07	4.73

$$\text{Yield of crude pectin (\%)} = \frac{\text{Amount of pectin obtained} \times 100}{\text{Total amount of substrate powder}}$$

3.4.4 Estimation of pectin as calcium pectate

Pectin content in the substrates was estimated as calcium pectate following the method given by Ranganna (2000) (Appendix X).



Citrus peel waste



Banana pseudo stem



Willey Huller mill



Coffee pulp waste



Deseeded Sunflower head



Pineapple fruit waste



Wheat bran



Jackfruit rind waste

Plate 2: Preparation of agro wastes for solid state fermentation experiment

3.4.5 Solid state fermentation (SSF) studies

3.4.5.1 Fermentative organisms and sporulation medium

The pure cultures of the selected efficient isolates and the reference fungus *Aspergillus niger* MTCC1344 were used for the SSF studies. All the fungal cultures were raised on slants containing Czapek dox yeast extract agar (CYA) sporulation medium. The sporulation medium was prepared and the pH adjusted to 5.0 with 1M HCl/1M NaOH.

3.4.5.2 Process variables

The following process variables were studied for optimization of solid state fermentation (SSF) for maximum enzyme production using the best substrate along with wheat bran as the standard check.

1. Moisture content of the substrate (%): 55, 60, 65, 70 and 75.
2. Inoculum (spore) load : 1×10^5 spores g^{-1} and 1×10^7 spores g^{-1}
3. Substrate particle size (mm) : 2.0 and 4.0.
4. Incubation period : 2, 4, 6 and 8 days
5. pH : 3.0, 4.0, 5.0, 6.0 and 7.0
6. Temperature ($^{\circ}C$): 20, 30, 35 and 40.
7. Nitrogen sources: Ammonium sulfate and soybean meal at 1% and 2% each.
8. Carbon sources: Glucose and sucrose at 1% and 2% each.
9. Salts: $ZnCl_2$ and $MnSO_4$ at 1% and 2% each.

The experiment was conducted for the selection of the optimum particle size of substrates for maximum pectinases production. The particle size of 2 mm and 4 mm of the substrate was obtained by sieving the powdered substrate using mechanical sievers with 2 mm and 4 mm mesh size. The different moisture levels (55-75%) of the substrate were maintained by adding sterile distilled water. Five different pH levels (3.0, 4.0, 5.0, 6.0 and 7.0) of the medium were evaluated for the maximum enzyme production. The respective pH of substrates was adjusted accordingly with either 0.01 M HCl or 0.01 M NaOH. The temperature range from 20 to 50 $^{\circ}C$ was maintained in the BOD incubator throughout the incubation period. Similarly, effect of different salts, carbon sources and nitrogen sources at 1 per cent and 2 per cent level were studied as given by (Teixeira *et al.*, 2000). Four fermentation periods namely 2,4,6,8 days were given to each treatment to arrive at the best period of fermentation under pre-optimized conditions.

3.4.5.3 Solid state fermentation process

SSF process was carried out in sterile 250 mL Erlenmeyer flasks. Experiments were performed using powder of wheat bran, coffee husk; citrus peel, deseeded sunflower head and banana pseudostem as the sole carbon sources. Attempts were also made to study the effect of combination of substrates on enzyme production by supplementing with pectin rich wastes such as citrus peel, jackfruit rind and pineapple peel in the ratio of 2:1:1. Three replicates were maintained for each substrate. The aliquots were drawn on 2nd, 4th, 6th and 8th day of incubation period and for performing various assays.

The SSF was carried out as follows: 10 g of substrate in a flask was mixed with adequate quantity of sterile water to maintain the moisture content along with the salt solution. The pH of substrates was adjusted accordingly with either 0.01 M HCl or 0.01 M NaOH and autoclaved for 15 min at 121 $^{\circ}C$ and cooled. The spore load was adjusted in the range from 1×10^5 to 1×10^7 spores mL^{-1} in the homogenous spore suspension. From this suspension, 1 mL was drawn and inoculated into each flask to solid substrate at different temperature ($^{\circ}C$) and incubated for respective period. For proper aeration, flasks were intermittently shaken (Plate 3). In order to estimate the total protein, polygalacturonase, pectin esterase and pectin lyase, the samples were drawn and properly mixed with sterile distilled water (1:3, w/v). The mixture, thus, obtained was gently shaken and filtered using a coarse filter paper. The obtained filtrate was centrifuged and the resulting crude enzyme extract was used for conducting these assays. In the above studies the optimized factor was adopted in the subsequent experiments.

3.4.5.4 Scale up studies

Under all optimized conditions, the best fungal isolates producing pectinase was cultivated on 1 kg of the substrate using plastic trays of size (35 cm length, 30 cm width and 6 cm height) and the enzyme extracted was quantified using the method of Singh *et al.*, (1999).

3.4.5.5 Sample harvesting

After 6 days of incubation, experimental trays were harvested. The extract from SSF trays was prepared by mixing a known quantity of fermented material with distilled water (1: 3, w/v) for 30 min on a rotary shaker (180 rpm). The fermented biomass sample was filtered using a muslin cloth and the filtrate was centrifuged at 10,000 rpm for 10 min at 4°C temperature to remove the spores of the organism. The supernatant was filtrated again on Whatman no.1 filter paper and stored aseptically under refrigerated conditions to prevent contamination. The crude enzyme extract, thus, obtained was subjected to purification after enzyme assay.

3.4.6 Purification and characterization of pectinase enzyme

3.4.6.1 Ammonium sulfate fractionation

The cell free extract (30 mL) of the enzyme produced by the selected efficient fungal isolates was subjected to precipitation using ammonium sulfate starting from 20 per cent up to 90 per cent with 10 per cent increase each time (Appendix XI). The precipitated protein was removed by centrifugation at 10,000 rpm for 30 min at 4°C and the supernatant was subjected for purification with further addition of ammonium sulfate to remove other unknown proteinaceous material (Afifi *et al.*, 2002). Ammonium sulfate precipitated pellets were dissolved in 0.01M Tris-HCl buffer (pH 6.0).

3.4.6.2 Dialysis

After ammonium sulfate precipitation, the enzyme salt solution was dialyzed against the 0.01M Tris-HCl buffer (pH 6.0) for 24 h at 4°C to remove ammonium salt with changes of the buffer at every 4 h interval.

The dialysis was carried out using cellulose tubing (dialysis sacks) which retains proteins with Molecular weight higher than 12,000 Da. They were obtained from Sigma-Aldrich, USA. The tubes measured a length of 30 cm and flat width of 25 mm with inflated diam. of approx. 16 mm open ended.

3.4.7 Gel filtration chromatography

3.4.7.1 Pretreatment of Sephadex G-100

Sephadex G-100 (5.0 g) (M/s. Sigma-Aldrich) was suspended in 50 mL distilled water placed in a boiling water bath for 5 h and the solution stirred occasionally. After cooling, distilled water was decanted and the swelled gel was equilibrated with 1M Tris-HCl running buffer (pH 6.0).

The dialyzed enzyme fraction was further purified as per the method of Keller *et al.* (2006) with certain modifications. It was loaded on to Sephadex G- 100 column (50 x 2.0 cm, bed volume 80 mL) and eluted with 1M Tris-HCl running buffer (pH 6.0) (Appendix XII) at the flow rate of 30 mL h⁻¹. Many fractions of 2 mL per tube were collected and the fraction in which protein present was detected. The fractions bearing high protein were collected and evaluated for pectinase activity. The fractions showing higher enzyme activity were pooled together for further characterization.

3.4.7.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis was carried out following the protocol given by Sambrook and Russel (2001).

3.4.7.3 Preparation of the Polyacrylamide Gel

The glass plates were assembled as per the manufacturer's instructions. Ten mL of 10 per cent (Appendix XIII) polyacrylamide gel was poured into the gap between the glass plates leaving sufficient space for the stacking gel. SDS (10 %) was poured on the top of the polyacrylamide gel in order to make the surface smooth and level. The gel was left for 30 min or until it solidified. After solidification, the SDS layer was removed by washing with water for two to three times. About 4 mL of 5 per cent stacking gel was poured on the resolving gel after inserting the comb. After the polymerization, the comb was carefully removed and wells washed with the deionized water for two to



General view of SSF fermentation set up



Clear view of proteolytic fungal growth on citrus peel in SSF experiment



Scale up studies using tray fermentors



Growth of *Aspergillus niger* RBF 96 on citrus peel

Plate 3: Solid state fermentation experimental set up

three times to remove the unpolymerized acrylamide. While pouring the resolving gel and stacking gel, enough care was taken to avoid air bubble formation in the SDS-PAGE.

3.4.7.4 Preparation of sample for the SDS- PAGE

The sample for the SDS-PAGE was prepared freshly as described in the section 3.4.6 and 3.4.7 and diluted to get the equal concentration of total protein. To this supernatant, equal volume of the loading dye (Appendix XIII) was added and heated upto 95°C for 10 minutes. To each well, about 10 µg of the sample and equal volume of dye (v/v) mixture were loaded.

3.4.7.5 Running of the Polyacrylamide Gel Electrophoresis

After loading the dye, the apparatus was submerged in the running buffer and rest of the parts were assembled as per the manufacturer's instruction. Initially, the voltage of the current was kept at 70 mV and when protein entered the resolving gel the voltage was increased upto 120 mV. The gel was run upto the end of the resolving gel, for about 4 h. After sufficient run, the glass plates were carefully removed and the gel transferred to a tray containing the staining solution.

3.4.7.6 Staining and De-staining of Polyacrylamide Gel

After running the polyacrylamide gel electrophoresis, the gel was carefully removed and placed in the staining solution (Appendix XIII). The gel was stained for 3 h in shaking condition. After staining with coomassie brilliant blue, the excess dye was removed by destaining solution (Appendix XIII) under shaking condition. The de-staining solution was replaced for two to three times until the clear blue colour bands appeared. The gel was sealed in polyethylene bag and stored at 4°C. The purity of the enzyme was checked by comparing with the standard protein marker (M/s. genetix Biotech Asia pvt. ltd. with approximate protein size of 10- 250 kDa) bands and purified enzyme and crude enzyme bands for determination of the molecular weight.

3.4.8 Characterization of the pure enzyme

3.4.8.1 Optimum Temperature

The maximum activity of the pure enzyme was determined at different incubation temperatures which ranged from 4, 10, 20, 30, 40, 50, 60, and 70°C for 30 min.

3.4.8.2 Optimum pH of the pure enzyme

The pH optimum was determined using the same assay method with 0.1 M sodium acetate buffer (for the pH range 3.0-5.0), phosphate buffer (for the pH range 6.0-7.0) and 0.1 M Tris – HCl buffer (for the pH 8.0-9.0). The pure enzyme was incubated at different pH values at 50°C for 10 min and the activity measured under the standard conditions.

3.4.8.3 K_m values of the purified enzymes

The Michaelis constants (K_M and V_{MAX} values) of pectinases were determined by measuring the activity reaction rates. The K_m value was determined from Lineweaver-Burk plot (Appendix XIV) by using pectin as substrate at concentrations of 0.2 to 1.0 mg mL⁻¹.

3.5 Application of pectinase in orange and guava fruit juice clarification

3.5.1 Pectinase in clarification of cloudy juices

Pectin rich fruits like orange and guava were extracted and clarified by adding pectinase at different concentrations following the method prescribed by Alkorta *et al.* (1998).

3.5.2 Procurement of raw materials

Orange and guava fruits were purchased from Dharwad city fruit market. The pectinases purified in this study were used to extract and clarify the juices. The pectinolytic enzymes procured from M/s. Hi-Media were used as the standard check.

3.5.3 Juice extraction and clarification

Orange and guava fruits were washed using clean water, peeled and mashed using a lab scale manually operated juicer. Their TSS and pH were recorded. The pulp (100 g) was incubated with different concentrations of the pectinase enzyme singly and in combination at 50°C temperature, with constant stirring, for different time intervals. Later on, the samples were kept in boiling water for 5

min to inactivate the enzymes and after cooling, the juices were filtered, measured and subjected to physico-chemical analysis.

3.5.4. Juice yield

The volume of the juice obtained after filtration was measured. The juice recovery in per cent (g/100g of fresh pulp) was calculated using the formula,

$$\text{Per cent juice yield} = \frac{\text{Weight of the filtrate}}{\text{Weight of the sample taken}} \times 100$$

3.5.5 Treatment details

1. Different concentrations of the efficient fungal pectinase and commercial pectinase enzymes are used for treating both orange and guava fruits juices with three replications for each treatment in completely randomized Design (CRD) experiment.
2. The pectinase enzyme treated orange and guava juices were incubated for 60 min and 120 min at 50°C to obtain the maximum clarification.

Treatments (enzyme U 100 g ⁻¹ fruits pulp)
T ₁ - No enzyme
T ₂ - Polygalacturonase (PG) 50 U
T ₃ - Pectin lyase (PL) 50 U
T ₄ - Pectin esterase (PE) 50 U
T ₅ - PG+PL+PE 50 U (16.66 U each)
T ₆ - Polygalacturonase (PG) 100 U
T ₇ - Pectin lyase (PL) 100 U
T ₈ - Pectin esterase (PE) 100 U
T ₉ - PG+PL+PE 100 U (33.33 U each)
T ₁₀ - Commercial PG 50 U
T ₁₁ - Commercial PL 50 U
T ₁₂ - Commercial PE 50 U
T ₁₃ - Commercial PG+PL+PE 50 U (16.66 U each)
T ₁₄ - Commercial PG 100 U
T ₁₅ - Commercial PL 100 U
T ₁₆ - Commercial PE 100 U
T ₁₇ - Commercial PG+PL+PE 100 U (33.33 U each)

3.5.5.1 Physical and chemical analysis of the juice

3.5.5.2 Clarity

Clarity of the juices was determined by measuring the transmittance at 660 nm using a spectrophotometer, with distilled water as the reference.

3.5.5.3 Estimation of titrable acidity

The titratable acidity of the juice was determined and expressed as per cent citric acid (Sreenath and Santhanam, 1992).

3.5.5.4 Estimation of Total soluble solids

The total soluble solid (TSS) in the juice was determined using a hand refractometer and expressed as °Brix (Yokotsuka, 1990).

3.5.5.6 Sensory evaluation

The sensory evaluation of the enzyme treated juice was done by trained taste panel consisting of six members for colour, flavour and taste following hedonic scoring system (Appendix XV) as described by Meilgaard *et al.* (2007) was followed in this evaluation.

3.6 Statistical analysis

The data averaged into respective parameter requisites were subjected to suitable transformation wherever required. After proper analysis, data were accommodated in the tables as per the needs of objectives for interpretation of results. Computer software in MS Excel was used for analysis. The standard procedures in agricultural statistics given by Gomez and Gomez (1976) were consulted throughout. Factorial, Complete Randomized Design (CRD) was applied and to determine the level of significance, the data obtained for each parameter was subjected to Analysis of Variance (ANOVA) technique.

EXPERIMENTAL RESULTS

The present investigation was undertaken to isolate, characterized and screened fungi for pectinase production. Attempts were also made to screen agro residues for pectinase production under SSF conditions. Various process parameters were optimized for maximum production of pectinase; and its application for extraction and clarification of orange and guava juices were explored. The results of these experiments are presented in this chapter.

4.1 Isolation of pectinolytic fungi

Pectinase producing fungi were isolated from different fruit and vegetable wastes using pectin agar medium following selective enrichment and isolation technique. The details of the waste sample used for isolation, the number of isolates obtained and the code numbers assigned to the individual isolates are furnished in Table 4. Totally, as many as 100 isolates were obtained (Plate 4). From the vegetable wastes, three isolates were from rotten tomato (RT), four from rotten beans (RB), 14 from rotten green chilli (RGC), eight from rotten carrot (RC), and five from fruit and vegetable waste market yard soil (S). Using fruit wastes like decayed jackfruit rind (DJFR), five isolates were obtained. Similarly, three from decayed pineapple fruit waste (DPW), four each from rotten guava fruit (RGF) and decayed sapota fruit (DSF), rotten seven each from decayed lemon peel (DLP) and rotten papaya (RP), two each from decayed pomegranate fruit waste (DPFW), banana fruit peel (RBFP) and coffee husk waste (CHW), 12 each from rotten citrus fruit (RCF), rotten apple fruit (RAF) and six from rotten ber fruit (RBF) were obtained.

4.1.1 Primary screening of isolates for pectinase activity on pectin agar medium

Based on visual observations of clear zone formation by the fungal isolates on pectin agar medium, when stained with 50 mM iodine solution, the pectinase potency index values were calculated by measuring the corresponding clear zone and colony diameter of the isolate. The details of the results of pectinase potency index of the isolates are presented in Table 5, Fig. 4 and Plate 5.

Totally, 48 isolates were found positive for pectinase activity. Amongst these, 10 possessed potency index of more than three. These included: RBF96 (3.25), RC27 (3.21), DLP51 (3.21), RGC14 (3.13), RAF81 (3.13), RBFP92 (3.09), RAF80 (3.08), RAF88 (3.08), RCFW68 (3.04) and RC25 (3.01), while the reference strain *Aspergillus niger* MTCC1344 resulted in 2.99. As many as 20 isolates produced a pectinase potency of more than two. They were: RBF95 (2.89), RAF86 (2.83), RCFW64 (2.80), RBF98 (2.79), RCFW65 (2.64), DPW40 (2.53), DPFW62 (2.43), DPW42 (2.39), DLP47 (2.32), RC29 (2.30), DJFR37 (2.28), RC23 (2.24), DPW41 (2.16), RC28 (2.16), RB7 (2.10), DJFR39 (2.10), DJFR36 (2.10), RGC15 (2.09), RC22 (2.06) and RAF90 (2.03). The remaining 18 isolates had a potency index values less than two namely, RB5 (1.69), RAF89 (1.54), RAF87 (1.50), RGC9 (1.49), DSF76 (1.48), RCFW70 (1.48), RGF43 (1.48), RCFW72 (1.47), RBFP91 (1.47), RBF100 (1.46), RBF99 (1.44), RGC16 (1.42), DJFR35 (1.40), RB4 (1.37), RCFW73 (1.31), RGC20 (1.26), DLP49 (1.25) and S33 (1.23).

4.1.2 Quantitative estimation of pectinolytic activity of the isolates

After preliminary screening of the isolates, quantitative estimation of pectinolytic activities of the isolates was done under submerged fermentation conditions. All the pectinase positive isolates including the reference strain *A. niger* MTCC1344 were grown in pectin broth to estimate pectinolytic enzymes viz., polygalacturonase (PG), pectin lyase (PL) and pectin esterase (PE).

The data on pectinolytic profiles of the fungal isolates are presented in Table 6 and Fig 5. All the isolates produced multiple enzymes. Of all the isolates, RBF96 showed the maximum polygalacturonase activity (189.33 U mL^{-1}), followed by DLP51 (167.32 U mL^{-1}), RC27 (165.50 U mL^{-1}), RAF81 (162.94 U mL^{-1}), RGC14 (162.51 U mL^{-1}) as compared to the reference strain *A. niger* MTCC1344 (153.32 U mL^{-1}). The lowest PG production was observed in the isolate RGC9 (106.41 U mL^{-1}).

With regard to pectin lyase production, DLP51 showed the highest activity (372 U mL^{-1}) followed by RAF88 (294.67 U mL^{-1}), RC23 (282 U mL^{-1}) as compared to the reference strain *A. niger* MTCC1344 (156 U mL^{-1}) (Table 6 and Fig. 6). The least pectin lyase production was observed in the isolate RBF100 (39 U mL^{-1}).

The data pertaining to pectin esterase production by the pectinolytic isolates in Table 6 and Fig. 7 showed that RC27 produced the maximum pectin esterase activity (375 U mL^{-1}), followed by

RC25 (350 U mL⁻¹), RBF99 (335 U mL⁻¹) and DSF76 (333 U mL⁻¹) as compared to the reference strain *A. niger* MTCC1344 (310 U mL⁻¹). The least PL activity was seen in the isolate RB7 (118.33 U mL⁻¹).

4.1.3 Identification of the pectinase positive fungal isolates based on colony morphology, growth characters and microscopic observations

The detailed results of the colony characteristics of all the pectinolytic isolates on Czapek dox yeast extract agar medium (CYA), their microscopic features and the growth of these isolates at different temperatures are furnished in Table 7 and Plate 6. The microscopic examination of the isolates was performed using a compound microscope, attached with an Image Analyser. All the strains were identified upto the species level by referring to the Hand Book of Soil Fungi (Nagamani *et al.*, 2006). Out of 48 isolates, as many as 38 belonged to the *Aspergillus* genus (RB4, RB5, RGC9, RGC15, RGC16, RGC20, RC22, RC25, RC27, RC28, RC29, S33, DJFR35, DJFR36, DJFR37, DPW40, DPW41, DPW42, RGF43, DLP47, DLP49, DPFW62, RCFW64, RCFW65, RCFW68, RCFW70, RCFW72, DSF76, RAF80, RAF81, RAF90, RBFP91, RBFP92, RBF95, RBF96, RBF98, RBF99 and RBF100). Eight isolates were identified as *Penicillium* (RB7, RGC14, RC23, DJFR39, DLP51, RCFW73, RAF86 and RAF89) and the remaining two as *Fusarium*.

4.1.4 Molecular characterization of the selected fungal isolates

The molecular identification of selected isolates was carried out by *In Silico* analysis of ITS rDNA region sequences of the isolates. Based on the potency index values and the pectinolytic enzyme production, 10 most efficient isolates namely RGC14, RC25, RC27, DLP51, RCFW68, RAF80, RAF81, RAF88, RBFP92 and RBF96 were selected for molecular identification. The isolate RBF96 (showing maximum PG activity), DLP51 (showing maximum pectin lyase activity), RC27 (showing the highest pectin esterase activity) and RAF88 which was tentatively identified as *Fusarium* were selected for partial sequencing of their PCR amplified ITS rDNA regions and the sequencing results are presented in Table 8 and Plate 7.

Based on maximum identity of the NCBI GenBank matching accession number and ITS rDNA partial sequences of the potent pectinolytic fungal isolates by using nucleotide BLAST search in the NCBI database, their molecular identification was confirmed. The detailed *In Silico* BLAST analysis results are furnished in Table 9. The pectinolytic fungal isolates namely RBF96, RC27, DLP51 and RAF88 were confirmed as *Aspergillus niger* KC119204.1, *Aspergillus oryzae* HF546392.1, *Penicillium citrinum* KC344966.1 and *Fusarium sporotrichioides* FR750924.1 respectively.

4.1.5 Phylogenetic analysis

The evolutionary history of 25 taxa was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 per cent bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches (Fig 8). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There were a total of 297 positions in the final dataset.

4.1.6 Optimization of culture parameters for maximum enzyme production by the most efficient fungal isolates under submerged conditions

Different culture parameters such as inoculum levels, pH of the medium, incubation temperatures, substrate levels and incubation time were optimized. *Aspergillus niger* RBF96 for the production of polygalacturonase (PG), *Penicillium citrinum* DLP51 for the production of pectin lyase (PL) and *Aspergillus oryzae* for pectin esterase (PE) production were finally selected for further studies. *A. niger* MTCC1344 was used as the reference strain for comparison purposes. The colony morphology and microscopic features of these strains are shown in Plate 8.

4.1.7 Effect of inoculum size on polygalacturonase production under submerged fermentation

The data presented in Table 10 indicated that production of polygalacturonase was significantly affected due to varied levels of inoculation of fungal spores in the growth medium. The

Table 4. List of fungi isolated from different fruit and vegetable wastes on pectin agar medium

Sl. No.	Particulars of the waste sample used	Code number of the isolates	Number of isolates
1	Rotten tomato(RT)	RT1, RT 2, RT 3	3
2	Rotten beans (RB)	RB4, RB 5, RB 6, RB 7	4
3	Rotten green chilli (RGC)	RGC 8, RGC 9, RGC 10, RGC 11, RGC 12, RGC 13, RGC 14, RGC 15, RGC 16, RGC 17, RGC18, RGC 19, RGC 20, RGC 21	14
4	Rotten carrot (RC)	RC 22, RC 23, RC 24, RC 25, RC 26, RC 27, RC 28, RC 29	8
5	Fruit and vegetable waste market yard soil (S)	S30,S31,S32,S33,S34	5
6	Decayed jackfruit rind (DJFR)	DJFR 35, DJFR 36, DJFR 37, DJFR 38, DJFR 39	5
7	Decayed pineapple waste (DPW)	DPW 40, DPW 41, DPW 42	3
8	Rotten guava fruit (RGF)	RGF 43, RGF 44, RGF 45, RGF 46	4
9	Decayed lemon peel (DLP)	DLP 47, DLP 48, DLP 49, DLP 50, DLP 51, DLP 52, DLP 53	7
10	Rotten papaya (RP)	RP 54, RP 55, RP 56, RP 57, RP 58, RP 59, RP 60	7
11	Decayed pomegranate fruit waste (DPFW)	DPFW 61, DPFW 62	2
12	Rotten citrus fruit waste (RCFW)	RCFW63, RCFW64, RCFW 65, RCFW 66, RCFW 67, RCFW 68, RCFW 69, RCFW 70, RCFW 71, RCFW 72, RCFW 73, RCFW 74	12
13	Decayed Sapota fruit (DSF)	DSF 75, DSF 76, DSF 77, DSF 78	4
14	Rotten apple fruit (RAF)	RAF 79, RAF 80, RAF 81, RAF 82, RAF 83, RAF 84, RAF 85, RAF 86, RAF 87, RAF 88, RAF 89, RAF 90	12
15	Rotten banana fruit peel (RBFP)	RBFP 91, RBFP 92	2
16	Coffee husk waste (CHW)	CHW 93, CHW 94	2
17	Rotten ber fruit (RBF)	RBF95, RBF96, RBF 97, RBF 98, RBF 99, RBF 100	6
Total number of isolates			100

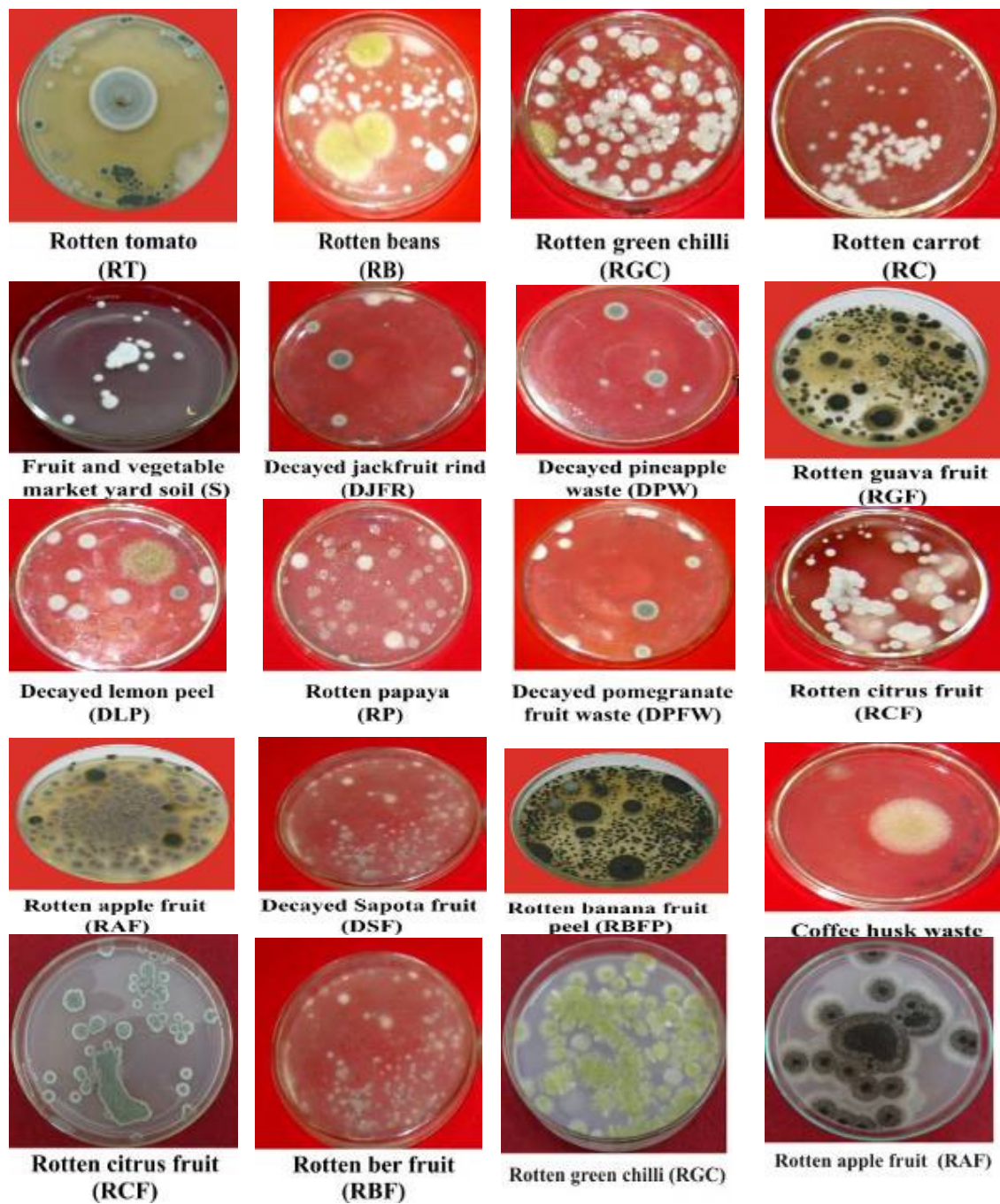


Plate 4: Occurrence of fungus on pectin agar medium from different fruit and vegetable waste samples

Table 5. Pectinase potency index values of the fungal isolates

Sl. No.	Code number of the isolate	Colony diameter (mm)	Zone + colony diameter (mm)	Potency index = (Zone dia+ colony dia/colony dia) (mm)
1	RT1	-	-	-
2	RT2	-	-	-
3	RT3	-	-	-
4	RB4	9.50	13.00	1.37
5	RB5	12.17	20.60	1.69
6	RB6	-	-	-
7	RB7	10.00	21.00	2.10
8	RGC8	-	-	-
9	RGC9	12.10	18.00	1.49
10	RGC10	-	-	-
11	RGC11	-	-	-
12	RGC12	-	-	-
13	RGC13	-	-	-
14	RGC14	8.00	25.00	3.13
15	RGC15	11.00	23.00	2.09
16	RGC16	16.00	22.67	1.42
17	RGC17	-	-	-
18	RGC18	-	-	-
19	RGC19	-	-	-
20	RGC20	10.00	12.60	1.26
21	RGC21	-	-	-
22	RC22	10.30	21.20	2.06
23	RC23	9.00	20.20	2.24
24	RC24	-	-	-
25	RC25	8.00	24.10	3.01
26	RC26	-	-	-
27	RC27	7.00	22.50	3.21
28	RC28	5.00	10.80	2.16
29	RC29	5.00	11.50	2.30
30	S30	-	-	-
31	S31	-	-	-
32	S32	-	-	-

*Contd....***Note:** - No halo zone, colony diameter not measured

Table 5 contd....

Sl. No.	Code number of the isolate	Colony diameter (mm)	Zone + colony diameter (mm)	Potency index = (Zone dia+ colony dia/colony dia)(mm)
33	S33	30.00	37.00	1.23
34	S34	-	-	-
35	DJFR35	15.80	22.10	1.40
36	DJFR36	12.60	26.40	2.10
37	DJFR37	12.00	27.40	2.28
38	DJFR38	-	-	-
39	DJFR39	12.60	26.40	2.10
40	DPW40	4.00	10.10	2.53
41	DPW41	5.00	10.80	2.16
42	DPW42	9.00	21.50	2.39
43	RGF43	20.00	29.50	1.48
44	RGF44	-	-	-
45	RGF45	-	-	-
46	RGF46	-	-	-
47	DLP47	12.50	29.00	2.32
48	DLP48	-	-	-
49	DLP49	15.00	18.70	1.25
50	DLP50	-	-	-
51	DLP51	8.50	27.30	3.21
52	DLP52	-	-	-
53	DLP53	-	-	-
54	RP54	-	-	-
55	RP55	-	-	-
56	RP56	-	-	-
57	RP57	-	-	-
58	RP58	-	-	-
59	RP59	-	-	-
60	RP60	-	-	-
61	DPFW61	-	-	-
62	DPFW62	11.50	28.00	2.43
63	RCFW63	-	-	-
64	RCFW64	3.50	9.80	2.80
65	RCFW65	9.10	24.00	2.64
66	RCFW66	-	-	-
67	RCFW67	-	-	-

Contd....

Note: - No halo zone, colony diameter not measured

Table 5 contd....

Sl. No.	Code number of the isolate	Colony diameter (mm)	Zone + colony diameter (mm)	Potency index = (Zone dia+ colony dia/colony dia)(mm)
68	RCFW68	7.10	21.60	3.04
69	RCFW69	-	-	-
70	RCFW70	12.20	18.10	1.48
71	RCFW71	-	-	-
72	RCFW72	18.00	26.50	1.47
73	RCFW73	24.00	31.50	1.31
74	RCFW74	-	-	-
75	DSF75	-	-	-
76	DSF76	12.20	18.10	1.48
77	DSF77	-	-	-
78	DSF78	-	-	-
79	RAF79	-	-	-
80	RAF80	8.80	27.10	3.08
81	RAF81	8.30	26.00	3.13
82	RAF82	-	-	-
83	RAF83	-	-	-
84	RAF84	-	-	-
85	RAF85	-	-	-
86	RAF86	9.60	27.20	2.83
87	RAF87	7.00	10.50	1.50
88	RAF88	10.00	30.83	3.08
89	RAF89	10.50	16.20	1.54
90	RAF90	6.50	13.20	2.03
91	RBF91	12.10	17.83	1.47
92	RBF92	8.10	25.00	3.09
93	CHW93	-	-	-
94	CHW94	-	-	-
95	RBF95	4.70	13.60	2.89
96	RBF96	8.00	26.00	3.25
97	RBF97	-	-	-
98	RBF98	10.40	29.00	2.79
99	RBF99	8.50	12.20	1.44
100	RBF100	13.00	19.00	1.46
101	<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	10.00	29.90	2.99

Note: - No halo zone, colony diameter not measured

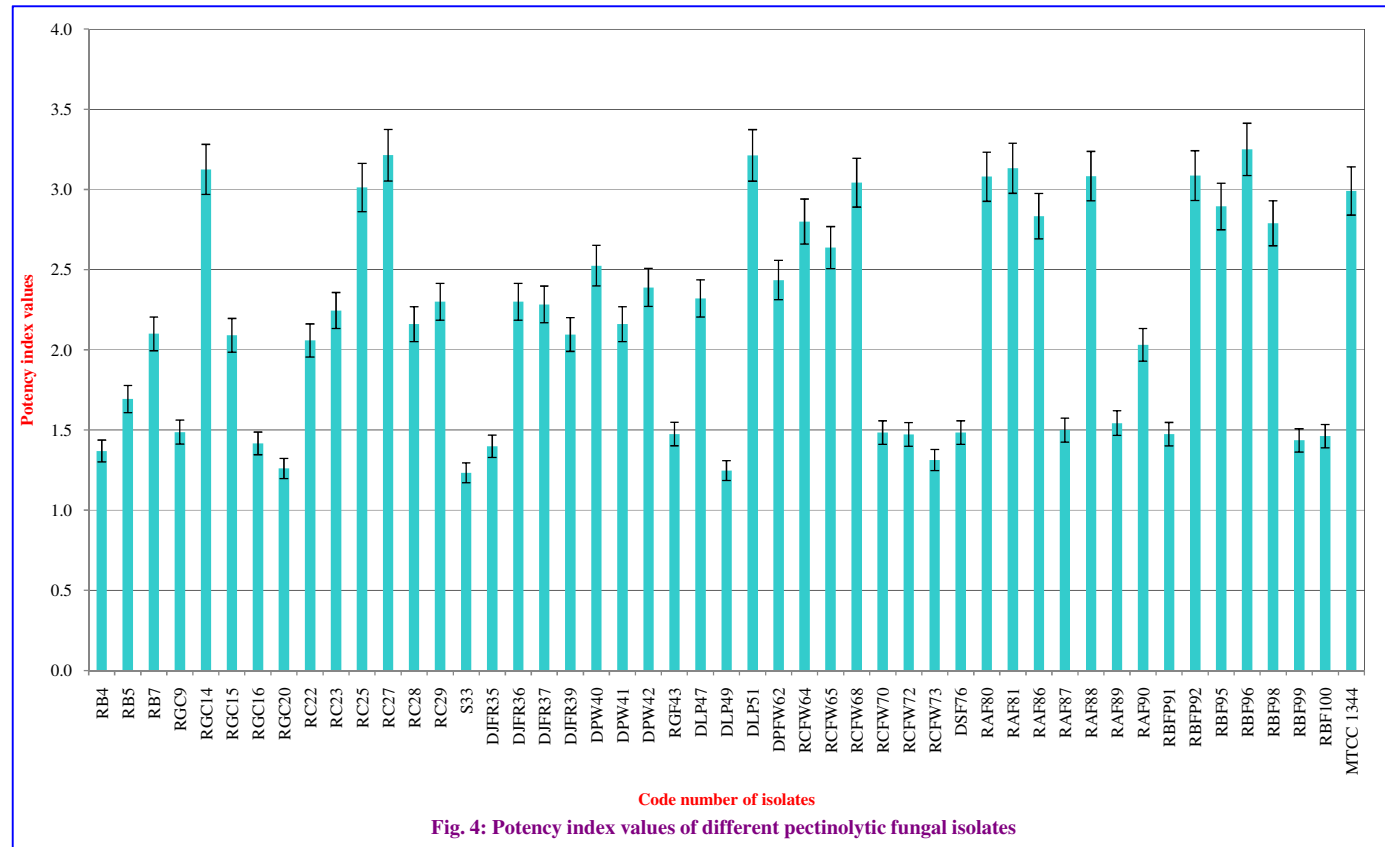


Fig. 4: Potency index values of different pectinolytic fungal isolates

Fig 4: Potency index values of different pectinolytic fungal isolates

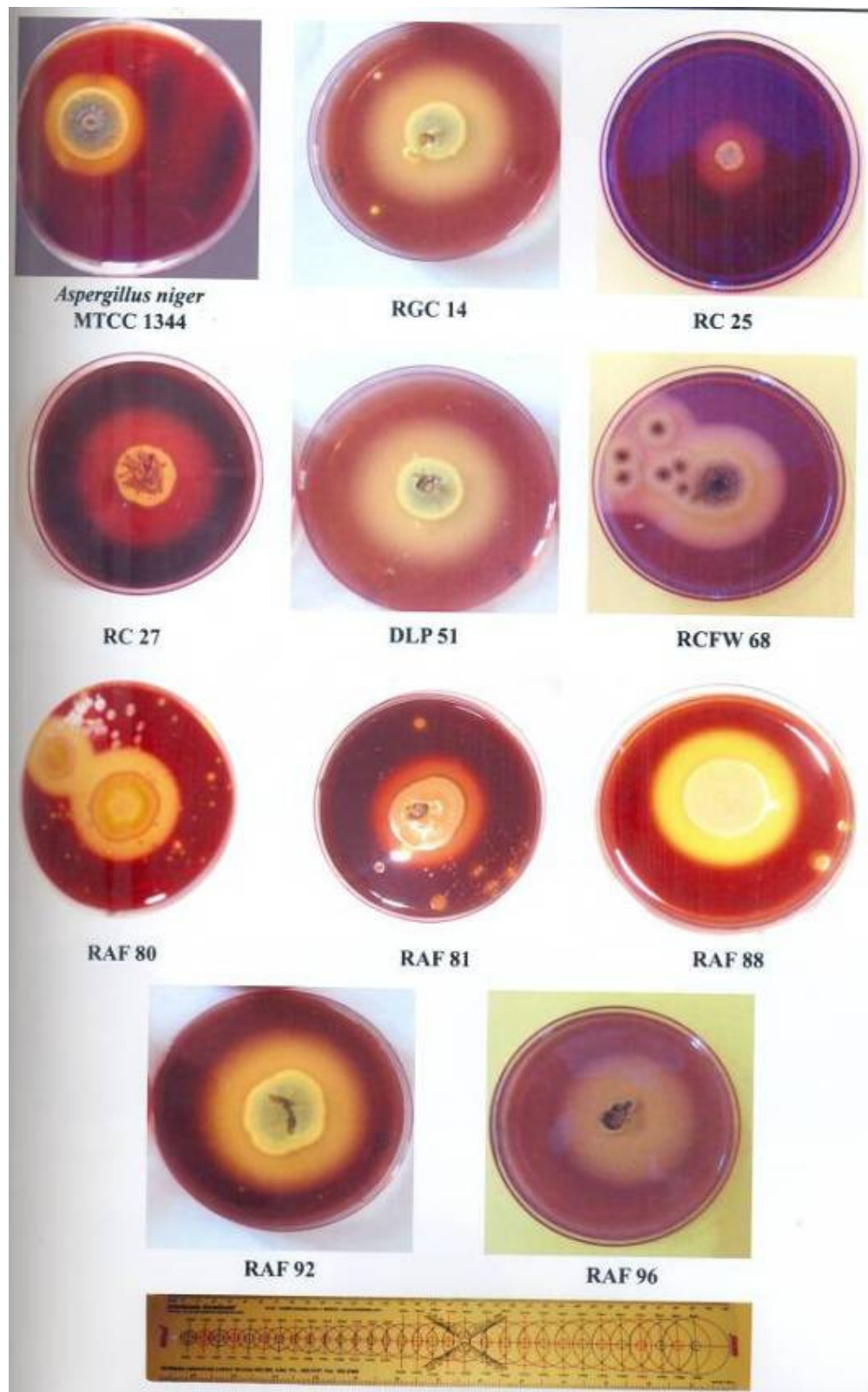


Plate 5: Qualitative plate assay of fungal isolates showing the clear zone formation on pectin agar medium

Plate 5 contd....

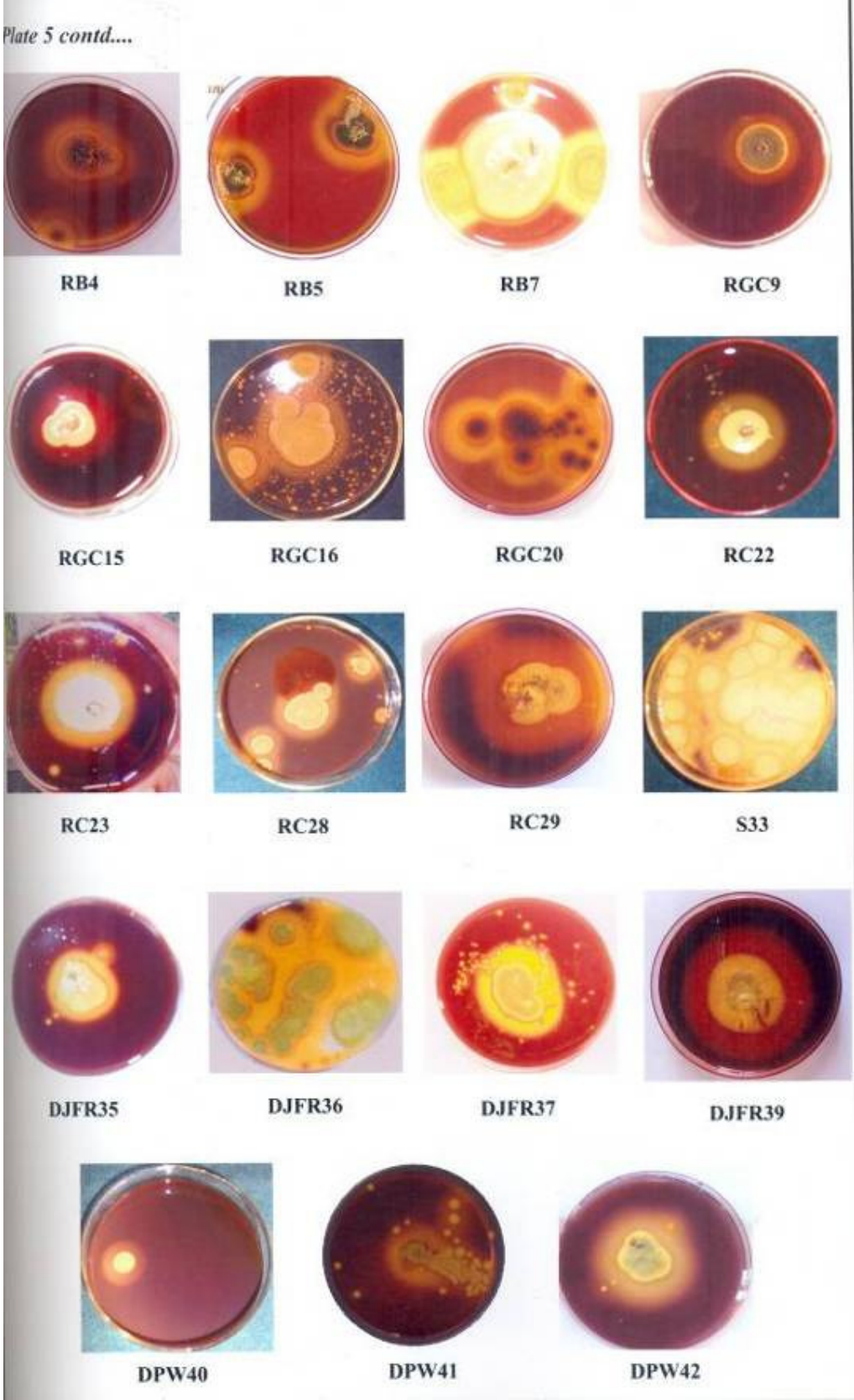


Plate 5 contd....



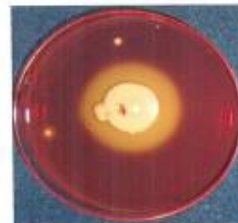
RGF43



DLP47



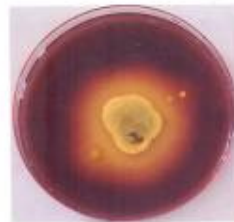
DLP49



DPFW62



RCF64



RCF65



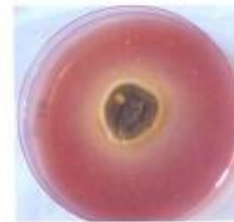
RCF73



RCF70



RCF72



DSF76



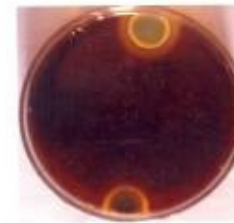
RAF86



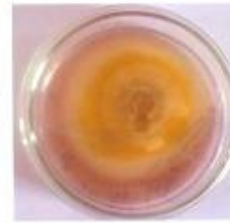
RAF87



RAF89



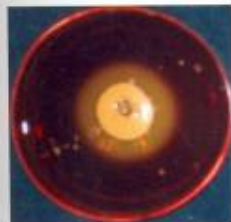
RAF90



RBFP91



RBF95



RBF98



RBF99



RBF100

Table 6. Pectinolytic profile of the pectinase positive fungal isolates

Sl. No.	Isolates	Polygalacturonase activity (U mL ⁻¹)	Pectin lyase activity (U mL ⁻¹)	Pectin esterase activity (U mL ⁻¹)
1	RB4	118.81	173.67	200.00
2	RB5	124.69	174.80	236.67
3	RB7	127.68	258.00	118.33
4	RGC9	106.41	142.67	300.00
5	RGC14	162.51	268.67	145.00
6	RGC15	127.47	181.00	228.33
7	RGC16	121.98	155.33	181.67
8	RGC20	117.75	159.00	178.33
9	RC22	125.97	156.00	165.00
10	RC23	130.67	282.00	144.33
11	RC25	153.22	189.67	350.00
12	RC27	165.50	56.00	375.00
13	RC28	128.86	169.33	158.33
14	RC29	128.96	190.67	158.33
15	S33	117.32	169.33	156.33
16	DJFR35	121.23	186.67	160.00
17	DJFR36	130.80	186.67	130.00
18	DJFR37	133.24	169.33	228.33
19	DJFR39	126.74	273.67	131.67
20	DPW40	137.51	205.67	218.33
21	DPW41	129.18	175.00	183.33
22	DPW42	134.95	173.67	190.00
23	RGF43	114.54	174.33	310.00
24	DLP47	134.95	202.33	303.33
25	DLP49	117.75	221.33	266.67
26	DLP51	167.32	372.00	136.67
27	DPFW62	136.66	238.67	165.00
28	RCFW64	143.92	184.00	171.67
29	RCFW65	137.30	203.33	171.67
30	RCFW68	154.60	191.00	173.33
31	RCFW70	115.46	185.67	188.33
32	RCFW72	121.91	192.00	171.67
33	RCFW73	119.88	256.00	133.33
34	DSF76	125.01	56.00	333.00
35	RAF80	156.85	200.67	160.00
36	RAF81	162.94	240.33	311.67
37	RAF86	143.39	196.67	131.67
38	RAF87	125.54	227.33	301.67
39	RAF88	158.66	294.67	330.00
40	RAF89	122.34	185.00	135.00
41	RAF90	143.60	183.67	296.67
42	RBFP91	125.33	193.67	250.00
43	RBFP92	156.31	195.33	320.00
44	RBF95	143.60	252.67	241.67
45	RBF96	189.33	251.00	276.67
46	RBF98	140.72	56.00	270.00
47	RBF99	119.67	256.00	335.00
48	RBF100	106.75	39.00	313.33
49	<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	153.32	156.00	310.00
S.Em±		2.00	5.70	8.10
CD at 1%		7.48	20.75	29.65

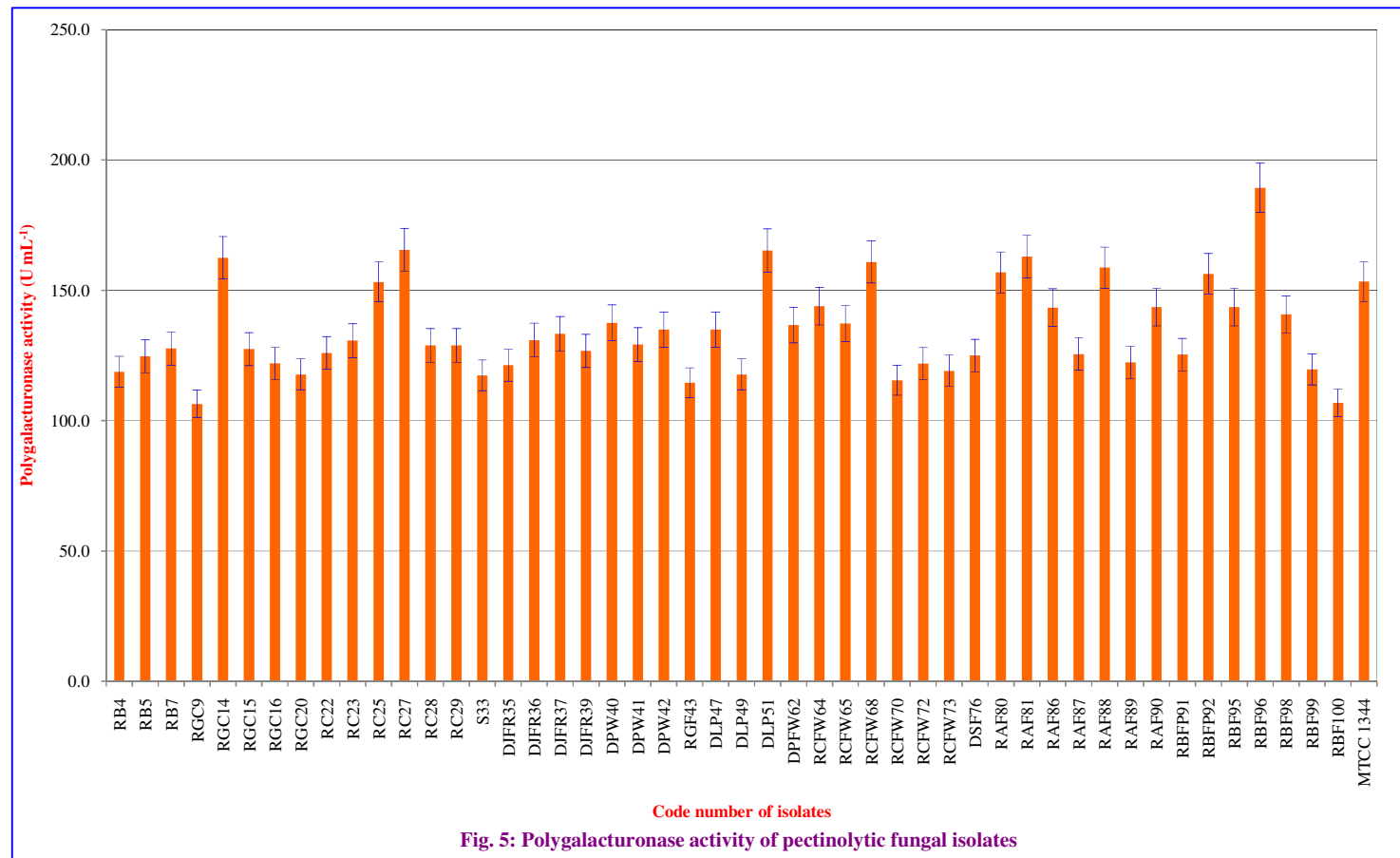


Fig 5: Polygalacturonase activity of pectinolytic fungal isolates

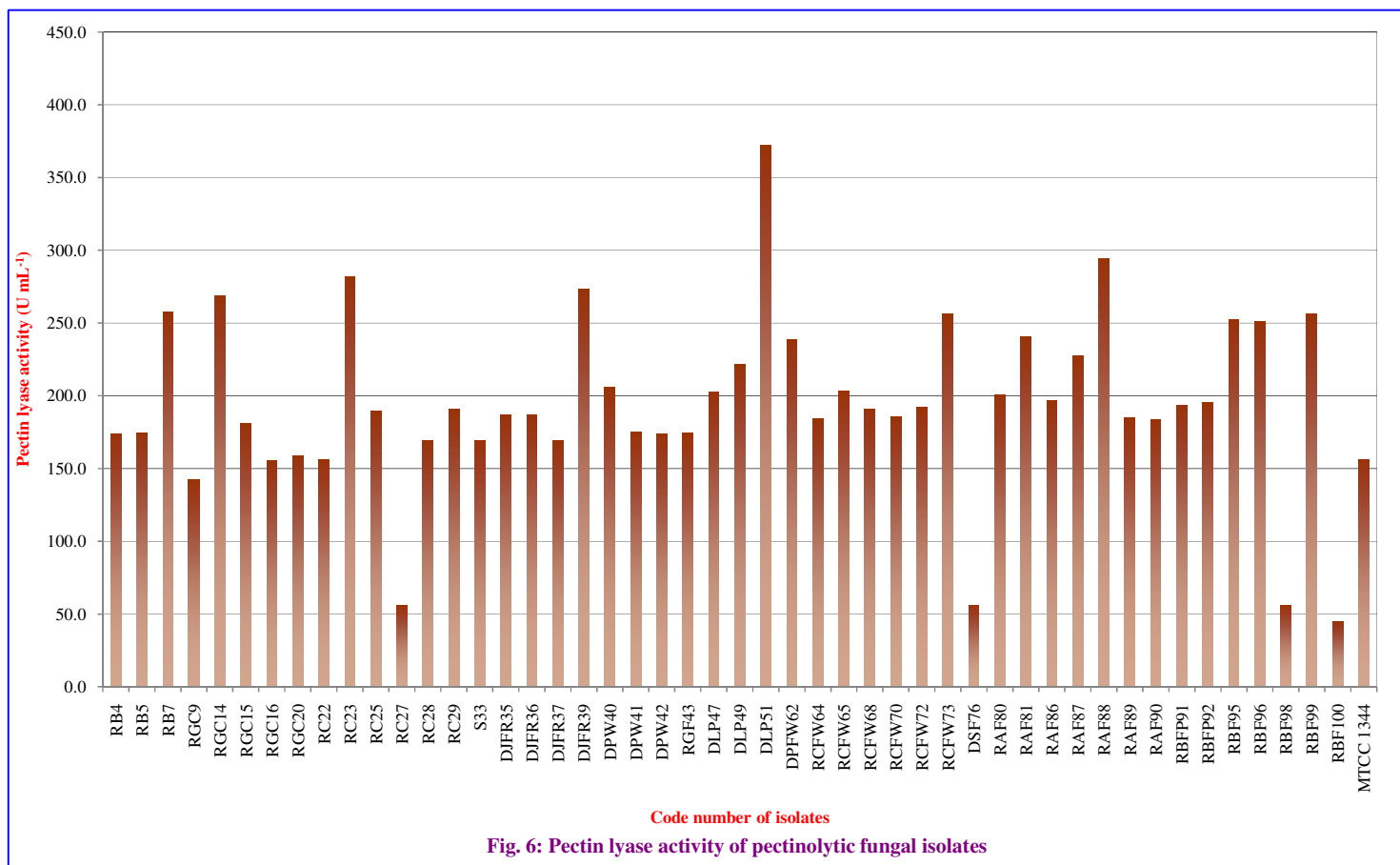


Fig 6: Pectin lyase activity of pectinolytic fungal isolates

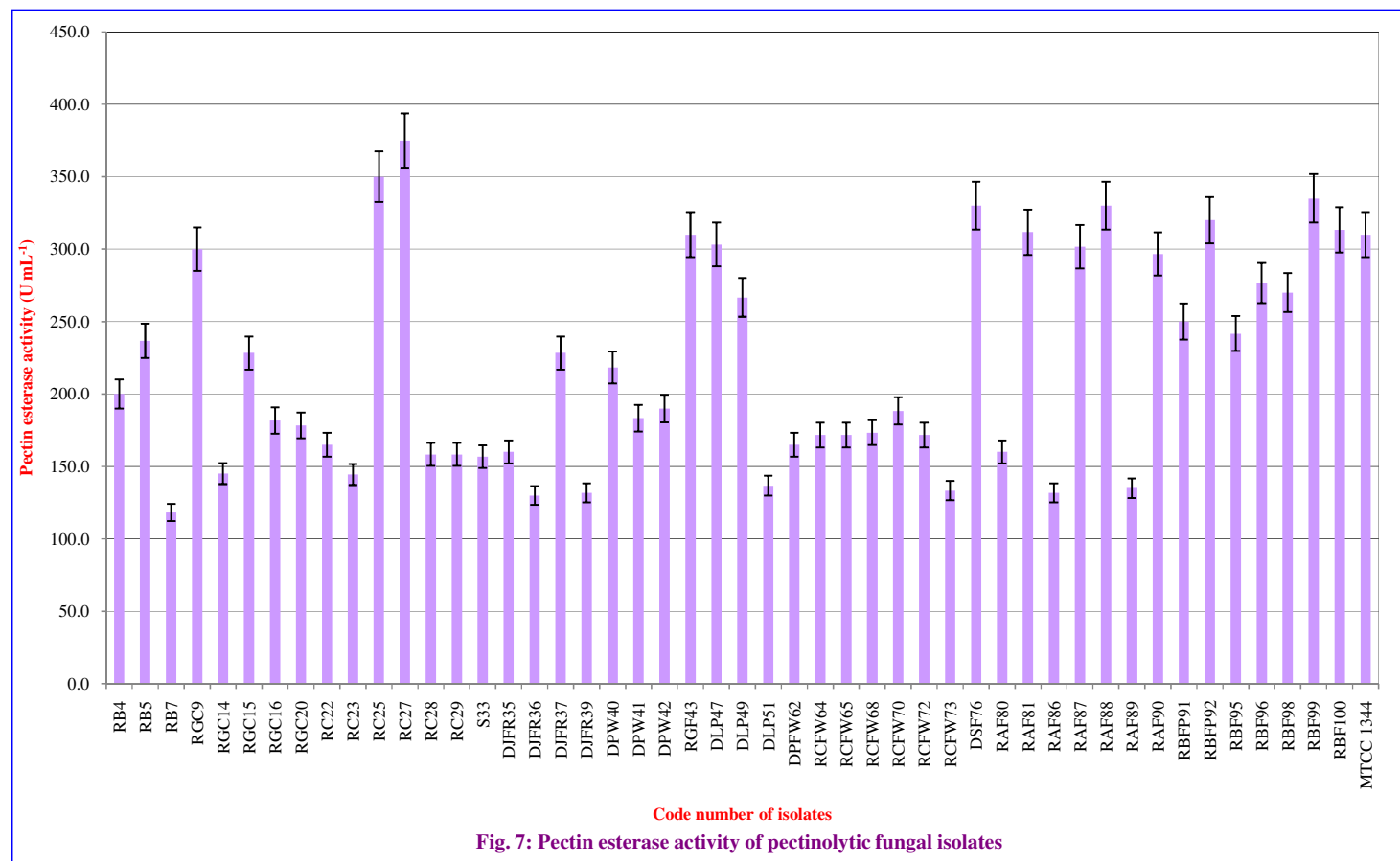


Fig 7: Pectin esterase activity of pectinolytic fungal isolates

Table 7. Tentative identification of the pectinase positive fungal isolates based on colony morphology, growth characters and microscopic observations

Sl. No.	Code number of the isolate	Colony characteristics on Czapek dox Yeast extract Agar (CYA)	Growth on Petri plate (90 X 15 mm dia)	Growth at temperature (°C)			Microscopic features	Probable fungi
				30	40	50		
1	RB4	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
2	RB5	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	—	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	<i>Aspergillus oryzae</i>
3	RB7	Bluish green to brownish olive coloured smaller radially sulcate colonies with sterile white margin, reverse-yellow, reddish brown, conidiation moderate, greyish turquoise or glaucous sky blue, exudates clear, pale yellow to reddish brown aerial part of colony consists of densely strong conidiophores except in the centre where tufts of aerial hyphae arise, mycelium white in peripheral areas, white to greyish at the centre	Covered the plate in 20 days	+	+	-	Conidiophores moderate in length, conidial chains in columns arising from each vertical, ampulliform phialides, conidia globose with heavy walls	<i>Penicillium citrinum</i>
4	RGC9	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
5	RGC14	Bluish green to brownish olive coloured smaller radially sulcate colonies with sterile white margin, reverse-yellow, reddish brown, conidiation moderate, greyish turquoise or glaucous sky blue, exudates clear, pale yellow to reddish brown aerial part of colony consists of densely strong conidiophores except in the centre where tufts of aerial hyphae arise, mycelium white in peripheral areas, white to greyish at the centre	Covered the plate in 20 days	+	+	-	Conidiophores moderate in length, conidial chains in columns arising from each vertical, ampulliform phialides, conidia globose with heavy walls	<i>Penicillium citrinum</i>
6	RGC15	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>

		form of mycelium						
7	RGC16	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
8	RGC20	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biserial phialides	<i>Aspergillus terreus</i>
9	RC22	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biserial phialides	<i>Aspergillus terreus</i>
10	RC23	Bluish green to brownish olive coloured smaller radially sulcate colonies with sterile white margin, reverse-yellow, reddish brown, conidiation moderate, greyish turquoise or glaucous sky blue, exudates clear, pale yellow to reddish brown aerial part of colony consists of densely strong conidiophores except in the centre where tufts of aerial hyphae arise, mycelium white in peripheral areas, white to greyish at the centre	Covered the plate in 20 days	+	+	-	Conidiophores moderate in length, conidial chains in columns arising from each vertical, ampulliform phialides, conidia globose with heavy walls	<i>Penicillium citrinum</i>
11	RC25	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biserial	<i>Aspergillus niger</i>
12	RC27	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	—	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	<i>Aspergillus oryzae</i>
13	RC28	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
14	RC29	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biserial	<i>Aspergillus niger</i>

Contd....

Sl. No.	Code number of the isolate	Colony characteristics on Czapek Yeast extract Agar (CYA)	Growth on Petri plate (90 X 15 mm dia)	Growth at temperature (°C)			Microscopic features	Probable fungi
				30	40	50		
15	S33	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
16	S35	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
17	DJFR36	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biseriate phialides	<i>Aspergillus terreus</i>
18	DJFR37	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>
19	DJFR39	Broadly spreading bluish green, becoming grey green when old, velvet like texture, reverse brownish coloured, produce exudates as droplets excreted by mycelium, sclerotia produced after three weeks of growth, produces floccose form of loose aerial mycelium	Covered the plate in 12 days	+	+	-	conidiophores longer in length conidial chains in columns arising from each vertical, ampulliform phialides, loosely divergent long conidia cylindrical to elliptical or slightly ovate in shape	<i>Penicillium italicum</i>
20	DPW40	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>
21	DPW41	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biseriate phialides	<i>Aspergillus terreus</i>
22	DPW42	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
23	RGF43	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>

		form of mycelium						
24	DLP47	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
25	DLP49	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
26	DLP51	Bluish green to brownish olive coloured smaller radially sulcate colonies with sterile white margin, reverse-yellow, reddish brown, conidiation moderate, greyish turquoise or glaucous sky blue, exudates clear, pale yellow to reddish brown aerial part of colony consists of densely strong conidiophores except in the centre where tufts of aerial hyphae arise, mycelium white in peripheral areas, white to greyish at the centre	Covered the plate in 20 days	+	+	-	Conidiophores moderate in length, conidial chains in columns arising from each vertical, ampulliform phialides, conidia globose with heavy walls	<i>Penicillium citrinum</i>
27	DPFW62	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biserial phialides	<i>Aspergillus terreus</i>
28	RCFW64	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
29	RCFW65	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
30	RCFW68	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biserial phialides	<i>Aspergillus terreus</i>

Contd....

Table 7 contd....

Sl. No.	Code number of the isolate	Colony characteristics on Czapek Yeast extract Agar (CYA)	Growth on Petri plate (90 X 15 mm dia)	Growth at temperature (°C)			Microscopic features	Probable fungi
				30	40	50		
31	RCFW70	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biseriate phialides	<i>Aspergillus terreus</i>
32	RCFW72	Rapidly spreading velvety, cinnamon-buff to wood brown coloured colonies plain or with radial furrows, reverse- dull brown, produces floccose forms of mycelium	Covered the plate in 10 days	+	+	-	Conidial heads long columnar, vesicles are dome like hemispherical, conidiophores longer in length, closely packed biseriate phialides	<i>Aspergillus terreus</i>
33	RCFW73	Broadly spreading bluish green, becoming grey green when old, velvet like texture, reverse brownish coloured, produce exudates as droplets excreted by mycelium, sclerotia produced after three weeks of growth, produces floccose form of loose aerial mycelium	Covered the plate in 12 days	+	+	-	conidiophores longer in length conidial chains in columns arising from each vertical, ampulliform phialides, closely divergent long conidia cylindrical to elliptical or slightly ovate in shape	<i>Penicillium italicum</i>
34	DSF76	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	—	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	<i>Aspergillus oryzae</i>
35	RAF80	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>
36	RAF81	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biseriate	<i>Aspergillus niger</i>
7	RAF86	Bluish green to brownish olive coloured smaller radially sulcate colonies with sterile white margin, reverse-yellow, reddish brown, ,conidiation moderate, greyish turquoise or glaucous sky blue, exudates clear, pale yellow to reddish brown aerial part of colony consists of densely strong conidiophores except in the centre where tufts of aerial hyphae arise, mycelium white in peripheral areas, white to greyish at the centre	Covered the plate in 20 days	+	+	-	Conidiophores moderate in length, conidial chains in columns arising from each vertical, ampulliform phialides, conidia globose with heavy walls	<i>Penicillium citrinum</i>
38	RAF87	Colonies white or pink, usually with a purple or reddish tinge, reverse colourless, produces floccose form of aerial mycelium	Covered the plate in 7 days	+	+	-	Conidiophores unbranched, mono phialidic, microconidia usually abundant, three septate oval or kidney shaped or sickle shaped macroconidia. Chlamydospores terminal or intercalary, globose, one or two celled in hyphae and conidia.	<i>Fusarium Sporotrichoides</i>
39	RAF88	Colonies white or pink, usually with a purple or reddish tinge, reverse colourless, produces floccose form of aerial mycelium	Covered the plate in 7 days	+	+	-	Conidiophores unbranched, mono phialidic, microconidia usually abundant, three septate oval or kidney shaped or sickle shaped	<i>Fusarium Sporotrichoides</i>

							macroconidia. Chlamydospores terminal or intercalary, globose, one or two celled in hyphae and conidia.	
40	RAF89	Broadly spreading bluish green, becoming grey green when old, velvet like texture, reverse brownish coloured, produce exudates as droplets excreted by mycelium, sclerotia produced after three weeks of growth, produces floccose form of loose aerial mycelium	Covered the plate in 12 days	+	+	-	conidiophores longer in length conidial chains in columns arising from each vertical, ampulliform phialides, closely divergent long conidia cylindrical to elliptical or slightly ovate in shape	<i>Penicillium italicum</i>
41	RAF90	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
42	RBF91	Colonies spreading rapidly with purple brown/black conidia, cream coloured colony, sclerotia produced occasionally, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 8 days	+	+	-	Conidial heads globose to subglobose, vesicles nearly globose, conidiophores 0.5-1mm in length, phialides uniseriate	<i>Aspergillus japonicus</i>
43	RBF92	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
44	RBF95	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
45	RBF96	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biserial	<i>Aspergillus niger</i>
46	RBF98	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	—	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	<i>Aspergillus oryzae</i>
47	RBF99	Rapidly spreading, colonies are colourless to yellow or velvety, dull blue green, reverse - colourless to varying in shades, produces tufted aerial mycelium up to felted floccose forms	Covered the plate in 8 days	+	+	+	Conidial heads columnar, densely crowded, flask shaped vesicles, conidiophores short in length, phialides uniseriate, sclerotia and cleistothesia absent.	<i>Aspergillus fumigatus</i>
48	RBF100	Rapidly spreading, colonies are olive yellow to old gold and finally shades of yellow brown, reverse-colourless, no exudates produced, submerged form of mycelium	Covered the plate in 10 days	+	+	—	Conidial heads radiate, conidiophores erect, thin walled and typically long, vesicles sub globose, phialides uniseriate in smaller heads, conidia elliptical, sclerotia often produced and dark coloured.	<i>Aspergillus oryzae</i>
49	<i>Aspergillus niger</i> MTCC 1344	Moderately growing colonies with carbon black /deep brownish black coloured conidia; reverse-colourless to pale yellow, exudates lacking, produced submerged form of mycelium	Covered the plate in 10 days	+	+	+	Conidial heads globose/radiate, vesicles nearly globose, conidiophores upto 3mm in length, phialides biserial	<i>Aspergillus niger</i>

Note:

+ : Growth - : No growth

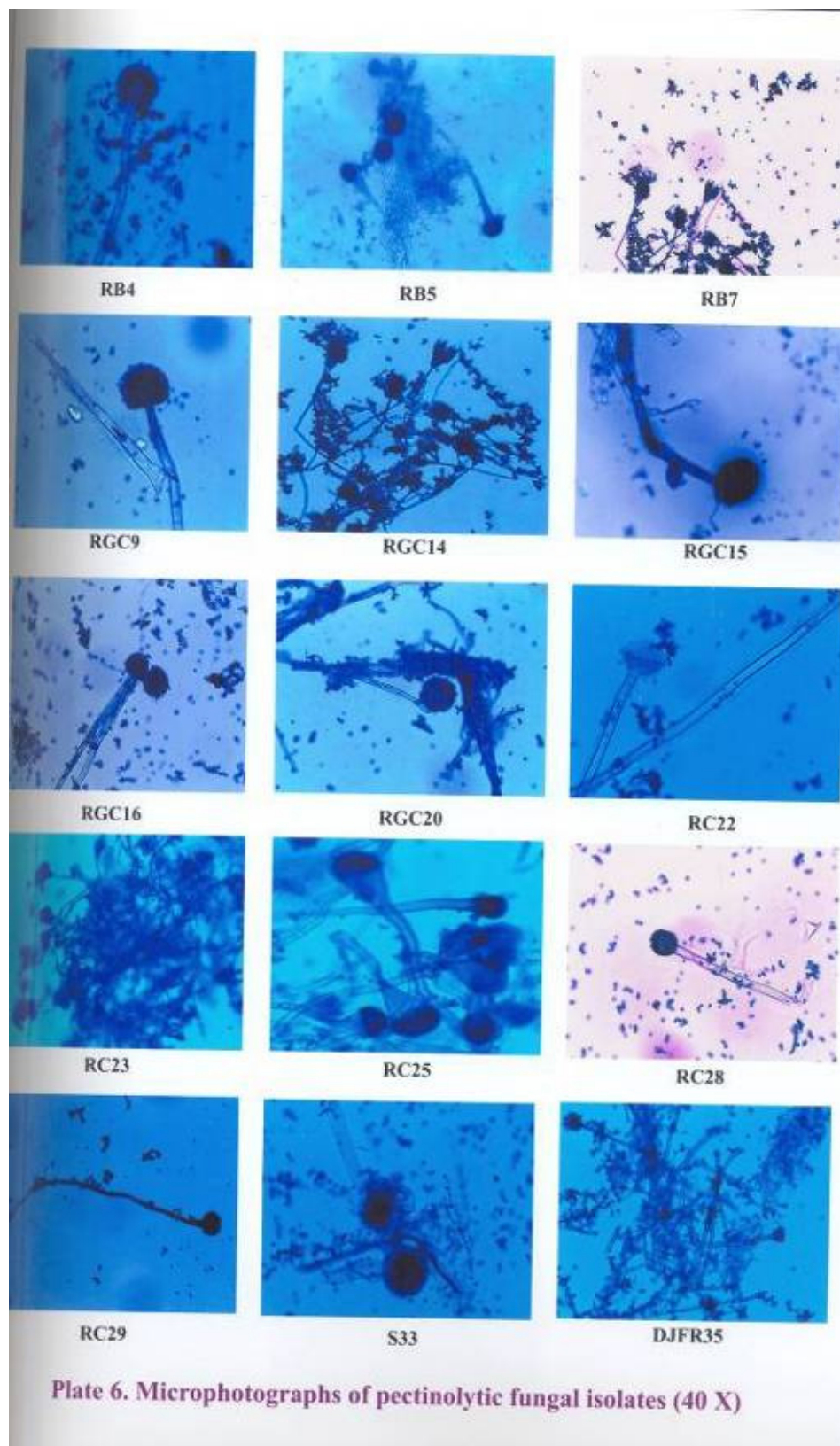
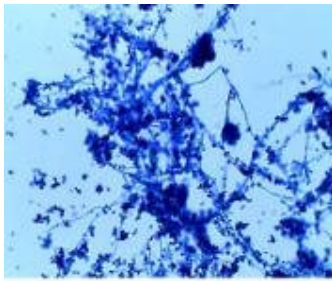
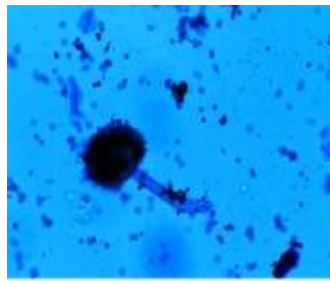


Plate 6. Microphotographs of pectinolytic fungal isolates (40 X)

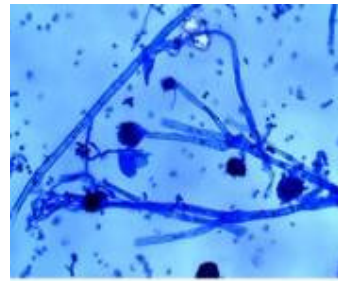
Plate 6 Contd....



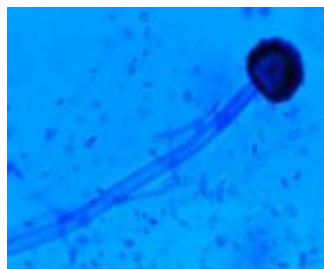
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DSF76



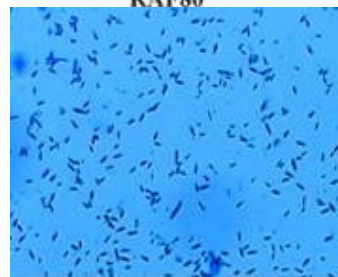
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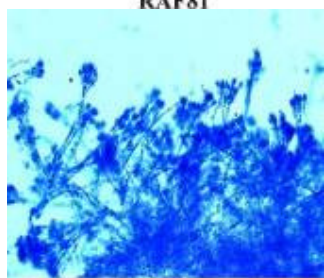
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RAF86



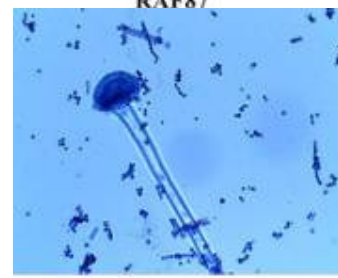
RAF87



RAF89



RAF90



RBF91



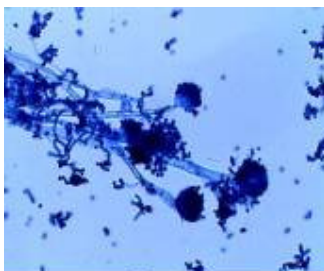
RBF92



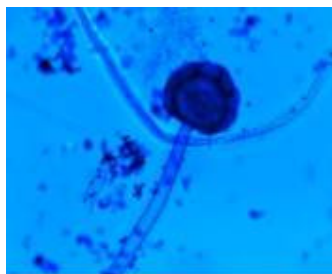
RBF95



RBF98



RBF99



RBF100



Aspergillus niger MTCC 1344

Table 8. ITS rDNA partial sequences of the potent pectinolytic fungal isolates

Sl. No.	Strain identifier code number	ITS1 (Internal transcribed spacer 1) rDNA partial sequences
1	RBF96	ATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGG GCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTG TTGGGTGCGCGTCCCCCTCTCCGGGGGGACGGGCCCCGAAAGGCAG CGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACAT GCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTTCTT TCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCA TATCAATAAGCGGAGGAA
2	RC27	TGATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGG GGGCATGCCTGTCCGAGCGTCATTGCTGCCATCAAGCACGGCTTGT GTGTTGGGTGTCGTCGTCCTCTCCGGGGGGGACGGGCCCCAAAGGC AGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCAC CCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTT TTCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC ATATCAATAAGCGGAGGAA
3	DLP51	AGGTTGGGGGTGCGCTGGCGCCGGCCGGGCCTACTAGAGCGGGTG ACGAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGCTGCC TTTCGGGCCCCGTCCCCCGGCGGGGGGGACGGGGCCCCAACACGCA AGCCGGGCTTGAGGGCACCAATGACGCTCGGACAGGCATGCCCTCC GGAATACCAGAGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACT GAATTCTGCAATTCACATTAGTTATCGCATTTGCTGCGTTCTTCATCG ATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTAATTTGTT ATAGGTCTCAGACTGCAACTTCAGACAGCGTTCAGGGGGGCCGTCG GCAGGGGCGGGGCAGCAGACTTTTTTTTTGTTTTTATTAGGGGGGGG GTGGGGGACGGGGGGGGGGGGGGGGGGAGGTTAACTATCCTAGC GTAGG
4	RAF88	TGGCCCCCACCATTACCTATAACGAGGTGTATGATTACTACGCTATGG AAGCTCGACGTGACCGCCAATCGATTTGGGGAACGCGGGTTACCGC GAGTCCCAACACCAAGCTGAGCTTGAGGGTTGAAATGACGCTCGAAC AGGCATGCCCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGA TTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTTGC TGCGTTCTTCATCGATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTT TGATTTATTTGTTTGTCTTACTCAGAAGTTCCACTAAAAACAGAGTTTA GGGGTCCTCGGGCGGGCCGTCCCGTTTTACGGGGCGCGGGCTGAT CCGCCGAGGCAACGTATAGGTATGTTACAGGGGTTTGGGAGTTGTA AACTCGGTAATGATCCCTCCGCAGGTTACCTACGGAAG

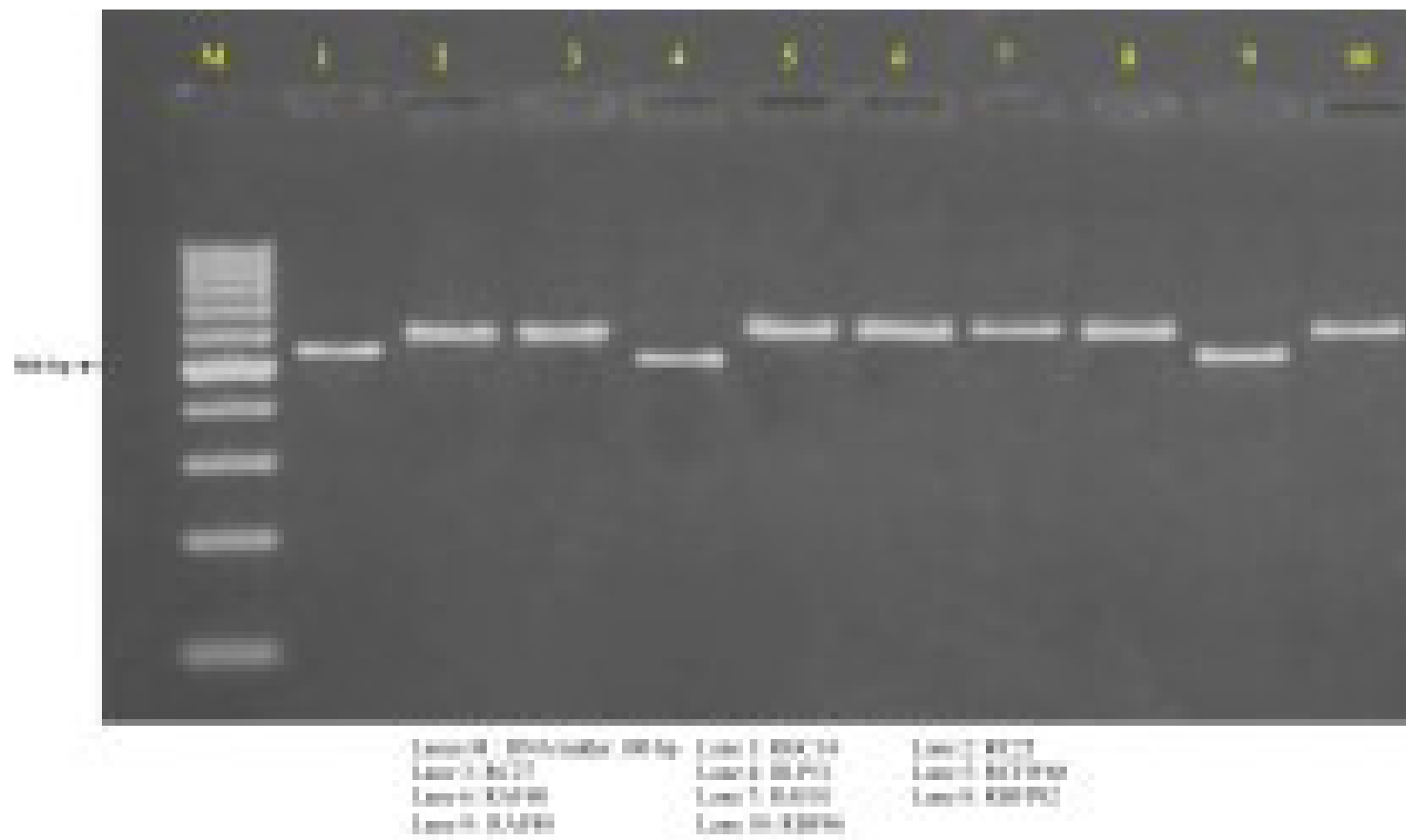


Plate 7: PCR amplification of DNA from pectinolytic fungal isolates with primers ITS1 and ITS4

Table 9. *In silico* BLAST analysis of the ITS rDNA sequences of potent pectinolytic fungal isolates for molecular identification

Code number of the isolate	Matching GeneBank Accession number	NCBI BLAST search description for the sequences producing significant alignments	Maximum score	Query cover	E value	Maximum Identity	Sequence identification
RBF96	KC119204.1	<i>Aspergillus niger</i> strain KAML02, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	549	100%	5e-153	100%	<i>Aspergillus niger</i>
RC27	HF546392.1	<i>Aspergillus oryzae</i> TUEF32 strain genomic DNA containing 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	549	100%	5e-153	100%	<i>Aspergillus oryzae</i>
DLP51	KC344966.1	<i>Penicillium citrinum</i> strain a2s6_6 , ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	909	100%	0.0	99%	<i>Penicillium citrinum</i>
RAF88	FR750924.1	<i>Fusarium sporotrichioides</i> strain MTCC: 7375, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene	869	100%	0.0	99%	<i>Fusarium sporotrichioides</i>

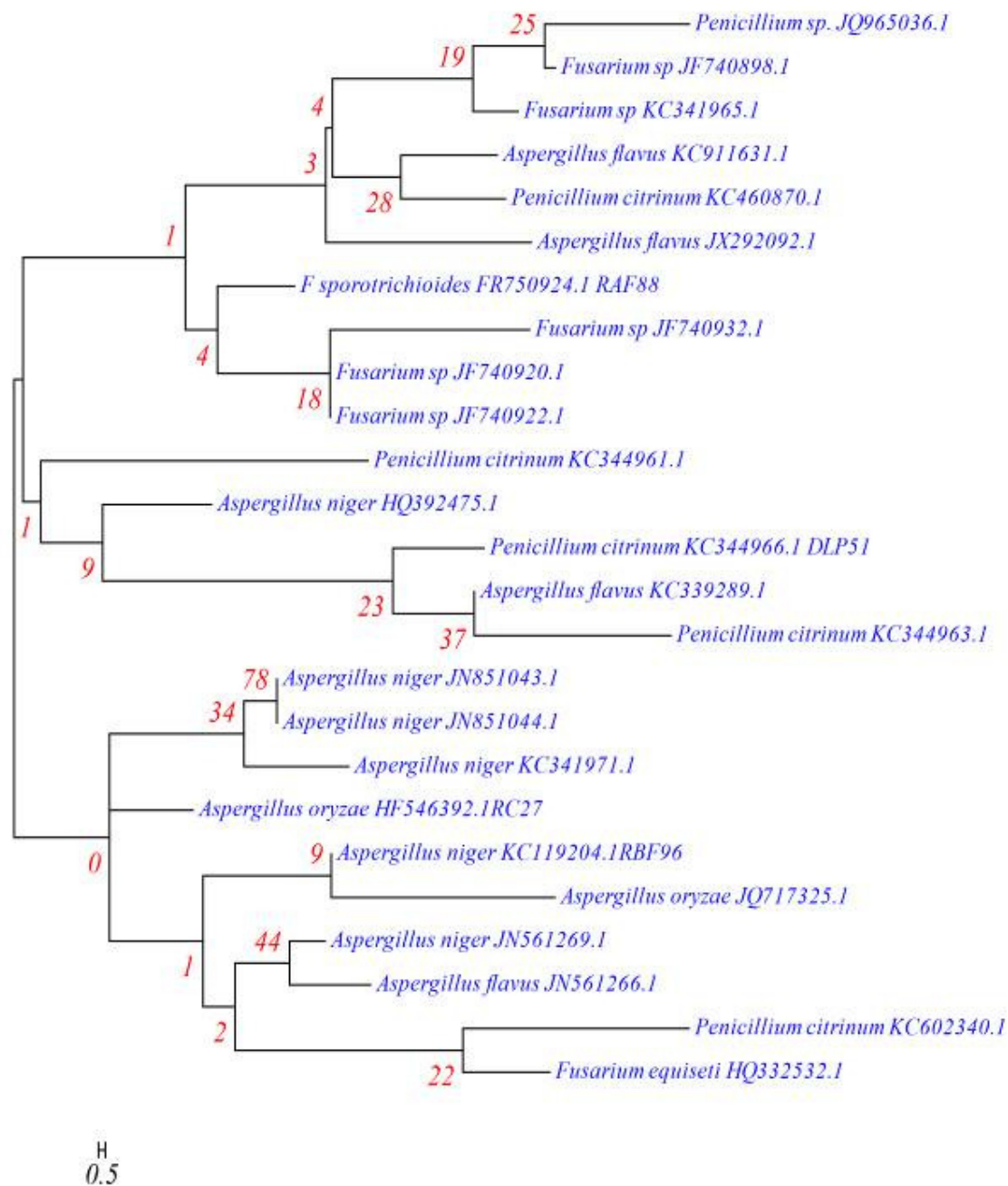
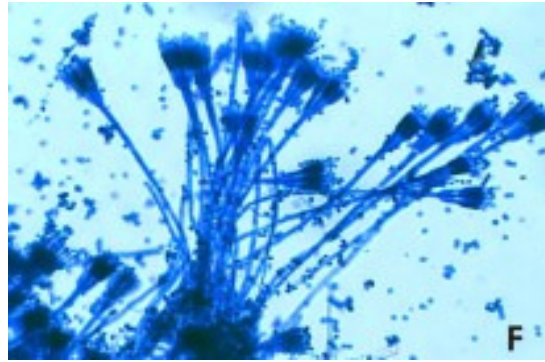
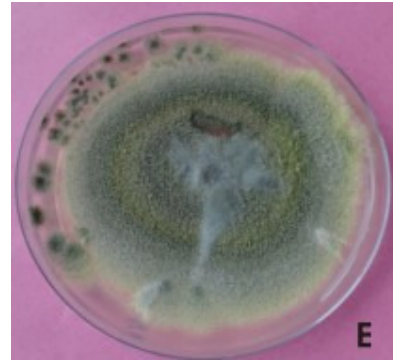
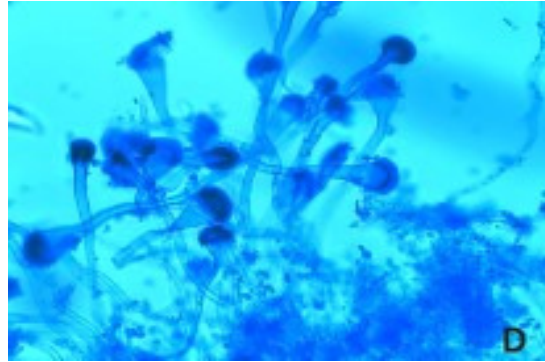
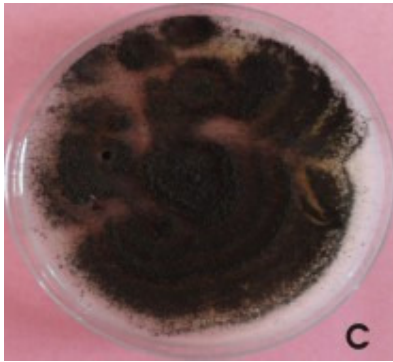
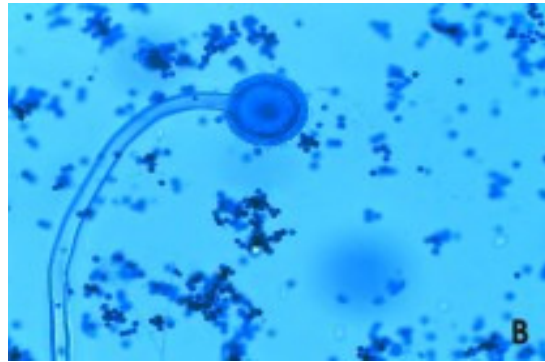
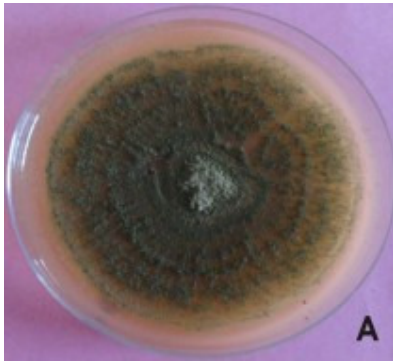


Fig 8: Colony characteristics and microscopic features of potent pectinolytic fungal isolates



Note- A: Colony of RC27 isolate on Czapek Yeast extract Agar (CYA) medium; B: Microscopic features of RC27; C: Colony of RB96 isolate on CYA medium; D: Microscopic features of RB96; E: Colony of DLP51 isolate on CYA medium; F: Microscopic features of DLP51; G: Colony of RAF88 on CYA medium; H: Microscopic features of RAF88

Plate 8: Colony characteristics and microscopic features of potent pectinolytic fungal isolates

two way interactions between pectinolytic fungi and inoculum levels, showed that *A. niger* RBF96 produced significantly higher polygalacturonase (219.88 U mL^{-1}) at 1×10^7 inoculum level when compared to 188.57 U mL^{-1} by the reference strain *Aspergillus niger* MTCC1344. The lowest PG activity (159.83 U mL^{-1}) was seen in the reference strain *A. niger* MTCC1344 at inoculum level of 1×10^5 spores mL^{-1} .

4.1.8 Effect of inoculum size on pectin lyase production by *P. citrinum* DLP51 under submerged fermentation

It was observed that the inoculum levels of the two fungal strains differed significantly in pectin lyase production (Table 11). The mean maximum activity of pectin lyase (295.20 U mL^{-1}) was observed at 1×10^7 (spores mL^{-1}) inoculum level which was significantly superior to other inoculum level 1×10^5 (spores mL^{-1}) which produced 238.50 U mL^{-1} pectin lyase. *P. citrinum* DLP51 produced the highest pectin lyase (343.00 U mL^{-1}) when compared to the reference strain *A. niger* MTCC1344 (247.40 U mL^{-1}) at 1×10^7 (spores mL^{-1}) inoculum level.

4.1.9 Effect of inoculum size on pectin esterase production by *A. oryzae* RC27 under submerged fermentation

The data on pectin esterase activity indicated that the inoculum levels significantly affected the enzyme activity (Table 12). The two way interactions between pectinolytic fungi and inoculum levels, showed that *A. oryzae* RC27 produced maximum pectin esterase (377.00 U mL^{-1}) at 1×10^7 (spores mL^{-1}) inoculum level as compared to 245.00 U mL^{-1} by the reference strain *A. niger* MTCC1344. The least pectin esterase activity (197.00 U mL^{-1}) was seen in the reference strain *A. niger* MTCC1344 at 1×10^5 (spores mL^{-1}).

4.1.10 Influence of pH on polygalacturonase production by *A. niger* RBF96 under submerged fermentation

The initial pH of the growth medium was varied from 3.0-7.0, to find out the optimum pH for maximum production of PG by *A. niger* RBF96 under submerged fermentation. The data presented in Table 13 showed that *A. niger* RBF96 produced maximum activity at pH 4.0 (187.83 U mL^{-1}) as compared to the reference strain *Aspergillus niger* MTCC1344 (152.79 U mL^{-1}). The least mean activity of polygalacturonase (145.12 U mL^{-1}) was observed for the reference strain *A. niger* MTCC1344 as compared to 163.34 U mL^{-1} by *A. niger* RBF96.

4.1.11 Effect of pH on pectin lyase production by *P. citrinum* DLP51 under submerged fermentation

The combined effect of pectinolytic fungi and pH levels was found to differ significantly for pectin lyase production (Table 14). *P. citrinum* DLP51 showed significantly the highest pectin lyase activity (187.83 U mL^{-1}) at pH 7.0 when compared to reference strain *A. niger* MTCC1344 which produced maximum pectin lyase (144.33 U mL^{-1}) at pH 6.0. The lowest pectin lyase activity was observed by the reference strain *A. niger* MTCC1344 (100.33 U mL^{-1}) at pH 3.0.

4.1.12 Influence of pH on pectin esterase production by *A. oryzae* RC27 under submerged fermentation

Data presented in Table 15 indicated that the interaction effects of pectinolytic fungi and the pH levels were found to vary significantly in production of pectin esterase. *A. oryzae* RC27 produced maximum pectin esterase (346.67 U mL^{-1}) at pH 5.0 as compared to 303.33 U mL^{-1} by the reference strain *Aspergillus niger* MTCC1344. The lowest pectin esterase activity (186.67 U mL^{-1}) was seen in the reference strain *Aspergillus niger* MTCC1344 at pH 3.0.

4.1.13 Effect of temperature on polygalacturonase production by *A. niger* RBF96 under submerged fermentation

The effect of different temperatures viz., 25, 30, 35 and 40°C on production of polygalacturonase enzyme was studied. The data presented in Table 16 revealed that significantly higher polygalacturonase activity of 305.14 U mL^{-1} was observed in *A. niger* RBF96 when compared to the reference strain *A. niger* MTCC1344 (152.79 U mL^{-1}) at 35°C . The significantly lower polygalacturonase activity was recorded in the reference strain *A. niger* MTCC1344 (136.76 U mL^{-1}) at 40°C . The highest mean maximum polygalacturonase activity (228.96 U mL^{-1}) was recorded in the

culture filtrates of fungi grown at 35°C temperature. Significantly lower mean polygalacturonase activity was observed at 40°C (146.75 U mL⁻¹).

4.1.14 Influence of temperature on pectin lyase production by *P. citrinum* DLP51 under submerged fermentation

The data pertaining to pectin lyase activity are presented in Table 17. The fungal strains, the reaction temperatures and their combined effect differed significantly with regard to pectin lyase production. It was observed that 35°C temperature resulted in significantly higher mean pectin lyase activity (247.67 U mL⁻¹). It was found to be significantly superior over other incubation temperature conditions. At this temperature, the observed pectin lyase activities were 351 U mL⁻¹ for *P. citrinum* DLP51 and 144.33 U mL⁻¹ for the reference strain *A. niger* MTCC1344.

4.1.15 Influence of temperature on pectin esterase production by *A. oryzae* RC27 under submerged fermentation

Data presented in Table 18 indicated that the interaction effects of pectinolytic fungi and the incubation temperature were found to vary significantly. The temperature 35°C was found to be optimum for pectinolytic fungi for higher pectin esterase activity. *A. oryzae* RC27 produced maximum pectin esterase (338.33 U mL⁻¹) at 35°C as compared to 306.67 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The lowest pectin esterase activity (180.00 U mL⁻¹) was seen in the reference strain at 40°C.

4.1.16 Polygalacturonase production by *A. niger* RBF96 under submerged fermentation at different substrate concentrations

The interaction between pectinolytic fungi and different substrate concentrations indicated a significantly higher polygalacturonase activity (Table 19). In presence of pectin at 1 per cent, *A. niger* RBF96 resulted in polygalacturonase activity of 198.30 U mL⁻¹ as compared to 188.58 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The significant mean maximum polygalacturonase activity, 193.44 U mL⁻¹ was recorded in the culture filtrates of the fungi grown at 1 per cent pectin concentration. It was found to be significantly superior over other levels of substrates studied and significantly lower mean polygalacturonase activity was observed at 0.1 per cent with an activity of 141.89 U mL⁻¹.

4.1.17 Pectin lyase production by *P. citrinum* DLP51 under submerged fermentation at different substrate concentrations

The results depicted in Table 20 showed that the combined interactions between substrate concentrations and pectinolytic fungal strains were found to differ significantly in pectin lyase production. The maximum mean activity of pectin lyase (337.33 U mL⁻¹) was at 1 per cent pectin concentration which was significantly superior to other levels. *P. citrinum* DLP51 produced the highest Pectin lyase (452.33 U mL⁻¹) compared to the reference strain *A. niger* MTCC1344 (222.33 U mL⁻¹) at 1 per cent pectin concentration. The lowest Pectin lyase activity (101.00 U mL⁻¹) was seen in the reference strain *A. niger* MTCC1344 at 0.1 per cent substrate concentration.

4.1.18 Pectin esterase production by *A. oryzae* RC27 under submerged fermentation at different substrate concentrations

The data pertaining to pectin esterase activity produced on different substrate concentrations by pectinolytic fungal strains is presented in Table 21. The pectin esterase activity varied significantly. One per cent pectin concentration was found to be optimum for pectinolytic fungi for higher pectin esterase activity. *A. oryzae* RC27 produced maximum pectin esterase (403.33 U mL⁻¹) at 1 per cent pectin concentration as compared to 386.67 U mL⁻¹ by the reference strain *A. niger* MTCC1344. It was also observed that as the pectin concentration was increased, the pectin esterase activity was found to decrease gradually. The lowest pectin esterase activity (120.00 U mL⁻¹) was observed in the reference strain *A. niger* MTCC1344 at 0.1 per cent substrate concentration.

4.1.19 Polygalacturonase production by *A. niger* RBF96 under submerged fermentation at different incubation periods

For testing the optimum incubation time of pectinolytic fungal strains for polygalacturonase production, the fermentation was carried out upto 8 days and the enzyme activity was examined at

Table 10. Effect of inoculum size on polygalacturonase production by *Aspergillus niger* RBF96 under submerged fermentation

Polygalacturonase activity (U mL ⁻¹)			
Inoculum size (Spores mL ⁻¹)	<i>Aspergillus niger</i> RBF96	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
1 x 10 ⁵	169.02	159.83	164.93
1 x 10 ⁷	219.88	188.57	204.51
Mean	194.67	174.76	
		S.Em±	CD at 1%
Strains (A)		0.75	3.24
Inoculum size (B)		0.37	1.62
A x B		0.53	2.29

Table 11. Influence of inoculum size on pectin lyase production by *Penicillium citrinum* DLP51 under submerged fermentation

Pectin lyase activity (U mL ⁻¹)			
Inoculum size (Spores mL ⁻¹)	<i>Penicillium citrinum</i> DLP51	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
1 x 10 ⁵	283.40	193.60	238.50
1 x 10 ⁷	343.00	247.40	295.20
Mean	313.20	220.50	
		S.Em±	CD at 1%
Strains (A)		1.99	8.60
Inoculum size (B)		0.99	4.30
A x B		1.40	6.08

Table 12. Effect of inoculum size on pectin esterase production by *Aspergillus oryzae* RC27 under submerged fermentation

Pectin esterase activity (U mL ⁻¹)			
Inoculum size (Spores mL ⁻¹)	<i>Aspergillus oryzae</i> RC27	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
1 x 10 ⁵	316.00	197.00	256.50
1 x 10 ⁷	377.00	245.00	311.00
Mean	346.50	221.00	
		S.Em±	CD at 1%
Strains (A)		1.87	4.04
Inoculum size (B)		0.93	8.08
A x B		1.32	5.71

Table 13. Polygalacturonase production by *Aspergillus niger* RBF96 under submerged fermentation as influenced by different pH levels

Polygalacturonase activity (U mL ⁻¹)			
pH levels	<i>Aspergillus niger</i> RBF96	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
3	164.54	143.17	153.86
4	187.83	152.79	170.31
5	162.40	149.48	155.94
6	156.31	143.39	149.85
7	145.63	136.76	141.20
Mean	163.34	145.12	
		S.Em±	CD at 1%
Strains (A)		0.17	0.70
pH levels (B)		0.27	1.11
A x B		0.38	1.57

Table 14. Effect of pH on pectin lyase production by *Penicillium citrinum* DLP51 under submerged fermentation

Pectin lyase activity (U mL ⁻¹)			
pH levels	<i>Penicillium citrinum</i> DLP51	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
3	218.00	100.33	159.17
4	249.67	123.00	186.33
5	299.67	132.00	215.83
6	312.33	144.33	228.33
7	351.67	135.00	243.33
Mean	286.27	126.93	
		S.Em±	CD at 1%
Strains (A)		0.58	2.38
pH levels (B)		0.92	3.76
A x B		1.30	5.32

Table 15. Influence of pH on pectin esterase production by *Aspergillus oryzae* RC27 under submerged fermentation

Pectin esterase activity (U mL ⁻¹)			
pH levels	<i>Aspergillus oryzae</i> RC27	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
3	253.33	186.67	220.00
4	308.33	280.00	294.17
5	346.67	303.33	325.00
6	300.00	260.00	280.00
7	220.00	200.00	210.00
Mean	285.67	246.00	
		S.Em±	CD at 1%
Strains (A)		1.26	5.14
pH levels (B)		1.99	8.13
A x B		2.82	11.50

Table 16. Influence of temperature on polygalacturonase production by *Aspergillus niger* RBF96 under submerged fermentation

Polygalacturonase activity (U mL ⁻¹)			
Temperature (°C)	<i>Aspergillus niger</i> RBF96	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
20	158.56	139.33	148.94
30	187.94	148.62	168.28
35	305.14	152.79	228.96
40	156.74	136.76	146.75
Mean	202.09	144.38	
		S.Em±	CD at 1%
Strains (A)		0.82	3.44
Temperature (B)		1.16	4.87
A x B		0.55	2.30

Table 17. Pectin lyase production by *Penicillium citrinum* DLP51 under submerged fermentation as influenced by temperature

Pectin lyase activity (U mL ⁻¹)			
Temperature (°C)	<i>Penicillium citrinum</i> DLP51	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
20	216.33	123.00	169.67
30	303.33	135.00	219.17
35	351.00	144.33	247.67
40	249.67	100.33	175.00
Mean	280.08	125.67	
		S.Em±	CD at 1%
Strains (A)		2.33	9.82
Temperature (B)		3.30	13.89
A x B		1.56	6.55

Table 18. Influence of temperature on pectin esterase production by *Aspergillus oryzae* RC27 under submerged fermentation

Pectin esterase activity (U mL ⁻¹)			
Temperature (°C)	<i>Aspergillus oryzae</i> RC27	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
20	213.33	216.67	215.00
30	310.00	270.00	290.00
35	338.33	306.67	322.50
40	220.00	180.00	200.00
Mean	270.42	243.33	
		S.Em±	CD at 1%
Strains (A)		7.40	31.16
Temperature (B)		10.47	44.07
A x B		4.94	20.78

Table 19. Polygalacturonase production by *Aspergillus niger* RBF96 under submerged fermentation at different substrate concentrations

Polygalacturonase activity (U mL ⁻¹)			
Substrate Concentration (%)	<i>Aspergillus niger</i> RBF96	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
0.1	146.38	137.40	141.89
0.5	185.80	149.80	167.80
1.0	198.30	188.58	193.44
1.5	194.67	165.82	180.25
2.0	178.43	157.17	167.80
Mean	180.72	159.75	
		S.Em±	CD at 1%
Strains (A)		0.14	0.60
Substrate Concentration (B)		0.23	0.95
A x B		0.33	1.34

Table 20. Pectin lyase production by *Penicillium citrinum* DLP51 under submerged fermentation at different substrate concentrations

Pectin lyase activity (U mL ⁻¹)			
Substrate Concentration (%)	<i>Penicillium citrinum</i> DLP51	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
0.1	215.00	101.00	158.00
0.5	361.00	149.00	255.00
1.0	452.33	222.33	337.33
1.5	248.33	199.00	223.67
2.0	202.00	123.67	162.83
Mean	295.73	159.00	
		S.Em±	CD at 1%
Strains (A)		0.48	1.96
Substrate Concentration (B)		0.76	3.10
A x B		1.07	4.39

Table 21. Pectin esterase production by *Aspergillus oryzae* RC27 under submerged fermentation at different substrate concentrations

Pectin esterase activity (U mL ⁻¹)			
Substrate Concentration (%)	<i>Aspergillus oryzae</i> RC27	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
0.1	200.00	120.00	160.00
0.5	363.33	303.33	333.33
1.0	403.33	386.67	395.00
1.5	380.00	366.67	373.33
2.0	246.67	280.00	263.33
Mean	318.67	291.33	
		S.Em±	CD at 1%
Strains (A)		1.18	4.82
Substrate Concentration (B)		1.87	7.63
A x B		2.65	10.79

every two days intervals. The results depicted in Table 22 showed that *A. niger* RBF96 produced the highest polygalacturonase (192.10 U mL^{-1}) at 6th day of incubation.

4.1.20 Pectin lyase production by *P. citrinum* DLP51 under submerged fermentation at different incubation periods

The incubation time of the fungal strains for pectin lyase production was found to differ significantly (Table 23). The mean maximum activity of pectin lyase (267.00 U mL^{-1}) was observed at 6th day after incubation which was significantly superior to other incubation periods. *P. citrinum* DLP51 produced the highest pectin lyase (340.67 U mL^{-1}) when compared to the reference strain *A. niger* MTCC1344 (210.00 U mL^{-1}) at 6th day of fermentation.

4.1.21 Pectin esterase production by *A. oryzae* RC27 under submerged fermentation at different incubation periods

There was an increase in PE activity with an increase in incubation time up to 6 days and declined thereafter in both the strains (Table 24). The highest pectin esterase activity (353.33 U mL^{-1}) was shown by *A. oryzae* RC27 as compared to 343.33 U mL^{-1} by the reference strain *A. niger* MTCC1344.

4.2 Solid State Fermentation (SSF) studies for optimization of culture parameters for maximum pectinase production

4.2.1 Polygalacturonase (PG) production by *A. niger* RBF96 under SSF

4.2.2 Effect of different moisture levels of substrates

The data presented in Table 25 indicated a significant difference in PG production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other. A mean maximum of 454.12 U mL^{-1} PG was produced by *A. niger* RBF96 as compared to 266.23 U mL^{-1} by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly highest average PG activity of 416.19 U mL^{-1} , followed by wheat bran (394.63 U mL^{-1}), sunflower head (365.11 U mL^{-1}), citrus peel + jackfruit rind + pineapple peel (363.23 U mL^{-1}), coffee husk (340.59 U mL^{-1}) and the lowest mean was observed in banana pseudo stem (281.31 U mL^{-1}).

The PG production varied with moisture content of substrates. The mean highest PG activity (473.11 U mL^{-1}) was noticed at 65 per cent moisture level of the substrates, which was significantly superior over 70 per cent (423.52 U mL^{-1}), 60 per cent (356.77 U mL^{-1}), 75 per cent (278.12 U mL^{-1}) and 55 per cent (269.35 U mL^{-1}) moisture levels of substrates.

The production of PG as influenced by pectinolytic fungi and different moisture levels indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 at 65 per cent moisture in citrus peel (733.88 U mL^{-1}) followed by wheat bran (709.89 U mL^{-1}), sunflower head (685.18 U mL^{-1}) and citrus peel + jackfruit rind + pineapple peel (527.46 U mL^{-1}). Whereas, 70 per cent moisture content was found optimum with the highest PG activity in coffee husk (685.18 U mL^{-1}) and banana pseudo stem (592.36 U mL^{-1}). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity at 65 per cent moisture in citrus peel (478.14 U mL^{-1}) followed by wheat bran (457.54 U mL^{-1}), citrus peel + jackfruit rind + pineapple peel (450.41 U mL^{-1}) and sunflower head (428.79 U mL^{-1}). Whereas, 70 per cent moisture content was found optimum for PG activity in case of coffee husk (401.36 U mL^{-1}) and banana pseudo stem (342.92 U mL^{-1}).

4.2.3 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.3.1 Effect of different moisture levels of substrates

The data pertaining to the pectin lyase production (Table 26) indicated significant variations due to individual effects such as pectinolytic fungi, moisture levels of the substrates and different substrates. The interactions of these individual factors with one another as well as their combined interactions were also found to be significant.

Both the pectinolytic fungi differed statistically with each other in terms of PL production. A mean maximum of 206.69 U mL^{-1} PL was produced by *P. citrinum* DLP51 as compared to 177.30 U mL^{-1} by the reference strain *A. niger* MTCC1344. Citrus peel showed significantly higher average PL activity of 220.90 U mL^{-1} , followed by wheat bran (211.17 U mL^{-1}), sunflower head (199.26 U mL^{-1}),

citrus peel + jackfruit rind + pineapple peel (187.93 U mL^{-1}), coffee husk (187.44 U mL^{-1}) and the lowest mean for PL production was found in banana pseudo stem (145.54 U mL^{-1}). All these substrates differed significantly over one another for PL production. The PL production varied with moisture content of substrates. The highest mean PL activity (276.99 U mL^{-1}) was noticed at 65 per cent moisture level, which was significantly superior over 70 per cent (240.25 U mL^{-1}), 60 per cent (186.37 U mL^{-1}), 55 per cent (158.40 U mL^{-1}) and 75 per cent (97.97 U mL^{-1}) moisture levels.

The production of PL as influenced by pectinolytic fungi and different moisture levels of substrates indicated varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 at 65 per cent moisture in citrus peel (379.67 U mL^{-1}) followed by wheat bran (360.77 U mL^{-1}), sunflower head (350.87 U mL^{-1}) and citrus peel + jackfruit rind + pineapple peel (257.24 U mL^{-1}). Whereas, 70 per cent moisture was found optimum in coffee husk (338.53 U mL^{-1}) and banana pseudo stem (294.47 U mL^{-1}). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity at 65 per cent moisture in citrus peel (273.33 U mL^{-1}) followed by citrus peel + jackfruit rind + pineapple peel (450.41 U mL^{-1}), wheat bran (273.25 U mL^{-1}), sunflower head (261.44 U mL^{-1}), coffee husk (251.81 U mL^{-1}) and banana pseudo stem (202.64 U mL^{-1}) respectively. The lowest PL production (58.33 U mL^{-1}) was seen in banana pseudo stem at 70 per cent moisture level in case of the reference strain *A. niger* MTCC1344.

4.2.3 Pectin esterase production by *A. oryzae* RC27 under SSF

4.2.3.1 Effect of different moisture levels of substrates

The data concerning to pectin esterase production (Table 27) indicated significant variations due to individual effects such as pectinolytic fungi, moisture levels of the substrates and different substrates. The interactions of these individual factors with one another as well as their combined interactions were found to be significant.

The pectinolytic fungal strains differed significantly with each other. The mean maximum of 281.96 U mL^{-1} PE was produced by *A. oryzae* RC27 as compared to 265.39 U mL^{-1} by the reference strain *A. niger* MTCC1344. Citrus peel showed significantly higher average (363.40 U mL^{-1}), followed by citrus peel + jackfruit rind + pineapple peel (304.70 U mL^{-1}), wheat bran (296.40 U mL^{-1}), sunflower head (270.40 U mL^{-1}) and coffee husk (245.90 U mL^{-1}). The lowest mean for PE production was found in banana pseudo stem (160.63 U mL^{-1}). All these substrates differed significantly over one another for PE production.

The PE production varied with moisture content of substrates also. The highest mean PE activity (382.36 U mL^{-1}) was noticed at 65 per cent moisture level which was significantly superior over 70 per cent (335.17 U mL^{-1}), 60 per cent (263.61 U mL^{-1}), 55 per cent (207.06 U mL^{-1}) and 75 per cent (179.67 U mL^{-1}) moisture levels of substrates.

The production of PE as influenced by pectinolytic fungi and different moisture levels of substrates indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 at 65 per cent moisture in citrus peel (511.67 U mL^{-1}) followed by wheat bran (455.00 U mL^{-1}), citrus peel + jackfruit rind + pineapple peel (443.33 U mL^{-1}) and sunflower head (435.00 U mL^{-1}). Whereas, 70 per cent moisture content was found optimum in coffee husk (406.67 U mL^{-1}) and banana pseudo stem (316.67 U mL^{-1}). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity at 65 per cent moisture in citrus peel (470.00 U mL^{-1}) followed by citrus peel + jackfruit rind + pineapple peel (411.67 U mL^{-1}), wheat bran (390.00 U mL^{-1}) and sunflower head (370.00 U mL^{-1}). Whereas, 70 per cent moisture level was found optimum for highest PE activity in case of coffee husk (336.67 U mL^{-1}) and banana pseudo stem (253.33 U mL^{-1}). The lowest PE production (58.00 U mL^{-1}) was seen in the substrate banana pseudo stem at 70 per cent moisture level in the reference strain *A. niger* MTCC1344.

4.2.4 Polygalacturonase (PG) production by *A. niger* RBF96 under SSF

4.2.4.1 Effect of different particle sizes of substrates

There was a significant difference in PG production due to different particle sizes (Table 28).

All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other. A mean maximum of 578.96 U mL^{-1} PG was produced by *A. niger* RBF96 as compared to 451.11 U mL^{-1} by the reference strain *A. niger* MTCC1344. Citrus peel showed significantly higher average PG activity of 557.86 U mL^{-1} , followed by wheat bran (555.01 U mL^{-1}), citrus peel + jackfruit rind + pineapple peel (518.87 U mL^{-1}), sunflower

head (514.29 U mL⁻¹) and banana pseudo stem (505.94 U mL⁻¹). The lowest mean for PG production was found in coffee husk (438.26 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with the substrate particle sizes also. The highest mean PG activity (652.13 U mL⁻¹) was found in 2 mm substrate particle size which was significantly superior over 4 mm substrate particle size (377.94 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and different particle sizes indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 in 2 mm particle size of citrus peel (859.20 U mL⁻¹) followed by wheat bran (810.27 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (774.80 U mL⁻¹), sunflower head (736.66 U mL⁻¹), banana pseudo stem (651.51 U mL⁻¹) and coffee husk (571.06 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity in 2 mm substrate particle size of citrus peel (647.66 U mL⁻¹) followed by wheat bran (589.54 U mL⁻¹), sunflower head (583.99 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (544.24 U mL⁻¹), banana pseudo stem (532.81 U mL⁻¹) and coffee husk (523.84 U mL⁻¹).

4.2.5 Pectin lyase production by *Penicillium citrinum* under SSF

4.2.5.1 Effect of different particle sizes of substrates

The data presented in Table 29 indicated a significant difference in pectin lyase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PL production. The mean maximum of 313.51 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 219.81 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 327.29 U mL⁻¹, followed by wheat bran (295.00 U mL⁻¹), sunflower head (275.83 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (265.17 U mL⁻¹) and coffee husk (209.83 U mL⁻¹). The lowest mean for PL production was found in banana pseudo stem (205.33 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with substrate particle size. The highest mean PL activity (301.71 U mL⁻¹) was found in 2 mm substrate particle size which was significantly superior over 4 mm substrate particle size (231.61 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and different substrate particle size indicated varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 in 2 mm substrate particle size of citrus peel (434.83 U mL⁻¹) followed by wheat bran (426.00 U mL⁻¹), sunflower head (354.67 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (344.67 U mL⁻¹), coffee husk (209.83 U mL⁻¹) and banana pseudo stem (205.33 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity RBF96 in 2 mm substrate particle size of citrus peel (301.67 U mL⁻¹) followed by sunflower head (285.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (282.67 U mL⁻¹), wheat bran (244.33 U mL⁻¹), banana pseudo stem (226.83 U mL⁻¹) and coffee husk (196.67 U mL⁻¹).

4.2.6 Pectin esterase production by *A. oryzae* RC27 under SSF

4.2.6.1 Effect of different particle sizes of substrates

The data presented in Table 30 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PE production. The mean maximum of 267.72 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 232.97 U mL⁻¹ by the reference strain *A. niger* MTCC1344. All these substrates differed significantly over one another for PE production. The substrate citrus peel showed significantly higher average PE activity of 360.83 U mL⁻¹, followed by sunflower head (271.50 U mL⁻¹), wheat bran (263.33 U mL⁻¹), coffee husk (227.50 U mL⁻¹) and banana pseudo stem (198.50 U mL⁻¹). The lowest mean for PE production was found in citrus peel + jackfruit rind + pineapple peel (180.42 U mL⁻¹). The PE production varied with substrate particle size. A mean highest PE activity (295.97 U mL⁻¹) was found in 2 mm substrate particle size which was significantly superior over 4 mm substrate particle size (204.72 U mL⁻¹).

The production of PE as influenced by pectinolytic fungi and different substrate particle size indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 in 2 mm

substrate particle size of citrus peel (460.00 U mL⁻¹) followed by in wheat bran (333.33 U mL⁻¹), sunflower head (326.00 U mL⁻¹), coffee husk (271.67 U mL⁻¹), banana pseudo stem (265.00 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (248.33 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity RBF96 in 2 mm substrate particle size of citrus peel (340.00 U mL⁻¹) followed by in sunflower head (295.00 U mL⁻¹), wheat bran (286.67 U mL⁻¹), coffee husk (285.00 U mL⁻¹), banana pseudo stem (234.00 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (206.67 U mL⁻¹).

4.2.7 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.7.1 Effect of different inoculum size

The data presented in Table 31 indicated a significant difference in PG production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PG production. A mean maximum of 596.04 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 427.97 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PG activity of 548.46 U mL⁻¹, followed by wheat bran (534.45 U mL⁻¹), sunflower head (524.75 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (513.04 U mL⁻¹) and coffee husk (512.03 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (453.13 U mL⁻¹).

All these substrates differed significantly over one another for PG production. The PG production varied with inoculum size. The highest mean PG activity (649.51 U mL⁻¹) was found in 1x10⁷ inoculum size which was significantly superior over 1x10⁵ inoculum size (374.49 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and different inoculum size indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 at 1x10⁷ inoculum size in citrus peel (814.86 U mL⁻¹) followed by in citrus peel + jackfruit rind + pineapple peel (779.93 U mL⁻¹), wheat bran (774.8 U mL⁻¹), coffee husk (773.84 U mL⁻¹), sunflower head (766.08 U mL⁻¹) and banana pseudo stem (686.34 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity at 1x10⁷ inoculum size in citrus peel (558.13 U mL⁻¹) followed by in sunflower head (554.28 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (541.46 U mL⁻¹), wheat bran (523.84 U mL⁻¹), coffee husk (506.63 U mL⁻¹) and banana pseudo stem (503.96 U mL⁻¹).

4.2.8 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.8.1 Effect of inoculum size

The data presented in Table 32 indicated a significant difference in pectin lyase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PL production. A mean maximum of 332.39 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 220.78 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 312.67 U mL⁻¹, followed by wheat bran (292.75 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (291.25 U mL⁻¹), sunflower head (277.25 U mL⁻¹) and coffee husk (259.83 U mL⁻¹). The lowest mean for PL production was found in banana pseudo stem (226.75 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with inoculum size. The highest mean PL activity (340.08 U mL⁻¹) was found in 1x10⁷ inoculum size which was significantly superior over 1x10⁵ inoculum size (213.08 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and different inoculum size indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 at 1x10⁷ inoculum size in citrus peel (431.67 U mL⁻¹) followed by wheat bran (424.67 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (394.67 U mL⁻¹), sunflower head (384.33 U mL⁻¹), coffee husk (375.00 U mL⁻¹) and banana pseudo stem (315.00 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity at 1x10⁷ inoculum size in citrus peel (373.33 U mL⁻¹) followed by wheat bran (314.33 U mL⁻¹), sunflower head (305.00 U mL⁻¹), coffee husk (276.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (282.67 U mL⁻¹) and banana pseudo stem (214.33 U mL⁻¹).

Table 22. Polygalacturonase production by *Aspergillus niger* RBF96 under submerged fermentation at different incubation periods

Polygalacturonase activity (U mL ⁻¹)			
Incubation time (Days)	<i>Aspergillus niger</i> RBF96	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
2	136.34	143.17	139.75
4	165.18	187.19	176.18
6	192.10	164.86	178.48
8	156.53	145.84	151.18
Mean	162.54	160.27	
		S.Em±	CD at 1%
Strains (A)		0.52	2.20
Incubation time (B)		0.74	3.11
A x B		0.35	1.47

Table 23. Pectin lyase production by *Penicillium citrinum* DLP51 under submerged fermentation at different incubation periods

Pectin lyase activity (U mL ⁻¹)			
Incubation time (Days)	<i>Penicillium citrinum</i> DLP51	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
2	214.00	98.00	156.00
4	311.00	191.33	251.17
6	340.67	210.00	275.33
8	202.33	144.00	173.17
Mean	267.00	160.83	
		S.Em±	CD at 1%
Strains (A)		0.71	2.99
Incubation time (B)		1.00	4.23
A x B		0.47	1.99

Table 24. Pectin esterase production by *Aspergillus oryzae* RC27 under submerged fermentation at different incubation periods

Pectin esterase activity (U mL ⁻¹)			
Incubation time (Days)	<i>Aspergillus oryzae</i> RC27	<i>Aspergillus niger</i> MTCC1344 (Reference strain)	Mean
2	126.67	206.67	166.67
4	203.33	300.00	251.67
6	353.33	343.33	348.33
8	280.00	216.67	248.33
Mean	240.83	266.67	
		S.Em±	CD at 1%
Strains (A)		5.79	24.36
Incubation time (B)		8.18	34.45
A x B		3.86	16.24

Table 25. Polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation at different moisture content of substrates

Strains	Moisture (%)	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	55	399.33	373.29	341.61	314.73	261.66	361.99	454.12
	60	568.07	546.67	512.70	428.92	368.88	490.24	
	65	733.88	709.89	685.18	489.20	423.86	527.46	
	70	492.35	474.55	448.68	657.47	592.36	465.24	
	75	371.97	355.83	323.29	300.50	247.06	356.75	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)	55	250.68	230.55	199.18	174.43	110.57	214.12	266.23
	60	252.53	232.80	203.19	181.57	123.39	219.81	
	65	478.14	457.54	428.79	254.08	191.39	450.41	
	70	330.82	302.76	277.02	401.36	342.92	296.73	
	75	284.09	262.37	231.42	203.59	151.04	249.53	
Mean		416.19	394.63	365.11	340.59	281.31	363.23	
Mean	55	269.35						
	60	356.77						
	65	473.11						
	70	423.52						
	75	278.12						
		S.Em±			CD at 1%			
Strains (A)		4.983			18.449			
Moisture levels (B)		7.878			29.170			
Substrates (C)		8.630			31.955			
A x B		11.142			41.253			
B x C		19.298			71.453			
A x C		12.205			45.191			
A x B x C		27.291			101.049			

Table 26. Pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation at different moisture content of substrates

Strains		Moisture (%)	Pectin lyase activity (U mL ⁻¹)						
			Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Penicillium citrinum</i> DLP 51		55	195.67	172.70	161.07	157.53	111.20	142.87	206.69
		60	227.00	207.73	201.13	187.87	144.47	186.72	
		65	379.67	360.77	350.87	239.43	204.87	257.24	
		70	295.00	304.07	263.53	338.53	294.47	227.67	
		75	131.67	111.40	101.47	93.20	47.20	103.67	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)		55	179.67	174.81	166.44	156.14	109.64	173.07	177.30
		60	201.33	192.07	188.55	175.95	141.67	181.96	
		65	273.33	268.55	261.44	251.81	202.64	273.25	
		70	213.33	211.15	199.55	185.28	140.95	209.42	
		75	112.33	108.48	98.55	88.61	58.33	120.73	
Mean			220.90	211.17	199.26	187.44	145.54	187.93	
Mean	55		158.40						
	60		186.37						
	65		276.99						
	70		240.25						
	75		97.97						
			S.Em±			CD at 1%			
Strains (A)			0.799			2.960			
Moisture levels (B)			1.264			4.680			
Substrates (C)			1.385			5.127			
A x B			1.788			6.619			
B x C			3.096			11.464			
A x C			1.958			7.250			
A x B x C			4.379			16.212			

Table 27. Pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation at different moisture content of substrates

Strains	Moisture (%)	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	55	258.33	200.00	173.33	148.33	58.00	201.67	281.96
	60	313.33	250.00	218.33	203.33	111.67	275.00	
	65	511.67	455.00	435.00	355.00	260.00	443.33	
	70	466.67	403.33	380.00	406.67	316.67	416.67	
	75	275.00	218.33	191.67	168.33	80.67	213.67	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)	55	353.33	280.00	253.33	189.00	100.67	268.33	265.39
	60	411.67	335.00	311.67	233.33	143.33	356.67	
	65	470.00	390.00	370.00	288.33	198.33	411.67	
	70	315.67	249.00	210.67	336.67	253.33	266.67	
	75	258.33	183.33	160.00	130.00	83.33	193.33	
Mean		363.40	296.40	270.40	245.90	160.63	304.70	
Mean	55	207.06						
	60	263.61						
	65	382.36						
	70	335.17						
	75	179.67						
			S.Em±		CD at 1%			
Strains (A)			0.741		2.742			
Moisture levels (B)			1.171		4.335			
Substrates (C)			1.283		4.749			
A x B			1.656		6.131			
B x C			2.868		10.619			
A x C			1.814		6.716			
A x B x C			4.056		15.018			

Table 28. Effect of substrate particle size on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	Substrate particle size (mm)	Polygalacturonase activity (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Aspergillus niger</i> RBF 96	2	859.20	810.27	736.66	571.06	651.51	774.80	578.96
	4	452.15	470.95	479.44	307.60	364.54	469.35	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	647.66	589.54	583.99	523.84	532.81	544.24	451.11
	4	336.12	349.26	308.24	330.14	360.05	307.49	
Mean		557.86	555.01	514.29	438.26	505.94	518.87	
Mean	2	652.13						
	4	377.94						
			S.Em+			CD at 1%		
Strains (A)			0.94			3.57		
Substrate particle size (B)			0.94			3.57		
Substrates (C)			1.63			6.19		
A x B			1.33			5.06		
B x C			1.63			6.19		
A x C			2.31			8.76		
A x B x C			3.26			12.38		

Table 29. Effect of substrate particle size on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	Substrate particle size (mm)	Pectin lyase activity (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Penicillium citrinum</i> DLP 51	2	434.83	426.00	354.67	310.33	294.67	344.67	313.51
	4	345.00	325.67	275.33	217.00	184.67	249.33	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	301.67	244.33	285.00	196.67	226.83	282.67	219.81
	4	227.67	184.00	188.33	115.33	222.67	184.00	
Mean		327.29	295.00	275.83	209.83	205.33	265.17	
Mean	2	301.71						
	4	231.61						
			S.Em+			CD at 1%		
Strains (A)			3.22			12.22		
Substrate particle size (B)			3.22			12.22		
Substrates (C)			5.58			21.16		
A x B			4.55			17.28		
B x C			5.58			21.16		
A x C			7.89			29.92		
A x B x C			11.16			42.32		

Table 30. Effect of substrate particle size on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Substrate particle size (mm)	Pectin esterase activity (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Aspergillus oryzae</i> RC27	2	460.00	333.33	326.00	271.67	265.00	248.33	267.72
	4	360.00	246.67	235.00	173.33	155.00	138.33	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	340.00	286.67	295.00	285.00	234.00	206.67	232.97
	4	283.33	188.33	230.00	185.00	133.33	128.33	
Mean		360.83	263.33	271.50	227.50	198.50	180.42	
Mean	2	295.97						
	4	204.72						
			S.Em+			CD at 1%		
Strains (A)			0.83			3.13		
Substrate particle size (B)			0.83			3.13		
Substrates (C)			1.43			5.42		
A x B			1.17			4.43		
B x C			1.43			5.42		
A x C			2.02			7.67		
A x B x C			2.86			10.85		

Table 31. Influence of inoculum size on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	Inoculum size (Spores g ⁻¹)	Polygalacturonase activity (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Aspergillus niger</i> RBF 96	1 x 10 ⁵	466.04	466.25	452.15	460.16	295.07	406.96	596.04
	1 x 10 ⁷	814.86	774.80	766.08	773.84	686.34	779.93	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)	1 x 10 ⁵	354.82	307.49	326.51	307.49	327.15	323.84	427.97
	1 x 10 ⁷	558.13	523.84	554.28	506.63	503.96	541.46	
Mean		548.46	534.45	524.75	512.03	453.13	513.04	
Mean	1 x 10 ⁵	374.49						
	1 x 10 ⁷	649.51						
			S.Em+			CD at 1%		
Strains (A)			0.53			2.00		
Inoculum size (B)			0.53			2.00		
Substrates (C)			0.91			3.46		
A x B			0.74			2.82		
B x C			0.91			3.46		
A x C			1.29			4.89		
A x B x C			1.82			6.91		

Table 32. Pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation as influenced by different inoculum sizes

Strains	Inoculum size (Spores g ⁻¹)	Pectin lyase activity (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Penicillium citrinum</i> DLP51	1 x 10 ⁵	293.67	280.00	276.67	271.00	271.00	275.00	332.39
	1 x 10 ⁷	431.67	424.67	384.33	375.00	315.00	394.67	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)	1 x 10 ⁵	152.00	152.00	143.00	117.33	106.67	222.67	220.78
	1 x 10 ⁷	373.33	314.33	305.00	276.00	214.33	272.67	
Mean		312.67	292.75	277.25	259.83	226.75	291.25	
Mean	1 x 10 ⁵	213.08						
	1 x 10 ⁷	340.08						
			S.Em+			CD at 1%		
Strains (A)			3.24			12.30		
Inoculum size (B)			3.24			12.30		
Substrates (C)			5.62			21.31		
A x B			4.59			17.40		
B x C			5.62			21.31		
A x C			7.94			30.14		
A x B x C			11.24			42.62		

4.2.9 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.9.1 Effect of inoculum size

The data presented in Table 33 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PE production. A mean maximum of 233.97 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 223.39 U mL⁻¹ by the reference strain *A. niger* MTCC1344. All these substrates differed significantly over one another for PE production. The substrate citrus peel showed significantly higher average PE activity of 320.83 U mL⁻¹, followed by wheat bran (263.75 U mL⁻¹), sunflower head (248.83 U mL⁻¹), coffee husk (203.75 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (192.67 U mL⁻¹). The lowest mean for PE production was found in banana pseudo stem (142.25 U mL⁻¹). The PE production varied with inoculum size. The highest mean PE activity (303.97 U mL⁻¹) was found in 1x10⁷ inoculum size which was significantly superior over 1x10⁵ inoculum size (153.39 U mL⁻¹).

The production of PE as influenced by pectinolytic fungi and different inoculum size indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 when inoculated at 1x10⁷ inoculum size in citrus peel (473.33 U mL⁻¹) followed by wheat bran (391.67 U mL⁻¹), sunflower head (381.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (273.33 U mL⁻¹), coffee husk (265.00 U mL⁻¹) and banana pseudo stem (156.67 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity at 1x10⁷ inoculum size in citrus peel (343.33 U mL⁻¹) followed by wheat bran (323.33 U mL⁻¹), sunflower head (314.33 U mL⁻¹), coffee husk (290.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (234.00 U mL⁻¹) and banana pseudo stem (201.67 U mL⁻¹).

4.2.10 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.10.1 Effect of different incubation period

The data presented in Table 34 indicated a significant difference in polygalacturonase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PG production. A mean maximum of 457.14 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 352.38 U mL⁻¹ by reference strain *A. niger* MTCC1344.

The substrate citrus peel showed significantly higher average PG activity of 459.97 U mL⁻¹, followed by wheat bran (420.18 U mL⁻¹), sunflower head (409.99 U mL⁻¹), banana pseudo stem (396.74 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (380.09 U mL⁻¹), and lowest mean for PG production was found in coffee husk (361.59 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with incubation period. The incubation periods analysed for production of PG indicated that the mean maximum was 540.53 U mL⁻¹ at 6 days incubation period followed by 4 days (410.64 U mL⁻¹), 8 days (360.74 U mL⁻¹) and 2 days (307.12 U mL⁻¹). The extension of incubation periods upto 8 days with this fungus did not increase the PG activity. All these incubation days differed significantly with each other.

The production of PG as influenced by pectinolytic fungi and different incubation period indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 at 6 days incubation period in citrus peel (667.96 U mL⁻¹) followed by sunflower head (664.33 U mL⁻¹), banana pseudo stem (658.88 U mL⁻¹), wheat bran (643.71 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (605.46 U mL⁻¹) and coffee husk (446.06 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity at 6 days incubation period in citrus peel (493.60 U mL⁻¹) followed by wheat bran (479.93 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (477.15 U mL⁻¹), coffee husk (465.50 U mL⁻¹), sunflower head (443.92 U mL⁻¹) and banana pseudo stem (439.86 U mL⁻¹). The lowest quantity of PG production was noticed in citrus peel + jackfruit rind + pineapple peel (214.43 U mL⁻¹) at 2 days incubation period.

Table 33. Effect of inoculum size on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Inoculum size (Spores g ⁻¹)	Pectin esterase (U mL ⁻¹)						
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	Mean
<i>Aspergillus oryzae</i> RC27	1 x 10 ⁵	258.33	155.00	115.00	115.00	93.33	130.00	233.97
	1 x 10 ⁷	473.33	391.67	381.00	265.00	156.67	273.33	
<i>Aspergillus niger</i> MTCC1344 (Reference strain)	1 x 10 ⁵	208.33	185.00	185.00	145.00	117.33	133.33	223.39
	1 x 10 ⁷	343.33	323.33	314.33	290.00	201.67	234.00	
Mean		320.83	263.75	248.83	203.75	142.25	192.67	
Mean	1 x 10 ⁵	153.39						
	1 x 10 ⁷	303.97						
			S.Em+			CD at 1%		
Strains (A)			0.82			3.11		
Inoculum size (B)			0.82			3.11		
Substrates (C)			1.42			5.38		
A x B			1.16			4.40		
B x C			1.42			5.38		
A x C			2.01			7.61		
A x B x C			2.84			10.77		

Table 34. Polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation during different incubation periods

Strains	Incubation time (days)	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	2	365.34	310.80	323.95	234.41	295.20	342.53	457.14
	4	480.25	529.07	416.46	372.55	502.47	477.18	
	6	667.96	643.71	664.33	446.06	658.88	605.46	
	8	544.35	439.54	469.24	319.67	407.60	454.28	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	320.52	343.22	298.09	330.57	306.42	214.43	352.38
	4	394.88	386.98	359.95	378.75	348.84	280.35	
	6	493.60	479.93	443.92	465.50	439.86	477.15	
	8	412.83	228.22	303.96	345.20	214.65	189.36	
Mean		459.97	420.18	409.99	361.59	396.74	380.09	
Mean	2	307.12						
	4	410.64						
	6	540.53						
	8	360.74						
		S.Em±					CD at 1%	
Strains (A)		0.92					3.41	
Incubation time (B)		1.30					4.83	
Substrates (C)		1.59					5.91	
A x B		1.84					6.82	
B x C		2.25					8.36	
A x C		3.18					11.82	
A x B x C		4.50					16.72	

4.2.11 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.11.1 Effect of different incubation periods

The data presented in Table 35 indicated a significant difference in pectin lyase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PL production. A mean maximum of 196.68 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 121.31 U mL⁻¹ by the reference strain *A. niger* MTCC1344.

The substrate citrus peel showed significantly higher average PL activity of 217.29 U mL⁻¹, followed by wheat bran (210.87 U mL⁻¹), sunflower head (182.97 U mL⁻¹), coffee husk (120.54 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (105.41 U mL⁻¹), and the lowest mean for PL production was found in banana pseudo stem (96.50 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with the incubation period. The incubation periods analysed for production of PL indicated that the mean maximum was 222.48 U mL⁻¹ at 6 days incubation period followed by 4 days (155.41 U mL⁻¹), 8 days (153.18 U mL⁻¹) and 2 days (104.93 U mL⁻¹). The extension of incubation periods upto 8 days with this fungus did not increase the PL activity. All these incubation days differed significantly with each other.

The production of PL as influenced by pectinolytic fungi and different incubation periods indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 at 6 days incubation period in citrus peel (433.10 U mL⁻¹) followed by in wheat bran (410.61 U mL⁻¹), sunflower head (362.18 U mL⁻¹), coffee husk (196.77 U mL⁻¹), banana pseudo stem (163.06 U mL⁻¹), and citrus peel + jackfruit rind + pineapple peel (162.31 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity at 6 days incubation period in citrus peel (180.86 U mL⁻¹) followed by wheat bran (172.97 U mL⁻¹), sunflower head (165.82 U mL⁻¹), coffee husk (149.18 U mL⁻¹), banana pseudo stem (142.03 U mL⁻¹), and citrus peel + jackfruit rind + pineapple peel (130.87 U mL⁻¹). The lowest quantity of PL production was noticed in citrus peel + jackfruit rind + pineapple peel (61.57 U mL⁻¹) at 2 days incubation period.

4.2.12 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.12.1 Effect of different incubation periods

The data presented in Table 36 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Pectinolytic fungal strains differed statistically with each other in terms of PE production. A mean maximum of 292.50 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 260.49 U mL⁻¹ by the reference strain *A. niger* MTCC1344.

The substrate citrus peel showed significantly higher average PE activity of 371.32 U mL⁻¹, followed by wheat bran (310.42 U mL⁻¹), sunflower head (278.75 U mL⁻¹) and coffee husk (274.29 U mL⁻¹), banana pseudo stem (221.04 U mL⁻¹). The lowest mean for PE production was found in citrus peel + jackfruit rind + pineapple peel (203.13 U mL⁻¹). All these substrates differed significantly over one another for PE production.

The PE production varied with the incubation period. The incubation periods analysed for production of PE indicated that the mean maximum was 388.61 U mL⁻¹ at 4 days incubation period followed by 6 days (268.08 U mL⁻¹), 8 days (247.06 U mL⁻¹) and 2 days (202.21 U mL⁻¹). The extension of incubation periods upto 8 days with this fungus did not increase the PE activity. All these incubation days differed significantly with each other.

The production of PE as influenced by pectinolytic fungi and different incubation periods indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 at 4 days incubation period in citrus peel (520.00 U mL⁻¹) followed by wheat bran (511.67 U mL⁻¹), sunflower head (475.00 U mL⁻¹), coffee husk (420.00 U mL⁻¹), banana pseudo stem (353.33 U mL⁻¹), and citrus peel + jackfruit rind + pineapple peel (335.00 U mL⁻¹).

Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity at 4 days incubation period in citrus peel (468.33 U mL⁻¹) followed by coffee husk (408.33 U mL⁻¹), wheat bran (315.00 U mL⁻¹), sunflower head (303.33 U mL⁻¹), banana pseudo stem (281.67 U mL⁻¹), and citrus

peel + jackfruit rind + pineapple peel (271.67 U mL⁻¹). The lowest quantity of PE production was noticed in citrus peel + jackfruit rind + pineapple peel (95.00 U mL⁻¹) at 2 days incubation period.

4.2.13 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.13.1 Effect of pH

The pectinolytic fungi were grown at different pH values such as 3.0, 4.0, 5.0, 6.0 and 7.0 to determine the optimum pH conditions for maximum polygalacturonase activity. The data presented in Table 37 indicated a significant difference in polygalacturonase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PG production. A mean maximum of 395.37 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 325.31 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PG activity of 497.70 U mL⁻¹, followed by wheat bran (420.92 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (383.30 U mL⁻¹), sunflower head (356.86 U mL⁻¹) and coffee husk (253.98 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (249.26 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with different pH levels of the fermentation medium. The highest mean PG activity was noticed at pH 4.0 with 435.89 U mL⁻¹ which was significantly superior over pH 5.0 (386.31 U mL⁻¹), pH 3.0 (365.12 U mL⁻¹), pH 6.0 (331.49 U mL⁻¹) and pH 7.0 (282.87 U mL⁻¹) in the fermentation medium.

The production of PG as influenced by pectinolytic fungi and different pH levels of the fermentation medium indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 at pH 4.0 in citrus peel (618.28 U mL⁻¹) followed by wheat bran (539.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (502.83 U mL⁻¹), sunflower head (473.26 U mL⁻¹), banana pseudo stem (368.91 U mL⁻¹) and coffee husk (361.67 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity at pH 4.0 in citrus peel (534.63 U mL⁻¹) followed by wheat bran (456.84 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (420.29 U mL⁻¹) and sunflower head (394.38 U mL⁻¹), coffee husk (280.25 U mL⁻¹) and banana pseudo stem (280.33 U mL⁻¹).

4.2.14 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.14.1 Effect of pH on fermentation

The pectinolytic fungi were grown at different pH such as 3.0, 4.0, 5.0, 6.0 and 7.0 to determine the optimum pH conditions for maximum pectin lyase activity. The data presented in Table 38 indicated a significant difference in pectin lyase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PL production. A mean maximum of 462.48 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 357.14 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 284.76 U mL⁻¹, followed by wheat bran (261.03 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (250.13 U mL⁻¹), sunflower head (244.18 U mL⁻¹) and coffee husk (224.27 U mL⁻¹). The lowest mean for PL production was found in banana pseudo stem (173.97 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with different pH levels of the fermentation medium. The highest mean PL activity was noticed at pH 7.0 with 275.25 U mL⁻¹ which was significantly superior over pH 6.0 (260.98 U mL⁻¹), pH 5.0 (247.14 U mL⁻¹), pH 4.0 (220.99 U mL⁻¹) and pH 3.0 (194.25 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and different pH levels of the fermentation medium indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 at pH 7.0 in citrus peel (429.00 U mL⁻¹) followed by wheat bran (406.67 U mL⁻¹), sunflower head (389.67 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (371.67 U mL⁻¹), coffee husk (367.33 U mL⁻¹) and banana pseudo stem (318.53 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity at pH 4.0 in citrus peel (215.00 U mL⁻¹) followed by in citrus peel + jackfruit rind + pineapple peel (193.27 U mL⁻¹), wheat bran (189.00 U mL⁻¹), sunflower head (171.50 U mL⁻¹), coffee husk (150.67 U mL⁻¹) and banana pseudo stem (100.73 U mL⁻¹).

Table 35. Pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation during different incubation periods

Strains	Incubation time (days)	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	2	165.33	176.19	121.67	92.63	94.43	80.70	196.68
	4	235.72	231.18	229.46	164.13	127.29	101.35	
	6	433.10	410.61	362.18	196.77	163.06	162.31	
	8	335.29	319.00	200.57	102.81	105.99	108.60	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	114.33	110.16	89.53	85.97	66.65	61.57	121.31
	4	153.67	155.97	138.47	115.98	107.21	104.44	
	6	180.86	172.97	165.82	149.18	142.03	130.87	
	8	132.57	116.04	128.24	97.43	103.71	87.89	
Mean		217.29	210.87	182.97	120.54	96.50	105.41	
Mean	2	104.93						
	4	155.41						
	6	222.48						
	8	153.18						
		S.Em±					CD at 1%	
Strains (A)		0.23					0.85	
Incubation time (B)		0.33					1.21	
Substrates (C)		0.40					1.48	
A x B		0.46					1.71	
B x C		0.56					2.09	
A x C		0.80					2.96	
A x B x C		1.13					4.19	

Table 36. Pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation during different incubation periods

Strains	Incubation time (days)	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	2	254.89	216.67	185.00	166.67	148.33	178.33	292.50
	4	520.00	511.67	475.00	420.00	353.33	335.00	
	6	356.67	338.33	285.00	261.67	243.33	180.00	
	8	313.33	368.33	315.00	196.67	186.67	210.00	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	2	351.67	225.00	200.00	261.67	143.33	95.00	260.49
	4	468.33	315.00	303.33	408.33	281.67	271.67	
	6	386.67	253.33	211.67	250.33	245.00	205.00	
	8	319.00	255.00	255.00	229.00	166.67	150.00	
Mean		371.32	310.42	278.75	274.29	221.04	203.13	
Mean	2	202.21						
	4	388.61						
	6	268.08						
	8	247.06						
		S.Em±					CD at 1%	
Strains (A)		0.73					2.71	
Incubation time (B)		1.03					3.83	
Substrates (C)		1.26					4.69	
A x B		1.46					5.42	
B x C		1.78					6.63	
A x C		2.52					9.38	
A x B x C		3.57					13.26	

Table 37. Influence of pH on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	pH	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	3	521.33	450.04	387.78	275.64	278.82	410.44	395.37
	4	618.28	539.00	473.26	361.67	368.91	502.83	
	5	562.67	478.82	419.56	301.16	311.23	445.33	
	6	501.54	426.08	358.75	259.52	253.99	389.27	
	7	464.76	390.34	321.85	224.28	216.27	347.55	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	3	481.78	406.72	337.23	236.21	230.66	364.78	325.31
	4	534.63	456.84	394.38	280.25	280.33	420.29	
	5	492.87	411.90	350.41	246.28	241.26	374.29	
	6	433.87	355.92	292.91	202.25	184.47	319.29	
	7	365.22	293.58	232.43	152.58	126.63	258.92	
Mean		497.70	420.92	356.86	253.98	249.26	383.30	
Mean	3	365.12						
	4	435.89						
	5	386.31						
	6	331.49						
	7	282.87						
			S.Em+		CD at 1%			
Strains (A)			0.340		1.259			
pH (B)			0.538		1.990			
Substrates (C)			0.589		2.180			
A x B			0.760		2.815			
B x C			1.317		4.875			
A x C			0.833		3.083			
A x B x C			1.862		4.281			

Table 38. Effect of pH on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	pH	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	3	295.58	277.00	256.83	233.00	182.20	263.13	316.79
	4	325.33	307.00	286.33	270.33	217.95	299.28	
	5	379.67	352.67	338.17	318.50	251.33	317.66	
	6	381.67	365.00	349.33	331.67	279.07	342.00	
	7	429.00	406.67	389.67	367.33	318.53	371.67	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	3	182.33	153.67	136.94	118.83	85.00	146.52	162.66
	4	204.33	171.33	160.00	138.00	92.07	179.93	
	5	212.33	189.00	170.00	150.67	99.07	186.60	
	6	222.33	199.00	183.00	163.67	113.73	201.27	
	7	215.00	189.00	171.50	150.67	100.73	193.27	
Mean		284.76	261.03	244.18	224.27	173.97	250.13	
Mean	3	194.25						
	4	220.99						
	5	247.14						
	6	260.98						
	7	275.25						
			S.Em±		CD at 1%			
Strains (A)			0.318		1.177			
pH (B)			0.503		1.861			
Substrates (C)			0.551		2.038			
A x B			0.711		2.632			
B x C			1.231		4.558			
A x C			0.779		2.883			
A x B x C			1.741		4.761			

4.2.15 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.15.1 Effect of pH

The data presented in Table 39 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PE production. A mean maximum of 351.22 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 294.89 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PE activity of 415.50 U mL⁻¹, followed by wheat bran (354.83 U mL⁻¹), sunflower head (329.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (304.50 U mL⁻¹) and banana pseudo stem (295.33 U mL⁻¹). The lowest mean for PE production was found in coffee husk (224.33 U mL⁻¹). All these substrates differed significantly over one another for PE production.

The PE production varied with different pH levels of the fermentation medium. The highest mean PE activity was noticed at pH 5.0 with 380.14 U mL⁻¹ which was significantly superior over pH 4.0 (346.67 U mL⁻¹), pH 6.0 (331.25 U mL⁻¹), pH 3.0 (278.06 U mL⁻¹) and pH 7.0 (266.81 U mL⁻¹) of the fermentation medium.

The production of PE as influenced by pectinolytic fungi and different pH levels of the fermentation medium indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 at pH 5.0 in citrus peel (508.33 U mL⁻¹) followed by in wheat bran (445.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (411.67 U mL⁻¹), sunflower head (405.00 U mL⁻¹), banana pseudo stem (375.00 U mL⁻¹) and coffee husk (318.33 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 also showed the highest PE activity at pH 5.0 in citrus peel (475.00 U mL⁻¹) followed by wheat bran (400.00 U mL⁻¹), sunflower head (370.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (333.33 U mL⁻¹), banana pseudo stem (315.00 U mL⁻¹) and coffee husk (246.67 U mL⁻¹).

4.2.16 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.16.1 Effect of temperature

The influence of different incubation temperatures viz., 20, 30, 35 and 40°C on polygalacturonase enzyme production in solid state fermentation was studied. The data presented in Table 40 indicated a significant difference in polygalacturonase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PG production. The mean maximum of 462.48 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 357.14 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PG activity of 452.73 U mL⁻¹, followed by wheat bran (428.08 U mL⁻¹), sunflower head (413.31 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (404.35 U mL⁻¹) and coffee husk (397.97 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (362.42 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with incubation temperatures. The highest mean PG activity (524.99 U mL⁻¹) was noticed at 35°C temperature which was significantly superior over 30°C (414.50 U mL⁻¹), 40°C (370.94 U mL⁻¹) and 20°C (328.81 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and incubation temperatures indicated a varied PG activity. The 222.48 highest PG activity was recorded by *A. niger* RBF96 at 35°C temperature for citrus peel (712.47 U mL⁻¹) followed by sunflower head (663.58 U mL⁻¹), coffee husk (659.95 U mL⁻¹), wheat bran (602.36 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (556.10 U mL⁻¹) and banana pseudo stem (420.52 U mL⁻¹). Whereas, the reference strain *A. niger* MTCC1344 showed the highest PG activity at 35°C incubation temperature in case of citrus peel (493.60 U mL⁻¹) followed by citrus peel + jackfruit rind + pineapple peel (461.66 U mL⁻¹), wheat bran (458.73 U mL⁻¹), and sunflower head (455.35 U mL⁻¹), coffee husk (421.49 U mL⁻¹) and banana pseudo stem (394.03 U mL⁻¹).

4.2.17 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.17.1 Effect of temperature

The influence of different incubation temperatures viz., 20, 30, 35 and 40°C on pectin lyase enzyme production in solid state fermentation was studied. The data presented in Table 41 indicated a significant difference in pectin lyase production. All the individual factors and their two way and

three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PL production. A mean maximum of 195.78 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 115.41 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 217.29 U mL⁻¹, followed by wheat bran (210.87 U mL⁻¹), sunflower head (182.97 U mL⁻¹), coffee husk (120.54 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (105.41 U mL⁻¹). The lowest mean for PL production was found in banana pseudo stem (96.50 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with incubation temperatures. The highest mean PL activity (220.00 U mL⁻¹) was noticed at 35°C temperature which was significantly superior over 40°C (154.04 U mL⁻¹), 30°C (144.04 U mL⁻¹) and 20°C (104.30 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and incubation temperatures indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 at 35°C temperature for citrus peel (432.47 U mL⁻¹) followed by wheat bran (402.86 U mL⁻¹), sunflower head (386.89 U mL⁻¹), coffee husk (189.14 U mL⁻¹), banana pseudo stem (162.39 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (160.13 U mL⁻¹). Whereas, the reference strain *A. niger* MTCC1344 showed the highest PL activity at 35°C incubation temperature in case of citrus peel (180.19 U mL⁻¹) followed by wheat bran (175.60 U mL⁻¹), sunflower head (166.36 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (138.29 U mL⁻¹), coffee husk (133.13 U mL⁻¹) and banana pseudo stem (112.55 U mL⁻¹).

4.2.18 Pectin esterase activity by *Aspergillus oryzae* RC27 under SSF

4.2.18.1 Effect of temperature

The influence of different incubation temperatures viz., 20, 30, 35 and 40°C on pectin esterase enzyme production in solid state fermentation was studied. The data presented in Table 42 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PE production. A mean maximum of 289.38 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 262.07 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PE activity of 373.63 U mL⁻¹, followed by wheat bran (313.33 U mL⁻¹), sunflower head (286.46 U mL⁻¹), coffee husk (276.75 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (221.67 U mL⁻¹). The lowest mean for PE production was found in banana pseudo stem (182.50 U mL⁻¹). All these substrates differed significantly over one another for PE production. The PE production varied with incubation temperatures. The highest mean PE activity (382.36 U mL⁻¹) was noticed at 35°C temperature which was significantly superior over 30°C (272.50 U mL⁻¹), 40°C (253.72 U mL⁻¹) and 20°C (194.31 U mL⁻¹).

The production of PE as influenced by pectinolytic fungi and incubation temperatures indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 at 35°C temperature for citrus peel (521.67 U mL⁻¹) followed by wheat bran (505.00 U mL⁻¹), sunflower head (478.33 U mL⁻¹), coffee husk (411.67 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (366.67 U mL⁻¹) and banana pseudo stem (346.67 U mL⁻¹). The reference strain *A. niger* MTCC1344 also showed the highest PE activity at 35°C incubation temperature in case of citrus peel (460.00 U mL⁻¹) followed by coffee husk (381.67 U mL⁻¹), wheat bran (320.00 U mL⁻¹), sunflower head (305.00 U mL⁻¹), banana pseudo stem (290.00 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (201.67 U mL⁻¹).

4.2.19 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.19.1 Effect of nitrogen sources

The pectinolytic fungi were cultured by adding the nitrogen sources viz., ammonium sulfate and soybean meal at one per cent and two per cent concentrations for studying their impact on the production of polygalacturonase. The data presented in Table 43 indicated a significant difference in polygalacturonase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PG production. The mean maximum of 635.21 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 503.38 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PG activity of 706.69 U mL⁻¹, followed by sunflower head (673.12 U mL⁻¹), wheat bran (659.31 U mL⁻¹), coffee husk (469.58 U mL⁻¹) and citrus peel + jackfruit rind + pineapple

peel (468.77 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (438.25 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with the addition of nitrogen sources into the fermentation medium. The highest mean PG activity (683.57 U mL⁻¹) was noticed in soybean meal at two per cent, which was significantly superior over ammonium sulfate at two per cent (585.86 U mL⁻¹), ammonium sulfate at one per cent (571.77 U mL⁻¹) and soybean meal at one per cent (524.68 U mL⁻¹). The lowest PG activity was observed in control flasks (480.58 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and nitrogen sources in the fermentation medium indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 for soybean meal at two per cent in citrus peel (910.20 U mL⁻¹) followed by wheat bran (893.54 U mL⁻¹), sunflower head (846.10 U mL⁻¹), coffee husk (714.48 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (698.66 U mL⁻¹) and banana pseudo stem (429.68 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 also showed the highest PG activity for soybean meal at two per cent in citrus peel (820.25 U mL⁻¹) followed by wheat bran (811.70 U mL⁻¹), sunflower head (739.37 U mL⁻¹), coffee husk (550.69 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (531.71 U mL⁻¹) and banana pseudo stem (425.79 U mL⁻¹).

4.2.20 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.20.1 Effect of nitrogen sources

The pectinolytic fungi were cultured by adding the nitrogen sources namely ammonium sulfate and soybean meal at one per cent and two per cent concentrations for studying their impact on the production of pectin lyase. The data presented in Table 44 indicated a significant difference in pectin lyase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PL production. The mean maximum of 482.16 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 446.48 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 489.78 U mL⁻¹, followed by sunflower head (488.48 U mL⁻¹), wheat bran (483.75 U mL⁻¹), coffee husk (457.24 U mL⁻¹), banana pseudo stem (435.83 U mL⁻¹), and lowest mean for PG production was found in citrus peel + jackfruit rind + pineapple peel (430.82 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with the addition of nitrogen sources into the fermentation medium. The highest mean PL activity (538.07 U mL⁻¹) was noticed in soybean meal at two per cent which was significantly superior over ammonium sulfate at two per cent (495.60 U mL⁻¹), soybean meal at one per cent (479.79 U mL⁻¹) and ammonium sulfate at one per cent (443.11 U mL⁻¹). The lowest PL activity was observed in control flasks (365.02 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and nitrogen sources in the fermentation medium indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 for soybean meal at two per cent in citrus peel (698.77 U mL⁻¹) followed by in wheat bran (640.89 U mL⁻¹), sunflower head (596.65 U mL⁻¹), coffee husk (521.25 U mL⁻¹) and banana pseudo stem (501.06 U mL⁻¹). The lowest PL activity was observed in citrus peel + jackfruit rind + pineapple peel (466.59 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity for soybean meal at two per cent in citrus peel (547.22 U mL⁻¹) followed by in sunflower head (537.32 U mL⁻¹), wheat bran (514.77 U mL⁻¹), coffee husk (500.22 U mL⁻¹), banana pseudo stem (480.03 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (474.78 U mL⁻¹).

4.2.21 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.21.1 Effect of nitrogen sources

The pectinolytic fungi were cultured by adding the nitrogen sources namely ammonium sulfate and soybean meal at one per cent and two per cent concentrations for studying their impact on the production of pectin esterase. The data presented in Table 45 indicated a significant difference in pectin esterase production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PE production. The mean maximum of 522.04 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 476.94 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PE activity of 570.28 U mL⁻¹, followed by wheat bran (517.36 U mL⁻¹), sunflower head (500.35 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (484.40 U mL⁻¹) and

coffee husk (475.67 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (448.90 U mL⁻¹). All these substrates differed significantly over one another for PE production.

The PE production varied with addition of nitrogen sources into the fermentation medium. The highest mean PE activity was noticed in ammonium sulfate at two per cent with 559.25 U mL⁻¹ which was significantly superior over soybean meal at two per cent (518.39 U mL⁻¹), ammonium sulfate at one per cent (510.64 U mL⁻¹) and soybean meal at one per cent (506.42 U mL⁻¹). The lowest PE activity was observed in control flasks (402.77 U mL⁻¹).

The production of PE as influenced by pectinolytic fungi and nitrogen sources in the fermentation medium indicated varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 in ammonium sulfate at two per cent in citrus peel (734.67 U mL⁻¹) followed by wheat bran (659.67 U mL⁻¹), sunflower head (618.00 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (559.67 U mL⁻¹) and banana pseudo stem (549.67 U mL⁻¹). The lowest PE activity was observed in coffee husk (499.67 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity in wheat bran (601.33 U mL⁻¹), followed by sunflower head (591.33 U mL⁻¹), citrus peel (586.33 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (548.00 U mL⁻¹), banana pseudo stem (508 U mL⁻¹) and coffee husk (496.33 U mL⁻¹).

4.2.22 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.22.1 Effect of carbon sources

The pectinolytic fungi were cultured by adding different carbon sources viz. glucose and sucrose at one and two per cent concentrations for studying their impact in the production of polygalacturonase. The data presented in Table 46 indicate a significant difference in PG production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PG production. A mean maximum of 628.15 U mL⁻¹ PG was produced by *A. niger* RBF96 as compared to 495.28 U mL⁻¹ by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PG activity of 707.40 U mL⁻¹, followed by sunflower head (671.65 U mL⁻¹), wheat bran (669.01 U mL⁻¹), coffee husk (457.80 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (452.24 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (412.19 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with addition of carbon sources into the fermentation medium. The highest mean PG activity was noticed in sucrose at two per cent with 685.37 U mL⁻¹ which was significantly superior over glucose at two per cent (591.92 U mL⁻¹), glucose at one per cent (530.48 U mL⁻¹), sucrose at one per cent (526.47 U mL⁻¹). The lowest PG activity was observed in the control (474.33 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and carbon sources in the fermentation medium indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 in presence of sucrose at two per cent in wheat bran (912.00 U mL⁻¹) followed by citrus peel (895.33 U mL⁻¹), sunflower head (847.90 U mL⁻¹), coffee husk (716.27 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (700.46 U mL⁻¹). The lowest PG activity in banana pseudo stem (431.47 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity for sucrose at two per cent in wheat bran (822.04 U mL⁻¹) followed by citrus peel (813.49 U mL⁻¹), sunflower head (741.16 U mL⁻¹), coffee husk (552.49 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (460.94 U mL⁻¹). The lowest PG activity was seen in banana pseudo stem (417.10 U mL⁻¹).

4.2.23 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.23.1 Effect of carbon sources

The pectinolytic fungi were cultured by adding the carbon sources namely glucose and sucrose at one and two per cent concentrations for studying their impact in the production of pectin lyase. The data presented in Table 47 indicate a significant difference in PL production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PL production. A mean maximum of 544.33 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 339.53 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly higher average PL activity of 509.80 U mL⁻¹, wheat bran (484.42 U mL⁻¹), followed by sunflower head (442.15 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (436.40 U mL⁻¹) and coffee husk (433.80 U mL⁻¹). The lowest

Table 39. Effect of pH on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	pH	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	3	418.33	321.67	311.67	188.33	278.33	300.00	351.22
	4	476.67	398.33	368.33	293.33	310.00	343.33	
	5	508.33	445.00	405.00	318.33	375.00	411.67	
	6	320.00	386.67	355.00	348.33	328.33	378.33	
	7	386.67	323.33	283.33	153.33	306.67	290.00	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	3	353.33	276.67	250.00	125.00	273.33	240.00	294.89
	4	423.33	366.67	348.33	223.33	275.00	291.67	
	5	475.00	400.00	370.00	246.67	315.00	333.33	
	6	423.33	361.67	333.33	203.33	286.67	250.00	
	7	370.00	268.33	265.00	143.33	205.00	206.67	
Mean		415.50	354.83	329.00	224.33	295.33	304.50	
Mean	3	278.06						
	4	346.67						
	5	380.14						
	6	331.25						
	7	266.81						
		S.Em±			CD at 1%			
Strains (A)		3.357			12.431			
pH (B)		5.309			19.655			
Substrates (C)		5.815			21.531			
A x B		7.507			27.797			
B x C		13.003			48.146			
A x C		8.224			30.450			
A x B x C		18.389			68.088			

Table 40. Influence of temperature on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	Temperature (°C)	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	20	359.52	372.30	323.95	295.20	308.77	359.73	462.48
	30	420.52	538.54	419.99	502.47	372.55	513.25	
	35	712.47	602.36	663.58	659.95	420.52	556.10	
	40	536.87	481.16	469.13	413.26	348.94	448.33	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	20	320.52	344.31	301.29	310.59	330.57	319.02	357.14
	30	358.45	392.92	359.31	349.69	378.75	367.63	
	35	493.60	458.73	455.35	421.49	394.03	461.66	
	40	419.88	234.37	313.90	231.10	345.20	209.08	
Mean		452.73	428.08	413.31	397.97	362.42	404.35	
Mean	20	328.81						
	30	414.50						
	35	524.99						
	40	370.94						
				S.Em±		CD at 1%		
Strains (A)				1.31		4.87		
Temperature (B)				1.85		6.88		
Substrates (C)				2.27		8.43		
A x B				2.62		9.74		
B x C				3.21		11.92		
A x C				4.54		16.86		
A x B x C				6.42		23.85		

Table 41. Effect of temperature on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	Temperature (°C)	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	20	198.33	175.52	110.17	96.33	71.33	78.42	195.78
	30	233.70	229.34	228.79	127.28	104.46	101.36	
	35	432.47	402.86	386.89	189.14	162.39	160.13	
	40	333.49	320.33	220.88	121.50	105.32	108.29	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	20	112.59	110.25	91.66	85.30	60.30	61.39	115.41
	30	112.04	157.19	130.53	115.31	84.32	104.14	
	35	180.19	175.60	166.36	133.13	112.55	138.29	
	40	135.47	115.84	128.43	96.33	71.33	91.26	
Mean		217.29	210.87	182.97	120.54	96.50	105.41	
Mean	20	104.30						
	30	144.04						
	35	220.00						
	40	154.04						
		S.Em±					CD at 1%	
Strains (A)		1.41					5.25	
Temperature (B)		2.00					7.43	
Substrates (C)		2.45					9.10	
A x B		2.83					10.50	
B x C		3.46					12.86	
A x C		4.89					18.19	
A x B x C		6.92					25.73	

Table 42. Influence of temperature on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Temperature (°C)	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	20	260.00	213.33	183.33	171.67	151.67	81.67	289.38
	30	308.33	370.00	311.67	200.00	190.00	211.67	
	35	521.67	505.00	478.33	411.67	346.67	366.67	
	40	351.67	340.00	285.00	258.33	246.67	180.00	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	20	353.33	223.33	203.33	256.67	136.67	96.67	262.07
	30	413.33	273.33	268.33	313.33	241.67	168.33	
	35	460.00	320.00	305.00	381.67	290.00	201.67	
	40	320.67	261.67	256.67	220.67	170.00	153.33	
Mean		373.63	313.33	286.46	276.75	221.67	182.50	
Mean	20	194.31						
	30	272.50						
	35	382.36						
	40	253.72						
		S.Em±					CD at 1%	
Strains (A)		0.80					2.99	
Temperature (B)		1.14					4.22	
Substrates (C)		1.39					5.17	
A x B		1.61					5.97	
B x C		1.97					7.32	
A x C		2.78					10.35	
A x B x C		3.94					14.63	

Table 43. Effect of nitrogen sources on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	Nitrogen sources	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	Control	701.00	594.33	542.33	404.00	302.44	521.67	635.21
	Ammonium sulphate at 1%	765.44	668.86	611.06	381.57	554.15	445.99	
	Ammonium sulphate at 2%	843.43	785.20	807.64	599.84	785.45	570.38	
	Soybean meal at 1%	730.61	681.57	752.08	510.31	456.67	547.49	
	Soybean meal at 2%	910.20	893.54	846.10	714.48	429.68	698.66	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	475.65	443.58	431.00	298.99	307.35	282.56	503.38
	Ammonium sulphate at 1%	601.44	536.27	590.76	457.75	406.67	307.64	
	Ammonium sulphate at 2%	621.74	588.41	741.93	413.99	380.11	408.07	
	Soybean meal at 1%	597.17	589.69	668.96	364.22	334.27	373.56	
	Soybean meal at 2%	820.25	811.70	739.37	550.69	425.79	531.71	
Mean		706.69	659.31	673.12	469.58	438.25	468.77	
Mean	Control	480.58						
	Ammonium sulphate at 1%	571.77						
	Ammonium sulphate at 2%	585.86						
	soybean meal at 1%	524.68						
	soybean meal at 2%	683.57						
		S.E.m±			CD at 1%			
Strains (A)		2.396			8.873			
Nitrogen sources (B)		3.789			14.030			
Substrates (C)		4.151			15.369			
A x B		5.359			19.841			
B x C		9.282			34.366			
A x C		5.870			21.735			
A x B x C		13.126			48.600			

Table 44. Influence of nitrogen sources on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	Nitrogen sources	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	Control	417.83	385.17	353.68	390.89	361.83	356.83	482.16
	Ammonium sulphate at 1%	455.22	450.77	497.60	453.40	433.21	458.79	
	Ammonium sulphate at 2%	535.22	511.00	538.87	537.32	477.85	462.86	
	Soybean meal at 1%	606.55	501.55	491.65	464.18	443.99	453.37	
	Soybean meal at 2%	698.77	640.89	596.65	521.25	501.06	466.59	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	308.90	380.18	374.63	380.96	344.67	324.67	446.48
	Ammonium sulphate at 1%	350.62	455.22	502.65	424.84	413.80	421.20	
	Ammonium sulphate at 2%	492.07	512.55	525.32	433.99	456.68	440.83	
	Soybean meal at 1%	485.43	485.43	466.47	465.40	445.21	448.25	
	Soybean meal at 2%	547.22	514.77	537.32	500.22	480.03	474.78	
Mean		489.78	483.75	488.48	457.24	435.83	430.82	
Mean	Control		365.02					
	Ammonium sulphate at 1%		443.11					
	Ammonium sulphate at 2%		495.60					
	soybean meal at 1%		479.79					
	soybean meal at 2%		538.07					
			S.Em±			CD at 1%		
Strains (A)			0.816			3.022		
Nitrogen sources (B)			1.291			4.779		
Substrates (C)			1.414			5.235		
A x B			1.825			6.758		
B x C			3.161			11.705		
A x C			1.999			7.403		
A x B x C			4.471			16.554		

Table 45. Effect of nitrogen sources on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Nitrogen sources	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	Control	541.67	306.67	425.00	466.67	296.67	366.67	522.04
	Ammonium sulphate at 1%	564.67	564.67	464.67	474.67	489.67	553.00	
	Ammonium sulphate at 2%	734.67	659.67	618.00	499.67	549.67	559.67	
	soybean meal at 1%	583.00	599.00	493.00	493.00	426.33	508.00	
	soybean meal at 2%	611.33	611.33	616.33	521.33	508	554.67	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	482.83	406.95	403.50	418.33	348.33	370.00	476.94
	Ammonium sulphate at 1%	539.67	471.33	439.67	449.67	426.33	448.00	
	Ammonium sulphate at 2%	586.33	601.33	591.33	496.33	508	548.00	
	soybean meal at 1%	484.67	468.00	484.00	453.00	449.67	453.00	
	soybean meal at 2%	574.00	484.67	468.00	484.00	486.33	483.00	
Mean		570.28	517.36	500.35	475.67	448.90	484.40	
Mean	Control		402.77					
	Ammonium sulphate at 1%		510.64					
	Ammonium sulphate at 2%		559.25					
	soybean meal at 1%		506.42					
	soybean meal at 2%		518.39					
		S.Em±			CD at 1%			
Strains (A)		0.540			1.999			
Nitrogen sources (B)		0.854			3.160			
Substrates (C)		0.935			3.462			
A x B		1.207			4.469			
B x C		2.091			7.741			
A x C		1.322			4.896			
A x B x C		2.957			10.948			

Table 46. Polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation as influenced by different carbon sources

Strains	Carbon sources	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	Control	637.40	588.37	658.88	383.37	209.23	454.28	628.15
	Glucose at 1%	767.23	670.65	612.85	417.10	555.94	447.79	
	Glucose at 2%	845.22	787.00	809.43	601.63	787.24	572.18	
	Sucrose at 1%	732.40	683.37	753.88	512.10	304.23	549.28	
	Sucrose at 2%	895.33	912.00	847.90	716.27	431.47	700.46	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	477.18	460.94	423.64	300.78	309.15	284.36	495.28
	Glucose at 1%	623.54	590.20	454.28	363.62	389.59	309.43	
	Glucose at 2%	674.71	592.55	743.73	415.78	336.00	333.79	
	Sucrose at 1%	598.96	591.49	670.76	314.86	381.90	409.86	
	Sucrose at 2%	822.04	813.49	741.16	552.49	417.10	460.94	
Mean		707.40	669.01	671.65	457.80	412.19	452.24	
Mean	Control	474.33						
	Glucose at 1%	530.48						
	Glucose at 2%	591.92						
	Sucrose at 1%	526.47						
	Sucrose at 2%	685.37						
		S.Em±				CD at 1%		
Strains (A)		3.228				11.951		
Carbon sources (B)		5.104				18.897		
Substrates (C)		5.591				20.700		
A x B		7.218				26.724		
B x C		12.501				46.287		
A x C		7.906				29.275		
A x B x C		17.679				65.460		

Table 47. Influence of carbon sources on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	Carbon sources	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	Control	455.18	412.33	402.33	423.56	387.33	423.19	544.33
	Glucose at 1%	510.17	482.72	430.91	570.89	387.67	478.18	
	Glucose at 2%	597.99	582.29	657.20	649.86	457.72	566.00	
	Sucrose at 1%	689.60	680.10	485.64	478.29	447.00	657.61	
	Sucrose at 2%	796.74	783.61	578.24	566.27	532.42	758.83	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	266.46	217.33	241.61	234.27	178.08	234.47	339.53
	Glucose at 1%	327.52	246.33	276.38	269.03	197.93	295.53	
	Glucose at 2%	335.19	291.63	300.88	293.53	260.67	303.20	
	Sucrose at 1%	298.91	336.10	308.92	301.57	193.34	266.92	
	Sucrose at 2%	820.25	811.70	739.37	550.69	408.07	380.11	
Mean		509.80	484.42	442.15	433.80	345.02	436.40	
Mean	Control		375.60					
	Glucose at 1%		394.66					
	Glucose at 2%		401.50					
	Sucrose at 1%		408.70					
	Sucrose at 2%		629.20					
			S.Em±			CD at 1%		
Strains (A)			4.632			17.152		
Carbon sources (B)			7.324			27.119		
Substrates (C)			8.023			29.708		
A x B			10.358			38.352		
B x C			17.941			66.428		
A x C			11.347			42.013		
A x B x C			25.372			93.944		

mean for PL production was found in banana pseudo stem (345.02 U mL^{-1}). All these substrates differed significantly over one another for PL production.

The PL production varied with addition of carbon sources into the fermentation medium. The highest mean PL activity was noticed for sucrose at two per cent with 629.20 U mL^{-1} which was significantly superior over sucrose at one per cent (408.70 U mL^{-1}), glucose at two per cent (401.50 U mL^{-1}) and glucose at one per cent (394.66 U mL^{-1}). The lowest PL activity was observed in control (375.60 U mL^{-1}).

The production of PL as influenced by pectinolytic fungi and carbon sources in the fermentation medium indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 for sucrose at two per cent in citrus peel (796.74 U mL^{-1}), followed by wheat bran (783.61 U mL^{-1}), citrus peel + jackfruit rind + pineapple peel (758.83 U mL^{-1}), sunflower head (578.24 U mL^{-1}) and coffee husk (566.27 U mL^{-1}). The lowest PL activity was in banana pseudo stem (532.42 U mL^{-1}). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PL activity for sucrose at two per cent in citrus peel (820.25 U mL^{-1}), followed by wheat bran (811.70 U mL^{-1}), sunflower head (739.37 U mL^{-1}), coffee husk (550.69 U mL^{-1}) and banana pseudo stem (408.07 U mL^{-1}). The lowest PL activity was observed in citrus peel + jackfruit rind + pineapple peel (380.11 U mL^{-1}).

4.2.24 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.24.1 Effect of carbon sources

The pectinolytic fungi were cultured by adding the carbon sources namely glucose and sucrose at one and two per cent concentrations for studying their impact in the production of pectin esterase. The data presented in Table 48 indicated a significant difference in PE production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PE production. A mean maximum of 529.61 U mL^{-1} PE was produced by *A. oryzae* RC27 as compared to 401.03 U mL^{-1} by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly highest average PE activity of 572.67 U mL^{-1} , followed by wheat bran (525.80 U mL^{-1}), coffee husk (461.49 U mL^{-1}), sunflower head (442.15 U mL^{-1}) and banana pseudo stem (408.70 U mL^{-1}). The lowest mean for PE production was found in citrus peel + jackfruit rind + pineapple peel (357.10 U mL^{-1}). All these substrates differed significantly over one another for PE production.

The PE production varied with the addition of carbon sources into the fermentation medium. The mean highest PE activity was noticed for glucose at two per cent (559.56 U mL^{-1}) which was significantly superior over glucose at one per cent (469.21 U mL^{-1}), sucrose at two per cent (464.69 U mL^{-1}) and sucrose at one per cent (450.53 U mL^{-1}). The lowest PE activity was observed in control (382.61 U mL^{-1}).

The production of PE as influenced by pectinolytic fungi and carbon sources in the fermentation medium indicated a varied PE activity. The highest PE activity was recorded by *A. oryzae* RC27 for glucose at two per cent in citrus peel (735.00 U mL^{-1}), followed by wheat bran (716.67 U mL^{-1}), sunflower head (690.00 U mL^{-1}), coffee husk (635.00 U mL^{-1}) and banana pseudo stem (611.67 U mL^{-1}). The lowest PE activity was observed in citrus peel + jackfruit rind + pineapple peel (568.33 U mL^{-1}). Similarly, the reference strain *A. niger* MTCC1344 showed higher PE activity for glucose at two per cent in citrus peel (553.33 U mL^{-1}), followed by wheat bran (543.00 U mL^{-1}), coffee husk (451.33 U mL^{-1}), sunflower head (450.33 U mL^{-1}) and banana pseudo stem (408.07 U mL^{-1}). The lowest PE activity was seen in citrus peel + jackfruit rind + pineapple peel (345.33 U mL^{-1}).

4.2.25 Polygalacturonase production by *Aspergillus niger* RBF96 under SSF

4.2.25.1 Effect of salts

The effect of salts on polygalacturonase production by pectinolytic fungal strains in solid state fermentation medium was studied by incorporating zinc chloride and manganese sulfate at one and two per cent concentrations. A perusal of data in Table 49 indicated a significant difference in PG production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PG production. A mean maximum of 701.99 U mL^{-1} PG was produced by *A. niger* RBF96 as compared to 581.42 U mL^{-1} by the reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly highest average PG activity of 699.75 U mL^{-1} , followed by citrus peel + jackfruit rind + pineapple peel (675.91 U mL^{-1}), wheat bran (659.57 U mL^{-1}), sunflower head (647.76 U mL^{-1}) and coffee husk

(587.54 U mL⁻¹). The lowest mean for PG production was found in banana pseudo stem (576.41 U mL⁻¹). All these substrates differed significantly over one another for PG production.

The PG production varied with addition of salts into the fermentation medium. The highest mean PG activity was noticed for manganese sulfate at two per cent with 711.62 U mL⁻¹ which was significantly superior over zinc chloride at two per cent (698.10 U mL⁻¹), manganese sulfate at one per cent (671.67 U mL⁻¹) and zinc chloride at one per cent (603.59 U mL⁻¹). The lowest PG activity was observed in control (523.56 U mL⁻¹).

The production of PG as influenced by pectinolytic fungi and salts in the fermentation medium indicated a varied PG activity. The highest PG activity was recorded by *A. niger* RBF96 for manganese sulfate at two per cent in citrus peel (984.08 U mL⁻¹), followed by citrus peel + jackfruit rind + pineapple peel (896.46 U mL⁻¹), wheat bran (886.82 U mL⁻¹), sunflower head (864.1 U mL⁻¹) and banana pseudo stem (727.35 U mL⁻¹). The lowest PG activity was observed in coffee husk (639.42 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PG activity for manganese sulfate at two per cent in citrus peel (720.29 U mL⁻¹) followed by wheat bran (683.11 U mL⁻¹), sunflower head (672.67 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (670.33 U mL⁻¹) and coffee husk (643.67 U mL⁻¹). The lowest PG activity was noticed in banana pseudo stem (592.00 U mL⁻¹).

4.2.26 Pectin lyase production by *Penicillium citrinum* DLP51 under SSF

4.2.26.1 Effect of salts

The effect of salts on pectin lyase production by pectinolytic fungal strains in solid state fermentation medium was studied by incorporating the zinc chloride and manganese sulfate at one per cent and two per cent each. The perusal of data in Table 50 indicated a significant difference in PL production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PL production.

A mean maximum of 485.29 U mL⁻¹ PL was produced by *P. citrinum* DLP51 as compared to 262.11 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly highest average PL activity (423.71 U mL⁻¹) followed by sunflower head (395.52 U mL⁻¹), wheat bran (390.04 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (365.94 U mL⁻¹) and coffee husk (340.15 U mL⁻¹). The lowest mean for PL production was found in banana pseudo stem (330.10 U mL⁻¹). All these substrates differed significantly over one another for PL production.

The PL production varied with the addition of salts into the fermentation medium. The highest mean PL activity was noticed for zinc chloride at two per cent with 470.46 U mL⁻¹ which was significantly superior over manganese sulfate at two per cent (430.04 U mL⁻¹), zinc chloride at one per cent (388.45 U mL⁻¹) and manganese sulfate at one per cent (357.06 U mL⁻¹). The lowest PL activity was observed in control (222.47 U mL⁻¹).

The production of PL as influenced by pectinolytic fungi and salts in the fermentation medium indicated a varied PL activity. The highest PL activity was recorded by *P. citrinum* DLP51 for manganese sulfate at two per cent in wheat bran (700.18 U mL⁻¹), followed by citrus peel (599.14 U mL⁻¹), sunflower head (595.72 U mL⁻¹), coffee husk (581.27 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (575.89 U mL⁻¹). The lowest PL activity was observed in banana pseudo stem (469.25 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed highest PL activity for zinc chloride at two per cent in citrus peel (424.21 U mL⁻¹) followed by sunflower head (411.99 U mL⁻¹), citrus peel + jackfruit rind + pineapple peel (390.70 U mL⁻¹), coffee husk (321.90 U mL⁻¹) and wheat bran (302.48 U mL⁻¹). The lowest PL activity was noticed in banana pseudo stem (286.99 U mL⁻¹).

4.2.27 Pectin esterase production by *Aspergillus oryzae* RC27 under SSF

4.2.27.1 Effect of salts

The effect of salts on pectin esterase production by pectinolytic fungal strains in solid state fermentation medium was studied by incorporating the zinc chloride and manganese sulfate at one and two per cent concentrations. A perusal of data in Table 51 indicated a significant difference in PE production. All the individual factors and their two way and three way combinations varied significantly. Both the pectinolytic fungi differed statistically with each other in terms of PE production. A mean maximum of 517.28 U mL⁻¹ PE was produced by *A. oryzae* RC27 as compared to 424.84 U mL⁻¹ by reference strain *A. niger* MTCC1344. The substrate citrus peel showed significantly highest average PE activity (569.83 U mL⁻¹) followed by wheat bran (525.43 U mL⁻¹), sunflower head (472.77

U mL⁻¹), coffee husk (458.93 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (407.93 U mL⁻¹). The lowest mean for PE production was found in banana pseudo stem (391.47 U mL⁻¹). All these substrates differed significantly over one another for PE production.

The PE production varied with addition of salts into the fermentation medium. The highest mean PE activity was noticed for zinc chloride at two per cent with 518.69 U mL⁻¹ which was significantly superior over manganese sulfate at two per cent (517.58 U mL⁻¹), manganese sulfate at one per cent (463.14 U mL⁻¹) and zinc chloride at one per cent (460.58 U mL⁻¹). The lowest PE activity was observed in control (395.31 U mL⁻¹).

The production of PE as influenced by pectinolytic fungi and salts in the fermentation medium indicated a varied PE activity. A highest PE activity was recorded by *A. oryzae* RC27 for zinc chloride at two per cent in citrus peel (672.67 U mL⁻¹), followed by wheat bran (634.67 U mL⁻¹), sunflower head (608.00 U mL⁻¹), coffee husk (553.00 U mL⁻¹) and citrus peel + jackfruit rind + pineapple peel (486.33 U mL⁻¹). The lowest PE activity was observed in banana pseudo stem (461.33 U mL⁻¹). Similarly, the reference strain *A. niger* MTCC1344 showed the highest PE activity for zinc chloride at two per cent in citrus peel (648.33 U mL⁻¹), followed by wheat bran (538.00 U mL⁻¹), sunflower head (445.33 U mL⁻¹), coffee husk (446.33 U mL⁻¹) and banana pseudo stem (409.67 U mL⁻¹). The lowest PE activity was observed in citrus peel + jackfruit rind + pineapple peel (340.33 U mL⁻¹).

4.3 Ammonium sulfate precipitation of the crude pectinase enzyme

A perusal of data in Table 52 indicated that there was an increase in the protein recovery with an increase in the concentration of the ammonium sulfate up to 80 per cent. At 80 per cent level, 37.88 per cent, 39.45 per cent, 37.40 per cent and 30.45 per cent of protein was precipitated with maximum specific activities of 18.85 U µg⁻¹, 10.50 U µg⁻¹, 12.11 U µg⁻¹ and 16.13 U µg⁻¹ in *Aspergillus niger* RBF96, *Penicillium citrinum* DLP51, *Aspergillus oryzae* RC27 and *Aspergillus niger* MTCC1344 (reference strain) respectively. Increasing the ammonium sulfate to 90 per cent saturation decreased the yield; specific activity and purification fold in all the crude enzymes. This indicated that pectinase enzyme could be precipitated and isolated at 80 per cent ammonium sulfate saturation.

4.3.1 Purification of polygalacturonase produced by *Aspergillus niger* RBF96

The crude enzyme produced by *A. niger* RBF96 had total enzyme activity (27306 U), total protein (2312 µg) and the specific activity of 11.81 U µg⁻¹ protein when compared to total enzyme (14700 U), total protein (1750 µg) and the specific activity of 8.4 U µg⁻¹ in the reference strain *A. niger* MTCC1344 (Table 53). The purification steps resulted in enhanced specific activity; the ammonium sulfate precipitation resulted in 16515 U of the total enzyme activity with specific activity of 18.85 U µg⁻¹ protein by *A. niger* RBF96, when compared to 8500 U of the total enzyme activity with specific activity of 15.94 U µg⁻¹ protein in the reference strain. After purification through gel filtration chromatography using Sephadex G-100, the maximum specific activity was 83.06 U µg⁻¹ protein in case of *A. niger* RBF96 compared to 27.63 U µg⁻¹ protein in *A. niger* MTCC1344.

These treatments increased the purification by many folds; 1.59 folds purification was achieved after ammonium sulfate precipitation in *A. niger* RBF96 compared to 2.20 folds in *A. niger* MTCC1344. Whilst, the maximum purification was observed after gel filtration (7.03 fold). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield was reduced to 37.88% after ammonium sulfate precipitation which was further reduced to 14.04%, after gel chromatography separation.

4.3.2 Purification of pectin lyase produced by *Penicillium citrinum* DLP51

The crude enzyme produced by *P. citrinum* DLP51 had a total enzyme activity (20300 U), total protein (2800 µg) and the specific activity of 7.25 U µg⁻¹ protein when compared to total enzyme (14700 U), total protein (1750 µg) and the specific activity of 8.4 U µg⁻¹ in the reference strain (Table 54). The purification steps resulted in enhanced specific activity; the ammonium sulfate precipitation resulted in 13450 U of the total enzyme activity with specific activity of 10.50 U µg⁻¹ protein by *P. citrinum* DLP51, when compared to 8500 U of the total enzyme activity with specific activity of 15.94 U µg⁻¹ protein in the reference strain. The maximum specific activity was 44.13 U µg⁻¹ protein after gel filtration chromatography through Sephadex G-100 in case of *P. citrinum* DLP51 compared to 22.87 U µg⁻¹ protein in the reference strain.

Table 48. Effect of carbon sources on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Carbon sources	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	Control	511.00	497.67	475.67	411.67	351.67	371.67	529.61
	Glucose at 1%	680.00	568.33	505.00	590.00	463.33	492.67	
	Glucose at 2%	735.00	716.67	690.00	635.00	611.67	568.33	
	Sucrose at 1%	580.00	528.33	500.00	421.33	423.33	401.67	
	Sucrose at 2%	646.67	571.67	530.00	485.00	466.67	458.33	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	465.00	353.33	300.00	381.67	278.33	184.00	401.03
	Glucose at 1%	519.67	456.33	394.55	429.67	341.33	189.67	
	Glucose at 2%	553.33	543.00	450.33	451.33	414.67	345.33	
	Sucrose at 1%	489.67	503.00	376.33	399.67	344.67	271.33	
	Sucrose at 2%	546.33	519.67	393.00	456.33	391.33	288.00	
Mean		572.67	525.80	461.49	466.17	408.70	357.10	
Mean	Control	382.61						
	Glucose at 1%	469.21						
	Glucose at 2%	559.56						
	Sucrose at 1%	450.53						
	Sucrose at 2%	464.69						
		S.Em±				CD at 1%		
Strains (A)		0.568				2.105		
Carbon sources (B)		0.899				3.328		
Substrates (C)		0.984				3.645		
A x B		1.271				4.706		
B x C		2.201				8.151		
A x C		1.392				5.155		
A x B x C		3.113				11.527		

Table 49. Effect of mineral salts on polygalacturonase production by *Aspergillus niger* RBF96 under solid state fermentation

Strains	Salts	Polygalacturonase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel + Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus niger</i> RBF 96	Control	696.71	616.67	593.08	436.67	507.00	605.46	701.99
	Zinc chloride at 1%	717.27	668.55	681.70	592.16	552.95	688.73	
	Zinc chloride at 2%	838.00	759.29	774.21	730.30	660.22	806.18	
	Manganese sulphate at 1%	717.71	693.50	788.99	585.81	605.16	745.28	
	Manganese sulphate at 2%	984.08	886.82	864.10	639.42	727.35	896.46	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	522.35	508.68	405.67	494.25	419.00	477.15	581.42
	Zinc chloride at 1%	547.27	569.97	524.84	557.32	533.17	609.10	
	Zinc chloride at 2%	678.35	613.73	586.70	605.50	591.65	621.63	
	Manganese sulphate at 1%	575.48	595.42	585.67	590.25	575.59	638.80	
	Manganese sulphate at 2%	720.29	683.11	672.67	643.67	592.00	670.33	
Mean		699.75	659.57	647.76	587.54	576.41	675.91	
Mean	Control	523.56						
	Zinc chloride at 1%	603.59						
	Zinc chloride at 2%	698.10						
	Manganese sulphate at 1%	671.67						
	Manganese sulphate at 2%	711.62						
		S.Em±				CD at 1%		
Strains (A)		0.552				2.043		
Salts (B)		0.873				3.231		
Substrates (C)		0.956				3.539		
A x B		1.234				4.569		
B x C		2.137				7.913		
A x C		1.352				5.005		
A x B x C		3.023				11.191		

Table 50. Effect of mineral salts on pectin lyase production by *Penicillium citrinum* DLP51 under solid state fermentation

Strains	Salts	Pectin lyase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Penicillium citrinum</i> DLP 51	Control	432.10	407.66	357.63	180.76	170.85	198.48	485.29
	Zinc chloride at 1%	533.07	567.46	547.98	499.22	478.80	488.80	
	Zinc chloride at 2%	593.75	610.37	555.22	558.20	565.72	585.46	
	Manganese sulphate at 1%	508.63	422.10	455.93	425.04	447.69	446.18	
	Manganese sulphate at 2%	599.14	700.18	595.72	581.27	469.25	575.89	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	172.35	180.76	133.37	143.89	170.85	120.89	262.11
	Zinc chloride at 1%	276.35	284.76	237.37	247.89	274.85	224.89	
	Zinc chloride at 2%	424.21	302.48	411.99	321.90	286.99	390.70	
	Manganese sulphate at 1%	278.37	193.53	319.70	211.48	231.15	313.25	
	Manganese sulphate at 2%	419.09	231.15	340.36	231.83	204.80	314.81	
Mean		423.71	390.04	395.52	340.15	330.09	365.94	
Mean	Control	222.47						
	Zinc chloride at 1%	388.45						
	Zinc chloride at 2%	470.46						
	Manganese sulphate at 1%	357.06						
	Manganese sulphate at 2%	430.04						
			S.Em±			CD at 1%		
Strains (A)			0.280			1.035		
Salts (B)			0.442			1.637		
Substrates (C)			0.484			1.793		
A x B			0.625			2.315		
B x C			1.083			4.010		
A x C			0.685			2.536		
A x B x C			1.532			5.671		

Table 51. Effect of mineral salts on pectin esterase production by *Aspergillus oryzae* RC27 under solid state fermentation

Strains	Salts	Pectin esterase activity (U mL ⁻¹)						Mean
		Citrus peel	Wheat bran	Sunflower head	Coffee husk	Banana pseudo stem	Citrus peel +Jackfruit rind + Pineapple peel (2:1:1)	
<i>Aspergillus oryzae</i> RC27	Control	523.00	504.67	478.00	423.00	356.33	399.67	517.28
	Zinc chloride at 1%	598.00	592.67	523.00	508.00	381.33	481.33	
	Zinc chloride at 2%	672.67	634.67	608.00	553.00	461.33	486.33	
	Manganese sulphate at 1%	606.00	554.33	526.00	511.00	384.67	427.67	
	Manganese sulphate at 2%	653.00	597.67	556.00	540.00	492.67	484.33	
<i>Aspergillus niger</i> MTCC 1344 (Reference strain)	Control	456.67	381.67	340.00	337.33	263.33	280.00	424.84
	Zinc chloride at 1%	514.67	451.33	388.67	424.67	336.33	327.00	
	Zinc chloride at 2%	648.33	538.00	445.33	446.33	409.67	340.33	
	Manganese sulphate at 1%	484.67	484.67	371.33	394.67	386.33	369.67	
	Manganese sulphate at 2%	541.33	514.67	491.33	451.33	442.67	483.00	
Mean		569.83	525.43	472.77	458.93	391.47	407.93	
Mean	Control		395.31					
	Zinc chloride at 1%		460.58					
	Zinc chloride at 2%		518.69					
	Manganese sulphate at 1%		463.14					
	Manganese sulphate at 2%		517.58					
			S.Em±			CD at 1%		
Strains (A)			1.030			3.812		
Salts (B)			1.628			6.028		
Substrates (C)			1.783			6.603		
A x B			2.302			8.524		
B x C			3.988			14.764		
A x C			2.522			9.338		
A x B x C			5.639			20.880		

Table 52. Protein recovery in the pectinase crude extract of different fungi as influenced by different concentrations of ammonium sulfate

Saturation (%)	<i>Aspergillus niger</i> RBF96					<i>Penicillium citrinum</i> DLP51				
	Total activity (U)	Total protein (µg)	Specific activity (U µg ⁻¹)	Yield (%)	Purification folds	Total activity (U)	Total protein (µg)	Specific activity (U µg ⁻¹)	Yield (%)	Purification folds
20	1955	150	13.0	6.48	1.10	1400	180	7.70	6.42	1.06
30	2600	180	14.4	7.78	1.21	3215	415	7.14	14.82	1.06
40	6500	415	15.66	17.94	1.32	4500	600	7.50	21.42	1.03
50	7400	519	14.25	22.44	1.20	5700	763	7.47	27.25	1.03
60	13000	808	16.08	34.94	1.36	7150	909	7.86	32.41	1.08
70	14350	860	16.88	36.76	1.42	9430	1105	8.53	35.01	1.17
80	16515	876	18.85	37.88	1.59	13450	980	10.50	39.45	1.44
90	14100	805	17.51	34.81	1.48	8100	900	8.10	32.14	1.11

Saturation (%)	<i>Aspergillus oryzae</i> RC27					<i>Aspergillus niger</i> MTCC1344 (Reference Strain)				
	Total activity (U)	Total protein (µg)	Specific activity (U µg ⁻¹)	Yield (%)	Purification folds	Total activity (U)	Total protein (µg)	Specific activity (U µg ⁻¹)	Yield (%)	Purification folds
20	1764	900	1.96	32.72	0.28	1320	162	8.14	9.25	0.96
30	1915	816	2.34	29.67	0.34	1780	145	12.27	8.28	1.46
40	2655	951	2.79	34.50	0.41	2400	139	17.26	7.94	2.05
50	4900	570	8.59	20.70	1.26	3430	303	11.32	17.31	1.34
60	7500	845	8.87	30.72	1.30	4950	330	15.07	18.85	1.78
70	9300	1030	9.03	35.27	1.32	6840	424	15.44	24.22	1.92
80	11650	970	12.11	37.40	1.76	8500	533	16.13	30.45	2.20
90	9370	845	11.08	30.70	1.62	7450	480	15.52	27.40	1.84

Table 53. Purification of polygalacturonase produced by *Aspergillus niger* RBF96

Strains	Purification step	Total activity (U)	Total protein (□g)	Specific activity (U □g ⁻¹)	Yield (%)	Purification folds
<i>Aspergillus niger</i> RBF96	Crude Extract	27306	2312	11.81	100	1.0
	Ammonium sulphate fraction (80%)	16515	876	18.85	37.88	1.59
	Sephadex G-100	10217	123	83.06	14.04	7.03
<i>Aspergillus niger</i> MTCC1344 (Reference Strain)	Crude Extract	14700	1750	8.4	100	1.0
	Ammonium sulphate fraction (80%)	8500	533	15.94	30.45	2.20
	Sephadex G-100	5330	192.84	27.63	11.01	3.28

Table 54. Purification of pectin lyase produced by *Penicillium citrinum* DLP51

Strains	Purification step	Total activity (U)	Total protein (□g)	Specific activity (U □g ⁻¹)	Yield (%)	Purification folds
<i>Penicillium citrinum</i> DLP51	Crude Extract	20300	2800	7.25	100	1.0
	Ammonium sulphate fraction (80%)	13450	980	10.50	35	1.44
	Sephadex G-100	8650	196	44.13	20	6.08
<i>Aspergillus niger</i> MTCC1344 (Reference Strain)	Crude Extract	14700	1750	8.4	100	1.0
	Ammonium sulphate fraction (80%)	8500	533	15.94	30.45	2.20
	Sephadex G-100	4240	185.33	22.87	13.31	4.32

Table 55. Purification of pectin esterase produced by *Aspergillus oryzae* RC27

Strains	Purification step	Total activity (U)	Total protein (□g)	Specific activity (U □g ⁻¹)	Yield (%)	Purification folds
<i>Aspergillus oryzae</i> RC27	Crude Extract	18700	2750	6.8	100	1.0
	Ammonium sulphate fraction (80%)	11650	970	12.01	35.27	1.76
	Sephadex G-100	6480	213.67	30.32	15.34	5.73
<i>Aspergillus niger</i> MTCC1344 (Reference Strain)	Crude Extract	14700	1750	8.4	100	1.0
	Ammonium sulphate fraction (80%)	8500	533	15.94	30.45	2.20
	Sephadex G-100	3250	114	28.50	11.75	4.19

These steps increased the purification by many folds; 1.44 fold purification was achieved after ammonium sulfate precipitation in *P. citrinum* DLP51 compared to 2.20 folds in *A. niger* MTCC1344. Whilst, the maximum PL purification was observed after gel filtration (6.08 folds). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield was reduced to 35% after ammonium sulfate precipitation and 20%, after gel chromatography separation.

4.3.3 Purification of pectin esterase produced by *Aspergillus oryzae* RC27

The crude enzyme produced by *A. oryzae* RC27 had a total enzyme activity (18700 U), total protein (2750 µg) and the specific activity of 6.8 U µg⁻¹ protein when compared to total enzyme (11650 U), total protein (970 µg) and the specific activity of 12.01 U µg⁻¹ in the reference strain (Table 55). The purification steps resulted in enhanced specific activity; the ammonium sulfate precipitation resulted in 11650 U of the total enzyme activity with specific activity of 12.01 U µg⁻¹ protein by *A. oryzae* RC27, when compared to 8500 U of the total enzyme activity with specific activity of 15.94 U µg⁻¹ protein in the reference strain. The maximum specific activity (30.32 U µg⁻¹ protein) was obtained after gel filtration chromatography in case of *A. oryzae* RC27 compared to 28.50 U µg⁻¹ protein in the reference strain.

These steps increased the enzyme purification by many folds; 1.76 folds after ammonium sulfate precipitation in *A. oryzae* RC27 compared to 2.20 folds in *A. niger* MTCC1344. The maximum PE purification was observed after gel filtration (5.73 folds). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield was reduced to 35.27 per cent after ammonium sulfate precipitation and 15.34%, after gel chromatography through Sephadex G-100.

4.3.3 Characterization of pectinase enzymes

The purified pectinases were characterized for molecular mass, Michaelis-Menten kinetics (K_m and V_{max}) and pH and temperature optima.

4.3.3.1 Molecular weight

The crude enzyme preparations and purified fractions were subjected to SDS-PAGE to determine the molecular weight of the enzyme. The electrophoresis revealed the presence of multiple bands in the crude enzyme while a single band in the purified fractions. The molecular weight of the PG protein produced by *Aspergillus niger* RBF96 was 45 kDa (Plate 10), of PL protein produced by *Penicillium citrinum* DLP51 was 37 kDa and of PE protein produced by *Aspergillus oryzae* RC27 was 31 kDa. The molecular weights of the PG, PL and PE proteins produced by the reference strain were 35 kDa, 47 kDa and 33 kDa respectively (Plate 10).

4.3.3.2 K_m determination

Michaelis constant (K_m) and V_{max} of the purified pectinases were determined by measuring the reaction velocities at various concentrations of pectin. The data were plotted according to Lineweaver-Burk plot. The apparent K_m and V_{max} values for polygalacturonase from *Aspergillus niger* RBF96 were 0.155 U mL⁻¹ and 38.57 U mL⁻¹ respectively (Fig. 15) as compared to K_m (0.153 U mL⁻¹) and V_{max} (44.57 U mL⁻¹/h) obtained for polygalacturonase from the reference strain (Fig. 18).

The K_m (0.278 U mL⁻¹) and V_{max} (28.70 U mL⁻¹/h) (Fig. 16) values were obtained for pectin lyase from *Penicillium citrinum* DLP 51 as compared to K_m (0.161 U mL⁻¹) and V_{max} (58.87 U mL⁻¹/h) (Fig. 19) from the reference strain.

In case of pectin esterase from *Aspergillus oryzae* RC27, the apparent K_m and V_{max} values were 0.163 U mL⁻¹ and 38.57 U mL h⁻¹ (Fig. 17) when compared to 0.133 U mL⁻¹ and 56 U mL⁻¹/h in case of the reference strain (Fig. 20). In general, it was observed that the enzyme activity was increased with an increase in pectin concentration upto 0.9 U mL⁻¹.

4.3.4 Optimization of temperature for maximum polygalacturonase activity

The effect of various temperatures on the relative activity of the purified polygalacturonase from *Aspergillus niger* RBF96 was studied and the results are furnished in Table 56 and Fig.9. It was found that the enzyme exhibited maximum activity (774 U mL⁻¹) at 50°C while it was maximum in the reference strain at 40°C (630 U mL⁻¹). When the temperature increased above 50°C, the activity of the

enzyme was gradually reduced. Similarly, below 50°C, a gradual decline in the PG activity was observed.

4.3.5 Optimization of temperature for maximum pectin lyase activity

The data on the effect of various temperatures on the relative activity of purified pectin lyase from *Penicillium citrinum* DLP51 are presented in Table 57 and Fig. 10. It was found that the maximum activity (653 U mL⁻¹) was at 50°C. The reference strain showed maximum activity (385 U mL⁻¹) at 40°C. When the temperature was increased above 50°C, the activity was gradually reduced. At 90°C temperature, the PL found to be inactive. Similarly, below 50°C, a gradual decline in the PG activity was observed.

4.3.6 Optimization of temperature for maximum pectin esterase activity

The data on the effect of various temperatures on the relative activity of purified pectin esterase from *Aspergillus oryzae* RC27 are presented in Table 58 and Fig. 11. It was found that the PL exhibited maximum activity (575 U mL⁻¹) at 50°C as compared to the reference strain (550 U mL⁻¹). When the temperature was increased above 50°C, the activity of the enzyme was gradually reduced. At 90°C, the PE was inactive. Similarly, below 50°C, a gradual decline was observed.

4.3.7 Optimization of pH for maximum polygalacturonase activity

The effect of various pH values on the PG activity are presented in Table 59 and fig 12. The data indicated that the highest enzyme activity (712 U mL⁻¹) in *A. niger* RBF96 was found at pH 4.5 when compared to (673 U mL⁻¹) in the reference strain. The enzyme showed maximum stability at pH 4.0, 4.5 and 5.0. When the pH of the enzyme medium was increased up to 8.0; it showed a decline in the enzyme activity. Likewise, when the activity of PG was assayed at pH values lower than 4.5, it again showed a decreasing trend with a decrease in pH.

4.3.8 Optimization of pH for maximum pectin lyase activity

The results presented in Table 60 and Fig. 13. The data indicated that the highest PL activity (613 U mL⁻¹) in *Penicillium citrinum* DLP 51 was found at pH 6.5 compared to (370 U mL⁻¹) at pH 5.0 in the reference strain. The enzyme showed maximum stability at pH ranging from 5.5 to 7.0. When the pH was increased up to 8.0; it showed a decrease in the enzyme activity. Similarly, at pH values lower than 5.5, it showed a decreasing trend with a decrease in pH.

4.3.9 Optimization of pH for maximum pectin esterase activity

The results presented in Table 61 and Fig. 14 indicated that the highest enzyme activity (600 U mL⁻¹) in *Aspergillus oryzae* RC27 was at pH 5.0 compared to (530 U mL⁻¹) in the reference strain. The enzyme showed maximum stability at pH ranging from 4.0 to 5.0. When the pH was increased up to 8.0; it showed a decrease in the enzyme activity. Likewise, at pH values lower than 4.0, it showed a decrease in PE activity with a decrease in pH.

4.4 Extraction of orange and guava juice using pectinase enzyme

4.4.1 Optimization of incubation time and enzyme concentrations

The purified forms of polygalacturonase (PG), pectin lyase (PL) and pectin esterase (PE) produced during the study were compared with the commercial PG, PL and PE at different concentrations for the extraction of orange and guava juice. The effect of enzyme addition on the orange and guava juice yield at two different incubation periods was studied.

The results presented in Table 62 indicated a significant difference in juice recovery. All the individual enzymatic treatments, incubation periods and their combined interactions varied significantly. The mean maximum of 80.20 per cent juice recovery was observed at 120 min incubation which was significantly higher compared to 68.89 per cent at 60 min of incubation period. The higher average juice yield of 84.88 per cent was observed in combination of commercial (PG+PL+PE) at 100 U concentrations followed by combinations of the purified (PG+PL+PE) at 100 U concentrations resulting in 81.71 per cent juice yield as compared to control (50.54 per cent).

The performance of enzymatic treatments on orange fruit mash increased with increased levels of enzyme addition compared to control in terms of juice yield. After 60 min of incubation period, the orange pulp treated with PL at 100 U concentration resulted in the highest juice yield

(77.37%) followed by commercial (PG+PL+PE) at 100 U (77.27%). The minimum juice recovery was observed in the untreated orange pulp (50.37%).

After 120 min of incubation period, the orange pulp treated with commercial (PG+PL+PE) at 100 U concentration resulted in the highest juice yield (92.50%) followed by (PG+PL+PE) at 100 U (90.17%). The least juice recovery was observed in the untreated orange pulp (50.72%).

The results presented in Table 62 indicated a significant difference in guava juice recovery. All the individual enzymatic treatments, incubation periods and their combined interactions varied significantly. The mean maximum of 56.11 per cent juice recovery was observed at 120 min of incubation which is significantly higher compared to 49.12 per cent at 60 min of incubation period. The highest average juice yield of 64.05 per cent was observed in T₁₇ commercial (PG+PL+PE) at 100 U concentration, followed by combination of the purified (PG+PL+PE) at 100 U concentration (60.52%). The least (14.65%) was observed in the control.

After 60 min of incubation period the guava pulp treated with T₁₇ Commercial (PG+PL+PE) at 100 U concentration resulted in highest juice yield (58.84%) followed by commercial T₇ (PL at 100 U) (58.16%). The minimum juice recovery was observed in the untreated guava pulp (11.93%).

After 120 min of incubation period, the guava pulp with commercial (PG+PL+PE) at 100 U concentration (T₁₇) resulted in the highest juice yield (69.25%) followed by (PG+PL+PE) at 100 U (66.75%). The minimum juice recovery was observed in the untreated guava pulp (17.37%).

4.4.2 Effect of pectinase enzyme treatment on physico-chemical characteristics of orange and guava juice

After the extraction from enzyme treated orange and guava pulp the juice was analyzed for different parameters such as TSS, pH, titrable acidity and clarity.

4.4.2.1 Total soluble solids (TSS)

A perusal of the data presented in Table 63 revealed that there were significant differences among TSS (°Brix) values obtained with various enzyme treatments. The maximum TSS value (11.72) was observed in orange juice treated with combinations of (PG+PL+PE) at 100 U followed by commercial (PG+PL+PE) at 100 U concentration (11.45), while the minimum value was observed in case of control (11.10).

The maximum TSS value (11.10) was observed in guava juice treated with combination of commercial (PG+PL+PE) at 100 U (T₁₇) followed by (T₁₆) while the minimum value was observed in case of control (9.03) (Table 63). It was further observed that pectinase or combination of pectinases showed an increase in TSS with an increase in concentration of the enzyme.

4.4.2.2 pH of orange and guava juice

The mean values for pH of juice given in Table 63, indicated that enzymatic treatments with PG, PL and PE or their combination had significant effect on the pH of orange juice. The maximum pH was observed in the control (3.95), followed by T₉ (3.66). The least pH was observed in treatment T₁₇ (3.57). It was further observed from the results that pectinase and/or combination of pectinase showed a slight decrease in pH with increased levels of enzyme addition compared to control.

The mean values for pH of juice given in Table 63, indicated that guava pulp treatments with PG, PL and PE or their combination had significant effect on the pH of juice. T₁₇ (3.97), T₁₆ (3.98) and T₉ (4.02) showed a slight decrease in pH as compared to the control (4.18).

4.4.2.3 Titrable acidity of orange and guava juice

Titration acidity is an important factor for determining juice quality. It was noticed that titration acidity of juice varied significantly among the treatments (Table 63). The individual enzyme treatments alone did not affect titration acidity so much. However, combination of pectinase resulted in a slight increase in titration acidity. The maximum titration acidity (0.34%) was recorded each by commercial (PG+PL+PE) at 100 U and commercial PE at 100 U compared to untreated (0.30%).

The titration acidity of juice varied significantly among the treatment (Table 63). The individual enzyme treatments alone did not affect titration acidity so much. However, combination of pectinases resulted in a slight increase in titration acidity. Maximum titration acidity (0.59%) was recorded in T₁₇, followed by T₁₆ (0.57%), compared to titration acidity (0.42%) of untreated guava juice.

Table 56. Optimization of temperature for maximum polygalacturonase activity

Temperature (°C)	<i>Aspergillus niger</i> RBF96		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Relative activity (%)	Enzyme activity (U mL ⁻¹)	Relative activity (%)
4	94	12.14	70	11.11
20	114	14.72	110	17.46
30	139	17.95	230	36.50
40	320	41.34	630	100
50	774	100	555	89
60	684	88.37	380	60.30
70	134	17.31	150	23.80
80	22	2.84	54	8.57
90	-	-	-	-

Note: -: no activity

Table 57. Optimization of temperature for maximum pectin lyase activity

Temperature(°C)	<i>Penicillium citrinum</i> DLP 51		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Relative activity (%)	Enzyme activity (U mL ⁻¹)	Relative activity (%)
4	105	16.07	74	19.22
20	125	19.14	103	26.75
30	240	36.75	200	52
40	512	78.40	385	100
50	653	100	360	93.50
60	470	72	235	61.03
70	215	32.92	173	45.00
80	70	10.71	54	14.02
90	-	-	-	-

Note: -: no activity

Table 58. Optimization of temperature for maximum pectin esterase activity

Temperature (°C)	<i>Aspergillus oryzae</i> RC27		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Relative activity (%)	Enzyme activity (U mL ⁻¹)	Relative activity (%)
4	90	15.65	100	18.18
20	140	24.34	160	29.10
30	320	55.65	300	54.55
40	450	78.26	530	96.36
50	575	100	550	100
60	500	86.95	420	76.36
70	225	39.13	200	36.36
80	75	13.04	90	16.36
90	-	-	-	-

Note: -: no activity

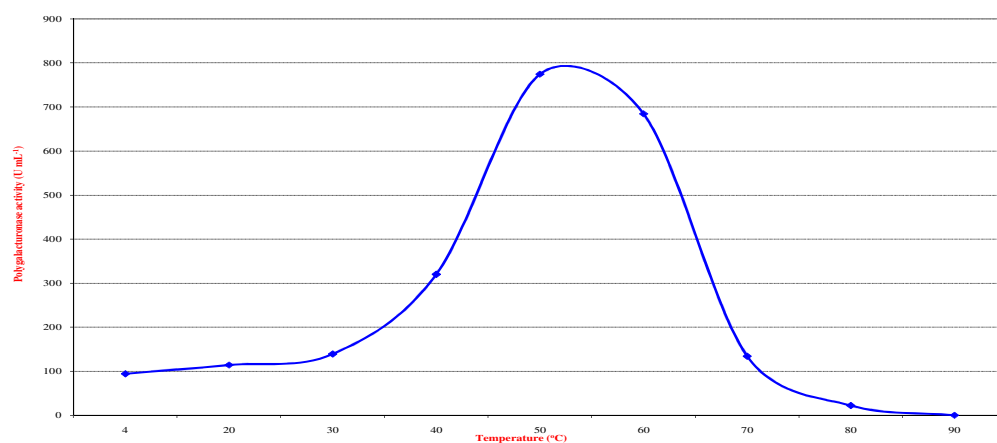


Fig. 9: Optimization of temperature for maximum polygalacturonase activity

Fig 9: Optimization of temperature for maximum polygalacturonase activity

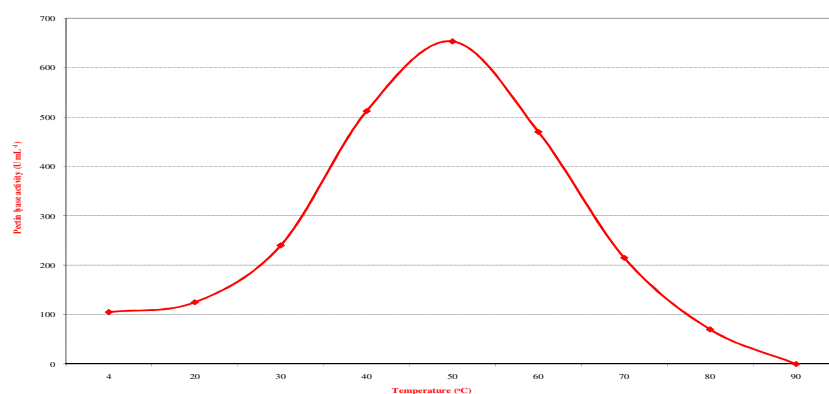


Fig. 10: Optimization of temperature for maximum pectin lyase activity

Fig 10: Optimization of temperature for maximum pectin lyase activity

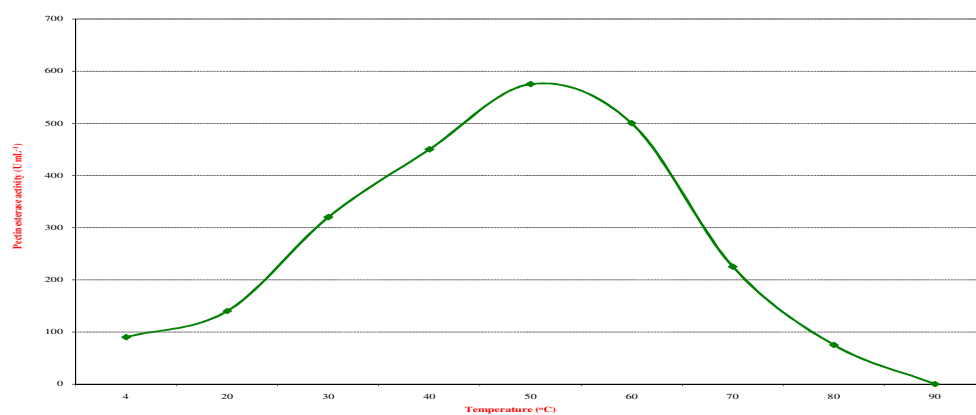


Fig. 11: Optimization of temperature for maximum pectin esterase activity

Fig 11: Optimization of temperature for maximum pectin esterase activity

Table 59. Optimization of pH for maximum polygalacturonase activity

pH	<i>Aspergillus niger</i> RBF96		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Stability (%)	Enzyme activity (U mL ⁻¹)	Stability (%)
3.0	317	44.52	212	31.50
3.5	490	68.82	449	66.71
4.0	700	98.31	650	96.58
4.5	712	100	673	100
5.0	650	91.20	621	92.27
5.5	605	84.97	580	86.18
6.0	407	57.16	415	61.66
6.5	365	51.26	350	52.00
7.0	240	33.70	257	38.18
7.5	130	18.25	91	13.52
8.0	84	11.80	44	6.53

Table 60. Optimization of pH for maximum pectin lyase activity

pH	<i>Penicillium citrinum</i> DLP 51		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Stability (%)	Enzyme activity (U mL ⁻¹)	Stability (%)
3.0	117	19.08	120	34.43
3.5	203	33.11	145	39.18
4.0	320	52.20	220	59.45
4.5	365	58.72	300	82
5.0	424	69.16	370	100
5.5	500	81.56	335	90.54
6.0	565	92.16	320	86.48
6.5	613	100	230	62.16
7.0	540	88.10	190	51.35
7.5	350	58	140	37.83
8.0	120	19.57	70	18.91

Table 61. Optimization of pH for maximum pectin esterase activity

pH	<i>Aspergillus oryzae</i> RC27		<i>Aspergillus niger</i> MTCC1344	
	Enzyme activity (U mL ⁻¹)	Stability (%)	Enzyme activity (U mL ⁻¹)	Stability (%)
3.0	240	40	200	37.73
3.5	380	63.33	375	70.75
4.0	550	91.66	450	84.90
4.5	570	95.00	500	94.33
5.0	600	100	530	100
5.5	520	86.66	480	90.56
6.0	405	67.70	370	69.81
6.5	350	58.33	300	56.60
7.0	210	35.00	180	34.00
7.5	150	25.00	130	24.50
8.0	60	10.00	70	13.20

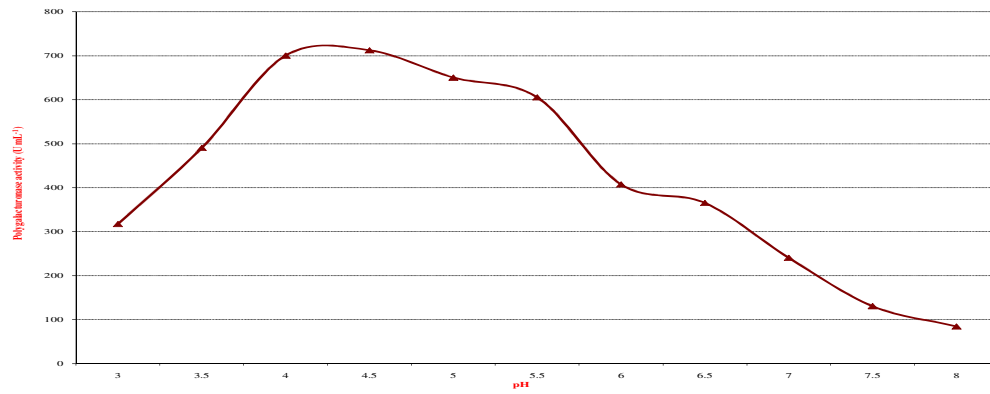


Fig. 12: Optimization of pH for maximum polygalacturonase activity

Fig 12: Optimization of pH for maximum polygalacturonase activity

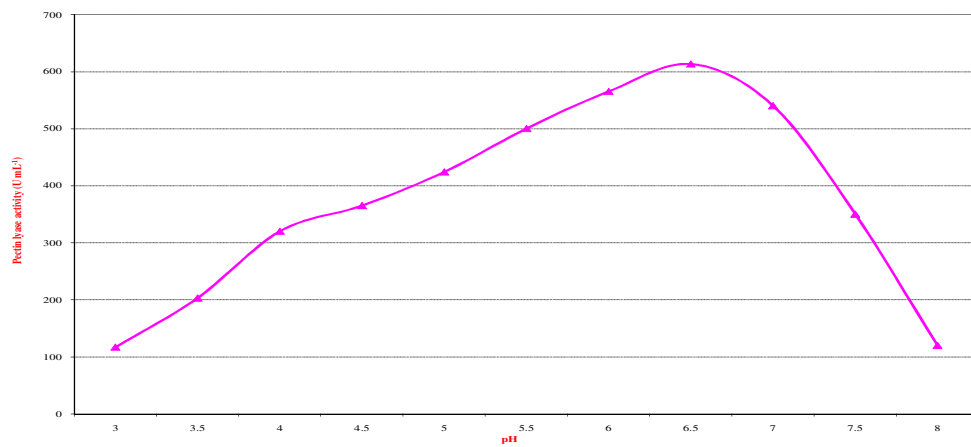


Fig. 13: Optimization of pH for maximum pectin lyase activity

Fig 13: Optimization of pH for maximum pectin lyase activity

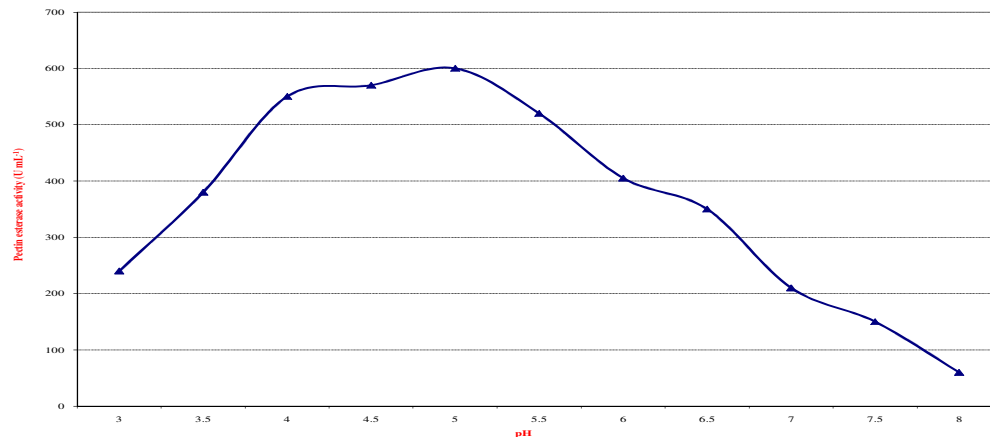


Fig. 14: Optimization of pH for maximum pectin esterase activity

Fig 14: Optimization of pH for maximum pectin esterase activity

Table 62. Extraction of orange and guava juice using pectinase enzymes

Treatments (enzyme U 100 g ⁻¹ pulp)	Orange			Guava		
	Juice recovery (%)			Juice recovery (%)		
	60 min. after incubation	120 min. after incubation	Mean (%)	60 min. after incubation	120 min. after incubation	Mean (%)
T ₁ - No enzyme	50.37	50.72	50.54	11.93	17.37	14.65
T ₂ - Polygalacturonase (PG) 50 U	61.51	75.37	68.44	42.31	51.67	46.99
T ₃ - Pectin lyase (PL) 50 U	60.30	77.77	69.03	41.86	54.16	48.01
T ₄ - Pectin esterase (PE) 50 U	68.53	80.60	74.57	50.13	56.92	53.53
T ₅ - PG+PL+PE 50 U	71.25	82.50	76.88	52.65	58.81	55.73
T ₆ - Polygalacturonase (PG) 100 U	74.58	81.37	75.98	53.93	56.02	54.97
T ₇ - Pectin lyase (PL) 100 U	77.37	81.92	79.64	57.98	58.16	58.07
T ₈ - Pectin esterase (PE) 100 U	70.50	83.50	77.00	52.39	60.08	56.23
T ₉ - (PG+PL+PE) 100 U	73.25	90.17	81.71	54.40	66.75	60.58
T ₁₀ - Commercial PG 50 U	60.50	77.50	69.00	41.82	54.50	48.16
T ₁₁ - Commercial PL 50 U	67.37	78.17	72.77	48.74	55.11	51.92
T ₁₂ - Commercial PE 50 U	63.83	83.43	73.63	45.15	59.55	52.35
T ₁₃ - Commercial (PG+PL+PE) 50 U	73.39	85.17	79.28	54.87	61.89	58.38
T ₁₄ - Commercial PG 100 U	74.70	81.00	77.85	56.19	56.98	56.58
T ₁₅ - Commercial PL 100 U	75.67	83.00	79.33	57.15	59.58	58.37
T ₁₆ - Commercial PE 100 U	70.83	82.77	76.80	52.32	59.33	55.82
T ₁₇ - Commercial (PG+PL+PE) 100 U	77.27	92.50	84.88	58.84	69.25	64.05
Mean	68.89	80.20		49.12	56.11	
	Treatments (A)	Incubation period (B)	A x B	Treatments (A)	Incubation period (B)	A x B
S.Em±	0.195	0.067	0.276	0.212	0.073	0.300
CD at 1%	0.731	0.251	1.033	0.795	0.273	1.125

Table 63. Physico-chemical characteristics of orange and guava juice treated with pectinase enzymes

Treatments (enzyme U 100 g ⁻¹ pulp)	Orange				Guava			
	TSS (°Brix)	pH	Titration acidity (%)	Clarity (% transmittance)	TSS (°Brix)	pH	Titration acidity (%)	Clarity (% transmittance)
T ₁ - No enzyme	11.10	3.95	0.30	17.67	9.03	4.18	0.42	4.46
T ₂ - Polygalacturonase (PG) 50 U	11.27	3.75	0.30	73.33	9.26	4.12	0.44	25.93
T ₃ - Pectin lyase (PL) 50 U	11.25	3.81	0.31	77.67	9.26	4.21	0.45	28.17
T ₄ - Pectin esterase (PE) 50 U	11.19	3.70	0.31	70.33	9.33	4.06	0.45	22.37
T ₅ - PG+PL+PE 50 U	11.28	3.65	0.32	81.07	9.41	4.04	0.47	31.53
T ₆ - Polygalacturonase (PG) 100 U	11.23	3.71	0.32	87.17	9.86	4.06	0.53	34.27
T ₇ - Pectin lyase (PL) 100 U	11.25	3.74	0.31	88.05	10.18	4.11	0.53	32.30
T ₈ - Pectin esterase (PE) 100 U	11.30	3.73	0.31	63.50	10.18	4.15	0.54	30.40
T ₉ - (PG+PL+PE) 100 U	11.72	3.66	0.30	96.28	10.68	4.02	0.53	36.67
T ₁₀ - Commercial PG 50 U	11.20	3.69	0.31	70.05	10.53	4.09	0.46	27.67
T ₁₁ - Commercial PL 50 U	11.25	3.74	0.30	74.17	10.23	4.13	0.46	29.47
T ₁₂ - Commercial PE 50 U	11.22	3.75	0.31	78.33	10.24	4.12	0.46	31.67
T ₁₃ - Commercial (PG+PL+PE) 50 U	11.25	3.70	0.32	79.13	10.60	4.06	0.48	34.73
T ₁₄ - Commercial PG 100 U	11.25	3.65	0.31	91.17	10.79	4.02	0.56	31.37
T ₁₅ - Commercial PL 100 U	11.25	3.66	0.32	89.52	10.83	4.08	0.57	33.70
T ₁₆ - Commercial PE 100 U	11.30	3.61	0.34	87.10	10.73	3.98	0.57	37.57
T ₁₇ - Commercial (PG+PL+PE) 100 U	11.45	3.57	0.34	95.43	11.10	3.97	0.59	40.28
S.E.m±	0.04	0.02	0.004	6.61	0.04	0.02	0.005	0.14
CD at 1%	0.16	0.07	0.01	25.52	0.14	0.08	0.019	0.55

4.4.2.4 Clarity

The juice transmittance reflects the clarity of juices. The data presented in Table 63 indicated that per cent transmittance of juice was affected significantly by the treatments. The maximum per cent transmittance (96.28%) was observed in T₉ (PG+PL+PE) at 100 U levels followed by T₁₇ i.e. commercial (PG+PL+PE) at 100 U. The lowest value (17.67 %) was observed in the untreated orange juice. It was further noticed that the individual enzyme treatments alone did not affect clarity. However, the combination of pectinases resulted in maximum transmittance.

In case of guava juice the maximum clarity (40.28%) was observed in T₁₇ (commercial PG+PL+PE) at 100 U levels followed by T₁₆ (commercial PG at 100 U) (37.57), and the minimum value (4.46) in the control treatment (Table 65). It was also noticed that the individual enzyme treatments alone did not affect clarity so much. However, the combination of pectinases resulted in maximum per cent transmittance.

4.4.2.5 Sensory Evaluation of clarified orange and guava juice

Sensory properties are among the most important factors influencing product acceptability; the product having good colour, flavour and taste, is accepted by the consumer. It is obvious from the Table 64 that treatments showed significant effect on colour, flavour, taste, sweetness and acceptability of the juice. The results of these parameters are furnished below.

4.4.2.6 Colour

The addition of pectinase significantly affected the colour and resulted in a more clear orange juice as compared to control. The combinations of enzymes resulted in more pronounced colour degradation than either of the enzymes used. It is evident from Table 64 that the untreated juice (T₁) attained the highest score of 8.2, followed by 8.00 in T₂ (PG at 100 U) and the least score (2.80) in T₈ (commercial PE at 100 U).

In case of guava juice also, the addition of pectinase significantly affected the colour and resulted in relatively more clear juice as compared to the control. It is evident from Table 64 that both T₁ and T₇ attained the highest score of 6.40 each. The least score (4.80) was observed in T₂.

4.4.2.7 Flavour

Flavour is combination of taste, odor and mouth feel. The addition of pectinase enzymes, alone as well as in combinations improved the flavour of the juice as shown in the Table 64. T₉ (commercial PG+PL+PE at 100 U) secured the highest score of 6.2 followed by 6.0 each in T₆, T₇ and T₈, while the least score (4.20) was observed in T₄ (PE at 100 U) (Table 63). Similarly, in clarified guava juice the treatments T₇ and T₈ obtained the values 6.80 each followed by T₉ (6.60) (Table 64). The least score (5.20) was observed in T₂ (PG at 100 U).

4.4.2.8 Sweetness

The sweetness of juice increased with the addition of enzymes. The results elucidated that the Enzyme addition had marked effect on the sweetness of juice. The sweetness score increased with the addition of enzymes alone or in combinations as evident from Table 64. T₅ and T₉ both showed the highest score of 6.4, followed by T₇ with 6.0. And, the least score (4.20) was in T₃. Similarly, in guava juice also, the sweetness score was increased with the addition of enzymes alone or in combinations, with T₉ showing the highest value (7.0) followed by T₈ (6.80). The least score (5.40) was observed in (T₁) untreated guava juice.

4.4.2.9 Overall acceptability

The enzyme addition brought out an improvement in the quality traits and the overall acceptability of orange juice. It is obvious from Table 64 that the combination of enzymes improved the overall acceptability to the highest value. T₅ and T₉ secured an overall acceptability score of 6.80 and 6.20, while the untreated control scored 5.80. The lowest score (4.80) was observed in T₃ (PL at 100 U). The results presented in Table 64 showed that the treatments containing combination of the enzymes improved the overall acceptability of guava juice. T₉ and T₅ secured the highest 6.80 and 6.40 scores. The lowest overall acceptability score (5.40) was observed in the untreated guava juice (T₁).

Table 64. Sensory attributes of orange and guava juice treated with pectinase enzymes

Treatments (enzyme U 100 g ⁻¹ pulp)	Orange				Guava			
	Colour	Flavour	Sweet ness	Overall accept ability	Colour	Flavour	Sweet ness	Overall accept ability
T ₁ - No enzyme	8.20	4.80	4.80	5.80	6.40	5.80	5.40	5.40
T ₂ - Polygalacturonase(PG) 100 U	8.00	4.80	5.00	6.00	4.80	5.20	5.80	5.80
T ₃ - Pectin lyase(PL) 100 U	7.60	5.00	4.40	4.80	5.60	5.40	6.00	5.80
T ₄ - Pectin esterase (PE) 100 U	7.20	4.20	4.60	5.40	6.20	5.40	6.40	5.60
T ₅ - PG+PL+PE 100 U	6.60	5.80	6.40	6.80	5.80	6.20	6.20	6.40
T ₆ - Commercial PG 100 U	3.40	6.00	5.80	5.80	5.40	5.60	5.80	6.00
T ₇ - Commercial PL 100 U	5.40	6.00	6.00	5.80	6.40	6.80	6.40	6.40
T ₈ - Commercial PE 100 U	2.80	6.00	5.20	5.80	5.60	6.80	6.80	6.40
T ₉ - Commercial PG+PL+PE 100 U	6.00	6.20	6.40	6.20	5.80	6.60	7.00	6.80

Note: Hedonic scale

- 9 - Like extremely
- 8 - Like very much
- 7 - Like moderately
- 6 - Like slightly
- 5 - Neither like nor dislike
- 4 - Dislike slightly
- 3 - Dislike moderately
- 2 - Dislike very much
- 1 - Dislike extremely

DISCUSSION

Pectinases are mixtures of several enzymes that act on pectic substances, which are structural polysaccharides in the middle lamella and primary cell wall of higher plants. Pectinases are important in processing fruits and vegetables and also in wine production. Pectinolytic enzymes, catalyzing the degradation of pectic substances, are of great industrial importance. The present study was designed to isolate, characterize and select fungi from diverse natural sources capable of producing pectinase. Attempts were also made to purify different pectinase enzymes produced by the efficient strains. Owing to the expensive medium ingredients, solid state fermentation (SSF) was tried using citrus peel, wheat bran, coffee husk, banana pseudo stem, deseeded sunflower head, jackfruit rind and pineapple peel as a carbon source in order to find out cheaper and suitable natural source for production of pectin degrading enzymes. The process parameters for maximum production of the enzymes by the selected strains were optimized. The purified enzyme was used for clarification of orange and guava fruit juices. The results obtained during the course of study are discussed in this chapter.

5.1 Isolation, screening and identification of pectinolytic fungi

Isolation is the first stage in the screening of microorganisms with potential industrial applications. The isolation procedure is so designed as to favor the growth of a specific organism possessing the industrially important characteristics. The ideal isolation procedure starts with the identification of the environmental source, which is probably richer in the desired types of microorganisms and also incorporating a simple test to distinguish them. In the present investigation, as many as 100 fungal isolates were selectively isolated by enrichment method using a medium containing pectin as the sole carbon source. These isolates were tested for pectin hydrolysis by the agar plate assay. On the basis of prominent zone of clearance on pectin agar plate, and the potency index values, a total of 48 isolates were selected for further studies. This screening method deserves attention due to the high number of isolates. Based on this agar plate assay, the best pectinolytic activity was observed in *A. niger* RBF96 (potency index of 3.25) isolated from rotten ber fruit, closely followed by *P. citrinum* DLP51 (potency index of 3.21) from decayed lemon peel. The pectin agar plate assay screening method, used in the study, has been widely employed for screening pectinase producing microorganisms (Soares *et al.*, 1999), and the results obtained are in agreement with the earlier reports. Souza *et al.* (2003) came out with 46 fungal isolates which were pectinase producers as tested following the pectin agar plate assay. Similarly, Phutela *et al.* (2005) also reported that strains of *Aspergillus fumigatus* isolated from decomposing orange peels possessed the similar potency index values.

Based on the colony morphology, microscopic features and biochemical characterization of the isolates and by referring to the Hand Book of Soil Fungi by Nagamani *et al.* (2006), the pectinolytic fungi were tentatively identified up to the generic level. Out of 48 isolates, as many as 38 belonged to genus *Aspergillus*; eight were identified under *Penicillium*, and two as *Fusarium*. Pectinase production has already been reported in *Aspergillus niger* (Diaz-Godinez *et al.*, 2001), *A. oryzae* (Fontana *et al.*, 2009) *Penicillium chrysogenum* (Banu *et al.*, 2010) and *Fusarium moniliforme* (Niture and Pant, 2004). Therefore, it may be inferred that the isolated pectinolytic fungus comes under the already well known groups of pectinolytic fungi belonging to *Aspergillus*, *Penicillium* and *Fusarium*.

The molecular identification of the selected isolates was carried out by analysis of ITS rDNA region by sequencing. Ten most efficient isolates viz., RGC14, RC25, RC27, DLP51, RCFW68, RAF80, RAF81, RAF88, RBF92 and RBF96, with the highest potency index values and the maximum pectinolytic enzyme activities, were subjected to molecular identification. *In Silico* BLAST analysis results revealed that isolates RBF96 and RC27 showed 100 per cent homology with the GeneBank Accession numbers KC119204.1 and HF546392.1, while the isolates DLP51 and RAF88 showed 99 per cent homology with the GeneBank Accession numbers KC344966.1 and FR750924.1. Based on homology of the NCBI GenBank matching accession number, the isolates RBF96, RC27, DLP51 and RAF88 were confirmed as *Aspergillus niger*, *A. oryzae*, *Penicillium citrinum* and *Fusarium sporotrichioides* respectively. Similarly, Baffi *et al.* (2012) investigated on the fungal diversity of fresh olive fruits, olive paste and olive pomace. They screened and quantified the enzymatic activities. Identification was based on comparison of their polymerase chain reaction (PCR) amplicons of the ITS1–5.8S–ITS2 ribosomal DNA region, followed by nucleotide sequence analysis, they identified the fungal isolates belonged to seven different genera (*Aspergillus*, *Penicillium*, *Rhizomucor*, *Mucor*, *Rhizopus*, *Lichtheimia* and *Galactomyces*). *Aspergillus fumigatus*, followed by *Galactomyces*

geotrichum, *Penicillium commune* and *Rhizomucor variabilis* var. *regularior* were the most frequent species.

5.1.1 Quantitative estimation of pectinolytic activity of the selected pectinolytic isolates

Different pectinase enzymes namely, polygalacturonase (PG), pectin lyase (PL) and pectin esterase (PE) produced by potent isolates were quantified under submerged fermentation conditions. *A. niger* RBF96 showed the maximum PG activity, while *P. citrinum* DLP51, showed the highest PL activity and RC27 the highest PE activity (Table 3). Similarly, Taskin *et al.* (2008) also screened 262 *Aspergillus* isolates from vineyards for pectinase production. *A. foetidus* var. *pallidus* Ege-K-730 and *A. aculeatus* Ege-K-355 presented the highest pectinase production. In submerged fermentation (SmF), strains Ege-K-730, Ege-K-355, Ege-K-635 and CFTRI 1047 produced the highest PG levels. In fact, the yield of PG (189.33 U mL^{-1}), obtained in this study, was much higher than those reported earlier (51.82 U mL^{-1}) by Gomes *et al.* (2011).

5.1.2 Optimization of culture parameters for maximum enzyme production under submerged conditions

In order to produce maximum enzyme yields, by the efficient fungal isolates, various culture parameters were optimized. The inoculum size is an important parameter in SSF for production of high yields of pectinase enzymes as it influences efficient substrate-microbe interaction. In this study, the optimized inoculum level was found to be 1×10^7 (spores mL^{-1}) for *A. niger* RBF96, *P. citrinum* DLP51 and *A. oryzae* RC27 which resulted in maximum polygalacturonase (219.88 U mL^{-1}), pectin lyase (343.00 U mL^{-1}) and pectin esterase (377.00 U mL^{-1}) respectively. Similarly, Padma *et al.* (2012); Mrudula and Anitharaj (2011) also observed that the inoculum size of 1×10^7 spores mL^{-1} as optimum inoculum level for maximum pectinase production by *Aspergillus awamori* MTCC9166 and *Aspergillus niger*. From all these studies and from the current investigations it may be inferred that an inoculum size of 1×10^7 spores mL^{-1} for pectinolytic fungi yields higher extracellular enzymes.

The pH of a medium is another important factor influencing enzyme production. The pH influences growth rate in a manner similar to its influence on enzyme activity. Hence, an experiment was conducted to determine pH optima of each fungal isolate known to produce pectin degrading enzyme. The result indicates that pH optima for production of each pectinase enzyme by potent isolates varied. These findings not only helped in knowing the pH optima for maximum yield of each enzyme by respective isolate but also, significance of such studies in harvesting the potentiality of isolates from environmental samples. The optimum pH for maximum PG production by *A. niger* RBF96 was found to be pH 4.0, while it was pH 7.0 for *P. citrinum* DLP51 for pectin lyase activity and pH 5.0 for *A. oryzae* RC27 for pectin esterase. Similar results of higher pectinase production under acidic to near neutral conditions were also reported by Patil and Dayanand (2006), Khairnar *et al.* (2009); Vasanthi and Meenakshisundaram (2012).

The temperature of incubation is another process parameter in enzyme production. The optimum temperature for maximum pectinase activity was 35°C in *A. niger* RBF96, *P. citrinum* DLP51 and *A. oryzae* RC27 for maximum production of PG, PL and PE respectively. The temperature at which a particular organism grows depends on its psychrophilic, mesophilic or thermophilic nature. Thus, the isolates of the present study may belong to mesophilic group. Similarly, Patil and Dayanand (2006) also reported 34°C as the optimum temperature for PG production by *A. niger* DMF27 under submerged conditions.

The minimum requirements in any fermentation media are suitable carbon source, bioavailable nutrients and absence of toxic or inhibitory constituents. Maximum PG, PL and PE were produced in the culture filtrates of the fungi grown in a medium containing 1 per cent pectin concentration. It was also observed that as the pectin concentration was increased, the pectin esterase activity was found to decrease gradually. This can be attributed to the phenomenon of catabolic repression, where galacturonic acid or any other metabolite produced from pectin is undergoing self catabolite repression. Similar enzyme inhibition at higher pectin concentration in the medium has been reported in *Aspergillus niger* MTCC 281 (Palaniyappan *et al.*, 2009).

Growth of fungi in any medium varies according to physical and chemical environmental factors. Cell doubling time increases with increasing organic complexity. Enzymes are predominantly produced after the exponential phase. The producer strains need to have shorter fermentation cycles, as this would be cost effective. In the present study, the maximum PG, PL and PE were produced on

6th day of incubation. Teixeira *et al.* (2000) also reported that polygalacturonase enzymatic activities in *Aspergillus japonicus* 586 were the highest at the end of 122 h.

5.2 Screening of agro wastes for pectinase production under solid state fermentation (SSF)

SSF involves the cultivation of microorganisms on a solid substrate, such as grains, rice, wheat bran and several pectin rich fruit wastes. This method was an alternative to the production of enzymes in liquid by submerged fermentation. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment. Any substrates can be utilized for the production of enzymes by SSF. However, selection of suitable substrate depends on many factors, which may be related to the cost and availability of the substrate. Hence, a number of locally available agro wastes were screened for pectinase production by the efficient fungal isolates. The abilities of these isolates namely *A. niger* RBF96, *Penicillium citrinum* DLP51, *A. oryzae* RC27 and the reference strain *A. niger* MTCC1344 were tested for the production of all the three enzymes using these agro wastes.

On the basis of the experimental results, citrus peel was found to be the best substrate for maximum enzyme activities. The maximum pectinase activity obtained was 984 U mL⁻¹. In fact, the enzyme yields were higher than even wheat bran, which is widely used as a substrate for production of many enzymes under SSF. Citrus peel is known to possess higher pectin content when compared to others. Substantial portion of commercial pectin is derived from citrus peel. On dry weight basis, citrus peel contains 12.89 per cent calcium pectate (Table 3), which might have induced the biosynthesis of pectinase enzyme.

In the literature, there have been many reports of maximum pectinase production using citrus peel. Phutela *et al.* (2005); Mrudula and Anitharaj (2011) have also reported citrus peel as the most efficient substrate for pectinase production. SSF process has been described for the production of pectinase by *Aspergillus niger* Aa-20 using lemon peel pomace (LPP) as the support and carbon source, in a solid-state bioreactor (Ruiz *et al.*, 2012).

5.2.1 Optimization of process parameters for maximum pectinase production under SSF

Particle size of the substrate is very important factor in SSF. In this study in general, the particle size of 2 mm was found superior to 4 mm for production of all the three enzymes. It is known that smaller particles have a larger surface area for the proliferation of the microorganisms. Although larger particles provide more efficient aeration and respiration, there is a reduction in the surface area and hence 4 mm size was inferior.

Moisture content plays a significant role in SSF, as it gives a good comparison between water availability and substrate swelling. It is also significant, as both oxygen availability and its diffusion depend on this. In the present study, in general, 65 per cent moisture was found to be optimum for production of all the three pectinase enzymes. These findings are in line with the earlier reports of Acuna-Arguelles (1995); Blandino *et al.* (2002); Maciel *et al.* (2011). It was also observed that as the moisture level was increased beyond 65 per cent, there was a drastic decrease in all the three enzymes production. The high moisture content might have resulted in low substrate porosity which, in turn, prevented oxygen penetration. Whereas, low moisture content might have led to poor accessibility of nutrients resulting in hampered microbial growth.

The effect of inoculum size on pectinase production was assessed. The data indicated that 1x10⁷ spores g⁻¹ was more effective than 1x10⁵ spores g⁻¹. Higher spore population is known to have better interaction with the substrate, resulting in higher enzyme production. Similarly, Padma *et al.* (2012) obtained the highest enzyme production in jack fruit rind and mango peel at 10⁷ spores g⁻¹ inoculum level.

The incubation time is generally dictated by the composition of the substrate and properties of the strain, such as the length of lag phase, enzyme production profile, initial inoculum level and others. In this study the optimum incubation time for maximum pectinase production was found to be 6 days. Maximal activities of PG (667.96 U mL⁻¹), PL (433.10 U mL⁻¹) and PE (520.00 U mL⁻¹) were observed on 6th day of incubation. These results are in agreement with the report of Diaz-Godinez *et al.* (2001). Patil and Dayanand (2006) also made similar observations. In their report, the maximum

pectinase production was produced on 6th day of cultivation using a medium with mango peels, plantain and banana peels. The decline in pectinase activity after reaching its maximum level of production may be due to catabolic repression, cessation of enzyme synthesis, or to the increased proteolysis in the culture (Sakellaris *et al.*, 1988). The lower enzyme activities during the initial fermentation period may be due to the added carbon sources causing catabolic repression in the initial stages of growth and after its consumption, the initiation of enzyme production usually starts. Drop in pH in the initial stages of growth does not favor the enzyme production. However, when the pH increases, the enzyme production will commence.

The pH of a medium is also extremely important if optimal productivity is to be achieved. The results of this study clearly shown that the maximum activity of polygalacturonase (618.28 U mL^{-1}) was at pH 4.0, while that of pectin esterase (429.00 U mL^{-1}) and Pectin lyase (508.33 U mL^{-1}) were at 5.0 and 7.0 respectively. In general, as the pH of the growth medium was increased, the enzyme production decreased. Similarly, in earlier studies also, pH 4.0, 5.0 and 7.0 were found to be optimum respectively for extracellular PG, PE and PL activities respectively in *Aspergillus niger* URM4645 under SSF using forage palm as the substrate (Maciel *et al.* (2011). Martos *et al.* (2009) also observed pH 4.1 as the optimal condition for PG production by *Aspergillus niger*, using response surface methodology under submerged fermentation.

Temperature is directly related to the metabolic activities of the microorganisms and affects the proper growth and product formation by the organism. Every organism has its own optimal temperature at which it grows and produces the desired product maximally. Hence, the maintenance of optimal temperature is a must. The thermal condition for maximal production of pectinase was studied. In general, the temperature of 35°C was found to be optimal for maximum activities of all the enzymes. Higher temperatures resulted in lower production, indicating that the enzymes were thermosensitive in nature. Likewise, Banu *et al.* (2010); Patil and Chaudhari (2010) also observed that *Penicillium chrysogenum* and *Penicillium* sp., showed maximum pectinase enzyme production at 35°C in the growth medium.

For better output from microorganisms, they need to be cultivated in a full fledged way, by providing the basic requirements including sources of energy, carbon and nitrogen, mineral elements for production of cell biomass, energy biosynthesis and cell maintenance. Most of the industrial organisms are chemo-organotrophs; therefore the most common sources of carbon and energy include carbohydrates, lipids and proteins. In the present study, glucose and sucrose were tested as carbon sources. The nitrogen source plays an important role in affecting the pH changes in the substrate during the fermentation. Higher titers of pectinases were observed when the medium was supplemented with sucrose at 2 per cent and soy meal (as N source) at 2 per cent. In SSF, presence of glucose and sucrose does not inhibit pectinase production; SSF is less affected by catabolic repression. This may be the reason for better production of pectinases.

The nitrogen sources tested such as soy meal and ammonium sulfate supported better pectinase activities. And, organic source was better than the inorganic source. This is in consonance with Arotupin (2011), who reported that organic nitrogen sources tend to support good growth of fungi more than the inorganic nitrogen sources. Vahidi *et al.* (2004) also observed good growth and antifungal activities when complex nitrogen sources (yeast extract and peptone) were used when compared to inorganic nitrogen source (NH_4Cl and NaNO_3). This implication may be that the organic nitrogen sources may serve as good growth stimulators. During growth, the fungi hydrolyze the organic nitrogen releasing mineral components and other growth factors in them into constituents which can be easily incorporated into the cellular metabolism. The precedence of organic nitrogen sources over inorganic substrates in PG production has hinted by Akhilesh *et al.* (2010) who reported that the best polygalacturonase production was obtained with *Mucor circinelloides* ITCC 6025 when casein hydrolysate and yeast extract were used together. Thus, the present study has shown that the enzyme biosynthesis could be further enhanced with the amendment of the culture medium with appropriate carbon and nitrogen sources.

Mineral nutrition is very much necessary for better growth and metabolism of the microorganisms. The effects of manganese and zinc on pectinase production were assessed. The results of the present investigation showed that there was a marked increase in all the three enzyme production due to the addition of salts when compared to control, indicating that they stimulated enzyme biosynthesis. Sakellaris *et al.* (1988) also obtained stimulatory results with MnCl_2 and ZnCl_2 on pectinase production.

It is evident from all these findings that parameters like pH, temperature, inoculum size, substrate moisture levels, particle size, incubation time including media supplementation still holds the most important consideration for obtaining higher enzyme yields from isolates grown on SSF.

5.3 Purification of pectinolytic enzymes

In order to characterize and study the properties of microbial pectinases, the enzymes must be purified. The analysis of enzyme activity in the crude extract does not indicate the isolated action or the presence of a multi-enzymic system working in synergy on the substrate degradation. The characterization of purified enzymes is important since it provides information about discrimination between the enzymic complex components, substrate degradation mechanisms, optimum activity conditions and enzyme synthesis regulation. Hence in this study, pectinases were purified to homogeneity (Plate 9). The enzymes were partially purified by addition of ammonium sulfate to the cell-free supernatant. Ammonium sulfate precipitation is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins, since large proteins tend to precipitate first while smaller ones will stay in solution. After protein has been precipitated and taken back up in buffer, the solution contained a lot of residual ammonium sulfate which was bound to the protein. Pectinase activity was found in the 80 per cent salt saturation fraction; these fractions were concentrated and dialyzed. After purification through gel filtration chromatography using Sephadex G-100, the maximum PG specific activity of $83.06 \text{ U } \mu\text{g}^{-1}$ protein was obtained in case of *A. niger* RBF96 compared to $27.63 \text{ U } \mu\text{g}^{-1}$ protein in the reference strain, with 7 folds increase in purification. The maximum specific activity in pectin lyase was $44.13 \text{ U } \mu\text{g}^{-1}$ protein in *P. citrinum* DLP5. About 1.44 fold purification was achieved after ammonium sulfate precipitation in *P. citrinum* DLP51 and 6.08 folds purification after gel filtration. Similarly, pectin esterase purification resulted in enhanced specific activity in *A. oryzae* RC27. The maximum specific activity ($30.32 \text{ U } \mu\text{g}^{-1}$ protein) was obtained after gel filtration chromatography in *A. oryzae* RC27 with 5.73 folds purification. The presence of residual pectin may interfere in the initial purification steps, particularly with ammonium sulfate precipitation. Gel permeation separates molecules on the basis of pore size. The smaller molecules diffuse into the gel more rapidly than the larger ones. Electrophoretic method is used for checking the purity and also for determining the molecular weight by comparing with the molecular weight of marker proteins.

When this purified pectinase was electrophoresed on 10% SDS-PAGE, a single band was observed, indicating the complete purification of the enzyme (Plate 10). Using standard protein markers, the size of the purified enzyme was confirmed. The molecular weight of the PG protein produced by *Aspergillus niger* RBF96 was 45 kDa, of PL protein produced by *Penicillium citrinum* DLP51 was 37 kDa and of PE protein produced by *Aspergillus oryzae* RC27 was 31 kDa. The molecular weights of the PG, PL and PE proteins produced by the reference strain were 35 kDa, 47 kDa and 33 kDa respectively.

It is important to determine the kinetic properties of the enzyme. The kinetic properties are K_m , V_{max} , thermal stability and pH. K_m , the substrate concentration which gives half of the maximal velocity possible at that enzyme concentration, is a constant characteristic for a particular enzyme, acting on a particular substrate. In this study, the kinetic parameters were determined against the substrate pectin.

The apparent K_m and V_{max} values for polygalacturonase of *A. niger* RBF96 were 0.155 U mL^{-1} and $38.57 \text{ U mL}^{-1}/\text{h}$ respectively (Fig. 15) as compared to 0.153 U mL^{-1} and $44.57 \text{ U mL}^{-1}/\text{h}$ obtained for polygalacturonase in the reference strain (Figs 18,19 and 20).

The K_m (0.278 U mL^{-1}) and V_{max} ($28.70 \text{ U mL}^{-1}/\text{h}$) (Fig. 16) values were obtained for pectin lyase of *Penicillium citrinum* DLP 51. In case of pectin esterase of *Aspergillus oryzae* RC27, the apparent K_m and V_{max} values were 0.163 U mL^{-1} and $38.57 \text{ U mL h}^{-1}$ (Fig. 17). In general, it was observed that the enzyme activity was increased with an increase in pectin concentration up to 0.9 U mL^{-1} . Here, it can be explained that the substrate has been binding to the high affinity sites up to the concentration of 0.9 U mL^{-1} , after which it might be to the low affinity site. This presumed to inhibit catalytic reaction. It is evident that the kinetic property of pectinase varied with the source of the enzyme. In the present study, the K_m value is low. This agrees with the results of Price and Stevens (1999). Another important observation was the diminished enzyme activity above $0.9 \mu\text{g mL}^{-1}$ substrate concentration which may be due to the inhibitory effect of the pectin on the enzymes.



Centrifugation



Filtration



Dialysis of ammonium sulphate saturated fraction



Gel filtration chromatography using Sephadex G100

Plate 9: Various steps in purification of pectinase enzymes

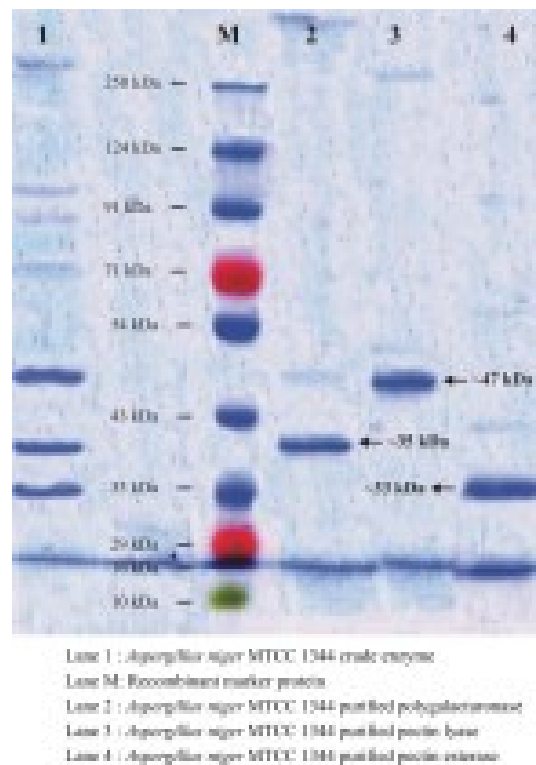
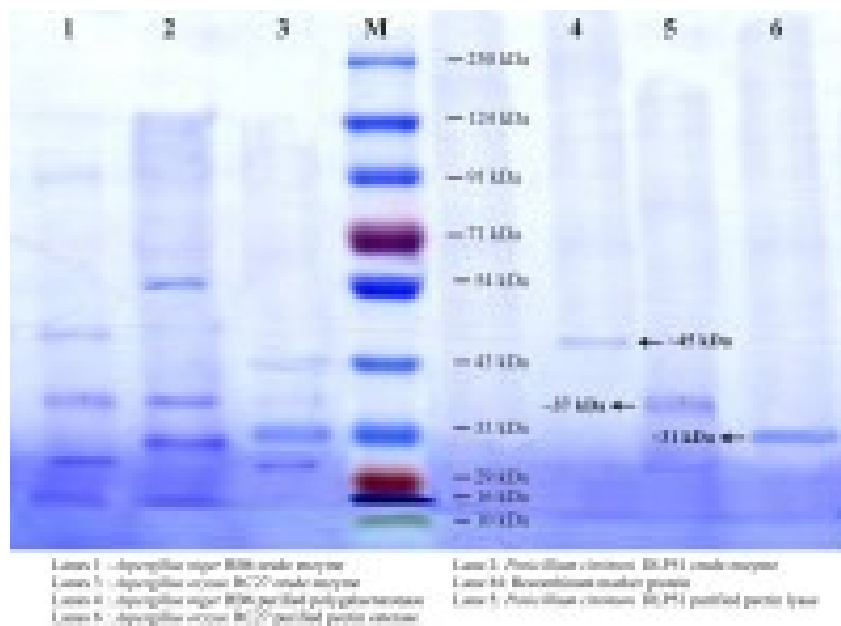


Plate 10: SDS-PAGE for determination of molecular weight of pectinase enzymes

5.3.1 pH and thermal stability of pectinases

Like all other chemical reactions, the velocity of the enzyme catalyzed reactions also increases with an increase in temperature. However, eventually a temperature is reached where the enzyme becomes unstable and begins to denature, at which point the reaction rate again declines. The results of the present investigation showed that the optimum temperature for the maximum activity of pectinase was 50°C, afterwards, a sudden drop in enzyme activity was observed. As the denaturation of the enzymic protein occurs at elevated temperatures, the enzyme activity decreased rapidly.

Enzyme deactivation and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes. Enhancing the stability and maintaining the desired level of activity over a long period are two important points considered for the selection and design of pectinases. The catalytic activity of many enzymes is markedly dependent on pH. It can exert its effect in different ways: on the ionization of groups in the enzymes active site, either on the ionization of groups in the substrate, or by affecting the conformation of either the enzyme or the substrate. The stability of pectinases is affected by pH and temperature. In this study, it was interesting to note that the PG showed maximum stability in the pH range 4.0 to 5.0, whereas PL and PE showed maximum stability at pH ranging from 5.5 to 7.0 and 4.0 to 5.0 respectively.

Changes in pH of a medium have been reported to affect the affinity of the enzyme for substrates. The pH of the medium in which the enzyme is exposed affects the ionization state of its amino acids which dictates the primary and secondary structure of the enzyme thus controlling its activity. The observed reduction in the enzyme activities of pectinolytic strains at pH values other than the optimal pH could also be attributed to a probable change in the state of the ionic groups involved in the maintenance of the active conformation of the enzymes. Extreme pH has been reported to initiate chemical reactions that could alter, cross-link or destroy amino residues of the protein molecules resulting in irreversible inactivation (Arotupin, 2011).

Similarly, Rashad *et al.* (2010) purified extracellular polygalacturonase (PG) produced from *Pleurotus ostreatus* NRRL-0366 on lemon Peel (*Citrus limonium*). The enzyme was purified to a homogenous form with about 27 folds purification and specific activity of 158 U mg⁻¹ proteins by two chromatographic steps in DEAE cellulose and sephacryl S-300. The extracellular PG was a monomer protein with a molecular weight of 21 kDa when determined by SDS gel electrophoresis and 20 kDa determined by gel filtration techniques. The optimal pH and temperature values were 3.5, 30°C respectively. The enzyme was stable at 30°C and pH (3.0-3.5); PG hydrolysed pectin and partially polygalacturonic acid. It exhibited K_m of 1.33 mg mL⁻¹ and V_{max} of 28.6 μmol min⁻¹. The pectin lyase produced by *Penicillium oxalicum* produced on mandarin peel meal on solid state fermentation was studied by Yadav and Shastri (2005). The partially purified pectinase showed the pH optima of 8.0 and was stable over pH range of 4.0-8.0. The optimum temperature of the thermolabile enzyme was 40°C. The K_m of the enzyme was found to be 2.08 mg mL⁻¹.

Kant *et al.* (2013) also characterized polygalacturonase from *A. niger* (MTCC 3323). The enzyme when precipitated with 60 per cent ethanol resulted in 1.68-folds purification. The enzyme was purified to 6.52-folds by Sephacryl S-200 gel-filtration chromatography. Homogeneity of the enzyme was checked by native-PAGE and its molecular weight was found to be 106 kDa. The purified enzyme showed maximum activity in the presence of polygalacturonic acid at temperature of 45°C, pH of 4.8, reaction time of 15 min. The enzyme was stable within the pH range of 4.0–5.5 for 1 h. The K_m and V_{max} values of the enzyme were found to be 0.083 mg mL⁻¹ and 18.21 μmol mL⁻¹ min⁻¹.

5.4 Application of pectinase enzyme

The exploitation of pectinases, has been well established in a variety of fruit juices and wine processing industries to increase the juice yield, clarification, promoting antioxidant formation and juice concentrate production. The addition of such exogenous enzymes also allows more specific degradation which is necessary to give a characteristic smooth texture, colour and increased level of reducing sugar. During extraction of juice from fruits, pectin passes into the juice and imparts cloudiness to it. The pectin is associated with other plant polymers and after crushing the fruit, it remains in the pulp and the cloudiness caused by them is difficult to be removed except by enzymatic hydrolysis. Hence, the pectinases produced by the strains developed during the study, were used to clarify juices of two fruits namely Orange and Guava (Plate 11).

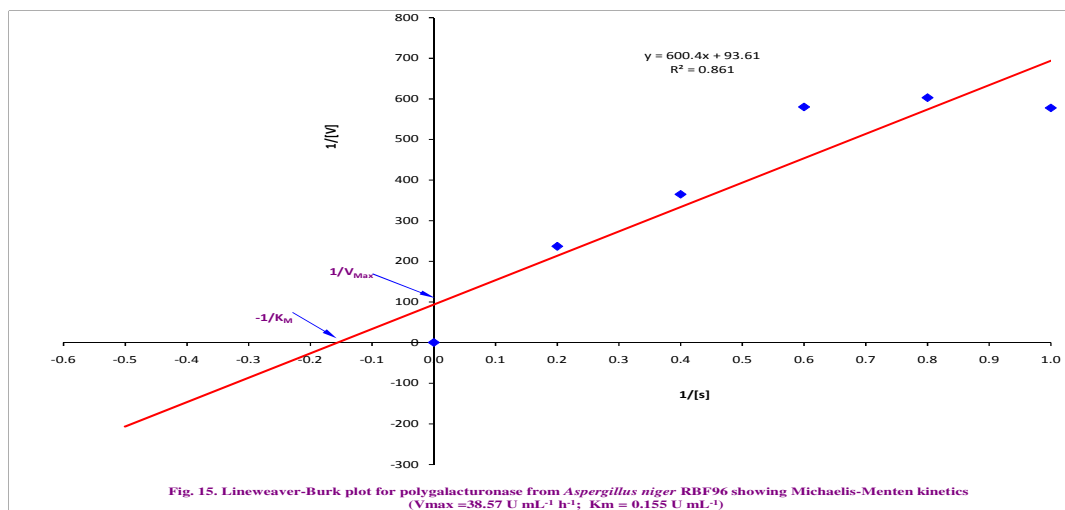


Fig 15: Lineweaver-Burk plot for polygalacturonase from *Aspergillus niger* RBF96 showing Michaelis-Menten kinetics

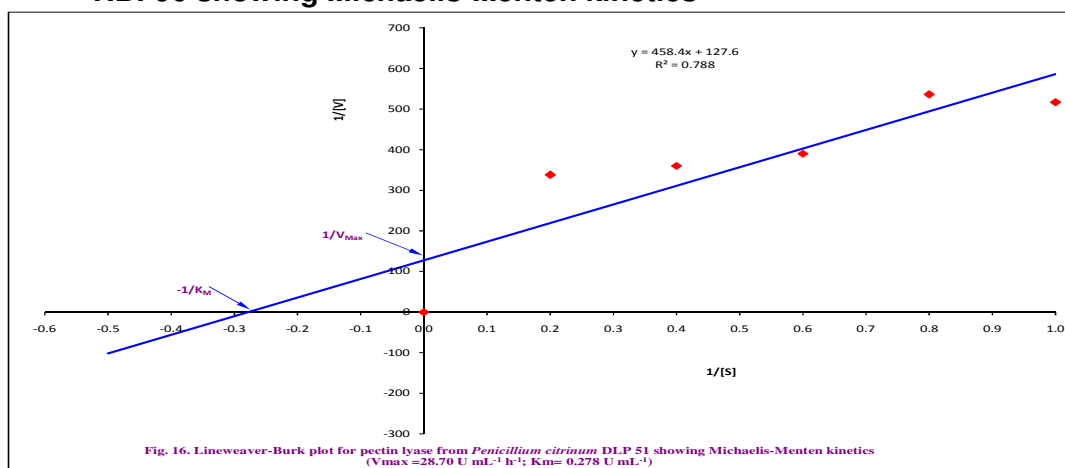


Fig 16: Lineweaver-Burk plot for pectin lyase from *Penicillium citrinum* DLP 51 showing Michaelis-Menten kinetics

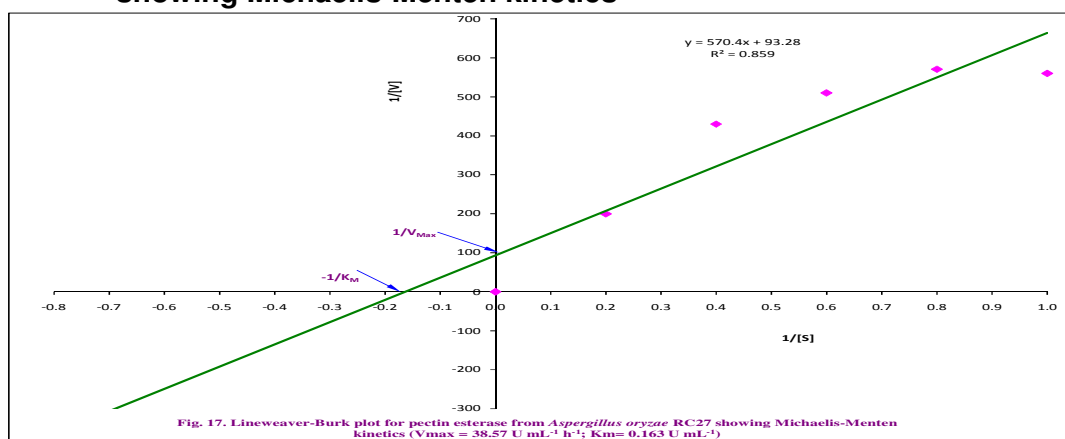


Fig 17: Lineweaver-Burk plot for pectin esterase from *Aspergillus oryzae* RC27 showing Michaelis-Menten kinetics

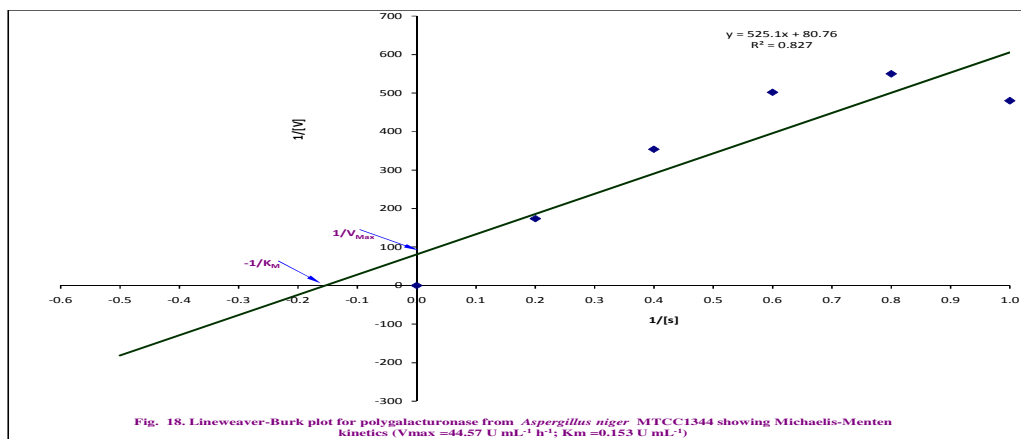


Fig 18: Lineweaver-Burk plot for polygalacturonase from *Aspergillus niger* MTCC1344 showing Michaelis-Menten kinetics

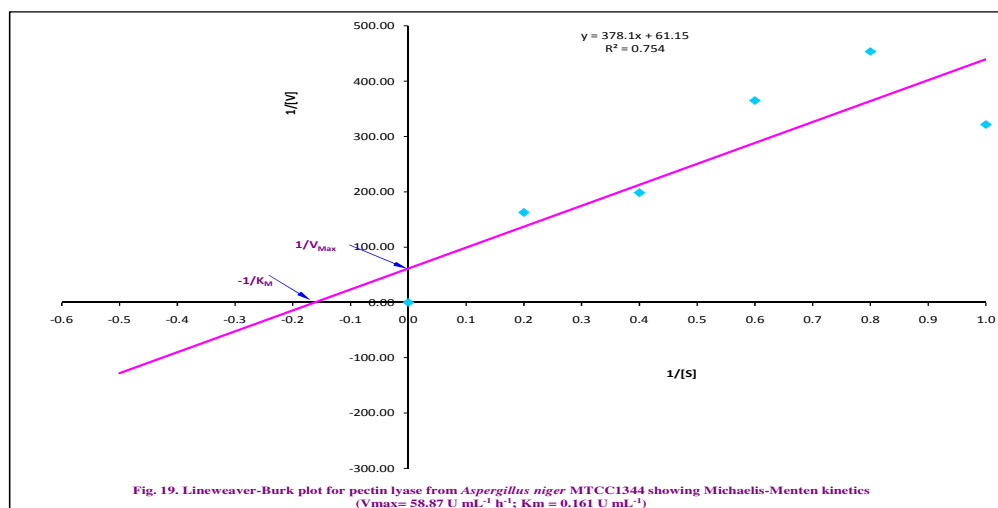


Fig 19: Lineweaver-Burk plot for pectin lyase from *Aspergillus niger* MTCC1344 showing Michaelis-Menten kinetics

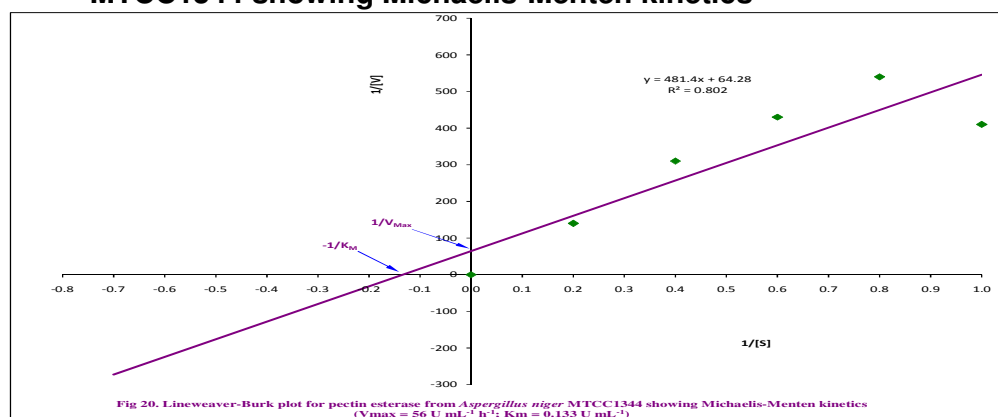


Fig 20: Lineweaver-Burk plot for pectin esterase from *Aspergillus niger* MTCC1344 showing Michaelis-Menten kinetics

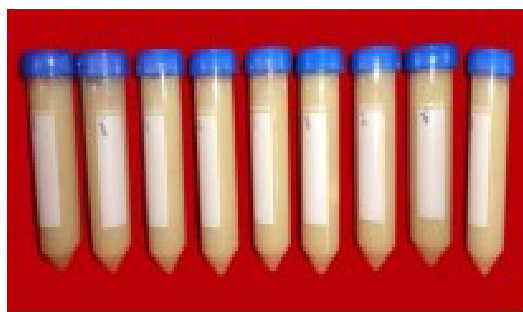


Orange and guava juice preparation using manually-operated juicer



Orange juice treated with pectinase enzymes

T ₁ - No enzyme	T ₇ - Polygalacturonase (PG) 100 U
T ₂ - Pectin lyase (PL) 100 U	T ₈ - Pectin esterase (PE) 100 U
T ₃ - PG+PL+PE 100 U	T ₉ - Commercial PG 100 U
T ₄ - Commercial PL 100 U	T ₁₀ - Commercial PE 100 U
T ₅ - Commercial PG+PL+PE 100 U	



Guava juice treated with pectinase enzymes



Fresh guava juice Clarified guava juice

Plate 11: Orange and guava fruit juices extraction and clarification using pectinase enzymes

Since temperature and incubation time are known to influence the action of pectinases in fruit juice processing, in the present study, the effect of incubation time at 50°C. on pectinase action on orange and guava juice was carried out. It was interesting to note that loss or decrease in cloudiness was observed. The ability to degrade the pectin by the pectinase was also comparable to that of commercial enzyme.

The enzymatic treatment of pulp resulted in considerable increase in juice recovery. It was that after 120 min of incubation period, the orange pulp treated with (PG+PL+PE) at 100 U concentrations resulted in the highest juice yield (90.17%) and the highest clarity (96.28%). Similarly in guava also after 120 min of incubation period, this treatment resulted in the highest juice yield (66.75%), and maximum clarity (37.57%). While it was a mere (4.46%) untreated juice.

The synergistic effects of PG, PL and PL enzymes resulted in depolymerisation of insoluble cell-wall polysaccharides that constitute the suspended solids after juice extraction. Long chains of pectin are broken down, resulting in more clear juice. Such type of action was also observed by Chen *et al.* (2000). They reported 50% more transmittancy and stability in the enzyme-treated juice as compared to the untreated juice. Shah (2007) also observed 93.53 per cent clarity in litchi juice. Kaur *et al.* (2004) reported increase in juice yield while treating apple, banana and grapes with PG produced by *Sporotrichum thermophile*.

Sandri *et al.* (2011) analysed the efficiency of fungal pectinolytic preparations produced in laboratory and commercial products used in the clarification process of apple, Butia palm fruit, blueberry, and grape juices. Time increase resulted in an improved clarification. When compared to the commercial products, the use of the crude preparation (TE1) resulted in similar clarification of apple and blueberry juices.

5.4.1 Sensory evaluation

Sensory properties are among the most important factors influencing product acceptability; the product having good colour, flavour and taste is accepted by the consumer. Colour is the first perception perceived by the consumer and discolouration may significantly affect the acceptability of any drink. Flavour is combination of taste, odor and mouth feel. Here, in the present study, the orange and guava juices treated with different enzymes were subjected to organoleptic evaluation by panel of six examiners. The addition of enzymes, alone as well as in combinations improved the flavour of the juice. Taste is the sensation of taste buds located on tongue. Taste may be sour, bitter, salty and sweet. These tastes alone or in combination may contribute to the consumer's acceptance. Pectinase enzymes, when used singly or in combinations showed positive hedonic effects on the taste of juice. The combination of enzymes (PG + PL+ PE) improved the overall acceptability, with the highest value of 6.80 in both orange and guava juices. The enzymatic treatment of orange and guava fruit pulp was found to be advantageous in various aspects. The enzymatic treatment of pulp resulted in considerable increase in juice recovery. The sensory evaluation of the juice extracted from enzyme treated pulp proved that the pectinase enzymes could be used to improve the sensorial attributes of fruit juices.

SUMMARY AND CONCLUSIONS

In the present study, an attempt was made to isolate, characterize and screen fungi for pectinase production. Studies were also conducted to screen agro residues for pectinase production under SSF conditions using the elite isolates. Various process parameters were optimized for maximum production of pectinase. Purification and characterization of the enzymes were carried out and their application in clarification of orange and guava juices were explored. The results obtained from various experiments are summarized in this chapter.

- As many as 100 fungal isolates were isolated from different fruit and vegetable wastes using pectin agar medium following selective enrichment and isolation technique. The primary screening for pectinase activity on pectin agar medium yielded 48 pectinase positive isolates based on the clear halo zone formation. Out of these, 10 possessed potency index of more than 3.0. These included: RBF96 (3.25), RC27 (3.21), DLP51 (3.21), RGC14 (3.13), RAF81 (3.13), RBFP92 (3.09), RAF80 (3.08), RAF88 (3.08), RCFW68 (3.04) and RC25 (3.01), while the reference strain *Aspergillus niger* MTCC1344 resulted in 2.99. As many as 20 isolates produced pectinase potency between 2.0 and 3.0. They were: RBF95 (2.89), RAF86 (2.83), RCFW64 (2.80), RBF98 (2.79), RCFW65 (2.64), DPW40 (2.53), DPFW62 (2.43), DPW42 (2.39), DLP47 (2.32), RC29 (2.30), DJFR37 (2.28), RC23 (2.24), DPW41 (2.16), RC28 (2.16), RB7 (2.10), DJFR39 (2.10), DJFR36 (2.10), RGC15 (2.09) and RC22 (2.06). The remaining 18 isolates had a potency index values less than two.
- The quantitative estimation of all the three pectinase enzymes viz, PG, PL and PE by these 48 isolates was done under submerged fermentation conditions. Of all the isolates, RBF96 showed the maximum polygalacturonase activity (189.33 U mL^{-1}), followed by DLP51 (167.32 U mL^{-1}) and RC27 (165.50 U mL^{-1}) as compared to the reference strain *A. niger* MTCC1344 (153.32 U mL^{-1}). DLP51 showed the highest pectin lyase activity (372 U mL^{-1}) followed by RAF88 (294.67 U mL^{-1}). RC27 produced the maximum pectin esterase activity (375 U mL^{-1}), followed by RC25 (350 U mL^{-1}).
- Based on the colony morphology, microscopic features and biochemical characterization, these pectinolytic fungi were tentatively identified up to the generic level. Out of 48 isolates, as many as 38 belonged to *Aspergillus* genus; eight were identified as *Penicillium*, and two as *Fusarium*.
- The molecular identification of these selected isolates was carried out by PCR amplification of ITS rDNA. *In Silico* BLAST analysis of the nucleotide sequences revealed that the isolates RBF96 and RC27 showed 100 per cent homology with the GeneBank Accession numbers KC119204.1 and HF546392.1, while the isolates DLP51 and RAF88 showed 99 per cent homology with the GeneBank Accession numbers KC344966.1 and FR750924.1. Based on homology of the NCBI GenBank matching accession number, the isolates RBF96, RC27, DLP51 and RAF88 were confirmed as *Aspergillus niger*, *A. oryzae*, *Penicillium citrinum* and *Fusarium sporotrichioides* respectively.
- The studies on optimization of culture parameters for maximum enzyme production by the most efficient fungal isolates under submerged conditions clearly revealed 1×10^7 spores mL^{-1} inoculum level as the optimum level for *A. niger* RBF96, *P. citrinum* DLP51 and *A. oryzae* RC27 to produce maximum PG, PL and PE.
- The optimum pH for maximum PG production by *A. niger* RBF96 was found to be pH 4.0, while it was pH 7.0 for *P. citrinum* DLP51 for pectin lyase activity and pH 5.0 for *A. oryzae* RC27 for pectin esterase. The optimum temperature for maximum pectinase activity was 35°C in *A. niger* RBF96, *P. citrinum* DLP51 and *A. oryzae* RC27 to produce maximum PG, PL and PE respectively.
- The maximum PG, PL and PE activities were observed at 1 per cent pectin concentration and 6 days of incubation was found to be optimum.
- A number of locally available agro residues were screened for pectinase production by the efficient pectinase producing fungi under SSF. Citrus peel was found to be the best substrate for maximum pectinase activities.
- The process parameters of SSF for maximum pectinase production were optimized. The highest PG, PL and PE activity was observed at 65 per cent moisture level in citrus peel,

wheat bran, sunflower head, jackfruit rind and pineapple peel, Whereas, 70 per cent moisture level was found optimum in coffee husk and banana pseudo stem. The substrate particle size of 2 mm was found superior to 4 mm for production of all the three enzymes.

- The inoculum level of 1×10^7 spores g^{-1} was more effective than 1×10^5 spores g^{-1} , and the optimum incubation time for maximum pectinase production was found to be 6 days. The maximal activity of polygalacturonase (618.28 U mL^{-1}) was at pH 4.0, while that of pectin esterase (429.00 U mL^{-1}) and Pectin lyase (508.33 U mL^{-1}) were at 5.0 and 7.0 respectively. The temperature of 35°C was found to be optimal for maximum activities of all the enzymes.
- Higher titers of pectinases were observed when the medium was supplemented with sucrose at 2% and soy meal (as N source) @ 2%.
- Pectinases were purified to homogeneity using various steps. After purification through gel filtration chromatography using Sephadex G-100, the maximum PG specific activity of $83.06 \text{ U } \mu\text{g protein}^{-1}$ was obtained in case of *A. niger* RBF96 with about 7 folds increase in purification. Maximum specific activity in pectin lyase was $44.13 \text{ U } \mu\text{g protein}^{-1}$ in case of *P. citrinum* DLP51. Similarly, about 6 folds and 5 folds increase in purification was observed in PL enzymes in *Penicillium citrinum* DLP51 and *Aspergillus oryzae* RC27 respectively.
- SDS-PAGE was done for the purified enzymes and their molecular weights determined. The molecular weight of the PG protein produced by *Aspergillus niger* RBF96 was 45 kDa, of PL protein produced by *Penicillium citrinum* DLP51 was 37 kDa and of PE protein produced by *Aspergillus oryzae* RC27 was 31 kDa. The molecular weights of the PG, PL and PE proteins produced by the reference strain were 35 kDa, 47 kDa and 33 kDa respectively.
- The apparent K_m and V_{max} values for polygalacturonase of *A. niger* RBF96 were 0.155 U mL^{-1} and $38.57 \text{ U mL}^{-1}/\text{h}$ respectively. The K_m (0.278 U mL^{-1}) and V_{max} ($28.70 \text{ U mL}^{-1}/\text{h}$) values were obtained for pectin lyase from *Penicillium citrinum* DLP51. In case of pectin esterase from *Aspergillus oryzae* RC27, the K_m and V_{max} values were 0.163 U mL^{-1} and $38.57 \text{ U mL h}^{-1}$.
- pH and thermal stability studies of pectinases also were carried out. PG showed from pH 4.0 to 5.0, whereas PL and PE showed maximum stability at pH ranging from 5.5 to 7.0 and 4.0 to 5.0 respectively. The optimum temperature for the maximum activity of pectinase was 50°C .
- Pectinases produced by the strains developed during the study were used to prepare and clarify juices of two fruits namely orange and guava. The enzymatic treatment of fruit pulps resulted in considerable increase in juice recovery. It was interesting to note that after 120 min of incubation period, the orange pulp treated with (PG+PL+PE) at 100 U concentrations resulted in the highest juice yield (90.17%) and the highest clarity (96.28%). Similarly, in guava also after 120 min of incubation period, this treatment resulted in the highest juice yield (66.75%) and the maximum clarity (37.57%).
- The sensory evaluation of the juices extracted from enzyme treated pulp proved that the pectinase enzymes could be used to improve the sensorial attributes of fruit juices.

In conclusion, *Aspergillus niger*, *Penicillium chrysogenum*, and *Aspergillus oryzae* RC27 were found efficient in the production of PG, PL and PE respectively.

Citrus peel was identified as the most suitable low-cost substrate for pectinase production under SSF. The use of agro-wastes for pectinase production will not only reduce the production costs of the enzyme but also help decrease pollution-load due to the agro-industrial waste. The substrate particle size of 2 mm, moisture level of 65 per cent, incubation temperature of 35°C , the level of inoculum of 1×10^7 spores g^{-1} and the incubation time of 6 days were found optimum for production of maximum pectinase enzymes. The use of mixture of all the three pectinase enzymes proved quite effective in improving the extraction of juices from the pulp of orange and guava as well as their clarification.

Future line of work

- The future efforts in pectinase research need to be focussed on elucidation of the regulatory mechanism of enzyme secretion at the molecular level and the mechanism of action of different pectinolytic enzymes towards pectic substrates. These studies can provide valuable

tools to manipulate microorganisms making them to produce efficient enzymes in higher amounts.

- There are number of industrial processes in which pectinases can be applied to improve the quality and yield of the final products. In this direction it is important to investigate on the production conditions and physico-chemical characteristics of the new enzymes at a larger scale.
- Further investigations on pectinases alone or in combination with other enzyme like cellulases, hemicellulases and amylases in many other industrial applications such as biofuels production can be attempted. Use of immobilized pectinase enzymes for treatment of pectin rich waste can also be explored.

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Appendix I: Pectin agar medium

Pectin	: 5.0 g
K ₂ HPO ₄	: 0.5 g
MgSO ₄ ·7H ₂ O	: 0.1 g
NaCl	: 0.2 g
CaCl ₂ ·2H ₂ O	: 0.2 g
FeCl ₃ ·6H ₂ O	: 0.01 g
Yeast extract	: 1.0 g
Agar	: 20.0 g
pH	: 5.0
Distilled water	: 1000 mL

***Note:** Ampicillin antibiotic @ 100 mgmL⁻¹ was added to the molten and cooled medium to prevent bacterial growth.

Appendix II: Composition of Czapek dox yeast extract agar (CYA)

Czapek concentrate	: 10.0 mL
K ₂ HPO ₄	: 1.0 g
Yeast extract	: 5.0 g
Sucrose	: 3.0 g
Agar	: 15.0 g
Distilled water	: 1.0 L
pH	: 6.0

Czapek Concentrate

NaNO ₃	: 30.0 g
KCl	: 5.0 g
MgSO ₄ ·7H ₂ O	: 5.0 g
FeSO ₄ ·7H ₂ O	: 0.1 g
Distilled water	: 100.0 mL
pH	: 6.0

Appendix III: 50 mM Iodine-potassium iodide solution (1000 mL)

Potassium iodide	: 12.7 g
Iodine	: 5.0 g
Distilled water	: 1000 mL
pH	: 5.0

Appendix IV: Polygalacturonase (PG) assay

Reagents

- 0.2 M Tris acetate buffer (pH 4.5)
- 0.01 M CaCl_2
- Polygalacturonic acid 1 %
- Alkaline copper tartrate reagent

Solution A: dissolved 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g of potassium sodium tartrate and 20 g anhydrous sodium sulfate in 80 mL water and made upto 100 mL.

Solution B: dissolved 15 g copper sulfate in a small volume of distilled water. Added one drop of sulfuric acid and made upto 100 mL.

- mix 4 mL of solution B and 96 mL of solution A before use

Arsenomolybdate solution

Dissolved 2.5 g ammonium molybdate in 45 mL water. Added 2.5 mL sulphuric acid and mixed well. Then added 0.3 g di-sodium hydrogen arsenate dissolved in 25 mL water. Mixed well and incubated at 37°C for 24-48 h.

Standard solution: Galacturonic acid 1 %

Procedure

1. The assay mixture was prepared with the following components
0.2 mL enzyme
0.2 mL 0.2 M tris-acetate buffer pH 4.5
0.1 mL 0.001 M CaCl_2
0.5 mL of 1.0 % solution of polygalacturonic acid (PGA)
2. Prepared a blank for each sample by boiling the reaction mixture before the addition of substrate
3. Incubated at 37°C for 1 h
4. Stopped the reaction by heating at 100°C for 3 min
5. 0.5 mL of the solution mixture was taken and analysed for reducing sugars by Dinitrosalicylic acid (DNSA) method.
6. Made up the volume in both sample and standard tubes to 2.0 mL with distilled water
7. Pipetted out 2.0 mL distilled water in a separate tube to set up a blank
8. Add 1.0 mL of alkaline copper tartrate and kept for 10 min
9. Cooled tubes and added 1.0 mL of Arsenomolybdate reagent to each of the tubes
10. Made up the volume in each tube to 10 mL with distilled water.
11. Read the absorbance of blue colour at 620 nm after 10 min

Run a series of standards using D- Galacturonic acid (0 to 500 $\mu\text{g mL}^{-1}$) and plot a graph.

Calculation: calculated the amount of reducing sugars present in the sample using the standard graph.

$$\text{Activity (U mL}^{-1}\text{)} = Q/(\text{TxVxC})$$

Where Q: Quantity of D-galacturonic acid released in μmoles during time T (4 Min)

V: Quantity of enzyme solution introduced (mL), in this case 0.2 mL

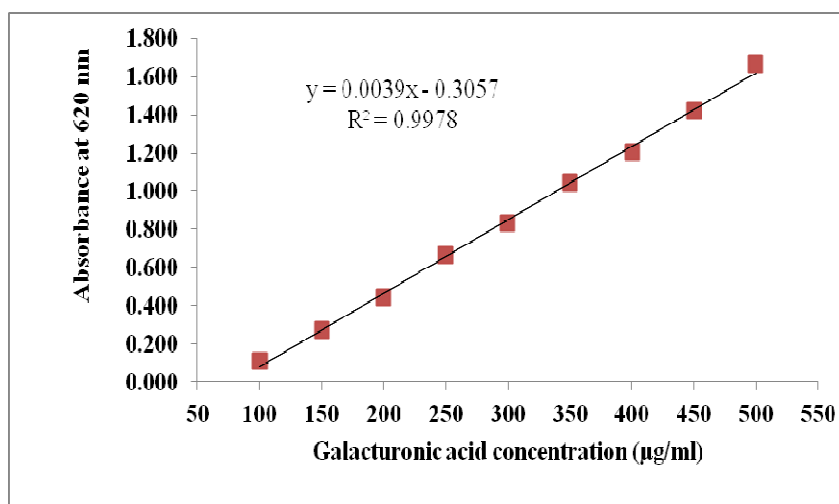
C: Concentration of the enzyme solution (g L^{-1}), in this case 1 g L^{-1}

$$\text{Units mg}^{-1} \text{ protein} = Q/(\text{TxVxC}) / \mu\text{g protein mL}^{-1} \text{ enzyme}$$

Spectrophotometer values at different D-galacturonic acid concentrations

Sl. No.	Galacturonic acid ($\mu\text{g mL}^{-1}$)	Absorbance at 620 nm
1	100	0.112
2	150	0.271
3	200	0.446
4	250	0.664
5	300	0.832
6	350	1.042
7	400	1.204
8	450	1.424
9	500	1.666

Fig 1.0: D-galacturonic acid standard curve



Appendix IX: CTAB extraction buffer (100 mL) for fungal

A) DNA isolation

10 % CTAB	: 20 mL
1 M Tris base	: 10 mL
4 M NaCl	: 35 mL
Mercapto ethanol (autoclaved)	: 1 mL
Poly Vinyl Pyrrolidone (PVP)	: 0.1 %
Make up the volume to 100 mL with sterile water	

B). DNA Agarose gel electrophoresis

Loading dye composition: Dissolve in 6.25 mL of water 0.25 g Bromophenol blue (BPB) 1.25 mL of 10 % SDS and 12.50 mL glycerol

Ethidium bromide

10 mg mL⁻¹ in distilled water, stored at 4 °C in dark bottle.

C) Recipe for 1 per cent Agarose gel (40 mL)

Agarose	: 400 mg
1x TAE	: 40 mL
EtBr (10 mg mL ⁻¹)	: 2 µl

D) 50x TAE (Tris- Acetate EDTA) composition

Tris base	: 242 g
Glacial acetic acid	: 57.1 mL
0.5 M EDTA (pH 8.0)	: 100 mL
Total volume 1000 mL with distilled water	

E) T₁₀ E₁ (pH 8.0)

Tris base	: 0.121 g 100 mL ⁻¹
EDTA	: 0.0372 g 100 mL ⁻¹

Appendix V: Pectin Lyase Assay

Pectin lyase was assayed by measuring the increase in absorbance at 550 nm according to method described by Albersheim and Killias (1962).

Reagents

- Tris HCl buffer, 0.05 M pH 8.5
- Pectin, 1.0 % (w/v) in tris buffer
- CaCl₂, 0.01 M in distilled water
- ZnSO₄, 7H₂O, 9.0 % (w/v) in distilled water
- NaOH, 0.5 M in distilled water
- Thiobarbituric acid, 0.04 M in distilled water
- Hydrochloric acid, 0.01 M

Procedure

The reaction mixture contained 5.0 mL of pectin solution, 1.0 mL of calcium chloride solution and an aliquot of enzyme solution (1.0 mL) in a final reaction volume of 10 mL made up with distilled water. After incubation for 2 hours at 30 °C, 0.6 mL of ZnSO₄ solution was added followed by 0.6 mL of NaOH. Precipitated protein and unused substrate were removed by centrifugation at 8000 rpm for 10 minutes and 5.0 mL of the supernatant was added to a mixture of thiobarbituric acid (3.0), hydrochloric acid (1.5) and distilled water (0.5 mL). The mixture was heated in a boiling water bath for

30 minutes before cooling and absorbance was read at 550 nm against a reference cuvette which contained the same reagent as the experimental cuvette but which was incubated for zero time before the addition of ZnSO₄ and NaOH.

Calculations:

$$\text{Units mL}^{-1} \text{ enzyme} = \frac{(A_{550\text{nm}} \text{ min.}^{-1} \text{ Test} - A_{550\text{nm}} \text{ min.}^{-1} \text{ Blank}) (10)(\text{df})}{(1.0) (1.0)}$$

10= Total volume (in milliliters) of assay

df = Dilution factor is 100.

1.0 = Change in A550 nm per minute at 30 °C as per the Unit Definition

1.0 = Volume (in millilitres) of enzyme used

$$\text{Units mg}^{-1} \text{ protein} = \frac{\text{Units mL}^{-1} \text{ enzyme}}{\mu\text{g protein mL}^{-1} \text{ enzyme}}$$

Appendix VI: Pectin esterase (PE) activity assay

The PE activity was estimated by the method of Kertesz (1955), which is based on the principle of measuring released carboxylic groups by titration against NaOH. Enzyme unit was expressed as amount of enzyme required to produce carboxylic groups to be titrated by one micro-equivalent of NaOH and the activity was expressed as μ moles of carboxylic acid produced mL⁻¹/μg protein as given below:

Materials

Solid salt mixture of NaCl and EDTA disodium salt 10:1 (w/w):dissolved 7.5g of the above mixture in 100mL water. **Pectin:** Prepared 2 % (w/w) pectin solution in 1.5M NaCl. From this stock solution, diluted accordingly to get 1% solution in 0.1M NaCl.

Enzyme extract

To the crude extract added 2.25g NaCl-EDTA salt mixture and stirred for 15 min and centrifuged at 10000 rpm for 15 min, collected the supernatant. Again stirred the residue with 20mL of salt mixture solution for 15min. Centrifuged and collected the supernatant. Repeated the extraction step and to the supernatant collected each time, added 10mL cold ethanol and collected the precipitate. Pooled the precipitate; dissolved in 1.5M NaCl and use as enzyme source.

Procedure

- 100mL of pectin (1%) solution was taken in a 500mL beaker and 30mL of 1M NaCl and 60mL distilled water were added. It was placed in a water bath at 30 °C and stirred continuously.
- Check the pH and adjust it to 7.5 by adding 0.1N NaOH with continuous mixing.
- Now 10mL of the enzyme solution was added and set the timer on.
- A constant pH of 7.5 of the reaction mixture was maintained by continuous addition of 0.1N NaOH for a definite period of time.
- The alkali consumption as a function of time was measured.
- A blank using the inactivated enzyme was run. The enzyme was inactivated by placing it in boiling water for 10 min.

$$\text{PE activity (U mL}^{-1}\text{)} = \frac{\text{Volume of NaOH used (mL) X N of NaOH X 1000}}{\text{Time (min) X Amount of enzyme used (mL)}}$$

$$\text{Volume of NaOH (mL) used X N of NaOH X 1000}$$

$$(U\text{ mL}^{-1}/\mu\text{g protein}) = \frac{\text{Time (min)} \times \text{Amount of enzyme used (mL)} \times \mu\text{g protein mL}^{-1} \text{ enzyme}}{\text{Time (min)} \times \text{Amount of enzyme used (mL)} \times \mu\text{g protein mL}^{-1} \text{ enzyme}}$$

Appendix VII: Total protein estimation by Lowry's method

Solution A

-0.143 N NaOH: 2.859 g in 500 mL distilled water

-0.135 N Na₂CO₃: 14.308 g in 500 mL distilled water

Solution B

-0.057M CuSO₄.5 (H₂O): 1.423 g 100 mL distilled water

Solution C

-0.124 M Na₂ tartrate 2(H₂O): 2.852 g in 100 mL distilled water

Lowry solution: 100+1+1 mL (Solution A+ Solution B+ Solution C)

Solution D: Folin- Ciocalteu Reagent (FCR) solution 1 N (2 mL FCR + 2 mL distilled water).

Spectrophotometer values at different Bovine serum albumin (BSA) concentration

Sl. No.	Bovine serum albumin($\mu\text{g mL}^{-1}$)	Absorbance at 600 nm
1	100	0.09
2	200	0.20
3	400	0.39
4	600	0.61
5	900	0.80
6	1200	1.15
7	1500	1.25

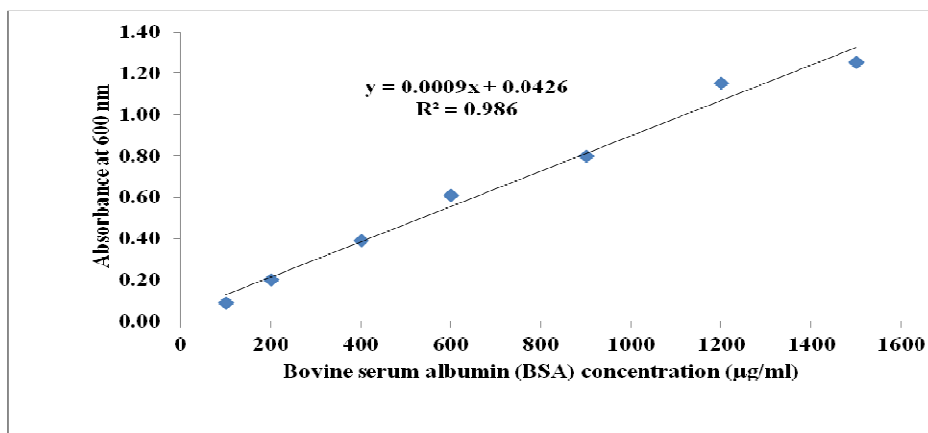


Fig 2.0: Standard calibration curve for total protein estimation by Lowry's method

Appendix VIII: Lactophenol Aniline Blue stain

Lactic Acid	: 250.0 mL
Glycerol	: 400.0 mL
Phenol	: 250.0 g
Aniline Blue	: 0.6 g
KOH (10 %)	: 100.0 mL
Distilled water	: 250.0 mL
pH	: 5.0

Appendix X: Estimation of pectin as calcium pectate

Dried pectin (200 mg) was weighed into a one litre beaker and was wetted with 2 or 3 mL of 100 per cent alcohol. Water (400 mL) was added with stirring to it. The solution was boiled and then cooled immediately. It was transferred into a 500 mL volumetric flask and the volume was made up. A known volume of aliquot (200 mL) was pipetted out each into two 1L beakers. Water (250 mL) was added and solution was neutralized with 1N Sodium hydroxide using phenolphthalein as indicator. Afterwards, 10 mL of 1N Sodium hydroxide was pipetted out in excess with constant stirring and was allowed to stand overnight. Next day, 50 mL of 1N Acetic acid was added and after 5 minutes 25 mL of 1N calcium chloride was added with stirring. The solution was allowed to stand for 1 hour, and then boiled for 2 min. The solution was filtered using previously prepared filter paper (filter paper was made wet in hot water, dried in oven at 102°C for 2 hours, cooled and weighed in a covered dish) and the precipitate obtained was washed thoroughly with boiling water. In an originally weighed dish, the filter paper containing calcium pectate was transferred and dried overnight at 100°C. The dish was cooled and final weight was taken.

Calcium pectate was calculated using the following formula,

$$\text{weight of calcium pectate} \times 500 \times 100$$

Calcium pectate (%) = -----

$$\text{mL of filtrate taken} \times \text{weight of sample taken}$$

Appendix XI: Amounts of solid ammonium sulfate (g) required to change the % saturation of 100 mL of solution

Final concentration (% saturation) of ammonium sulfate solution

[illegible]

Appendix XII: Gel filtration chromatography

Running buffer (pH 6.0)

	200 mL	Final Concentration
1M Tris base	: 2.0 mL	10 mM
0.5M EDTA	: 0.4 mL	1 mM
5.0 M NaCl	: 0.2 mL	5 mM
10 % SDS	: 2.0 mL	0.1%
Distilled water (sterilized):	196 mL	

Appendix XIII: Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS PAGE)

A) Acrylamide solution (30 per cent)

Acrylamide : 29.0 g

Bis-acrylamide : 1.0 g

Made up the volume to 100 mL using the distilled water and stored at 4°C (Do not autoclave).

B) 4X resolving gel buffer (pH 8)

Tris Cl (1.5 M) : 182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 8.8 using 1M HCl and final volume was made up to 1000mL.

C) 4X stacking gel buffer (pH 6.8)

Tris Cl (1.5 M) : 182.0 g of Tris base was dissolved in little amount of water and pH was adjusted to 6.8 using 1M HCl and final volume was made up to 1000mL.

D) Ammonium per sulfate (APS) 10%

(Add the preparation) It has to be prepared fresh, don't autoclave.

E) Tank Buffer

Tris Base : 25 mM

Glycine pH 8.3 : 250 mM

SDS : 0.1 per cent

F) SDS PAGE Gel

	Resolving gel 10 % (10mL)	Stacking Gel 5 % (4mL)
Water	: 4.0	2.70
30 % Acrylamide mix	: 3.3	0.67
1.5 M Tris (pH 8.8)	: 2.5	0.50
10% SDS	: 0.1	0.04
10 % APS	: 0.1	0.04
TEMED	: 0.004	0.004

G) Sample loading dye for SDS PAGE (for 2 mL)

Tris-HCl (100mM, pH 6.8) : 200 µl

Dithiothreitol (DTT) (200mM) : 100 µl

Glycerol (20 %) : 400 µl

Bromo phenol blue : 4 mg

SDS (4 %) : 800 µl

Deionized water made up to 2 mL

H) Staining solution (for one liter)

Coomassie brilliant blue	: 2.5 mg
Methanol	: 500 mL
Acetic acid	: 100 mL
Distilled water	: 400 mL

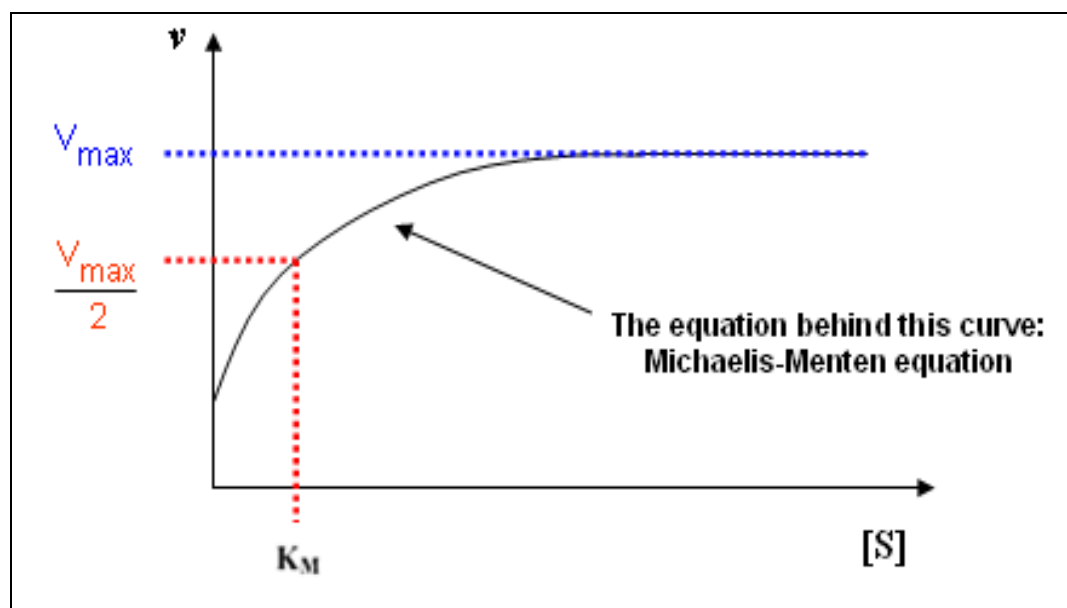
I) Destaining solution (for one litre)

Methanol	: 300 mL
Acetic acid	: 100 mL
Distilled water	: 600 mL

Appendix XIV: $V_{\max}$ and K_m of the Michaelis-Menten equation:

$V = V_{\max} \frac{[S]}{K_m + [S]}$, where V is the velocity of enzymatic reaction and [S] is the concentration of the substrate.

If we plot this function (the enzymatic velocity) as a function of the substrate concentration we will get the following figure:



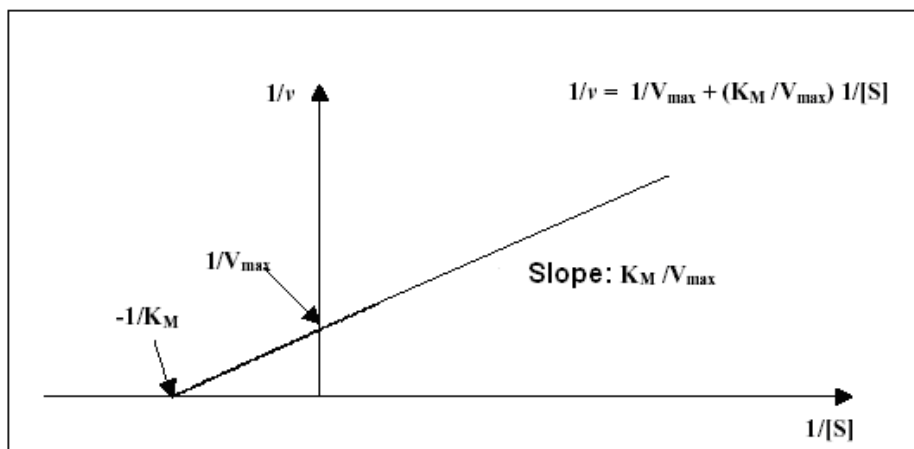
The reaction velocity, v , as a function of the substrate concentration, [S], for an enzyme. This relationship is called the Michaelis-Menten equation.

The Lineweaver- Burk equation is used to make the Michaelis-Menten equation linear by taking its reciprocal:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{\max} [S]} = \frac{K_m}{V_{\max} [S]} + \frac{[S]}{V_{\max} [S]} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

By using the Lineweaver-Burk equation we will be able to make a Lineweaver-Burk plot which is linear and where the intercept on the y-axis gives us: $1/V_{\max}$ (Set $[S]=0$ in the above equation (intercept on the y-axis) and you will see that $1/V=1/V_{\max}$), and the intercept on the x-axis gives us $-1/K_m$ (set $1/V=0$ in the above equation (intercept on the x-axis) and solve for $1/[S]$ and you will see that $1/[S]=-1/K_m$).



Lineweaver-Burk plot for an enzyme that shows upon Michaelis-Menten kinetics.

Worked example

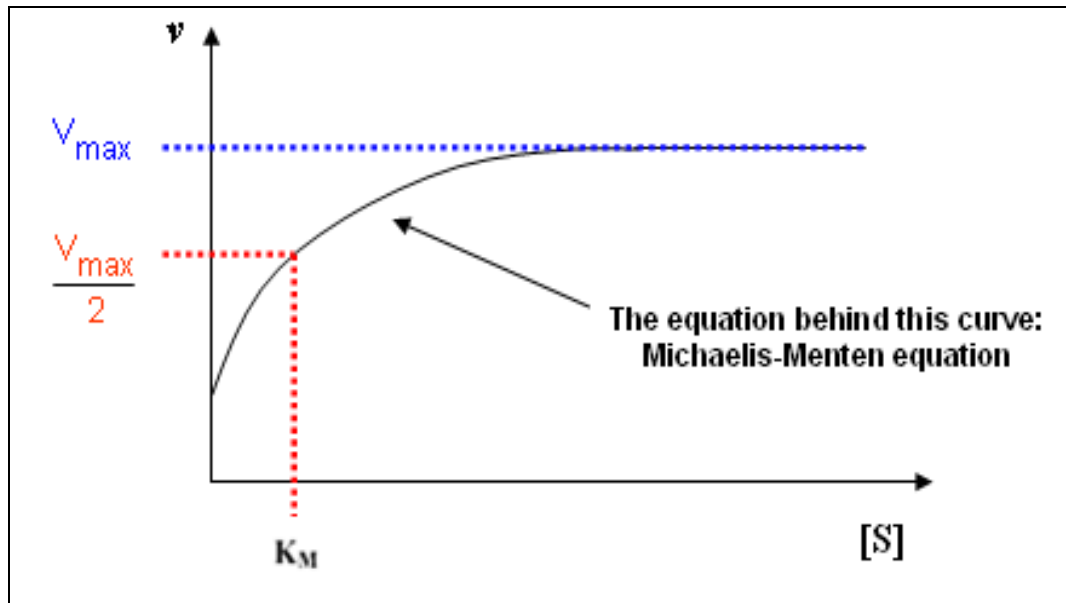
The equation for the linear regression is $y = kx + m$ where k is the slope of the line (you can see that my slope is 600.43) and m represents the intercept of the y-axis (my m -value is 93.61). The R^2 value is the square of the correlation coefficient which gives us a measure of the reliability of the linear relationship between the x and y values (values close to 1 indicate excellent linear reliability).

1. To determine the $V_{\max}$ value. The $V_{\max}$ value is the reciprocal of the intercept on the y-axis (which is equal to my m -value). The m -value is 93.61 ($1/V = 93.61$ and we calculate the $V_{\max}$: $1/93.61 = 0.01$). This means that my $V_{\max}$ value is 0.01 U sec^{-1} ($36 \text{ U mL}^{-1}/\text{h}$).
2. We will now determine the K_m value. We can see that the line intercept the x-axis at about -0.15. However, we can calculate this value more precisely by using the formula $y = kx + m$ and set $y=0$ ($0 = kx + m$) and solve this equation for $x=m/k$. K_m value is 0.155 U mL^{-1} .

Appendix XIV: $V_{\max}$ and K_m of the Michaelis-Menten equation:

$V = V_{\max} \frac{[S]}{K_m + [S]}$, where V is the velocity of enzymatic reaction and $[S]$ is the concentration of the substrate.

If we plot this function (the enzymatic velocity) as a function of the substrate concentration we will get the following figure:



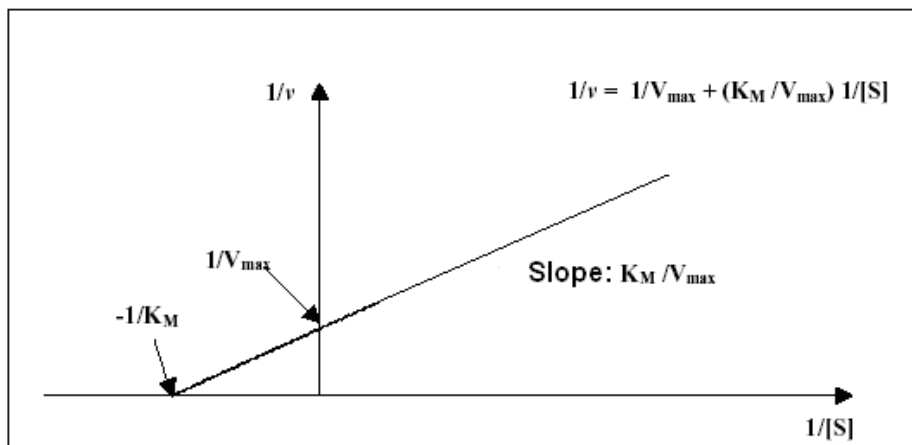
The reaction velocity, v , as a function of the substrate concentration, $[S]$, for an enzyme. This relationship is called the Michaelis-Menten equation.

The Lineweaver- Burk equation is used to make the Michaelis-Menten equation linear by taking its reciprocal:

$$\frac{1}{V} = \frac{K_m + [S]}{V_{\max} [S]} = \frac{K_m}{V_{\max} [S]} + \frac{[S]}{V_{\max} [S]} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \cdot \frac{1}{[S]} + \frac{1}{V_{\max}}$$

By using the Lineweaver-Burk equation we will be able to make a Lineweaver-Burk plot which is linear and where the intercept on the y-axis gives us: $1/V_{\max}$ (Set $[S]=0$ in the above equation (intercept on the y-axis) and you will see that $1/V=1/V_{\max}$), and the intercept on the x-axis gives us $-1/K_m$ (set $1/V=0$ in the above equation (intercept on the x-axis) and solve for $1/[S]$ and you will see that $1/[S]=-1/K_m$).



Lineweaver-Burk plot for an enzyme that shows upon Michaelis-Menten kinetics.

Worked example

The equation for the linear regression is $y = kx + m$ where k is the slope of the line (you can see that my slope is 600.43) and m represents the intercept of the y -axis (my m -value is 93.61). The R^2 value is the square of the correlation coefficient which gives us a measure of the reliability of the linear relationship between the x and y values (values close to 1 indicate excellent linear reliability).

3. To determine the $V_{\max}$ value. The $V_{\max}$ value is the reciprocal of the intercept on the y -axis (which is equal to my m -value). The m -value is 93.61 ($1/V = 93.61$ and we calculate the $V_{\max}$: $1/93.61 = 0.01$). This means that my $V_{\max}$ value is 0.01 U sec^{-1} ($36 \text{ U mL}^{-1}/\text{h}$).
4. We will now determine the K_m value. We can see that the line intercept the x -axis at about -0.15. However, we can calculate this value more precisely by using the formula $y = kx + m$ and set $y=0$ ($0 = kx + m$) and solve this equation for $x=m/k$. K_m value is 0.155 U mL^{-1} .

Appendix XV: Score card for sensory evaluation of orange and guava juice

Directions

Please examine these samples one by one and evaluate them for the following parameters on hedonic scale as given below. It is very important to rinse mouth thoroughly with clean water after evaluation of each sample.

Name of the judge :

Age :

Sex :

Signature :

Date:

Attribute	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇	T ₈	T ₉
Colour									
Flavour									
Sweetness									
Overall acceptability									

Hedonic scale

- 9 - Like extremely
- 8 - Like very much
- 7 - Like moderately
- 6 - Like slightly
- 5 - Neither like nor dislike
- 4 - Dislike slightly
- 3 - Dislike moderately
- 2 - Dislike very much
- 1 - Dislike extremely

Note

Colour: It is the first perception perceived by the consumer.

Taste: It is the sensation of taste buds located on tongue. Taste may be sour, bitter, salty and sweet.

Flavour: It is a combination of taste, odor and mouth feel.

BIOPROSPECTING OF PECTINASE ENZYMES FROM FUNGI

DUSHYANTHA D. K.

2013

Dr. K. S. JAGADEESH

Major Advisor

ABSTRACT

The present study was conducted to isolate, and characterize fungi from diverse natural sources capable of producing pectinases. Based on the preliminary screening of 100 isolates, 48 isolates were found positive for pectinase production. Out of them, isolates RBF96 (3.25), RC27 (3.21) and DLP51 (3.21) possessed the highest potency index values compared to the reference strain *Aspergillus niger* MTCC1344 (2.99). Following the PCR based molecular methods, the isolates were identified as *Aspergillus niger*, *Aspergillus oryzae* and *Penicillium citrinum* respectively. Solid state fermentation (SSF) was carried out using various locally available agro-wastes as the carbon source. Citrus peel was identified as the most suitable substrate. The particle size of 2 mm, moisture level of 65 per cent, incubation temperature of 35°C, inoculum level of 1×10^7 spores g⁻¹ and the incubation time of six days were found optimum. After purification the maximum specific activity of 83.06 U µg⁻¹ protein was obtained for polygalacturonase (PG) by *A. niger* RBF96 with seven fold increase in purification. Similarly, about six folds and five folds increases in purification were obtained for pectin lyase (PL) and pectin esterase (PE) in *P. citrinum* DLP51 and *A. oryzae* RC27 respectively. The molecular weights of PG, PL and PE, as determined by SDS-PAGE, were 45 kDa, 37 kDa and 31 kDa respectively. The purified PG showed maximum stability at pH 4.5, whereas, PL and PE showed at pH 6.5 and 5.0 respectively. The temperature of 50°C was found optimum. The orange pulp treated with (PG+PL+PE) at 100 U resulted in the highest juice yield (90.17%) and the highest clarity (96.28%). Similarly, in guava this treatment resulted in the highest juice yield (66.75%) and maximum clarity (37.57%). The organoleptic evaluation proved that pectinases could be used to improve the sensorial attributes of fruit juices.