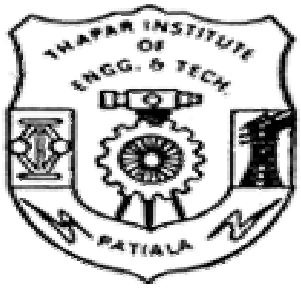
ISOLATION OF EXTRACELLULAR FUNGAL PECTINOLYTIC ENZYMES AND EXTARCTION OF PECTIN USING KINNOW WASTE AS SUBSTRATE

A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF

MASTER OF SCIENCE (BIOTECHNOLOGY)



Under the guidance of:

Dr. Pratima Khandelwal
Lecturer

Submitted by:

ROZA BALI (Ms.)
Roll No. 3010116

DEPARTMENT OF BIOTECHNOLOGY & ENV. SCIENCES

THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY

(DEEMED UNIVERSITY)

PATIALA –147 004, PUNJAB, INDIA

MAY, 2003

CANDIDATE'S DECLARATION

I here by declare that the work which is being presented in dissertation entitled, **'Isolation of Extracellular Fungal Pectinolytic Enzymes and Extraction of Pectin using Kinnow Waste as Substrate'** in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE IN BIOTECHNOLOGY**, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala is an authentic record of my own work during the period of 5 months from January 2003 to May 2003, under the supervision of Dr. Pratima Khandelwal, Lecturer, Department of Biotechnology and Environmental Sciences, Thapar Institute Engineering and Technology, Patiala. The matter embodied in this dissertation has not submitted by me for the award of any other degree or diploma.

Place: Patiala
Date: 15 May, 2003

Roza Bali
Author

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

Dr. Pratima Khandelwal
Supervisor
DBTES
Thapar Inst. of Engg. & Tech.
Patiala

Dr. Sunil Khanna
Prof & Head
DBTES
Thapar Inst. of Engg & Tech
Patiala

ACKNOWLEDGEMENT

I thank the almighty whose blessings have enabled me to accomplish my dissertation work successfully.

It is my pride and privilege to express my sincere thanks and deep sense of gratitude to ***Dr. Pratima Khandelwal****, Lecturer, Department of Biotechnology and Environmental Sciences, TIET, Patiala for her valuable advice, splendid supervision and constant patience through which this work was able to take the shape in which it has been presented. It was her valuable discussions and endless endeavors through which I have gained a lot. Her constant encouragement and confidence-imbibing attitude has always been a moral support for me.*

My sincere thanks to ***Dr. Sunil Khanna****, Head Department of Biotechnology and Environmental Sciences, for his immense concern throughout the project work.*

I owe a special word of thanks to all the faculty members of CORE and Department for their constant encouragement and support throughout this duration. I am very thankful to ***Dr. Reddy****,* ***Dr. Das, Dr. Ganguly*** *and* ***Mr. A.R. Chowdhary*** *for their help and guidance during my project work.*

I am also grateful to Mr. Ravi Dutt Yadav, Senior Scientific Assistant, DAS, TCRID for his help during my project work.

I feel lacunae of words to express my most heartfelt and cordial thanks to my friends, who have always been a source of inspiration for me and who stood by my side at the toughest times.

Finally, I wish to extend a warm thanks to everybody involved directly or indirectly with my work.

The whole credit of my achievements goes to my parents and my brother who were always there for me in my difficulties. It was their unshakable faith in me that has always helped me to proceed further.

DATE *ROZA BALI*

ABSTRACT

Kinnow consumption and processing results in generation of waste in form of peels and pomace. Value addition of such waste can result in extraction of important byproducts namely pectin, pectinases, peel oil and dietary fibers. Of these pectin and pectinases are of wide importance in food industry. Various types of fungal species have been reported to be employed for production of pectinases.As extracellular pectinases are easier to harvest and thus the scale up is cheaper and simpler. Thus in the present study, kinnow waste was used to generate extracellular fungal pectinases from natural sources along with extraction of pectin.

The selected potential fungal isolates from nature were screened based on qualitative estimation of their pectinolytic activity on selective media (pectin as only carbon source). Morphological examination of screened isolates revealed that Isolate#1 and Isolate#3 could be of *Rhizopus* genus and Isolate#2 could of *Aspergillus* genus. *Aspergillus oryzae* var *oryzae* was used as standard fungi in the present.

Quantitative estimation of total protein was made to know total enzymatic activities generated from isolates. It was done via SmF as well as SSF and pure pectin resulted in maximum activity among substrates, followed by peel powder and finally pomace. Optimal day of incubation for isolate #2 and isolate #3 was 9^{th} day and it was 6^{th} day for isolate #1 and standard, in both SmF and SSF.

In SmF, among all isolates, Isolate #3 was found to produce maximum total protein and all other enzymatic activities (Total protein-8.69mg/ml/10min, Pectinase-763.82 nmoles/ml/60min, Polygalacturonase activity- 99.83 nmoles/ml/10min andPL-1038.68nmoles/ml/15min), followed by isolate#1 and lastly isolate #2. In SSF, only peel powder was used as substrate and isolate #3 was again found best amongst all isolates in terms of total protein and all enzymatic activities also (Total protein-7.95mg/ml/10min, Pectinase-436.27 nmoles/ml/60min, Polygalacturonase activity- 52.39nmoles/ml/10min and Pectin lyase- 758.83nmoles/ml/15min). Standard fungus resulted in even higher total protein and enzymatic activities than isolate #3 in both SmF and SSF.

In general, SSF resulted in about 30% decrease in activities as compared to SmF.

Extraction of pectin resulted in 20% yield from kinnow peel powder, 80% ethanol was found to be suitable for extraction of pectin and 7.3% calcium pectate was obtained. Further attempts for characterization of isolates needs to be done before employing them in food industry application.

Date: May, 2003 (Roza Bali)

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INTRODUCTION

1. INTRODUCTION

Kinnow is one of the most important citrus crops of northern India especially of Punjab, which produces about 0.180 MMT of kinnow, accounting over 50% of the national produce **(Hindustan Times; May 7, 2003)**. Kinnow is highly relished as a fresh fruit owing to its rich organoleptic and thirst quenching properties and has a high appeal. Steps are already underway to harness the immense potential of this crop and tap the market, by attacking the major problem of bitterness in extracted kinnow juice. The attempts have been mainly based on curative approaches and of late, preventive approach is also being worked out.

Processing and utilization of kinnow into various products eventually leads to generation of waste in form of peels and pomace. Kinnow waste is conventionally biotransformed anaerobically into humus, although many valuable byproducts can be produced from the rich waste. In other words wealth can be derived from this waste by value addition and products such as pectin, peel oil, pomace powder, dietary fibers and predominantly pectinases can be easily harnessed. Of these products, pectin and pectinases have a wide global market.

Pectinases accounts for 10% of global industrial enzymes produced and their market is increasing day by day **(Stutzenberger, 1992)**. Pectinases are the group of enzymes, which cause degradation of pectin that are chain molecules with a rhamnogalacturonan backbone, associated with other polymers and carbohydrates. These pectinases have wide applications in fruit juice industry and wine industry. In fruit juice industry, it is used for clarification, where reduction in viscosity is caused which ultimately leads to formation of clear juice. They increase the yield of juices by enzymatic liquefaction of pulps; these pectinases also helps in formation of pulpy products by macerating the organized tissue into suspension of intact cells. In wine industry pectinases are mainly used for decreasing astringency by solubilizing anthocyanins without leaching out procyadin polyphenols, and pectinases also increase pigmentation by extracting more anthocyanins **(Tucker and Woods, 1991)**

Pectinases can be produced by both submerged and solid state fermentation (SSF). Submerged fermentation is cultivation of microorganisms on liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. SSF incorporates microbial growth and product formation on or within particles of a solid substrate **(Mudgett, 1986)** under aerobic conditions, in the absence or near absence of free water, and does not generally require aseptic conditions for enzyme production. Many filamentous fungi like *Aspergillus niger, Aspergillus. awamori, Penicillium restrictum, Trichoderma viride, Mucor piriformis and Yarrowia lipolytica*.etc are used in both submerged as well as solid state fermentation for production of various industrially important products such as citric acid, ethanol etc. Fungi like *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium expansum*, which are generally regarded as safe (GRAS) by United States Food and Drugs Administration (USFDA) are employed in food industry (**Pariza and Foster**, **1983).** Some bacteria *(Bacillus licheniformis, Aeromonas cavi, Lactobacillus* etc), yeasts like *Saccharomyces, Candida* and *Actinomycetes* like *Streptomycetes* are also used. Amongst these, the filamentous fungi are most commonly employed **(Pandey *et al*, 1999).**

Fungi can produce both intracellular as well as extracellular enzymes. All fungi are hetrotrophic, and rely on carbon compounds synthesized by other living organisms. Small molecules like mono disaccharides fatty acids and amino acids can easily pass through but for breaking down of larger complex compounds like pectin, fungi secrete extra cellular enzymes. It is well known that as compared to intracellular enzymes, the extra cellular enzymes are easier to be extracted. Intracellular enzymes require more time and costly chemicals for extraction **(Hankin and Anagnostakis, 1975).** Till date, substrates used for solid-state fermentation are materials of plant origin like grains rice, corn, root, tubers, and legumes. Apart from these, pomace, mango peels, orange waste like peels and other fruit and vegetable industry waste are also being in much use **(Smith and Aidoo, 1988)**

Kinnow waste also holds a promising substrate because of rich pectin content in peels. **Sudhakar and Miani (1992)** have reported 18.3% pectin in mandarin peels, which is quite close to the pectin content of apple pomace and is utilized for commercial extraction of pectin **(Girdharilal *et al*, 1998)**. As pectin is the ideal substrate for production of Pectinases, it was thought that attempts should be made to extract pectinase from kinnow waste after isolation of potential pectinolytic fungi from natural sources. Till now, no report is available on pectinase production using kinnow waste as substrate.

Also, pectin itself can be extracted from kinnow waste (mainly from peels) as a commercially important by-product. Apple, lemon, orange, mango, tomato, beet, carrots etc are the commonly used sources for extraction of pectin. Of these, mango peels, apple pomace, lemon pulp and orange pulp are most commonly used. Pectin finds wide application in the manufacture of many fruit products like jams, jellies, marmalades, preservatives etc and thus are indispensable to the fruit juice industry. It is also used as a thickening agent for sauces, ketchups, flavored syrups and as a texturing agent in fruit flavored milk desserts **(Girdharilal *et al*, 1998).**

In view of the above-mentioned points, the present investigation was undertaken and attempts were made to produce pectinases from the isolated fungi from natural sources and to extract pectin from kinnow waste as substrate. The following main objective was drawn to carry out the investigation:

- Harnessing extracellular fungal pectinolytic enzymes and pectin from kinnow waste.

REVIEW OF LITERATURE

2 REVIEW OF LITERATURE

India, with its current fruit production of around 32 MMT, accounts for about 8% of the world's fruit production **(www.fruitvegetable.com, 2003).** Citrus is the second important fruit crop after mango and banana in India. Kinnow is a member of citrus family and is one of the most important citrus crops of northern India especially of Punjab, which produces about 0.180 MMT of kinnows, accounting over 50% **(Hindustan Times; May 7, 2003)** of the national produce. Kinnow is mainly utilized as fresh fruit, 95% of kinnows are utilized as fresh fruits. Processing of kinnow faces major hindrance due to development of bitterness, as delayed bitterness is commonly known phenomenon in citrus fruits **(Sandhu *et al.*, 1990; Premi *et al.,* 1994)**. The attempts to handle the problem have been mainly based on curative approaches (debittering of juice) and of late preventive approach is also being worked out. By either means of utilizing kinnow as fresh or processed form, huge waste is and would be generated. Conventional means of utilization of waste has been via biotransformation of kinnow waste (mainly kinnow peels and kinnow pomace) into humus. Another useful way of exploitation of such waste could be by value addition whereby many useful products like pectinases, pectin, peel oil and dietary fibers can be obtained. Of these, pectin and pectinases have appreciable global importance.

Pectinases are frequently used in fruit and vegetable industry, and pectin is also employed widely in food industry. Peel oil finds many useful applications in both food and pharmaceutical industry. It is good for the skin Citrus *Solvent* is a biodegradable solvent occurring in nature as the main component of citrus peel oil. Citrus Solvents have pleasant aroma, & FDA-GRAS rating ("generally recognized as safe") makes it suitable to be used as solvent, *citrus solvent* can replace a wide variety of products, including mineral spirits, methyl ethyl ketone, acetone, toluene, glycol ethers, and of course fluorinated and chlorinated organic solvents. Dietary fibers are the most recent

value added product and are used as means of roughage. **(**www.realmilkpaint.com/citrus.html).

2.1 Pectinases

Pectinases are group of enzymes that attack pectin and depolymerise it by hydrolysis and transelimination as well as by de-esterification reactions, which hydrolyses the ester bond between carboxyl and methyl groups of pectin **(Ceci and Loranzo, 1998**). These enzymes act on pectin, a class of complex polysaccharides found in the cell wall of higher plants and cementing material for the cellulose network (**Thakur *et al*, 1997).** Pectinases account for 10% of the global industrial enzymes produced **(Stutzenberger, 1992)**

2.2 Classification

Pectinases are classified according to their mode of secretion as extracellular and intracellular pectinases.

An extracellular enzyme is excreted (secreted) outside the cell into the medium in which that cell is living. Extracellular enzymes usually convert large substrate molecules (i.e. food for the cell or organism) into smaller molecules that can then be more easily transported into the cell, whereas an intracellular enzyme operates within the confines of the cell membrane. Membrane proteins remain attached in some way to the cell membrane **(Hankin and Anagnostakis, 1975)**.

Both intracellular extracellular pectinases are classified on the mode of their attack on the galacturonan part of pectin molecules as shown in Figure 2.1 (**Pilnik and Voragen, 1993)**

2.2.1 Pectin methylesterases: (PE) (EC 3.1.11.1) It is a specific enzyme that acts gradually removing units of methanol and pectic acid from the terminal pectin chains (**Pilnik and Voragen, 1993)**

2.2.2 Pectin depolymerase: These are endopectinases attacking the links α 1-4 of pectin chains i.e. they attack in the middle of the chain and not from the terminals (**Pastore, 2001)**

2.2.2.1 Endopolygalaturonase: (Polygalacturonase: EC 3.2.2.15). They split s glycosidic linkages next to free carboxyl groups by hydrolysis.

2.2.2.2Pectate lyase or acid Endo-pectin transeliminase: Pectic acid lyase (EC4.2.2.2) splits glycosidic linkage next to free carboxyl groups by β-elimination. Endopectin transeliminase (EC 4.2.2.3) split glycosidic bonds of highly methylated pectin.

2.2.2.3 Pectin lyase: These enzymes act on highly methylated pectins.

2.2.3 Exoenzymes: These are enzymes, which release galacturonic acid from the terminal pectin chain. Only exo Polygalacturonases and exo PALs are known. *Rhizopus tritici, Gleosporium kake, Ciniothrium diplodella* and *Aspergillus niger* have been reported to produce exo-Polygalacturonases as well as endo Polygalacturonase **(Kawano *et al*, 1999).**

2.3 Properties of pectic enzymes

Pectin esterase, isolated from *Aspergillus niger* has molecular wt 39,000, isoelectric point 3.9 and optimum pH 4.5 **(Baron *et al,* 1980**) Polygalaturonase: extracted from tomato is having Molecular wt 100,000, Isoelectic point 8.6 and optimum pH 4.5. Pectin lyase is extracted from *Aspergillus niger and* is having Molecular wt of 35,400, isoelectric point 3.65 and optimum pH 6.00 (**Honderhoven,V, 1975).** Pectate lyase is extracted from *Bacillus subtilis* is having Molecular wt 33,000, isoelectric point 9.85 and optimum pH 8.5 (**Chesson *et al,* 1978).**

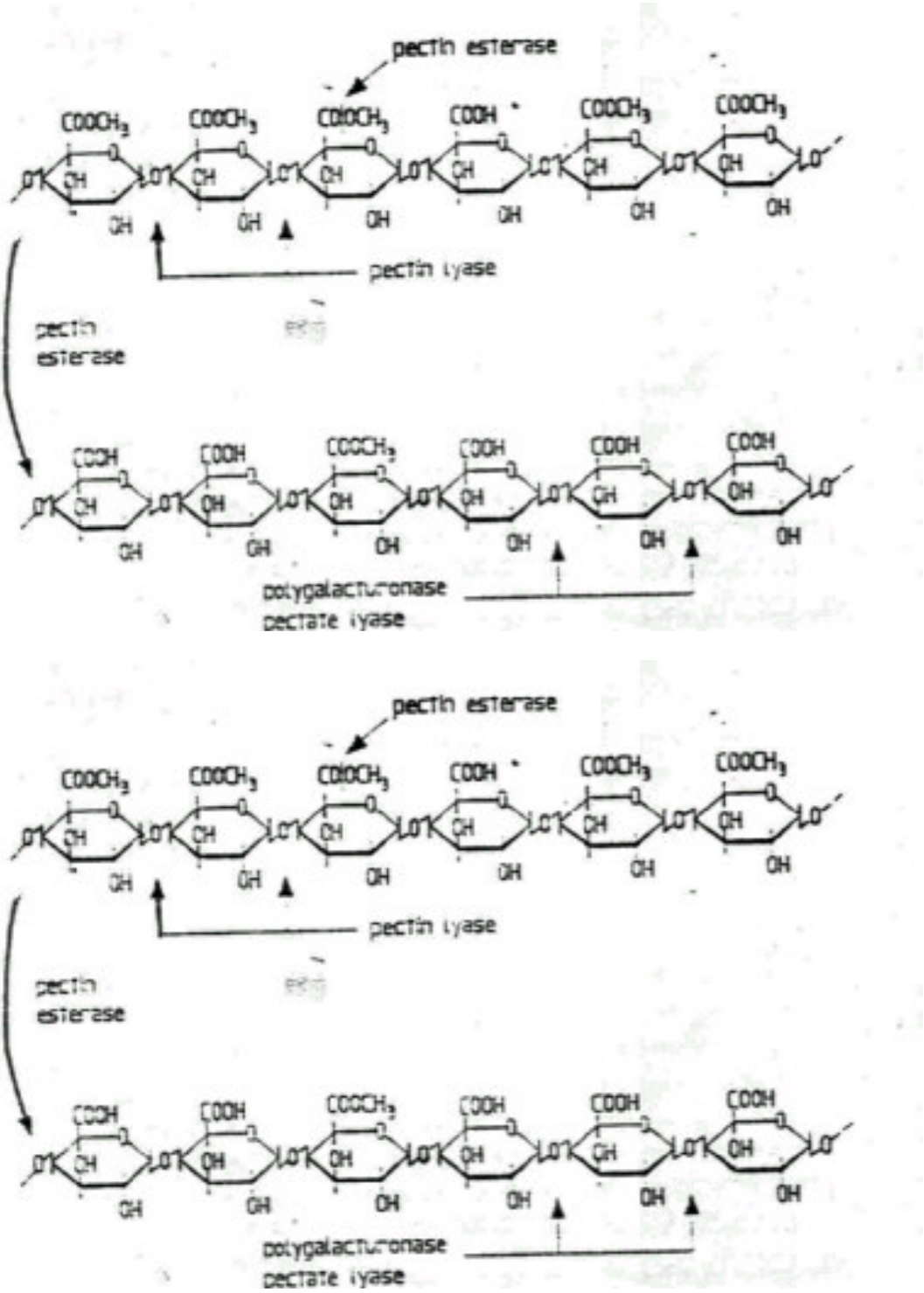

Figure 2.1 Mode of action of various types of pectinases on pectin

2.4 Occurrence of pectic enzymes

Table2.1 shows the presence of PE and PG in higher plants. These enzymes also are produced by microorganisms, which produce PAL and PL as well as shown in Table 2.2 **(Rombouts and Pilnik, 1980)**

Plant	PE	PG	
		Endo	Exo
Apple	+	-	+
Apricot	+	-	-
Banana	+	+	-
Berries	+	-	-
Lime	+	-	-
Mandarin	+	-	-
Orange	+	-	-
Grapes	-	-	-
Mango	+	+	-
Peach	-	+	+
Pear	+	-	+
Plums	+	-	-
Carrots	+	-	-
Leek	+	-	+
Pea	+	-	-
Potato	+	-	+
Tomato	+	+	+

Table 2.1 Occurrence of PE and PG in higher plants

Organism	PE	PG		PAL		PL
		Endo	Exo	Endo	Exo	
Aspergillus	++	++	++	+	-	+
Penicillium	+	+	-	-	-	+
Fusarium	+	+	-	-	-	-
Rhizopus	+	++	-	-	-	-
Sclerotina	+	+	-	-	-	+
Kluyveromyces	++	+	-	-	-	-
Bacillus	-	-	-	+++	-	-
Clostridium	+	+	-	-	+	-
Erwinia	-	-	+	+++	+	+
Pseudomonas	-	-	-	+++	-	-
Arthrobacter	-	-	-	+	-	-

Table 2 .2 Occurrence of pectinolytic enzymes in microorganisms

2.5 Pectinases production

Microbial enzymes are commercially produced either through submerged fermentation (SmF) or solid substrate fermentation (SSF) techniques. SmF techniques for enzyme production are generally conducted in stirred tank reactors under aerobic conditions using batch or fed batch systems. High capital investment and energy costs, and the infrastructural requirements for large-scale production make the application of SmF techniques in enzyme production,impractical in a majority of developing country environments **(www.fao.org).** Submerged fermentation is cultivation of microorganisms on liquid broth it requires high volumes of water, continuous agitation and generates lot of effluents. SSF incorporates microbial growth and product formation on or with in particles of a solid substrate under aerobic conditions, in the absence or near absence of free water and does not generally require aseptic conditions for enzyme production (**Mudgett, 1986 and Sanzo *et al*, 2001)**

2.5.1 Microorganisms commonly used in submerged and solid state fermentation for Pectinases production

Microorganisms are currently the primary source of industrial enzymes: 50 % originate from fungi and yeast; 35 % from bacteria, while the remaining 15 % are either of plant or animal origin. Filamentous microorganisms are most widely used in submerged and solid-state fermentation for pectinase production. Ability of such microbes to colonize the substrate by apical growth and penetration gives them a considerable ecological advantage over non-motile bacteria and yeast, which are less able to multiply and colonize on low moisture substrate (**Smith *et al,* 1988**). Among filamentous fungi three classes have gained the most practical importance in SSF; the phycomycetes such as genera *Mucor*; the ascomycetes genera *Aspergillus* and basidiomycetes especially the white and rot fungi **(Young *et al*, 1983)**. Bacteria and yeasts usually grow on solid substrates at the 40%to70% moisture levels **(Young *et al,* 1983).** Common bacteria in use are *(Bacillus licheniformis, Aeromonas cavi, Lactobacillus* etc and common yeasts in use are

Saccharomyces and Candida Pectinase production by Aspergillus strains has been observed to be higher in solid-state fermentation than in submerged process (**Solis *et al*, 1996)**

2.5.2 Substrate for fermentation: Medium require presence of bioavailable nutrients and absence of toxic or inhibitory constituents medium carbon, nitrogen, inorganic ions, growth factors are also required. For submerged fermentation besides carbon source, nitrogen growth factors media requires plenty of water. The most widely used substrate for solid state fermentation for pectinase production are materials of mainly plant origin, which include starchy materials such as grains, rice, corn, roots, tubers and legumes, and cellulosic lignin, proteins, and lipid materials (**Smith *et al,* 1988).** Agricultural and food processing wastes such as wheat bran, cassava, sugar beet pulp, Citrus waste, corn cob, banana waste, saw dust and fruit pomace (apple pomace) are the most commonly used substrates for SSF for pectinase production **(Pandey *et al*, 2002).**

2.6 Present status

Apple pomace has been reported to be an attractive raw material for production of pectinases by *Aspergillus foetidus* in solid-state cultures (**Hours *et al*, 1988).** In addition to apple pomace, other important fruit and vegetable processing waste come, from citrus, grape, cherries, berries, banana olives, peach, pear pineapple, kiwifruit, tomato, potato, asparagus, beans, peas, spinach, and sauerkraut processing industries (**Hang *et al,* 1979).** Orange finisher pulp (OFP) is byproduct of orange juice processing composed mainly of cell wall and membrane ruptured juice vesicles (**Braddock**, **1983). .** OFP was found to be a substrate for Polygalacturonase production by *Aspergillus oryzae* **(Braddock and Kesterson, 1975, Kesterson *et al*, 1976)** Under optimum conditions, maximum amount of 15g of Polygalacturonase and 200mg of PE per kg of solid medium was obtained (**Berovic *et al*, 1997).**

2.7 Comparison of solid and submerged fermentation for pectinase production:

Factor	Liquid Substrate Fermentation	Solid Substrate Fermentation
Substrates	Soluble Substrates (sugars)	Polymer Insoluble Substrates: Starch Cellulose Pectins Lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited Consumption of Water; low Aw. No effluent
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
pH control	Easy pH control	Buffered solid substrates

(Raimbault, 1998)

2.8 For several products Solid-State Fermentation offer advantages over fermentation in liquid broths/submerged fermentation: (Cook,P.E, 1994)

- Higher product yield
- Better product quality
- Cheaper product recover
- Cheaper technology (www.ftns.wau.nl/prock)
- Higher substrate concentration
- Less probability of contamination
- Lower capital in vestment

Disadvantages. Despite solid-state fermentation being both economically and environmentally attractive, their biotechnological exploitation has been rather limited (**Pandey *et al*, 1992; Aidoo *et al*, 1982)**

- Limitation on microorganism
- Medium heterogeneity
- Heat and mass transfer control growth measurement and monitoring
- Scale up problems

2.9 Uses of Pectinases

2.9.1. Fruit juice industry

2.9.1.1 Fruit juice clarification

Addition of pectinase lowers the viscosity and causes cloud particles to aggregate to larger units (break), which sediment and is removed easily by centrifugation. Indeed pectinase preparation once was known as filtration of enzymes. Careful experiments with purified enzyme have shown that this effect is reached either by a combination of PE and Polygalacturonase or by PL alone in the case of apple juice, which contains highly, esterified pectin (. >80%) (**Ishii *et al,* 1972),** PL alone does not perform as well (**Ishii *et al*, 1973).**

2.9.1.2 Enzymes treatment of pulp for juice extraction

In early period of use of pectinase for clarification, it was found first for black currents that enzyme treatment of the pulp before pressing improved juice and color yield **(Charley**, **1969).** Enzymatic pectin degradation yields thin free run juice and a pulp with good pressing characteristics **(Beltman *et al*, 1971).** In case of apples it has been shown that any combination of enzymes that depolymerize highly esterifies pectin (DE>90) can be successfully used **(Pilnik and Voragen, 1993).**

2.9.1.3 Liquefaction-

It is process in which pulp is liquefied enzymatically so pressing is not necessary. Viscosity decreases of stirred apple pulp during treatment with pectinases C1 cellulase and a mixture of the two-enzyme preparation.

Cellulase alone had little effect on pectin and solubilized only 22% of cellulose. Combined cellulose pectinase activities released 80% of the polysaccharide. A similar effect has been found for grapefruit segment membrane **(Pilnik and Voragen, 1993).**

2.9.1.4 Maceration

It is the process by which the organized tissue is transformed into a suspension of intact cells resulting in pulpy products used as a base material for pulpy juices And nectars as baby foods The aim of enzyme treatment is transformation of tissue into suspension of intact cells. This process is called enzymatic maceration (The so called macerases are enzyme preparation with only Polygalacturonase or PL activity). A very interesting use of enzymatic maceration is pulp for the production of dried instant potato mash Inactivation of endogenous PE is important for the maceration of many products **(Pilnik and Voragen, 1993).**

2.9.2 Wine industry

Pectolytic enzymes are added before fermentation of white wine musts, which are made from pressed juice without any skin contact in order to hasten clarification. Another application of pectolytic enzymes during wine making is associated with the technology of thermovinification. During heating the grape mash to 50^0 for few hours large amounts of pectin are released from the grape, this does not occur in traditional processing. It is therefore necessary to add a pectolytic preparation of the heated mash, so that the juice viscosity is reduced. An additional benefit from the process is that the extraction of anthocyanins is enhanced, probably due to a breakdown in cell structure by the enzyme, which allows the pigments to escape more readily and thus helps in color enhancement **(Tucker and Woods, 1991).**

2.9.3. Textile industry

In the textile industry pectinases are sometimes used in the treatment of natural fibers such as linen and ramie fibers (**Baracet *et al*, 1991).**

2.10 Pectin

Pectin was discovered in 1790 by Vauquelin and later (1825) crudely characterized by Braconnot. The structure of pectin is shown in Fig 2. 2

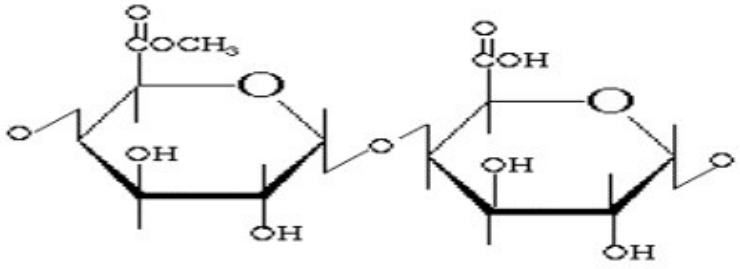

Fig 2. 2 Structure of pectin

Pectic substances (pectins) are chains, molecules with a rhamnogalacturonan backbone. This backbone consists of smooth α 1-4 galacturonan regions that are interrupted to a small extent by insertion of 1,2 linked α-l-rhamnoglycosyl residues and highly branched regions with an almost alternating rhamnogalaturonban chain. Side chains composed of neutral sugars are attached by glycosidic linkages to carbon atoms 3 and 4 of rhamnose units and carbon atoms 2 and 3 of galaturonic acid units having rhamnogalaturonan portion of pectin backbone a hairy character. Predominant sugars D-Galactose and L-arabinose are complex chains of considerable length. Acetyl groups are prominent in sugar beet pectins, which also carry feruloyl groups linked as esters to terminal galactose or arabinose units of side chains **(Pilnik and Voragen, 1993).**

2.11 Pectic substance nomenclature (www.cfs.purdue.edu)

Four types of pectic substances exists in nature, these are mentioned below:

Protopectin.	It is defined as high methyl ester pectin.
Pectinic acid.	It has intermediate methyl ester content and is soluble.
Pectin	It also is having intermediate methyl ester content but it is colloidal.
Pectic acid	It is having little methyl acid content.

2.12. Chemical constitution of pectin ((http://www.cfs.purdue.edu/)

The chemical constitution of pectin is given on the basis of Degree of methylation (DM) and Degree of esterification (DE).

Types of pectin	**(DM %)**	**DE (%)**
Protopectin	16	100
Normal pectin	8	50
Low methoxyl pectin	2-4	15-25

2.13 Types of pectin

Pectin as extracted normally has more than 50% of the acid units esterified, and is classified as "high methyl ester (HM) pectin" shown in Figure 2.3

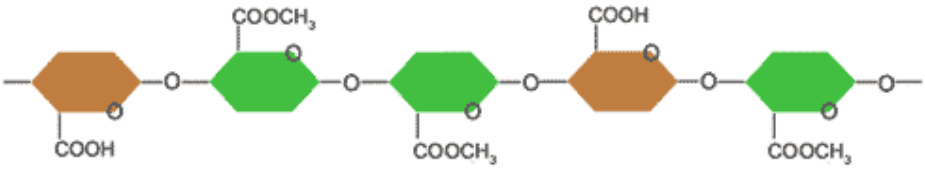

Figure 2.*3 HM p*ectin formula

The percentage of ester groups is called degree of esterification. High methyl ester pectins are classified in groups according to their gelling temperature as rapid set to slow set pectins.

Modification of the extraction process, or continued acid treatment, will yield low methyl ester (LM) pectin with less than 50% methyl ester groups as shown in Figure 2.4.

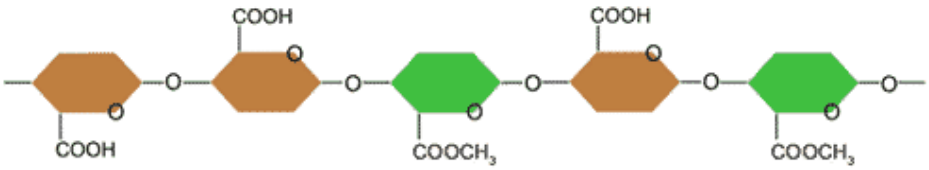

Figure 2.4 LM pectin formula

Some pectins are treated during manufacture with ammonia to produce amidated pectins Figure 2.5, which have particular advantages in some applications.

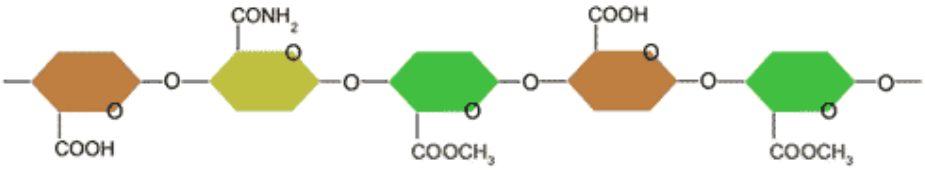

Figure 2.5 Amidated pectin (www.ippa.info)

2.14 Sources

Pectic substances occur in the middle lamella and the primary cell wall of higher plants and prominent in parenchymatous tissue. These are abundantly present in apple, lemon, orange, mango, tomato, beet, and carrots **(Pilnik and Voragen, 1993; Girdharilal *et al*, 1998) as shown in Table 2.3**

Material	**% pectin in fresh material**	**%pectin in dry wt basis**
Apple Pomace	1.5-2.5	15-18
Lemon Pulp	2.5-4.0	30-35
Orange Pulp	3.5-5.5	30-40
Beet Pulp	1.0	25-30

Table: 2.3 Sources of pectin

2.15 Manufacture of pectin

The first commercial production of a liquid pectin extract was recorded in 1908 in Germany, and the process spread rapidly to the United States, where a classic patent was obtained by Douglas (US Pat. 1.082,682, 1913). This was followed by a rapid growth of the pectin industry in the United States, and also somewhat later in Europe.

In recent years, the centre of production has moved to Europe and to citrus-producing countries like Mexico and Brazil **(www.ippa.info**) Most commercial pectin's come from "apple pomace" or waste from manufacture of apple products (10-15% pectin), citrus wastes (20-30% pectin' and from sugar beet processing. Pectin's are extracted by heating the plant materials in water (60-95^0 C) and at an acidic pH (2.5). The pectin are precipitated with ethanol and removed by centrifugation. **(www.life.uiuc.edu)**

2.16 Pectin from various sources yield and quality.

Pectin yield, methoxylated pectin and degree of esterification (DE) in different sources of pectin as observed by **Rao and Maini , 1999** is given below in Table 2.4

SOURCE	YIELD	MeO%	DE (%)
Apple pomace	17.0	8.9	74.9
Lime peels	32.0	8.6	63.2
Lemon peels	27.7	9.2	73.4
Sweet orange peels	17.8	7.7	57.0
Mandarin orange peels	18.4	9.5	64.9

Table 2.4 Sources of pectin along with respective MeO and DE

2.17 Uses of pectin

The pectin, when isolated from plant tissue play a significant role in the manufacture of many fruit products like jams, jellies, marmalades, preservatives etc and thus are indispensable to the fruit juice industry. It is also used as a thickening agent for sauces, ketchups, flavored syrups and as a texturing agent in fruit flavored milk desserts **(Girdharilal *et al*, 1998).** Pectin, in the diets of humans and lab animals, has been shown to increase the excretion of lipids, cholesterol and bile acids, and reduce serum cholesterol levels. Pectin operates by binding with bile acids, thereby decreasing cholesterol and fat absorption.

Pectin is also effective in causing regressions in, and preventing, gallstones. There is also evidence that the regular use of pectin may lessen the severity of diabetes. Along these lines, it has been suggested that fiber-depleted diets actually help cause diabetes mellitus. Other studies have shown that fiber and pectin as contained in this formula could lead to permanent changes in insulin requirements. To prevent the possibility of insulin overdose, diabetics should make their physician aware of the dietary change **(.http://www.metromkt.net/viable/1pectin;).**

MATERIALS AND METHODS

Materials and Methods

3.1 Material procurement

3.1.1 Kinnow as natural source of pectinolytic fungi

Rotten and fungus infected kinnow fruits were collected from different fruit shops/ vendors in Patiala in the month of January 2003.

3.1.2 Kinnow peels and Kinnow pomace powder

Kinnow peels and Kinnow pomace, which is juice extracted kinnow fruit, were collected from different fruit shops/vendors in Patiala and also from Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, where another project on kinnow fruit was going on during the course of present study.

Kinnow peel powder and pomace powder was made by following procedure -

Peels and pomace were first washed many a times with water to remove all adhering substances. Small pieces of peels were made using knife and these were then dried in tray drier at 50^0C for 24 hr with intermittent shaking. Similarly washed pomace was well spread uniformly and dried. The dried kinnow peels and pomace were then made to powder using a mechanical grinder (Philips India limited, Kolkata).

3.1.3 *Aspergillus oryzae var oryzae* (as standard)

Freeze dried culture of *Aspergillus oryzae var oryzae* (MTCC 3567) were collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

The culture was activated according to the following method mentioned as by suppliers:

1. Firstly, a mark was made on the ampoule near the middle of cotton wool with a sharp file. The surface around the mark was then disinfected with 100% alcohol.
2. The ampoule was then wrapped with thick cotton wool and marked area was broken, cotton plug was then carefully removed and 0.4ml sterile water was added to make a suspension.
3. The suspension was allowed to stand for 20 minutes, after 20 min, few drops of suspension were streaked onto Medium 117 (Annexure I) in number of petri plates and slants.
4. Rest of the suspension was transferred to 5 ml of Medium 117(broth) in a test tube.
5. The plates were then incubated in BOD at 25^0C for 5 days.
6. After the growth of fungi, fungal culture was maintained by subculturing the fungi on the same medium throughout the experimental period.

3.1.4 Chemicals, media and standard enzyme

All the chemicals used in the present investigation were of Analytical grade (AR grade). D-galacturonic acid and Disodium hydrogen arsenate were procured from Sigma, Aldrich Corp, MO, USA. Polygalacturonic acid, Neocuproine, pectin, Congo red dye were procured from Himedia Laboratories Limited, Mumbai.

Standard enzyme- Pectinase was procured from Himedia Laboratories Limited, Mumbai.

All the experiments for isolation, screening and pectinolytic enzyme production were done under sterile conditions and adequate safety measures were undertaken (Annexure IX)

3.2 Isolation of Pectinolytic fungi

Rotten and fungus infected kinnows were swabbed in 0.8% saline in an autoclaved stomacher bags. Dilutions upto10^{-8} was made and pour plating of higher dilutions namely $10^{-5,}$ 10^{-6}, 10^{-7}, and 10^{-8} was done using melted Czapek

agar (Annexure II). The above dilutions were plated in duplicates. The plates were then incubated at 30^0C for 12 days. The colonies thus isolated were then subcultured 3-4 times on Czapek agar till active growth of isolates were obtained, and thereafter maintained on the same media for further experimental work.

3.3 Screening of isolates having pectinolytic activity

3.3.1 Qualitative test for screening of isolates having pectinolytic activity

For screening purpose, Czapek agar having1% pure pectin was used as sole carbon source and Congo red (@150mg/l) was also added in medium, so as to clearly visualize the formed clear zones. Simultaneously, control plate was set having Congo red but without pectin or any other carbon source. Following methodology was adopted for screening of potential isolates -

Using a flamed and cooled cork borer, one disc of fungal hyphae from leading edge of actively growing colonies was cut on petri plate. With a flamed and cooled transfer needle, discs were then transferred to Czapek agar media having pectin as sole carbon source. Plates were then incubated at 30^0C for 12 days. Selection was done on the basis of formation of clear zones and the corresponding diameters were noted during that span of time. Morphological examination of isolates was done using Image Analyser having compound microscope.

3.4 Quantitative estimation of pectinolytic activity of screened pectinolytic isolates

Quantitative estimation of pectinolytic activity screened isolates was done on submerged as well as solid state fermentation.

3.4.1 Submerged Fermentation

In order to undertake submerged fermentation firstly pure pectin was used as substrate, subsequently it was replaced by kinnow peel powder and then finally by kinnow pomace powder. For each isolate, 2 flasks were prepared; one was treated as test flask and the other as control flask.

In every sterile flask, 1 disc of respective fungal isolate was added and the flasks were properly plugged. The flasks were then incubated in incubator shaker for 12 days at 30^0C at 250 rpm. Aliquots were withdrawn every 0, 3, 6, 9, 12 day for carrying out assays namely: Total protein, Pectinase assay, Polygalacturonase assay and Pectin Lyase assay according to methods explained in section 3.5.

Experiments were also conducted to know the effect of number of fungal discs (1, 2 & 3) as well as amount of pectin 1% and 2% on total protein using the above methodology.

3.4.2 Solid State Fermentation

Solid State Fermentation was carried out in sterile 250ml flasks. Experiments were performed using only kinnow peel powder as sole carbon source. For every isolate and standard fungus, experiments were set up using 5 sterile flasks. Aliquots were drawn after adequate dilution (10 times) on every 0, 3^{rd}, 6^{th}, 9^{th} and 12^{th} day.

Solid State Fermentation was carried as follows:

15g of substrate was taken and 8ml of sterile water was added. One disc of respective fungal hyphae was mixed to 5ml of sterile water and a suspension was made. From this suspension, 1ml was withdrawn and inoculated into each of the 5 flasks. The flasks were then incubated at 30^0C for respective period of time. For proper aeration, flasks were intermittently shaken. In order to estimate the Total Protein, Pectinase, Polygalacturonase and Pectin Lyase, 10ml of

sterile water was added and then properly mixed, the mixture thus obtained was gently shaken and was filtered using coarse filter paper. The obtained filtrate was then centrifuged and resulting supernatant was used for conducting the assays.

3.5 Assays

In order to quantify total protein and pectinolytic enzymes produced via submerged and solid state fermentation, following experiments were conducted:

3.5.1 Total protein content (BIURET METHOD)

5mg albumin/ml was used as protein standard

Reagents used: Biuret reagent (Annexure III)

Firstly standard curve with different concentrations of bovine albumin serum (BSA) namely-100µl, 150µl, 200µl, 250µl 300µl was made. For analysis of total protein, 3ml of biuret reagent was added to 2ml of test protein solution in a sterile test tube and the mixture was properly mixed. The tubes were then warmed at 37^0C for 10 min with shaking and finally the tubes were cooled and absorbance was noted at 540nm.

3.5.2 Pectinase assay

WBC Home manual/protocol index methodology was followed to estimate the pectinase activity.

Reagents used:

i) 0.1 M Phosphate buffer pH 5.0
ii) Color reagent A (Annexure IV)
iii) Color reagent B (Annexure V)
iv) D-galacturonic acid-1mg/ml
v) 0.5%Polygalacturonic acid substrate (Annexure VI)

1. In one test tube 6ml of substrate and 1ml of buffer was added and then properly mixed (reagent blank).
2. In second test tube 6ml substrate and 1ml of enzyme solution 0.1mg/ml was added (test sample).
3. In third test tube 6ml buffer and 1ml enzyme sample at 1mg/ml (sample blank) was taken.
4. For standard D- galacturonic acid, different concentration 0, 20, 40, 60, 80, 100, 120 µg of D-galacturonic acid was used.
5. The above three reaction tubes along with standard tubes were incubated at 37^0C water bath with shaking for 60min±1min. After incubation the tubes were immediately placed into ice water to stop reaction.100µl aliquot was drawn from each tube and was pipetted into another set of tubes also placed on ice water.
6. To each reaction tube and standard tube. 2ml color reagent A and 2ml color reagent B was added, and the mixture was mixed properly by inversion. The tubes were then placed into boiling water bath for 13min±1min. The tubes were then cooled and then 2ml water was added. Again proper mixing was done by inversion. The absorbance was noted at 450nm using water blank and disposable cuvettes were used throughout the assay owing to sticking of formed orange color in cuvettes.

3.5.3 Polygalacturonase (PG)

PG assay was carried by method as described **Baldwin and Pressey (1989)**.

1. In a test tube, 2ml of 0.1M Sodium acetate, .025ml of 0.15 M Sodium chloride, 0.5ml of 1% polygalacturonic acid and .05ml of enzyme solution was taken. Test tubes were then incubated at 37^0C for 15 min in water bath with shaking.
2. The above solution was then analyzed for reducing groups following the Arsenomolybdate method given by Nelson Somogyi (1952).

Reagents used-

- Alkaline copper tartarate (Annexure VII)
- Arsenomolybdate reagent (Annexure VII)

i) Aliquots of .1 or .2 ml of above solution was pipetted out in test tube.

ii) 0.2, 0.4, 0.6, 0.8, 1.0 ml of standard solution (100µg/ml glucose solution) were taken into series of tubes.

iii) The volume in both sample and standard tubes was made to 2ml with distilled water

iv) 2ml distilled water was pipetted out in separate test tubes to set up a blank.

v) 1ml of alkaline copper tartarate reagent was added to each tube

vi) The tubes were then placed in boiling water bath for 10 minutes and then cooled; and 1ml of arsenomolybdolic reagent was added to all test tubes taking full precautions

vii) The volume in each tube was then made to 10ml with water

viii) The absorbance of blue color was then determined at 620 nm after 10 min.

3.5.4 Pectin Lyase Assay

Pectin lyase was assayed by measuring the increase in absorbance at 235nm according to method described by **Albersheim and Killias (1962)**.

In a properly washed and cleaned test tube 0.2ml of 0.1M Sodium acetate (pH 5.5), 25ml of 0.12M sodium chloride, 0.5ml of 1% pectin (pH 5.5) and 0.05ml of enzyme solution was pippeted out and mixed. The tubes were then incubated at 37^0C for 15 min in water bath with shaking. 5ml of water was then added to the above reaction mixture and absorbance was then determined at 235nm.

3.6 Extraction and estimation of pectin

Pectin was extracted by method given by **Rao and Maini, (1999)** as shown in Figure 3.1. 15 g of peel powder was weighed and was taken in 150ml flask, to it 30 ml of dilute acid .05N Hydrochloric acid was added. Extraction was done by boiling the above mixture at 100^0C for 60 min. The marc was then separated from the extract after filtration. The extraction was performed 2

times. Filtrates obtained were then combined and then cooled. Two volumes of absolute alcohol were added to precipitate pectin. Pectin was also precipitated using different strengths of alcohol namely 90%, 80%, 70%, 60%, and 50%.

Yield of pectin was calculated by the following formula:

$$\text{Yield of pectin} = \frac{\text{Amount of pectin obtained} \times 100}{\text{Total amount of peel powder}}$$

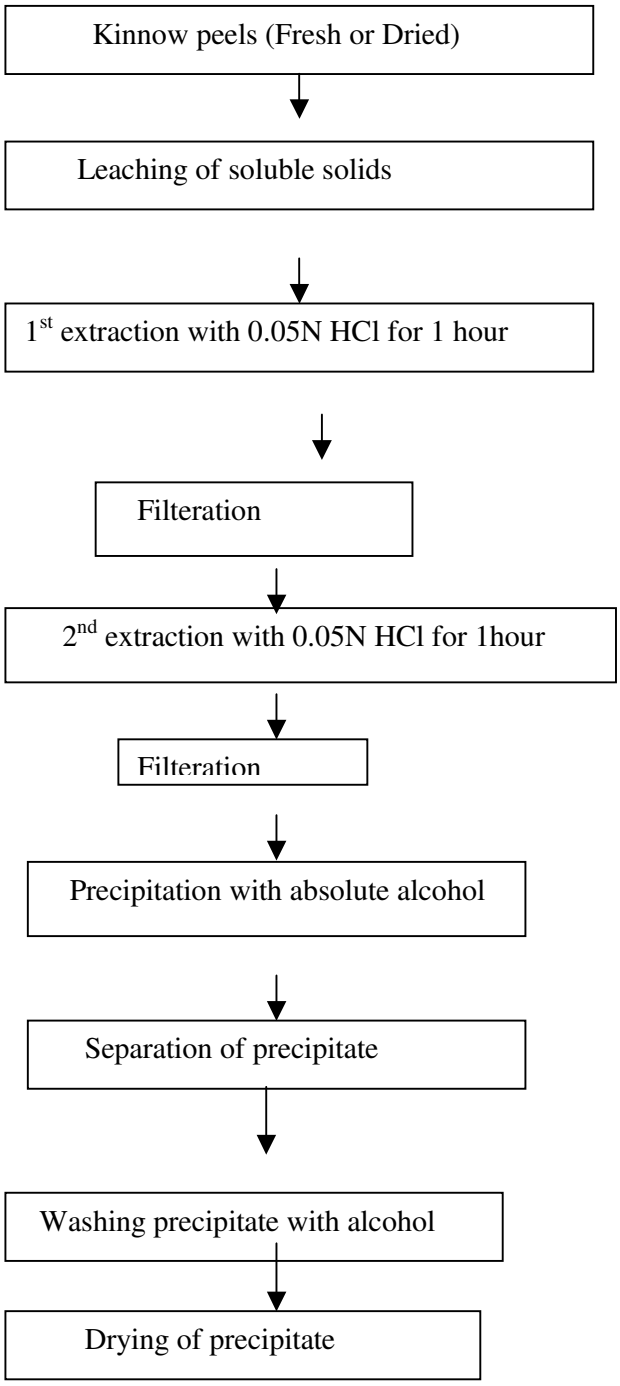

Figure 3.1 Flow diagram showing different stages of pectin production from kinnow peels (Rao and Maini, 1999)

3.6.1 Estimation of pectin (Ranganna, 2000)

The procedure followed was as follows:

1. 200mg of dried pectin was weighed into a 1-litre beaker and it was wetted with 2 or 3 ml of 100% alcohol.
2. 400ml of water was added with stirring to it.
3. The solution was then boiled and then cooled immediately.
4. Then it was transferred into a 500ml volumetric flask and volume was made up.
5. 200ml aliquot was pipetted out each into two 1l beakers.
6. 250ml water was added and solution was neutralized with 1N Sodium hydroxide using phenolphthalein as indicator Afterwards, 10ml of 1N Sodium hydroxide was pipette out in excess with constant stirring and was allowed to stand overnight.
7. Then next day 50ml of 1N Acetic acid was added and after 5minutes 25ml of 1N calcium chloride was added with stirring.
8. Solution was allowed to stand for 1 hour, and was then boiled for 2 min.
9. The solution was filtered using previously prepared filter paper (filter paper was made wet in hot water, dried in oven at 102^0C for 2 hours, cooled in a dessicator and weighed in a covered dish) precipitate obtained was washed thoroughly with boiling water.
10. In an originally weighed dish, the filter paper containing calcium pectate was transferred and was dried overnight at 100^0C.
11. Weighing dish was cooled in dessicator and then it was finally weighed.

% Calcium pectate was calculated using following formula:

$$\% \text{ Calcium Pectate} = \frac{\textit{Wt of calcium pectate x 500 x 100}}{\text{ml of filtrate taken x wt of sample taken for estimation}}$$

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The present investigation was undertaken in order to harness the valuable products namely fungal pectinases as well as pectin from waste obtained from processing/ utilization of kinnow fruits. As it is well reported that certain sporulating strains like *Aspergillus niger, Aspergillus oryzae, Rhizopus oryzae* etc have pectinolytic activity, the purpose was to screen similar isolates from nature and employee them for the production of pectinolytic enzymes. Only extracellular pectinases were targeted because in comparison to intracellular pectinases, extracellular are easier to harvest and scaling up work can be more easily attempted. Furthermore, as the isolated strains were to thrive only on pectin, pectin content of kinnow fruit was determined. The results of experiments performed to quantify enzymatic activity as well as amount of pectin are being systematically represented below.

4.1 Isolation of pectinolytic fungi

The collected rotten and fungus infected fruits were used as source of natural pectinolytic fungi and such fruits were collected in the month of January 2003. From the collected samples, eight potential fungi were screened for pectinolytic activity on selective media (Czapek media having pure pectin as the only carbon source) in duplicates, by taking higher dilutions from 10^{-5} to10^{-8}. Plates having Czapek media without any carbon source were treated as control, which showed no sign of growth. Based on visual observance, only three isolates gave clear zones of diameter 2.3mm to 4.1mm as shown in Plate 4.1 –4.3. In the first isolate, clear zone of diameter 2.9mm was observed on 4^{th} day of its incubation at 30^0C, for the second isolate clear zone of diameter 2.2mm was observed on 6^{th} day of its incubation, and for isolate#3 zone of 4.1mm was observed on 4^{th} day; thereafter excessive growth was seen in each plate thus covering entire plate by mycelia. On the basis of literature review, *Aspergillus oryzae* var *oryzae* and *Rhizopus oryzae* were treated as standard. Freeze dried culture of *R. oryzae* failed in being activated even after performing

repeated trials. Thus only *Aspergillus oryzae* var *oryzae* was treated as standard in the present study.

4.2 Morphological examination of the screened isolates.

Morphological examination of screened isolates was performed using Image Analyser with compound microscope. Isolate # 1 and Isolate # 3 showed clear aerial hyphae, and black colored spores were also found by macroscopical examination microscopic examination showed presence of sporangiospores in sporangia at the tip of fertile hyphae, the sporangiophore. Sporangiophores were arising from the node and sporangia were present at the end of sporangiophores. The swollen tip of sporangiophore columella, projecting into sporangia, was also seen. Rhizoid like structures were also found arising from node as shown in Plate 4.4 (a & b) and in Plate 4.6 (a & b). As these characters belong to *Rhizopus* genus from this observation it can be inferred that Isolate # 1 and Isolate # 3 belong to *Rhizopus* genus.

Isolate #2 showed definite zones of growth and mycelia were greenish. Spore bearing heads were seen as large and globular and were tightly packed. Chains of conidia were also seen. Conidia were greenish in color. From these observations, it can be inferred that Isolate # 2 might be belonging to *Aspergilus* genus (Plate4.5 a & b).

Isolate # 1

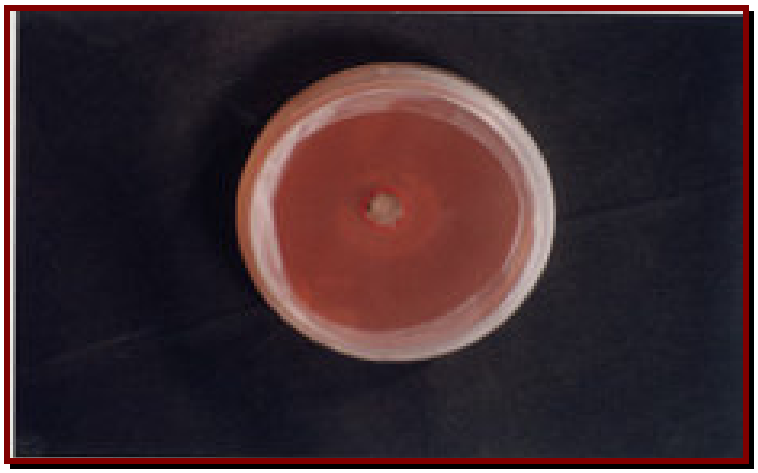

Plate 4.1a Clear zones as obtained in selective media*

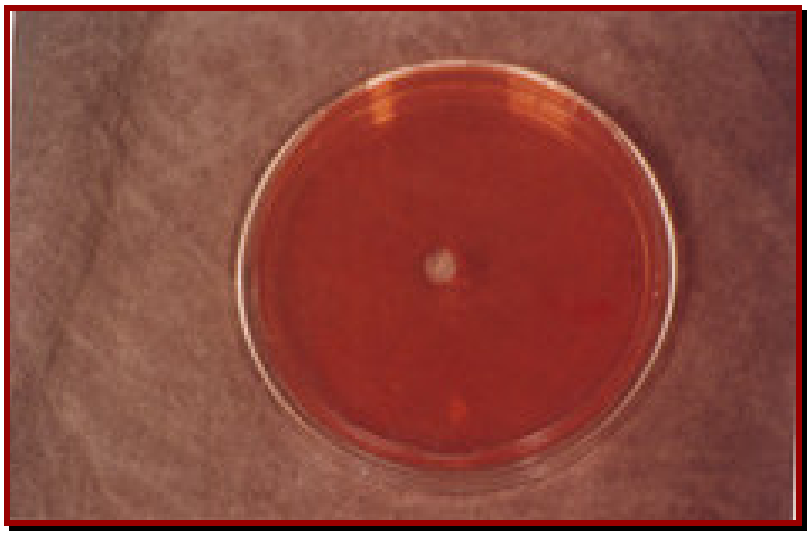

Plate 4.1b) Control Plate with selective media* (no zone formation)

*: Czapek Agar media (with pectin as sole carbon source)

Isolate # 2

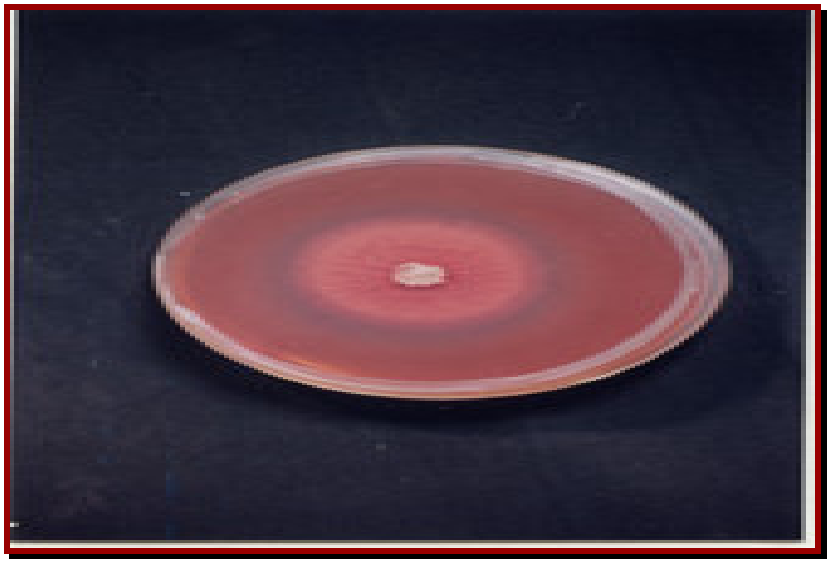

Plate 4.2 a) Clear zone as obtained in selective media*

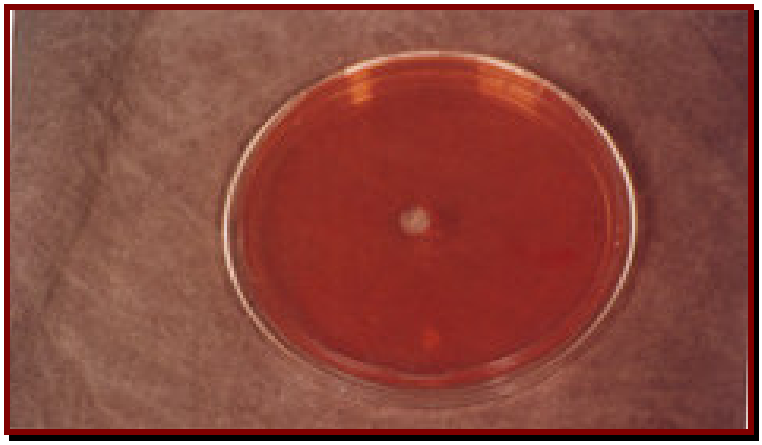

Plate 4.2 b) Control Plate with selective media* (no zone formation)

*: Czapek Agar media (with pectin as sole carbon source)

Isolate # 3

Plate4.3 a) Clear zone as obtained in selective media*

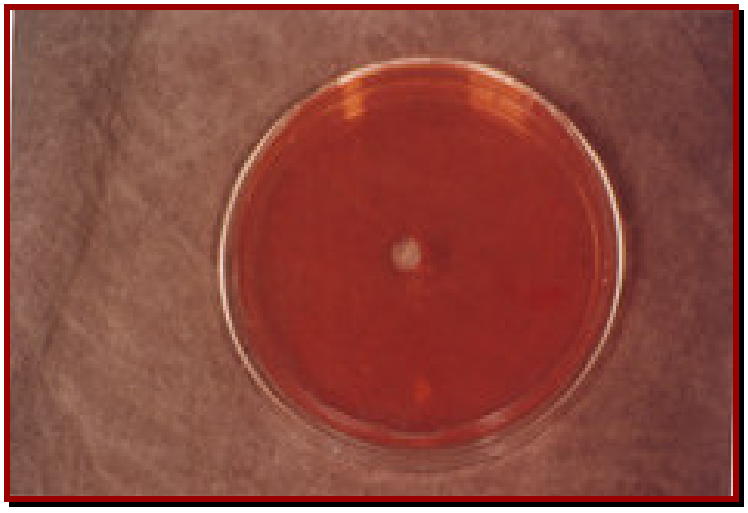

Plate 4.3 b) Control Plate with selective media* (no zone formation)

*: Czapek Agar media (with pectin as sole carbon source)

4.3 Quantitative estimation of pectinolytic activity of screened pectinolytic isolates.

After selecting the isolate, quantitative estimation of pectinolytic activity of isolates was done via submerged as well as solid state fermentation.

Suitable form of kinnow waste was processed as kinnow peel powder and kinnow pomace powder. Determination of total protein content was indicative of total enzyme activity of the extracts as because most enzymes are protein and during growth in the selective broth, they secrete extracellular enzymes. In order to use complex pectin, the enzyme must have caused its break down to simpler products and intracellular enzymes must have been retained within fungal cells.

As described in review of literature, pectinases constitute Pectin methyesterases, Polygalacturonase, Pectin Lyase and pectic acid lyase. As Polygalacturonase and Pectin lyase are most commonly used in fruit juice industry, PG and PL were also analyzed along with Pectinases.

4.3.1 Submerged fermentation (SmF)

The isolated strains and standard fungi *Aspergillus oryzae* var *oryzae* were grown in Czapek broth replacing sucrose by pure pectin.

4.3.1.1 Effect of number of discs and percent pectin on total protein

Czapek broth (80ml) having pure pectin as soul carbon source was inoculated in three different flasks with respectively 1, 2 and 3 discs, of each of the isolates. As it can be inferred from Table 4.1 that no difference in total protein was observed among the different extracts, collected from the inoculated flasks; it could be said that number of discs play no role on protein content

Similarly varying amount of pectin (1% and 2%) in media did not affect enzyme activity in terms of total protein as can be again seen from Table4.1

Thus in further course of experiments, only 1disc of either of isolated fungi as well as standard fungus and 1% pectin were used.

4.3.1.2 Total protein and enzyme activities of selected isolates.

For studying total protein content and enzymatic activities in SmF, isolated strains of fungi and standard fungi were grown in Czapek broth replacing sucrose first by pure pectin, followed by peel powder and finally by pomace powder.

4.3.1.2.1 Total protein

Total protein was determined by Biuret method. Aliquots withdrawn on every 3^{rd} day till 12^{th} day of incubation at 30^0C for total protein assay showed that among all isolates isolate#3 produced maximum protein (8.69 g/ml/10min) (Table 4.2) on 9^{th} day of its incubation, followed by Isolate#1 which showed maximum protein content (1.96 mg/ml/10min) on 6^{th} day of its incubation and then Isolate#2 (1.59mg/ml/10min) on 9^{th} day of its incubation. Also in each case maximum protein was obtained using pure pectin as substrate (Figure 4.1). It can also be seen from the Figure 4.1 that standard fungi produced maximum protein using all substrates and maximum total protein obtained was by using pure pectin (17.16mg/ml/10min), on 6thday of incubation, followed by peel powder (12.84mg/ml/10min) and then from pomace 8.85mg/ml/10min as can be seen Table4.2 and Figure 4.1.

Thus it can be inferred that maximum protein was produced using pure pectin as substrates (in all isolates and standard fungus). It could be attributed to the reason that pure pectin contains only pectin, whereas peel powder and pomace have many other constituents, all of which couldn't be supportive as substrate for protein production.

As per expectations, no absorbance/ activity was observed of either type in control. This suggests that fungi could thrive only on given carbon source.

The difference in optimal number of day for maximum total protein production among various isolates could be due to species/ subspecies/ varietal differences. It can also be inferred from Table 4.2 that protein content reduced after optimal day after gaining peak after 6th day for isolate#1 and standard fungi or 9thday for isolate#2 and isolate#3. Thus can be inferred that maximum metabolic activity corresponded to optimal incubation time.

4.3.1.2.2 Pectinase, Polygalacturonase (PG)& Pectin Lyase (PL)

For all the enzymatic activities, maximum activity was obtained via using pure pectin as substrate followed by peel powder and lastly by pomace (Table 4.3, 4.4, & 4.5). The activity corresponded to each substrate is being represented below.

For Isolate#1, maximum pectinase activity was obtained using pure pectin (210.37 nmoles/ml/60min), peel powder resulted in 178.16 nmoles/ml/60min activity and pomace gave 26.56 nmoles/ml/60min (Table 4.3 & Figure 4.2). PG activity was noted to be 72.66 nmoles/ml/10min, 38.22 nmoles/ml/10min and 21.66 nmoles/ml/10min in respective substrate extracts (Table 4.4 & Figure 4.3). PL activity obtained was found to be 136.53 nmoles/ml/15min using pure pectin for peel powder, 117.57 nmoles/ml/15min. It was worth noticing that no PL activity was obtained using pomace as substrate. (Table 4.5 and Figure 4.4).

Isolate #2 - Like isolate# 1, similar trends were seen for various activities using different substrates, and 9th day was the optimal day of incubation for almost all activities. Pectinase activity on pure pectin as substrate was observed to be 367.68 nmoles/ml/60min followed by 345.18 nmoles/ml/60min on peel powder and least was observed on pomace (51.17 nmoles/ml/60min) as shown in Table 4.3 and Figure 4.4). PG activity obtained was 69.61, 61.00 and 34.83 nmoles/ml/10min respectively on pure pectin, peel powder and pomace. (Table 4.4 and Figure 4.3). Again it is notable that PL activity was totally absent in isolate#2.

Isolate#3 showed maximum Pectinase activity corresponding to 763.82 nmoles/ml/60min on pure pectin, 407.50 on peel powder and 312.73 on pomace. Maximum PG activity produced on three substrates were 99.83 nmoles/ml/15min (pure pectin), 67.33 nmoles/ml/15min (peel powder) and 39.38 nmoles/ml/15min (pomace). PL activity noted was 1038.68 nmoles/ml/15min on pure pectin 890.85 nmoles/ml/15min and 697.50 nmoles/ml/15min respectively on peel powder and pomace powder respectively (Table 4.3, 4.4 and 4.5).

Aspergillus oryzae var *oryzae* (standard fungi) showed maximum of all activities among all 4 fungi and on all substrates. For standard fungus also, pure pectin was found to be the best substrate followed by peel powder and finally pomace and optimal day of incubation was the 6^{th} day. Maximum pectinase activities on three substrates was 980.50 nmoles/ml/60min, 574.29 nmoles/ml/60min, 443.53 nmoles/ml/60min. Maximum PG activities obtained were 102.61 nmoles/ml/10min, 87.00 nmoles/ml/10min and 57.55 nmoles/ml/10min respectively on pure pectin, peel powder and pomace (Table4.3, 4.4 and 4.5). Similarly PL activity noted was 1190.30 nmoles/ml/15min, 1163.77 nmoles/ml/15min and 784.71 nmoles/ml/15min.

Amongst the three isolates it can be thus inferred that Isolate # 3 was found to be the best isolate (Figure 4.5, 4.6, 4.7 and 4.8). But its activity was less 70%- 90% less than standard fungus for all activities on an average (Figure 4.9, 4.10, 4.11 and 4.12).

4.3.2 Solid-state fermentation (SSF)

As the basic aim of study was to use kinnow waste, kinnow peel powder for pectinolytic enzyme production, taking economics into considerations. Thus SSF may also be carried out, isolated strains and standard fungi were grown only on peel powder as substrate.

4.3.2.1 Total protein estimation.

For total protein content, it was found that standard fungus showed maximum of 12.10mg/ml/10min, followed by Isolate#3 which gave, 7.95mg/ml/10min, then by Isolate#1 (1.72 mg/ml/10min) and finally Isolate#2, which gave 1.51mg/ml/10min the comparison is shown in Table 4.6 and Figure 4.5

4.3.2.2Pectinase, Polygalacturonase (PG) and Pectin Lyase (PL)

Isolate#1 showed maximum pectinase activity of 151.98 nmoles/ml/60min on its 6th day of incubation at 30^{0}C, PG activity corresponding to 45.09-nmoles/ml/10min and PL activity of 190.24nmoles/ml/10min. were observed.

Isolate#2 showed maximum total protein 1.51 mg/ml/10min, Pectinase 170.93 nmoles/ml/60min maximum, PG activity 42.54 nmoles/ml/10min and no PL activity was noted.

Isolate#3 showed pectinase activity of 436.27nmoles/ml/60min, PG activity 52.39 nmoles/ml/15min and PL activity corresponded to 758.83 nmoles/ml/15min was observed.

Like in SmF, Standard fungus was found to have maximum pectinase, PG and PL activity corresponding to 512.01 nmoles/ml/60min, 61.33 nmoles/ml/15min and 2312.96nmoles/ml/15min respectively.

It may be thus concluded that Isolate # 3 was found to be best amongst three isolates for SSF in terms of total protein and all enzyme activities (Pectinase, PG and PL), though the values are less as compared to standard fungus (Table 4.7, 4.8 and 4.9).

4.4 Comparison of submerged fermentation (SmF) and solid state fermentation (SSF)

Comparing the concentration obtained for different assays from different isolates via submerged and solid state fermentation, it can be concluded that more total protein was produced by submerged than via solid state fermentation (Figure 4.9).

It can be inferred from Figure 4.5 that roughly 70%of activity of SmF was observed in SSF In general Pectinase activity was less in SSF than SmF, but Isolate#3 showed more pectinase activity in SSF (Figure 4.6). Isolate#3 showed lesser activity than standard in both SmF and SSF (Figure 4.10) It was also found that PG activity for optimal day of its incubation for each isolated fungi was more for SmF than SSF (Figure4.7). PL activity was found to be lesser in SmF than SSF for isolate#1 and standard, whereas isolate#3 showed the other way (Figure 4.8). Standard fungi showed more of PL activity in SSF than in SmF (Figure4.12).

4.5 Pectin

Attempts were also made to harness another byproduct pectin from kinnow waste, peel powder was taken as source for pectin extraction and method described by **Rao and Miani (1999**) was followed who recommended 2 volumes of extracting solution (.05N HCl), for extracting pectin from Mango peel but in the present examination, 5V of extracting solution was used when peel powder was used as source of pectin.

Though absolute alcohol has been recommended for precipitation of pectin, different other concentrations (50%, 60%, 70%, 80%, 90%) of ethanol was also used, 50% and 60% ethanol resulted in poor precipitation, some better precipitation was obtained by 70% ethanol of whose 1.8ml was used. Quite appreciable precipitation of pectin could be achieved by 80% ethanol pectin, which gives rise to thick gel of pectin and only 5 drops of it was used finally and 90% also gave precipitation equivalent to absolute alcohol. Thus in the present

study, 80% was used for precipitation pectin against absolute alcohol as recommended by **Rao and Miani (1999)**. Furthermore on average about 20% pectin from kinnow waste was obtained in present study. **Rao and Miani (1999**) reported 18.4% yield in orange mandarin peels. Thus yield obtained in present investigation is little higher to that reported by said workers. ***Lotha et al 1994*** reported 6-8% pectin from kinnow.

Pectin was also reported as calciumpectate and 7.37% calciumpectate was obtained in the present study.

4.6 Path Forward

The selected isolates in the present study would have to be thoroughly characterized before these could be utilized for fruit juice clarification, haze removal, etc. Thus once they are deemed as generally regarded as safe (GRAS) these could be then used in further course of study including scale up trials.

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

The prime objective of the present study was to extract the important byproducts, namely extracellular fungal pectinases and pectin, from kinnow waste. The findings of the study are being summarized below:

1. From the eight potential isolates collected from naturally infected kinnow fruits, 3 isolates were selected based on formation of the clear zones ranging from 2.3mm to 4.1mm on selective Czapek agar plate (Pectin as only carbon source).

2. *Aspergillus oryzae* var *oryzae* was used as only standard fungi in the study.

3. Morphological examination of screened isolates revealed that Isolate #1 and Isolate #3 could be of *Rhizopus* genus and Isolate #2 could be of *Aspergillus* genus. Further characterization is required to confirm the findings.

4. Quantitative estimation of pectinolytic activities of screened isolates was performed via SmF and SSF. For SmF substrates used were pure pectin, peel powder and pomace powder. Quantification of the total protein content and enzymatic activities revealed that 9^{th} day is the optimal day for Isolate #1 and Isolate #3 and 6^{th} day for isolate #2 and Standard fungi for both SmF and SSF.

5. SmF- There was no effect of number of discs and percentages of pectin on total protein content. Isolate#3 produced maximum total protein (8.69 mg/ml/10min). Among all isolates Isolate #3 was found to produce maximum total protein and all other enzymatic activities (Total protein-8.69mg/ml/10min, Pectinase-763.82 nmoles/ml/60min, Polygalacturonase activity- 99.83 nmoles/ml/10min andPL-1038.68nmoles/ml/15min), and it was

found that isolate#3 generate 70%-90% activity as observed in standard fungi. The standard was found to result in even higher amount of Total protein and all enzymatic activities.

6. Among the substrates, pure pectin resulted in maximum total protein and all enzymatic activities followed by peel powder and finally pomace.

7. It is also to be noted that no PL activity was noted in isolate #2 using pomace powder and PL activity was totally absent in isolate #3.

8. SSF was carried out only on peel powder and maximum total protein and enzymatic activities was found in Isolate #3 (Total protein-7.95 mg/ml/10min, Pectinase-436.27 nmoles/ml/60min, Polygalacturonase activity-52.39nmoles/ml/10min and PL-758.83nmoles/ml/15min), but standard resulted in even higher Total protein and all enzymatic activities, (total protein-12.10mg/ml/10min Pectinase-512.01nmoles/ml/60min, Polygalacturonase-41.83nmoles/ml/10min and PL-2312.96 nmoles/ml/10min).

9. In SSF also no PL activity was noted in isolate #2 on pomace powder and PL activity was totally absent in isolate #3.

10. Comparing the two types of fermentation it was observed that SmF produced more total protein and isolate#3 was found to produce more pectinase activity than SmF

11. Pectin was extracted from peel powder and it was found that 80% ethanol resulted in equivalent precipitation as observed in absolute alcohol. On an average 20% yield was pectin was obtained from it and 7.3% pectin in form of calcium pectate was obtained.

Thus it may be concluded that value addition may be incorporated in kinnow waste, consequently extracellular fungal pectinases could be easily extracted along with appreciable amount of pectin. Thorough characterization of selected isolates is next step before taking up scale up studies for final use in food industry.

ANNEXURES

ANNEXURE

ANNEXURE I

Medium 117

Composition of Czapek yeast extract agar (CYA)

Czapek cocncentrate	10.0ml
K_2HPO_4	1.0g
Yeast extract	5.0g
Sucrose	30.0g
Agar	15.0g
Disttiled water	1.0L

Czapek Concentrate

$NaNO_3$	30.0g
KCl	5.0g
$MgSO_4.7H_2O$	5.0g
$FeSO_4.7H_2O$	0.1g
Distilled water	100.0ml

ANNEXURE II

Czapek agar

Stock solution A	50.0ml
Stock solution C	50.0ml
Sucrose	30.0g
Zinc solution	1ml
Copper solution	1.0ml
Agar	20.0g
Distilled water	1 litre

Stock solution A

$NaNO_3$	4.0g
KCl	1.0g
$MgSO_4.7H_2O$	20.0mg
Distilled water	100.0ml

Zinc solution

$ZnSO_4.7H_20$	1.0g
Distilled water	100.0ml

Stock solution C

K_2HPO_4	2.0g
Distilled water	100.0ml

Copper solution

$CuSO_4.5H_2O$	0 .5g
Distilled water	100.0 ml

ANNEXURE III

Biuret reagent

Copper sulphate	3.0g
Sodium potassium tartarate	9.0g
(in 500ml of 0.2M/L Sodium hydroxide)	
Sodium iodide	5.0g

Make up to 1L with 0.2mol/litre Sodium hydroxide

ANNEXURE IV

Color reagent A

Sodium carbonate	40.0g (in 600ml water)
Glycine	16.0g
Copper sulphate pentahydrate	0 .450g

Make up to 1L with water

ANNEXURE V

Color reagent B

Neocuprine-HCl	1.2g

Make up to 1 L with water and store at 4^0C in a brown bottle

ANNEXURE VI

.5% Polygalacturonic acid substrate

Heated 500ml Phosphate buffer on hot plate. While heating slowly 2.5g polygalacturonic acid is slowly added. Heated and stirred until dissolved, cooled and stored at 4^0C.

ANNEXURE VII

Alkaline Copper tartarate

(A) Anhydrous sodium carbonate 2.5g
Sodium bicarbonate 2.0g
Potassium sodium tartarate 2.5g
Anhydrous sodium sulphate 20.0g
Dissolved in 80 ml water and make up to 100 ml

(B) Copper sulphate 15g.

Dissolved in small amount of water added 1 drop of Sulphuric acid and make up to 100ml. Mix 4mlof B and 96 ml of A.

ANNEXURE VIII

Arsenomolybdate reagent
Ammonium molybdate 2.5g
Sulphuric acid 2.5ml
Disodium hydrogen arsenate 0.3g(dissolved in 25ml water)
Mixed well and incubated at 37^0C for 24 to 48 hours

ANNEXURE IX

Biosafety precaution while performing experiments

1. Hand gloves and mouth covers were worn while performing all fungal experiments in sporulating rooms.
2. Sporulating room was properly fumigated before performing experiments.
3. Discarded the fungi by properly autoclaving the flasks after performing experiments.

Precautions taken during Nelson Somogyi method

1. Mouth covers and hand gloves were worn while handling dihydrogen sodium arsenate, as it is very carcinogenic.
2. Mouth pippeting was completely avoided.

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