# Pectolytic enzymes activity test from *Aspergillus awamori* strain (MTCC NO.-9644, 1344) using two different substrates in Solid State Fermentation

**Enrollment No.:111816, 111119**
**Name of Students: Sahil Jaswal, Akhil Rana**
**Name of the Supervisor: Dr. Garlapati Vijay Kumar**

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# CERTIFICATE

This is to certify that the work presented in this dissertation entitled **"Pectolytic enzymes activity test from *Aspergillus awamori* strain (MTCC NO.-9644, 1344) using two different substrates in Solid State Fermentation"** was carried out by **Akhil Rana and Sahil Jaswal** at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat (Solan) under my supervision towards the fulfillment of her Bachelor of Technology in Biotechnology. It is also certified that no part of this dissertation has been submitted elsewhere for the award of any degree or diploma.

**(Dr. Garlapati Vijay Kumar)**
Department of Biotechnology and Bioinfomatics
Jaypee University of Information Technology
Waknaghat, HP-173234, India.

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Name of students-Sahil Jaswal

Akhil Rana

## SUMMARY

Pectinolytic enzymes are widely produced by fungal and certain bacterial species to degrade food materials and extract nutrients out of them. Fungal strains hydrolysis pectic substances in their natural habitat through pectolytic enzymes. In this study, we have used two strains of Aspergillus awamori for production of pectinolytic enzyme in solid state fermentation. We have used two different substrates preparation:Apple pomace and Kinnow pomace in SSF process. Substrates were prepared by mixing with 1% rice husk, aftere which, substrate mixture was dried and grinded into smaller appropriate sized particles for carryinf SSF. The polygalactorunase enzyme produced is tested for its enzyme activity for both substrates growing in both of substrates under optimized conditions. After getting results of enzyme activity in normal recommended conditions, we tested for optimization of enzyme activity in two different substrates in different parameters of SSF for both strains. Enzymes were partially purifies by centrifugation after extracting crude extract from all testing samples. Centrifuged samples were then tested for absorbance and total reduced substances were tested using DNS test. BY getting or noting absorbance, total enzyme activity for all samples were formulated. Relative efficiency was known by carrying enzyme activity of enzyme extract from different test samples having different combinations of SSF parameters.


| **Signature of Student (s)** | **Signature of Supervisor** |
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| Date: | Date: |

# CHAPTER 1
# INTRODUCTION

## 1.1 Role of Industrial Biotechnology in Fruit Juice Industries

The increasing energy demands have focuses worldwide attention on the utilization of renewable resources, including agricultural and forest waste and residues. Major components of these wastes includes cellulose, starch, lignin and pectin. These materials have attracted various industries mainly including enzyme industries and juice or wine production industries. Several microbes are capable of using these waste substrates to produce various enzymes of industrial importance. Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations.

The production of fruit and vegetable juices is important both from the human health and commercial standpoints. The availability of nutritious components from fruits and vegetables to a wide range of consumers is thus facilitated throughout the year by the marketing of their juices. The production process of fruit and vegetable juices includes steps like extraction, clarification, and stabilization. Fruit contains pectin and other polysaccharides, so it may lead to fouling during filtration through membrane. Enzymatic treatment leads to degradation of pectin. Enzymatically clarified juice results in viscosity reduction and cluster formation, which facilitates separation through centrifugation or filtration. As a result, the juice presents higher clarity, as well as more concentrated flavor and color (Kaur et al., 2004).

Fruit juice clarification is done to maintain uniform product quality and for better juice concentration without gelling. It is done by degrading cell wall components and pectic substances of fruit colloidal particles in juice. Enzymes like pectinases, pectoglucoranases, xylanases are used which are isolated from cultures of various fungal strains and bacterial strains. In the juice production process, compounds such as pectin, fibers, semi-fibers, starch and proteins are needed to be clarified. However, the traditional process is an energy and time consuming task. By adopting membrane filtration technology, impressive yields and significant soluble solids removal rate can be achieved,

giving producers a more environmentally-friendly and a more profitable process application. Hyflux is the leading solution provider in the juice clarification industry with highly competitive membrane filtration products.

Fruit juice clarification is an important step in today's juice industry as juice with no pulp is preferred mostly. Pulp particles are basically removed by using enzymes such as pectin and so DO they avoid the coagulation of bittering particles within the juice. Fruit juice clarification is an important part of today's juice manufacturing companies . Except for citrus juices, most industrially processed fruit based beverages, including both fruit juices and wines, are clarified during processing in order to avoid undesirable turbidity, haze, and sediments in the final products. Visual perception of turbidity and haze in fruit juices is the result of light scattering caused by suspended substances. The immediate turbidity in freshly pressed fruit juices is generally considered to be a result of suspended pectin particles stemming from the plant cell walls, but other disrupted cell wall and cell materials may also contribute to juice turbidity.In contrast, the development of turbidity during cold storage, usually referred to as haze formation, is assumed to be caused by interactions between haze-active proteins and polyphenols that form insoluble multi-molecular structures.

Current juice clarification strategies, including those used in the large scale processing of e.g. apple, pear, black currant, cherry, cranberry, and grape juices, have been designed according to this comprehension. Industrial juice clarification procedures for these juices thus typically involve enzyme catalyzed depectinization and fining by addition of pectinases, gelatin. These treatments are followed up by filtrations and/or centrifugation. The turbidity-reducing action of pectinases is assumed to work mainly via electrostatic destabilization of suspended, negatively charged, pectin particles. The turbidity-causing pectin particles are otherwise retained in suspension as a result of charge repulsion between the particles. Upon enzymatic pectin hydrolysis their presumed proteinaceous core, which is positively charged even at the relatively low pH of juices, is exposed to react with the negatively charged pectin. Once this occurs to a significant extent, agglomeration occurs and the particles may be removed by centrifugation or filtration of

the juice. The fining treatment with gelatin–silica sol and/or bentonite presumably works by allowing unspecific binding of both haze-active polyphenols and proteins during the formation of colloid flocs . With gelatin–silica sol treatment additional cloud causing substances, e.g. pectin, pectin agglomerates, and other cell wall fragments, may be captured during the slow settling of the viscous, colloidal flocs that are formed mainly by the gelatin . The gelatin–silica sol step is slow because the settling of flocs may last for 6–18 h . Another problem with this treatment is that it subsequently requires use of vacuum rotary filtration systems involving use of diatomaceous earth as a filtering aid or sludge frame filters with kieselguhr to clarify and recover the juice . Both of these types of filtering aids are considered harmful and require special handling and disposal procedures. In the fruit processing industry cherry juice is known to be particularly mischievous in relation to incomplete sedimentation of the colloidal particles, which frequently results in prolonged processing times and juice losses. These problems have created a need for development of alternative juice clarification strategies, particularly with respect to cherry juice clarification.

Despite the mature state of the fruit juice industry, detailed knowledge about the components, precursors, and mechanisms involved in turbidity and haze formation in dark, berry fruit juice types, including cherry juice, is surprisingly scarce and only few studies have evaluated alternative juice clarification treatments. demonstrated that addition of a fungal acid protease to kiwifruit juice decreased the immediate turbidity and retarded the haze formation during cold storage. It have been found that treatment of cherry juice with an acid-stable protease, a pectinase, and Gallic acid improved cherry juice clarity and diminished haze levels during cold storage. The present study was undertaken to examine the efficiencies of various alternative clarification strategies on cherry juice clarification and notably to obtain further insight into the events and the possible causes of cherry juice turbidity and haze. This was done by monitoring pectin, protein, and phenols levels during clarification treatments and cold storage of the juice. We particularly targeted the application Enzymes hydrolyzing pectic substances, which contribute to the firmness and structure of plant cells, are known as pectin lytic enzymes or pectinases. Based on their mode of action, these enzymes include polygalacturonase

(PG), pectin esterase (PE), and lyases (pectinase (PL) and pectatelyase (PAL)). PG, PL, and PAL are depolymerizing enzymes, which split the α-(1,4)-glycosides bonds between galacturonic monomers in pectic substances either by hydrolysis (PG) or by β-elimination (PL, PAL). PG catalyzes the hydrolytic cleavage of the polygalacturonic acid chain while PL performs a trans eliminative split of pectin molecule, producing an unsaturated product. PE catalyzes the de-esterification of the methyl group of pectin, forming pectic acid. There are two types of PGases with different technological applications: exopolygalacturonases (exo-PG) that break down the distal groups of the pectin molecule, reducing chain length relatively slowly, and endopolygalacturonases (endo-PG) which act randomly on all the links in the chain, reducing molecular dimensions and viscosity more rapidly. Pectinolytic enzymes play an important role in food technology, mainly in the processing of fruit juices and wines and in the maceration of plant tissue. Maceration is a process by which organized tissue is transformed into a suspension of intact cells, resulting in pulpy products used in the food industry for the production of fruit nectars as pears, peaches, apricots, strawberries, and vegetables mashed such as potatoes, carrots, red pepper, and others that are used in babies and seniors foods. For such purposes, only the intercellular cementing material that holds together cells and some portion of primary plant cell walls should be removed without damage to adjacent secondary cell walls, to help avoid cell lysis, keeping nutritional properties of food. For this reason, cellulases in the enzyme mixture are undesirable.The stability of pectinases is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators).

Enhancing the stability and maintaining the desired level of activity over a long period are two important points considered for an efficient application of these enzymes.Pectinases used in the food industry are commercially produced by Aspergillus niger. Commercial preparations of fungal origin contain a complex mixture of different enzymes with pectinolytic activity, including PGases, lyases, the undesirable PE, and others enzymes. Yeasts have advantages compared to filamentous fungi, because they are unicellular, the growth is relatively simple, and usually yeasts do no secret PE. A yeast isolated from citrus fruit peels in the province of Misiones (Argentine) and identified as

Wickerhamomyces anomalus, recent reclassification of the species Pichia anomala, produced pectinolytic enzymes in liquid medium containing glucose and citrus pectin as carbon and energy sources and inductor, respectively. In the present work, enzymes produced by this wild yeast strain were characterized, and physicochemical properties of polygalacturonase were determined by the study of the effect of temperature and pH on its activity and stability, in order to evaluate the application of the supernatant in the maceration of potato tissues. Microbial production of pectinolytic enzymes is mainly from filamentous fungi, yeasts and filamentous and non-filamentous bacteria and is produced in two different techniques viz; submerged fermentation (SmF) and solid-state fermentation (SSF). SSF permits the use of agricultural and agro-industrial residues as substrates for enzyme production. As these residues are renewable and in an abundant supply, they represent a potential low cost raw material for microbial enzyme production.

**1.2 Pectinolytic enzymes:**

Pectinases are an enzyme group that catalyzes pectic substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. The well-known pectinolytic enzymes are homogalacturonan
degrading enzymes.

**a)Protopectinases**

Protopectinases solubilize protopectin forming highly polymerized soluble pectin. They are classified into two types: one reacts with the polygalacturonic acid region of protopectin, A type; the other with the polysaccharide chains that may connect the polygalacturonic acid chain and cell wall constituents, B type.

**b)Pectin Methyl Esterases (PME)**

Pectin methyl esterase or pectinesterase catalyzes deesterification of the methoxyl group of pectin forming pectic acid and methanol. The enzyme acts preferentially on a methyl ester group of galacturonate unit next to a non-esterified galacturonate unit. It acts before polygalacturonases and pectate lyases which need non-esterified substrates. It is classified into carbohydrate esterase family 8.

**c)Pectin Acetyl Esterases (PAE)**

Pectin acetyl esterase hydrolyses the acetyl ester of pectin forming pectic acid and acetate. It is classified into carbohydrate esterase families 12 and 13.

**d)Polymethylgalacturonases (PMG)**

Polymethylgalacturonase catalyzes the hydrolytic cleavage of 1,4-glycosidic bonds in pectin backbone, preferentially highly esterified pectin, forming 6-methyl-D-galacturonate.

**e)Polygalacturonases (PG)**

Polygalacturonase catalyzes hydrolysis of □-1,4-glycosidic linkages in polygalacturonic acid producing D-galacturonate.It is classified into glycosyl-hydrolases family 28. Both groups of hydrolase enzymes (PMG and PG) canact in an endo- or exo- mode. Endo-PG and endo-PMG catalyze random cleavage of substrate, exo-PG and exo-PMG catalyze hydrolytic cleavage at substrate non reducing end producing monogalacturonate or digalacturonate in some cases.

**f)Pectin Lyases (PL)**

Pectin lyase catalyzes the random cleavage of pectin, preferentially high esterified pectin, producing unsaturated methyl oligo galacturonates through trans elimination of glycosidic linkages. PLs do not have an absolute requirement of Ca2+ but they are stimulated by this and other cations. Up until now, all described pectin lyases are endo-PLs. Van Alebeek and coworkers conducted a detailed study of the action mode of pectin lyase A from *Aspergillus niger* which produces mono-, di-, tri- and tetragalacturonates, besides unsaturated di-, tri- and tetragalacturonates from methyl oligo galacturonates. Unsaturated monogalacturonates were not identified in the reaction products in any assay. Complete degradation of pectin substrate still requires enzymes that cleave the rhamno galacturonan chain.

**g)Rhamnogalacturonan Rhamnohydrolases**

RG rhamnohydrolase, rhamnogalacturonan Lrhamnopyranohydrolase or L-rhamnosidase catalyzes hydrolytic cleavage of the rhamnogalacturonan
chain at nonreducing end producing rhamnose. These enzymes are classified into glycosyl-hydrolase families 28, 78 and 106.

h) **Rhamnogalcturonan Galacturonohydrolases**

RG galacturonohydrolase catalyzes hydrolytic cleavage of the rhamnogalacturonan chain at nonreducing end producing monogalacturonate . It is classified into glycosyl-hydrolase family 28.

**i)Rhamnogalacturonan Hydrolases**

RG hydrolase randomly hydrolyses the rhamnogalacturonan chain producing oligogalacturonates.

**j)Rhamnogalacturonan Acetylesterases**

RG acetylesterase catalyzes hydrolytic cleavage of acetyl groups from rhamnogalacturonan chain. It is classified into carbohydrate esterase family 12.

**k) Xylogalacturonan Hydrolase**

Xylogalacturonase (EC 3.2.1.-) catalyzes hydrolytic cleavage of glycosidic linkages between two galacturonate residues in xylose-substituted rhamnogalacturonan chain, producing xylose-galacturonate dimers . These enzymes are classified into glycosyl-hydrolase family 28 .

### 1.2.1 MICROBIAL PECTINASES

Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, insects, nematodes, protozoan and plants. Microbial pectinases are important in the phyto pathologic process, in plant-microbe symbiosis and in the decomposition of dead plant material, contributing to the natural carbon cycle. Pectinases are abundantly produced by saprophytic fungi, and decaying plant tissue represents the most common substrate for pectinase-producing microorganisms. Plant attack by pathogenic microorganisms usually starts by pectinolytic enzymes attack since pectic substances are more accessible than other fibers in plant tissue.Several studies on microbial enzymes have shown the production of multiple pectinase forms which differ on molecular mass and kinetic properties. The production of multiple forms of enzymes improves the microorganism ability to adapt to environmental modifications. The cell wall is the first defense line of plants, having a crucial role in preventing infections. It consists mainly of the large biopolymers cellulose, hemicellulose, lignin and pectin; however, the composition can change according to the age and physiological conditions of the plant. In

such case, the pathogenic microorganism needs to produce a pool of enzymes to successfully infect the plant tissue.Pectic substances are rich in negatively charged or methyl-esterified galacturonic acid. The esterification level and the distribution of esterified residues along the pectin molecule change according to the plant life cycle and betweendifferent species. Thus, the ability of some phytopathogenic microorganisms to poduce a variety of pectinolytic enzymes that differ in their characteristics, mainly in their substrate specifity, can provide them with more efficacy in cell wall pectin degradation and consequently more success in the plant infection. Several polygalacturonases can be expressed from only one gene or from some different genes. Caprari and coworkers reported the production of four endo-polygalacturonases by the pathogenic fungus *Fusarium moniliforme* that differ in the extent of glycosylation of the same polypeptide arisen one gene. On the other hand, the polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. The PG isozymes present significant differences in substrate specificity, kinetic parameters and optimum pH. The polygalacturonases isozymes produced by the phytopathogenic fungus *Botrytis cinerea* have been isolated by using different fermentation conditions. Most of the plant cell wall degrading enzymes are encoded by a large multigenic family showing diverged expression pathways suggesting functional specialization. These genic families result from gene duplication and are linked in tandem.Since pectin cannot enter the cell, it has been suggested that compounds structurally related to this substrate might induce pectic enzyme productions by microorganisms. Low levels of constitutive activities may attack the polymeric substrate and release low molecular products which act inducers.

(SSF) have been successfully used in pectinase production by fungi and by bacteria. Submerged fermentation is a well developed system used in industrial scale to produce a large variety of microbial metabolites. SmF is technically easier than SSF and has been strongly developed from the 1940s onwards because of the necessity to produce antibiotics in large scale. On the other hand, solid state fermentation provides higher productivity due to larger enzyme yields. SSF processes simulate the living conditions of many higher filamentous fungi. Ascomycetes, basidiomycetes and deuteromycetes developed in terrestrial habitats on wet substrates. Higher fungi and their enzymes, as

well as spores or metabolites, are well adjusted to growth on solid wet substrates. For instance, fungal spores produced by SSF show higher stability, are more resistant to drying and exhibit higher germination rates for extended periods of time after freeze-drying than do spores produced by SmF. Solid state fermentation has only found restricted applications in processes using unicellular organisms. Despite the advantages, the industrial application of SSF is, at least at present, hard to envisage. The main obstacles are the low amenability of the process to regulation, the strongly heterogeneous fermentation conditions and the ensuing frequently unsatisfactory reproducibility of the results, difficult scale-up, the often unfeasible biomass determination and complicated product purification by downstream processes resulting from the use of heterogeneous organic growth substrates.

### 1.3 Enzyme production

Enzyme production is an important field of biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the use of the solid-state fermentation (SSF) technique to produce a wide variety of enzymes.SSF in comparison to SmF have shown higher productivities when using fungal strains for enzymes production.SSF has marked advantages over SmF in terms of productivity, concentration of the product and effluent generation. Besides higher product titres, low capital and recurring expenditure, lower waste water output, reduced energy requirement, absence of foam formation, it is simple and highly reproducible. These fermentation need simpler fermentation media, less fermentation space, absence of rigorous control of fermentation parameters, easier aeration, is economical to use even on smaller scale, have easier control of contaminants, applicability of using fermented solid directly, storage of dried fermented matter and lower cost of downstream processing. Also, the fermentation time is shorter and the degradation of the produced enzymes by undesirable proteases is minimized. SSF is a microbial process occurring mostly on the surface of solid materials, which can absorb or contain water, in the presence or absence of soluble nutrients. SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi, since they reproduce the natural living conditions of such fungi due to which they may be more

capable of producing certain enzymes with high productivity in comparison to SmF. SSF is a very promising cultivation technique for the production of industrially-relevant enzymes such as pectinase and polygalactorunase.

## 1.4 PECTIC SUBSTRATES:

Pectic substances are complex high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues. Three major pectic polyssacharides groups are recognized, all containing D-galacturonic acid to a greater or a lesser extent.

### 1. 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 % of the carboxyl groups methylated; pectinic acid has less than 75 % of the carboxyl groups methylated; pectic acid or polygalacturonic acid has no methyl esterified carboxyl groups. Frequently the word pectin is used as a generic name for pectic substances.

### 2. 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.

### 3. Rhamnogalacturonan II (RGII)

Despite its name, RGII is a homogalacturonan chain with complex side chains attached to the galacturonic residues.Vincken and coworkers have proposed a pectin molecule structure model in which HG and RGII are long side chains of RGI backbone. 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 microfibrils conferring rigidity on cell walls. During ripening the fruit enzymes alter the pectin structure by breaking the pectin backbone or side chains, resulting in a more soluble

molecule. Pectic subsances 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. 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 . Pectic polysaccharides have been used as bioactive food ingredients and as detoxifying agents. It is an adequade infant food supplement.

## CHAPTER 2
# REVIEW OF LITERATURE

### 2.1 Fungal Enzyme Production:

Enzyme production is a growing field of biotechnology. Annual world sales figures are close to a billion dollars with increasing number of patents and research articles related to this field. Most enzyme manufacturers produce enzymes using submerged fermentation (SmF) techniques with enzyme titers in the range of grams per liter. Such levels are a prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in a fermentation broth . There is, however, a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, mainly from mold origin, as indicated by the growing number of research papers in the literature and the marketing and development by a small but visible number of fermentation industries . Among the advantages for SSF processes it is often cited that enzyme titers are higher than in SmF, when comparing the same strain and fermentation broth. However, there is a scarcity of papers regarding physiological studies comparing SSF and SmF when trying to explain why microorganisms produce higher titers in the first kind of process as compared to the second. This lack of information makes difficult any assessment regarding the value of one process versus the other, thereby hindering the fundamental approach to process optimization and design for SSF technique in areas such as strain improvement, solid substrate engineering and process control (Singh A, Kumar S and Sharma HK (2012)).

(Romero-Gómez et al.) published data showing that three separate strains of *A. niger* produced higher titers of invertase and had higher observed productivity, $\Gamma$ obs, when cultured by SSF technique as compared to SmF technique.Díaz-Gód´ınez et al. have shown that *A. niger* produces much higher titers and have higher productivity in SSF system than in SmF system.Aguilar et al. studied tannase production by *A. niger* grown on finely ground samples of PUF (density of 113 g $l^{-1}$) with the purpose of measuring enzyme productivity by SSF under conditions where growth was limited by steric hindrances. This work was different to previous reports by Romero-Gómez et al.and

D´ıaz-God´ınez et al. who used regular commercial samples of PUF (density of 15 g l−1). Aguilar et al. compared SSF production of tannase grown in ground PUF to SmF production in shake flasks. Tannase titers were found to be much higher in SSF (14,000Ul−1) than in SmF (2800Ul−1).All of these certain researches have had shown the productivity and other economical benefits of SSF over Smf especially for production of fungal enzymes.Also the environmental factores of SSF resembles fungal cells natural habitat, which makes it easier for them to adapt and grow more efficiently and effectively.SSF culture seems to be working, in a natural way, as a fed batch culture with fast oxygenation but slow sugar supply. The process has the added advantage of being a static process without mechanical energy expenditures. In contrast, SmF cultures work as homogeneous systems requiring large energy expenditures to supply oxygen at fast enough rates to cope with high oxygen demand. There is the need in such processes for automated fed batch supply of substrates in order to avoid catabolite repression. Further study of SSF systems may profit from the use of microscopic techniques such as image analysis, microelectrodes, development of DNA probes with optical properties such as in situ hybridization of specific RNA messengers and the use of colored fusion proteins used as tags of specific protein products. Perhaps this way, heterogeneity of SSF systems will be transformed to its advantage to control microbial activity for enzyme production as compared to the handicap of homogeneous systems present in SmF cultures.

### 2.2 Pectinolytic enzymes production (Polygalactorunase)

The biotechnological potential of pectinolytic enzymes is well known due to their various industrial applications wherever degradation of pectic substances is required. This includes food related processes like fruit juice clarification, tissue maceration, wine clarification, coffee and tea fermentation and many others (Kashyap et al. 2001). It has been reported that pectinases have a share of almost 5% of global enzyme sales. Commercial pectinases used in food industry normally contain a mixture of enzymes that split pectic compounds; which traditionally includes PG (polygalacturonase), PL (pectin lyase) and PME (pectin methylesterase). Pectinolytic enzyme production occupies about 10% of the worldwide manufacturing of enzyme preparations. Industrial production of microbial pectinolytic enzymes is mainly done by filamentous fungi, especially

Aspergillus niger (Naidu and Panda 1998). Aspergillus species produce a large number of enzymes particularly involved in the degradation of pectic substances. However, the variety of enzyme sets differs between fungal species (Benoit et al. 2012). The koji molds, Aspergillus oryzae and Aspergillus sojae, are often associated with a long history of safe use in traditional food fermentations and several Aspergillus-derived food additive products have already obtained a GRAS (generally recognized as safe) status from regulatory authorities, which supports the potential of these strains as production organisms for pectinolytic enzymes used in food industry. Filamentous fungi are known to produce pectinolytic enzymes in submerged fermentation (SmF), as well as in solid-state fermentation (SSF) processes. Degradation and utilization of diverse biopolymers such as starch, cellulose or pectin enables cultivation of Aspergillus species on agricultural and agro-industrial residues, which can be used as low-cost substrates for microbial enzyme production in SSF processes (Nigam and Pandey 2009). The application of agricultural and agro-industrial by-products, such as apple pomace or sugar beet pulp, offers a wide range of alternative substrates and helps to solve disposal problems of these by-products. The apple pomace is mainly composed of insoluble carbohydrates such as cellulose, hemicelluloses and lignin. Traditionally, apple pomace and citrus peels are used as raw materials for pectin production. Citrus peels consist of approximately 24% of pectic substances on α-□-galacturonic acid basis. Alternatively, high content in pectins (20-25%), its availability and low-cost make sugar beet pulp a potential source of pectins. Sugar beet pulp is mainly composed of (% on dry basis) pectin, 28.7; cellulose, 20; hemicellulose, 17.5; protein, 9.0; and lignin 4.4. Since the pectin content of beet pulp or apple pomace is high it can be used for the microbial production of pectinolytic enzymes without adding pectinaceous material as inducer. However, the most potent solid agro-industrial by-product for biotechnological pectinolytic enzyme production, used in combination or without pectinase inducers, is wheat bran, which is composed predominantly of non-starch carbohydrates, starch and crude proteins. Utilization of low-cost agro-industrial residues offers potential benefits for SSF, which is attractive for implementation of sustainable bioprocesses. Further advantages of SSF processes are lower energy requirement associated with higher product yields and less wastewater production with lesser risk of bacterial contamination.

Nevertheless, there are many factors which have a critical influence on the process development in SSF, such as selection of microorganism and substrate or optimum physio-chemical and biological process parameters. In view of this, optimization of the SSF process for PG production by A. sojae was targeted applying statistical design tools. A previous study demonstrated already the potential of A. sojae for pectinase production in SSF. A further study about application of strain improvement methods revealed the increase of pectinase production by A. sojae applying a classical mutation and selection strategy. Carbohydrate active enzymes of the pectinase enzyme-complex were investigated comparing the production by two fungal strains of A. sojae under optimized conditions and characterizing the pectinolytic enzyme sets on the basis of their substrate degrading mode. Therefore, experiments were conducted comparing PG production with both inducer substrates at similar moisture levels, which was achieved by freeze-drying apple pomace or increasing the moisture content in sugar beet pulp. Higher PG activity was obtained utilizing sugar beet pulp, which was chosen as inducer substrate for further optimization investigations. Moreover, longer incubation time indicated further increase of PG activity, which was considered for optimization of this parameter. In addition, further HCl concentrations were tested in the range from 0.175 M to 0.2 M, which resulted in a positive effect on PG production applying HCl concentrations of about 0.2 M (data not shown). The combination of a cultivation medium including predominantly wheat bran and applying the moisture level with 0.2 M HCl concentration was already demonstrated for enhanced microbial enzyme production by filamentous fungus. PG production by A. sojae was optimized applying crude plant compounds, such as wheat bran and sugar beet pulp, as substrate in SSF. The present study demonstrated a great potential for cost-efficient pectinolytic enzyme production by Aspergillus sojae ATCC 20235. Production under optimized conditions in laboratory scale yielded high exo-PG activity and sugar beet pulp was identified as significant pectinase inducer substrate. Utilization of agricultural and agro-industrial by-products developed an attractive sustainable bioprocess for enzyme production.Optimized pectinolytic enzyme production yield of A. sojae ATCC 20235 was 6.9 times higher in comparison to A. sojae CBS 100928. High enzyme yield obtained by A. sojae ATCC 20235 under optimized

conditions will be a promising starting point for scale-up and Polygalacturonase purification studies.

### 2.3 Pectic enzymes and fruit juice industry

Pectic substances are responsible for the consistency, turbidity and appearance of fruit juice. In fact, the presence of pectic substances in fruit juices causes a considerable increase in their viscosity, thereby impeding the processes of filtration and subsequent concentration.Pectic enzymes have long been used to increase juice yield and to clarify juices. Actually, most pectic enzyme preparations are used in the fruit processing industry. Considering that pectic enzymes alone account for about one-quarter of the worlds food enzyme production.Fruit juice clarification, a process required to facilitate filtration and remove turbidity, is the oldest use of pectic enzymes. Degradation of pectin by these enzymes facilitates pressing and ensures high yields. After crushing soft fruits such as blackcurrants, raspberries and black cherries, these crude juices are often very viscous and the remaining solids very difficult to separate from the juice. The activity of pectic enzymes causes a drop in viscosity making it possible to extract juice by pressing. Pectic enzymes are also applied in the maceration and solubilization of fruit tissues to retain integrity of the cell wall in a process called liquefaction of tissues.Another relatively recent use of pectic enzymes is the selective treatment of citrus concentrates with endopolygalacturonase to give limited hydrolysis of the pectins so as to reduce viscosity (citrus juices tend to gel when concentrated).

### 2.4 Fruit Juice clarification:

Traditionally, the industrial utilization of pectic enzymes in fruit juice processing has been conducted in conventional batch reactors using soluble enzymes. Unfortunately, after each cycle of operation the enzymes can not be recovered for further use, and are inevitably present in the final product altering organoleptic properties. In this context, the immobilization of pectolytic enzymes has proven to be very advantageous for continuous industrial use. However, relatively little research has been carried out on the immobilization of pectic enzymes. Several authors have already reported the different

advantages that the immobilization of pectic enzymes offers. Most studies dealing with immobilized pectic enzymes can be classified into two groups :
(a)those concerning the immobilization of the whole complex of pectic enzymes, and (b) those showing the immobilization of separate purified pectic enzymes which were further studied for the effect of immobilization on their properties and mode of degradation of polymeric substrates.

In spite of this amount of research on immobilized pectic enzymes, the procedures proposed still show low immobilization yields, high support costs and methods too elaborate to be easily applied on an industrial scale. In fact, Alkorta ***et al.*** in their studies on nylon immobilized pectin lyase from P ***italicurn*** found relatively low percentage of activity, probably due to the existence of diffusional constraints that impeded both substrate access to the enzyme and the release of the product into the bulk phase where it was actually being monitored. Nonetheless, there are in the literature some positive results suggesting the vast potential of immobilized pectic enzymes for the clarification of fruit juices and other similar applications. For instance, Alkorta *et al.* reported that, in spite of the low yield values, the nylon-immobilized pectin lyase was capable of producing a 36% reduction in viscosity in a pectin solution (compared to a 46Y o viscosity reduction found with the free soluble enzyme), a value considered acceptable for the commercial application of this immobilized system in fruit juice processing. The main purpose of the clarification treatment employed in industria lapple juice processing is to eliminate constituents responsible for the turbidityand cloudiness in freshly produced juice. Application of ultrafiltration (UF) as an alternative to conventional processes for the clarification of fruit juices is gaining acceptance in the industry. However, these membrane techniques partially substituted the use of specific enzymes , and the adequacy of UF as a technique for preventing haze formation still requires further studies. The other important purpose of clarification is to remove substances that may cause haze and sediment formation during storage, at reconstitution of the concentrate or after bottling of the juice. Apple juice is one of the juices that may contain considerable amounts of starch, particularly at the beginning of the harvest season. Unripe apples contain as much as 15% starch, and up to 1% starch may be present in juice after milling and pressing. Starch is a common problem for apple juice processors, complicating

filtration and causing postprocess cloudiness.The process of depectinization involves the use of commercial enzymes,generally a blend of pectinases (e.g., pectinase, polygalacturonase, cellulase,pectin lyase) to degrade pectic substances. Starch-degrading enzymes such as amylase and amyloglucosidase are also commonly added during the depectinization stage. These enzymes degrade starch into smaller units and may contribute to postbottling haze formation by aggregating among themselves or through the formation of protein–starch complexes. Fining of apple juice also involves the use of gelatin and bentonite as fining agents (Stocké 1998). Differences in the nature of ionic charges of protein, polyphenols and the fining agents induce flocculation and sedimentation and result in the removal of these potential haze precursors from juices. Turbidity of opalescent apple juice is provided by particulate material that remains in suspension. The cell wall of fruit comprises a complex mixture of cellulose, hemicelluloses, pectins and proteins, which incorporate to pressed juice in different ways, providing cloudiness.

**Objectives of the present study were as follows:**

1. Culturing and sub culturing of fungal strains of *Aspergillus awamori* :
   MTCC: 9644 and 1344.
2. Screening of appropriate substrates for solid state fermentation by fungal strains and preparation of solid waste substrates for SSF.
3. Polygalactorunase and pectinolytic enzymes production in solid state condition
4. Polygalactorunase assay in previous recommended optimized conditions of SSF.
5. Process parameters optimization for polygalactorunase production in solid state fermentation.

CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials and Chemicals:

- Substrates: Apple Pomace and Kinnow Pomace having 1% rice bran in each of them. Substrates were procured from local market.
- Media constituents were procured from Himedia Laboratories (Mumbai, India) and all other chemicals used were analytical grade and obtained from SRL (Mumbai).
- Equipments used:
  Autoclave,Weighing balance,Spectrophotometer; Water bath, Centrifuge ,Incubator shaker ,Cold storage room, Incubator ,Laminar air flow unit

**Media preparation:**

**1. PDA medium**

| Ingredients amount in | (g/l) |
|---|---|
| Potatoes infusion from potatoes | 200.00 |
| Dextrose | 20.00 |
| Agar-agar | 15.00 |

**2. Potato Dextrose Broth**

| Ingredients amount in | ( g/l) |
|---|---|
| Potatoes infusion from potatoes | 200.00 |
| Dextrose | 20.00 |

- **DNS preparation:**

| **Ingredients** | **amount in g/l** |
|---|---|
| Distilled water | 1000.0 ml |
| 3, 5 Dinitrosalicylic acid | 10.6 g |
| NaOH | 19.8 g |

**3.3 Substrates preparation:**

Two substrates were procured from the market : Apple pomace and Kinnow pomace with 1percent rice husk each Substrates were mixed with rice husk and dried at 70degree celcius for 48hours in hot air oven . After drying the substrates were grinded in electric mixer and sieved .

| Kinnow pomace | Apple pomace |
|---|---|

Fig 1 Substartes used for pectinolytic enzymes

## 3.4 Methodology

### 3.4.1 Revival and Culturing of procured strains of *Aspergillus awamori* from IMTECH(MTCC):

*Aspergillus awamori* strains MTCC no.:9644 & 1344 were procured from IMTECH, Chandigarh and were revived on selective Czapex dox medium. Czapex dox medium was used as it is acidic in nature, provides protection from any bacterial growth. The strains were cultured for 7 and 5 days respectively in broth medium. And revived cultures were further subcultured in Potato Dextrose Broth. Some sub-cultures of fungal strains were carried on slants made of potato dextrose agar. After subculturing, the glycerol stocks of strains were also taken for storage purpose.

### 3.4.2 Solid state fermentation using Aspergillus strains:

5mg of each substrates preparations were taken in 2 flasks each. 10ml of czapex dox broth was added in the flasks for some moisture.The subcultured strains of A. awamori were added in each substrate(1ml). That is, each strain was added in both of the substrates.The flasks were then kept under recommended conditions of 30 degree celcius for 7 days. Flasks were checked each day for growth.

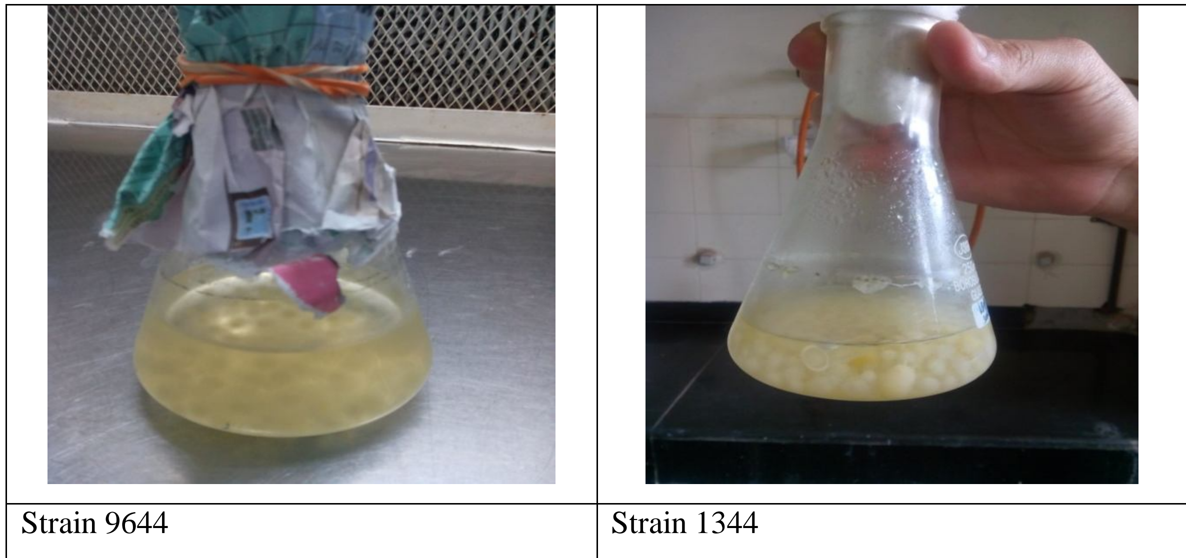
### 3.4.3 Selection of parameters for production of polygalacturonase through solid state fermentation:

8 flasks were taken and half of them were containing one kind of substrate and other half containing second substrate. 4 of the flasks were inoculated with first strain and other four from second strain.The enzyme extract was taken from each flask after particular regular interval for five days.Absorbance was taken and optimization of enzyme activity in SSF conditions was checked.

We took eight different samples containing both of the substrates in half of them respectively. Four of the flasks were kept at 30degree celcius and other four at 35 degree celcius.The enzyme extract was taken from different samples at different intervals of time to check the optimized conditions and parameters for different samples and strains to produce Polygalactorunase enzyme.

# CHAPTER 4
# RESULTS AND DISCUSSION

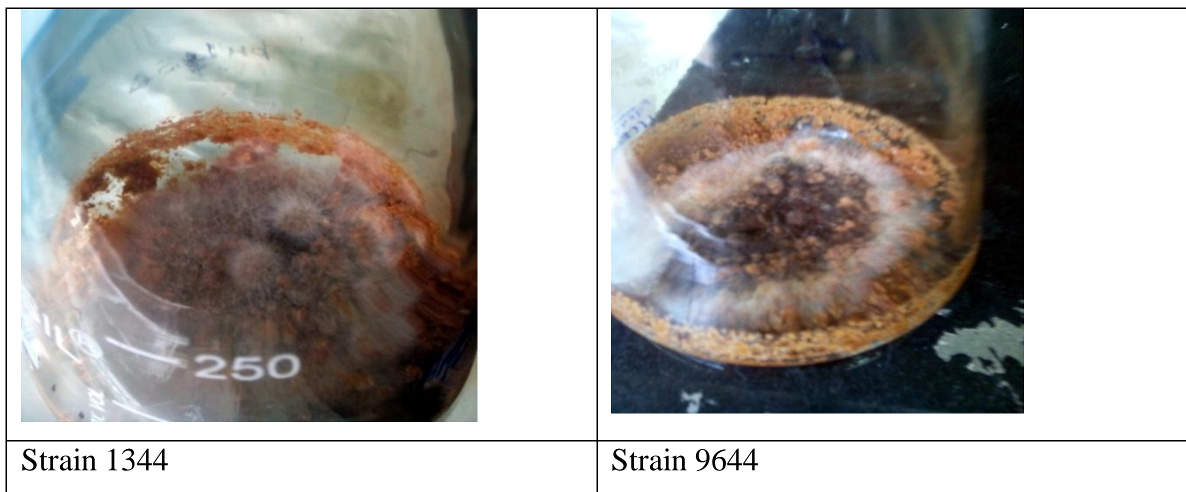
**1.Sub culturing:** 9644 and 1344 were revived in czapex dox broth and subcultured in PDB for 7 days +7days.



| Strain 9644 | Strain 1344 |
|---|---|

It was observed that the strain no. 9644 were having black mold, having cilia and the growth was slow whereas in the case of strain no 1344,it was observed that fungi had white mold, no cilia growth and the growth was faster.

2.**Fermentation**:substrate was added into the flasks and inoculated with subcultures and left for 1 week.



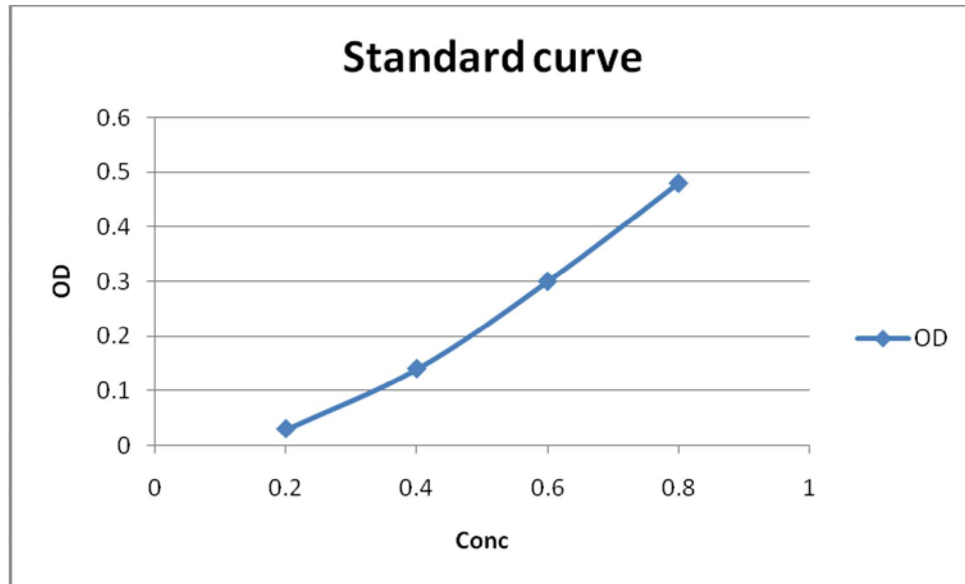

| Strain 1344 | Strain 9644 |
|---|---|

5.Enzyme extraction after adding 10 ml distilled water in samples and centrifugation of crude extract taken from samples for 10 min. at 10000rpm.:

6.Centrifuged samples of crude extract of extracellular enzymes

These samples were further tested for absorbance to check enzyme activity.

**3.Enzyme activity after SSF of substrates at different intervals of time**




St. curve of Beta-polygalactorunic acid by DNS method(1mg/ml).

Absorbance and enzyme activity at different time intervals for 8 different samples to check optimized value of enzyme activity at standard temperature for two strains in two different substrates in SSF:

Table-1

| | 48HR | | | 72HR | | | | 96HR |
|---|---|---|---|---|---|---|---|---|
| Kinnow(I) | 3.46 | | Kinnow(I) | 2.7 | | | Kinnow(I) | 3.17 |
| Apple(I) | 3.34 | | Apple(I) | 3.4 | | | Apple(I) | 3.7 |
| Kinnow(II) | 3.2 | | Kinnow(II) | 2.86 | | | Kinnow(II) | 3.08 |
| Apple(II) | 3.16 | | Apple(II) | 3.06 | | | Apple(II) | 3.3 |

(I)-Strain of A. awamori MTCC:9644

(II)-Strain of A. awamori MTCC:1344

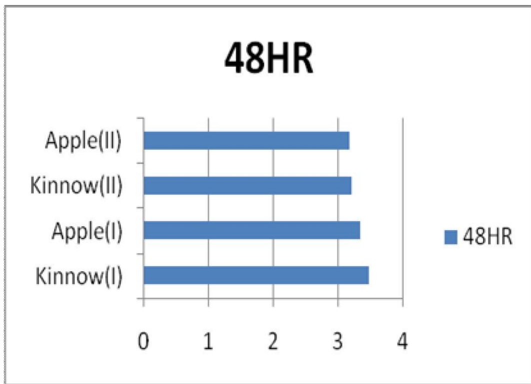



Fig.1

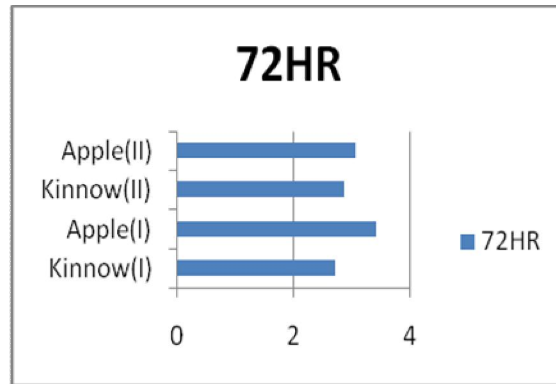



Fig.2

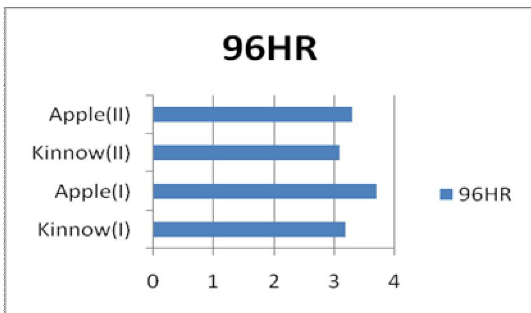



Fig.3

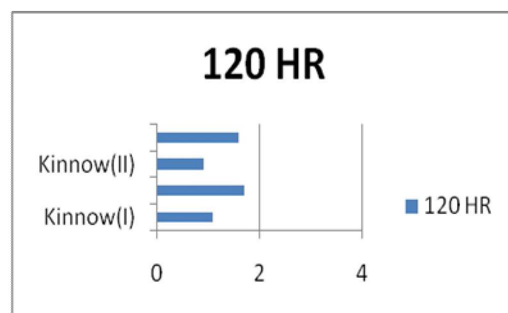



Fig.4

- Fig-1,2,3,4;showing bar graphs of relative absorbance of test samples of enzyme extracts at four different time intervals.

**7.Process parameters optimization for polygalactorunase activity:**

8 different samples containing 2 strains in 4 each flasks and two substrates in 4 each respectively. 4 flasks were kept at 26.7 degree celcius and 4 were kept at 35 degree celcius and absorbance for enzyme activity was checked at different time intervals for particular different samples. To check optimized value of parameters of SSF for different samples and both strains for efficient polygalactorunase activity.

Table-2

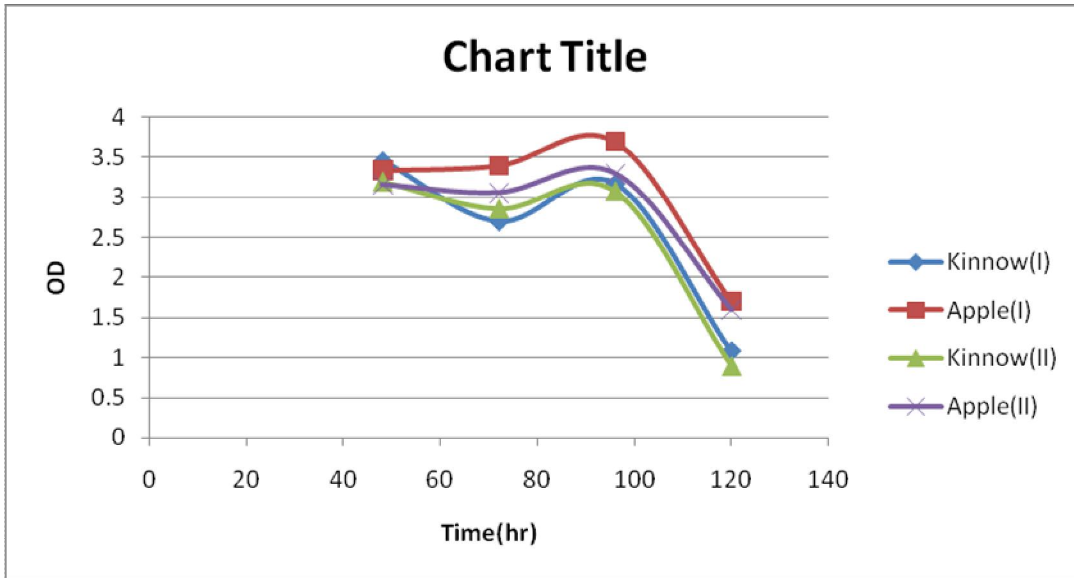
| | | 48hrs | | 72hrs | | 96 hrs |
|---|---|---|---|---|---|---|
| temp. | | OD | temp. | | OD | OD |
| 27.5(°C) | kinnow(I) | 3.2 | 30(°C) | Apple(I) | 2.4 | |
| 35(°C) | kinnow(II) | 2.8 | 35(°C) | Apple(I) | | 1.7 |

(1)-having strain of MTCC:9644

(2)-Having strain of MTCC: 1344

**Discussions:**

Results from enzyme extract absorbance has shown the activity of pectinolytic enzyme(polygalactorunase) for two different substrates and strains combination in SSF at different time intervals. The table 1 values of absorbance being interpreted in graph as:

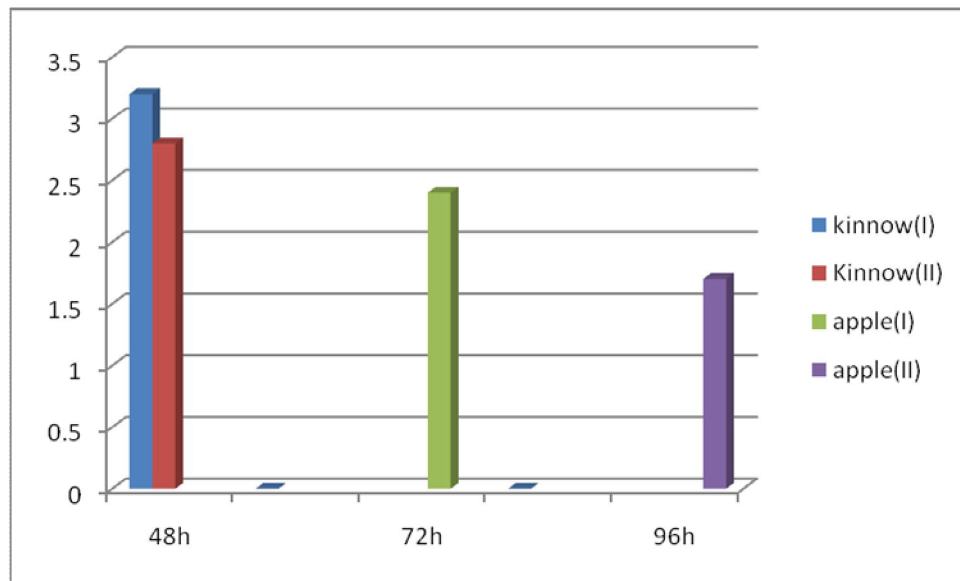



**The results and interpretation of results of graph table-1 are:**

1. This graph shows that strain (1) of A. awamori is showing most enzyme activity at the start of fermentation that is; at 48 hrs, as compared to strain (II) with both the substrates.
2. Apple pomace is showing more enzyme activity at consistent level with both strains as compared to kinnow substrate at the start of SSF;at 48hrs to 72hrs.

3. Kinnow substrate samples with both strains shows enzyme activity less than apple substrate at start as well as end of SSF time period for strains.
4. Enzyme activity for strain (I) is more at 48hrs to 72hrs. as compared to strain(II) which shows more enzyme activity from 72hrs to 96hrs for both substrates.
5. On average enzyme activityin both substrates with both strains is most around 72 hrs mark.

Results from values of table-2 which shows the polygalactorunase activity efficiency at different parameters of SSF for different samples tested shows the efficiency of both strains at two different temperatures at 3 different time intervals to know optimized value of enzyme activity for different samples in SSF. Table -2 has led to the graph as:




The bar graph has following interpretations:

I. Strain(I) & (II), both have optimized value of polygalactorunase activity with kinnow substrate at 48 hrs. And strain(I) has more enzyme activity with kinnow as compared to strain(II).

II. Apple pomace has shown more enzyme activity at 27degree celcius at 72hrs as compared to 35 degree celcius at 96hrs.

# CONCLUSIONS

Pectinolytic enzymes including Polygalactorunase are major industrially important enzymes that find great application in food, feed biotechnology sectors. Current study propose the optimization of bioprocess conditions for improving pectinolytic enzyme by Fungal strains in solid state fermentation (SSF) using two different substrates for both strains. A comparison of enzyme production by two different strains in SSF on two fifferent substrates under optimized and test conditions was studied. Differences in enzyme activity by different combination of strain and substrate was observed. Further studies were carried out by changing few SSF parameters for different test samples containing all 8 combinations of two substrates with two strains. Optimation of enzyme production by both strains on two both substrates under particular changed parameters was carried out. Enzyme activity carried shown strong ineteraction between changed paramerts like substrate, time, temperature,etc; and fungal strains enzyme production.

However, change in enzyme activity was not found be significantly different under particular time interval and temperature for strains, but there was significant change at particular time intereval with two different substrates.

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