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Pectinase from *Bacillus subtilis* strain Btk27: Optimization of cultural conditions,
Characterization of the enzyme and its potential application in coffee processing

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Pectinase from *Bacillus subtilis* strain NBT-15: Optimization of cultural conditions, Characterization of the enzyme and its potential application in coffee processing

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Abstract

The demand for enzymes in the global market is projected to rise at a fast pace in recent years. There has been a great increase in industrial applications of pectinase owing to their significant biotechnological uses. For this reason, this study was undertaken with aims of; isolating and screening microorganisms from coffee pulp, molecular identification of the potential pectinolytic isolates and optimizing pectinase production both on submerged fermentation and solid state fermentation. Moreover, characterizing the enzyme and proving its potential application in demucilisation of coffee. In the present investigation 95 isolates were isolated and screened for their pectinolytic activity from coffee pulp samples. The isolates with high potential pectinase activity were identified molecularly by sequencing 16s rDNA region of the isolates. The bacterium, Bacillus subtilis strain Btk27 was selected for production optimization of the enzyme based on its outstanding features. The production of pectinase was enhanced by 656% (in YEP) and 159% (in wheat bran) times on submerged fermentation and solid state fermentation, respectively. In addition, optimization steps were carried out to make the production of pectinase enzyme cost effective and commercially viable. The maximum pectinase production was observed using YEP (submerged fermentation) and Wheat bran (solid state fermentation) at initial pH of 6.5, at 37⁰C and by supplementing the medium with 3mM MgSO₄.7H₂O. The maximum Pectinase activity was achieved at pH 7.5 and 50⁰C. Also, the enzyme activity was found stimulated with Mg²⁺ and Ca²⁺ metal ions. Moreover, it was stable on EDTA, Trixton-100, Tween 80 and Tween 20. The enzyme, Km and Vmax values were identified as 1.879mg/ml and 149.6 U respectively. The potential application of the enzyme for coffee processing was studied, and it is found that complete removal of mucilage from coffee beans within 24 hours of treatment indicating the potential application in coffee processing.

Keyword: Coffee pulp, Coffee processing, *Bacillus subtilis* strain Btk27, Microbial Pectinase, Submerged fermentation , Solid state fermentation

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LIST OF ABBREVIATIONS AND ACRONYMS

BLAST -Basic Local Alignment Search Tool

EDTA- Ethylene-Diamine Tetra Acetic Acid

mM -Millimololar

nm -Nanometer

OD -Optical Density

PCR- Polymerase Chain Reaction

SDS -Sodium Dodecyl Sulphate

SmF -Submerged State Fermentation

SSF -Solid state fermentation

U -Pectinase activity unit

U/g -Unit per gram

U/ml -Unit per milliliter

YEP-Yeast extract pectin

µg -Micro gram

µl -Micro liter

TAE -Tris-acetate-EDTA

1 INTRODUCTION

Biotechnological answers for environmental sustainability are modern solutions that help in the growth of the nation and are a boon for the welfare of human beings for the present and forthcoming generations. Biotechnology operations for enzyme production is no longer an academic, it is potentially useful alternative proposition for the future (Mishra *et al.*, 2011). Enzymes are natural catalysts. They are produced by living organisms to increase the rate of an immense and diverse set of chemical reactions required for life. Furthermore, their ability to perform very specific chemical transformations has made them increasingly useful in industrial processes (Li *et al.*, 2012).

Enzymes are employed in a diverse array of applications in industries and scientific research, ranging from the degradation of various natural substances in the starch processing, detergent and textile production to the manipulation of DNA/RNA in biotechnology research. Global enzymes market is estimated to rise 7 percent at a healthy pace to 8.0 billion dollar in 2015 (Li *et al.*, 2012). Today's enzyme technology mostly depends on microbes like bacteria and actinomycetes. Potential microorganisms are highly susceptible to genetic manipulations and hence provide ample scope for strain improvement and for further investigation (Mishra *et al.*, 2011). Ecofriendly biotechnological processes seem to be very important as far as the modern society is concerned for which microbial enzymes are recognized as efficient tools. Thus we attempted a study to screen and report pectinase producing microbes.

Pectinolytic enzymes can be applied in various industrial sectors wherever the degradation of pectin is required for a particular process. Several microorganisms have been used to produce different types of pectinolytic enzymes (Jayani *et al.*, 2010). Microbial pectinases account for

25% of the global food and industrial enzyme sales (Jayani *et al.*, 2005; Murad and Azzaz, 2011) and their market is increasing day by day. These are used extensively for fruit juice clarification, juice extraction, manufacture of pectin free starch, refinement of vegetable fibers, degumming of natural fibers, wastewater treatment, and as an analytical tool in the assessment of plant products (Alkorta *et al.*, 1998; Singh *et al.*, 1999). Pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying pectins (Carr, 1985). They are also used in coffee fermentation to remove mucilaginous coat from coffee beans (Sieiro *et al.*, 2012; Hoondal *et al.*, 2002).

Parentetically, Ethiopia is the original home of *Coffea arabica L.*, and thus, possesses the largest diversity in coffee genetic resources (Mayne *et al.*, 2002). Coffee is critical to the Ethiopian economy, since over 25% of the Ethiopia population depends on coffee for its livelihood (IMF, 2006). As per the past few years data, coffee production accounted on average for about 5% of Gross Domestic Product (GDP). Though, Ethiopian exports continue to be dominated by basic commodities, share of coffee in total exports has shrunk from 53% to 31% during 2000–2012 (IMF, 2014). Coffee processing in Ethiopia is executed by both dry and wet processing methods, of which sun drying is widely practiced by farmers and it accounts for 71% while washing in coffee preparation accounts 29% of the total (Musebe *et al.*, 2007).

Regardless of the importance of the crop, poor postharvest processing techniques largely contribute to the decline in coffee quality (Bayetta *et al.*, 1998; Behailu *et al.*, 2008). The traditional processing practices employed by producers have imparted a negative impact on Ethiopian coffee quality. So far, few research attempts have been made to optimize with regard to fermentation for wet processing of coffee (Behailu *et al.*, 2008). Conventional coffee

processing uses water to remove mucilage from coffee beans by natural fermentation. Quite often the mucilage breakdown is not complete even after 36-72 hour of fermentation. If the coffee beans are fermented for long hours, stinker beans (over fermented beans) develop. Most quality defects of coffee are attributed to incomplete mucilage removal and uncontrolled fermentation (Avallone, *et al*, 2001).

Owing to the enormous potential of pectinase in various sectors of industries whenever degradation of pectin is needed, it is important to undertake research in screening of microorganisms for pectinase and investigate optimal conditions for production of microbial pectinase. Use of pectinase in coffee processing, improves coffee quality by completely removing mucilage from coffee beans.

Therefore, this study was conducted with the aims of screening pectinolytic microorganisms from coffee pulp, optimization of pectinase production and testing the potential application of pectinase in removal of mucilage from coffee beans.

2 OBJECTIVES OF THE STUDY

2.1 General objective of the Study

The main objective of this study was to screen potential pectinolytic microorganisms from coffee pulp, optimize production, characterize and testing its potential application in coffee processing.

2.2 Specific objectives of the Study

The specific objectives are the following:

- a) To isolate microorganisms from Coffee pulp,
- b) To screen the isolated microorganisms for their pectinase activity,
- c) To identify potential pectinolytic isolates by molecular methods,
- d) To optimize Pectinase production both on Solid state fermentation and Submerged fermentation,
- e) To characterize the produced pectinase enzyme, and
- f) To test the pectinase in small scale demulsification of coffee beans.

3 REVIEW OF RELATED LITERATURES

3.1 Pectic Substances

The plant cell wall is a complex macromolecular structure that surrounds and protects the cell, and is a distinguishing characteristic of plants essential to their survival. As a consequence of limited mobility, plants are plastic in their ability to withstand a variety of harsh environmental conditions and to survive attack by pathogens and herbivores. The structure formed by the polysaccharides, proteins, aromatic, and aliphatic compounds of the cell wall enables plants to flourish in diverse environmental niches. Cell wall structure is continually modified to accommodate the developmental stage and the environmental condition. The plant cell lays down the middle lamella and the primary wall during initial growth and expansion of the cell. In many cells, the wall is thickened and further strengthened by the addition of a secondary wall. The primary wall is thought to contribute to wall structural integrity, cell adhesion, and signal transduction (Caffall and Mohnen, 2009).

The structural constituents of a young plant cell wall are cellulose, hemicellulose and pectic substances. The cellulose micro fibrils provide strength to the cell wall, while hemicelluloses and pectic substances act as the cementing substance for the cellulose network. Pectins or pectic substances contribute to complex physiological processes like cell growth and cell differentiation and so determine the integrity and rigidity of plant tissue. Polysaccharides from cell walls of ripe pears were reported to contain 11.5% pectic substances, 16.1% lignin, 21.4% glucosan, 3.5% galactan, 1.1% mannan, 21% xylan and 10% arabinan (Horikoshi, 1999).

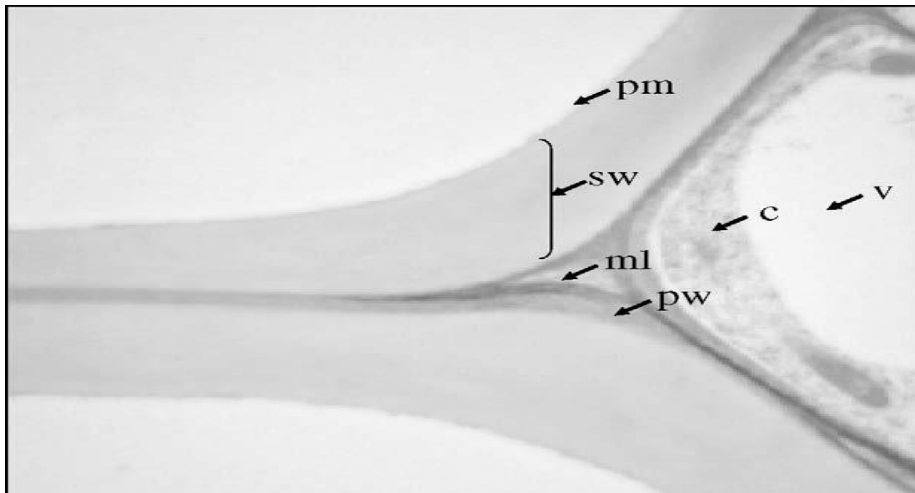


Figure 1: The cell wall of Arabidopsis thaliana, middle lamella (ml), primary wall (pw), secondary wall (sw) of the metaxylem. plasma membrane (pm), cytosol (c) and vacuole (v) (Caffall and Mohnen, 2009).

Pectic substances are polysaccharides of high molecular weight, with a negative charge, appearing mostly in the middle lamella and the primary cell wall of higher plant (Osborne, 2004). They are formed by a central chain containing a variable amount although in high proportion of galacturonic acid residues linked through α -(1-4) glycosidic bonds partially esterified with methyl groups. This molecule is known as pectin, while the demethylated molecule is known as polygalacturonic acid or pectic acid. Pectin was discovered in 1790 by Vauquelin and later in 1825 crudely characterized by Braconnot (Caffall and Mohnen, 2009).

Compared with young, actively growing tissues, lignified tissues have a low content of pectic substances. The content of the pectic substances is very low in higher plants usually less than 1%. They are mainly found in fruits and vegetables, constitute a large part of some algal biomass (up to 30%) and occur in low concentration in forestry or agricultural residues (Horikoshi, 1990).

3.1.1 Classification of Pectic Substances

Based on the type of modifications of the backbone chain, The American Chemical Society classified pectic substances into four main types as protopectin, pectic acid, pectinic acid and pectin (Alkorta *et al.*, 1998; Abalos *et al.*, 2002; Kashyap *et al.*, 2001).

3.1.1.1 Protopectin

This is a parent pectic substance and upon restricted hydrolysis yields pectin or pectinic acid. Protopectin is occasionally a term used to describe the water-insoluble pectic substances found in plant tissues and from which soluble pectic substances are produced (Kilara, 1982).

3.1.1.2 Pectic Acids

These are the galacturonans that contain negligible amounts of methoxyl groups. Normal or acid salts of pectic acid are called pectates.

3.1.1.3 Pectinic Acids

These are the galacturonans with various amounts of methoxyl groups. Pectinates are normal or acid salts of pectinic acids. Pectinic acid alone has the unique property of forming a gel with sugar. The salts of pectinic acids are either normal or acid pectinates. Under suitable conditions, pectinic acids are capable of forming gels with sugars and acids or if suitably low in methoxyl content with certain metallic ions (Kilara, 1982).

3.1.1.4 Pectin (polymethylgalacturonate)

It is polymeric material in which, at least, 75% of the carboxyl groups of the galacturonate units are esterified with methanol. It confers rigidity on cell wall when it is bound to cellulose in the cell wall. Pectins are the soluble polymeric materials containing pectinic acids as the major component. They can form insoluble protopectins with other structural polysaccharides and proteins located in the cell wall (Kashyap *et al.*, 2001). Pectin substance consists of pectin and pectic acid. Demethylated pectin is known as pectic acid or polygalacturonic acid.

3.1.2 Structure of Pectic substances

Pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1→4) linkage. The side chains of the pectin molecule consist of L-rhamnose, arabinose, galactose and xylose. The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by Sodium, Potassium or Ammonium ions (Limberg *et al.*, 2000; Kuhad *et al.*, 2004).

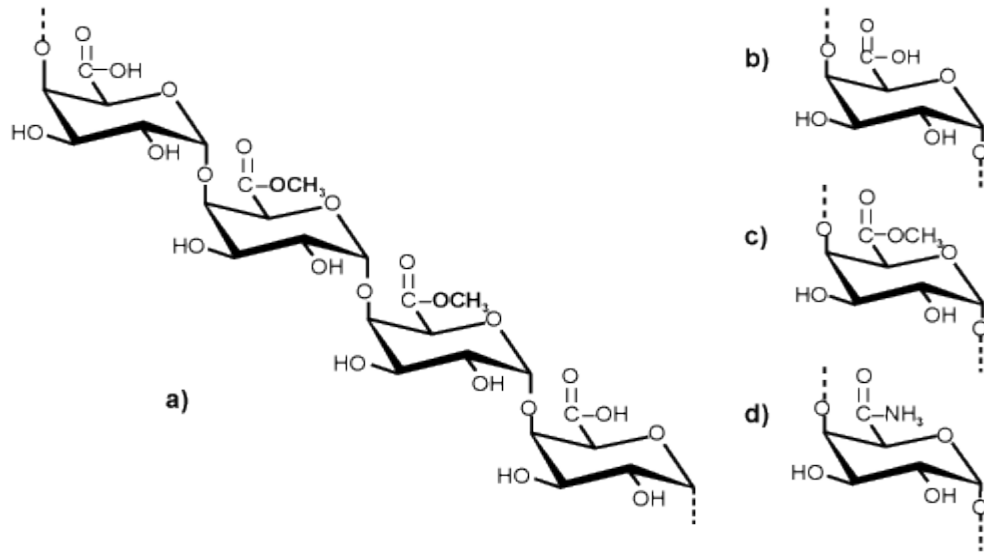


Figure 2: (a) A repeating segment of pectin molecule and functional groups: (b) carboxyl; (c) ester; (d) amide in pectin chain (Sundar *et al.*, 2012).

It is one of the most complex bio-macromolecules in nature and it can be composed of as many as 17 different monosaccharides, with at least seven different polysaccharides (Jayani, *et al.*, 2005). The structural classes of the pectic polysaccharides include homogalacturonan (HG), xylogalacturonan (XGA), apiogalacturonan (AGA), rhamnogalacturonan II (RG-II), and rhamnogalacturonan I (RG-I) (Neill, 2001).

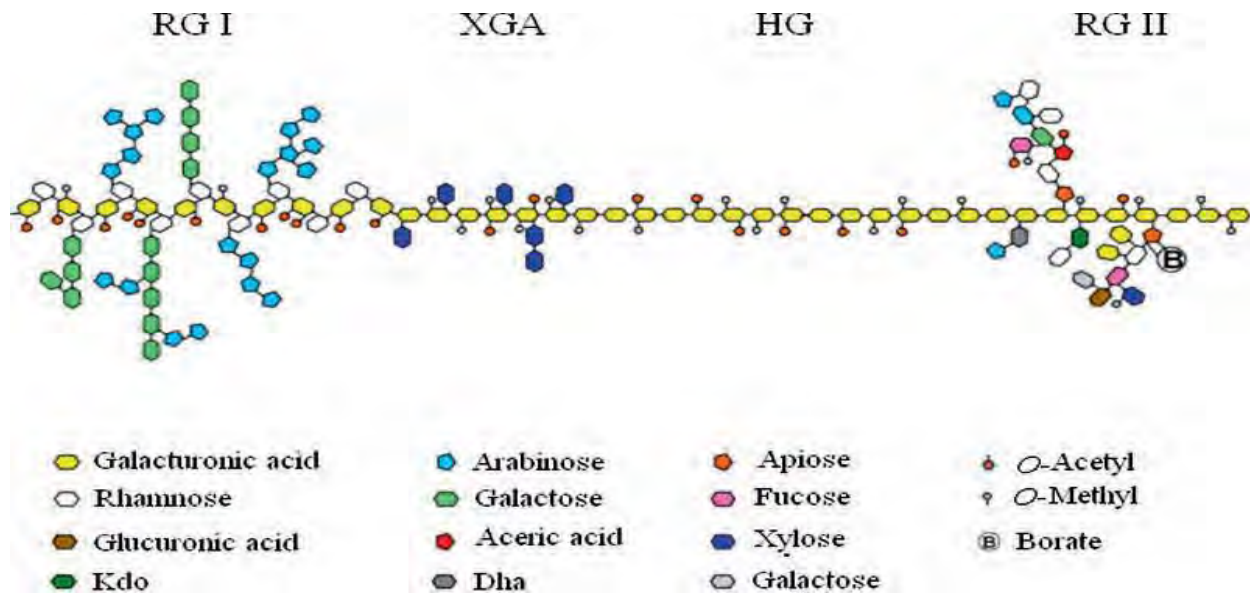


Figure 3: Schematic structure of pectin. Pectin consists of four different types of polysaccharides, and their structures are shown. Kdo, 3-Deoxy-d-manno-2-octulosonic acid; DHA, 3-deoxy-d-lyxo-2-heptulosaric acid. (Jesper *et al.*, 2010).

3.1.3 Applications of Pectins

Pectins show widespread commercial use, especially in the textile industry and in the food industry as thickener, texturizer, emulsifier, stabilizer, filler in confections, dairy products, and bakery products etc. In the food sector, it is primarily used as a gelling agent, replacing sugars and/or fats in low-calorie food and as nutritional fiber (Sakai *et al.*, 1993). It is also studied for its potential in drug delivery, in the pharmaceutical industry and is interesting as a dietary supplementation to humans due to its possible cholesterol-lowering effect. These polysaccharides are which can also reduce the toxicity of these and make their activity longer lasting without altering their therapeutic effects (Morris *et al.*, 2010). Pectin also has a potential in making biodegradable films. Despite these applications, pectins are similar to cellulose and hemicelluloses, in converting common waste materials to soluble sugars, ethanol and biogas (Hutnan *et al.*, 2000).

3.2 Pectinase

Pectinases are a heterogeneous group of related enzymes that hydrolyze the pectin substances, present mostly in plants. Pectic enzymes are widely distributed in nature and are produced by bacteria, yeast, fungi and plants (Lang and Dornenburg, 2000; Whitaker, 1990). In plants, pectic enzymes are very important since they play a role in elongation and cellular growth as well as in fruit ripening (Sakai, 1992; Whitaker, 1990). Pectolytic activity of microorganisms plays a significant role, firstly, in the pathogenesis of plants since these enzymes are the first to attack the tissue (Whitaker, 1990). In addition, they are also involved in the process of symbiosis and the decay of vegetable residues (Hoondal *et al.*, 2002; Lang and Dornenburg, 2000). Thus by breaking down pectin polymer for nutritional purposes, microbial pectolytic enzymes play an important role in nature (Yadav *et al.*, 2009). These enzymes are inducible, produced only when needed and they contribute to the natural carbon cycle (Hoondal *et al.*, 2002).

3.2.1 Classification of Pectinases

According to the cleavage site, pectinases are divided into three groups: (1) hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); (2) lyase/trans-eliminases comprising pectinlyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); (3) esterase consisting Pectin esterase, PE (EC 3.1.1.11) (Yadav *et al.*, 2009; Visser *et al.*, 2004).

3.2.1.1 Pectinesterase (PE)

Pectin methyl esterase or pectinesterase (EC 3.1.1.11) 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 (Kashyap *et al.*, 2001).

3.2.1.2 Polygalacturonase (EC 3.2.1.15)

Polygalacturonases (PGases) are the pectinolytic enzymes that catalyse the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge (Kashyap *et al.*, 2001).

3.2.1.3 Pectatelyase (EC 4.2.2.2)

Pectatelyase (PGL) cleaves glycosidic linkages preferentially on polygalacturonic acid forming unsaturated product through transelimination reaction. PGL has an absolute requirement of Ca^{2+} ions. Hence it is strongly inhibited by chelating agents as EDTA (Jayani *et al.*, 2005).

3.2.1.4 Pectin lyase (EC 4.2.2.10)

Pectin lyase catalyzes the random cleavage of pectin, preferentially high esterified pectin, producing unsaturated methyloligogalacturonates through transelimination of glycosidic linkages. PLs do not have an absolute requirement of Ca^{2+} but they are stimulated by this and other cations (Jayani *et al.*, 2005).

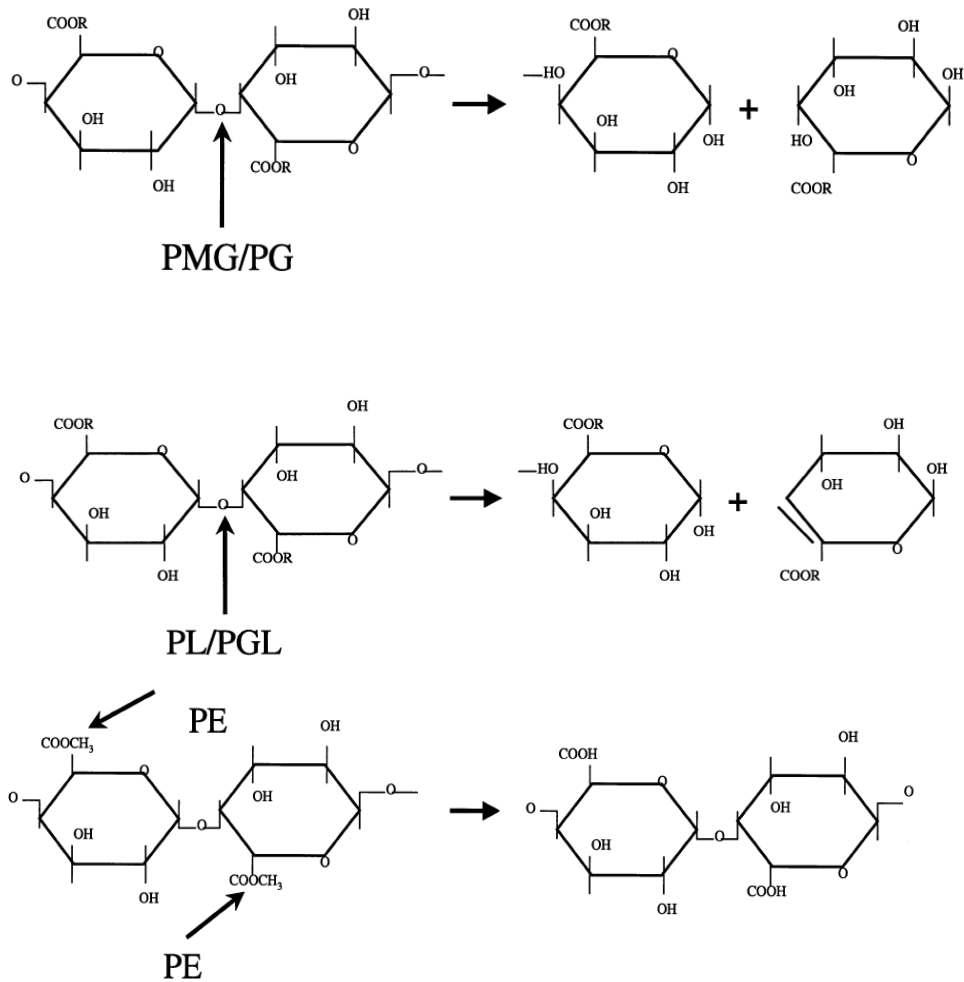


Figure 4: Mode of action of Pectinases; PMG is polymethylgalacturonase, PG is polygalacturonase, PL is pectin lyase, PE is pectinesterase. R group is H in case of PG, CH₃ in case of PGL. R group is CH₃ in case of PL, H in case of PGL (Gopalan and Veeramani, 1994).

3.2.2 Production of Microbial Pectinase

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 origin (Boopathy, 1994). The microbial world has shown to be very heterogeneous in its ability to synthesize different types of pectolytic enzymes with different mechanisms of action and biochemical properties (Gummadi and Panda, 2003). There are two fermentation techniques for

pectinases production, as many other enzymes (Murad and Foda, 1992) these techniques are Solid State Fermentation (SSF) and submerged fermentation (SmF).

Solid state fermentation is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can be used as carbon and energy source. This process occurs in the absence or near absence of free water in the space between substrate particles. In this system, water is present in the solid substrate whose capacity for liquid retention varies with the type of material (Pandey *et al.*, 2000). In contrast, in submerged fermentation (SmF) the nutrients and microorganisms are both submerged in water.

Approximately 90% of all industrial enzymes are produced in SmF, frequently using specifically optimized, genetically manipulated microorganisms (Pandey *et al.*, 2001). In this respect SmF processing offers an insurmountable advantage over SSF. SSF has several advantages over SmF system such as higher concentration of products, less effluent generation, requirement for simple equipments (Pandey *et al.*, 1994). The price of commercially available enzymes which are produced mostly by submerged fermentation is usually too high for agro-biotechnological applications. An alternative technique of enzyme production is solid state cultures (Kawano *et al.*, 1999).

Microbial production of pectinases has been studied during recent years (Kashyap *et al.*, 2001). Pectinase production has been reported from bacteria including actinomycetes (Beg *et al.*, 2000), yeast and fungi. However, almost all the commercial preparations of pectinases are produced from fungal sources (Singh *et al.*, 1999). *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Gummadi and Panda, 2003 Jayani, *et al.*, 2005; Murad and Azzaz, 2011).

Most extracellularly induced enzymes are known to be synthesized in higher quantities when inducers are present in the cultivation medium (Alkorta *et al.*, 1998; Lang and Dornenburg, 2000). The production of pectolytic enzymes using different sources and the effect of physical parameters such as temperature, aeration rate and type of fermentation were investigated and reported in literature. Pectolytic enzymes have been reported to be induced by several substances. In many cases pectin itself has been used. Many investigators had used complex media such as beet sugar, wheat bran, ground nut meal, citrus fruit peels etc (Kilara, 1982; Hoondal *et al.*, 2002). Higher cost of the production is perhaps the major constraint in commercialization of new sources of enzymes. Though, using high yielding strains, optimal fermentation conditions and cheap raw materials as a carbon source can reduce the cost of enzyme production for subsequent applications in industrial processes (Murad and Azzaz, 2011).

There are many studies that have been conducted related to the characterization of different microbial pectic enzymes concerning their mechanisms of action and biochemical properties. The optimal pH that these enzymes may act range between 3.5-11, while the optimal temperatures vary between 40-75 °C (Gummadi and Panda, 2003; Kashyap *et al.*, 2001).

3.2.3 Biotechnological Applications of Microbial Pectinases

Application of enzymes in biotechnological process has expanded considerably in recent years. In food and related industry, major importance was being attached to the use of enzymes in upgrading quality, increasing yields of extractive processes, product stabilization, and improvement of flavor and by product utilization (Arora, 2006). Pectinases or pectinolytic enzymes are today one of the upcoming enzymes of the commercial sector. It has been reported that microbial pectinases account for 25% of the global food enzymes sales (Jayani *et al.*, 2005).

On the bases of their applications, pectinases are mainly of two types: acidic pectinases and alkaline pectinases (Jayani, *et al.*, 2005; Murad and Azzaz 2011).

3.2.3.1 Acidic Pectinases

Acidic pectic enzymes used in the fruit juice industries and wine making often come from fungal sources, especially from *Aspergillus niger* (Kashyap *et al.*, 2001). Potential applications of Acidic pectinase are briefly described below.

Fruit juice clarification/extraction

Fruit juice clarification/extraction is one among the important applications of acidic pectinases. Fruit juices contain colloids that may lead to fouling problem during filtration process and these colloids are basically polysaccharides such as pectin and starch (Rai *et al.*, 2004). Pre-treatment of juices with pectinases is performed to lower the amount of pectin present and to decrease the viscosity of the juice, which in turn accelerates the subsequent filtration process. Also, it helps to increase the clarity of the juice.

Tissue maceration

Tissue maceration is another important application of acidic pectinases in which organized tissue is transformed into a suspension of intact cells and it is significant in the food industry as well as in the field of biotechnology. The process can be applied for the liquefaction and saccharification of biomass, isolation of protoplasts.

Wine processing

Wine processing industry also recognizes the importance of acidic pectinases (Roldan *et al.*, 2006), where the enzyme can be applied at different stages. The addition of pectinases during crushing of the fruits increases the juice yield and also accelerates the release of anthocyanins into the juice. Pectinase treatment at the pre-fermentation or fermentation stage, settles out suspended particles. After fermentation, enzyme is added to the wine to increase its clarity and filtration rate (Kashyap *et al.*, 2001).

3.2.3.2 Alkaline pectinases

Alkaline pectinases are mainly used in the degumming and retting of fiber crops and pretreatment of pectic wastewater from fruit juice industries. These enzymes come mostly from bacterial sources (Kashyap *et al.*, 2001). In the industrial sector, alkaline pectinases, mainly from *Bacillus spp.* are applied for the following purposes.

Paper and pulp industry

During paper making, pectinase can depolymerise pectins and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Reid and Ricard, 2000).

Retting and degumming of plant bast fibers

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle, e.g. Ramie and sun hemp. The fibers contain gum, which must be removed before its use for textile making. The chemical degumming treatment is polluting, toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanases presents an eco-

friendly and economic alternative to the above problem (Kapoor *et al.*, 2001). Pectinases have been used in retting of flax to separate the fibers and eliminate pectins (Hoondal *et al.*, 2002).

Textile processing and bio-scouring of cotton fibers

Pectinases have been used in conjunction with amylases, lipases, cellulases and hemi-cellulases to remove sizing agents from cotton in a safe and eco-friendly manner, replacing toxic caustic soda used for the purpose earlier. Bio-scouring is a novel process for removal of noncellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose degradation (Hoondal *et al.*, 2000).

Pectic waste water treatment

The wastewater from the citrus-processing industry contains pectinaceous materials that are barely decomposed by microbes during the activated-sludge treatment have tried to develop a new wastewater treatment process by using an alkalophilic microorganism. Pretreatment of these wastewaters with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Hoondal *et al.*, 2000).

Animal feed

Pectinases are used in the enzyme cocktail, used for the production of animal feeds. This reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (Jayanin *et al.*, 2005).

Oil extraction

Citrus oils such as lemon oil can be extracted with pectinases. They destroy the emulsifying properties of pectin, which interferes with the collection of oils from citrus peel extracts (Scott, 1978).

Purification of plant viruses

Pectinases have also been reported to work on purification of viruses. But they are yet to be commercialized. When virus particle is restricted to phloem, to release the virus from the tissues, alkaline pectinases and cellulases are used. This gives very pure preparations of the virus (Reid and Richard, 2004).

Coffee and tea fermentation

Fermentation of coffee using pectinolytic microorganisms is done to remove the mucilage coat from the coffee beans and to enhance the tea fermentation and foam forming property of tea. Fungal pectinases are also used in the manufacture of tea. Enzyme treatment accelerates tea fermentation, although the enzyme dose must be adjusted carefully to avoid damage to the tea leaf (Soresen *et al.*, 2000).

4 MATERIAL AND METHODS

Most of the studies were carried out in the Microbial and Molecular Biotechnology Laboratories of the Institute of Biotechnology and Mycology Laboratory of Cellular and Molecular Biology Department, Addis Ababa University. DNA sequencing was carried out at BecA-ILRI, Kenya.

4.1 Sample Collection

A total of 30 coffee husk samples were collected between August 2013 and September 2013 from coffee cherry processing site in Gomma, Jimma zone of Oromia, Ethiopia. The sample collected area is located in Oromia National Regional State and it is one of the 13 weredas in Jima zone for coffee production. It is located 390 km south west of Addis Ababa and about 50 km west of the Jima Zone capital (Jima). The altitude of this area ranges from 1,380 to 1,680 meters above sea level; however, some points along the southern and western boundaries have altitudes ranging from 2229 to 2870 meters.

About 100 g of each sample was collected aseptically using UV-rays sterilized polythene bags. The sample containing bags were sealed and stored into 4⁰C refrigerator in Mycology Laboratory, Addis Ababa University until the time of the analysis.

4.2 Serial Dilution

Ten gram (10 g) of Coffee Husk from each sample were pooled out and homogenized in sterile 90 ml distilled water antiseptically. The homogenized samples were agitated for 1 hour at 120 rpm on INFORS HT Ecotron- Incubator shaker, then serially diluted until dilution 10⁻⁴ and 10⁻⁵.

4.3 Media preparation

Starch Casein Agar media were prepared by dissolving soluble starch - 10g, casein - 0.3g, KNO_3 - 2g, NaCl - 2g, K_2HPO_4 - 2g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.05g, CaCO_3 - 0.02g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.01g and Agar- 15 g in 1000ml of distilled water in 2 L Erlenmeyer flask. The pH of the medium was adjusted to 7.0 ± 0.5 using digital pH meter (OAKTON-pH110) and the medium was boiled. The flask was sterilized at a temperature of 121°C for 15 minutes. Finally, about 20 - 25 ml of the sterilized Starch casein agar media was poured on sterile Petri plates in the microbiological hood and allowed to solidify at room temperature.

Nutrient Agar media were prepared by dissolving 28 g of Nutrient agar in 1000ml of distilled water in 2 L Erlenmeyer flask. The pH of the medium was adjusted to 7.0 ± 0.5 using digital pH meter (OAKTON-pH110) and the mixture was boiled. The flask was sterilized at a temperature of 121°C for 15 minutes. Finally, about 20 - 25 ml of the sterilized nutrient agar media was poured on sterile Petri plates in the microbiological hood and allowed to solidify at room temperature.

Malt Extract Agar media were prepared by dissolving 50 g of malt extract agar powder in 1000ml of distilled water in 2 L Erlenmeyer flask. The pH of the medium was adjusted to 7.0 ± 0.5 using digital pH meter (OAKTON-pH110) and the mixture was boiled. The flask was sterilized at a temperature of 121°C for 15 minutes. After sterilization, the medium allowed to cool up to 50°C and supplemented with $1 \mu\text{g/mL}$ of Chloramphenicol to eliminate the growth of microorganisms other than fungus. Finally, about 20 - 25 ml of the sterilized malt extract agar media was poured on sterile Petri plates in the microbiological hood and allowed to solidify at room temperature.

4.4 Isolation of Actinomycetes

For the isolation of Actinomycetes, 0.1ml aliquots of samples from appropriate dilutions were inoculated onto sterilized and solidified Starch Casein Agar medium using spread plate method (SCAM). Inoculated plates were incubated aerobically at 30⁰C for 3 days – 2 weeks in the INFORS HT Ecotron incubator.

4.5 Isolation of Bacteria

For the isolation of bacteria, 0.1 ml aliquots of samples from appropriate dilutions were inoculated onto sterilized and solidified Nutrient Agar medium by spread plate method. Inoculated plates were incubated aerobically at 30⁰C for 24-48 hours in the INFORS HT Ecotron incubator.

4.6 Isolation of Fungi

For the isolation of Fungi, 0.1 ml aliquots of samples from appropriate dilutions were inoculated on sterilized and solidified Malt Extract agar medium by spread plate method. Inoculated plates were incubated at 30⁰C for 3-8 days in the INFORS HT Ecotron incubator.

4.7 Purification and preservation of cultures

Different colonies were randomly picked from countable plates (Starch Casein Agar media (SCAM) for actinomycetes, Nutrient Agar (NA) for bacteria and Malt extract Agar (MEA) for fungi) and purified by repeated streaking on SCAM, NA, and MEA agar plates for actinomycetes, bacteria and fungi respectively. Pure cultures of each group of microorganisms were then streaked on slants of respective media, and stored at 4⁰C for further study.

4.8 Presumptive screening of Isolates for the Pectinase Activity

The isolates were preliminarily screened for pectinase activity using Pectinase screening Agar Medium (PSAM). The pH of the medium was adjusted to 5.5 ± 0.5 before sterilization and then, autoclaved at 121°C for 15 min. Finally, 20- 25 ml of the media was poured on sterile Petri dishes in the microbiological hood and allowed to solidify at room temperature. All isolates were streaked into this media and incubated at 30°C for 24 hour to 2 weeks. At the end of the incubation period, the plates were flooded with 50 mM Potassium iodide – Iodine solution. A clear halo zone around the colonies indicates the ability of an isolate to produce pectinase (Beg *et al.*, 2000).

4.9 Primary Screening of efficient Pectinase Producing isolates

All the Pectinase positive isolates were screened by inoculating them into the above-mentioned screening media. Using a flamed and cooled cork borer, a disc of actively growing pectinase positive isolate was taken and transferred to the center of screening media and then, incubated at 30°C for 24 hours to 2 weeks. The ratio of the clear zone diameter to colony diameter during that span of time was measured in order to select isolates with highest pectinase activity. The largest ratio is assumed to contain the highest activity. Those isolates with highest ratio were selected for further screening.

4.10 Secondary Screening of efficient Pectinase Producing isolates

Isolates with highest clear zone diameter to colony diameter ratio in the pectin-agar plates were subjected to submerged fermentation using YEP (Yeast Extract Pectin) medium. The pH of the medium, YEP, was adjusted to 7.0 ± 0.5 before sterilization and then, autoclaved at 121°C for 15

minutes. A volume of 50 mL YEP medium in 250 mL Erlenmeyer flask was inoculated with 1% (v/v) inoculum. The inoculated flasks were incubated at 30 °C on an INFORS HT Ecotron incubator shaker at 120 rpm. Samples from inoculated flasks were collected at regular intervals of 24 h and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was used for assaying the enzyme activity. The enzyme activity was assayed using Sodium acetate buffer, pH 6.5. Those isolates with highest activities were selected for further screening.

4.11 Tertiary Screening of efficient Pectinase Producing isolates

In order to identify isolates with broad pH activity, the screened and selected isolates with highest pectinase activity were subjected again to submerged fermentation for further screening. The growth media and conditions were maintained as mentioned above. The enzyme activity was assayed both in Acidic and Basic buffers using Sodium acetate buffer, pH 5.5 and Tris-HCl buffer, pH 8.5 respectively. The isolate with highest activity in both buffers was selected for further study.

4.12 Pectinase Enzyme Assay

The Pectinase enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by dinitrosalicylic acid reagent (DNS) method (Miller, 1959). For enzyme assay, 1.5 mL of freshly grown culture was taken and centrifuged at 10,000 rpm for 5 min. The supernatant (100 μ l) from the culture broth was served as the source of the enzyme. In addition, substrate was prepared by mixing 0.5 % (w/v) citrus pectin in 0.1 M of pH 7.5 phosphate buffer.

From the prepared substrate, 900 μl was added into three clean labeled test tubes; one for enzyme, one for enzyme blank and one for reagent blank. Then, 100 μl of crude enzyme was added into test tube labeled as enzyme, 100 μl of distilled water was added into test tube labeled as reagent blank while, test tube labeled as enzyme blank remained as it was. Then, the test tubes were incubated at 50⁰C for 10 min in the water bath. After incubation 2000 μl of dinitrosalicylic acid reagent (DNS) was added into the all test tubes to stop the reaction. Meanwhile, into test tube labeled as enzyme blank 100 μl of crude enzyme was added after the DNS. Then, all the test-tubes were placed in a boiling water-bath (92⁰C) for 10 min. Finally, the tubes were cooled and optical density (OD) was measured using spectrophotometer (JENWAY 6300 UV/Vis) at 540 nm. Enzyme activity was measured against enzyme blank and reagent blank. The enzyme unit was defined as the amount of enzyme that catalyzes μmol of galacturonic acid per minute ($\mu\text{mol min}^{-1}$) under the assay conditions.

Relative activity was calculated as the percentage of enzyme activity of the sample with respect to the sample for which maximum activity was obtained.

$$\text{Relative Activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Maximum enzyme activity (U)}}$$

4.13 Preparation of D-Galacturonic acid standard curve

In order to express the enzyme activities into Enzyme unit, standard curve of D-galacturonic acid was plotted. The stock concentration of 5mg/ml D- galacturonic acid, Phosphate buffer pH 7.5 and 0.5 % w/v Citrus pectin (substrate) solutions were prepared. A mixture of 100 μl of 5mg/ml D- galacturonic acid and Phosphate buffer pH 7.5 with different concentrations as listed below in Table 2 were filled into test tubes. Substrate of 900 μl and 2000 μl of dinitrosalicylic acid reagent

(DNS) subsequently poured and the reaction mixture was boiled for 10 min. Finally, the tubes were cooled and the absorbance measured by spectrophotometer at OD 540 nm. The relationship between D-galacturonic acid concentration and OD 540 nm is plotted to get standard linear equation using GraphPad Prism 5 software.

Table 1: Standard concentrations of D-Galacturonic acid cid for determination of unit of Pectinase

S. No.	5mg/ml of D-Galacturonic acid (µl)	Buffer(µl)	mg/ml of D-Galacturonic acid	µM of D-Galacturonic acid
1	0	100	0	0.0
2	10	90	0.5	2.6
3	20	80	1	5.2
4	30	70	1.5	7.7
5	50	50	2.5	12.9
6	75	25	3.75	19.3

From the graph the linear regression equation was derived as $y = 15.889x - 1.4066$. The regression coefficient was $R^2 = 0.992$ which describes the concentrations of D-galacturonic acid and OD 540 nm readings were strongly positively correlated (Figure 5). Based on the regression equation the Pectinase enzyme activity expressed in units as:

$$\text{Enzyme Units} = \frac{(15.889 \times \text{OD540nm} \times \text{Dilution factor}) - 1.4066}{\text{Time of incubation}}$$

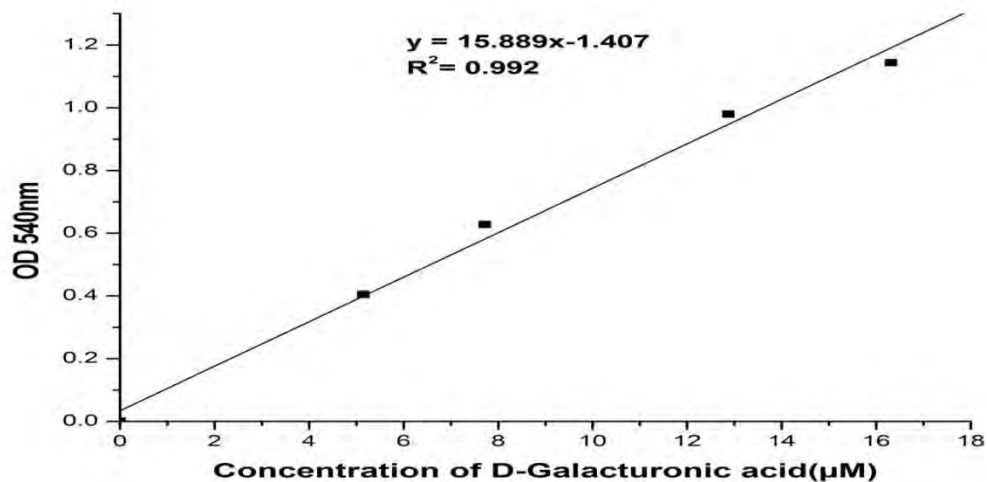


Figure 5: D-Galacturonic Acid Standard Curve

4.14 Molecular Identification of the isolates

The potential isolates which were screened and selected on the primary phase were identified using molecular techniques.

4.14.1 Genomic DNA extraction

The potential isolates were inoculated into 50 ml of LB broth (Luria-Bertani) and incubated at 37°C for 24-48 hours. After incubation, centrifuged at 5000 rpm, 4°C, for 10 min and cells were collected for genomic DNA extraction. The genomic DNA of the isolates was extracted by using the Bacterial Genomic DNA extraction kit according to the manufacturer's protocol (QIAGEN - QIAamp DNA Mini Kit). After DNA extraction, the next molecular studies were carried out at BeCA-ILRI, Kenya.

4.14.2 Quantification and Qualification of DNA by Agarose Gel Electrophoresis

The extracted DNA was subjected to Agarose gel electrophoresis along with the marker DNA (DNA Ladder) to confirm the quality and quantity. 1g of agarose weighted and poured into 100ml of 1X TAE buffer to make 1% agarose gel. The solution was heated in a microwave oven until agarose gets dissolved. The agarose solution cooled down for about 5 minute and 0.5µg/mL of ethidium bromide (EtBr) added. Then, the agarose solution poured into a gel tray with the well comb in place and it was allowed to solidify. After solidified, the comb was removed and the gel was placed in an electrophoresis tank consisting of 1X TBE buffer. About 3 µl of the extracted DNA was mixed with 2 µl of the gel loading dye (Bromophenol blue) and it was loaded into the agarose gel wells. The gel was then electrophoresed at 100 volts for about 45 min and it was observed in a gel documentation system.

4.14.3 PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene of the isolates were amplified using two universal primers, forward Primer-27F (5' - AGA GTT TGA TCM TGG CTC AG -3') and reverse primer-1492R (5'- CGG TTA CCT TGT TAC GAC TT -3'). The AccuPower® Taq PCR PreMix (10 µl master mix containing 10X *Taq*buffer, 10 mM dNTPs, 25 mM of MgCl₂, 1 U of *Taq*DNA polymerase), 1.5 µl of forward primer (27F), 1.5 µl of Reverse primer (1492R), 2 µl of Genomic DNA and 5 µl of PCR grade water were added into eppendorf tubes. Then, the tubes were placed into PCR machine and the amplification done. The thermal cycler was programmed for 35 cycles as follows: **1. Initial denaturation** 94°C for 2 min **2. Amplification** – 94°C for 45 sec(denaturation), 56°C for 1 min (annealing), 72°C for 1 min and 30 sec (extension), **3. Final extension** - 72°C for 5 min, then hold at 4°C until needed.

4.14.4 Quantification and qualification of amplified DNA samples by NanoDrop

The amplified DNA might contain impurities of RNA and protein that can interfere in the DNA analysis. About 1µl of isolated DNA sample placed into the NanoDrop instrument and Quantification and qualification of amplified DNA was done.

4.14.5 Gene Sequencing and Analysis

The PCR products were purified using QIAGEN - QIAquick PCR purification Kit and sent for sequencing. The purified PCR products were sequenced using ABI 3100 DNA sequencer. The sequencing was performed using both 27F and 1492R primers. The obtained sequence data were compared with known sequences in the GenBank using the basic local alignment search tool of the national center for biotechnology information (NCBI). Species were identified based on the percentage similarity with the known species sequences in the data base.

4.15 Preliminary Characterization of Pectinase

In order to identify the optimum conditions, the pectinase from selected isolate was preliminary characterized for its optimum pH and optimum temperature.

4.15.1 Optimum pH for Activity of Pectinase

Optimum pH for activity of pectinase was assayed in the cell-free supernatant in a range of pH values (pH 4.0–10.5) by preparing substrate (citrus Pectin, 0.5%, w/v) in 0.1 M of different buffers, such as Sodium acetate buffer, pH 4.0–6.5; Phosphate buffer, pH 6.0–7.5; Tris-HCl buffer, pH 7.5–8.5; and Glycine NaOH buffer, pH 8.5–10.5. 100 µl of crude enzyme was mixed with 900 µl of substrate in different buffers and incubated at 50⁰ for 10 minutes in the water bath.

After incubation 2000 μ l of dinitrosalicylic acid reagent (DNS) was added and the reaction mixture boiled for another 10 min. Finally, the tubes were cooled and optical density (OD) was measured using spectrophotometer at 540 nm.

4.15.2 Optimum Temperature for Activity of Pectinase

Optimum temperature for the Pectinase Activity was assayed at different temperatures 30–80°C by 5°C interval at optimized pH. The suitably diluted enzyme sample (100 μ l) was incubated with 900 μ l of Citrus Pectin (0.5%, w/v) for 10 minutes. After incubation 2000 μ l of dinitrosalicylic acid reagent (DNS) was added and the tubes were placed in a boiling water-bath for another 10 min. Finally, the tubes were cooled and optical density (OD) was measured using spectrophotometer at 540 nm.

4.16 Optimization of Growth Medium for Maximal Pectinase Activity

The production optimization of pectinase enzyme from selected isolate was carried out both on solid state fermentation (ssf) and submerged fermentation (smf).

4.16.1 Inoculum Preparation

Fresh culture of the screened and selected isolate for pectinase production was inoculated into sterilized YEP medium with pH of 7.0 ± 0.5 . The inoculated flask was incubated at 30°C on a rotary shaker at 120 rpm. Culture was grown in 50 ml media in 250 ml Erlenmeyer flasks. This seed culture was used for subsequent experiments.

4.16.2 Effect of Nutrient Media

The effect of nutrient media on the production of pectinase using submerged fermentation was studied by using different nutrient media such as Yeast extract, Luria-Bertani broth, Nutrient broth, Peptone, Trypton soybean meal, and Malt extract. Each nutrient media was supplemented at 1% (w/v) concentration with 0.25 % (w/v) Apple pectin. The pH of the nutrient media was adjusted to 7.0 ± 0.5 and sterilized. 50 mL of nutrient media in 250 mL Erlenmeyer flasks were inoculated with 1 % (v/v) of Btk27 inoculum and incubated at 30°C, 120 rpm, on an incubator shaker for 48 hour. After incubation, samples were collected and centrifuged at 10,000 rpm for 5 min at 4 °C. The supernatant was used for measuring the enzyme activity. The Pectinase activity was determined in the supernatant as U/ml.

Effect of Concentration of Nutrient Media

To examine the effect of concentration of optimized nutrient media for maximal enzyme production, the nutrient media were studied at different concentrations (0.5% - 4 %w/v).

4.16.3 Effect of Agro Residues (Substrate)

To study the potential of agro residues for pectinase production using solid state fermentation, variety of agro residues (Coffee pulp, Orange peel, and lemon peel and wheat bran) were used as solid substrate. In 250 ml conical flask, 5.0 g of each agro residue was moistened by 60% of distilled water and autoclaved at 121° for 15 minute. The flasks were inoculated with 2.0 ml of overnight-grown seed culture, mixed well to evenly distribute the inoculum and incubated at 37°C for 48 h.

4.16.4 Extraction of Pectinase from solid substrate

Extraction of Pectinase from ssf was done according to the method of Xiroset *al.* (2008). After 48 h of incubation 50ml of distilled water was added into the solid substrate, shaken the flasks for 1 h at 120 rpm on orbital shaker thoroughly and slurry is formed. Then, the flasks were kept at 4°C for 30 min under static conditions to facilitate the enzyme extraction. The slurry was centrifuged at 10,000g for 10 min at 4°C, and the clear supernatant was collected to assay the pectinase activity. The Pectinase activity was determined in the supernatant as U/g of solid substrate used.

4.16.5 The effect of moisture content

To study the effect of moisture content on the production of pectinase enzyme using ssf, the optimized solid substrate was moistened at 45%, 55%, 65%, 75% and 85% moisture content using distilled water before sterilization. Then, the autoclaved substrate was inoculated with 2ml of inoculum and incubated at 37⁰C for 48 h. After the end of incubation the pectinase activity was determined.

4.16.6 Effect of pH of growth Media

The pH of the optimized nutrient media and agro-residue was adjusted to pH that ranges from 4.0-9.0 with 0.5 intervals before sterilization. The sterilized nutrient medium and solid substrate were inoculated and incubated at 37⁰C, 120 rpm (for smf only), for 48 hours.

4.16.7 Effect of Temperature

The sterilized optimized nutrient medium and agro- residue were inoculated and incubated at various temperatures such as 25°C, 30°C, 37°C, 40°C, 45°C and 50°C at 120 rpm(for smf only) for 48 h to study the effect of temperature on enzyme production.

4.16.8 Effect of Agitation

To study the effect of Agitation on smf production of pectinase, the optimized nutrient media was inoculated and incubated at different speeds such as static (0), 120 rpm, 150 rpm and 180 rpm at optimized temperature.

4.16.9 Effect of Inoculum Size

To examine the effect of inoculums size on pectinase production, the optimized nutrient media and agro-residue were inoculated with various inoculum sizes such as 0.5%v/v, 1%v/v, 2%v/v, 3%v/v and 4%v/v for smf and 5%v/v, 10%v/v, 15%v/v and 20%v/v for ssf and followed by incubating at optimized conditions.

4.16.10 Effect of Salts

To study the effect of salts on pectinase production, the optimized nutrient media and agro-residue were supplemented with 3mM of various salts such as: CaCl₂.2H₂O, MgSO₄.7H₂O, CuCl₂.2H₂O, CoCl₂.2H₂O, ZnCl₂, FeSO₄.7H₂O and NaCl. In ssf, these salts were dissolved in the distilled water which was used for adjusting moisture level before incorporating them into the solid substrate.

Effect of Concentration of Salt

To study the effect of concentration of optimized salt for maximal enzyme production, it was studied at different concentrations such as 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM, 4.0 mM and 5.0 mM.

4.16.11 Effect of Carbon sources

To examine the effect of carbon sources on Pectinase production both in smf and ssf, various carbon sources such as dextrose, fructose, arabinose, galacturonic acid, galactose, sucrose, and xylose were supplemented into optimized nutrient media and agro residues at a concentration of 1% w/v along with 0.25% Apple pectin (in case of smf). In case of ssf, these carbon sources were dissolved in the distilled water which was used for adjusting moisture level before incorporating them into the solid substrate.

Effect of Concentration of Carbon Source

To observe the effect of concentration of optimized carbon source for maximal enzyme production, the optimal carbon source was supplemented at different concentrations such as 0.5% w/v, 0.75% w/v, and 1.0% w/v in the production media.

4.16.12 Effect of Nitrogen Sources

The effect of Nitrogen sources on pectinase production both in smf and ssf were studied by supplementing various organic and inorganic nitrogen sources, namely casein, peptone, tryptone, glycine, urea, ammonium chloride, ammonium nitrate, ammonium sulfate of 1% (w/v) into optimized nutrient media and agro residue. In case of ssf, these nitrogen sources were dissolved

in the distilled water which was used for adjusting moisture level before incorporating them into the solid substrate.

Effect of Concentration of Nitrogen Source

To study the effect of concentration of optimized Nitrogen source for maximal enzyme production, the optimal nitrogen source was used at different concentrations of 0.25%, 0.5%, 0.75%, 1.0%, 2%, 3% and 4% w/v in the production media.

4.16.13 Effect of Vitamins

To examine the effect of vitamin on pectinase production both in smf and ssf, the optimized medium and agro-residue were sterilized and supplemented with different concentrations of multivitamin solution such as 0.1%v/v, 0.2%v/v, 0.3%v/v and 0.4%v/v.

4.16.14 Effect of Time of Incubation

To study the effect of incubation time, the optimized YEP media and agro residue were inoculated and incubated at optimized conditions. With 12 hour interval Aliquots of samples were taken and pectinase activity assayed.

4.17 Characterization of Pectinase

The selected isolate was grown at optimized physiochemical parameters and conditions on ssf. After the end of incubation period the pectinase enzyme form solid substrate was extracted and characterized.

4.17.1 Effect of Substrate on Pectinase activity

The effect of substrate on pectinase enzyme activity was determined by incubating 100 μl of suitably diluted enzyme with 900 μl of different substrates like Apple pectin, Citrus pectin, Xylan and Galactose. These substrates were prepared in 0.1M of phosphate buffer (pH 7.5) with 0.5% w/v concentration. The reaction mixture was incubated at 50⁰C for 10 minute and the enzyme activity was assayed.

4.17.2 Effect of pH on Pectinase activity

The effect of pH on pectinase activity was determined by incubating 900 μl of substrate at different pH with 100 μl of suitably diluted enzyme at 50⁰C for 10 min and followed by assaying the enzyme activity. Substrate (0.5% w/v Citrus Pectin) was prepared at different pH values (pH 4.5–9.5) using different buffers (0.1 M) such as sodium acetate buffer, pH 4.5-6.0, phosphate buffer, pH 6.0–7.9, Tris-HCl buffer, pH 7.5–9.0, and glycine NaOH buffer, pH 8.5–10.0.

4.17.3 Effect of Temperature on Pectinase activity

The effect of temperature on pectinase enzyme was evaluated by incubating the reaction mixture (900 μL of substrate at different pH with 100 μl of suitably diluted enzyme) at different temperatures in the range of 30-80⁰C for 10 min with 5⁰C interval and the enzyme activity was assayed.

4.17.4 Effect of Surfactants and inhibitors on Pectinase Activity

The effect of surfactants and inhibitors including mercaptoethanol, EDTA- Ethylene-Diamine Tetra Acetic Acid (1 mM), SDS -Sodium Dodecyl Sulphate (1%, w/v), Tween (20 and 80; 0.1%, v/v), and Triton X-100 (0.1%, v/v) on pectinase enzyme activity was studied by directly incorporating them in the enzyme substrate system. And then, the reaction mixture was incubated at 50°C for 10 min and the enzyme activity was assayed.

4.17.5 Effect of Metal Ions on Pectinase Activity

The effect of metal ions on pectinase activity was studied by directly incorporating them into the enzyme substrate system at a final concentration of 5 mM. Metal ions which were examined for their effect were Ca^{2+} , Mg^{2+} , Co^{2+} , Cu^{2+} , Fe^{3+} , and Mn^{2+} . The reaction mixture was incubated at 50°C for 10 min and the enzyme activity was assayed.

4.17.6 Determining of enzyme stability

The degree of enzyme stability under optimized temperature and optimized pH was studied by incubating the reaction mixture at various time intervals ranging 30 min, 60 min, 90 min, 120 min, 150 min and 180 min.

4.17.7 Michaelis-Menten Constant (Km) and Vmax Values

The Km and Vmax values were determined by measuring the reaction velocity at different concentrations of the substrate (Citrus Pectin). First stock solution of citrus pectin which was 10mg/ml concentration prepared with appropriate buffer (Phosphate - pH 7.5). Then the stock solution was diluted by appropriate volume of buffer to make the final mg/ml citrus pectin

concentrations which listed on Table 3. The appropriate mg/ml citrus pectin (900 μ l) was incubated with 100 μ l of suitably diluted enzyme at 50⁰C for 10 minute and the pectinase enzyme activity assayed.

Table 2: mg/ml concentrations of Citrus Pectin to determine The Km and Vmax values

Volume of stock solution (μl)	Volume of buffer (μl)	mg/ml of Citrus Pectin
180	720	2
360	540	4
540	360	6
720	180	8
900	0	10

The relationship between Substrate (mg/ml of citrus pectin) and Velocity (pectinase enzyme activity) was plotted using GraphPad Prism 5 software. The Km and Vmax values were calculated using non-linear regression.

4.18 Removal of mucilage from Coffee Beans using pectinase

Fresh coffee beans were harvested and pulped manually. The pulped beans were soaked with the enzyme mixture under static conditions until the mucilage was removed. Complete demucilisation was observed by hand feel as per traditional method, finally the demucilised coffee beans were washed and sun dried. To compare the enzymatic demucilisation with natural fermentation, the pulped coffee beans were soaked with water without enzyme addition.

4.19 Data Analysis

All statistical analyses were performed using experimental results which were expressed as means \pm SD of three parallel replicates. Mean of the results were compared using post- hoc multiple comparison analysis performed using Tukey homogenous test using GraphPad Prism 5 software at a significance level of $p < 0.05$. The results were analyzed using Origin pro 8 data analysis and GraphPad Prism 5 desktop version software. The sequence analyses were performed by NCBI and EMBI web based bioinformatics tools.

5 RESULTS

5.1 Isolation of microorganisms from coffee pulp

To isolate microorganisms from the collected coffee pulp samples, serial dilution, pour plating and streak plating isolation techniques were used. Subsequently, the isolates were sub cultured into their respective selective growth media until pure cultures were isolated. In total, ninety five (95) isolates were identified from thirty coffee pulp samples. Based on characterization on the selective growth media, the isolates were grouped as Actinomycete (21.06%), Bacteria (65.26%) and Fungi (13.68%). For identification purpose, the isolates were designated by prefix-“Btk” and followed by their isolate numbers.

5.2 Screening isolates for pectinase production

5.2.1 Presumptive screening

Subsequent to isolation and purification, the isolates were assessed for pectinase activity using Pectinase Screening Agar Medium (PSAM). Among the Ninety five isolates, 31.58% showed colonies surrounded by clear zones which indicate the presence of pectinase activity.

5.2.2 Primary Screening

To identify isolates with higher pectinase activity, the ratio between clear zone diameter and colony diameter was calculated. The highest ratio observed was 4.7 ± 1.2 by isolate Btk27. Those isolates which scored higher than or equal to 2.0 ± 1.5 (Mean \pm SD) were considered as high enzyme producers, which accounts for 33.3% of the pectinase positive isolates (Table 3). These isolates were selected for molecular identification and further screening.

Table 3: Primary screening: Isolates clear zone to colony diameter ratio

S. No.	Isolate	Clear zone to colony diameter ratio*	Taxonomy
1	Btk5	2.9±1.1	B
2	Btk23	3.5±1.8	B
3	Btk25	2.7±0.3	B
4	Btk26	3.4±0.7	B
5	Btk27	4.7±1.2	B
6	Btk59	3.5±0.8	B
7	Btk71	2.7±1.3	B
8	Btk73	2.0±1.5	B
9	Btk81	2.7±0.3	B
10	Btk95	2.8±0.9	A

- * Values are Mean ±SD of triplicates.
- B: Bacteria
- A: Actinomycete

5.2.3 Secondary Screening

In order to further screen the selected isolates from primary screening, submerged fermentation test was performed. Accordingly, subsequent to incubation in YEP media, aliquot samples were taken and assayed for their pectinase activity at 6.5 pH Sodium Acetate buffer. The highest enzyme activities, observed were 7.5±1.17 and 7.5±0.52 (U/ml) by isolates Btk25 and Btk27 respectively (Table 4). The lowest observed enzyme activity was 0.02±0.01 (U/ml) by isolate

Btk71. The enzyme activity higher than or equal to 5.2 ± 0.5 (U/ml) was considered and shown by isolates Btk23, Btk26, Btk27 and Btk25, these isolates were selected for tertiary screening.

Table 4: Secondary Screening: pectinase activity (Unit/ml)

S. No.	Strain no.	Enzyme activity*
1	Btk5	3.4 ± 1.12^b
2	Btk23	6.6 ± 1.61^{ba}
3	Btk25	7.5 ± 1.2^a
4	Btk26	5.2 ± 0.5^{ba}
5	Btk27	7.5 ± 0.5^{ab}
6	Btk59	1.0 ± 0.4^b
7	Btk71	0.02 ± 0.01^b
8	Btk73	3.1 ± 1.9^b
9	Btk81	0.14 ± 0.01^b
10	Btk95	0.1 ± 0.01^b

- **Values are mean \pm S.D. of 3 replicates.*
- *Values followed by different superscripts are significantly different at ($P < 0.05$)*
- *Values followed by same superscripts are not significantly different at ($P < 0.05$)*

5.2.4 Tertiary screening

The selected isolates Btk23, Btk26, Btk27 and Btk25 were further subjected for tertiary screening. At this stage, subsequent to submerged fermentation, aliquot samples were taken and assayed for their pectinase activity both at Sodium Acetate pH 5.5 and Tris-HCL pH 8.5 buffers. The isolates pectinase activity at pH of 5.5 was not significantly different from one another

(Table 5). However, at pH 8.5 the pectinase activity of isolate Btk27 was significantly higher than the other isolates (Table 5). Therefore, isolate Btk27 was selected for further study.

Table 5: Tertiary screening: pectinase activity (Unit/ml)

S. No.	Strain no.	Enzyme Activity at Buffer pH 5.5*	Enzyme Activity at Buffer pH 8.5*
1	Btk23	2.53±0.03 ^a	1.0±1.12 ^b
2	Btk25	3.05±0.38 ^a	0.8±0.72 ^b
3	Btk26	3.52±1.17 ^a	2.5±0.14 ^b
4	Btk27	5.05±1.01 ^a	6.1±1.05 ^a

- **Values are mean ± S.D. of 3 replicates.*
- *Values followed by different superscripts are significantly different at (P<0.05)*
- *Values followed by same superscripts are not significantly different at (P<0.05)*

5.3 Molecular Identification of the isolates

The amplified PCR products were purified and sequenced. The obtained sequence data (appendix 14) were blasted in NCBI data base and the likely microorganisms (with lower E value, higher identity percentage and maximum total score) were identified (Table 6).

Table 6: Blast (Basic Local Alignment Search Tool) table

Isolate	Closest match	Gene Bank Accession No.	% Similarity	Taxonomy	Habitat	Reference
Btk5	<i>Bacillus methylotrophicus</i> strain EGY-SCJ5	KC573497	97	<i>Bacillus</i>	Marine water Sediment, Egypt	Amer <i>et al.</i> , 2013
Btk23	<i>Bacillus pumilus</i> strain B7	KF641839	95	<i>Bacillus</i>	Camel rumen, China	Zeng and Zheng, 2013
Btk25	<i>Bacillus subtilis</i> strain NBT-15	HQ244501	97	<i>Bacillus</i>	Penut, China	Yue <i>et al.</i> , 2010
Btk26	<i>Bacillus amyloliquefaciens</i> strain ASAG1	FJ597542	95	<i>Bacillus</i>	Stored corn, China	Dai <i>et al.</i> , 2008
Btk27	<i>Bacillus subtilis</i> strain NBT-15	HQ244501	97	<i>Bacillus</i>	Penut, China	Yue <i>et al.</i> , 2010
Btk59	<i>Exiguobacterium</i> sp. Y11	EF177690	95	<i>Exiguobacterium</i>	Salt Mine, China	Chen <i>et al.</i> , 2006
Btk71	<i>Pusillimonas ginsengisoli</i> strain DCY28	EF672089	96	<i>Pusillimonas</i>	Soil, South Korea	Srinivasan <i>et al.</i> , 2010
Btk73	<i>Bacillus methylotrophicus</i> strain IHB B 7249	KJ767354	97	<i>Bacillus</i>	<i>Camellia sinensis</i> (tea), India	Gulati <i>et al.</i> , 2014
Btk81	<i>Bacillus</i> sp. SVUNM	JX119240	74	<i>Bacillus</i>	Mica mines, India	Prasada and Paramageetham, 2012
Btk95	<i>Staphylococcus</i> sp. NR7	EU784844	88	<i>Staphylococcus</i>	Sausage, China	Zhao and Chen, 2008

5.4 Preliminary Characterization of Pectinase from isolate Btk27

In order to identify the optimum conditions for the pectinase activity from isolate Btk27, the enzyme was preliminarily characterized for its optimum pH and optimum temperature. The optimum pH was identified by assaying pectinase activity at ranges of pH and the maximum pectinase activity was at pH 7.5 - phosphate buffer (Appendix 3). The optimum temperature was identified by assaying the pectinase activity at ranges of temperature and the maximum activity of pectinase was observed at 50⁰C respectively (Appendix 4).

5.5 Optimization of growth media for maximal pectinase production

5.5.1 Nutrient media

In order to identify the best nutrient media for production of pectinase on submerged fermentation, six different kinds of nutrient media which are tabulated on Table 7 were used. The highest pectinase production was recorded using yeast extract (10.1±1.4 U/ml). Furthermore; the production of pectinase in yeast extract was significantly higher than any of the other media (Table 7). The effect of Yeast Extract concentration for production of pectinase studied and the maximum activity obtained was at 1%w/v Yeast Extract concentration (Appendix 7). Therefore, yeast extract at 1% (w/v) was used in the subsequent smf experiments.

Table 7: Effect of nutrient media on pectinase production

S. No.	Nutrient media	Enzyme activity (U/ml) *
1	Yeast extract	10.1±1.4 ^a
2	Luria-Bertani	6.0±0.8 ^c
3	Peptone	6.3±0.2 ^b
4	Tryptone soybean meal	5.0 ±1.4 ^c
5	Malt extract	4.8±0.1 ^c
6	Nutrient broth	3.5±0.1 ^c

- *Values are mean ± S.D. of 3 replicates.
- Values followed by different superscripts are significantly different at ($P < 0.05$)
- Values followed by same superscripts are not significantly different at ($P < 0.05$)

5.5.2 Agro residues

To study the effect of Agro-residues on pectinase production using solid state fermentation, different agro residues which are listed on Table 8 were inoculated with isolate Btk27. The maximum enzyme activity was achieved among the studied agro-residues was using wheat bran which showed 800.0±16.2 U/g enzyme production. In contrast, the lowest pectinase production was 93.4 ± 7.3 U/g from coffee husk (Table 8). Production of pectinase using coffee pulp, lemon peel and orange peel was not significantly different. Therefore, the subsequent ssf studies were carried out using wheat bran as substrate.

Table 8: Effect of Agro-Residues on Pectinase production

Agro Residues	Enzyme activity (U/g) *
Coffee	93.4±7.3 ^a
Lemon	136.8±51.1 ^a
Orange	113.6±2.4 ^a
Wheat bran	800.0±16.2 ^b

- **Values are mean ± S.D. of 3 replicates.*
- *Values followed by different superscripts are significantly different at (P<0.05)*
- *Values followed by same superscripts are not significantly different at (P<0.05)*

5.5.3 Moisture content

In order to study the effect of moisture content on pectinase production using solid state fermentation, the wheat bran was moistened at a range of 35 - 85% moisture content using distilled water. The maximum pectinase production was at 75% initial moisture content (Figure 6).

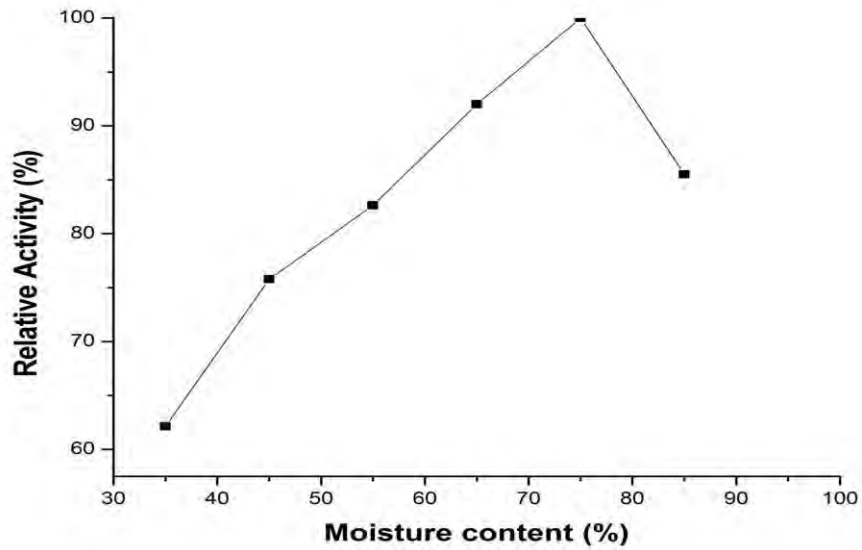


Figure 6: Effect of initial moisture content on Pectinase production

5.5.4 Initial pH of growth Media

To study the effect of initial pH of growth media both on smf and ssf pectinase production, YEP and wheat bran were adjusted to a pH range of 4.0 - 9.0. After 48 hour incubation samples were taken and assayed for enzyme activity. The maximum pectinase activity attained was at the 6.5 initial pH of YEP and wheat bran (Figure 7). The subsequent studies were performed at initial pH of 6.5.

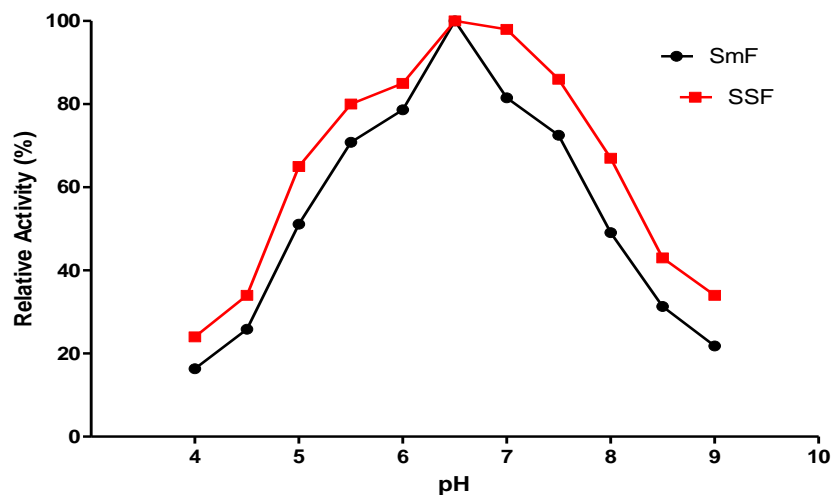


Figure 7: Effect of initial pH of growth media on pectinase production

5.5.5 Effect of Temperature

The wheat bran with appropriate moisture content and YEP with initial pH of 6.5 were inoculated with isolate Btk27, and incubated at various temperatures as tabulated on Table 9. The maximum pectinase production was observed at incubation temperature of 37⁰C for both smf and ssf (Table 9). Thus, the succeeding studies were performed at incubation temperature of 37⁰C.

Table 9: Effect of Incubation temperature on pectinase production

	SmF	SSF
Incubation temperature (°C)	Relative activity (%)	Relative activity (%)
25	56.3 ^a	50.3 ^a
30	76.7 ^b	74.2 ^b
37	100.0 ^c	100.0 ^c
40	84.0 ^b	86.0 ^b
45	42.0 ^d	52.1 ^a
50	0.1 ^e	8.3 ^d

- Values followed by different superscripts are significantly different at ($P < 0.05$)
- Values followed by same superscripts are not significantly different at ($P < 0.05$)

5.5.6 Effect of agitation

To determine the optimum agitation speed, isolate Btk27 was inoculated in YEP and incubated at different speeds of agitation as tabulated on Table 10. An enzyme activity of 13.1 ± 1.8 U/ml was recorded at 120 rpm which was the highest. In contrast, 7.2 ± 0.4 U/ml was found to be the lowest at the agitation speed of 0 rpm (Table 10).

Table 10: Effect of agitation speed on pectinase production

Agitation(rpm)	Enzyme Units*	Relative Activity (%)
0	7.2±0.4 ^a	55.0
120	13.1±1.8 ^b	100.0
150	11.7±1.2 ^b	89.3
180	11.1±0.6 ^b	84.7

- **Values are mean ± S.D. of 3 replicates.*
- *Values followed by different superscripts are significantly different at (P<0.05)*
- *Values followed by same superscripts are not significantly different at (P<0.05)*

5.5.7 Effect of inoculum Size

In order to determine the optimum inoculum size for pectinase production, YEP was inoculated with various isolate Btk27 inoculum sizes. The highest pectinase production 15.4±0.4 U/ml was achieved at 1%v/v inoculum size. There was a trend of decreasing pectinase production at lower and higher than 1%v/v inoculum size (Table 11). The inoculum size of 1% (v/v) was selected as optimum inoculum size, and the subsequent smf studies were performed at this size.

Meanwhile, wheat bran was also inoculated at different inoculum sizes (Table 11), to study the effect of inoculum size for pectinase production on ssf. The maximum pectinase production achieved was 1018.1±47.8 U/g using 10 % (v/v) inoculums size (Table 11).

Table 11: Effect of inoculum size on pectinase production

SmF			SSF		
Inoculum Size (%)	Enzyme activity*	Relative activity (%)	Inoculum Size (%)	Enzyme activity*	Relative activity (%)
0.5	13.9±1.0 ^a	90.3	5	817.4±6 ^a	80.3
1	15.4±0.4 ^a	100.0	10	1018.1±47.8 ^b	100.0
2	14.2±0.2 ^a	92.2	15	841.8±60.4 ^a	82.7
3	10.2±1.4 ^b	66.2	20	735.8±48.0 ^a	72.3

- *Values are mean ± S.D. of 3 replicates.
- Values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at ($P < 0.05$).

5.5.8 Effect of salts on pectinase production

YEP and Wheat bran were supplemented with 3.0 mM of different salts (Table 12) to study their effects on productivity of pectinase. In case of SmF, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and NaCl significantly enhanced the enzyme production compared to the control. Both $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ significantly increased pectinase activity by three folds. The maximum pectinase production attained was 54.0 ± 2.5 U/ml by supplementing YEP with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Table 12).

In case of SSF, supplementing wheat bran with $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl showed enhanced trend of pectinase production although not significant. The maximum pectinase production observed was 1169.7 ± 147.8 U/g by supplementing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Table 12). However, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ significantly reduced pectinase production. The lowest pectinase activity achieved was 566.9 ± 51.0 U/g by $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

Since, the presence of $MgSO_4 \cdot 7H_2O$ boosted robust pectinase production; the concentration of $MgSO_4 \cdot 7H_2O$ was optimized to 3mM (appendix 10). For that reason, the subsequent smf and ssf studies were performed by supplementing 3mM of $MgSO_4 \cdot 7H_2O$ into YEP and wheat bran.

Table 12: Effect of salts on pectinase production

	SmF		SSF	
Metal Ions	Enzyme activity (U/ml) *	Relative Activity (%)	Enzyme activity (U/g) *	Relative Activity (%)
CaCl₂·2H₂O	48.8±4.2 ^a	302.5	1159.1±100.1 ^a	115.3
CoCl₂·6H₂O	40.4±3.6 ^b	252.5	974.0±41.7 ^{ab}	96.9
FeSO₄·7H₂O	8.8±1.3 ^c	55.0	766.1±91.2 ^b	76.2
MgSO₄·7H₂O	54.0±2.5 ^a	337.5	1169.7±147.8 ^a	116.3
NaCl	44.6±5.1 ^{ab}	278.8	1025.6±135.1 ^a	102.3
ZnSO₄·7H₂O	14.3±0.4 ^c	89.4	566.9±51.0 ^b	56.4
Control	16.0±1.4 ^c	100.0	1005.4±47.8 ^a	100.0

- **Values are mean ± S.D. of 3 replicates.*
- *Values followed by different superscripts are significantly different at (P<0.05).*
- *Values followed by same superscripts are not significantly different at (P<0.05).*
- *Control-without any salt supplement*

5.5.9 Effect of carbon sources

Since the production of pectinase can also be influenced by carbon sources, YEP and wheat bran were supplemented with 1% of different carbon sources (Table 13) along with 3.0 mM of $MgSO_4 \cdot 7H_2O$ to study their effect. In case of SmF, supplementing with carbon sources (except for sucrose) significantly decreased the pectinase production (Table 13). The highest pectinase

production achieved was on the control. Therefore, the subsequent smf studies were carried out on YEP in the presence of 0.25 % apple pectin without any other carbon source.

In case of SSF, the highest activity attained was 1172.3±24.68 U/g in the control which was not supplemented by any carbon source (Table 13). Therefore, the subsequent sssf studies were also carried out without any carbon source supplementation.

Table 13: Effect of carbon sources on pectinase production

Carbon sources	SmF		SSF	
	Enzyme activity (U/ml) *	Relative Activity (%)	Enzyme activity (U/g) *	Relative Activity (%)
Arabinose	8.5±2.1 ^a	14.7	769.2±99.54 ^a	65.6
Dextrose	43.4±2.9 ^b	75.2	1045.7±316.2 ^a	89.2
Fructose	42.1±0.2 ^b	73.0	946.1±186.0 ^a	80.7
Galactose	14.1±5.3 ^{ac}	24.4	1045.7±232.2 ^a	89.2
D-Galacturonic Acid	20.9±7.0 ^c	36.2	666.5±280.7 ^a	56.9
Pectin	-	-	836.5±100.3 ^a	71.4
Sucrose	52.0±3.4 ^d	90.1	781.9±233.3 ^a	66.7
Xylose	13.6±1.6 ^a	23.6	707.2±62.17 ^a	60.3
Control	57.7±6.04 ^d	100.0	1172.3±24.68 ^a	100.0

- *Values are mean ± S.D. of 3 replicates.
- Values followed by different superscripts are significantly different at (P<0.05).
- Values followed by same superscripts are not significantly different at (P<0.05).
- Control-without any carbon supplement.

5.5.10 Effect of nitrogen sources

To study the effect of nitrogen sources on smf and ssf pectinase production, YEP and wheat bran were supplemented with different nitrogen sources at 1% (w/v) (Table 14). In case of smf, the highest pectinase production was at 67.7 ± 4.7 U/ml by supplementing Yeast extract with casein. The other tasted nitrogen sources significantly decreased pectinase production (Table 14). Subsequently, the concentration of casein was optimized for optimum pectinase production which was to be 0.5 % w/v (Appendix 9). Therefore, the following smf studies were undertaken by supplementing YEP with 0.5 % w/v casein.

In case of SSF, the highest pectinase production observed was 1261.2 ± 64.0 U/g when supplemented with ammonium sulphate (NH_4SO_4). However, the effect was not significant (Table 14).

Table 14: Effect of nitrogen sources on pectinase production

Nitrogen Sources	SmF		SSF	
	Enzyme activity (U/ml) *	Relative Activity	Enzyme activity (U/g) *	Relative Activity (%)
NH ₄ Cl	44.7±4.8 ^a	73.4	975.7±184.0 ^a	84.1
NH ₄ NO ₃	29.4±1.6 ^b	48.3	1230.0±30.2 ^a	106.0
NH ₄ SO ₄	34.9±2.4 ^b	57.3	1261.2±64.0 ^a	108.7
Casein	67.7±4.7 ^c	111.2	1008.6±19 ^a	87.0
Glycine	33.9±0.7 ^b	55.7	970.5±43.0 ^{ab}	83.7
Peptone	33.8±2.4 ^b	55.5	933.4±31.8 ^{ab}	80.5
Urea	32.2±3.7 ^b	53.0	884.7±83.4 ^b	76.3
Yeast Extract	-	-	1076.9±17.2 ^a	92.8
Control	60.9±1.1 ^c	100.0	1160.0 ±2.50 ^a	100.0

- *Values are mean ± S.D. of 3 replicates.
- Values followed by different superscripts are significantly different at ($P < 0.05$)
- Values followed by same superscripts are not significantly different at ($P < 0.05$).
- Control-without any nitrogen source supplement

5.5.11 Effect of vitamins

To study the effect of vitamins on smf and ssf pectinase production, multivitamin solution was incorporated into YEP and Wheat bran. There was no significant effect on smf pectinase production, however, significant decreasing enzyme production was observed on ssf (Table 15).

Table 15: Effect of vitamins on pectinase production

Vitamin(μ l)	SmF		SSF	
	Enzyme activity (U/ml) *	Relative Activity (%)	Enzyme activity (U/g) *	Relative Activity (%)
50	65.2 \pm 3.6 ^a	98.3	858.6 \pm 50.9 ^a	67.5
100	68.6 \pm 7.5 ^a	103.5	746.0 \pm 80.3 ^a	58.6
150	64.8 \pm 5.8 ^a	98.0	814.9 \pm 16.3 ^a	64.0
200	69.6 \pm 6.5 ^a	105.0	787.8 \pm 48.9 ^a	61.9
Control	66.3 \pm 1.2 ^a	100	1272.4 \pm 25.5 ^b	100.0

- *Values are mean \pm S.D. of 3 replicates.
- Values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at ($P < 0.05$).
- Control- without any vitamin supplement.

5.5.12 Effect of incubation period

To study the effect of incubation period on smf and ssf pectinase production, the inoculated YEP and Wheat bran were assayed at 24 hours of interval for pectinase activity. Accordingly, the highest pectinase enzyme production on both smf and ssf was achieved at 48 hours of incubation (Figure 8). Beyond 48 hour of incubation the production of pectinase both on smf and ssf, declined (Figure 8).

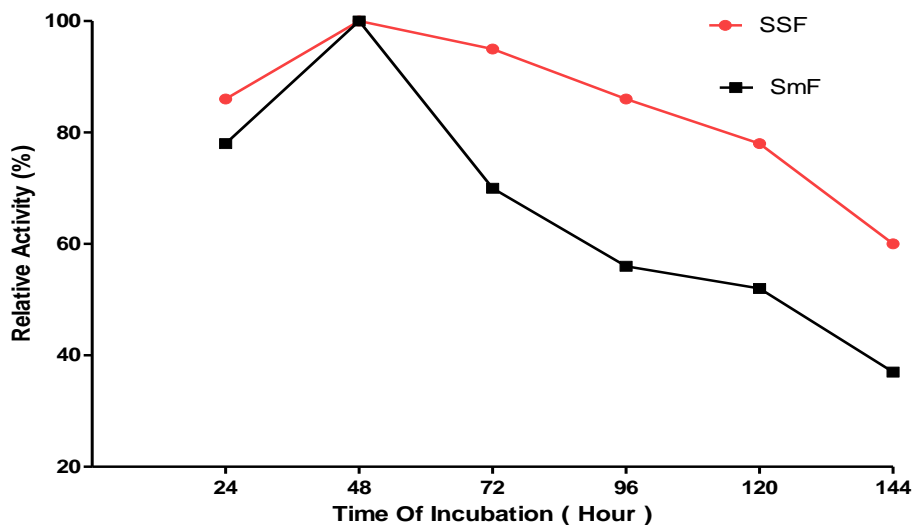


Figure 8: Effect of Time of Incubation on Pectinase production

5.6 Characterization of crude pectinase

Pectinase production was carried out on solid state fermentation using isolate Btk27 at optimized parameters. The produced pectinase enzyme was characterized on the following factors.

5.6.1 Effect of substrate

The effect of substrate specificity on the activity of pectinase enzyme was determined by incubating the pectinase enzyme with different substrates (Table 16). The highest activity was observed when Citrus pectin was used as substrate. The effect of citrus pectin was significantly higher than the other tasted substrates.

Table 16: Effect of substrate specificity on pectinase activity

Substrate	Relative Activity (%)
Apple Pectin	34.7 ^a
Citrus Pectin	100.0 ^b
Xylan	54.8 ^c
Galactose	0.0 ^d

- Values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at ($P < 0.05$).

5.6.2 Effect of pH

The effect of pH on pectinase activity was studied by incubating reaction mixture (citrus pectin and pectinase) at different pH values (pH 4.5–9.5). It was observed that the pectinase enzyme from isolate Btk27 had highest activity at pH of 7.5 (Figure 9).

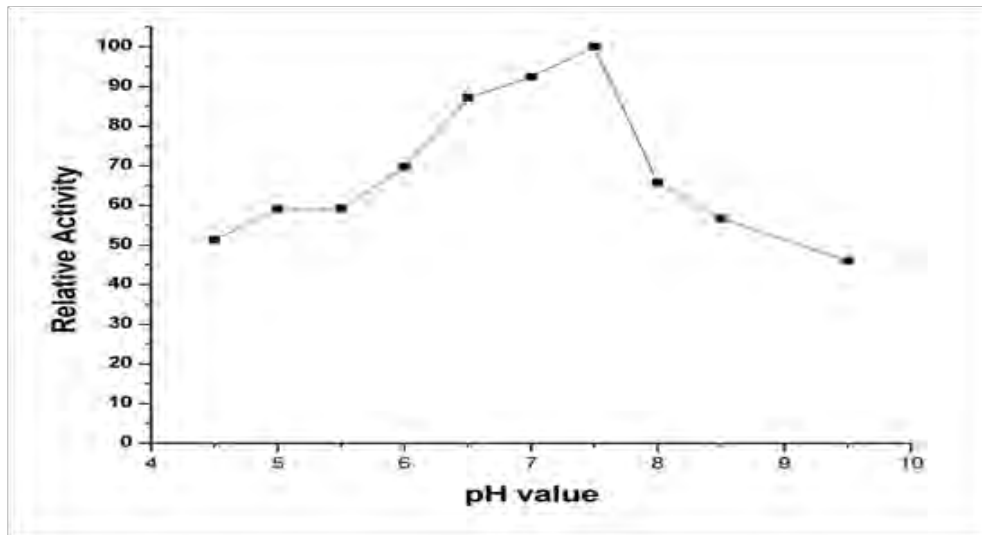


Figure 9: Effect of pH on pectinase activity

5.6.3 Effect of temperature

The effect of temperature on pectinase enzyme was evaluated by incubating the reaction mixture at different temperatures in the range of 30 - 80°C. The maximum pectinase activity observed was at 50°C (Figure 10).

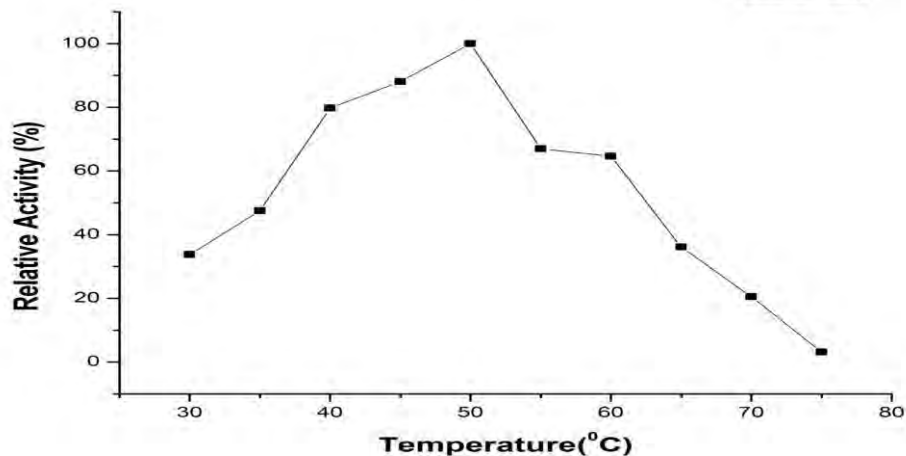


Figure 10: Effect of temperature on pectinase activity

5.6.4 Effect of inhibitors and surfactants on pectinase activity

The effect of surfactants and inhibitors on pectinase activity was studied by directly incorporating them into the enzyme substrate system. Among the tasted surfactants and inhibitors; EDTA, Trixton-100, Tween 80 and Tween 20 enhanced the pectinase activity with relative activity of 165.3, 134.9, 100.4 and 153.6 (%) respectively. It was observed that the presence of Meracptoethanol and SDS in the enzyme substrate system decreased pectinase activity significantly (Table 17).

Table 17: Effect of inhibitors and surfactants on pectinase activity

Surfactant and Inhibitor	Relative Activity (%)
Control	100 ^a
EDTA	165.3 ^b
Meracptoethanol	0.0 ^c
SDS	54.84 ^d
Trixtion-100	134.9 ^{ab}
Tween 20	153.6 ^b
Tween 80	100.4 ^a

- Values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at ($P < 0.05$).

5.6.5 Effect of metal Ions

The effect of metal ions on pectinase activity was studied by directly incorporating them into the enzyme substrate system at a final concentration of 5 mM. The highest relative activities observed were 136.7% and 132.4% in the presence of Mg^{2+} and Ca^{2+} metal ions respectively. The lowest activity observed was with the presence of Mn^{2+} metal ion (Table 18). However, the effect of these tested metal ions on pectinase activity was not significant.

Table 18: Effects of metal ions on pectinase activity

Metal Ion	Relative Activity (%)
CaCl ₂	132.4 ^a
CoCl ₂	127.2 ^a
FeCl ₂	120.1 ^a
MgCl ₂	136.7 ^a
MnCl ₂	74.19 ^a
Control	100.0 ^a

- Values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at ($P < 0.05$).
- Control- there is no any metal ion on the enzyme-substrate system.

5.6.6 Enzyme stability

The stability of pectinase enzyme under optimized temperature and pH was studied by incubating the reaction mixture at various time intervals (Figure 11). It was observed that the enzyme was stable with 100% relative activity until 60 min of incubation. However, beyond 60 min of incubation, the enzyme activity declined.

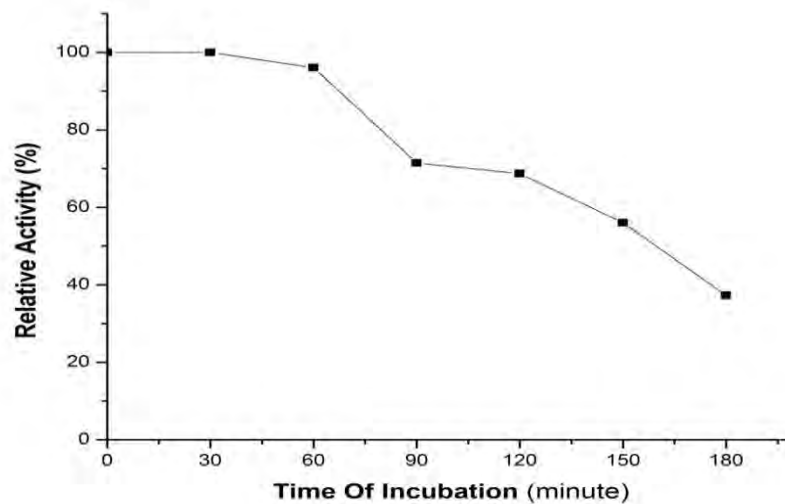


Figure 11: Enzyme Stability

5.6.7 Michaelis-Menten Constant (Km) and Vmax Values

The Km and Vmax values of the enzyme were determined by measuring the reaction velocity at different concentrations of the substrate (Citrus Pectin). The relation between reaction velocity and the substrate concentration was analyzed with non-regression analysis. The regression coefficient (R^2) was equal to 0.999 which describes the concentrations of Citrus Pectin and velocity (enzyme activity) readings were positively correlated. From, the non-regression analysis, Km and Vmax values were identified as 1.879 mg/ml and 149.6 U/g respectively.

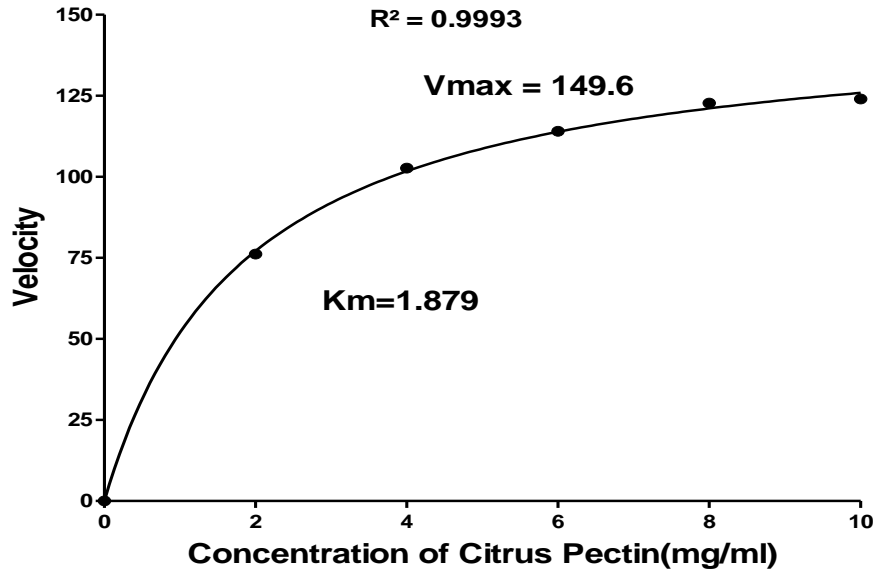


Figure 12: Michaelis-Menten Kinetics

5.7 Potential application of Pectinase on Demucilisation of Coffee Beans

Fresh coffee beans were harvested and pulped manually. The pulped beans were grouped into two and one group soaked into water that contained crude pectinase whereas the other group subjected to natural fermentation. Complete demucilisation was observed within 24 hours of

incubation on pectinase treated coffee beans (Figure 13B). However, in case of natural fermentation demucilisation was not completed even within 36 hour of fermentation (Figure 13A).



Figure 13: Removal of mucilage from coffee beans using natural fermentation(A) and using Pectinase enzyme(B)

6 DISCUSSIONS

Pectinase producing microorganisms have due advantage over other sources because they can be subjected to genetic and environmental manipulations to increase yield (Bhardwaj and Garg, 2014). In this study, potential pectinase producing microorganisms were isolated using serial dilution, pour plating and streak plating techniques from coffee pulp. Nayak *et al.*, (2012) and Sumitha *et al.*,(2013) were also followed similar procedures to isolate microorganisms from coffee husk and pulp. The presence of proteins, pectin, other sugars and minerals and its high humidity favors the rapid growth of pectinolytic microorganisms. Moreover, the organic nature of the material makes it an ideal substrate for microbial processes for the production of value-added products (Pandey *et al.*, 2000).

Emerging new applications underline the importance of screening pectinase producing microorganisms with novel properties, greater enzyme activity and large-scale production of these enzymes (Singh and Mandal, 2012). In this study, the isolates were subjected into plate agar and submerged fermentation screening methods to identify potent isolate with highest enzyme activity and enzyme activity with broad pH ranges. Hitha and Girija (2014) used plate agar screening method to screen native isolates for pectinase activity. Similarly, Mehta *et al.*, (2013) screened bacterial strains, isolated from soil and samples of vegetable using plate agar and submerged fermentation screening methods.

The potential isolates for pectinase production were identified using molecular techniques. Among the molecularly identified isolates about 70 % of the isolates were under genus *Bacillus*. According to Priest (1977), there was a widespread distribution of pectinolytic activity throughout the genus of *Bacillus*. Some works also had been done to produce pectinase by many

strains of these genus (El-Shishtawy *et al.*, 2014; Hou *et al.*, 2011; Kashyap, *et al.*, 2000; Namasivayam *et al.*, 2011; Roosdiana *et al.*, 2013). Namasivayam *et al.*, (2011) stated that *Bacillus sp.* can produce large quantities of extracellular pectinase enzyme.

The capacity of microorganisms to produce extracellular enzymes is influenced by environmental conditions such as temperature, pH, aeration, inoculums and the presence of inducer or repressor substrates (El-Refai *et al.*, 1984). In this study, optimum parameters that affect the pectinase enzyme production have been standardized and diligent optimization steps were carried out to make the production of pectinase enzyme to be cost effective and commercially viable. Since, to meet the growing industrial demands for pectinase, it is necessary to improve yield without increasing the cost of production.

Among the tasted nutrient media, the highest production of pectinase on submerged fermentation was 10.1 ± 1.4 U/ml using Yeast Extract. Kashyap *et al.*, (2000) also reported that the combination of Yeast Extract with pectin to be the best medium for pectinase production. In addition, *Bacillus shaericus* MTCC 7542 produced maximum polygalactouronase when grown on mineral medium containing yeast extract (Jayani, *et al.*, 2010). Yeast extract is the best nutrient media for pectinase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components (Perez and Perez, 2009).

Among the tested Agro-residues for pectinase production, maximum enzyme production on solid state fermentation achieved was 800.0 ± 16.2 U/g from wheat bran. In the same way, Namasivayam *et al.*, (2011) working on *B. cereus* isolated from market solid waste reported that pectinase production was enhanced by wheat bran. Of the various substrates reported in the literature, wheat bran has been the prime among all (Pandey *et al.*, 2000). Wheat bran

characterized by its better air circulation, loose particle binding and efficient penetration and cheaper, therefore it showed a better prospect economically in fermentation processes (El-shishtawy *et al.*, 2014).

Moisture is one of the most important parameter in solid state fermentation (ssf) that influences the growth of the organism and thereby enzyme production. Moisture is reported to cause swelling of the substrates, thereby facilitating better utilization of the substrate by microorganisms (El-shishtawy *et al.* 2014). The maximum pectinase production from *Bacillus subtilis* strain Btk27 was recorded at 75% initial moisture content. Kashyap *et al.* (2003) also reported that 75% initial moisture content for enhanced production of pectinase by *Bacillus sp.* DT7. The moisture level in ssf process varies between 70-80% for bacteria (Pandey, *et al.*, 2000). Any further increase in moisture content resulted in the decrease of enzyme yields may be due to clumping of solid particles which results in the decrease of interparticle space and diffusion of nutrients. In contrast, the low moisture content leads to the decreased solubility of nutrients present in the wheat bran thereby decreased enzyme yields (Nagendra and Chandrasekharan, 1996).

The initial pH of the fermentation medium plays a vital role in determining the level of metabolite synthesis. The stability of the microbial metabolite is also dependent on the hydrogen ion concentration of the medium (Kunamneni, 2005). In the present study, the maximum pectinase production attained both on SSF and SmF was at 6.5 initial pH. The optimum pH in both cases of fermentation was similar. This may be due to the fact that the optimum pH for the production of pectinase is more related to the optimum conditions required for the growth of specific microorganism employed to conduct the fermentation than other factors, so it may have

remained in a particular range for some microorganism, irrespective of the type of fermentation (Khairnar *et al.*, 2009). Banu *et al.*, (2010) also found that *P. chrysogenum* exhibited maximum polygalacturonase production at initial pH of 6.5. *Bacillus sphaericus* (MTCC 7542) showed maximum production at 6.8 initial pH of medium (Jayani *et al.*, 2010).

The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition and protein denaturation (Singh and Mandal, 2012). The maximum pectinase production from *Bacillus subtilis* strain Btk27 on both SmF and SSF was observed at 37°C. Similarly, Namasivayam *et al.*, (2011) reported that an optimum temperature for maximum activity of pectinase from *B. cereus* to be 37°C. Ahlawat *et al.*, (2008), also reported that the optimum temperature for pectinase production was 37°C whereas no other temperature was suitable to such extent for growth and enzyme secretion.

Agitation plays a vital role in oxygen and nutrient transfer in a submerged fermentation. In this regard, agitation increased pectinase production of *Bacillus subtilis* strain Btk27 significantly. Kashyap *et al.*, (2000) reported that aeration has a significant influence on the pectinase production by *Bacillus sp.* DT7. Darah *et al.*, (2013) explained that at lower agitation speed, the inadequate mixing of the broth towards the later stages of growth affected the enzyme synthesis, while the drop in enzyme activity at higher agitation speeds was due to shearing effect on the cells.

The initial load of microorganisms also influences the final level of the enzyme synthesized. In this study, the maximum enzyme production observed was at 1%v/v inoculum size and at 10%v/v in case of smf and ssf respectively. Beyond these inoculum sizes the pectinase production were declined. Similarly, Ahlawat *et al.*, (2008), reported that smf pectinase

production by *Bacillus subtilis* SS at inoculum size of 1% (v/v) was much higher compared to 2% (v/v). Kashyap *et al.*, (2003) also reported that, 10 % (v/v) of an inoculum size was an optimum for SSF production of pectinase using *Bacillus* sp. DT7. Adequate nutrient supply could be the reason of the higher enzyme production with optimum inoculum size. Also, the pectinase production reduction beyond optimum inoculum size could be due to rapid depletion of nutrients and development of oxygen stress resulting from a high microbial load.

Pectinase production on SmF was greatly enhanced by supplementing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl into growth media. Especially, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ increased pectinase production by three folds. Likewise, Kashyap *et al.*, (2000) reported more than three-fold increase in pectinase production by supplementing MgSO_4 and CaCl_2 . While, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl also increased SSF pectinase production but their effect was not significant. Banu *et al.*, (2010) observed little effect of Mg^{2+} and Ca^{2+} on pectinase production from *Penicillium chrysogenum*.

An adequate supply of carbon as energy source is critical for optimum growth of the organism and its metabolism. In the present study, the maximum pectinase production observed both on submerged fermentation and solid state fermentation were on the controls. Supplementing carbon sources decreased pectinase production both on SmF and SSF. According to Ahlawat *et al.*, (2008) low enzyme production with other carbon sources is may be because of catabolite repression. Glucose is known to repress the transcription of genes encoding enzymes required for the utilization of alternative carbon sources; some of these genes are also repressed by other sugars such as galactose, sucrose, arabinose and the process is known as catabolite repression (Crotti *et al.*, 1999; Beg *et al.*, 2001). Solís -Pereira *et al.*, (1993) reported the lower

polygalacturonase production when free sugars were added to the medium compared to the presence of pectin as the sole carbon source. Similarly, Fawole and Odunfa, (2003) found that pectin and polygalacturonic acid promoted the production of pectic enzyme. Phutela *et al.*, (2005) also stated that pure pectin and wheat bran supported maximum pectinase production. Moreover, the same carbon supplements except starch caused repressive effect on pectinase production by *B. licchenformis* (Rehman *et al.*, 2012).

Nitrogenous compounds are utilized by the microbial cells for the synthesis of nucleotides, amino acids, proteins and other metabolites (Padhiar and Bhattacharya, 2011). Nitrogen supplements, when incorporated into the production medium, facilitate better biomass production and subsequently higher metabolite secretion. In this study, the maximum pectinase production on smf attained was 67.7 ± 4.7 U/ml by supplementing Casein as nitrogen source. Whereas, among the tasted nitrogen sources on ssf, ammonium sulphate (NH_4SO_4) and ammonium nitrate (NH_4NO_3) slightly increased the pectinase productivity but their effect was not statistically significant when compared to the control. Correspondingly, Thakur *et al.*, (2010) reported that combination of casein and yeast extract gave high yield of polygalacturonase from *Mucor circinelloides* ITCC 6025. Jayani *et al.*, (2010) while working on *Bacillus sphaericus* (MTCC 7542) reported that a combination of yeast extract and casein also gave high polygalacturonase activity. With regard to ssf, Fawole and Odunfa (2003) found that ammonium sulphate and ammonium nitrate were good nitrogen sources for pectic enzyme production from *A. niger*. Moreover, Sarvamangala and Dayanand (2006) revealed that ammonium sulphate did influence production of pectinase positively in solid-state conditions.

In present study, there was no any significant effect of multivitamin supplement on smf pectinase production. However, it significantly decreased ssf pectinase production. According to Kashyap *et al.*, (2003b), pectinase production was enhanced by 65.8% when multivitamin solution was added to wheat bran. Similarly, Kashyap *et al.*, (2000), reported that supplementing multivitamin solution increased *Bacillus sp.* DT7 smf pectinase production by 61 %. However, the results of this study are in contrast with the above reports. This could be due to the multivitamin solution in this study contained $ZnSO_4 \cdot 7H_2O$ as a component. Zinc ion concentrations of 10^{-5} - 10^{-7} M are required for optimal bacterial growth of most microorganisms in vitro. However, it is claimed that high zinc ion concentrations may have some antibacterial properties (Södeberg *et al.*, 1990).

The time of fermentation had a profound effect on microbial product formation (Murad and Azzaz, 2011). The level of enzyme production varies with the time duration of the fermentation process. The pectinase production from *Bacillus subtilis* strain Btk27 was increased continuously until 48 hours of incubation. After 48 hours of incubation the pectinase activity was decreased. Siddiqui *et al.*, (2012) reported that the maximum polygalacturonase from *Rhizomucor Pusillus* observed at 48 hour of incubation. The reduction in pectinase production after 48 hours might be the result of change in pH during fermentation, denaturation or decomposition of enzyme due to interaction with other components of medium and depletion of nutrients in the medium (Soares *et al.*, 1999; Palaniyappan *et al.*, 2009). Relatively, 48 hours of incubation for high pectinase production is short incubation time, thus, it is good prospect for industrial application.

The maximum pectinase activity observed when citrus pectin used as substrate. Similarly, Celestino *et al.*, (2006) reported that novel pectinase enzyme from *Acrophialo phoranainiana*

showed the highest substrate activity on citrus pectin. Thus, it can be inferred that pectinase have high affinity for citrus pectin than others which are used in this study.

The optimum pH for pectinase activity was recorded at pH 7.5. Reports have shown that pectinase activity to be highest around alkaline pH (Namasivayam *et al.*, 2011; Kumar and Sharma, 2012). Similar study on *B. stearothermophilus* showed optimum pectinase activities at pH 7.5 (Torimiro, 2013). Moreover, Pectinase from *Bacillus sp.* DT7 was maximally stable under alkaline conditions of pH 7.5-8.5(Kashyap *et al.*, 2000). Therefore, this pectinase will have potential applications whenever alkaline pectin degradation is needed such as in coffee processing, paper and pulp industry and Pectic waste water treatment.

The maximum pectinase activity observed at 50°C, with further increase of temperature, the pectinase activity was decreased. This may be a result of thermal denaturation of the enzyme possibly due to disruption of non-covalent linkages, including hydrophobic interactions (Amin *et al.*, 2013). Likewise, Phutela *et al.*, (2005) reported an optimum temperature of 60°C for thermophilus *A. fumigates* pectinase. Alana *et al.*, (1990) also reported that *Pencillium italicum* pectinase activity increase up to 50°C. The result might indicate that pectinase from *Bacillus subtilis* strain Btk27 is thermophilic enzyme.

Surfactant agent stability of the enzyme is one of the important parameters enabling enzymes to be used in different types of industries. In this study, the pectinase activity was stimulated on EDTA, Trixton-100, Tween-20 and Tween-80, whereas SDS significantly decreased pectinase activity. Moreover, Meracptoethanol completely inhibited pectinase activity. Li et al., (2012) reported that Tween-80 and Tween-20 stimulated the Polygalacturonase activity. Zu-ming and Hong-xun, (2008) stated also surfactants such as Tween-80 and Tween-20 had stimulatory

effects on pectinase activity. In contrary, Amid *et al.* (2014) reported that SDS, Triton-100 and Tween-20 significantly reduced, Meracptoethanol significantly increased and EDTA no significant effect on thermo-alkaline pectinase. According to Zohdi and Amid, (2013) most of the surfactants which interact with proteins cause distinct electrostatic and hydrophobic regions and alter the secondary or tertiary structure of enzymes. The stimulatory effect of some surfactants may be probably that the surfactants might improve the turnover number of pectinase by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the aqueous medium (Kapoor *et al.*,2000). Since, *Bacillus subtilis* strain Btk27 was stable in most of surfactants and inhibitors it could be applicable in various industries whenever pectin degradation is needed.

Among the metal ions, Mg^{2+} , Zn^{2+} , Co^{2+} and Fe^{2+} increased pectinase activity whereas Mn^{2+} decreased the pectinase activity, however their effect was not significant. Metal ions like Ca^{2+} and Mg^{2+} might play a vital role in maintaining the active confirmation of alkaline endo polygalactouranase to stimulate the activity (Li *et al.*, 2008). Alana *et al.*, (1990) reported that Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+} did not affect pectin lyase activity of *P. italicum* at 5 mM. This discrepancy in the divalent metal ion preference suggested that the enzymes might have differential flexibility in the active site. Qasim *et al.*, (2003) reported that metal ions such as Mg^{2+} and Ca^{2+} might play a vital role in maintaining the active confirmations of the alkaline pectinase to stimulate the activity.

Pectinase from *Bacillus subtilis* strain Btk27 was stable with 100% relative activity until 60 minutes of incubation. However, above 60 minutes of incubation the enzyme activity declined. Nadaroglu *et al.*, (2010) reported that purified enzyme was stable and retained its full activity

until 1 hour incubation period but the activity was reduced to 20% after 1 hour incubation. Gummadi and Panda, (2003) stated that the stability of pectinases is affected by both physical parameters (pH and temperature) and chemical parameters (inhibitors or activators). The thermal inactivation of enzymes is always due to denaturation of enzyme (Andrade *et al.* 2011).

In enzymatic reaction, the kinetic parameter is also important, which describes enzyme efficiency. In this study, V_{max} and K_m value were 149.6U and 1.88 mg/ml, respectively. Saad *et al.*, (2007) reported a K_m of 1.88 mg/mL and V_{max} of 0.045 mole/mL/min for *Mucor rouxii*. Celestino *et al.*, (2006) also reported that *Acrophialophora nainiana*, had a K_m value of 4.22 mg/ml. Moreover, Sarkar D *et al.*, (2014) reported that *P. chrysogenum* had K_m and V_{max} values of 1.0 mg/mL and 78 U, respectively. Pectinase from *Bacillus subtilis* strain Btk27 relatively has the highest affinity for substrate due to its lowest K_m ; it also has the highest utility of pectin substrate as a result of its highest V_{max} . As a result of this high binding of pectinase from *Bacillus subtilis* strain Btk27 with pectin substrate, small quantity of the enzyme will digest a considerably high amount of substrate. This may therefore reduce the cost for the enzyme in industrial use.

Pectinase are used in coffee processing to remove the mucilaginous coat from the coffee beans (Kashyap *et al.*, 2001). However, there is no any reported application of pectinase in Ethiopia for processing coffee until to date. In this study, pectinase was applied in small scale coffee processing, and it was observed that complete removal of mucilage from coffee beans within 24 hours of incubation. Murthy and Naidu, (2011) reported complete demulsification of Robusta coffee within 36 hour of incubation. The enzyme treatment significantly reduces the fermentation time and holds up coffee quality lose due to traditional coffee processing.

7 CONCLUSIONS

Based on the results of this study, the following conclusions were drawn:

- Coffee pulp is a good source for pectinase producing microorganisms.
- Based on the molecular identifications, *Bacillus* is a potential genus for pectinase production at the same time, *Bacillus subtilis* strain Btk27 had high pectinase activity
- The production of pectinase from *Bacillus subtilis* strain Btk27 was enhanced greater than 656% (in YEP) and 159% (in wheat bran) on Smf and SSF respectively
- Higher Pectinase production was observed under solid state fermentation than submerged fermentation
- The pectinase from *Bacillus subtilis* strain Btk27 was alkaline, thermophilic and stable with many of tested surfactants.
- It was observed that the pectinase from *Bacillus subtilis* strain Btk27 has huge promising potential in removal of mucilage from coffee beans
- It also emphasizes the environmentally friendly applications of microbial pectinases thereby revealing their underestimated potential.

8 RECOMMENDATIONS

In light of the results and conclusion reached, the following are suggested:

- Further enzyme production optimization studies should be implemented using statistical models such as using Response surface methodology to study the interaction of factors.
- Further purification steps will allow enhancing the enzyme activity and studying specific characteristics.
- Further studies have to be performed on scaling up of the pectinase production process.
- Since, the potential of this pectinase is promising in coffee processing, further in depth comparative studies of traditional and enzymatic methods of coffee processing studies have to be performed.

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10 APPENDIX

Appendix 1: Media and multivitamin solution compositions

1. **Starch Casein Agar medium (SCAM):**g/L: soluble starch - 10, casein - 0.3, KNO₃ - 2, NaCl - 2, K₂HP0₄ - 2, MgSO₄.7H₂O - 0.05, CaCO₃ - 0.02, FeSO₄.7H₂O - 0.01, Agar-15
2. **Pectinase screening Agar Medium (PSAM) :** g/L: Pectin - 5, KH₂P0₄ - 4, Yeast Extract - 1, NH₄SO₃ - 2, Na₂HP0₄ - 6, MgSO₄.7H₂O - 0.200, and CaCl₂ - 0.001, FeSO₄.7H₂O - 0.01 Agar – 15
3. **Nutrient Broth** g/L: Peptone 10, Beef extract 3, Sodium-chloride 5, For nutrient agar, 15
4. **Luria-Bertani** g/l: Tryptone 10, Yeast Extract 5, NaCl 10, **For LB agar**, 15 of agar
5. **Yeast extract pectin(YEP):** g/l: Yeast extract 10, Pectin 2.5
6. **A multivitamin solution:** /ml Vitamin A - 500 I.U., Cholecalciferol (vitamin D3) - 40 I.U., Ascorbic acid - 3.8mg, Dexpanthenol - 0.3 mg, Niacin (Vitamin B3) - 2.3 mg , Thiamine (Vitamin B1) - 0.3 mg, Riboflavin (Vitamin B2) - 0.25 mg, Pyridoxine (Vitamin B6) - 0.1mg, Zinc sulphate - 3.3 mg, Manganese Suplphate - 0.01mg, Lysine HCl - 1 mg and Calcim lactate - 3 mg
7. **TAE (Tris-acetate-EDTA) - 50X stock:** /l; Tris-base: 242 g, Acetate (100% acetic acid): 57.1 ml, EDTA: 100 ml 0.5M sodium EDTA, Add dH2O up to one litre. To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of deionised water.
8. **Tryptone soybean meal:** g/l ; Pancreatic digest of casein-15, Papaic digest of soyabean meal-5, Sodium chloride-5, Agar-15

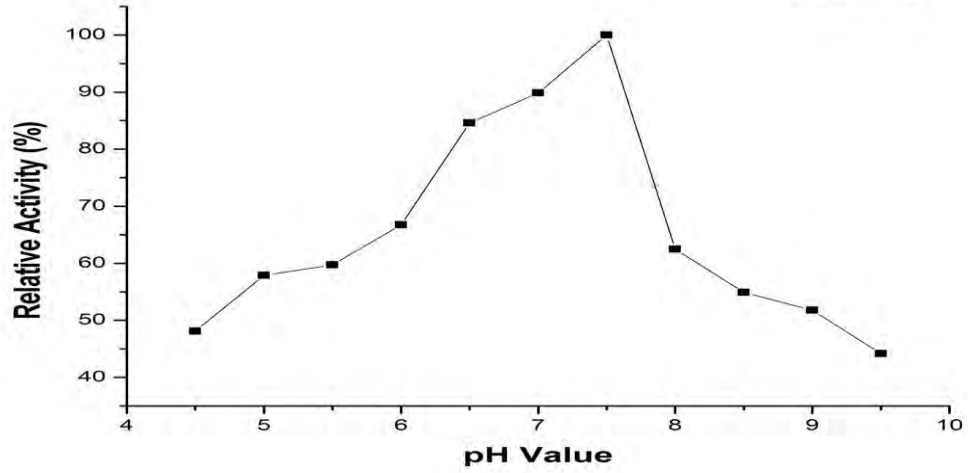
Appendix 2: List of isolated isolates from coffee pulp

Sr. No.	Isolate	Group	Pectinase	Sr. No.	Isolate	Group	Pectinase	Sr. No.	Isolate	Group	Pectinase
1	Btk1	B	-	33	Btk33	F	+	64	Btk64	A	-
2	Btk2	B	-	34	Btk34	F	+	65	Btk65	A	-
3	Btk3	B	-	35	Btk35	B	+	66	Btk66	A	-
4	Btk4	B	-	36	Btk36	B	+	67	Btk67	A	-
5	Btk5	B	+	37	Btk37	B	-	68	Btk68	B	-
6	Btk6	A	+	38	Btk38	B	-	69	Btk69	B	-
7	Btk7	F	+	39	Btk39	B	-	70	Btk70	B	-
8	Btk8	F	-	40	Btk40	B	-	71	Btk71	B	+
9	Btk9	F	-	41	Btk41	B	+	72	Btk72	B	-
10	Btk10	F	+	42	Btk42	B	-	73	Btk73	B	+
11	Btk11	F	-	43	Btk43	B	+	74	Btk74	B	-
12	Btk12	F	+	44	Btk44	B	-	75	Btk75	B	-
13	Btk13	F	+	45	Btk45	B	-	76	Btk76	B	-
14	Btk14	F	+	46	Btk46	B	-	77	Btk77	B	-
15	Btk15	B	-	47	Btk47	B	-	78	Btk78	B	-
16	Btk16	B	-	48	Btk48	B	-	79	Btk79	B	-
17	Btk17	B	-	49	Btk49	B	-	80	Btk80	B	-
18	Btk18	B	-	50	Btk50	B	-	81	Btk81	B	+
19	Btk19	B	-	51	Btk51	B	-	82	Btk82	A	-

20	Btk20	B	-	52	Btk52	B	+	83	Btk83	B	+
21	Btk21	B	-	53	Btk53	B	-	84	Btk84	B	-
22	Btk22	B	-	54	Btk54	A	+	85	Btk85	B	-
23	Btk23	B	+	55	Btk55	A	-	86	Btk86	B	-
24	Btk24	A	-	56	Btk56	A	-	87	Btk87	B	-
25	Btk25	B	+	57	Btk57	A	-	88	Btk88	B	-
26	Btk26	B	+	58	Btk58	A	-	89	Btk89	B	-
27	Btk27	B	+	59	Btk59	B	+	90	Btk90	B	-
28	Btk28	A	+	60	Btk60	A	-	91	Btk91	B	-
29	Btk29	A	-	61	Btk61	A	-	92	Btk92	B	-
30	Btk30	F	-	62	Btk62	A	+	93	Btk93	A	+
31	Btk31	F	-	63	Btk63	A	+	94	Btk94	A	+
32	Btk32	F	-				-	95	Btk95	A	+

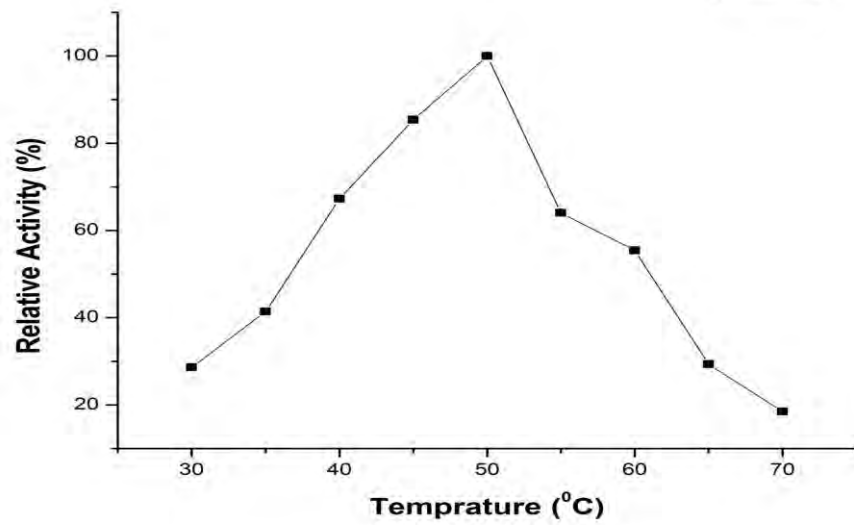
Letter A stands for Actinomycete, B stands for Bacteria, F stands for Fungi

+ stands for pectinase positive, - stands for pectinase negative



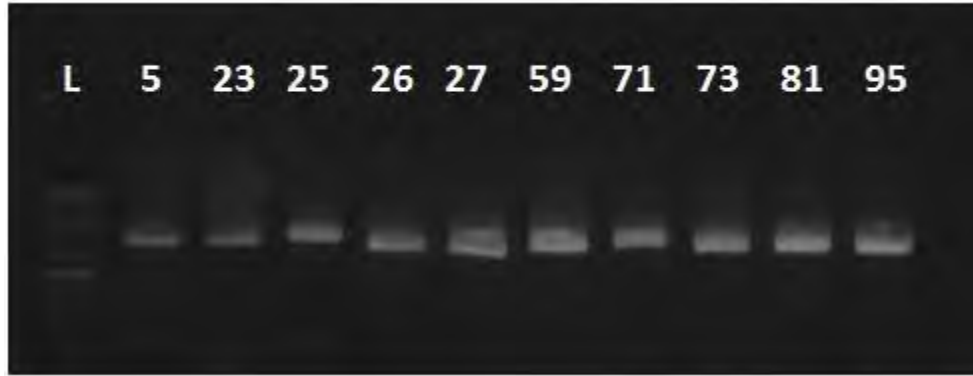
Appendix 3: Preliminary characterization of Pectinase from Btk27 isolate- Optimum pH for

Pectinase activity



Appendix 4. Preliminary characterization of Pectinase from Btk27 isolate- Optimum

Temperature for pectinase activity



Appendix 5: Quantification and qualification of DNA samples- Agarosegele electrophoresis, L- Ladder DNA, 5-Btk5, 23-Btk23, 25-Btk25, 26-Btk26, 27-Btk27, 59-Btk59, 71-Btk71, 73-Btk73, 81-Btk81, 95-Btk95

Appendix 6: Quantification and qualification of amplified 16srDNA by NanoDrop

Isolate	DNA concentration(ng/μl)	260/280	260/230
Btk5	120.5	2.41	1.33
Btk23	72.1	1.441	0.788
Btk25	137.2	2.744	1.485
Btk26	106.6	2.133	1.167
Btk27	124.6	2.491	1.334
Btk59	178.9	3.577	1.947
Btk71	44.6	0.893	0.474
Btk73	135	2.701	1.466
Btk81	116.9	2.338	1.272
Btk95	237.5	4.751	2.564

Appendix 7: Pectinase production optimization- Effect of Concentrations of Yeast Extract (%) on SmF

Yeast extract (%)	Enzyme activity
0.5	5.3±0.6
1	10.2±0.4
2	7.2±2.7
3	6.1±1.0
4	3.8±1.9

Values are mean ± S.D. of 3 replicates.

Appendix 8: Pectinase production optimization - Effect of Concentration of Pectin on SmF

Concentration of Pectin (%)	Relative Activity (%)
0.25	100
0.5	98.1
0.75	92.3
1	80.0

Values are mean ± S.D. of 3 replicates.

Appendix 9: Pectinase production optimization - Effect of Concentration of Casein on SmF

Nitrogen Source % (w/v)	Relative Activity (%)
0.25	90.6
0.5	100.0
1	98.2
2	88.1

Values are mean ± S.D. of 3 replicates.

Appendix 10: Pectinase production optimization - Effect of Concentration of Metal Ion

MgSO₄.7H₂O(mM)	Enzyme activity SmF	Enzyme activity SSF
1	87.5±16.7	887.3±167.0
2	95.9±8.8	971.5±88.0
3	115.9±3.5	1171±34.0
4	114.1±11.6	1154±116.0
5	89.6±4.5	908.5±45.0

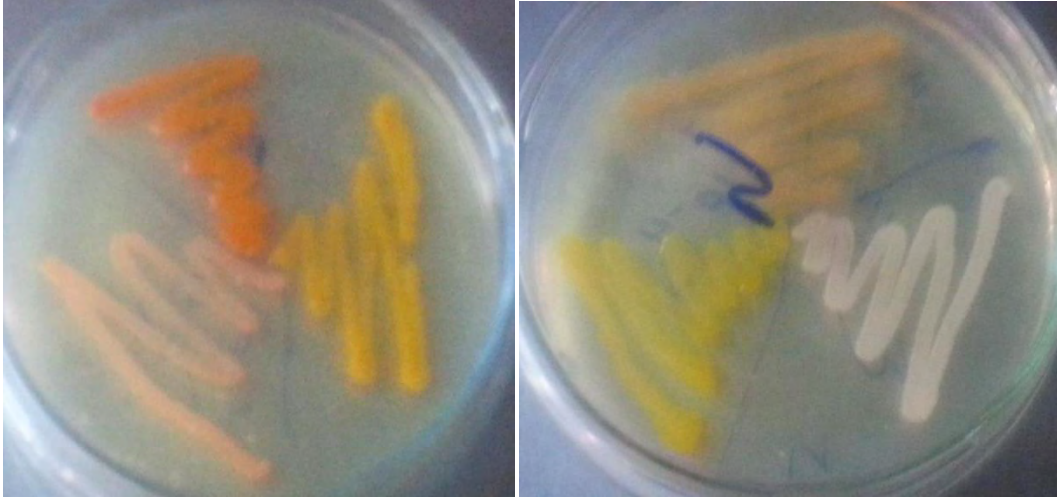
Values are mean ± S.D. of 3 replicates

Appendix 11: Pectinase production optimization - Effect of Concentration of Ammonium

Sulphate

NH₄SO₄ (1% w/v)	Relative Activity (%)
0.5	100.0
1	98.3
2	88.0
3	78.7

Values are mean ± S.D. of 3 replicates



Appendix 12: Some of the isolates on Petri plate



Appendix 13: Clear zones around the colony indicating pectinase positive isolates