ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCE

SCHOOL OF MEDICINE

DEPARTMENT OF BIOCHEMISTRY

TITLE: PRODUCTION AND CHARACTERIZATION OF FUNGAL EXTREMOPHILIC PECTINASE FOR FOOD PROCESSING AND AGRO INDUSTRIAL WASTE UTILIZATION

A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Medical Biochemistry

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Declaration

This is to certify that this thesis prepared by Ayetenew Abita, entitled: "Production and Characterization of Fungal Extremophilic Pectinase for Food Processing and Agro-Industrial Waste Utilization" and submitted in partial fulfillment of the requirement for Degree of Master of Science in Medical Biochemistry compliance with the regulation of the University and meets the accepted standard with respect to originality and quality.

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ABSTRACT

Enzymes are increasingly used in different industrial processes across the globe because of their extreme efficiency and highly specific bio-catalytic activities. Pectinases are among the most industrially important enzymes. Despite the great potential of these enzymes, however, their industrial applications have been hampered mainly owing to undesirable property in terms of stability in extreme conditions when they are exposed during industrial processing. Therefore, this study was undertaken with the aims of isolating and screening fungal extremophiles from Ethiopian extreme environment and optimizing pectinase production using submerged fermentation. In the present study, 30 fungal strains were isolated and screened for their pectinolytic activity from Lake Shalla and Sodere Spring Waters and from those 18 were filamentous fungi and 12 were yeasts. Five potential isolates were used for further study. Pectinase production was studied on mango peel, banana peel, orange peel and wheat straw as media in submerged fermentation. Higher production of pectinase was obtained from orange peel and then different production conditions were optimized on it. The pectinase activity was assayed with 3, 5-dinitrosalysalic acid method. In this study, the maximum pectinase activity was achieved at pH 8.0 and 60 °C. Also, the activities of enzymes were found to be stimulated by Mg^{2+} and Ca^{2+} metal ions. The kinetics of the enzymes was studied and the enzymes K_m and V_{max} values were identified from Lineweaver-Burk plot as 2.196, 1.972, 2.103, 1.418, 1.199 mg/mL and 69.93, 68.97, 68.97, 65.36, 64.10 U/mL for isolate SL-1, SL-4, SS-2, SS-5 and SH-1 respectively.

Key Words: *Enzyme, Extremophile, Lineweaver-Burk plot, Kinetics, Pectinase, Submerged fermentation,*

DEDICATION

I would like to dedicate this thesis to Misganaw Dereje, Bawoke Abita, Demeku Gelaw, and Yibeltal Abita.

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ACRONYMS AND ABBREVIATIONS

^{o}C	- Degree Celsius
DNS	- Dinitrosalicyclic acid
nm	- Nanometer
OD	- Optical density
PL	- Pectate lyase
PE	- Pectin esterase
PPase	- Protopectinases
HG	-Homogalacturonan
µL	-Micro liter
U	-Pectinase activity unit
PG	-Polygalacturonase
PMG	-Polymethylgalacturonase
RG	-Rhamnogalacturonan
SmF	-Submerged Fermentation
U/mL	-Unit per milliliter

1. INTRODUCTION

1.1 Background

Enzymes are biocatalysts produced by all living organisms such as plants, animals, human beings including microorganisms which help them to carry out their metabolic reactions. They are generally proteins in nature. The only exceptions are Ribozymes that are made up of nucleic acids (Sani and Krishnaraj, 2017). Enzymes have indirectly been utilized almost since the beginning of human history (Priya and Sashi, 2014a), though their first discovery was in the second half of the nineteenth century. After thier discovery, they have been extensively used in several industrial processes (Hoondal *et al*., 2002), because of their extreme efficiency and highly specific bio-catalytic activities (Caldwell, 2002), including chemoselectivity, regioselectivity, streoselectivity and enantioselectivity (Choi *et al.*, 2015).

Microorganisms and their enzymes have ample industrial, medical and environmental appl ications. These processes frequently involve exposure to extremes of temperature, pressure, ionic strength, pH and organic solvents. Indeed, there is a continuing need to isolate microorganisms/enzymes which are able to function and catalyze specific reactions under these imposed conditions (Herbert, 1992). Despite the great potential of enzymes, however, their industrial applications have been hampered mainly owing to undesirable property in terms of stability. To overcome such limitations, a variety of approaches have been attempted, including screening of enzymes from natural sources (extremophiles), random mutations, and immobilization (Choi *et al.*, 2015).

Extremophiles are strange microorganisms that can propagate and flourish in extreme environments (Gomes and Steiner, 2004), which were earlier well-thought-out too unfavorable to support life (Gerday and Glansdorff, 2007). The extreme conditions may be high or low temperature, high or low pH, high salinity, high metal concentrations, very low level of nutrient, very low water activity, ionizing radiation, UV light, high pressure and low oxygen tension (Gerday and Glansdorff, 2007; Gomes and Steiner, 2004). The possibility that the basic traits of the genetic code originated under these mentioned environmental conditions has been raised (Gerday and Glansdorff, 2007).

Most of the harsh environments are full of extremophiles. These extremophiles mostly adapted to such extreme conditions by secreting new macromolecules including enzymes with

conformational change. Most pectinolytic enzymes are naturally extremozymes, which acts best at acidic conditions. Pectinases are extensively used in fruit juice extraction, textile processing, degumming of plant bast fibres, retting of plant fibres, waste water treatment, tea and coffee fermentation, paper and pulp industry, animal feed and in oil extraction (Gundala and Chinthala, 2017). Accumulation of evidence reveals, industrial pectinases are exposed to extreme processing conditions for instance in sugar extraction and temperatures higher in biotech industries with temperatures ranging from 30 to 70 ^{o}C. Thus for effective utilization of it the choice of better thermo-stable pectinase become essential (Rebello *et al*., 2017).

Pectin is a methoxylated galacturonic acid polymer which provides integrity and rigidity to the plant tissues and present as a complex macromolecular structure in between the cell walls. The degradation of pectinolytic substances are attained by the synergistic and combined action of various pectinolytic enzymes. Pectinolytic enzymes which act on the homogalacturonan smooth regions are classified into three groups namely: Pectin lyase (EC 4.2.2.10) which cleave the bonds present in pectin by beta- elimination; Polygalacturonase (EC 3.2.1.15) which hydrolyses the glycosidic linkages present in pectin; Pectin methylestrase (EC 3.1.1.6) which helps to remove methyl groups in pectin (Kumar and Suneetha, 2016).

There are different sources of pectinolytic enzymes; including bacteria, fungi, plant (Rebello *et al*., 2017), and no evidence was found in animal (Yadav *et al*., 2009). Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. Over the past century, there has been a tremendous increase in awareness of the effects of pollution, and public pressure has influenced both industry and government (Hoondal *et al.,* 2002). Pectinase plays a role in food industries and agro-waste utilization. Most of the pectinase producers belongs to fungal populations among them *Aspergillus sp* are the most notable pectinase producers (Jayani *et al*., 2005).

Pectinases are among the most important industrial enzymes (Bajpai, 2018). About 45% of juice industries utilize food processing enzymes including pectinases (Kumar and Suneetha, 2016). Application of pectinases in various fields shows the potential for green process (Rebello *et al*., 2017). Most pectic enzyme preparations are used in the fruit processing industry (Priya and Sashi, 2014b) and pectic enzymes alone account for about one quarter of the world's food enzyme production (Priya and Sashi, 2014b; Ruiz *et al*., 2017). Fungal pectinases are among the

most important industrial enzymes and are of great significance with wide range application (Priya and Sashi, 2014b).

Despite the great potential of enzymes, however, their industrial applications have been hampered mainly owing to undesirable property in terms of stability. To overcome such limitations, a variety of approaches have been attempted, including screening of enzymes from natural sources (extremophiles), random mutations, and immobilization (Choi *et al*., 2015). Microbes which adapt extreme condition express enzymes which are stable in these conditions. From those, pectinase producing microbes are the one (Jayani *et al*., 2005). Ethiopia has various ecological zones, from cold to hot environment and microbes are found to grow and multiply everywhere (Priya and Sashi, 2014a). Having these opportumity, fungal strains capable of expressing pectinase enzyme in extreme environments such as alkaline, saline area and hot spring water, which may have vital role in food processing and agro-industrial waste utilization was studied. No related work has been reported in the extremophilic fungal pectinase in Ethiopia. Therefore, the main role of this research was to access new opportunities in Ethiopian extreme environment such as Lake Shala and Sodere spring water for extremophilic fungal enzyme pectinase which can be utilized for food processing and agro-industrial waste utilization.

1.2. Objectives of the Study

1.2.1. General objective

To produce fungal extremophilic pectinase for food processing and agro-industrial waste utilization and characterize optimal conditions for production and enzyme activity

1.2.2. Specific objectives

- To isolate and screen pectinase producing fungal extremophile,
- To quantify pectinase production from extremophile fungi using different agro-industrial/ agricultural residues as substrates,
- To determine and optimize pectinase production conditions,
- To determine the optimum pH and temperature of pectinase,
- To characterize the produced pectinase with different conditions, and
- To determine the kinetic parameters (K_m and V_{max})of the enzyme

2. LITERATURE REVIEW

2.1. Pectinic Substances

Pectic substances are galacturonic acid-containing polysaccharides assembled in the plant cell wall and middle lamella together with other structural biopolymers such as cellulose, hemicelluloses, proteins, and lignin (Ruiz *et al*., 2017). They are glycosidic macromolecules of high molecular weight that are widespread in plant kingdom (Alkorta *et al*., 1998). They found in the cell walls of higher plants, where they function as a hydrating agent and cementing material for the cellulosic network (Thakur *et al*., 1997). They form the major components of the middle lamella (Priya and Sashi, 2014a), a thin layer of adhesive extracellular material found between the primary walls of adjacent young plant cells (Alkorta *et al*., 1998; Thakur *et al*., 1997, Priya ; Sashi, 2014a) and relays signals from the outside environment (Cankar *et al*., 2014).

Naturally, pectin is the most complex macromolecule and heterogeneous in both chemical structure and molecular weight. They consist of three major polysaccharides with a back bone of galacturonic acid residues linked by α (1→4 linkages) and D-galacturonic acid residues usually referred as galacturonans. These are homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (Sani and Krishnaraj, 2017, Suryam and Charya, 2018). Homogalacturonan (HG) is a linear polymer consisting of 1, 4-linked α-D-GalA, whilst rhamnogalacturonanI (RGI) consists of the repeating disaccharide [1→4)-α-D-GalA- (1→2)-α-L-Rha-(1→] to which a variety of different glycan chains (principally arabinan and galactan) are attached to the Rha residues. The confusingly named rhamnogalacturonan II (RGII) has a backbone of HG rather than RG, with complex side chains attached to the GalA residues (Willats *et al*., 2006).

The chemical structure of pectin depends on the origin, location in the plant and extraction method, been composed of acidic polymers, known as homogalacturonan, rhamnogalacturonan and xylogalacturonan (Albuquerque *et al*., 2016) with several neutral sugars/polymers such as arabinans, galactans arabinogalactans, L-rhamnose, and xylose attached as side chains (Bajpai, 2018; Albuquerque *et al*., 2016). Figure 1 shows, the highly schematic model of pectin available today. The carboxylgroups of galacturonic acid are partially esterified by methyl groups (Priya and Sashi, 2014a; Bajpai, 2018). Demethylated pectin is known as pectic acid (pectate) or

polygalacturonic acid (Priya and Sashi, 2014a). Galacturonic acid comprises approximately 70% of pectin, and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position (Mohnen, 2008).

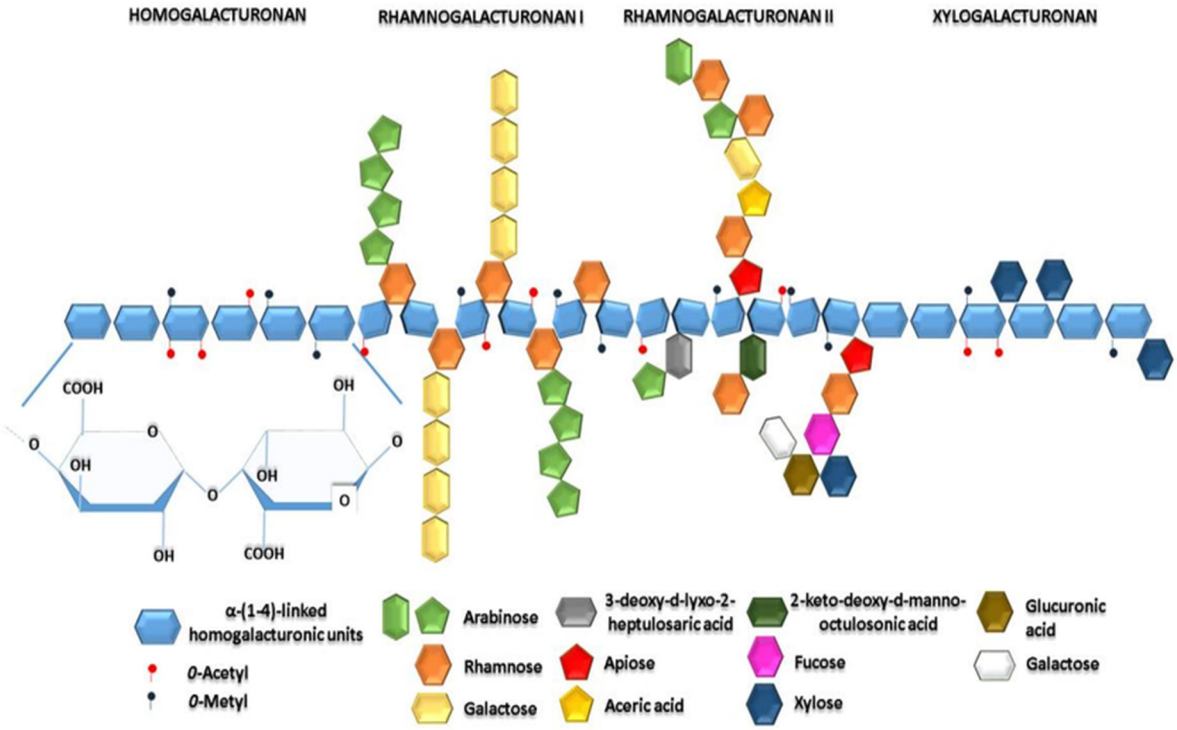

Figure 1: Schematic Structure of Pectin, the relative abundance of the different types of pectin varies, but homogalacturonan and rhamnogalacturonan I are considered the major components, while xylogalacturonan and rhamnogalacturonan II are minor components (Albuquerque *et al.*, 2016)

According to the American Chemical Society (Thakur et al., 1997; Sani and Krishnaraj, 2017), based on the type of modifications of the backbone chain (Kashyap *et al*., 2001), pectinic substances are classified into four main types: protopectins, pectinic acids, pectins and pectic acid (Priya and Sashi, 2014a; Alkorta *et al*., 1998; Sani and Krishnaraj 2017; Kashyap *et al*., 2001). Only protopectins are water insoluble, whereas the other three are either total or partially soluble in water (Alkorta *et al*., 1998).

Protopectins are a water insoluble pectic substances present in plant tissues (Sani and Krishnaraj, 2017) and hydrolysis by enzyme propectinase of protopectin yields pectin or pectic acids (Zhang *et al*., 2018). Pectic acids are galacturonans that contain negligible amounts of methoxyl groups. Normal or acid salts of pectic acids are called pectales. Pectinic acid are the

galacturonans that contains methoxy groups (75%). Pectinotes are normal or acid salts of pectinic acids. Normal or acid salts of pectinic acids are reffered to as pectinates. Pectin (Polymethyl galactenate): It is a polymeric material in which 75% of the carboxyl groups of the galacturonate units are esterified with methanol. It is located in the cell wall and gives rigidity to the cell wall (Sani and Krishnaraj, 2017).

2.2. Pectinase

Pectinases are group of enzymes that attack pectin and depolymerize it by hydrolysis and trans elimination as well as by de-esterification reaction (Suryam and Charya, 2018). Therefore, "pectinase" is a generic name for a family of enzymes that catalyze hydrolysis of the glycosidic bonds in pectic polymers (Bajpai, 2018). They are a big group of enzymes that break down pectic polysaccharides of plant tissues into simpler molecules like galacturonic acids (Hoondal *et al.*, 2002; Pedrolli *et al*., 2009). This enzyme splits polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages (Priya and Sashi, 2014a). Pectinases are inducible, *i.e*., produced only when needed (Hoondal *et al*., 2002). Many plant-pathogenic bacteria and fungi are known to produce pectolytic enzymes useful for invading host tissues. Moreover, pectinases are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (Soares *et al.*, 1999). It has long been used to increase yields and clarity of fruit juices (Soares *et al*., 1999; Ketipallyand Ram, 2018a).

Since pectic substances are a very complex macromolecule (Zhang *et al.*, 2018), various pectinolytic enzymes are required to degrade them completely (Pedrolli *et al*., 2009). Pectinases can be divided into depolymerizing and deesterifying enzymes following criteria such as cleavage type of glycoside linkages, reaction mechanisms (exo- or endotypes), and esterification degree. Furthermore, deesterifying enzymes are classified according to type of ester group (methyl, acetyl, or feruloyl ester). Deesterifying enzymes comprise pectin methylesterase, pectin acetylesterase, ferulic acid esterase, and rhamnogalacturonan acetylesterase. Other pectinases are the oxidases (*i.e*., galacturonic acid oxidase) and those responsible for the metabolism of degradation products of pectin. Such classification refers to the mode of action of pectinases on water-soluble substrates (Ruiz *et al*., 2017).

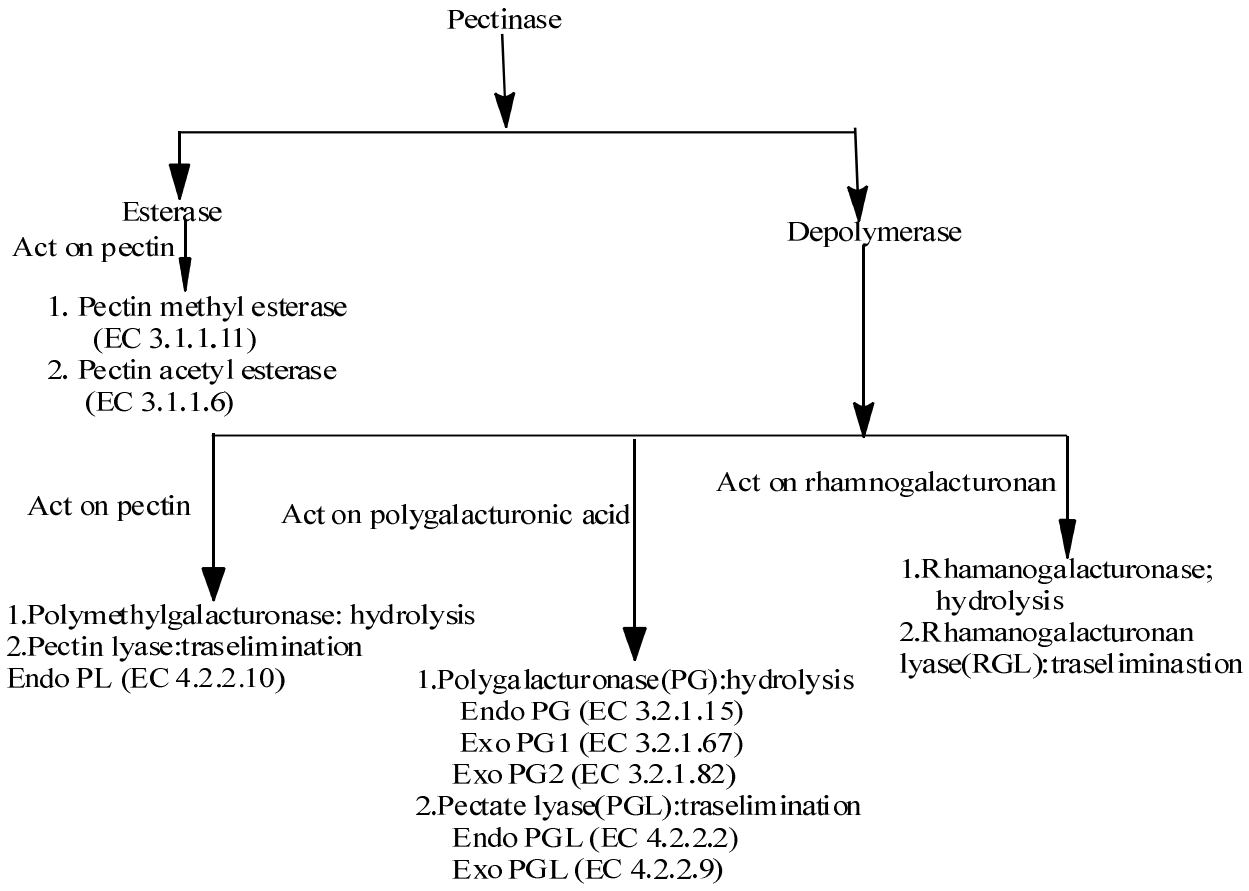

Figure 2: Classification of different pectinases based on their reaction with different pectin substances (Sharma *et al*., 2013).

Traditionally, breaking down of pectic substances was done through the use of well-known pectic enzymes (pectin methylesterase, polygalacturonase, pectin lyase, etc.) able to degrade only the smooth regions (Ruiz *et al*., 2017; Pedrolli *et al*., 2009). This is because, in the past, ´pectinases´ were described only as those enzymes that acted on homogalacturonan chains (Ruiz *et al*., 2017). Scientist discovered the differences in their cleavage mode and specificity and came to classify into two main groups such as pectinases that act on pectin smooth region or on pectin hairy regions (Pedrolli *et al*., 2009). Hence, pectinases are classified by the structural region that cleaves along the chemical structure of pectic substances (Ruiz *et al*., 2017). Indeed, several new enzymes have now been reported, which degrade parts of the hairy region (Ruiz *et al.,* 2017; Pedrolli *et al.,* 2009*)*, such as rhamnogalacturonan hydrolase, rhamnogalacturonan lyase, etc (Ruiz *et al*., 2017).

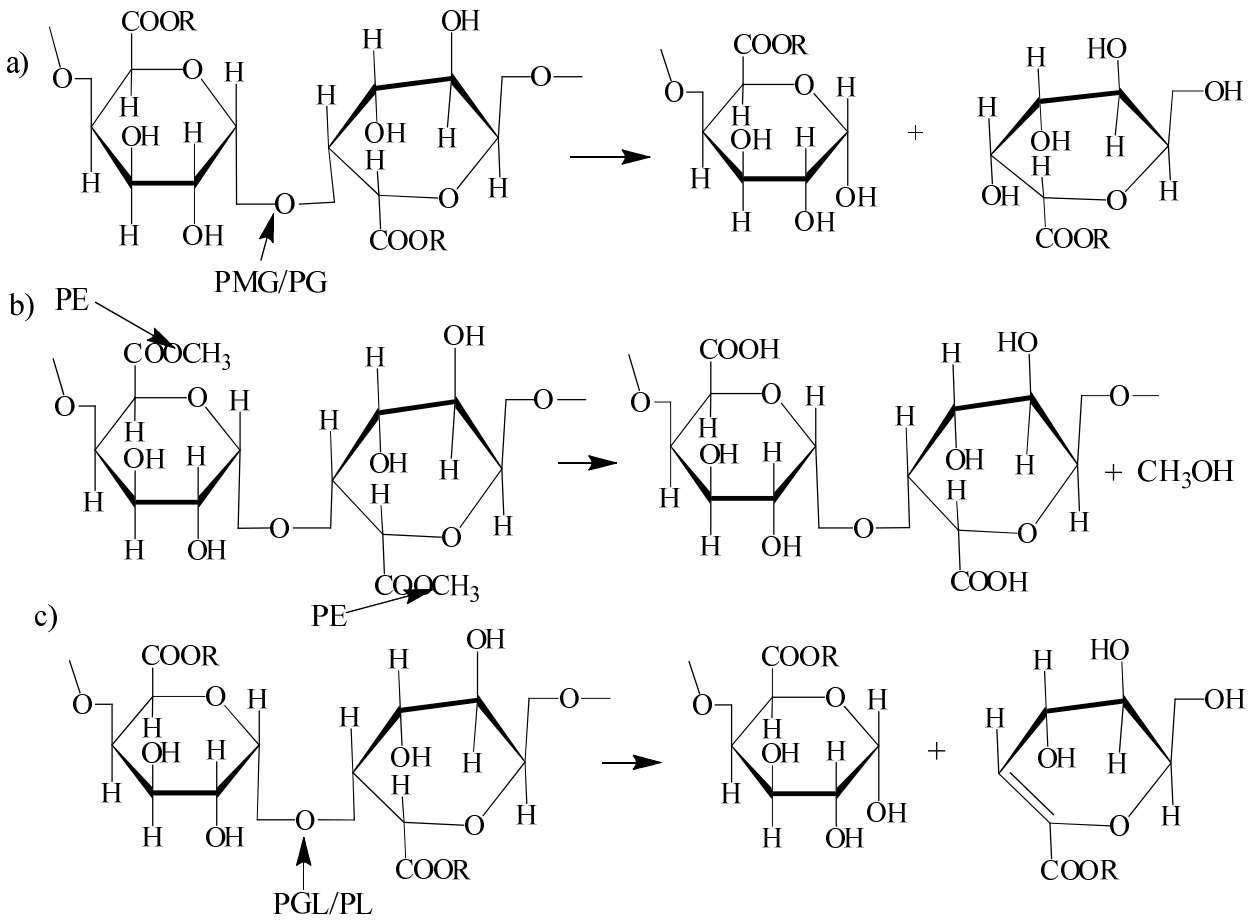

Figure 3: Different types of pectinases and their mode of action on pectic substances, the arrow indicates the place where the pectinase react with the pectic substances: (a) R= H for polygalacturonase (PG) and CH3 for polymethylgacturonase (PMG); (b) Pectin esterase (PE); and (c) R=H for pectate lyase (PGL) and CH3 for pectin lyase (PL) (Pedrolli et al., 2009).

2.3. Applications of Pectinases

Pectinases are among the most important industrial enzymes (Bajpai, 2018). About 45% of juice industries utilize food processing enzymes including pectinases (Kumar and Suneetha, 2016). Application of pectinases in various fields shows the potential for green process (Rebello *et al*., 2017). Most pectic enzyme preparations are used in the fruit processing industry (Priya and Sashi, 2014b) and pectic enzymes alone account for about one quarter of the world's food enzyme production (Priya and Sashi, 2014b; Ruiz *et al*., 2017). Microbial pectinase have tremendous potential to offer mankind. Fungal pectinase are among the most important industrial enzymes and are of great significance with wide range application (Priya and Sashi, 2014b).

Preparations containing pectin-degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectars and in the clarification of fruit juices and wines. They are widely used in industry for retting of natural fibers and extraction of oils from vegetable, citrus peels (Soares *et al.*, 1999), textile processing and bio-scouring of cotton fibers, retting and degumming of plant bast fiber, waste water treatment, coffee and tea fermentation, animal feed, purification of plant viruses, oil extraction, beverages, and wines (Zeni *et al*., 2011; Rebello *et al*., 2017).

2.3.1. Pectinases in the textile industry

Textile processing has benefited greatly in both environmental and product quality aspects through the use of enzymes (Hoondal *et al*., 2002). Pectinases have been used to remove sizing agents from along with amylases, lipases, cellulases and hemicellulases with specific enzymes (Sani and Krishnaraj, 2017). The use of enzymes has decreased the use of harsh chemicals in the textile industry, resulting in a lower discharge of waste chemicals to the environment, improving both the safety of working conditions for textile workers and the quality of the fabric (Hoondal *et al*., 2002).

Pectinases are also used for degumming of plant bast fibers (Sani and Krishnaraj, 2017)**.** The most upcoming application of pectinolytic enzymes is their use in the degumming of plant fibers such asramie, sunn hemp, jute, flax and hemp. The removal of heavily coated, non-cellulosic gummy material from the cellulosic part of plant fibers is called 'degumming' and is necessary prior to the industrial utilization of fibers (Hoondal *et al.*, 2002). Pectinases are used in degumming of textile fibres (Sani and Krishnaraj, 2017) in combination with xylanases which is an ecofriendly and economical alternative to the chemical degumming (Sani and Krishnaraj, 2017; Hoondal *et al*., 2002).

2.3.2. Pectinases in pulp and paper industry

With the advancement of biotechnology and increased reliance of paper and pulp industries on the use of microorganisms and their enzymes for bio-bleaching and paper making, the use of enzymes other than xylanases and ligninases, such as mannanase, pectinases, and α-galactosidase is increasing in the paper and pulp industries in many countries (Hoondal *et al.*, 2002). During papermaking, pectinase can depolymerize polymers of galacturonic acids (Hoondal *et al*., 2002; Sani and Krishnaraj, 2017), and subsequently lower the cationic demand of pectin solutions

(Hoondal *et al*., 2002; Jayani *et al*., 2005) and the filtrate from peroxide bleaching of thermomechanical pulp (Hoondal *et al*., 2002; Sani and Krishnaraj, 2017). Pectinases in combination with xylanases are primarily used in the paper industry as a bio-bleaching agent (Hoondal *et al*., 2002; Rebello *et al.*, 2017). Unlike the conventional chemical bleaching agents, the use of enzymes is found to be eco-friendly, less harsh and good in improving the quality of the paper. Biological bleaching using pectinases in combination with xylanases brighten the paper and improve its physical properties (Rebello *et al.*, 2017).

2.3.3. Pectinases in agriculture

Pectinases are used in the production of animal feeds to reduce the feed viscosity thereby to increase the absorption of nutrients (Sani and Krishnaraj, 2017; Jayani *et al.*, 2005). Pectinases can be used in the purification of plant virus. When the virions are restricted to phloem, to purify the virion particles alkaline pectinases and cellulases can be used (Sani and Krishnaraj, 2017).Pectinases can be used in the purification of plant virus (Sani and Krishnaraj, 2017; Hoondal *et al*., 2002). Knowledge about a virus prior to purification is very limited. Very pure preparations of viruses are required in order to carry out chemical, physical, and other biological studies. There are numerous purification procedures that can be adapted to many of the viruses that infect plants. So that, there are several different purification systems that can be selected for use according to the type of virus (Hoondal *et al.*, 2002). In those cases in which the virus is restricted to the phloem, certain enzymes, such as alkaline pectinases and cellulases, can be used to liberate the virus from the tissues (Hoondal *et al*., 2002; Sani and Krishnaraj, 2017; Rebello *et al*., 2017).

Pectinases are used in poultry feed as enzyme supplementation to improve weight gain and feed conversion significantly. Usually, a feed enzyme preparation is a multi-enzyme cocktail containing glucanases, xylanases, proteinases, pectinases and amylases (Hoondal *et al*., 2002). Enzyme addition reduces viscosity, which increases absorption of nutrients, liberates nutrients either by hydrolysis of non-degradable fibers, or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (Hoondal *et al*., 2002; Jayani *et al*., 2005).

2.3.4. Pectinases in the food and beverage industry

In food industry, biocatalysis have been used to produce raw material and final products for long time (Choi *et al.*, 2015). Among them, pectic enzymes alone account for about one

quarter of the world's food enzyme production (Sani and Krishnaraj, 2017; Ruiz *et al*., 2017; Priya and Sashi, 2014b). The largest industrial application of pectinases is in fruit juice extraction and clarification (Jayani *et al.*, 2005). It contributes to the fruit juice turbidity and viscosity (Suryam and Charya, 2018; Rosales *et al*., 2018; Jayani *et al*., 2005). They are industrially employed in the extraction, clarification, and concentration of fruit juices; the clarification of wines; and the extraction of oils, flavors, and pigments from plants. A mixture of pectinases and amylases are used to clarify fruit juices (Rosales *et al*., 2018). Treatment of fruit pulps with pectinases also showed an increase in fruit juice volume from banana, grapes and apples (Sani and Krishnaraj, 2017; Jayani *et al.*, 2005). Pectinases in combination with other enzymes viz., cellulases, arabinases and xylanases have been used to increase the pressing efficiency of the fruits for juice extraction. Vacuum infusion of pectinases has a commercial application to soften the peel of citrus fruits, to replace hand cutting for the production of canned segments and to pickle processing where to avoid excessive softening during fermentation and storage (Sani and Krishnaraj, 2017).

Alkaline pectinases which are produced by bacterial *Bacillus* species, some fungi and yeasts are used for coffee and tea fermentation (Rebello *et al.*, 2017; Hoondal *et al*., 2002). Pectinase treatment accelerates tea fermentation and also destroys the foam forming property of instant tea powders by destroying the pectins (Hoondal *et al*., 2002; Sani and Krishnaraj, 2017). Pectinase from *A. niger, Byssochlamys fulva* and *Mucor circinelloids* used for fermentation of tea leaves from *Camellia sinensis* plant increases tea quality by increasing production of phenolic compounds (Rebello *et al*., 2017). Pectinolytic microorganisms are used in the fermentation of coffee to remove the mucilaginous coat from the coffee beans. Pectinases are sometimes added to remove the pulpy bean layer consisting of pectic substances (Hoondal *et al*., 2002). They are also used in coffee fermentation to remove mucilaginous coat from coffee beans (Sani and Krishnaraj, 2017). The role of cellulase and hemicellulase enzyme preparations in enhancing digestion has also been exploited to help in digestion of the mucilage (Hoondal *et al*., 2002).

Enzymes have been widely used for decades in the beverage industry to enhance clarification, color extraction, and overall juice yield, including applications on apple, cherry, currant, kiwifruit, and raspberry (Reynolds *et al*., 2018). Pectinolytic enzymes can be utilized in wine and brewing industries for the improvement of chromaticity and stability, juice yields,

clarity, color, phenolic extraction and aroma of red wines (Reynolds *et al*., 2018; Sani and Krishnaraj, 2017). Pectinolytic enzymes are added during brewing processing to improve the visual characteristics (color and turbidity) red wine (Sani and Krishnaraj, 2017; Jayani *et al*., 2005). Enzymatically treated red wines also showed greater stability (Sani and Krishnaraj, 2017).

Pectinolytic enzymes can be used in oil extraction (Hoondal *et al*., 2002; Sani and Krishnaraj, 2017). Plant cell wall-degrading enzyme preparation has begun to be used in olive oil preparation. The enzyme is added during the process of grinding of olives by which easy removal of oil is accomplished in subsequent separation procedures (Hoondal *et al.*, 2002). Oils such as lemon oil also can be extracted with pectinolaytic enzymes. They destroy the emulsifying properties of pectin which interferes with the collection of oils from citrus peel extracts (Sani and Krishnaraj, 2017; Hoondal *et al*., 2002).

Food processing industries release waste water that contains pectin as by-product (Sani and Krishnaraj, 2017; Kashyap *et al*., 2001). Conventionally, the treatment of wastewater from citrus processing industries containing pectic substances is carried out in multiple steps, including physical dewatering, spray irrigation, chemical coagulation, direct activated sludge treatment and chemical hydrolysis, which leads to formation of methane. In addition to environmental pollution from the use of chemicals, this has several disadvantages, such as the high cost of treatment and longer treatment times. Thus, an alternative, cost-effective, and environmentally friendly method is the use of pectinases from bacteria, which selectively remove pectic substances from the wastewater (Hoondal *et al*., 2002). Pretreatment of these waste waters with pectinolytic enzymes removes pectinaceous material and turn into suitable for decomposition by activated sludge treatment (Sani and Krishnaraj, 2017; Hoondal *et al*., 2002). An extracellular endopectate lyase (optimally active at pH 10.0) from an alkalophilic soil isolate, *Bacillus sp. GIR 621*, was used effectively to remove pectic substances from industrial wastewater (Hoondal *et al*., 2002; Kashyap *et al*., 2001).

2.4. Sources of Pectinases

Pectolytic enzymes are widespread in nature (Hoondal *et al*., 2002) and are produced by bacteria, fungi, plants (Rebello *et al*., 2017; Hoondal *et al*., 2002), yeasts, insects, nematodes, and protozoa (Hoondal *et al*., 2002). Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants (Pedrolli *et al.*, 2009; Priya and Sashi, 2014b). Pectolysis is one of the most important processes for plants, as it plays a role in cell elongation (Hoondal *et al*., 2002)

and growth, as well as in fruit ripening (Rebello *et al*., 2017; Hoondal *et al*., 2002). Microbial pectolysis is important in plant pathogenesis, symbiosis, and decomposition of plant deposits; thus, by breaking down pectin polymer for nutritional purposes, microbial pectolytic enzymes play a hugely important role in nature. The enzymes are inducible, *i.e*., produced only when needed, and they contribute to the natural carbon cycle (Hoondal *et al*., 2002).

2.4.1. Microbial pectinases

Microbes are the best source of enzymes because they allow an economical technology with low resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources. Pectinases are produced by both prokaryotic microorganisms, which primarily synthesize alkaline pectinases, and by eukaryotic microorganisms such as fungi synthesize acid pectinases (Kohli and Gupta, 2015). Microbial pectinases are used frequently for its ease of production and unique physicochemical properties (Rebello *et al*., 2017). Microbial pectinase have tremendous potential to offer mankind (Priya and Sashi, 2014b). They are increasingly becoming important producer of pectinases for both technical and economic point of view (Priya and Sashi, 2014a). Various genera of microorganisms can produce pectinases of potential applications (Moubasher *et al*., 2016). Several species of microorganisms such as *Bacillus, Erwinia, Kluyveromyces, Streptomycetes, Actinomycetes, Aspergillus, Rhizopus, Xanthomonas, Trichoderma, Pseudomonas, Penicillium,* and *Fusarium* are known as predominant producers of pectinases (Zeni *et al*., 2011; Rebello *et al*., 2017).

2.4.1.1. Fungal pectinase

The enzymes preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3.0 to 5.5 (Soares *et al*., 1999). Fungal pectinase are among the most important industrial enzymes and are of great significance with wide range application including in juice clarification, textile processing, degumming of plant bast fibers, treatment of pectic wastewaters, papermaking and coffee and tea fermentations. Therefore, the biotechnological potential of pectinolytic enzymes from fungi has drawn a great deal of attention from various researchers worldwide (Priya and Sashi, 2014b).

According to (Priya and Sashi, 2014a) different pectinase producing fungal strains were discovered including *Aspergillus niger, Aspergillus versicolor, Aspergillus flavus,*

Fusarium oxysporum, Rhizopus stolonifer, Mucor racemous, Mucor hiemalis, Penicillium jensen i, Penicillium citrinum and Trichoderma viride and the plate assay shows, maximal pectinolytic activity by *Aspergillus niger, Penicillium jenseni* and closely followed by *Penicillium citrinum.* Strains including *A. niger*, A. *wnetii, A. oryzae and Rhizopus sp.* are found to be suitable organism for industrial pectinase production (Sani and Krishnaraj, 2017).

2.4.1.2. Bacterial pectinases

Pectinolytic enzymes are found in plant pathogenic bacteria (Sharma *et al.*, 2013). Some of the pectinolytic bacteria include *Bacillus spp., clostridium spp., and pseudomonas spp* (Prathy usha and Suneetha, 2011). Although highly productive bacterial strains producing polygalacturon ate lyase are known, still pectinases are not produced commercially from them (Sani and Krishn araj, 2017).

2.4.2. Non-microbial pectinases

The wide range of biotechnological applications of pectinolytic enzymes lead to a constant search for novel enzymes that are more compatible to the industrial requirements in improving the efficiency of pectin processing. Although pectinases from microbial sources have been extensively studied, endogenous insect pectinases were only recently discovered and their physicochemical properties remain largely unexplored (Habrylo *et al*., 2018).

In a nutshell, pectinases are group of enzymes which catalyze the degradation of complex heteropolysaccharide called pectin. Pectinases are used in numerous green industrial aplications. However, application of pectinases requires exposure of extreme conditions such as high temperature and high or low pH. For example application of pectinase in coffee and tea fermentation, degumming of natural fiber, pulp and paper industry, textile processing and purification of plant virus results exposure to alkaline environment. Antagonistically, application of pectinases in juice processing and wine industry results exposure to acidic environment. But, enzymes are unstable in these imposed conditions. Hence, to overcome stability of pectinase a variety of approaches have been attempted including screening of enzymes from natural sources (extremophiles), random mutations, and immobilization. Therefore, this study emphasizes to:

i. Isolate and screen pectinase producing fungi from alkaline saline and hot environment such as Lake Shalla and Sodere spring water
ii. Optimize pectinase production without increasing the cost; characterize the activity of the enzyme and also to study the kinetics of the produced pectinases.

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The soil and water samples were taken from Lake Shala which is found in Oromia Regiona l State, Ethiopia. Lake Shala is an alkaline soda lake with pH ranging from 8.31 to 10.3 (Klempe rer and Cash, 2007). The Lake is surrounded by alkaline (pH ranging from 8.1 to 8.8) hot spring water temperature ranges from 54-70 ^{o}C (Baumann *et al.*, 1975). The hot spring water sample was taken from Sodere hot spring water which is also found in the Oromia Regional State, Ethiopia. Sodere spring water hot with temperature and pH of 62 ^{o}C and 7.1 respectively (Gizaw, 1996).

3.2. Sample Collection

The soil and water samples were collected from Lake Shalla, and the other water samples were taken from Sodere Spring Water, Oromia Regional State, Ethiopia. About 20 g of each soil samples was collected aseptically using UV-rays sterilized polythene bags and about 20 mL of water samples was taken by autoclaved sterile bottle. The sample taken from Shala soil, Shala water and Sodere hot spring water were designated as SS, SL and SH respectively followed by its sample number. The samples containing bags and bottles were sealed, transported and then stored at 4 ^{o}C in a refrigerator at Microbial Biotechnology Research Laboratory, Ethiopian Institute of Agricultural Research until the time of the analysis.

3.3. Serial Dilution

One gram (1g) of soil samples and 1 mL of water samples from each samples were pooled out and homogenized in sterile 9 mL distilled water aseptically. The homogenized samples were agitated well using vortex mixer, and then serially diluted 10^4 and 10^5 times.

3.4. Media Preparation for Filamentous Fungi Isolation

Czapek-dox agar medium was prepared by dissolving (10 g) citrus pectin, (3 g) Di-ammonium orthophosphate, (2.0 g) K_2HPO_4, (0.1 g) $MgSO_4$ and (20 g) agar in 1000 mL of distilled water in 2 L Erlenmeyer flask (Ketipally and Ram, 2018b). The flask was sterilized at a temperature of 121 ^{o}C for 15 minutes and to restrict the bacterial growth Streptomycin (100 mg/L) was added to the media composition (Adeleke *et al*., 2012). Finally, about 20 - 25 mL of

the sterilized Czapek-dox agar media was poured on sterile petri plates in the microbiological hood and allowed to solidify at room temperature.

3.5. Media Preparation for Yeast Isolation

For isolation and screening of yeast a mineral medium containing citrus pectin was used as a sole carbon source: (10.0 g/L) citrus pectin, (1.0 g/L) yeast extract, (15.0 g/L) agar, (0.2 g/L) KH_2PO_4, (0.05 g/L) $CaCl_2$, (1.0 g/L) $(NH_4)_2SO_4$, (0.05 g/L) $MgSO_4.7H_2O$ was prepared as described by (Merín *et al*., 2011). The flask was sterilized at a temperature of 121 ^oC for 15 minutes and to restrict the bacterial growth Streptomycin (100 mg/L) was added to the media composition (Adeleke *et al*., 2012). Finally, about 20 -25 mL of the sterilized mineral agar media was poured on sterile petri plates in the microbiological hood and allowed to solidify at room temperature.

3.6. Isolation and Purification of Filamentous Fungi

The filamentous fungus was isolated by using pectin as sole carbon source Czapek-dox agar media as described insection **3.4**. The samples were serially diluted as described in section **3.3**. To isolate fungal extremophile 50 µL of 10^{-4} and 10^{-5} of serially diluted samples were taken and spread over the surface of semi-solid Czapek-dox agar media. Inoculated plates were incubated at 30 ^oC for 3-8 days in the INFORS HT Ecotron incubator. Different colonies were randomly picked from countable plates and the filamentous fungus isolate were purified by successive striking technique on Czapek-dox agar media. Colonies with distinct morphologies were randomly selected and the cultures were stored in the corresponding isolation broth described above containing 20% glycerol, until further analysis.

3.7. Isolation and Purification of Yeast

From water and soil samples, yeasts were isolated by spreading about 50 µL of 10^4 and 10^5 times serially diluted samples over semi solid mineral medium prepared as described in section **3.6**. Inoculated plates were incubated at 30 ^oC for 3-8 days in the INFORS HT Ecotron incubator. Different colonies were randomly picked from countable plates and yeasts were purified by successive striking technique. Colonies with distinct morphologies were randomly selected and the cultures were stored in the corresponding isolation broth described above containing 20% glycerol, until further analysis.

3.8. Primary Screening of Filamentous Fungi for Pectinolytic Activity

Potent producers of pectinase enzyme were screened by using modified pectin agar medium which is called Czapek-dox agar medium by using pectin as sole carbon source. The media was prepared as described in section 3.4 and point inoculated at the center of the petri plate. Then, inoculated plates were incubated at 30 ^{o}C for 72 hours in the INFORS HT Ecotron incubator. After incubation, they were flooded with iodine-potassium iodide solution (1.0 g) Iodine, (5.0 g) potassium-Iodide in 330 mL distilled water) on the culture plates and the plates were observed for zone of hydrolysis (Soares et al., 1999; Ketipally and Ram, 2018b). 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 potency index was assumed to contain the highest activity. Then after, isolates with highest ratio were selected for further screening.

$$\text{Potency Index} = \frac{(\text{Zone diameter} + \text{Colony diamter})}{\text{Colony diamter}}$$

3.9. Primary Screening of Yeasts for Pectinase Activity

Potent producers of pectinase enzyme were screened by using modified pectin agar medium by using pectin as sole carbon source. The media was prepared as described in section 3.5 and point inoculated at the center of the petri plate. Then, inoculated plates were incubated at 30 ^{o}C for 72 hours in the INFORS HT Ecotron incubator. After incubation, flooded with iodine-potassium iodide solution (1.0 g) Iodine, (5.0 g) potassium-iodide in 330 mL distilled water) on the culture plates and the plates were observed for zone of hydrolysis (Soares et al., 1999; Ketipally and Ram, 2018b). The ratio of the clear zone diameter to colony diameter during that span of time was measured to select isolates with highest pectinase activity as explained in the above equation.

3.10. Secondary Screening of Potential Pectinase Producing Fungus

In order to identify isolates with pectinase activity at alkaline pH, the screened and selected isolates with highest hydrolysis zone were subjected to submerged fermentation for further screening by using modified Czapek-dox broth medium. The composition of modified Czapek-dox broth medium was (10 g) citrus pectin, (3 g) di-ammonium orthophosphate, (2.0 g) K_2HPO_4, (0.1 g) $MgSO_4$, Distilled water-1000 mL (Ketipally and Ram, 2018b). The broth medium was autoclaved at 121 ^{o}C for 20 minutes. A volume of 50 mL Czapek-dox broth medium in 250 mL

Erlenmeyer flask was inoculated with 200 µL freshly cultured inoculum. The inoculated flasks were incubated at 30 ^{o}C on an INFORS HT Ecotron incubator shaker at 120 rpm. Samples from inoculated flasks were taken after 72 hours and centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant was used for assaying the enzyme activity. The enzyme activity was assayed in 0.1 M Tris-HCl buffer, pH 8.5 and at temperature of 70 ^{o}C. Isolates having highest activity were considered for further study.

3.11. Preparation of Dinitrosalicylic Acid (DNS)

The dinitrosalicylic acid (DNS) reagent was prepared by dissolving 5 g of dinitrosalicylic acid in 250 mL of distilled water at 80 ^{o}C. When this solution reaches room temperature, 100 mL of 2 N NaOH and 150 g of potassium sodium tartarate-4-hydrate were added and the volume was completed with distilled water to 500 mL (Gonçalves *et al.,* 2010).

3.12. Preparation of D-Galacturonic Acid Standard Curve

In order to express the enzyme activities into Enzyme unit, standard curve of D-galacturonic acidwas plotted. The stock concentration of 5 mg/mL D- galacturonic acid, Phosphate buffer pH 7.5 solutions were prepared. A mixture of 1000 µL of 5 mg/mL D-galacturonic acid and Phospahte buffer pH 7.5 with different concentrations as listed below in Table 1 were filled into test tubes. Then 2000 µL of dinitrosalicylic acid reagent (DNS) was subsequently poured and the reaction mixture was boiled for 10 min at 92 ^{o}C. Finally, the tubes were cooled and the absorbance was measured at wavelength of 540 nm. The relationship between D-galacturonic acid concentration and OD 540 nm was plotted to get standard linear equation using Microsoft Excel.

Table 1: Standard concentrations of D-galacturonic acid for determination of unit of pectinase

Number	5 mg/mL D-galacturonic acid (µL)	Buffer (µL)	D-galacturonic acid (ppm)	D-galacturonic acid (mg/mL)
1	0	1000	0	0.0
2	100	900	500	0.5
3	200	800	1000	1.0
4	300	700	1500	1.5
5	400	600	2000	2.0
6	500	500	2500	2.5
7	600	400	3000	3.0
8	700	300	3500	3.5

From the graph, the linear regression equation was derived as **y** = 0.5383x +0.028**.** The regression coefficient was R^2= 0.9937 which pronounces the concentrations of D-galacturonic acid and OD540 nm readings were powerfully positively correlated (Figure 4). Based on the regression equation, the amount of D-galacturonic acid (mg/mL) released based on the action of enzyme was expressed as follows:

$$[D_\ galacturonic\ acid]\ mg/mL\ = \frac{((OD540)_0.028) * \ dilution\ factor}{0.5383}$$

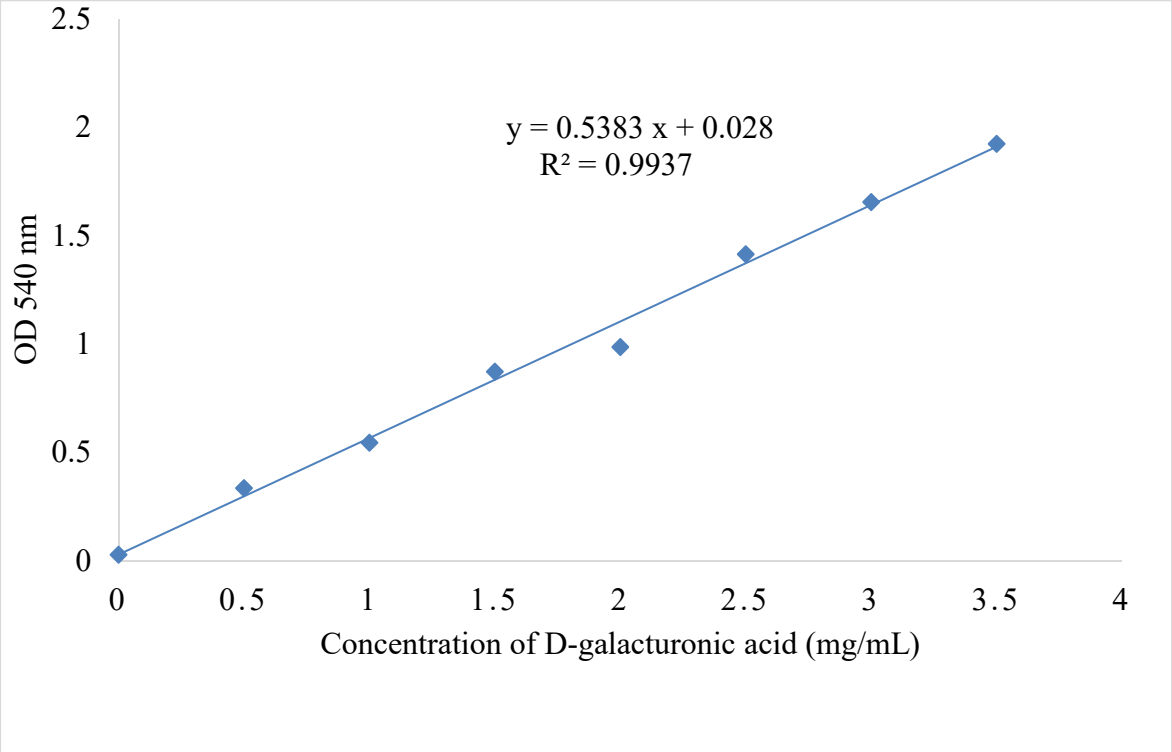

Figure 4: D-Galacturonic acid standard curve

3.13. 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, 15 mL of freshly grown culture was taken and filtered and centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant (100 μL) 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 8.0 Tris-HCl buffer.

From the prepared substrate, 900 µL was added into clean labeled test tubes; one for enzyme blank, the other for enzyme. Then, 100 µL of crude enzyme was added into test tube labeled as enzyme and test tube labeled as enzyme blank remained as it was. Then, the test tubes were incubated at 60 ^{o}C for 10 min in water bath. After incubation, 2000 µL of dinitrosalicylic acid reagent (DNS) was added into all the 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 (92 ^{o}C) for 10 min. Finally, the tubes were cooled and optical density (OD) was measured using visible spectrophotometer (Novaspec III Visible Spectrophotometer, Amersham Biosciences, Sweden) at wavelength of 540 nm. This is because

D-galacturonic acid reduces 3, 5-dinitrosalycylic acid into 3-amino-5-nitrosalycycilc acid which strongly absorbs at 540 nm (Figure 5). Enzyme activity was measured against enzyme blank. The enzyme unit was defined as the amount of enzyme that catalyzes µmol of D-galacturonic acid per minute (μmol min^{-1}) under the assay conditions.

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

$$\text{Relative Activity} = \frac{\text{Activity of sample(U)X100}}{\text{Maximum enzyme activity(U)}}$$

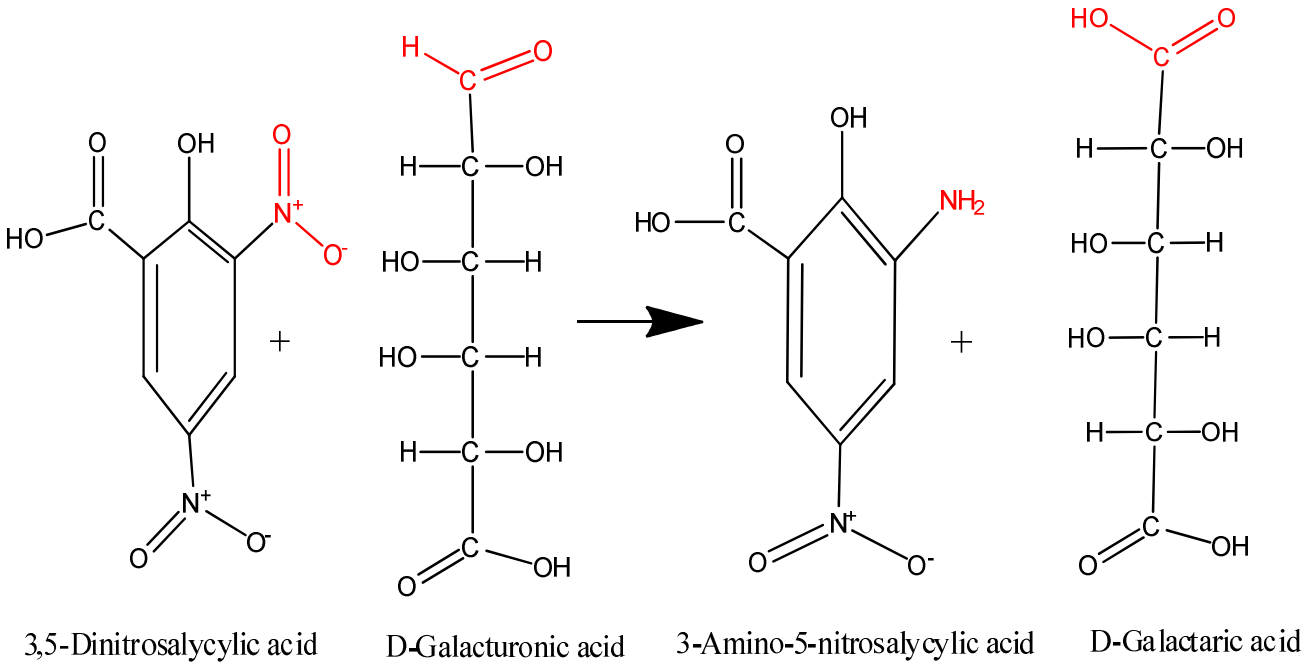

Figure 5: Oxidation of D-galacturonic acid

3.14. Preliminary Characterization of Pectinase

In order to identify the optimum conditions, the pectinase from selected isolates were preli minary characterized for its optimum pH and optimum temperature.

3.14.1. 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 (pH4.5-10.5) by preparing substrate (citrus pectin, 0.5%, w/v) in 0.1 M of dif ferent 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 70 ^{o}C for 10 minutes in the water bath. After incubation 2000 µL 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 visible spectrophotometer (Novaspec III Visible Spectrophotometer, Amersham Biosciences, Sweden) at 540 nm.

3.14.2. Optimum temperature for activity of pectinase

Optimum temperature for the pectinase activity was assayed at different temperatures 30–90 °C by 5 ^{o}C interval at optimized pH. 100 µL crude enzymes were incubated with 900 µL of citrus pectin (0.5%, w/v) for 10 minutes. After incubation 2000 µL of dinitrosalicylic acid reagent (DNS) was added and the tubes were placed in a boiling water-bath at 92 ^{o}C for another 10 min. Finally, the tubes were cooled and optical density (OD) was measured using visible spectrophotometer (Novaspec III Visible Spectrophotometer, Amersham Biosciences, Sweden) at 540 nm.

3.15. Pectinase Production Optimization

3.15.1. Inoculum preparation

Fresh culture of the screened and selected isolate for pectinase production was inoculated into sterilized Czapek-dox agar medium with pH of 7.0±0.5. The inoculated flask was incubated at 30 ^{o}C on a rotary shaker at 130 rpm. Culture was grown in 50 mL media in 250 mL Erlenmeyer flasks. This seed culture was used for subsequent experiments.

3.15.2. Preparation of the agro-residue

The agro based materials, *i.e.,* orange, banana, mango were collected from a local fruit Addis Ababa Market and peeled while the wheat straw was purchased from Holleta Local Market. Orange peels, mango peels, banana peels and wheat straw were oven dried at 60 ^{o}C. The oven dried agro-residues were grinded with Cyclotec Grinder Machine (Cyclotec 1093, Foss Analytical Co.,Ltd) to 1.19 mm particle size.

3.15.3. Effect of different agro- residues on pectinase production

To study the potential of agro residues for pectinase production using submerged fermentation, variety of agro-residues (orange peel, banana peel, mango peel and wheat straw) were used as substrates. For comparison, Czapek-dox broth medium was prepared by dissolving

citrus (10 g) pectin, (3 g) di-ammonium orthophosphate, (2.0 g) K_2HPO_4, and (0.1 g) $MgSO_4$ in 1000 mL of distilled water in 2 L Erlenmeyer flask. Each of the agro residues were taken instead of pectin in modified pectin media explained above. In 250 mL conical flask, 100 mL of each agro residue and Czapek-dox broth medium were autoclaved at 121 ^{o}C for 15 minute. The flasks were inoculated with 200 µL of isolate and incubated with shaker incubator at 130 rpm, 30 °C for 72 h. For enzyme assay, 15 mL of freshly grown culture was taken and filtered and centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant was used for enzyme assay and the activities of all isolates were measured as described above.

3.15.4. Effect of pH of growth media

The pH of the orange peel substrate was adjusted to pH that ranges from 3.5 to 10.5 with 1.0 interval before sterilization. The sterilized nutrient medium were inoculated and incubated at 30 ^{o}C, 120 rpm using submerged fermentation (SmF) for 72 hours.

3.15.5. Effect of temperature on pectinase production

The sterilized optimized orange was inoculated and incubated at various temperatures such as 20 ºC, 30 ºC, 40 ºC, 50 ºC, 60 ºC and 70 ºC at 130 rpm using SmF for 72 hours to study the effect of temperature on enzyme production.

3.15.6. Effect of nitrogen sources

The effect of Nitrogen sources on pectinase production in SmF was studied by supplementing various nitrogen sources namely casein, peptone, urea, ammonium sulfate, ammonium chloride of 1% (w/v) into orange peel agro residue as a substrate.

3.15.7. Effect of time of incubation

To study the effect of incubation time, the optimized orange peel was inoculated and incubated at 40 ^{o}C and 130 rpm. With 48 hour interval pectinase activity was assayed for selected isolates.

3.15.8. Effect of sodium chloride

The effect of sodium chloride on pectinase production using submerged fermentation was studied by directly incorporating it into orange peel agro residue at a final concentration of 2%, 4%, 6%, and 8% (w/v) incubated at 130 rmp and 40 ^{o}C.

3.16. Characterization of Pectinase

The selected isolates were grown at optimized physiochemical parameters and conditions on submerged fermentation. After the end of incubation period, 15 mL of freshly grown culture was taken and filtered and centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. The supernatant was used for enzyme assay and the activities of all isolates were measured as described above.

3.16.1. 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 60 °C 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.

3.16.2. 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 pH= 8.0 with 100 µL of crude enzyme) at different temperatures in the range of 30-90 ^{o}C for 10 min with 5 ^{o}C interval and the enzyme activity was assayed.

3.16.3. Effect of substrate on pectinase activity

The effect of substrate on pectinase enzyme activity was determined by incubating 100 µL of crude enzyme with 900 µL of different substrates like citrus pectin, polygalacturonic acid. These substrates were prepared in 0.1 M of Tris-HCl buffer (pH 8.0) with 0.5% w/v concentration. The reaction mixture was incubated at 60 ^{o}C for 10 minute and the enzyme activity was assayed.

3.16.4. Effect of sodium chloride

The effect of sodium chloride on pectinase activity was studied by directly incorporating it into the enzyme substrate system at a final concentration of 2%, 4%, 6%, and 8% (w/v).

3.16.5. 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+}, Zn^{2+} and Mn^{2+}. The reaction mixture was incubated at 60 °C for 10 min and the enzyme activity was assayed.

3.16.6. Determination of enzyme stability

The degree of enzyme stability under optimized temperature of 60 °C and optimized pH of 8.0 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.

3.16.7. Michaelis-Menten constant (K_m) and V_{max} values

The K_m and V_{max} values were determined by measuring the reaction velocity at different concentrations of the substrate (Citrus Pectin). First stock solution of citrus pectin *i.e* 10 mg/mL concentration was prepared with appropriate buffer (0.1 M Tris-HCl - pH 8.0). Then the stock solution was diluted by appropriate volume of buffer to make the final mg/mL citrus pectin concentrations listed in Table 2. The appropriate mg/mL citrus pectin (900 µL) was incubated with 100 µL of suitably diluted enzyme at 60 °C for 10 min and the pectinase enzyme activity assayed.

Table 2: mg/mL Concentrations of citrus pectin to determine the K_m and V_{max} 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 excel software. The reaction speed V_{max} and K_m were determined according to (Lineweaver and Burk, 1934) by plotting of the reciprocal of the observed velocity (1/V) against the reciprocal of the substrate concentration (1/[S]).

3.17. Data Analysis

The study was carried out experimentally in triplicate. The results were expressed as the mean of data obtained from triplicate experiments ±SD. The data were fed to Microsoft Excel

and data were analyzed by analysis of variance using general Linear Model procedure of SAS software (SAS, 2003). Differences among means with $P < 0.05$ were accepted as statistically significant. Tukey multiple comparison were deployed to separate treatment means. However, the effect of substrate on enzyme activity was compared by paired student T-test and analyzed by using Microsoft Excel 2010.

4. RESULTS

4.1. Isolation of Microorganisms

In total, thirty (30) isolates were identified from 15 samples. Based on characterization on the selective growth media, the isolates were grouped as Yeast (40%) and Filamentous Fungi (60%). For identification purpose, the isolates were designated by prefix-"SH" Sodere Spring Water, "SS" Shala Lake Soil, and "SL" Shala Lake Water followed by their isolate numbers for filamentous fungus and "PSH" Sodere Spring Water, "PSS" Shala Lake Soil, "PSL" Shala Lake water followed by their isolate numbers were used for yeasts.

4.2. Screening Isolates for Pectinase Production

4.2.1. Primary screening

To identify isolates with higher pectinase activity, the ratio between product clear zone diameter and colony diameter to colony diameter was calculated. The highest ratio observed was 5.3±0.44 by isolate SS-2. Those isolates which scored higher than or equal to 3.7±0.36 (Mean ±SD) were considered as high enzyme producers, which accounts for 30% of the pectinase positive isolates as showed Table 3. These isolates were selected for further screening.

Table 3: Primary screening: isolates (clear zone +colony diameter ratio) to colony diameter ratio.

Number	Isolate	*Potency index	Number	Isolate	*Potency Index
1	SL1	4.2 ± 0.25	16	SS5	5.1±0.55
2	SL2	2.8 ± 0.36	17	SH1	5.0±0.15
3	SL3	2.8 ± 0.58	18	SH2	3.7±0.36
4	SL4	5.0 ± 0.25	19	PSL-1	1.3±0.17
5	SL5	2.4 ± 0.15	20	PSL-2	1.2±0.25
6	SL6	2.6 ±0.12	21	PSL-3	1.3±0.25
7	SL7	3.2 ±0.15	22	PSL-4	2.5±0.50
8	SL8	2.2 ±0.25	23	PSL-5	2.8±0.76
9	SL9	3.2 ±0.15	24	PSL-6	2.3±0.96
10	SL10	4.4 ±0.32	25	PSS-1	1.7±0.25
11	SL11	2.9 ±0.58	26	PSS-2	1.2±0.15
12	SS1	4.5 ±0.15	27	PSS-3	2.8±0.76
13	SS2	5.3 ±0.44	28	PSS-4	2.8±1.04
14	SS3	3.8±0.45	29	PSH-1	2.8±0.76
15	SS4	2.4 ±0.1	30	PSH-2	2.1±0.90

- *Values are Mean ±SD of triplicate measurements*.

4.2.2. Secondary screening

In order to further screen the selected isolates from primary screening submerged fermentation test was performed. At this stage, subsequent to submerged fermentation, aliquot samples were taken and assayed for their pectinase activity using Tris-HCL pH 8.5 with temperature of 70 ^{o}C. Five isolates which showed higher pectinase activity were selected for this study (Table 4).

Table 4: Secondary screening: pectinase activity (Unit/mL).

Number	Strain number	*Enzyme activity (U/mL)	Relative Activity (%)
1	SL-1	20.42±0.07[bc]	94.19
2	SL-4	20.74 ±0.11[b]	95.68
3	SL-10	11.45 ±0.03[e]	52.81
4	SS-1	9.65± 0.62[f]	44.51
5	SS-2	20.16 ±0.05[bc]	92.97
6	SS-3	9.35 ±0.04[f]	43.11
7	SS-5	20.12±0.03[c]	92.81
8	SH-1	21.68 ±0.05[a]	100.00
9	SH-2	12.48±0.04[d]	57.55

- **Values are mean ± SD of 3 replicates*
- *The same superscript letters at the top right side of the values are not significantly different with in column at (P < 0.05).*

4.3. Preliminary Characterization of Pectinase

In order to identify the optimum conditions for the pectinase activity from isolate SL-1, SL-4, SS-2, SS-5 and SH-1, 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 achieved at pH 8.0-Tris-HCL buffer (Appendix 3). The optimum temperature was identified by assaying the pectinase activity at ranges of temperature and the maximum activity of pectinase was attained at 60 ^{o}C (Appendix 4).

4.4. Optimization of Growth Media for Maximal Pectinase Production

4.4.1. Agro residues

To study the effect of agro-residues on pectinase production using submerged fermentation, different agro residues which are listed on Table 5 were inoculated with five

potential isolates. Orange peel achieved the maximum pectinase production with isolate SL-1 which is 23.48±0.01 U/mL (Table 5). In contrast, the lowest pectinase production was from mango peel which is 0.84±0.05 U/mL (Table 5). Production of pectinase using orange peel, mango peel, banana peel, and wheat bran was significantly different in all isolates except isolate SS-2 which showed no statistical significant in banana and mango peel. However, production of pectinase using commercial pectin as a carbon source showed highest pectinase production compared to all agro residues which is statically significant (Table 5). Therefore, the subsequent SmF studies were carried out using orange peel as substrate.

Table 5: Effect of agro-residues on pectinase production

	***Enzyme activity (U/mL)**				
Strain number	**Orange peel**	**Banana peel**	**Mango peel**	**Wheat bran**	**Control**
SL-1	$^{a}23.48\pm0.01^{b}$	$^{b}2.53\pm0.02^{e}$	$^{c}2.27\pm0.08^{d}$	$^{b}9.19\pm0.34^{c}$	$^{c}29.03\pm0.07^{a}$
SL-4	$^{d}21.16\pm0.04^{b}$	$^{c}2.21\pm0.07^{d}$	$^{e}0.84\pm0.05^{e}$	$^{ab}9.52\pm0.01^{c}$	$^{d}27.89\pm0.04^{a}$
SS-2	$^{e}18.49\pm0.05^{b}$	$^{d}1.97\pm0.01^{d}$	$^{d}1.86\pm0.03^{d}$	$^{a}10.20\pm0.05^{c}$	$^{c}29.02\pm0.04^{a}$
SS-5	$^{c}22.33\pm0.05^{b}$	$^{c}2.18\pm0.03^{d}$	$^{b}2.77\pm0.21^{d}$	$^{c}5.19\pm0.46^{c}$	$^{a}33.05\pm0.04^{a}$
SH-1	$^{b}23.28\pm0.02^{b}$	$^{a}9.98\pm0.05^{c}$	$^{a}6.28\pm0.02^{e}$	$^{b}9.10\pm0.31^{d}$	$^{b}29.44\pm0.08^{a}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05).*
- *Control- Czapek-dox broth medium instead of agro-residue*

4.4.2. Initial pH of growth media

To study the effect of initial pH of growth media on SmF pectinase production on orange peel pH was adjusted to a range of 3.5 - 10.5. After 72 hour incubation, samples were taken and assayed for enzyme activity. The maximum pectinase activity was attained at initial pH of 6.5 orange peels (Figure 6). Hence, the subsequent studies were performed at initial pH of 6.5.

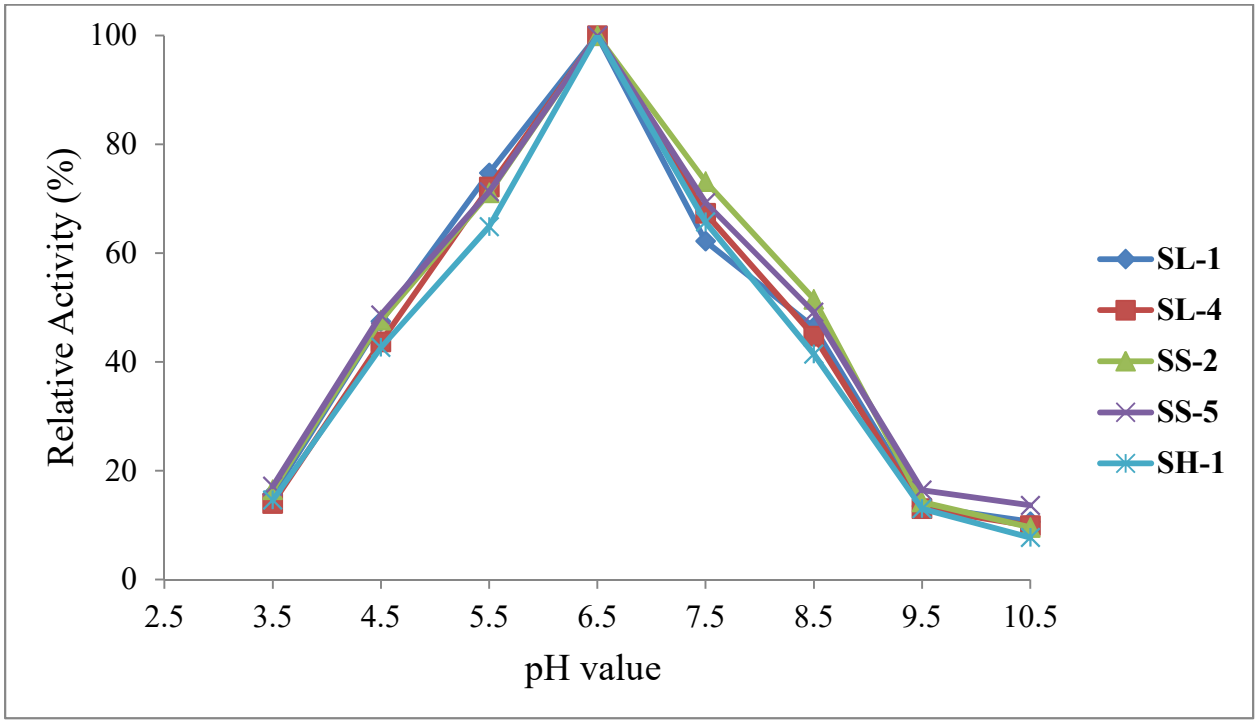

Figure 6: Effect of initial pH of growth media on pectinase production

4.4.3. Effect of temperature

To study the effect of temperature of growth media on submerged fermentation (SmF) pectinase production orange peel were incubated in temperature ranges of 20 ^{o}C to 70 ^{o}C. After 72 hour of incubation, aliquot of samples were taken and assayed for enzyme activity. The maximum pectinase activity was attained at a temperature of 40 ^{o}C (Figure 7). Hence, the subsequent studies were performed at 40 ^{o}C.

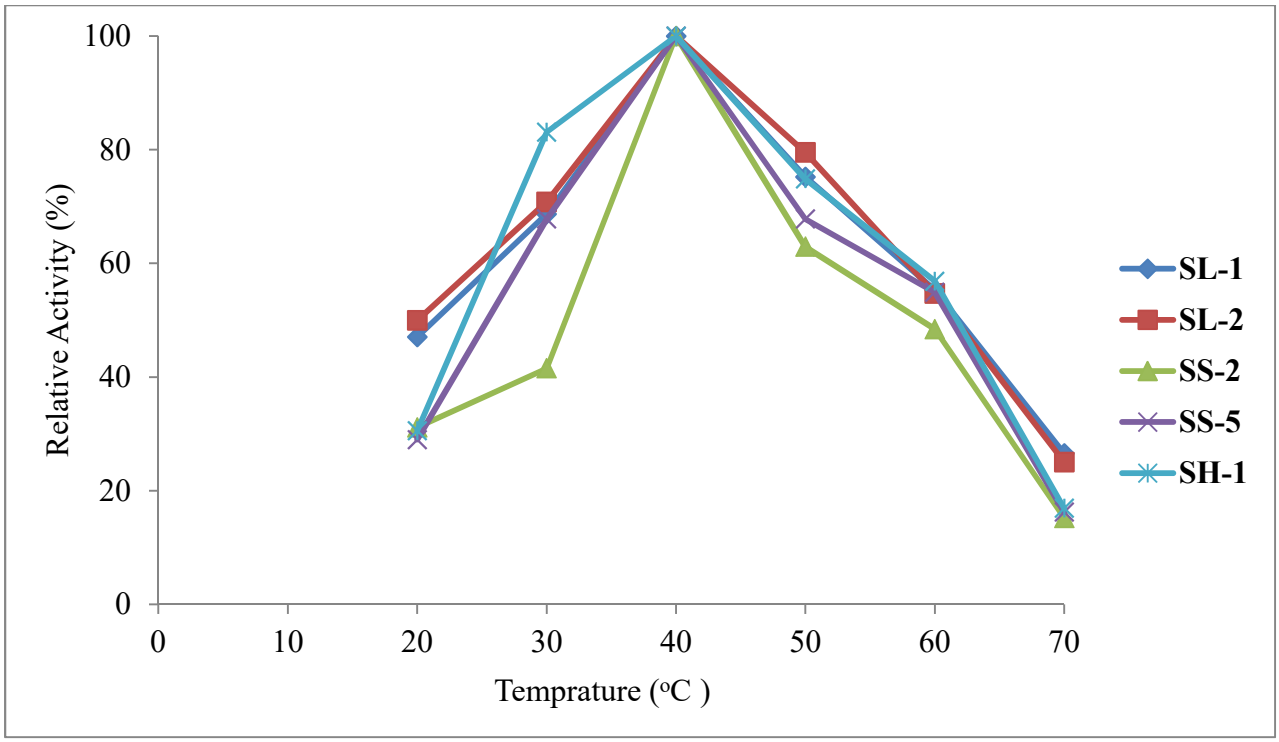

Figure 7: Effect of incubation temperature on pectinase production.

4.4.4. Effect of incubation period

To study the effect of incubation period on pectinase production, the inoculated orange peel broth was assayed at 48 hours of interval for pectinase activity. Accordingly, the highest pectinase enzyme production was achieved at 96 hrs of incubation (Figure 8; Appendix 19). However, beyond 96 hrs of incubation the production of pectinase were declined as showed in all isolates considered in this study (Figure 8; Appendix 19).

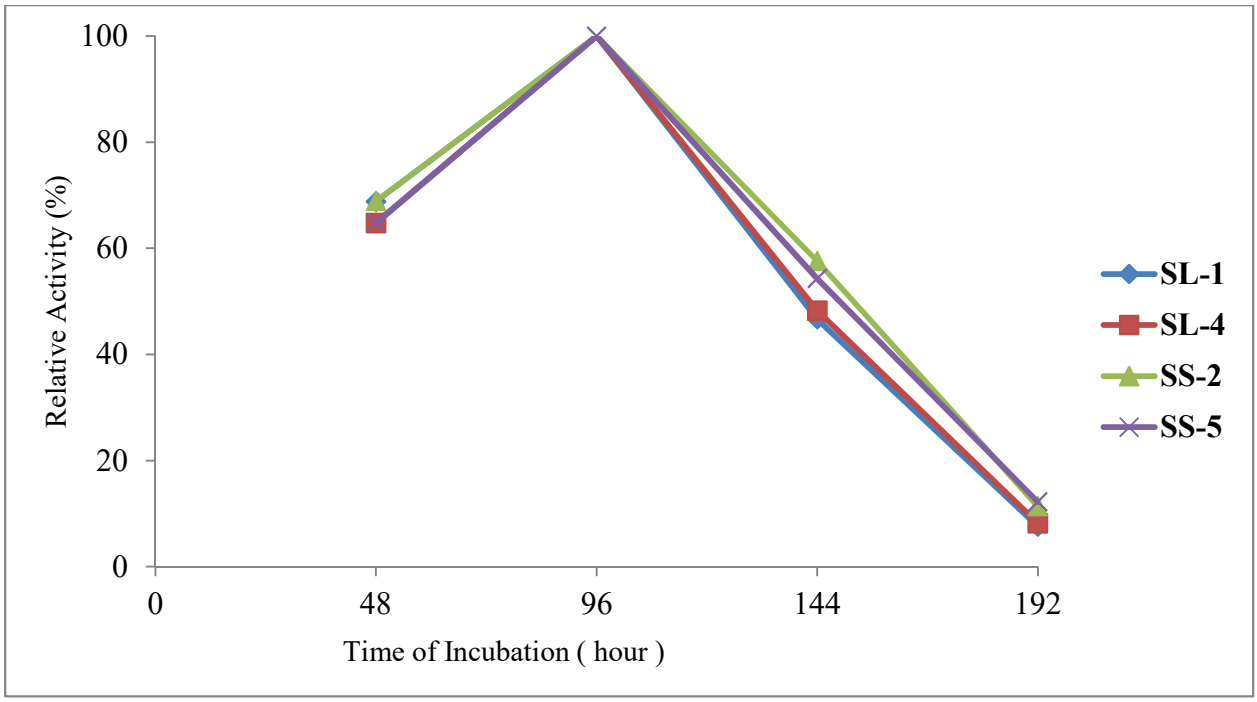

Figure 8: Effect of time of incubation on pectinase production

4.4.5. Effect of nitrogen sources

To study the effect of nitrogen sources for pectinase production using submerged fermentation, orange peel were supplemented with different nitrogen sources at 1% (w/v) (Table 6). The highest pectinase production was at 41.09±0.02 U/mL by supplementing orange peel with $(NH_4)_2SO_4$. Compared to orange peel supplement tested without nitrogen source, orange peel supplemented to all nitrogen sources except NH_4Cl significantly increased the production of pectinase (Table 6). However, compared to all nitrogen sources casein and $(NH_4)_2SO_4$ showed noticeable amount of pectinase production in all potential isolates considered in this study (Table 6).

Table 6: Effect of nitrogen sources for pectinase production

Nitrogen Source	*Pectinase activity (U/mL)				
	SL-1	SL-4	SS-2	SS-5	SH-1
Casein	$^{b}35.22\pm0.02^{b}$	$^{b}31.04\pm0.06^{d}$	$^{b}27.73\pm0.07^{e}$	$^{b}35.49\pm0.08^{a}$	$^{b}34.91\pm0.09^{c}$
Peptone	$^{c}24.54\pm0.06^{c}$	$^{d}24.35\pm0.03^{d}$	$^{c}24.66\pm0.07^{c}$	$^{d}25.32\pm0.05^{b}$	$^{c}26.64\pm0.03^{a}$
Urea	$^{c}23.82\pm0.04^{e}$	$^{e}24.00\pm0.06^{d}$	$^{d}24.38\pm0.08^{c}$	$^{c}26.12\pm0.05^{a}$	$^{d}25.09\pm0.07^{b}$
NH_4Cl	$^{d}22.00\pm0.72^{b}$	$^{c}24.73\pm0.03^{a}$	$^{e}21.55\pm0.03^{c}$	$^{e}20.76\pm0.03^{bc}$	$^{e}21.94\pm0.04^{b}$
$(NH_4)_2SO_4$	$^{a}41.09\pm0.02^{a}$	$^{a}37.03\pm0.07^{d}$	$^{a}32.35\pm0.07^{e}$	$^{a}39.07\pm0.09^{c}$	$^{a}40.73\pm0.04^{b}$
Control	$^{d}22.63\pm0.06^{b}$	$^{c}24.82\pm0.03^{a}$	$^{f}21.34\pm0.05^{d}$	$^{e}20.88\pm0.02^{e}$	$^{e}21.82\pm0.04^{c}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05).*
- *Control- no any nitrogen source in orange peel substrate*

4.4.6. Effect of sodium chloride concentration

To study the effect of sodium chloride concentration for pectinase production using submerged fermentation, orange peel was supplemented with different concentration of sodium chloride as showed Table 7. With initial addition of sodium chloride concentration *i.e.* at 2%, pectinase production was found to be slightly stimulated but further addition of sodium chloride inhibited pectinase production from three isolates such as SS-2, SS-5 and SH-1. However, pectinase productions from SL-4 and SL-1 strains were found to be especially stimulated with increase in concentrations of sodium chloride, but further increase declines the production of pectinase (Table 7). The highest pectinase activity was found 24.52±0.01 U/mL from strain SL-1 at 4% concentration of sodium chloride (Table 7).

Table 7: Effect of sodium chloride concentration on pectinase production

Concentration of NaCl (%)	*Pectinase activity (U/mL)				
	SL-1	SL-4	SS-2	SS-5	SH-1
2	$^{c}21.50\pm0.03^{d}$	$^{d}19.59\pm0.02^{e}$	$^{a}23.74\pm0.04^{c}$	$^{a}24.05\pm0.02^{b}$	$^{a}24.38\pm0.04^{a}$
4	$^{a}24.52\pm0.01^{a}$	$^{c}21.62\pm0.02^{e}$	$^{c}22.63\pm0.04^{d}$	$^{b}23.58\pm0.01^{b}$	$^{c}23.05\pm0.08^{c}$
6	$^{b}22.06\pm0.11^{c}$	$^{a}23.74\pm0.06^{a}$	$^{d}22.10\pm0.04^{c}$	$^{c}22.63\pm0.06^{b}$	$^{d}21.34\pm0.06^{d}$
8	$^{c}21.63\pm0.21^{b}$	$^{b}22.11\pm0.04^{a}$	$^{e}20.89\pm0.11^{c}$	$^{d}21.58\pm0.11^{b}$	$^{e}20.06\pm0.06^{d}$
Control	$^{d}20.70\pm0.03^{d}$	$^{e}19.17\pm0.11^{e}$	$^{b}23.12\pm0.05^{c}$	$^{b}23.43\pm0.03^{b}$	$^{b}23.80\pm0.12^{a}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05).*
- *Control- no sodium chloride on orange peel substrate*

4.5. Characterization of Crude Pectinase

Pectinase production was carried out with submerged fermentation using the isolates; SL-1, SL-4, SS-2, SS-5 and SH-1 at optimized parameters. The produced pectinase enzymes were characterized based on the factors listed in the following subsections.

4.5.1. 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 all selected isolates had highest activity at pH of 8.0 (Figure 9).

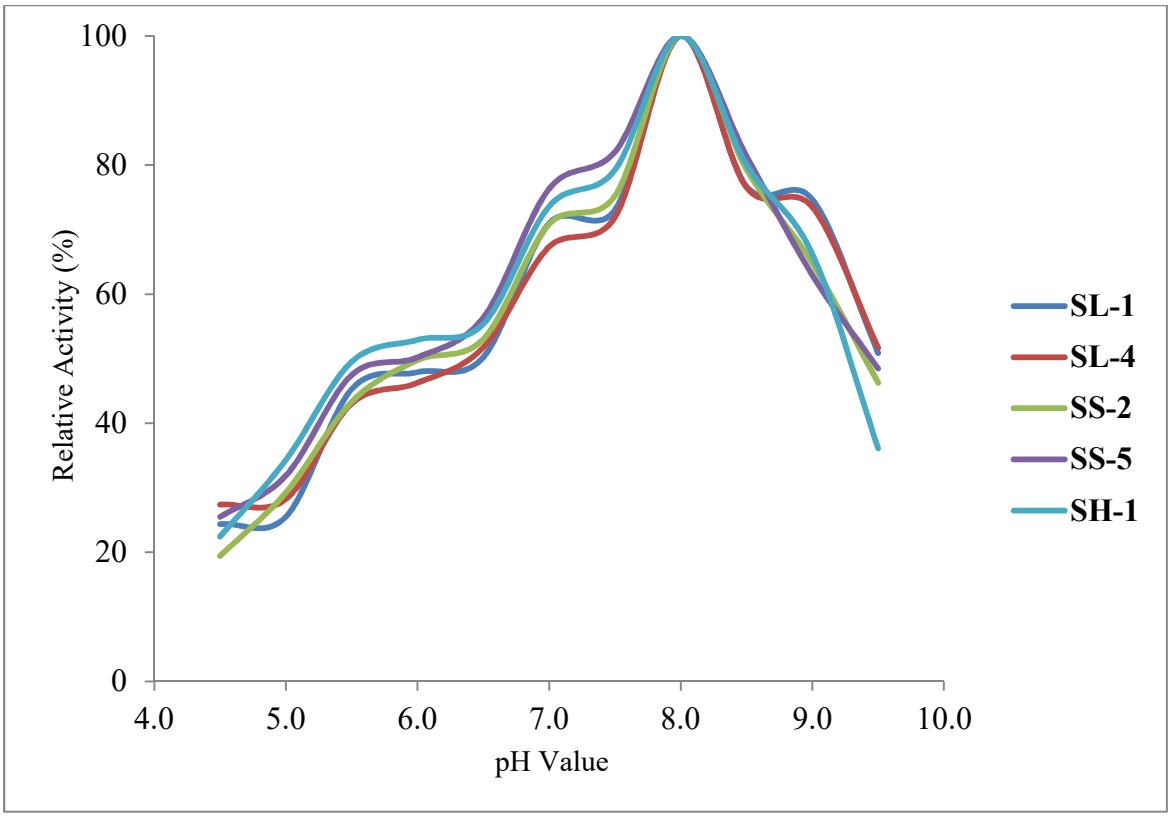

Figure 9: Effect of pH on pectinase activity

4.5.2. Effect of temperature

The effect of temperature on pectinase enzyme was evaluated by incubating the reaction mixture at different temperatures in the range of 30-90 ^{o}C. The maximum pectinase activity observed was at 60 ^{o}C (Figure 10).

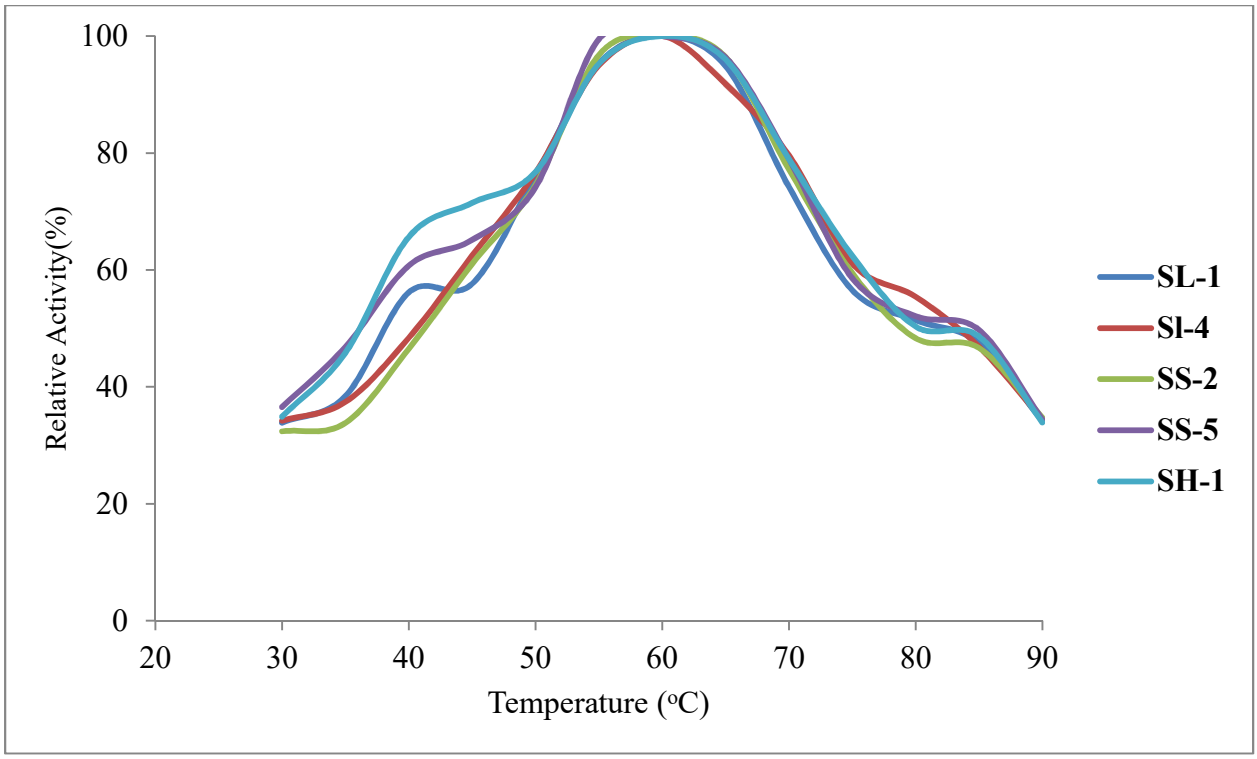

Figure 10: Effect of temperature on pectinase activity

4.5.3. Effect of substrate on pectinase activity

The effect of substrate on pectinase enzyme activity was determined by incubating 100 µL of crude enzyme with 900 µL of different substrates like citrus pectin, polygalacturonic acid. These substrates were prepared in 0.1 M of Tris-HCl buffer (pH 8.0) with 0.5% w/v concentration. The reaction mixture was incubated at 60 ^{o}C for 10 minute and the enzyme activity was assayed. The crude enzyme showed significantly higher activity when polygalacturonic acid was used than citrus pectin as a substrate in all isolates (Table 8).

Table 8: Effect of substrate on pectinase activity

Number	Isolates	* Pectinase activity (U/mL)	
		Citrus pectin	Polygalacturonic acid
1	SL-1	$^{a}38.40\pm0.05^{b}$	$^{b}43.06\pm0.08^{a}$
2	SL-4	$^{c}37.43\pm0.04^{b}$	$^{bc}42.98\pm 0.09^{a}$
3	SS-2	$^{e}36.48\pm0.05^{b}$	$^{d}40.27\pm0.07^{a}$
4	SS-5	$^{d}36.91\pm0.06^{b}$	$^{a}43.28\pm0.08^{a}$
5	SH-1	$^{b}38.00\pm0.16^{b}$	$^{c}42.85\pm0.03^{a}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05).*

4.5.4. Effect of sodium chloride

The effect of sodium chloride on pectinase activity was studied by directly incorporating it into the enzyme substrate system at a final concentration of 2%, 4%, 6%, and 8% (w/v) in 0.1 M of Tris-HCl buffer (pH 8.0) with 0.5% w/v concentration. Initially, increasing the concentration of sodium chloride in the enzyme substrate system increases the activity of pectinase from isolate SL-1 and SL-4 (Table 9). But pectinase activities form isolate SS-2, SS-5 and SH-1 were found to be slightly stimulated with the initial addition of sodium chloride (2% NaCl), but further increase inhibited enzyme activity. The highest pectinase enzyme activities observed were 28.80±0.06 U/mL, 28.47±0.03 U/mL and 28.22±0.23 U/mL in the presence of 2%, 4%, and 6% sodium chloride concentrations for SH-1, SS-5 and SL-4 respectively. However, the lowest activity was observed at higher concentration of sodium chloride (Table 9).

Table 9: Effects of sodium chloride concentration on pectinase activity

	*Pectinase activity (U/mL)				
Sodium chloride (%)	SL-1	SL-4	SS-2	SS-4	SH-1
2	$^{b}25.80\pm0.04^{d}$	$^{c}23.32\pm0.03^{e}$	$^{a}29.45\pm0.09^{b}$	$^{a}29.89\pm0.26^{a}$	$^{a}28.80\pm0.06^{c}$
4	$^{a}27.51\pm0.01^{b}$	$^{b}25.56\pm0.03^{d}$	$^{c}27.04\pm0.05^{c}$	$^{c}28.47\pm0.03^{a}$	$^{c}26.95\pm0.01^{c}$
6	$^{d}24.43\pm0.15^{d}$	$^{a}28.22\pm0.23^{a}$	$^{c}27.16\pm0.11^{b}$	$^{d}26.91\pm0.11^{b}$	$^{d}25.94\pm0.06^{c}$
8	$^{e}24.04\pm0.25^{d}$	$^{b}25.60\pm0.06^{b}$	$^{d}25.07\pm0.13^{c}$	$^{d}26.58\pm0.02^{a}$	$^{e}23.70\pm0.04^{d}$
Control	$^{c}25.04\pm0.04^{d}$	$^{d}22.75\pm0.03^{e}$	$^{b}28.72\pm0.03^{b}$	$^{b}29.06\pm0.04^{a}$	$^{b}28.40\pm0.05^{c}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05).*
- *Control- no sodium chloride on enzyme substrate system*

4.5.5. Effect of metal ions

The effect of metal ions on pectinase activity was studied by directly incorporating metal ions into the enzyme substrate system at a final concentration of 5 mM. The highest pectinase activities observed were 50.19±0.04 U/mL and 50.43±0.04 U/mL in the presence of $Mg^{2+}$and Ca^{2+} metal ions respectively in isolate SH-1 but the difference was not statistically significant. The lowest activity observed was 16.06±0.05 U/mL in the presence of Mn^{2+} metal ion (Table 10).

Table 10: Effects of metal ions on pectinase activity

	*Pectinase activity (U/mL)				
Metal ions	SL-1	SL-4	SS-2	SS-5	SH-1
Mg^{2+}	$^{a}47.62\pm0.10^{d}$	$^{a}50.02\pm0.01^{a}$	$^{a}47.91\pm0.04^{c}$	$^{a}49.49\pm0.09^{b}$	$^{a}50.19\pm0.04^{a}$
Zn^{2+}	$^{e}31.05\pm0.03^{d}$	$^{d}31.57\pm0.06^{a}$	$^{e}31.06\pm0.04^{d}$	$^{e}31.40\pm0.02^{b}$	$^{d}31.23\pm0.06^{c}$
Mn^{2+}	$^{f}16.06\pm0.05^{e}$	$^{e}19.16\pm0.07^{c}$	$^{f}17.97\pm0.02^{d}$	$^{f}21.38\pm0.02^{a}$	$^{e}20.30\pm0.10^{b}$
Co^{2+}	$^{d}36.51\pm0.07^{d}$	$^{c}37.42\pm0.18^{ab}$	$^{c}36.91\pm0.05^{c}$	$^{c}37.72\pm0.10^{a}$	$^{c}37.50\pm0.08^{b}$
Ca^{2+}	$^{b}46.38\pm0.03^{d}$	$^{b}49.00\pm0.05^{c}$	$^{b}44.88\pm0.08^{e}$	$^{b}49.22\pm0.08^{b}$	$^{a}50.43\pm0.04^{a}$
Control	$^{c}38.40\pm0.05^{a}$	$^{c}37.43\pm0.04^{c}$	$^{d}36.48\pm0.05^{e}$	$^{d}36.91\pm0.06^{d}$	$^{b}38.00\pm0.16^{b}$

- **Values are mean ± SD of 3 replicates.*
- *The same superscript letters at the top right side of the values are not significantly different with in rows at (P < 0.05).*
- *The same superscript letters at top left side of the values are not significantly different with in columns at (P < 0.05)*
- *Control- no metal ion on the enzyme-substrate system*

4.5.6. 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 30 min of incubation. However, beyond 30 min of incubation, the enzyme activity declined. Isolates SL-1 and SH-1 showed relative activity of nearly 100% at 60 min of incubation (Figure 11).

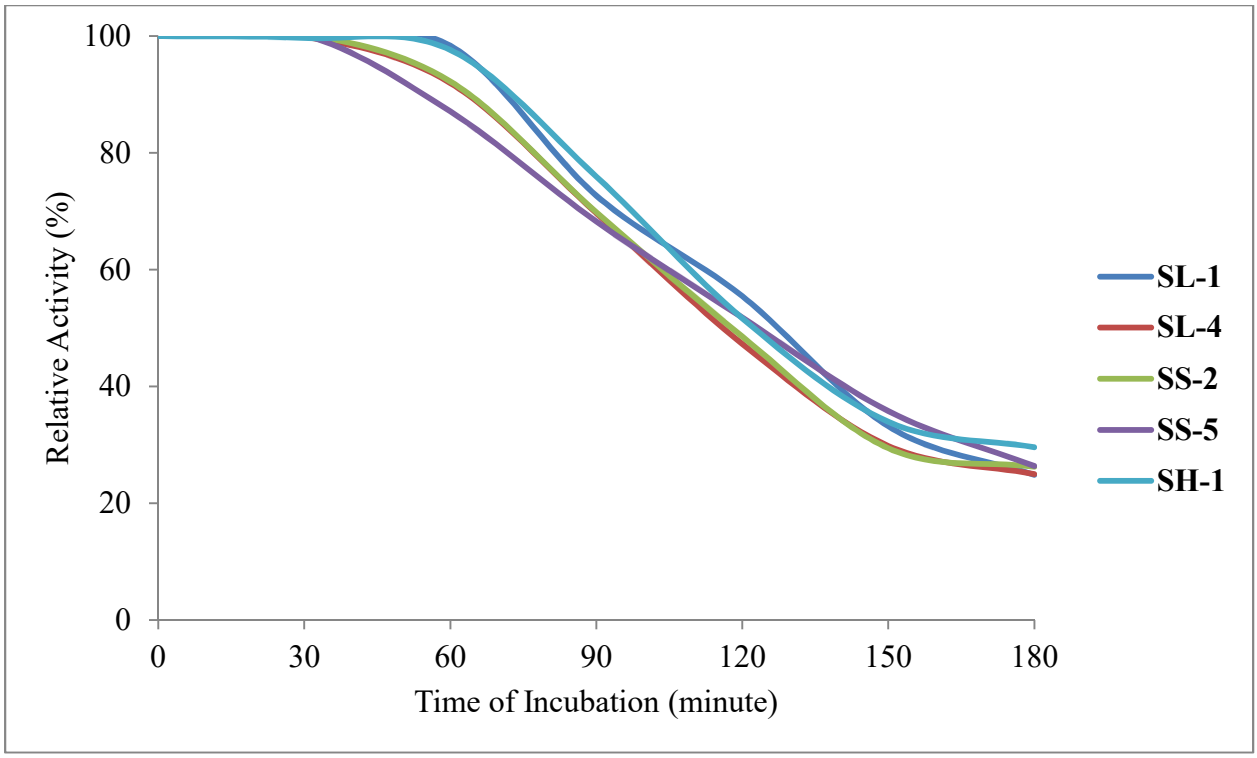

Figure 11: Enzyme stability

4.5.7. Michaelis-Menten constant (K_m) and V_{max} values

The K_m and V_{max} values of the enzymes from five potential isolates were determined by measuring the reaction velocity at different concentrations of the substrate (citrus pectin). The reaction speed V_{max} and K_m were determined by plotting of the reciprocal of the observed velocity (1/V) against the reciprocal of the substrate concentration (1/[S]) (Lineweaver and Burk, 1934). The regression coefficients (R^2) were found to be 0.9992, 0.9954, 0.9907, 0.9981 and 0.9978 for SL-1, SL-4, SS-2, SS-5 and SH-1 respectively which showed that the reciprocal concentrations of citrus pectin and velocity (enzyme activity) readings were positively correlated. From Lineweaver-Burk plot, K_m and V_{max} values were identified as 2.196, 1.972, 2.103, 1.418, 1.199 mg/mL and 69.93, 68.97, 68.97, 65.36, 64.10 U/mL for isolate SL-1, SL-4, SS-2, SS-5 and SH-1 respectively as showed in Appendix 6-10. Indeed, to appreciate the relationship between reaction velocity to concentration, reaction velocity versus substrate concentration graph was plotted. The plot showed initial increase in velocity of the reaction when increasing concentration of substrate. However, further increase in concentration of the substrate doesn't increase the velocity of the reaction sharply as was initially (Figure 12).

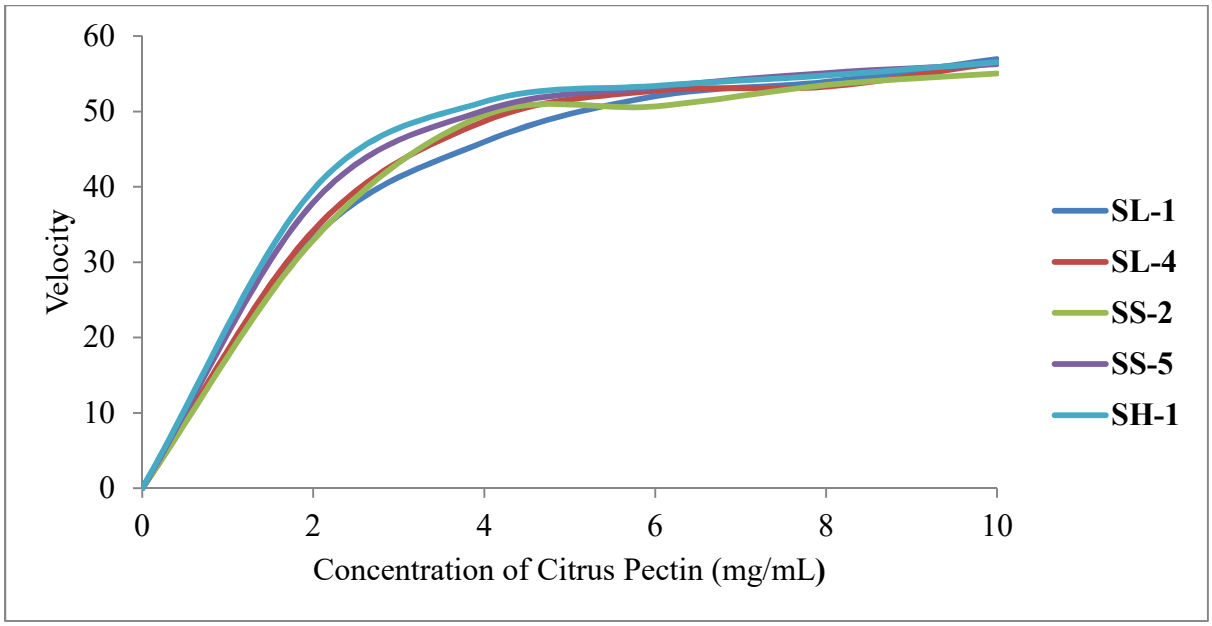

Figure 12: Michaelis-Menten kinetic curve of pectinase

5. DISCUSSION

Fungi have a diverse spectrum of extracellular enzymes. In nature, extracellular enzymes primarily serve to procure nutrients for the survival and growth of the fungi (Steudler *et al.*, 2019). Pectinases are among the most important industrial extracellular enzymes (Bajpai, 2018) , which account for about one quarter of the world's food enzyme production (Priya and Sashi, 2014; Ruiz et al., 2017). Fungal pectinase are remarkably the most important industrial enzymes with great significance having a wide ranges applications (Priya and Sashi, 2014). In this study, potential pectinase producing fungal extremophiles were isolated using serial dilution, pour plating and streak plating techniques from soil and water of Lake Shalla and from Sodere Spring water. Similar techniques were also followed to isolate pectinase producing fungi by (Ketipally and Ram, 2018; Merín *et al*., 2011).

Isolation is the first stage in the screening of microorganisms with potential industrial applications. The isolation procedure was designed to favor the growth of a specific organism possessing the industrially important characteristics. The ideal isolation procedure starts with the identification of the environmental source, which is probably richer in the desired types of microorganisms and also incorporating a simple test to distinguish them. In the present investigation, as many as 30 fungal isolates were selectively isolated by enrichment method using a medium containing pectin as the sole carbon source. However, 18 were found to be filamentous fungi and 12 of them were yeasts. Similar techniques for isolation of pectinase producing microorganism using pectin as sole carbon source were previously utilized (Merín *et al*., 2011; Ketipally and Ram, 2018).

Underlining the evolving application of pectinolytic enzymes, the isolated pectinase producing microbes were screened with superior enzyme activity of the enzyme. Thus, isolates were subjected into plate agar screening method using citrus pectin as a sole source of carbon and hydrolysis zone were used to segregate the highest pectinase producing. All the strains were positive for pectinase activity in a cup-plate assay, as evidenced by clear hydrolysis halos. Researchers Souza *et al*., (2003) applied similar techniques of isolation and screening of pectinolytic fungi and reported that all isolates were found to be pectinase enzyme producing. Others Munir *et al*., (2019); Ketipally and Ram, (2018) used plate agar screening method to screen native fungal isolates for pectinase activity. Isolates showing relatively higher clear zone to colony diameter ratio were subjected to submerged fermentation screening method to

identify potent isolate with highest enzyme activity at pH 8.5 and reaction temperature of 70 ^{o}C and the five isolates showed relatively higher enzyme activity *i.e* isolates with relative activity greater than 90% were considered in this study. Patil and Dayanand, (2006); Munir *et al*., (2019) employed the same techniques to screen pectinolytic fungi based on the production of pectinase on submerged fermentation using synthetic medium.

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 (Oumer and Abate, 2018). In this study, optimum parameters that affect the pectinase enzyme production have been standardized and thorough optimization steps were carried out to make the production of pectinase enzyme to be cost effective and commercially feasible. Hence, to meet the emerging industrial demands of pectinase, it is vital to advance the yield without increasing the cost of production.

Among the tested agro-residues for pectinase production, maximum enzyme production on submerged fermentation achieved was 23.48±0.01 U/mL from orange peel. In the same way, Mrudula and Anitharaj, (2011) working on *Aspergillus negur,* investigated that pectinase production was enhanced by orange peel. Of the various substrates reported in the literature, orange peel was found to be primarily suitable among all (Steudler *et al.*, 2019). According to Kaur and Gupta, (2017), orange peel is a chief source of pectin containing about 20-30% pectic substances which used as a good inducer of pectinase.

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 (Oumer and Abate, 2018). In the present study, the maximum pectinase production was attained SmF at 6.5 initial pH. The optimum pH for all selected potential isolates was found to be 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 microorganism irrespective of the type of microbes. Oumer and Abate, (2018) also found that *Bacillus subtilis strain Btk 27* exhibited maximum pectinase production at initial pH of 6.5. (Kaur et al., 2019) reported the maximum production of pectinase and xylanase from bacterium at pH 7.0 simultaneously. Similar study conducted by Ketipally and Ram, (2018) for the production of pectinase from *Aspergillus oryzae RR103* repotted maximum production at pH 6.5.

The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition and protein denaturation. The maximum pectinase production from all potential isolates considered in this study was observed at a temperature of 40 ^{o}C. Similar study conducted on thermophilic fungus *Thermomucor indicae-seudaticae N31* reported that ma ximum productio-n of pectinase was at 45 to 50 °C (Martin *et al.*, 2010). Moreover, Namasivayam *et al.*, (2011) reported that the optimum temperature for maximum production of extracellular pectinase by *Bacillus cereus* isolated from market solid waste 37 °C temperature. Others 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. Fungal stains having growth optima range from 37 to 45 ^{o}C are considered to be thermo-tolerant (Hoffmann *et al*., 2007). Therefore, fungal strains considered in our study are thermo-tolerant.

Nitrogenous compounds are utilized by the microbial cells for the synthesis of nucleotides, amino acids, proteins and other metabolites (Oumer and Abate, 2018). Nitrogen source has a great significance in fungal growth and enzyme production (Ketipally and Ram, 2018). Therefore, supplementing nitrogen into the production medium accelerates biomass production and subsequently results in higher metabolite secretion. In this study, the maximum pectinase activity on submerged fermentation was 41.09±0.02 U/mL when orange peel was supplemented with $(NH_4)_2SO_4$ as nitrogen source. Among the tested nitrogen sources on submerged fermentation, casein and $(NH_4)_2SO_4$ showed noticeable amount of pectinase production in all potential isolates considered in this study though orange peel supplemented to all nitrogen sources except NH_4Cl significantly increased the production of pectinase. Similarly, pectinase production from *Bacillus subtilis strain Btk 27 was* stimulated by casein on submerged fermentation and $(NH_4)_2SO_4$ on solid state fermentation Oumer and Abate, (2018); Adeleke *et al*., (2012) also reported stimulation of $(NH_4)_2SO_4$ for pectinase production from fungi. Moreover, a lab Ketipally and Ram, (2018) reported enhanced production of pectinase with the addition of peptone.

Growth of fungi in any medium varies according to physical and chemical environmental factors. Cell doubling time increases with increasing organic complexity. Enzymes are predominantly produced after the exponential phase (Dushyantha *et al.*, 2013). Thus, the level of enzyme production varies with the time of duration in the fermentation processes (Oumer and

Abate, 2018) . However, the producer strains needed to have shorter fermentation cycles for the purpose of their cost effectiveness. The pectinase production from all selected isolates was increased continuously until 96 hours of incubation. After 96 hours of incubation, the pectinase activity was decreased. A gradual increase in the production of pectinase from *Aspergillus oryzae RR103* and maximum production was reported at 192 hour of incubation (Ketipally and Ram, 2018). In addition, a group Adeleke *et al*., (2012) reported maximum production of polygalacturonase on the 5th day. The reduction in pectinase production after 96 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 (Oumer and Abate, 2018) . Relatively, 96 hours of incubation for high pectinase production is short incubation time, thus, it has good prospect for industrial application.

The concentration of sodium chloride affects the pectinase production. In this study, the production of pectinase was evaluated by incorporating different concentration of sodium chloride in the orange peel substrate. Gradual increase of pectinase production was observed when increasing the concentration of the salt (Table 7). However, further increase in concentrations leads to decrease in enzyme production but strain SL-4 stably increases pectinase production at all concentrations. Higher amount of pectinase production was recorded at 2% sodium chloride concentration for strain SS-2, SS-5 and SH-1 among the potential isolates considered in the present study. Two researches Ketipally and Ram, (2018) reported that 2% sodium chloride concentration is suitable for pectinase production from *Aspergillus oryzae RR103*. In contrast, the suitable sodium chloride concentration for strain SL-4 was 8%. A study conducted by Gummadi *et al*., (2007) on pectinase production by halotolerant yeast, *Debaryomyces nepalensis NCYC 3413* reported maximum pectinase production up to 2 M sodium chloride concentration. It was also reported that the growth rate of *Thalassospira frigidphilosprofundus* was not affected up to 12 g/L NaCl concentration and thereafter the growth decreased and the maximum Polygalacturonase production was found at 8 g/L NaCl concentration (Rekha *et al.*, 2013).

The activity of enzyme is known to be affected by pH, temperature, concentration of substrate, type of substrate and the presence of cofactors such as metal ions (Oumer and Abate, 2017). In this study, the pectinase enzymes produced from submerged fermentation of selected isolate were characterized and their optimum conditions were determined.

Specificity of the enzyme to their substrate is one of the peculiar properties of the enzyme. It is known that pectinases are specific to different source of their substrate. In this study, the maximum pectinase activity was observed when polygalacturonic acid was used as substrate compared to citrus pectin. Similarly, Evangelista *et al*., (2018) reported the highest specific activity of Exo-polygalacturonase from *Bacillus licheniformis* for polygalacturonic acid and approximately 5 times lower activity for citrus pectin. Moreover, cold-active and alkaline pectate lyase from Antarctic bacterium was reported to have high affinity to polygalacturonic acid (Tang *et al*., 2019). Thus, it can be inferred that the enzymes produced from all potential isolates considered in this study have high affinity for polygalacturonic acid than citrus pectin.

The pH of the medium greatly affects the enzyme activity. Changes in pH of a medium have been reported to affect the affinity of the enzyme for substrates (Oumer and Abate, 2017). The pH of the medium in which the enzyme is exposed affects the ionization state of its amino acids which dictates the primary and secondary structure of the enzyme thus controlling its activity. The observed reduction in the enzyme activities of pectinolytic strains at pH values other than the optimal pH could also be attributed to a probable change in the state of the ionic groups involved in the maintenance of the active conformation of the enzymes. Extreme pH has been reported to initiate chemical reactions that could alter, cross-link or destroy amino acid residues of the protein molecules resulting in irreversible inactivation (Dushyantha *et al.*, 2013).

The pH of the medium greatly affects the enzyme activity. Moreover, optimum pH is required for the maximum activity of the enzyme. In the present study, the effects of pH on pectinase activity of all selected pectinolytic enzyme producing isolates were determined. So that, the maximum pectinase activity for all isolates considered in this study was recorded at pH 8.0. Similar study conducted on a thermo-stable pectate lyase from *Aspergillus luchuensis var. saitoi* showed optimum pectinase activities at pH 8.0 (Kamijo *et al*., 2019) . Moreover, pectinase from Antarctic bacterium was reported to have maximum activity at pH 10 (Tang *et al*., 2019). Therefore, this pectinase will have potential applications whenever alkaline pectin degradation is needed such as in coffee processing, textile processing, animal feed processing, plant virus purification process, paper and pulp industry and also for pectic waste water treatment.

Like all other chemical reactions, the velocity of the enzyme catalyzed reactions also increases with an increase in temperature. However, eventually a temperature is reached where the enzyme becomes unstable and begins to denature, at which point the reaction rate again

declines (Dushyantha *et al.*, 2013). In the present study, the maximum pectinase activities were observed at 60 ^{o}C, with further increase in reaction 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 (Oumer and Abate, 2017). On the other hand, Okonji *et al*., (2019) reported an optimum temperature of 60 ^{o}C of pectinase produced from *Aspergillus fumigatus* and Evangelista *et al*., (2018) also reported the maximum activity *of* Exo-polygalacturonase from *Bacillus licheniformis* at temperature of 60 ^{o}C. Oumer and Abate, (2017) reported the maximum pectinase activity at temperature of 50 °C produced from Bacillus *subtilis strain Btk 27*. Moreover, others Martins *et al.,* (2002) also reported the maximum activity of thermo-stable pectinases at a temperature of 65 ^{o}C from thermophilic *Thermoascus aurantiacus*. The result might indicate that pectinase from all isolates considered in this study are thermophilic enzymes.

Since the strains were taken from halophilic environment, the effect of sodium chloride concentration on pectinase activity was studied by incorporating 2%, 4%, 6% and 8% NaCl into the enzyme substrate system. In the current study, initial increase in the activity of pectinase was observed in all potential isolates while further increase in concentration of the salt declines the activity of the enzyme. The activity of pectinase produced from SL-4 was exceptionally stimulated the by 6% sodium chloride by 124.04% times (Appendix 15). Similar study by Okonji *et al*., (2019) found stimulation by initial increase in concentration of sodium chloride but further increase declined the activity of pectinase produced from *Aspergillus fumigatus*.

Among the metal ions, Mg^{2+} and Ca^{2+} increased pectinase activity whereas Mn^{2+} Zn^{2+} and Co^{2+} decreased the pectinase activity; their effect was also statistically significant though no statistical significance difference was observed between Ca^{2+} and Mg^{2+} in isolate SH-1 but Co^{2+} exceptionally stimulated pectinase isolated from SS-2 and SS-5 strains . In contrary, lowest activity of pectinase was observed in the presence of Mn^{2+} compared to other metal ions considered isolates in this study. However, Zn^{2+} does not lower significantly the activity of pectinase produced by isolate SL-4. Metal ions like Ca^{2+} and Mg^{2+} might play a vital role in maintaining the active confirmation of alkaline endo polygalactournase to stimulate the activity (Beg and Gupta, 2003; Li *et al*., 2008). According to Kamijo *et al*., (2019) 5.0 mM Zn^{2+} lowered the activity of thermo-stable pectate lyase from *Aspergillus luchuensis var. saitoi.* Antagonistically, Tang *et al.*, (2019) reported that $Ca^{2+,}$ $Mg^{2+,}$ $Zn^{2+,}$ Co^{2+} and Mn^{2+} inhibit the

activity of cold-active and alkaline pectate lyase from Antarctic bacterium at 5 mM. This discrepancy in the divalent metal ion preference suggested that the enzymes might have differential flexibility in the active site.

Enzyme deactivation and stability are considered to be the major constraints in the rapid development of biotechnological processes. Stability studies also provide valuable information about structure and function of enzymes. Enhancing the stability and maintaining the desired level of activity over a long period are two important points considered for the selection and design of pectinases. The catalytic activity of many enzymes is markedly dependent on pH. It can exert its effect in different ways: on the ionization of groups in the enzymes active site, either on the ionization of groups in the substrate, or by affecting the conformation of either the enzyme or the substrate. The stability of pectinase is affected by pH and temperature (Dushyantha *et al.*, 2013).

In the current study, the stability of pectinase enzyme under optimized temperature and pH was studied by incubating the reaction mixture at various time intervals. Pectinase from all potential isolates considered in the study were stable with 100% relative activity until 30 minutes of incubation. However, above 30 minutes of incubation the enzyme activity declined. In contrary, isolate SL-1 and SH-1 showed relative activity nearly 100% at 60 minutes of incubation. Therefore, pectinase produced form SL-1 and SH-1, are relatively more stable compared to other potential isolates considered in this study. Okonji *et al.*, (2019) reported enzyme extracted from *Aspergillus fumigatus was fully* active at its optimum temperature until 45 minutes and further incubation declined the activity of enzyme. A study conducted on pectinase activity from *Bacillus subtilis strain Btk 27* was found fully active until 1 hour incubation but, further incubation declines its activity (Oumer and Abate, 2017). The thermal inactivation of enzymes is always due to denaturation of enzyme (Amin *et al*., 2013). This study reveals that, pectinase produced from isolates such as SL-1 and SH-1 were thermally stable. These isolates may be important for high temperature requiring industrial processes compared to other isolates considered in the present study.

In enzymatic reactions, the kinetic parameter is also important, which describes the efficiency of enzymes. In this study, lowest K_m value was recorded in enzyme produced from isolate SH-1 which is 1.199 mg/mL. However, highest V_{max} was attained in pectinase enzyme produced from isolate SL-1 which is 69.93 U/mL. Pectinase from SH-1 has relatively the highest

affinity for substrate due to its lowest K_m; but SL-1 has the highest utility of pectin substrate as a result of its highest V_{max}. As a result of this, due to high binding ability of pectinase from strain SH-1 to pectin as 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. In contrary, K_m value of pectinase produced from SL-1 was relatively higher than all other isolates. Therefore, it can be assumed that SL-1 has relatively lower affinity to its substrate. Tang *et al*., (2019) reported kinetics parameters like K_m of 0.93 g/L and V_{max} of 33.60 μmol min^{-1} for pectinase isolated from Antarctic bacterium. Moreover, Oumer and Abate, (2017) pectinase K_m and V_{max} values of 1.879 mg/mL and 149.6 U, respectively. Compared to pectinase produced in the present study, pectinase from strain SH-1 has relatively the highest affinity for its substrate due to its lowest K_m. Thus, highest binding ability of pectinase from strain SH-1 with pectin substrate, will lead to the requirement of small quantity of pectinase enzyme to digest considerably high amount of its substrate. This may, therefore, reduce the cost for the pectinase enzyme in industrial use.

6. CONCLUSION

The pectinase enzyme producing fungal strains were isolated from soil and water of Lake Shala and Sodere spring water using pectin as sole carbon source. The isolated fungal strains were screened with their pectinolytic activity and since the sole carbon source used during isolation was pectin all were found to express pectinase. After screening five potential isolates were considered in this study. Agricultural waste materials such as orange peel, banana peel, mango peel and wheat straw were studied for pectinase production using five isolates and orange peel was found to have promising carbon source for pectinase production in all isolates. Since using agricultural by product as a substrate for pectinase is both environmental friendly and cost effective, the five fungal isolates were studied for optimization of their pectinase production using orange peel. The production and optimization studies revealed that the fungal isolates optimum temperature and pH was 40 °C, pH 6.5 using orange peel (as a carbon source) and 96 hours of incubation for higher pectinase enzyme production. In addition, pectinase production using submerged fermentation was found to be stimulated by nitrogen source like casein and ammonium sulfate and sodium chloride salt also stimulated pectinase production. However, very high concentrations of sodium chloride stimulate only strain SL-4 for its pectinase production.

Pectinase from five isolates were characterized and their kinetics was also studied. The optimum temperature and pH was 60 ^{o}C and 8.0 respectively and thus, this enzymes are both alkalophilic and thermophilic. The enzymes were also found to be thermally stable, stimulated by Ca^{2+} and $Mg^{2+,}$ inhibited by $Zn^{2+,}$ Mn^{2+} and Co^{2+}, stimulated by initial increase in concentration of sodium chloride and inhibited by further increasing in concentration of sodium chloride, and were very specific to polygalacturonic acid. The kinetics study reveals that the highest affinity for its substrate is due to its lowest K_m. Thus, highest binding ability of pectinase from these isolates with pectin substrate will lead to the requirement of small quantity of pectinase enzyme to digest considerably high amount of its substrate. This may, therefore, reduce the cost for the pectinase enzyme in industrial use.

7. RECOMMENDATION

Depending on the results and conclusion reached in this study further purification steps shall be performed which will allow enhancing the enzyme activity and studying specific characteristics. In addition, immobilization of the enzyme shall be implemented which will enhance the stability of the enzyme. Since the produced pectinases active at alkaline environment, application of the enzyme in alkaline pectinase requiring processes such as in coffee fermentation, tea fermentation, textile processing, pulp and paper processing ought to be done. Last but not least, further enzyme production optimization studies needed be employed using statistical models such as using Response Surface Methodology (RSM) to study the interaction between factors.

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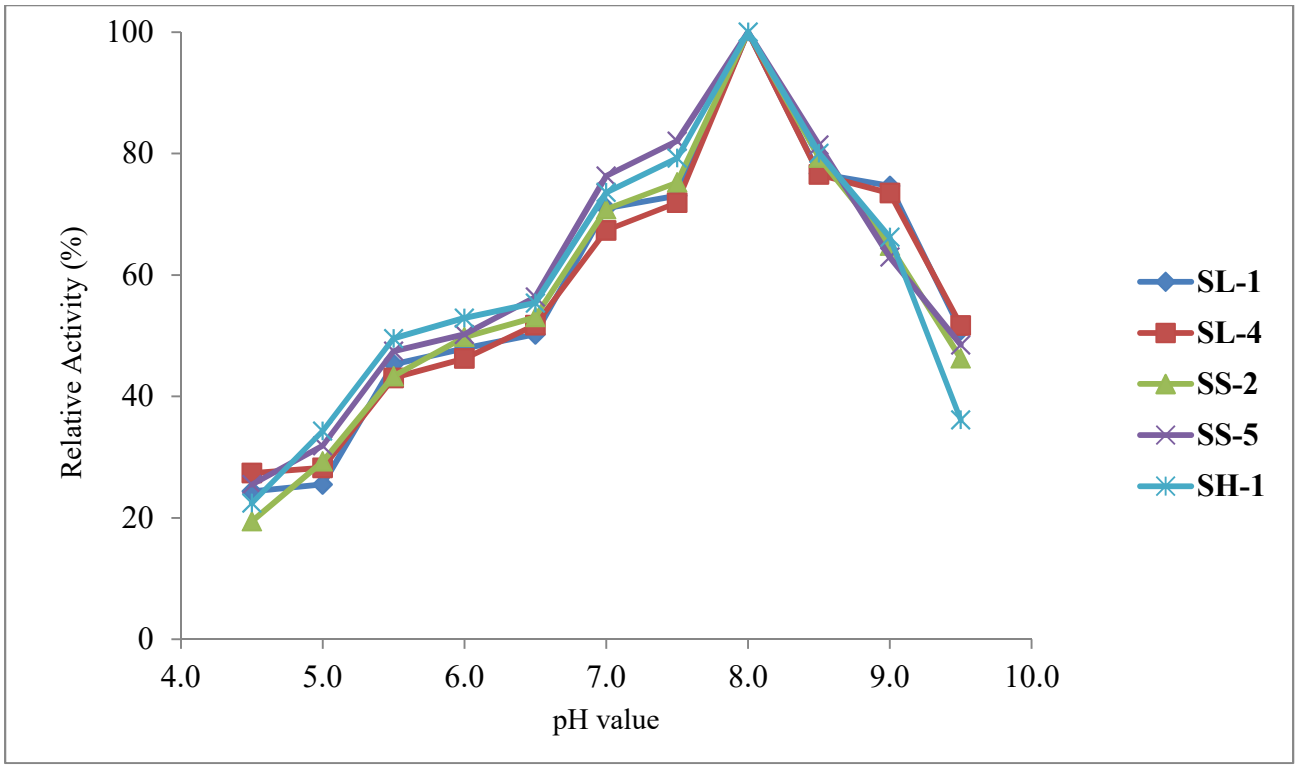
9. APPENDIX

Appendix 1: Media Composition

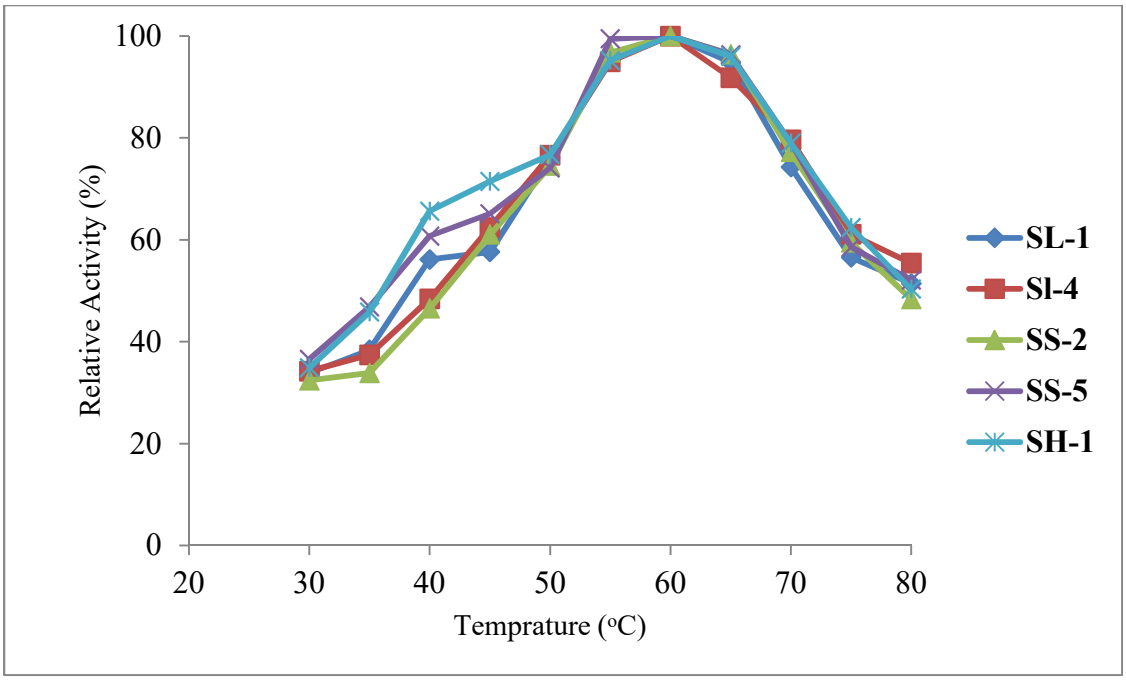
1. **Czapek-dox agar medium**: (10.0 g) citrus pectin, (3.0 g) Di-ammonium orthophosphate, (2.0 g) K_2HPO_4, (0.1 g) $MgSO_4$, (15.0 g) agar, (1000 mL) distilled water
2. **Modified mineral medium for yeast isolation, purification and pectinolytic screening**: (10.0 g/L) citric pectin, (1.0 g/L) yeast extract, (15.0 g/L) agar, (0.2 g/L) KH_2PO_4, (0.05 g/L) $CaCl_2$; (1.0 g/L) $(NH_4)_2SO_4$, (0.05 g/L) $MgSO_4.7H_2O$
3. **Iodine-Potassium Iodide Solution** : (1.0 g) iodine, (5.0 g) potassium iodide in 330mL distilled water

Appendix 2: List of isolates for pectinase production

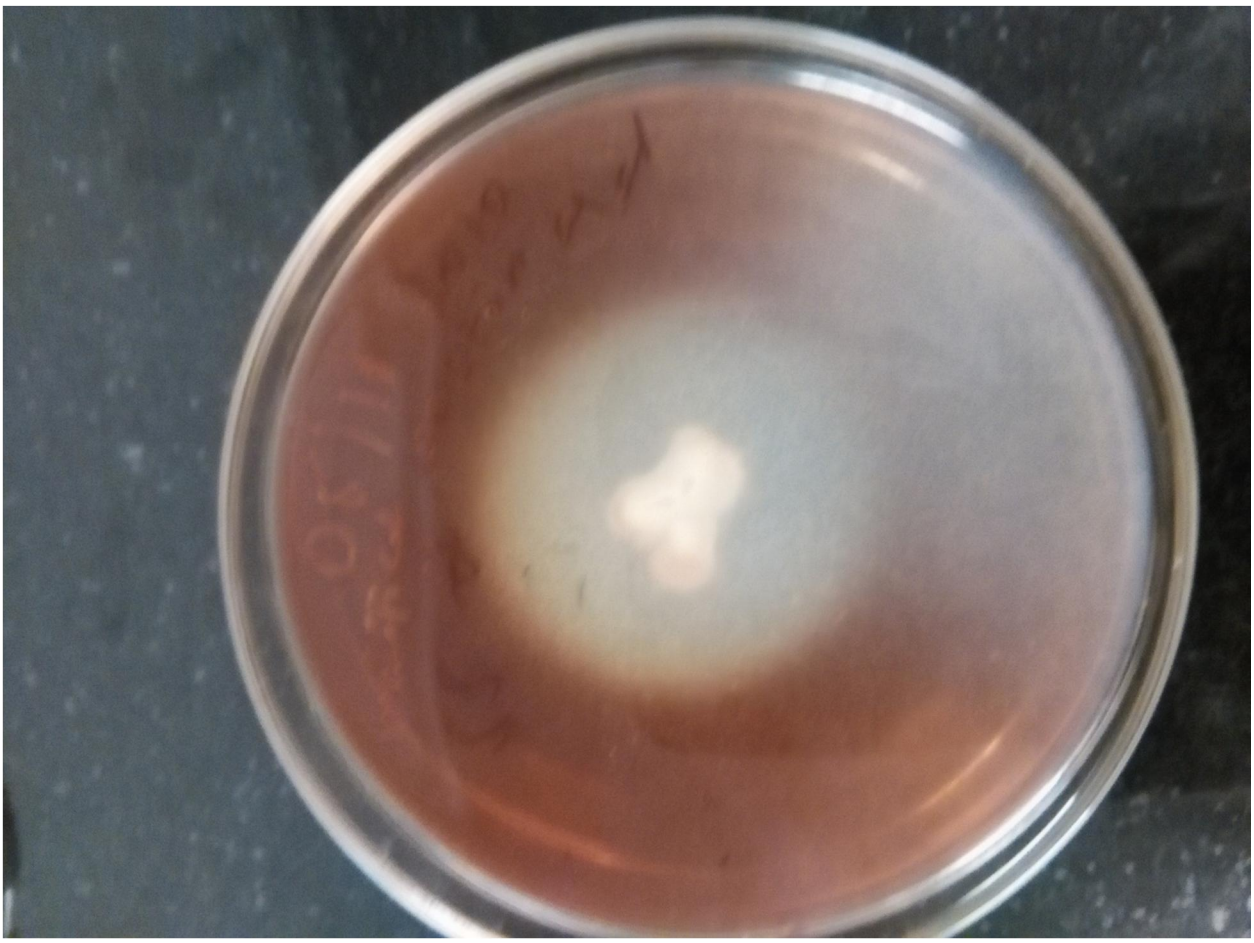
No.	Strain no.	Classification	No.	Strain no.	Classification
1	SL-1	Filamentous Fungi	16	SS-5	Filamentous Fungi
2	SL-2	Filamentous Fungi	17	SH-1	Filamentous Fungi
3	SL-3	Filamentous Fungi	18	SH-2	Filamentous Fungi
4	SL-4	Filamentous Fungi	19	PSL-1	Yeast
5	SL-5	Filamentous Fungi	20	PSL-2	Yeast
6	SL-6	Filamentous Fungi	21	PSL-3	Yeast
7	SL-7	Filamentous Fungi	22	PSL-4	Yeast
8	SL-8	Filamentous Fungi	23	PSL-5	Yeast
9	SL-9	Filamentous Fungi	24	PSL-6	Yeast
10	SL-10	Filamentous Fungi	25	PSS-1	Yeast
11	SL1-1	Filamentous Fungi	26	PSS-2	Yeast
12	SS-1	Filamentous Fungi	27	PSS-3	Yeast
13	SS-2	Filamentous Fungi	28	PSS-4	Yeast
14	SS-3	Filamentous Fungi	29	PSH-1	Yeast
15	SS-4	Filamentous Fungi	30	PSH-2	Yeast



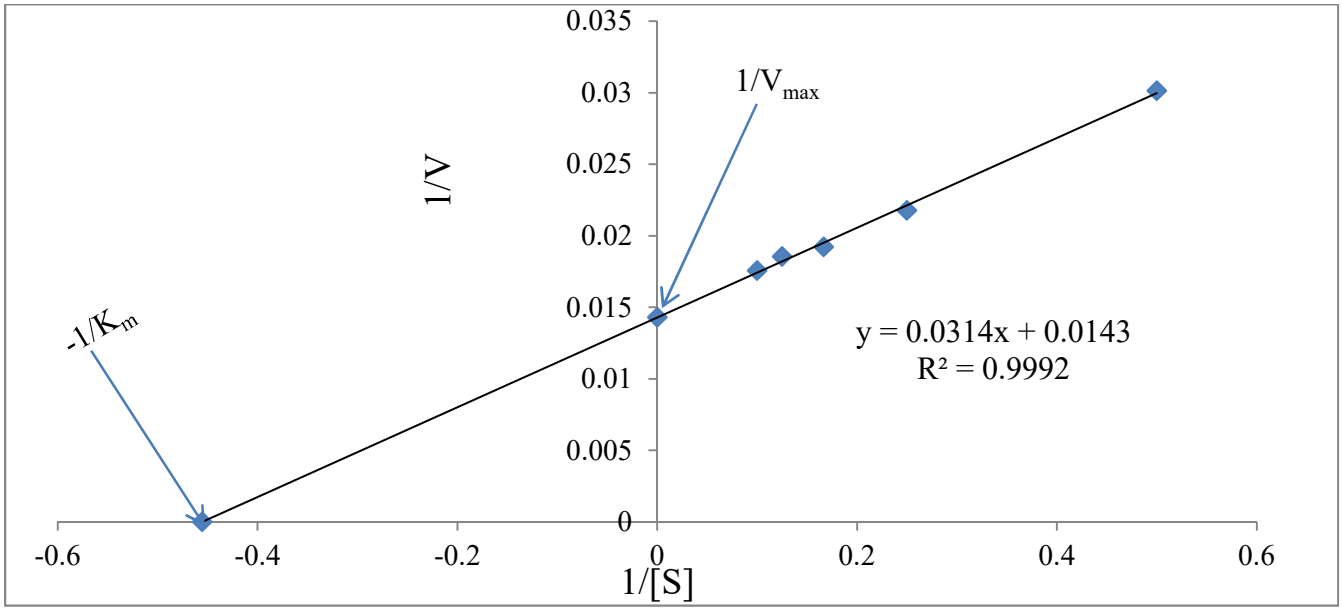
Appendix 3: Preliminary characterization of pectinase from five potential isolates- optimum pH for pectinase activity



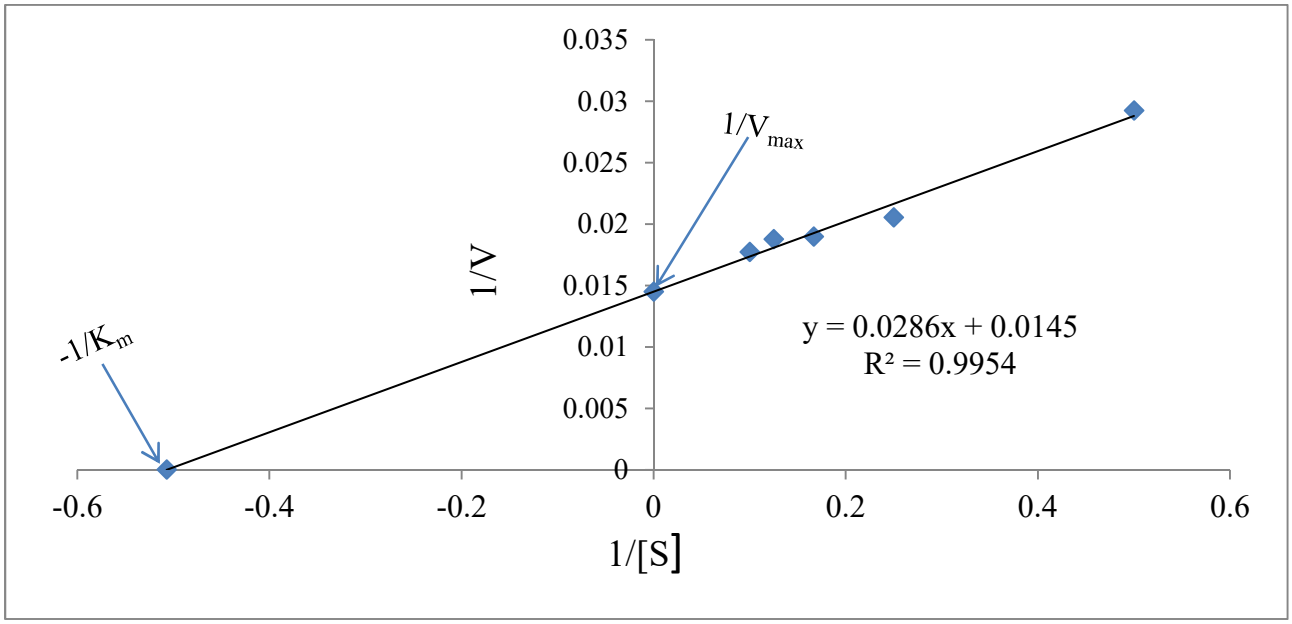
Appendix 4: Preliminary characterization of pectinase from five potential isolates- optimum temperature for pectinase activity



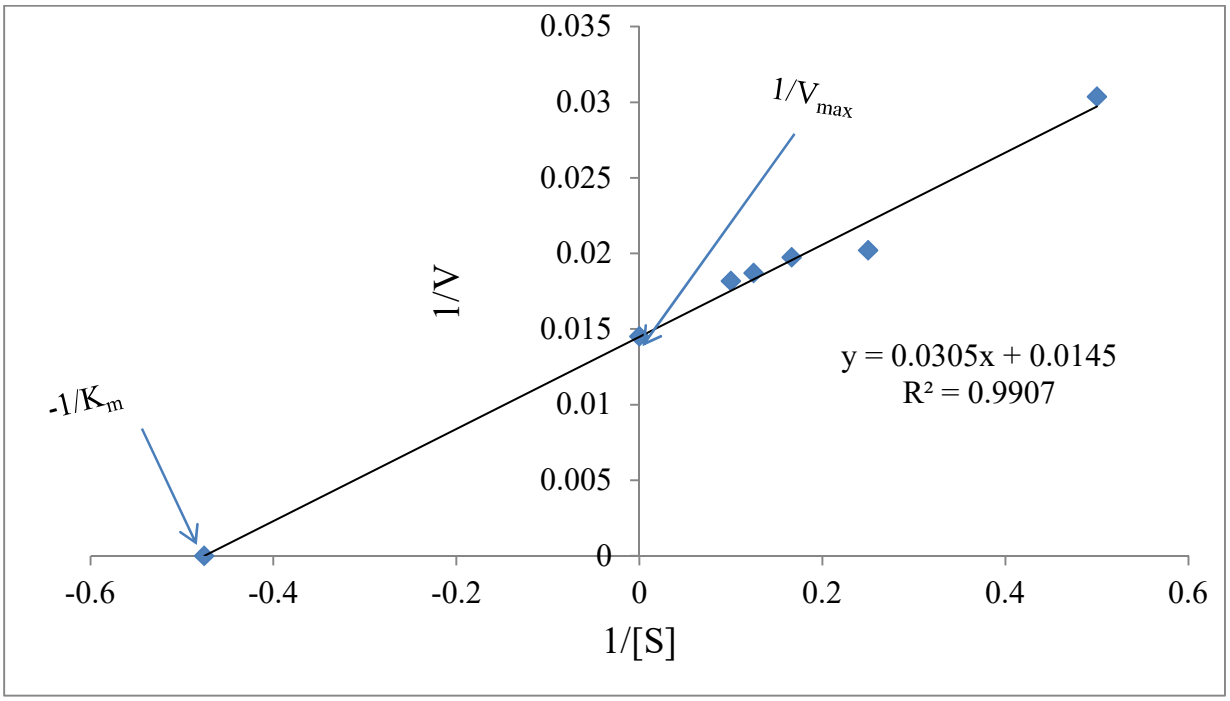
Appendix 5: Clear zones around the colony for isolate SS-2



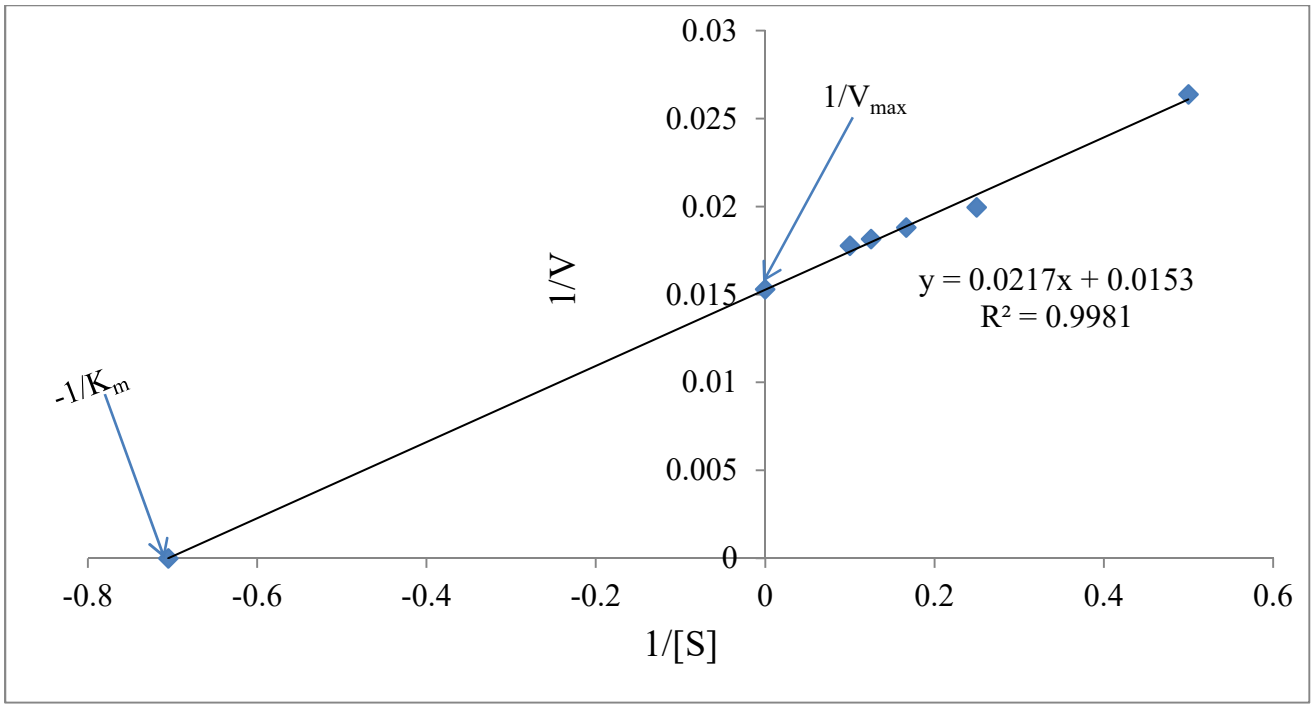
Appendix 6: Lineweaver-Burk plot for isolate SL-1 showing Michaelis-Menten kinetics



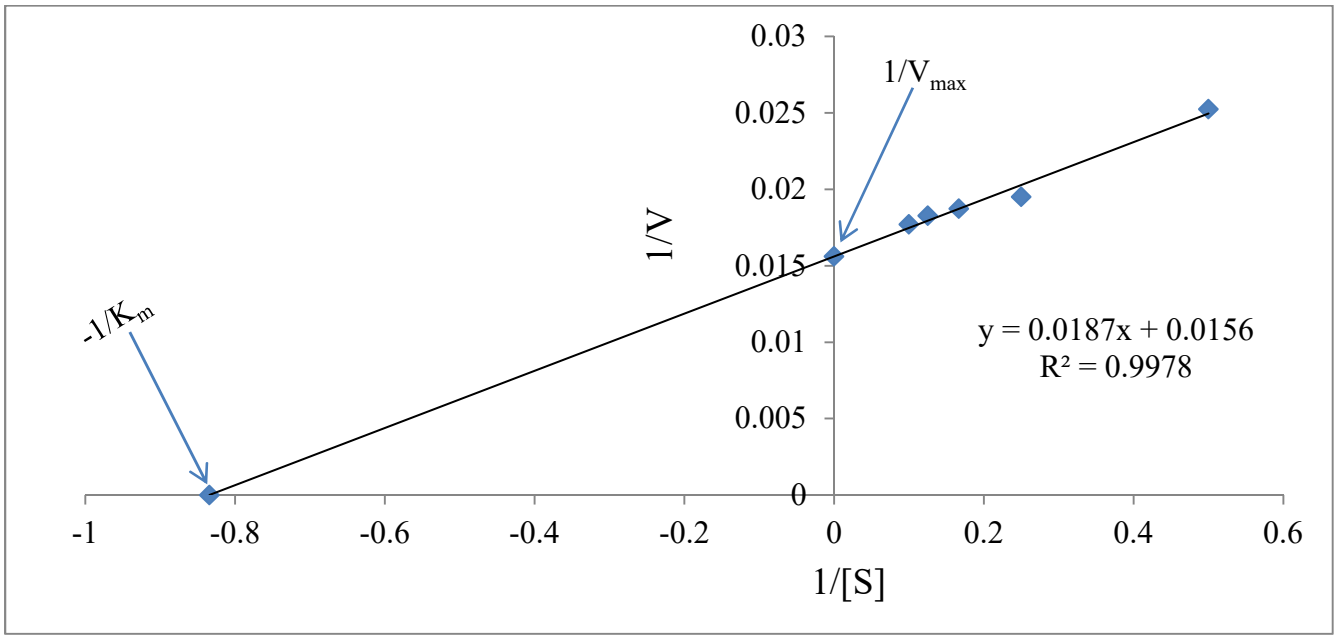
Appendix 7: Lineweaver-Burk plot for isolate SL-4 showing Michaelis-Menten kinetics



Appendix 8: Lineweaver-Burk plot for isolate SS-2 showing Michaelis-Menten kinetics



Appendix 9: Lineweaver-Burk plot for isolate SS-5 showing Michaelis-Menten kinetics



Appendix 10: Lineweaver-Burk plot for isolate SH-1 showing Michaelis-Menten kinetics

Appendix 11: List of K_m, V_{max}, slope and R^2 for pectinase enzyme from five potential isolates

Strain No.	**K_m**	**V_{max}**	**Slope (K_m/ V_{max})**	**R^2**
SL-1	2.196 mg/mL	69.93 U/mL	0.0314	0.9992
SL-4	1.972 mg/mL	68.97 U/mL	0.0286	0.9954
SS-2	2.103 mg/mL	68.97 U/mL	0.0305	0.9907
SS-5	1.418 mg/mL	65.36 U/mL	0.0217	0.9981
SH-1	1.199 mg/mL	64.10 U/mL	0.0187	0.9978

Appendix 12: Pectinase production using submerged fermentation

Appendix 13: Effect of nitrogen sources for pectinase production

Nitrogen Source	Relative activity (%)				
	SL-1	SL-4	SS-2	SS-5	SH-1
Casein	155.63	125.06	129.94	169.97	159.99
Peptone	108.44	98.11	115.56	121.26	122.09
Urea	105.26	96.70	114.25	125.10	114.99
NH_4Cl	97.22	99.64	100.98	99.43	100.55
$(NH_4)_2SO_4$	181.57	149.19	151.59	187.12	186.66
Control	100.00	100.00	100.00	100.00	100.00

- *Control- no any nitrogen source in orange peel substrate*

Appendix 14: Effect of sodium chloride concentration on pectinase production

Concentration of NaCl (%)	Relative activity (%)				
	SL-1	SL-4	SS-2	SS-5	SH-1
2	103.86	102.19	102.68	102.65	102.44
4	118.45	112.78	97.88	100.64	96.85
6	106.57	123.84	95.59	96.59	89.66
8	104.49	115.34	90.35	92.10	84.29
Control	100.00	100.00	100.00	100.00	100.00

- *Control- no sodium chloride on orange peel substrate*

Appendix 15: Effects of sodium chloride concentration on pectinase activity

	Relative activity (%)				
Sodium Chloride (%)	SL-1	SL-4	SS-2	SS-4	SH-1
2	103.04	102.51	102.54	102.86	101.41
4	109.86	112.35	94.15	97.97	94.89
6	97.56	124.04	94.57	92.60	91.34
8	96.01	112.53	87.29	91.47	83.45
Control	100.00	100.00	100.00	100.00	100.00

- *Control- no sodium chloride on enzyme substrate system*

Appendix 16: Effects of metal ions on pectinase activity

	Relative activity (%)				
Metal ions	SL-1	SL-4	SS-2	SS-5	SH-1
Mg^{2+}	124.01	133.64	131.33	134.08	132.08
Zn^{2+}	80.86	84.34	85.14	85.07	82.18
Mn^{2+}	41.82	51.19	49.26	57.92	53.42
Co^{2+}	95.08	99.97	101.18	102.19	98.68
Ca^{2+}	120.78	130.91	123.03	133.35	132.71
Control	100.00	100.00	100.00	100.00	100.00

- *Control- no any metal ion on the enzyme-substrate system*

Appendix 17: V_{max} and K_m of the Michaelis-Menten equation:

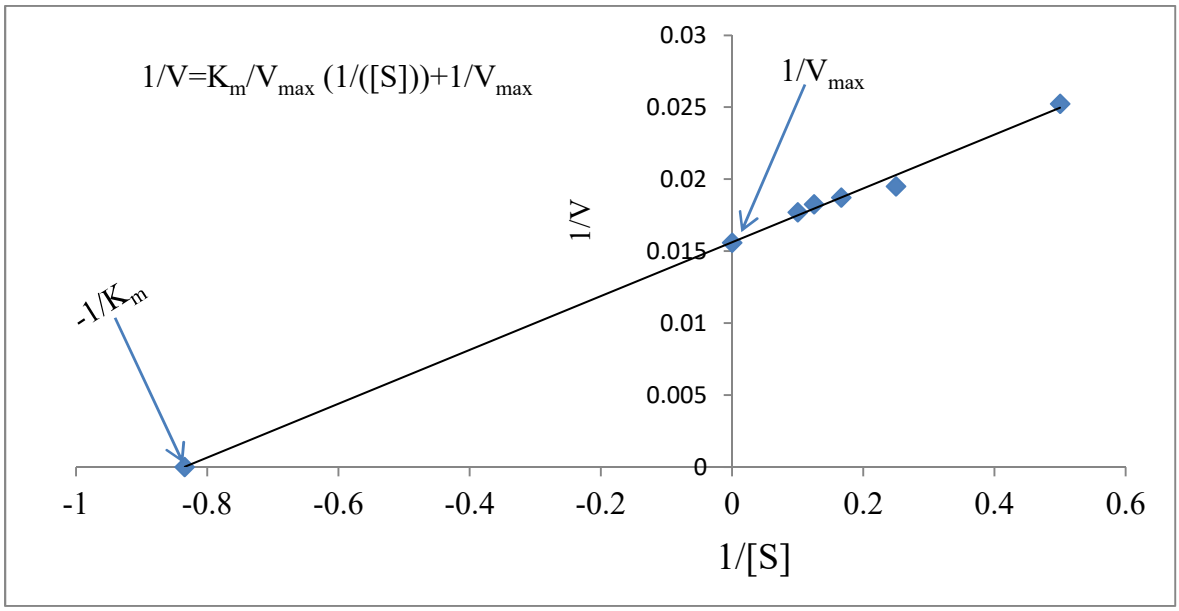
$$V = Vmax\frac{[S]}{Km + [S]}$$

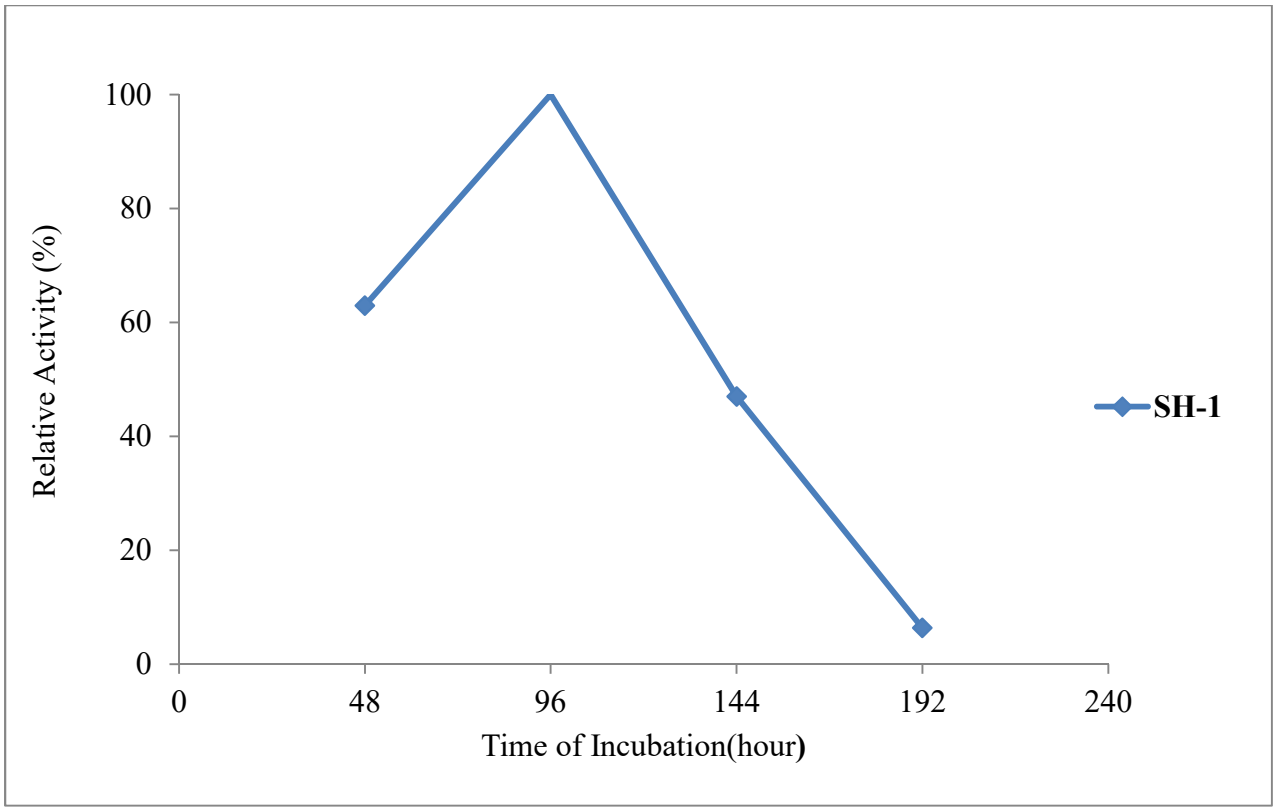
Where V is the velocity of enzymatic reaction and [S] is the concentration of the substrate.

Appendix 18: Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{Km}{Vmax}\left(\frac{1}{[S]}\right) + \frac{1}{Vmax}$$

The Lineweaver- Burk equation is used to make the Michaelis-Menten equation linear by taking its reciprocal as plotted below.





Appendix 19: Effect of time of incubation on pectinase production for SH-1