Pectinase Production by Bacteria from the Caeco-Colic Region of the Rabbit Hind Gut

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A Thesis submitted for the degree of

Doctor of Philosophy (PhD)

School of Science

Monash University

2015

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Notice 1

Notice 2

Abstract

Several bacterial species in the caeco-colic region of rabbit gut have been identified as pectinases producers that degrade pectin substances. Pectinases are used in food, pulp/paper and textile industries. This study aimed to isolate, identify pectinolytic bacteria in rabbit gut, select and optimize best producers followed by partial purification and characterization of extracellular crude pectinases produced.

Initially, pectinolytic bacterial species from caecum and colon of high fiber fed rabbits were isolated and characterized. *Bacillus subtilis, B. pumilus, B. ginsengihumi, B. megaterium, B. licheniformis* and *Paenibacillus* spp. were identified. Profile of extracellular pectinases showed that pectin-methyl esterase (PME) was rarely produced, followed by polygalacturonase (PG) while pectin lyase (PL) was most commonly and substantially produced by strains isolated. *B. subtilis 1E7* was the best producer of PG and PL.

Pectinase production by *B. subtilis* 1E7 was optimized using response surface methodology for influence of cultural temperature and agitation as independent variables in a central composite design for allocation of treatment combinations which generated 13 experimental runs. The PG and PL responses were analysed using Design Expert 8.0 and Stat-Ease statistical software to validate the responses by graphical and numerical approaches. Second order polynomial was fitted and adequate for PG ($p<0.01$) and PL ($p<0.01$) productions while R^2 values were 0.9215 and 0.9746 respectively. Predicted PG and PL productions were 0.56U/ml and 111.49U/ml respectively under cultural conditions of 38°C; 190rpm which agreed with practical experiment.

Pectinase produced by *B. subtilis* 1E7 in yeast pectin broth at predicted conditions was purified using ammonium sulphate (40 – 90% saturation) with subsequent dialysis, gel filtration and anion exchange chromatography, desalting was by gel filtration on sephadex C-

25. The partially purified pectinase was characterised for pectinolytic performance and stability under certain physico-chemical conditions. Catalytic performance of the pectinase was influenced by temperature, pH and substrate concentrations of the reaction mixtures with maximum PG activity at 60°C, pH 9 and 10mg/ml respectively while PL activity optimised at 60°C, pH 10 and 16mg/ml respectively. The pectinase showed stability at broad range of temperature (40°C-70°C) and pH (7-12). V_{max} and K_m values were 0.53U/ml, 1.29mg/ml respectively for PG and 64.52U/ml, 4.13mg/ml respectively for PL. PG and PL activities were significantly enhanced in the presence of Ca^{2+}. However, while PG showed no effect in the presence of Mg^{2+}, Co^{2+}, Glycine and Mercapto-ethanol, slight inhibition was observed in Hg^{2+}, Fe^{3+} and EDTA. Similarly, PL showed no effect in Mg^{2+}, Co^{2+}, glycine and Mercapto-ethanol but the inhibitory effects of Hg^{2+} and Fe^{3+} were significant while the extreme inhibitory effect of the chelating EDTA on the lyase suggested that pectate lyase was present. Liquid chromatography-Mass spectroscopy analysis confirmed presence of polygalacturonase and pectate lyases produced by a *B. subtilis* in our partially purified pectinase.

In summary, a novel *B. subtilis* 1E7 strain that produced alkaline and thermo-stable pectinase with alkaliphilic and thermophilic activity showing resistance towards some known potent inhibitors such as Hg^{2+}, glycine and mercapto-ethanol were identified from rabbit gut. These characteristics indicated structural strength of the pectinase and its suitability for industrial use.

Keywords: Alkaline, alkaliphilic, *Bacillus subtilis* 1E7, caeco-colic, LC-MS, lyase, pectinase, polygalacturonase, RSM, thermophylic, thermo-stable

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

General Declaration

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 3 manuscripts prepared for submissiom to appropriate journals. The core theme of the thesis is Pectinase Production by Bacteria from the Caeco-Colic Region of the Rabbit Hind Gut. The ideas, development and writing up of all the manuscripts in the thesis were the principal responsibility of myself, the candidate, working within the School of Science under the supervision of Professor Gary A. Dykes and Dr Siow Lee Fong.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. In the case of Chapter 2, Chapter 3 and Chapter 4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication status	Nature and Extent of candidate's contribution
Chapter 2	Catalytic capacity of extracellular pectinases production by bacteria isolated from the caeco-colic region of rabbit hind gut	To be submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 3	Optimisation of nutritional and cultural conditions for pectinase production by *Bacillus subtilis* (1E7) from rabbit gut using response surface methodology	To be submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 4	Purification and characterisation of pectinases produced by *B. subtilis* (1E7) from the caeco-colic region of rabbit hind gut	To be submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.

I have renumbered sections of the prepared manuscripts in order to generate a consistent presentation within the thesis.

Student's Signature: [redacted] **Date:** 18/12/2015

Main Supervisor's Signature: [redacted] **Date:** 18/12/2015

Acknowledgements

My sincerest gratitude to my supervisors, Prof. Gary Dykes and Dr. Siow Lee Fong for the consistent guidance, understanding and support throughout my research. I am especially indebted to Prof. Gary Dykes for his professionalism in scientific research, patience in guidance and ceaseless encouragement even when I strongly feel hopeless. I cannot thank him enough because I genuinely feel and believe that I got in him, the best I needed on my path to the fulfilment of my PhD degree.

My gratitude goes to the members of my Review Panel: Dr. Adeline Ting Su Yien, Dr. Choo Wee Sim and A/P Song Keang Peng for their advices and suggestions. I recognize and appreciate Prof. Steven Langford, Head of School, Science, for his efforts and interventions in times of need. Amreeta Sarjit, Diane Sunira Daniel, Darshini Sandragasan and Mr. Ragavan Murugiah are deeply acknowledged for their tireless technical assistance all the time. Thanks to Ms. Bawani Veeriah and her staff for administrative assistance. Profound appreciation to Prof. Iekhsan Othman and Syafiq, School of Medicine Monash University Malaysia for their assistance. Many thanks to Mr. Adebisi Abimbola, Akinsanya Adewale, my lab mates and GD research team for their love.

I thank Pastor Success Ejoba, members of Winners Chapel Malaysia and my root, Bishop & Rev. Mrs Mike Bamidele as well as members of Victory Chapel Omowest Osogbo Nigeria for their spiritual assistance. Lots of thanks to my dear wife, Awe Elizabeth Monisola and children for their encouragements, supports and care of home front in these four years. I thank the Federal Polytechnic Ede for the privilege to study abroad. Above all thanks to my God who said it in 1996 and performed it in His time who also renewed my strength to undertake this rigorous academic exercise at my age.

List of Abbreviations

Am. Ph. Di	Ammonium phosphate dibasic Solution
ANOVA	Analysis of variance
AS	Ammonium sulphate
BSA	Bovine serum Albumin
CCD	Central composite design
CFU	Colony forming unit
CP	Crude pectinase
DEAE	Diethylaminoethyl
ddH_2O	Double distilled water
DM	Dry matter
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
EARC	East Asia Rabbit Company
EC	Enzyme Commission of the International Union of Biochemistry
EDTA	Ethylenediaminetetraacetic acid
GF	Gel filtration
HPLC	High pressure liquid chromatography
IEC	Ion Exchange Chromatograhy
KI-I_2	Potassium iodide-iodine solution
LC-MS	Liquid chromarography – mass spectroscopy
MARP	Monash Animal Research Platform
ME	Mercapto-ethanol
mM	Millimolar
MUAEC	Monash University Animal Ethics Committee
NCBI	National Center for Biotechnology Information
OD	Optical density

Pept	Peptone solution
PG	Polygalacturonase
PL	Pectin lyase
PME	Pectin methyl esterase
QTOF	Quadrupole-time-of-flight
RPM	Revolutions per minute
RSM	Response surface methodology
SH	Sulfhydryl
S. Nit.	Sodium Nitrate solution
SPSS	Statistical Package for the Social Sciences
TBA	Thiobarbituric acid
TC	Total count
Yst	Yeast solution
YPA	Yeast pectin Agar
YPB	Yeast pectin broth

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CHAPTER 1

Literature review

1.1 Introduction

Pectin is an important constituent of the dietary fiber of the plant cell wall and is found predominantly in the middle lamella of leaves (Fonty and Gouet, 1989; Gidenne and Lamothe, 2002). Fiber is generally responsible for the rigidity and toughness of plants and allows them to withstand the osmotic pressure generated within the cells. Many sources of fiber ranging from forage (Garcia et al., 1995) to agriculture by-products (Perez de Ayala et al., 1991) are present in our environment and the mechanisms by which their constituents (including pectin) can be broken down in nature and commercially are of interest.

1.1.1 Pectic Molecules

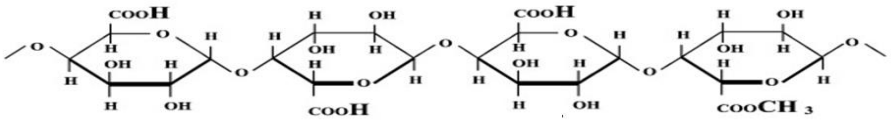
Pectic molecules are regarded as the intracellular cement between plant cells, which bind the cells together, and they are therefore largely responsible for the structural integrity and cohesion of plant tissues (Pedrolli et al., 2009). Pectic substances are a family or group of polysaccharides containing D-galacturonic acid monomers (as galacturonans and rhamnogalacturonans) in different amounts. Galacturonans are linear polymers of D-galacturonic acids and can be seen as the smooth regions in the molecular structure of a pectic substance. The carboxyl group of the galacturonic acid residue is vulnerable to esterification by acetyl and methyl groups to a larger or lesser extent (McFeeters et al., 1992; Pedrolli et al., 2009). Rhamnogalacturonans are formed when the C-6 carbon of galactate carries a carboxyl group linked to a side chain of rhamnose units. Pectic substances have been variously classified based on the modifications of the constituent backbone chains (Kashyap et al., 2001). They may be classified as follows:

Protopectin: This is the water insoluble pectic substance found in intact tissues of plants. It is generally regarded as the parent pectic substance which when hydrolysed yields pectic acids or pectin.

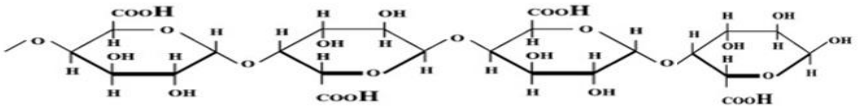
Pectic acid: This is a soluble polymer of galacturonans with a negligible amount of esterified carboxyl groups. Polypectate or pectates are those pectic acids with carboxyl groups in the salt form.

Pectinic acids. These are polygalacturonan chains that contain no more than 75% methylated galacturonate units. The salt form of this substance is known as pectinates.

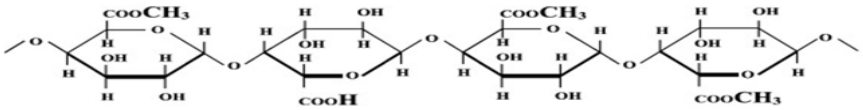
Pectin: Is a pectic substance having a substantial fraction (at least 75%) of the carboxyl groups of galacturonic acids esterified. Figure 1 shows the structural formula of different esterification levels in galacturonan chains. It should be noted that the word "pectin" is commonly used when referring to all the pectic substances (McFeeters et al., 1992; Jayani et al., 2005; Pedrolli et al., 2009)



Low Esterified Pectin



Pectic acid



High Esterified Pectin

Figure 1. Structural formula of levels of esterifications in pectic substances- Modification of Jayani et al. (2005)

1.1.2 Occurrence of pectic substances

Pectic polymers exist as insoluble protopectin in actively growing plant tissues and unripe fruits. At maturation and ripening, protopectin is converted to a more soluble pectin (Alkorta et al., 1998; Pedrolli et al., 2009). Fogarty and Kelly (1983) established the pectin contents of some foods such as grapes (0.2-1%), apple (0.5-1.6%), grape fruit (1.6-4.5%), lemon (3.0-4.0%), lemon seeds (6.0%), turnip (10%), lemon pulp (25%), sugar beet pulp (25-30%), lemon rind (32%), leguminous plant e.g. alfalfa (5-10%), orange peel and juice sacs (16-29%). Gidennes and Lebas (2002) stated that the total pectins of cotyledons of legume seeds (such as soy beans, peas, faba beans and white lupines) reach 4-14% of total dry matter. From the data reported above it can be seen that pectin is an important component of plant tissues and fibers.

1.1.3 Herbivorous mode of feeding

Herbivores are animals that feed on plant foliage, twigs, grass and similar fibers of plant origin. Herbivores are made up of a large number of wild animal species as well as some common domestic species including cattle, goats, sheep, horse, rabbits and mice. Herbivorous animals do not have the ability to digest the plant fiber components in their diet unaided. This is because this group of animals do not have the ability to produce the fibrolytic enzymes required to digest the fiber. For this reason, plant fiber is not usable to any appreciable extent by simple-stomached animals because a large part of the carbohydrates in the plant fibers consist of sugar monomers joined together in complexes by β-links. These herbivores have the challenge of effectively utilizing and digesting dietary fibers (lignin, cellulose, hemicellulose and pectins) which limits the values of forage and fiber feeds to them (Ahmad and William, 2001). Plant forage and fiber utilization by herbivores is possible through the symbiotic association that exist between them and fibrolytic microorganisms resident in

regions of the gut. The gut region which harbour these microorganisms include the rumen in ruminants, and the caecum and colon in non-ruminants. Microorganisms in these gut regions are able to ferment the fibrous materials in the animal gut and in so doing release nutrients and energy which the animals can use.

Due to the anatomical location of the rumen, ruminants are referred to as pre-gastric fermenters. McSweeney et al. **(**1999**)** stated that grazing ruminants often rely on highly fibrous diets as sources of energy but in many cases greater than fifty percent of the dietary fiber passes through the digestive tract in an un-degraded form. These authors further pointed out that this feature is worse with respect to tropical grasses as digestibility is inversely related to fiber content and that the higher lignin content of tropical forage reduces fiber digestion. Important enzymes required for the digestion of fibrous materials include cellulases, pectinases and xylanases (which are commonly referred to as fibrolytic enzymes). Non-ruminant herbivores ferment feed fiber in the same way as ruminants but the fermentation takes place in the hind gut and particularly in the caeco-colic region and therefore Beleaguer et al. (2005) described these animals as post-gastric fermenters.

1.1.4 Nutrition and fiber digestion in rabbits

Rabbits are herbivores that exhibit post-gastric fermentation of their fibrous feed components. Stone and Cozen (1975) in their description of anatomy of rabbits stated that bacteria which live in the caecum and colon convert dietary fibers to sugars. As a result of the post-gastric (after the enzyme digestion area) location of the caecum and colon (Figure 2), utilization of microbial biomass is a challenge for rabbits. Torrallardona et al. (2003) demonstrated that some absorption of microbial amino acid still take place in post-gastric fermentors. This claim was corroborated by the work of Belenguer et al. (2004) who found that about fourteen percent of total microbial amino acid can be absorbed in rabbits. This finding suggests that

these products of caeco-colic fermentation were not lost but, rather, are absorbed by other means.

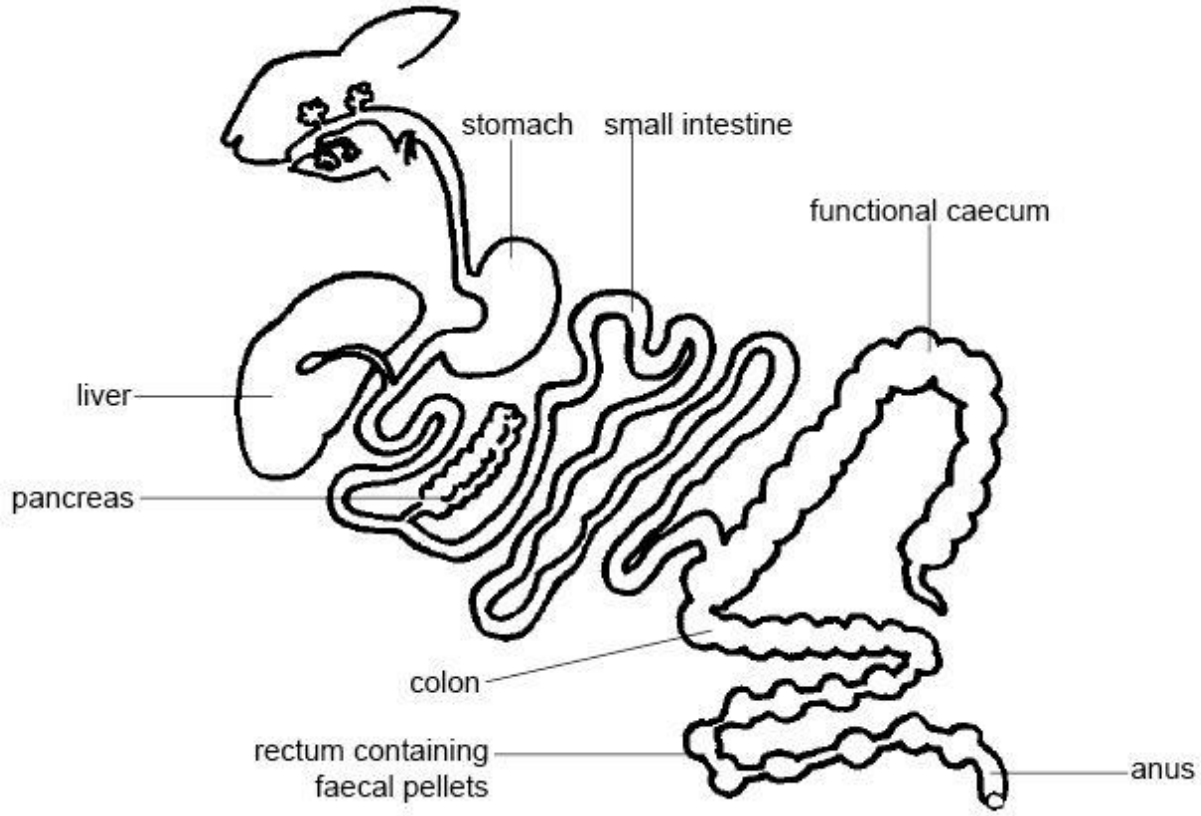


Figure 2. The Digestive System in Rabbit (*Oryntalagus caniculus*) -Stone and Cozen (1975)

Published literature (Kleessen et al., 1997; Garcia et al., 2000; Belenguer et al., 2005) indicate that rabbits excrete two types of faeces, namely soft and hard faeces. Soft faeces is a semi-liquid product rich in microbial proteins/amino acids, sugars and microorganisms. This high quality microbial protein material has been named 'cecotroph' (Garcia et al., 1995; Garcia et al., 2000; Ahmad & Wilman, 2001; Belenguer et al., 2002; Sakaguchi, 2003; Abecia, 2005; Romero et al., 2009; Combes et al., 2011). In a deliberate attempt to maximize intake of microbial proteins, rabbits re-ingest the self-produced caecotroph in a practice termed 'caecotrophy'. Personal communications with some domesticated rabbit keepers

showed many are unaware of soft faeces and caecotrophy, while others suggested that the soft faeces is produced at night. Rabbits ingest the soft faeces soon after production to allow the recycling of caecal microbial proteins and amino acid (Belenguer et al., 2005). The digesta comes out again as hard faeces which is the faecal balls that are seen in rabbit cages. There are a number of reports on the physical, chemical and biological analysis of rabbit diets and the contents of their gut. Rabbits are healthier when fed with high fibre feed. The intake of high fiber feed not only increases gut health but also allows for, and boosts, caecal microbial activity (Garcia et al., 2000; Bennegadi et al., 2003). Low fiber meals may result in caeco-colic infection in rabbits which in turn can lead to diarrhea, "mucoidenteropathy" syndrome, and associated mortality and morbidity.

Reports by various workers (Hall, 1952; Gidenne & Lamothe, 2002; Belenguer et al., 2002; Gidenne & Licois, 2005; Romero et al., 2009; Combes et al., 2011) have shown the presence of a variety of anaerobic, facultative and aerobic bacteria in the caecum of rabbits. McFeeters et al. (1992) indicated that pectin degrading bacteria in rabbit gut include, but are not limited to, bacteria of the genera *Achromobacter, Aeromonas, Arthrobacter, Agrobacterium, Bacillus, Clostridium, Erwinia, Flavobacterium* and *Pseudomonas.* Soares et al. (1999) and Janani et al. (2011), in separate reports, described twelve *Bacillus* spp that screened positive for pectinase production. Previous findings (Bennegadi et al., 2003) indicated that the nature of feed can greatly influence the composition of the caecal ecosystem. Boulharouf et al. (1991), Marounek et al. (1995), Gidenne (1997), Gidenne & Lamothe (2002) and Gidenne et al. (2007) have investigated fibrolytic enzyme activity in the caecal content of rabbit gut. It is noteworthy that cellulolytic, xylanolytic and pectinolytic enzyme activities have been observed in the caecal content but most of the authors indicated that pectinolytic activity was the highest of these. This signifies that the pectinolytic enzymes are the hydrolase most strongly involved in the digestion of fibre in the rabbit gut.

1.1.5 Pectinolytic enzymes

Pectinolytic enzymes are biological catalysts that degrade pectic substances. Pectinases are heterogenous group of enzymes which are grouped into two types based largely on their mechanisms of actions. The different types of pectinases described are esterases, also known as demethoxylases, which deesterify/demethylate pectic substances to pectic acid or pectinic acid and depolymerases which hydrolyse pectin, pectinic acid and pectic acid molecules to either short chains oligo-galacuronates or completely to the galacturonic acid subunits (Favela-Torres et al., 2006).

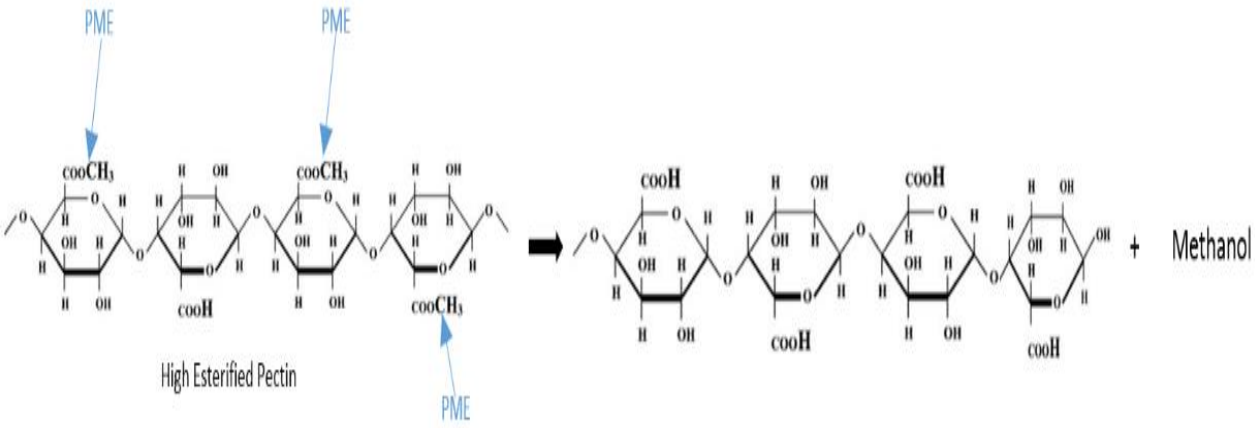


Figure 3. The catalytic activity of (PME) on esterified pectin- Modification of Jayani et al. (2005)

Demethoxylase pectinase, which is also called pectinmethyl esterase (PME), catalyses the de-esterification of the methyl/ester linkages of the galacturonan backbone of pectic substances to release pectic acid and methanol (Figure 3). The resulting pectic acid is then acted upon by depolymerases (Prades et al, 1999; Favela-Torres et al., 2006). The depolymerase enzymes consist of two types of pectinases which act on pectic substances either by hydrolysis or by a trans-eliminative split of the glycosidic bonds. These includes the hydrolases and the lyases. Hydrolases also referred to as polygalacturonases (PG) are able to catalyse the hydrolytic

clearage of the α-1, 4-glycosidic linkage in polygalacturonic acid chain with the introduction of water molecules at the oxygen bridge (Figure 4). Of the pectinases, PG is the most extensively studied.

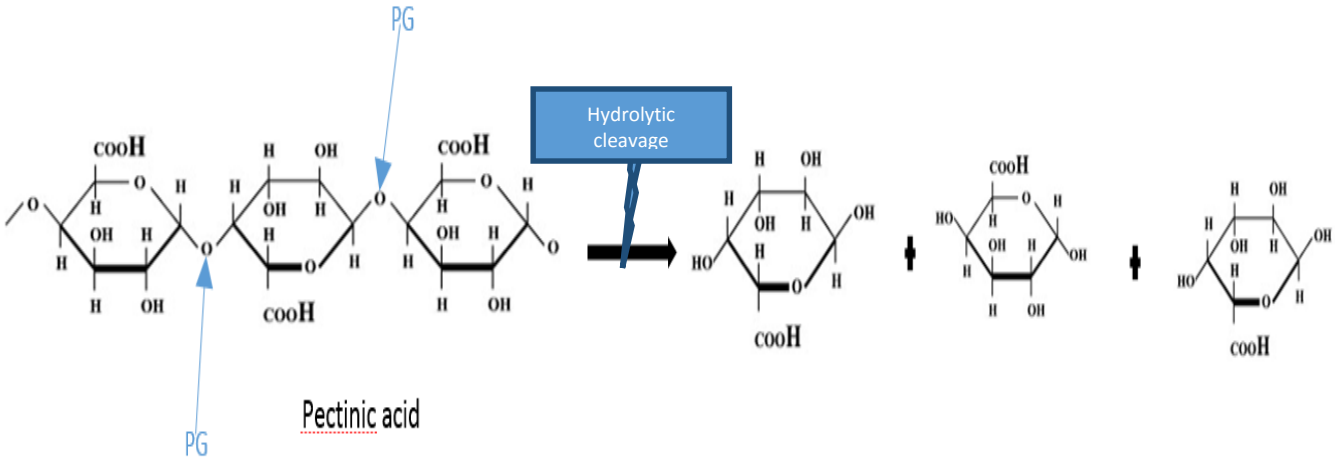


Figure 4. Catalytic activity of PG on pectinic acid polymer- Modification of Jayani et al. (2005)

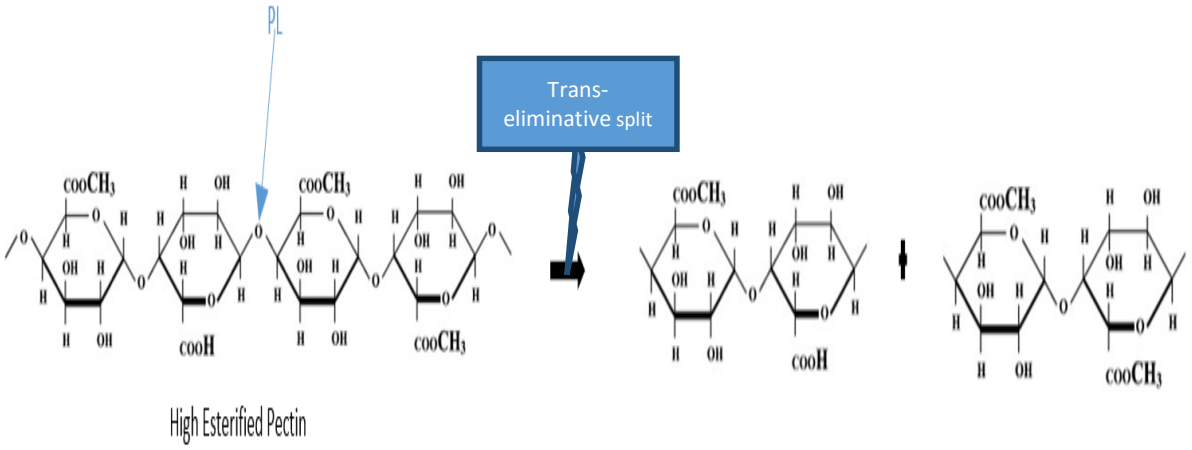


Figure 5. Catalytic activity of the lyases on esterified pectin- Modification of Jayani et al. (2005)

The lyases on the other hand comprise of pectate lyase and pectin lyase (PL). These are depolymerase enzymes that result in non-hydrolytic breakdown of pectic substances by a trans-eliminative split (Figure 5). The lyases do this by breaking the glycosidic linkages at C-

4 and simultaneously eliminate H from C-5, which lead to the production of a 4:5 unsaturated products. These products are in form of short chains of pectic substances, either esterified (methyl-oligo-galacturonates) or un-esterified (oligo-galacturonates) depending on the extent of the esterification of the initial substrates (Jayani et al., 2005; Pedrolli et al., 2009). Both pectin and pectate lyases are able to hydrolyse peptic substances of high esterification (Soriano et al., 2006) and no methanol (toxic chemical) is produced. Both pectinases exhibit optimal activity at 40-50^{0}C while the pH optimal is in alkaline range (7.5-10.0) (Jayani et al., 2005). Pectate lyase has an absolute requirement for calcium ions (Ca^{2+}). This is the reason why it is inhibited by chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA). Pectin lyase may be stimulated in presence of Ca^{2+} but this ion is not absolutely required for its catalysis. Pectin lyase, as compared to pectate lyase, will not be severely inhibited by EDTA (Jayani et al., 2005)

1.1.6 Microbial production of pectinases

Pectinases are produced by plants and microorganisms while animals are not capable of producing pectinases. Extracellular pectinolytic enzymes are produced by species of bacteria, fungi, yeasts and nematodes. The pectinases produced by microbes are important in the phytophathologic process of the microbes (Ramos et al., 2010) and decomposition of plants tissues (Pedrolli et al., 2009). Studies have shown that different types of pectinolytic enzymes are produced by different strains of microbes (both bacteria and fungi) (Kumar and Shama, 2012). Colonisation of senescent plant tissues increase at maturity and pectinases produced by colonising microbes break the tissue down result in liquefaction of the pectic substances that hold plant cell wall together and causing tissue maceration. Microbes produce various types of the pectinases which are responsible for their pathogenesis in plants. Studies have implicated certain fungal genera including *Aspergillus, Colletotrichum, Geotrichum, Rhizopus, Fusarium* and *Penicillium* in production of hydrolases (polygalacturonase) (Debing

et al., 2006). Bacterial producers of polygalacturonase tend to be species of *Bacillus, Erwinia, Pseudomonas, Xanthomonas* and *Clostridium* (Kashyap et al., 2001; Kluskens et al., 2005; Li et al., 2005; Sharma and Satyanarayana, 2006). Pectate lyases are produced predominantly by bacterial genera such as *Bacillus, Pseudomonas, Xanthomonas, Amycolata* and *Clostridium* (Kashyap et al., 2000; Gummadi and Panda, 2003; Li et al., 2005; Soriano et al., 2006). Pectin lyase is reported to be produced by bacterial genera such as *Bacillus, Clostridium, Erwinia* and *Bacteroides* (Kashyap et al., 2000; Soriano et al., 2000; Sirotek et al., 2001; Kashyap et al., 2003). All commercial microbial pectinases currently used are produced by species of *Aspergillus, Penicillium* and *Rhizopus* (Ismail, 1996; Moyo et al., 2003*).* The use of bacteria in pectinase production is of current and increasing interest. While pectinases from fungi are acidiphilic, those produced by bacteria are alkaliphilic in nature.

Pectinase production by bacteria is extracellular with the enzyme present in the culture supernatant in the absence of associated cell lysates or intact cells (Apoorvi and Vuppu, 2012). Microbial pectinases are generally studied in-vitro by isolating and purification of the crude enzyme. A number of *Bacillus* species have been screened and shown to produce extracellular pectinases (Chawanit et al., 2007; Janani et al., 2011) and further steps have been taken to optimise their pectinase production. Recently, optimisation of biotechnological process using statistical modeling in experimental designs is employed in maximization of pectinase productions (Chawanit et al., 2007). This approach is considered better than the conventional practices of varying one factor at a time, because unlike the conventional method, mathematical modeling considers the combined effects of all the process variables and how they interact towards the products response (Nwabueze, 2010). Ahlawat et al., (2009) reported pectinase production in submerged fermentation by *B. subtilis* was optimised at an alkaline pH of 9.5 and a 37^{o}C incubation temperature with a 200rpm culture agitation. Jayani et al. (2005) described optimal pectin lyase production by a *Bacillus* strain in the

alkaline range (7-10) and at 40-50^{o}C incubation. Al Balaa et al. (2014) reported the production of pectate lyase by a soil isolate of *B. subtilis* at 37^{0}C, 150 rpm, pH 6.8 conditions. These authors also reported that pectinases produced by *Bacillus* species were generally stable under alkaline and high temperature conditions (pH 7-11 and 50-70^{o}C respectively).

In the field of biotechnology, enzymes productions are growing as a result of ever increasing needs for enzymes in industrial processes (Bayoumi et al., 2008). Microorganisms have the characteristic feature of producing an array of enzymes which are known of industrial importance (Aboaba, 2009). Activity of crude enzymes does not indicate either an isolated action or a result of synergy in a multi-enzymic system on substrate degradation. Hence, purification and characterization of enzymes is an important research line which brings to focus, the actions of a component enzyme in a multi-enzyme system with regards to its substrate degradation mechanisms, optimum activity conditions and stability (Pedrolli et al., 2009). Crude extracellular enzymes of microbes are purified to homogeneity and are characterized for improved activity towards their use(s) in industrial processes. Production and purification of pectinases have been routinely carried out through the processes of ammonium sulphate precipitation and various chromatography (gel filtration (GF), ion-exchange (IEC), affinity) techniques (Yadav et al., 2008; Hamdy, 2006). Martins et al., (2007) isolated PG from *Thermoascus aurantiascus* with 21 fold specific activity increase and 24.6% yield using Sephadex G-75 gel filtration, followed by ion exchange chromatography with SP-Sepharose. Kashyap et al. (2000) reported a purification procedure for pectin lyase from *Bacillus* sp DT7 by ammonium sulphate precipitation followed by IEC with DEAE-sephacel and GF using Sephadex G-150 columns. They attained purification yield of 131.8 fold and increased specific activity from 53U/mg to 1433U/mg. Yadav et al., (2008) purified pectin lyase produced by *A. flavus* in three steps of ammonium sulphate precipitation, DEAE-cellulose (IEC) and Sephadex G-100 gel filtration column

chromatography. The PL produced was to 58 purification fold and the yield was 10.3% of the initial activity. Purified enzymes are often characterized in order to determine the stability and appropriateness in their applications in industrial processes.

1.1.7 Application of pectinases

It has been reported by Pedrolli et al. (2009) that pectinase production represents about 10% of the world production of commercial enzymes. Jayani et al. (2005) suggested that microbial pectinases account for 25% of global food enzymes sales. These figures indicate that pectinases are widely used in the food industries and this is particularly the case for the production of juices, fruit drinks and wines (Semenova et al., 2006). In spite of the fact that pectinases are known to play a major negative role with respect to post harvest rotting of fruits and vegetables, their positive commercial applications in fruit and vegetable processing and the manufacturing industries are substantial (Favelas-Torres et al., 2006). As earlier indicated, fungal pectinases are acidiphilic while bacterial pectinases are alkaliphilic in nature. This means that a particular pectinase is required for a specific purpose based on the prevalent pH of the process. At present, virtually all pectinases used for industrial applications are produced by moulds (Jayani et al., 2010). Commercial pectinase products from bacterial strains have been comparatively poorly studied. Of the bacterial species suggested for commercial pectinase production, members of the genus *Bacillus* stand out (Jayani et al., 2005). While fungal pectinases are effective at low pH (3.0-6.0), bacterial pectinases have optimal activity at pH ranges above neutrality. The prevailing properties of fungal pectinases have made them suitable for fruit juices which have lower pH but they are not suitable for preparations that require higher pH ranges (Soares et al., 1999). Pectinases produced by species of bacteria are more active under higher pH ranges and this suggests they be more useful for bio-scouring processes as compared to fungal pectinases (Wang et al., 2007).

Fruits often used for juice production include lemon, oranges, tomatoes, pineapples, guava, bananas, papaya and mangoes. The use of pectinases in juice extraction from fruits is by adding calculated quantities of enzyme to crushed (peeled, warmed 60-65^{0}C for 15 minutes to inactivate innate enzymes and cooled) pulp which is mixed well. The highly viscous pectin rich juice will remain bound to the pulp producing a jellified mass which makes extraction difficult. By addition of pectinases the viscosity of the juice is reduced significantly, thereby improving the pressing and increasing yields. In the enzymatic processing, the juice is made clearer by the breakdown of the pectin component that allows suspended particles to settle down and also eliminate undesirable changes in colour, bouquet and stability of juice. Pectinase treatment of fermented wines clarifies the must and settles out many suspended particles, often with some contaminating microbes (Kashyap et al., 2001).

Alkaline pectinases produced principally by bacteria are used mainly in the degumming and retting of fibers, in textile industries and in the pre-treatment of wastewaters received from fruit processing industries, as well as in paper making (Jayani et al. 2005). Ramie fibers, an excellent natural textile, occurs bound to ramie gum which consists mainly of pectin. The need to degum the fibers to meet the requirement for textile production is important. In classical degumming a hot alkaline (12-20% NaOH) solution is used. This method is expensive and poses serious environmental issues. Degumming with pectinase or pectinase producing bacteria ensures adequate degumming, reduced chemical consumption and guarantees environmental protection (Kashyap et al., 2001). Pectate lyase is reported to be effective in degumming (Hoondal et al., 2002). Pectinases have been used also as a replacement to toxic caustic soda in the removal of sizing agents from cotton. Bacterial pectinases are capable of depolymerising pectin and thereby improving the hydrophilicity, absorbency and whiteness of the textile materials without affecting the cellulose backbone and thus avoiding fiber damage (McNeil et al., 1984; Rouette et al., 2001). The pectic

wastewater effluent received from citrus-processing industries requires pre-treatment in order to render it suitable for decomposition during activated-sludge treatment. Conventionally, treatment involves physical dewatering, spray irrigation, chemical coagulation, direct activated sludge treatment and chemical hydrolysis which leads to formation of methane. This procedure is expensive, cumbersome and can pose a threat to the environment due to use of chemicals. The use of bacterial pectinases which selectively removes pectic substances from the wastewater is a better alternative. This involves using alkaline pectinase or alkaliphilic pectinolytic microbes to facilitate the removal of pectin component of the wastewater thereby render it suitable for decomposition by activated sludge treatment. This procedure is cheaper and safer for the environment (Hoondal et al., 2002). Studies showed that extracellular pectate lyase produced by *Bacillus* sp GIR 621 have been used for effective removal of pectic substances from wastewater (Kashyap et al., 2001). Paper making involves a continuous filtration of dilute suspension of fiber fragments, which is also known as fines and the fillers ($CaCO_3$) that form into sheets of paper. In this process, a cationic polymer is added to pulp as a retention aid to keep fines and filler particles in the paper sheet and also speed up water drainage. Alkaline peroxide bleaching of pulps solubulises troublesome polysaccharides, including pectin, which specifically interferes in this process by forming complexes with the cationic polymer (cationic demand) making it an ineffective retention aid. Addition of pectinase or pectinolytic bacteria at this stage will depolymerise the pectin and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Kashyap et al., 2001). Pectinases have been blended into animal feeds which serves to reduce feed viscosity and increases liberation of nutrients either by hydrolysis of non-biodegradable fibers or the release of nutrients blocked by the fibers. This in turn reduces the amount of faeces (Hoondal et al., 2000) as a result of greater fibrolytic activity.

1.1.8 Background of Study

Due to the unusual digestive system of rabbits and the effects of dietary fiber, Gidenne and Lebas (2002) suggested that the fibrolytic activity of caecal bacteria is focused on pectic substrates. It has also been established that colonization of rabbit gut by pectinolytic flora (10^8-10^7cfu/g) is greater as compared to cellulolytic flora (10^6-10^7cfu/g) (Forsythe and Parker, 1985; Boulharouf et al., 1991; Siroteck et al., 2001; Gidenne and Lebas, 2002). This is likely to be because uronic acids are important factors that modulate the fermentative activity in the caecum and caecal pH (Garcia et al., 2000). Gidenne and Lebas (2002) showed that feed ingredients rich in pectin and hemicelluloses are particularly well digested by rabbits. Previous studies on rabbits with respect to fibre digestion have focused on microbial population, nutrition evaluation, gut health, caecotrophy production, caecal content analysis and the gut enzyme activity. No attention has been given to the area of physiological studies of the microbiota and the *in-vitro* microbial synthesis of pectinolytic enzymes and this has resulted in limited information being available in this area. The potential use of pectinolytic bacteria from the caeco-colic region of rabbits for commercial production of enzymes has also not been investigated. This study is therefore undertaken to isolate symbiotic bacteria that produce pectinases (with emphasis on polygalacturonase, lyases and esterases) in the caeco-colic region of rabbit hind gut, select the best bacterial strain for in-vitro production of the pectinases and optimise the culture conditions to maximise production. The crude pectinases produced will be partially purified and their activity will be characterised.

1.1.9 Objectives of the Study

1. To isolate and identify bacteria that produce pectinase in the caeco-colic contents of rabbit fed with high fiber meals.
2. To assess the potentials of the bacterial isolates for pectinases (PG, PL, PME) production.

3. To optimize (nutritional and growth conditions) pectinase production by the selected bacterial isolate for maximum pectinase yield.
4. To partially purify and characterize the pectinases obtained and test the effects of physico-chemical conditions on the partially purified enzyme.
5. To determine stability of the partially purified pectinase to temperature (heat) and chemicals.

The first and second objectives are reported in Chapter 2, the third in Chapter 3 and the 4th and 5th objectives in Chapter 4.

CHAPTER 2

Catalytic capacity of extracellular pectinases produced by bacteria isolated from the caeco-colic region of rabbit hind gut

The work presented in this chapter represents the following manuscript in preparation for submission to an appropriate journal

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of chapter 2, the nature and extent of my contributions to the work was the followings:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Siow Lee Fong	SLF was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate'	[redacted]	**Date**
Signature		**18/12/2015**

Main Supervisor'	[redacted]	**Date**
Signature		**18/12/2015**

2.1 Introduction

Pectin is considered to be one of the principal constituents that make up plant cell walls and dietary fiber. It is found in high concentration as an integral part of the middle lamella of leaves where it acts as a cementing substance between adjacent cells (Kashyap et al., 2001; Janani et al., 2011). Pectic substances are complex macromolecules containing α-1-4-linked D-galacturonic acid residues. The carboxyl group of the galacturonic acid is vulnerable to esterification by acetyl or methyl groups (Pedroli et al., 2009). Based on the levels of esterification, pectic substances can exist in nature as pectic acid (almost zero esterification), pectinic acids (≤ 75% esterification) or pectin (≥ 75% esterification) (Jayani et al., 2005).

Rabbits (*Oryntalagus caniculus*), which are herbivores, are not capable of producing enzymes that digest fibers but symbiotic microbes resident in the gut of rabbits aid in fiber digestion (Ahmad and Wilman, 2001; Sirotek et al., 2001). Rabbits are able to digest and utilize fibers in the caecum and colon (caeco-colic region) by post-gastric fermentation. The caeco-colic region of rabbits is an ecosystem in which a wide array of microorganisms degrade the fiber content of the feed consumed by rabbit. Reports by various authors (Sirotek et al., 2001; Gidenne and Fortun- Lamothe, 2002; Belenguer et al., 2002; Romero et al., 2009; Combes et al., 2011) have shown the presence of bacteria in the rabbit gut and specifically pectinase producing bacterial species such as members of the genera *Bacillus*, *Bacteroides* and *Clostridium*. Several studies (Boulharouf et al., 1991; Marounek et al., 1995; Gidenne and Fortun-Lamothe, 2002; Gidenne et al., 2007) have investigated the fibrolytic enzyme activity in the ceacal contents of rabbit gut. These studies identified cellulolytic, xylanolytic and pectinolytic enzyme activities in this gut region. Pectinolytic activity has, however, been reported to be the greatest of these in the caecum and colon of rabbits (Gidenne, 1997; Sirotek et al., 2001).

Pectinases are a group of related enzymes which can be divided into: depolymerising polygalacturonase (PG), which hydrolyses the α-1,4glycosidic (galacturonic acid-galacturonic acid) bonds in pectic substances that is un- or poorly-esterified; pectin lyase (PL), which catalyses β-elimination reactions between methylated galacturonates; and pectinmethyl esterase (PME), which demethoxylates methylated pectin molecules (Soares et al., 1999).

Pectinolytic enzymes are widely used in food and beverage industries, particularly in the production of juices, fruit drinks and wines (Semenova et al., 2006). They also play a major role in the pulping processes for paper making. Pectinases currently used in industrial processes are generally produced by moulds, and in particular *Aspergillus* spp., but reports of pectinases production by bacterial species are limited (Jayani et al., 2010). In view of the potential application of pectinolytic enzymes there is a need to further explore alternative sources of pectinases. This study aimed to isolate, identify pectinolytic bacteria from the digesta in the caeco-colic region of rabbit hind gut and subsequently assess the potential of the isolates for the production of three pectinases (PG, PL and PME) to establish the best producer of these pectinases among the isolated bacteria.

2.2 Materials and Methods

2.2.1 Collection of digesta

Three weaned (8 week old) New Zealand White rabbits obtained from the East Asia Rabbit Corporation (EARC), Semenyih, Selangor, Malaysia were used in this research work. They were housed in the Monash University, Malaysia animal facility where they were fed *ad-libitum* with high fiber diet supplied by EARC and with continuous supply of water from a drinker for a minimum of three weeks before being sacrificed. Care and sacrifice of the animals were in accordance with the recommendations of Monash University Animal Ethics committee (MUAEC) (Approval Number MARP/2012/004). The caeco-colic (ceacum and

colon) region of the rabbit gut was aseptically excised and the content (digesta) was pressed out into sterile screw capped plastic tubes for further analysis.

2.2.2 Isolation of bacteria

One gram of the digesta from the caecum and colon of each animal was serially diluted in 0.1% (w/v) peptone water (Peptone; Oxoid, UK) and spread plated on Plate Count Agar (PCA; Oxoid, UK) and Yeast Pectin Agar (YPA; Soares et al., 1999) (See YPA in Appendix I) followed by aerobic incubation at 37ºC for 48 hours to establish the Total Count (TC) and Pectinolytic Count (PC), respectively.

2.2.3 Selection of pectinolytic bacterial colonies

All distinct bacterial colonies on the YPA were considered to have the ability to utilise pectin, as this is the sole carbon source in the medium, and were selected for further work. The selected colonies were repeatedly streaked for purity on Nutrient Agar (NA; Oxoid, UK) and the pure cultures were maintained on same until required for use.

2.2.4 Identification of bacterial isolates

Initially bacteria were characterized based on morphological, physiological and biochemical tests according to the standard methods previously used by Janani et al., (2011). Molecular identification of pectinase producing bacterial species was carried out by amplification of their 16S rDNA using primers 63f and 1387r using the method of Marchesi et al., (1998). The final products were sent for Sanger sequencing at 1st Base (Singapore). The DNA sequences were trimmed using BioEdit Sequence Alignment Editor Release 7.0.9 to eliminate vector sequences and the identities of the bacteria were established using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Chou et al., 2014)

2.2.5 Qualitative screening of isolates

Qualitative assays were carried out with the isolates to evaluate the potential of isolated colonies for pectinolysis. Bacterial colonies were stab-inoculated into YPA agar using a

sterile needle. Cultures were then incubated at 37ºC for 48 hours. At the end of incubation period the cultures were flooded with potassium iodide-iodine ($KI-I_2$) solution according to the protocol of Soares et al. (1999) and Janani et al. (2011). The diameter of any clear halos produced around the bacteria colonies were measured and considered as evidence of pectinolysis. The width of halos was measured to rank the capacity of the isolates for pectinolytic enzyme production according to the Janani et al. (2011) (ranking is shown in Appendix II).

2.2.6 Quantitative determination of pectinase activity

Quantification of pectinase activity was carried out by the submerged fermentation (SmF) method using 20ml Yeast Pectin Broth (YPB) medium (YPA used above but without agar) (pH7) in 100ml Schott bottles. The medium was inoculated with 1ml of standardized (OD ~0.7 at 600nmλ) inoculum of each bacterial isolate and was incubated at 37ºC on a shaker (150rpm) for 48hours (Ahlawat et al., 2009). The culture was terminated by centrifuging at 10,000*xg* for 10 minutes in 4ºC. The supernatant obtained was regarded as a crude pectinase which was investigated for enzymatic activity.

The crude pectinases were screened by assaying for PG, PL and PME. The assay for PG activity was based on the measurement of the released reducing groups using dinitrosalicylic acid (DNS) reagent (Miller, 1959) as adapted by Soares et al. (1999) and Kashyap et al. (2000). The lyases activity assay was based on the chromogenic reaction of the product of pectin breakdown with thiobarbituric acid (TBA) (Nedjma et al., 2001; Hayrunnisa et al., 2010; Ramos et al., 2010). The PME activity assay was based on the titrimetric method used by Arotupin et al. (2008). In all the assays D-(+)-galacturonic acid monohydrate was used as the standard. One unit (U) of pectinase activity was expressed as the quantity of enzyme that generates the release of 1µmol of galacturonic acid per minute under specific assay conditions (Jayani et al., 2010).

2.2.7 Statistical analysis

In all cases, treatments were conducted in triplicates and data were expressed as mean ± standard deviation. Significant differences between treatments were determined by a one-way analysis of variance (ANOVA) with Tukey's test used to determine the significance of pair-wise differences at a $p<0.05$ level. All analysis was achieved using SPSS (IBM Corporation, Armonk, New York).

2.3 Results and Discussion

2.3.1 Analysis of feed

A high fiber intake helps rabbits to stay healthy, encourages the caeco-colic microbiota development and fibrolytic activity (Gidenne and Licois, 2005). Physicochemical analysis of feed sample dry matter (DM; Appendix III) used in this work showed that it had a relatively low moisture (10.35±0.09% DM), a satisfactory crude protein (16.75±0.05% DM) and high crude fiber (23.20±0.1% DM) contents. These parameters met the content requirements for a high fiber feed suitable for gut health and caeco-colic fermentation in growing rabbits (Gidenne et al., 2000; Gidenne, 2003; Romero et al., 2009). All the rabbits in the experiment showed signs of good health until sacrificed (Gidenne et al., 2001).

2.3.2 Caeco-colic bacterial counts and identification

Bacterial growth was observed both on the PCA and PYA under aerobic conditions. Since PYA medium contains citrus pectin as the only carbon source, it is assumed that the bacterial strains growing on it were able to utilize pectin and that they are likely to be pectinolytic bacteria. The mean total count of bacteria in the caecal content ($10.83 \pm 1.2 \times 10^7$cfu/ml) and in the colon ($9.20 \pm 1.1 \times 10^7$cfu/ml) were not significantly ($p>0.05$) different from each other. In addition, there was no significant ($p>0.05$) difference in the pectinolytic bacterial count between the contents of caecum ($8.77 \pm 1.1 \times 10^7$cfu/ml) and that of the colon (7.03 ± 1

x 10^7cfu/ml). This result is in accordance with the previous observations of bacterial enumerations in the rabbit gut by Boulahrouf et al. (1991). These authors reported the same PC (10^7cfu/g) in both the caecum and colon. This may be because bacterial cells in the caecum move across with the digesta into the colon because both gut areas have the same pH and moisture content. A total of twenty-seven bacterial colonies displaying pectinolytic activity were isolated from the YPA plates of the highest sample dilution showing growth. All of the isolates were aerobic, Gram positive, motile and spore forming rods. These characteristics are consistent with the observations of Janani et al. (2011) who identified their pectinolytic isolates from a waste dump as *Bacillus* spp. Five *Bacillus* species, namely, *B. subtilis, B. ginsengihumi, B. megaterium, B. licheniformis*, *B. pumilus*, and *Paenibacillus* spp. were identified using 16S rDNA in this study (Table 1). The twenty seven bacterial isolates consisted of 20 different strains of *B. subtilis,* 1 strain of *B. ginsengihumi*, 1 of *B. pumilus*, 2 strains each of *B. megaterium,* and *B. licheniformis* while the *Paenibacillus* spp could only be identified to genus level. In the present study, it is evident that *B. subtilis* strains are the principal culturalble aerobic pectinolytic bacteria in the rabbit caeco-colic hind gut.

2.3.3. Qualitative screening of bacterial isolates for pectinolysis

The isolated colonies of the bacterial strains tested for hydrolysis on pectin plate agar formed visible halos around colonies in reaction to flooding with KI-I_2 solution, which is consistent with previous report of Soares et al. (1999) and Janani et al. (2011). These halos indicated pectinolytic activity by the bacterial cultures with the width of the halos proportional to pectinase production. Table 1 lists the width of the halos formed around different bacterial strains isolated. Categorising pectinase production by the bacterial species was based on rating the halo width (mm) as follows: 0mm (non-producer), 1-5mm (poor), 6-15mm (moderate) and above 15mm (strong producers) according to the method used by Soares et al.

(1999) and Janani et al. (2011). Generally, it can be observed that *B. subtilis* strains were strong producers of the pectinase (Appendix III) in the rabbit gut. The results were in agreement with the work of Soares et al. (1999) and Janani et al. (2011) in which certain *Bacillus* spp appeared strong producers of pectinase among other bacterial species isolated from soil samples.

Table 1: Identification and qualitative screening of bacterial isolates for pectinase production

Isolate	Identity	Halo diameter (mm)	Pectinase production rating
1E1	*B. subtilis*	19	Strong Producer
1E2	*B. subtilis*	20	Strong Producer
1E3	*B. subtilis*	26	Strong Producer
1E4	*Paenibacillus* spp.	2	Poor Producer
1E5	*B. subtilis*	26	Strong Producer
1E6	*B. subtilis*	18	Strong Producer
1E7	*B. subtilis*	26	Strong Producer
1E8	*B. subtilis*	21	Strong Producer
1E9	*B. subtilis*	23	Strong Producer
1E10	*B. ginsengihumi*	0	Non Producer
2E1	*B. subtilis*	26	Strong Producer
2E2	*B. megaterium*	13	Moderate Producer
2E3	*B. megaterium*	25	Strong Producer
2E4	*B. subtilis*	26	Strong Producer
2E5	*B.subtilis*	25	Strong Producer
2E7	*B. licheniformis*	13	Moderate Producer
2E9	*B. subtilis*	24	Strong Producer
3E1	*B. subtilis*	24	Strong Producer
3E2	*B. licheniformis*	16	Strong Producer
3E3	*B. subtilis*	26	Strong Producer
3E4	*B. subtilis*	25	Strong Producer
3E5	*B. subtilis*	26	Strong Producer
3E6	*B. subtilis*	25	Strong Producer
3E7	*B. subtilis*	25	Strong Producer
3E8	*B. subtilis*	25	Strong Producer
3E9	*B. subtilis*	26	Strong Producer
3E10	*B. pumilus*	0	Non Producer

*Pectinase production rating based on halo width: 0mm - non-producer, 1-5mm - poor, 6-15mm – moderate producers, above 15mm - strong producers

Table 2: Production of PG, PL and PME by the pectinolytic bacteria in pectin culture extract

Isolates	Identity	PG (U/ml)	PL(U/ml)	PME(U/ml)
1E 1	*B. subtilis*	0.15 ± 0^{b}	75.86 ± 0.0^{j}	0
1E 2	*B. subtilis*	0.48 ± 0.01^{f}	75.86 ± 0.0^{j}	0
1E 3	*B. subtilis*	0.27 ± 0.01^{d}	55.91 ± 0.04^{h}	0
1E 4	*Paenibacillus* spp.	0.1 ± 0.01^{a}	4.07 ± 0.35^{b}	0.184 ± 0^{b}
1E 5	*B. subtilis*	0.27 ± 0.01^{d}	55.91 ± 0.04^{h}	0
1E 6	*B. subtilis*	0.33 ± 0.02^{e}	61.05 ± 0.31^{i}	0
*1E 7	*B. subtilis*	$*0.61\pm0.04^{g}$	$*91.09\pm0.04^{k}$	*0
1E 8	*B. subtilis*	0.37 ± 0.02^{e}	76.07 ± 0.04^{j}	0
1E 9	*B. subtilis*	0.27 ± 0.01^{d}	61.05 ± 0.0^{i}	0
1E 10	*B. ginsengihumi*	0.2 ± 0.02^{c}	2.04 ± 0.35^{a}	0
2E 1	*B. subtilis*	0	36.98 ± 0.06^{g}	0
2E 2	*B. megaterium*	0	4.07 ± 0.35^{b}	0.184 ± 0^{b}
2E 3	*B. megaterium*	0	30.19 ± 0.0^{f}	0
2E 4	*B. subtilis*	0.15 ± 0.0^{b}	25.04 ± 0.04^{e}	0
2E 5	*B.subtilis*	0	28.33 ± 0.0^{f}	0
2E 7	*B. licheniformis*	0	9.2 ± 0.0^{c}	0
2E 9	*B. subtilis*	0.14 ± 0.1^{bc}	31.63 ± 0.04^{f}	0
3E 1	*B. subtilis*	0	52.2 ± 0.04^{h}	0
3E 2	*B. licheniformis*	0	19.9 ± 0.04^{de}	0
3E 3	*B. subtilis*	0	52.82 ± 0.04^{h}	0
3E 4	*B. subtilis*	0	55.91 ± 0.04^{hi}	0
3E 5	*B. subtilis*	0	13.52 ± 0.06^{d}	0
3E 6	*B. subtilis*	0	53.23 ± 0.04^{h}	0.13 ± 0^{a}
3E 7	*B. subtilis*	0	31.42 ± 0.0^{g}	0
3E 8	*B. subtilis*	0.20 ± 0.01^{c}	0	0
3E 9	*B. subtilis*	0	53.64 ± 0.0^{h}	0
3E 10	*B. pumilus*	0.31 ± 0.01^{e}	8.17 ± 0.04^{c}	0

$^{a\text{-}k}$ indicate significant differences ($p<0.05$) in pectinase production among the bacteria species. * = Highest production

2.3.4 Quantitative screening of bacterial isolates for pectinolysis

It has been established in the literature that rabbit caecal bacteria produce extracellular pectinases in their environment (Sirotek et al. 2001; Gidenne and Lebas 2002; Apoorvi and Vuppu 2012) and as a result, the culture supernatants from these bacteria are able to catalyse the degradation of pectic substances. Levels of production of PG by the different strains identified in the present study are shown in Table 2. A number of the isolates were found to be non-producers of PG. Among the producers, production levels ranged between 0.1 ± 0.01U/ml (1E4) to 0.61 ± 0.04U/ml (1E7) which was considered low but *B. subtilis* 1E7 was the best producer of PG. The lowest level of PME activity by the caeco-colic bacteria is expected because its product of pectin degradation (methanol) is toxic (Pedrolli et al. 2009) to rabbits and its accumulation can be injurious to the tender tissues of the gut. The low production of PG could be because the complementary action of PME is required to demethylate the esterified pectin molecules in order for the PG produced to function effectively (Tans-Kersten et al. 1998) and therefore it is expected that since PME is very low, PG production will be equally low. On the average, the level of PL production by most of the bacterial strains in the present study was higher than reported in *R. oryzae* (Hamdy 2005). In this study, it is evident that *B. subtilis* strains are the principal culturable aerobic pectinolytic bacteria in the rabbit caeco-colic hind gut. The high level of lyase production by the bacteria species in the caeco-colic biotope suggests that it plays the most important role in the pectinolytic function compared to the PG and PME screened. Jayani et al. (2005), Soriano et al. 2006 and Sinitsyna et al. (2007) reported that lyase is the only pectinase that is able to hydrolyse the α-1, 4-glycosidic bonds of highly esterified pectic substances without prior de-esterification process by the PME enzyme. Most importantly, the product of its depolymerisation is not toxic. The best producer bacteria (*B. subtilis*, 1E7, accession number

KT 713393, Appendix IV) in this study appeared to be novel because of its capacity to produce most, the PL of high activity.

2.4 Conclusion

Of the twenty seven bacterial strains screened, no single bacterium was able to produce all the three pectinases (PG, PL, and PME) in substantial quantities. This work is the first to evaluate the profile of the three pectinases production by the caeco-colic bacteria. Overall, PL was produced by the greatest number of bacterial strains while PME was rarely produced. Relatively, PL activity was the highest compared to PG and PME, which means that pectin catalysis with the lyases is the most predominant. All *B. subtilis* isolates were strong producers of pectinases indicating that they may play a major role in the pectinolytic activity in the rabbit gut. *Paenibacillus* spp. 1E4 and *B. megaterium* 2E2 displayed the PME activity but even this activity was relatively very low. Of all the bacterial isolates, strain *B. subtilis*, 1E7, accession number KT 713393 (Appendix 4) appeared to be the best producer of PG and PL pectinases. Most of the bacterial strains and especially *B. subtilis* 1E7 produced PL substantially with activity that was higher than previously reported for *R. oryzae* and *Bacillus* sp. DT-7. This work suggests that some *B. subtilis* strains and particularly 1E7, isolated from the caeco-colic region of the rabbit hind gut, could be good alternatives to some of the microbial sources currently used as commercial pectinase producers.

CHAPTER 3

Optimisation of nutritional and cultural conditions for pectinase production by *B. subtilis* 1E7 from rabbit gut using response surface methodology.

The work presented in this chapter represents the following manuscript in preparation for submission to an appropriate journal

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of chapter 2, the nature and extent of my contributions to the work was the followings:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Siow Lee Fong	SLF was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate' Signature	[redacted]	**Date 18/12/2015**

Main Supervisor' Signature	[redacted]	**Date 18/12/2015**

3.1 Introduction

Pectinases are a group of related enzymes which naturally degrade pectic substances that are often found in plant cell walls (Kashyap et al, 2000). Pectinolytic enzymes consist mainly of the polygalacturonases (PG), EC 3.2.1.15, pectin lyases (PL), EC 4.2.2.10 and pectinmethyl esterases (PME), EC 3.1.1.11, which differ in the form of pectic substances they prefer as substrate and the products formed. PG breaks the α-1, 4 bonds between polygalacturonates (un-esterified pectin) giving rise to galacturonic acid units (Jayani et al., 2005). PME attacks the alkyl side groups in an esterified pectic polymer thereby de-esterifying the methyl/ester linkages to release acidic pectins and methanol. The pectin produced is then acted upon by PG. The PL is the most unique of the three pectinases because it is able to degrade pectin of any degree of esterification without prior actions of other enzymes. PL action is by trans-eliminative split of the glycosidic linkages in the pectic polymer molecules (Alana et al, 1990; Pedrolli et al, 2009). Pectinolytic enzymes have been found to be produced by bacteria (Ahlawat, et al., 2009), moudls (Yadav et al., 2008) and yeasts (Moyo et al., 2003).

The significant industrial uses of pectinases can be seen in the food industry, in the treatment of waste waters and the purification of raw cotton in textile (Kashyap et al., 2000). PG, PL and PME are the most commonly used pectinases in industries and account for about 10% of total industrial enzymes used globally (Mandhania et al., 2010). Pectinases produced by moulds, particularly, *Aspergillus* species, are frequently used for commercial purposes in industries (Acuna-Arguelles et al, 1995). However, as a result of inherently better properties, pectinases produced by bacteria, particularly by *Bacillus* species, are fast gaining acceptance (Kashyap et al, 2000). Due to increased demand for pectinases in industrial applications there is need to maximise production of bacterial pectinases. The conventional method of "change-one-factor-at-a-time" is a classical technique used in bio-process optimisation. This method involves varying a single factor or one independent variable at a time while keeping

all others at specific levels (Bas and Boyaci 2007; Wang et al. 2007). The major disadvantage of this technicque is that the result does not encompass possible interactive effects among the variables which may lead to unreliable outcomes and errors (Oh et al. 1995). The response surface methodology (RSM) approach will be used in this work, based on factorial designs and regression analysis will more efficiently deal with the multifactorial interactions in optimisation of the experimental responses. However, the initial conventional method is used to identify the variable factors that can influence optimisation of a production. This present study is aimed at maximising the production of extracellular polygalacturonase (PG) and pectin lyase (PL) pectinases by *B. subtilis* 1E7, which best produced the pectinases in the rabbit gut, through optimisation of the nutrient and culture conditions using the response surface methodology.

3.2 Materials and Methods

3.2.1 Source and maintenance of bacteria culture

The *Bacillus subtilis* IE7 strain showed highest pectinase production (0.61U/ml, PG and 91.09U/ml, PL) from the caeco-colic region of rabbit hind gut compared to other bacteria species present in earlier work (data shown in chapter 2). It was screened and selected as the most promising producer of the pectinases (PG and PL) among other bacteria species. Cells were maintained in 20% (v/v) glycerol in -80^{o}C (Toit et al., 1998). Short term maintenance was on nutrient agar (NA) (Oxoid, England) at 4^{o}C.

3.2.2 Inoculum preparation

The inoculum was prepared in a 20ml nutrient broth (Oxoid, England) in a 100ml Scott bottle which was incubated overnight at 37^{o}C on a rotary shaker incubator at 150 revolutions per minute (rpm) agitation. Culture suspension was standardised by adjusting the optical density (OD) to ~0.7 at 600nm (21.7 ±2 x10^{8} cfu/ml) in a Cole Parmer 1100RS Spectrophotometer.

3.2.3 Pectinase Production

Yeast pectin broth (YPB) medium used in the pectinase production is composed of KCl 0.5g; $MgS0_4.7H_2O$, 0.5g; K_2HPO_4,1g; $FeS0_4.7H_2O$, 0.01g; yeast extract (Difco England), 2g; 10g citrus pectin (Sigma-Aldrich), and 1000ml distilled water, (Janani et al., 2011; Reddy et al., 2012). The medium was adjusted to initial pH of 10 with 0.1M NaOH (Ahlawat et al., 2009). A 20mL volume of the YPB medium in 100ml Scott bottle was inoculated with the standardised (OD ~ 0.7) inoculum and was grown for 48 hours in an incubator shaker at 37^oC and 150rpm.

3.2.4 Effects of nutritional and cultural conditions on pectinase production by *B. subtilis* 1E7

The experiments were carried out using conventional method of "change-one-factor-at-a-time" whereby a single condition (independent variable) is varied but fixed all others at specific levels.

3.2.4.1 Effects of nitrogen sources

Nitrogen content of the YPB was supplemented at equimolar (0.2% w/v) by sodium nitrate, ammonium sulphate dibasic, peptone and yeast extract as sole nitrogen source on different occasions. All the nitrogen compounds used were chosen based on reports in literature in which they were found to have influenced pectinase production. The combination of yeast extract:peptone (0.75%) was as used based on the method of Ahlawat et al. (2009)

3.2.4.2 Effects of incubation temperature, initial pH of medium, agitation speed and inoculum size

Pectinase production was carried out under different culture conditions in order to derive the level required for maximum enzyme production. Incubation temperature was put at intervals

of 30, 35, 40, 45 and 50^{o}C while the initial pH of production medium was at 6, 7, 8, 9, and 10. The shaker incubator was put at agitation speed of 100, 150, 200, 250 and 300 rpm while the inoculum sizes used were 1ml, 2ml, 3ml, 4ml and 5ml of bacterial cell suspension standardised at OD ~0.7 at 600nm ($21.7\pm2x10^{8}$ cfu/ml). All production runs were carried out with 20ml YPB medium in 100ml Scott bottles.

3.2.5 Preparation of crude pectinases

The supernatant obtained from the culture centrifuged at 10,000x*g* for 10 mins at 4^{o}C was regarded as the crude pectinase which was used in the activity assay of the pectinases.

3.2.6 Assays for pectinases

Crude pectinases were made to react with citrus pectin (Sigma Aldrich) solution and the activity was measured by quantifying the reducing groups liberated in the process. In the assay of PG, the released reducing groups were measured by using the dinitrosalicylic acid (DNS) reagent (Soares et al. 1999). The chromogenic reaction of the pectin breakdown product with 0.04M thiobarbituric acid (TBA) and 0.2M dilute hydrochloric acid was the bases for PL activity assay as previously used by Nedjma et al. (2001) and Hayrunnisa et al. (2010). In both cases of activity assays galacturonic acid monohydrate was used as the standard. One unit of pectinase activity was expressed as the milligram galacturonic acid released per millilitre of the enzyme per minute under specific assay conditions (Jayani et al. 2010).

3.2.7 Experimental design to optimize pectinase production using RSM

From the conventional methods of pectinase optimisation, the effects of each factor has been found and used as a bases for determining the independent variable and the levels for the

Table 3: The three level combinations of the independent process variables used in the CCD

Independent Variables	Code	Actual range and levels		
		-1	0	+1
Agitation (rpm)	X_1	100	200	300
Temperature (°C)	X_2	30	40	50

factorial experimental design. A central composite design (CCD) was used to allocate treatment combinations regarding the experimental design.

Table 4: Treatment combinations and pectinolytic activity in a CCD experimental Runs

Runs	Coded Variable levels		Mean Responses[a]	
	X_1	X_2	y^1	y^2
1	0	0	0.57	107.96
2	1	1	0.00	13.93
3	1	-1	0.04	73.40
4	0	0	0.57	107.96
5	0	0	0.57	107.96
6	0	-1	0.43	102.41
7	0	0	0.57	107.96
8	1	0	0.00	79.57
9	-1	1	0.036	97.88
10	-1	0	0.32	109.61
11	0	1	0.15	59.20
12	0	0	0.57	107.96
13	-1	-1	0.11	84.92

[a] Mean of triplicate responses, y^1, Polygalacturonases, PG, activity (U/ml) ; y^2, Pectin lyases, PL, activity (U/ml).

Two independent factors, agitation speed (X_1rpm) and cultural temperature ($X_2{}^oC$) were used to generate factorial combinations at three levels that led to thirteen sets of experimental runs which represented nine experimental combinations and five replicates at the center point as described by Nwabueze (2010). These are shown in Table 1 and Table 2. The pectinase activity of PG and PL (y^1 and y^2 respectively) were taken as the dependent variables/responses of the experiment runs (Table 2). The second order model used to predict the optimum responses were expressed in the following equations.

$$y^1 = b_0+b_1x_1+ b_2x_2+ b_{12}x_1x_2+ b_{11}x^2{}_1+ b_{22}x^2{}_2 \quad \text{……..(1)}$$

$$y^2 = b_0+b_1x_1+ b_2x_2+ b_{12}x_1x_2+ b_{11}x^2{}_1+ b_{22}x^2{}_2 \quad \text{……..(2)}$$

where y^1 and y^2 are the predicted responses of PG and PL activities respectively and b_0 is the coefficient at off-set terms

The analysis of variance (ANOVA) was performed by using Design Expert, version 8.0, and statistical software (Stat-Ease Inc. Minneapolis, MN,USA) in order to validate the responses by graphical and numerical analysis.

3.3 Results and Discussion

Pectinase productions by *Bacillus* spp for industrial purposes is becoming an important area of investigation because pectinases of bacterial origin often have inherent advantages such as greater stability and optimum activity at higher pH and temperature ranges. Bacterial pectinases, unlike fungal ones, are considered better in depolymerising pectin to improve the absorbency and whiteness of textile materials without affecting the cellulose backbone thus avoiding fiber damage (Ahlawat et al., 2009). Agrawal (2005) earlier reported that an enzymatic scouring process with alkaline pectinases produced by bacteria achieve better hydrophilicity. Maximizing enzymes production by optimization of the microbial cultural

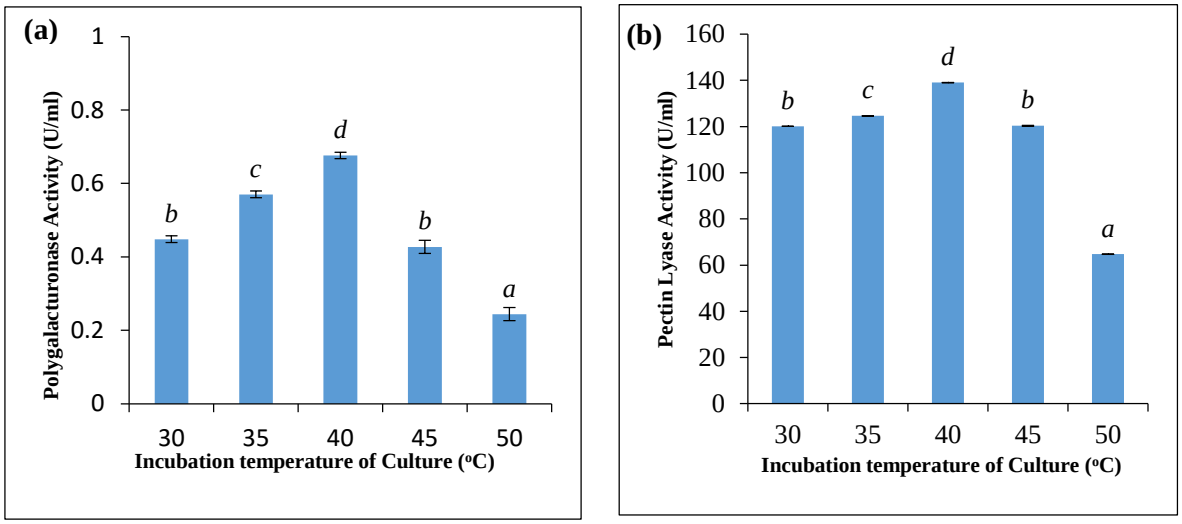
parameters is of utmost importance in industrial enzymology (Ahlawat et al., 2009). In the present study, production of PG and PL by *B.subtilis* 1E7 was optimized using the response surface methodology.

3.3.1 Effects of culture conditions on pectinase production

3.3.1.1. Cultural temperature

In this result, Figure 6(a) shows that PG productivity increased to an optimal value of 0.7U/ml at a 40^{o}C cultural incubation temperature while there was significantly ($p<0.05$) decreased production as the temperature increased to 45^{o}C and 50^{o}C. The PL production as shown in Figure 6(b) also exhibited increased production to maximum of 139 U/ml at 40^{o}C and decreased to 65 U/ml at a 50^{o}C incubation temperature. The incubation temperature observed for maximum pectinase production was similar to those previously reported. Bayoumi et al. (2008) observed 37^{0}C as the optimal temperature for pectinase production by some strains of *B. firmus* isolated from decaying potato peels. In a similar report, Kashyap et al. (2003) recorded an optimum yield of 4600U/ml of pectinase at 37^{0}C in solid state fermentation with a *Bacillus* sp DT7 (a soil isolate). In another report, Ahlawalat et al. (2009) observed optimum production of pectinase (151U/ml) by *B. subtilis* SS at 37^{0}C while other temperatures, lower and higher, were not suitable for growth and enzyme secretion. Farooqui (2012) observed an exception in a strain of *B. subtilis* from soil which maximized pectinase production at 35^{0}C. These are mesophilic bacteria which suggest that they grow best at these temperature ranges (35^{0}- 40^{0}C) which are the temperatures at which the metabolic activities of the bacterial cells were optimum. More importantly, the *B. subtilis* 1E7 in this work was isolated from the rabbit body where temperature was about 39 ±1^{0}C (temperature monitor data not presented), lower and higher temperatures would cause decrease in growth and metabolic activities of the bacteria. Proteins generally suffer a degree of denaturation at

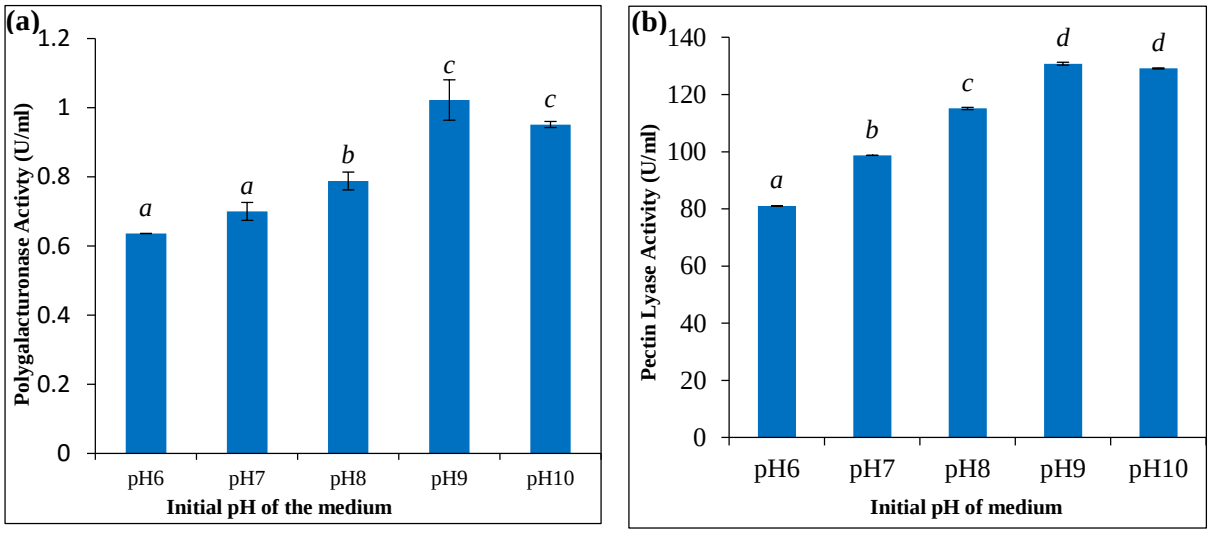
temperatures above 40^0C. Those may be responsible for the decreased activity at temperatures higher than 40^0C.



Letters *a-d* indicate significant difference ($p< 0.05$) in the activity of pectinases produced by *B. subtilis* in culture extract

Figure 6. Effect of incubation temperature on pectinases production by *B. subtilis* IE7. (a) PG (b) PL

3.3.1.2. Initial cultural pH



Letters *a-d* indicate significant difference ($p< 0.05$) in the activity of pectinases produced by *B. subtilis* in culture extract

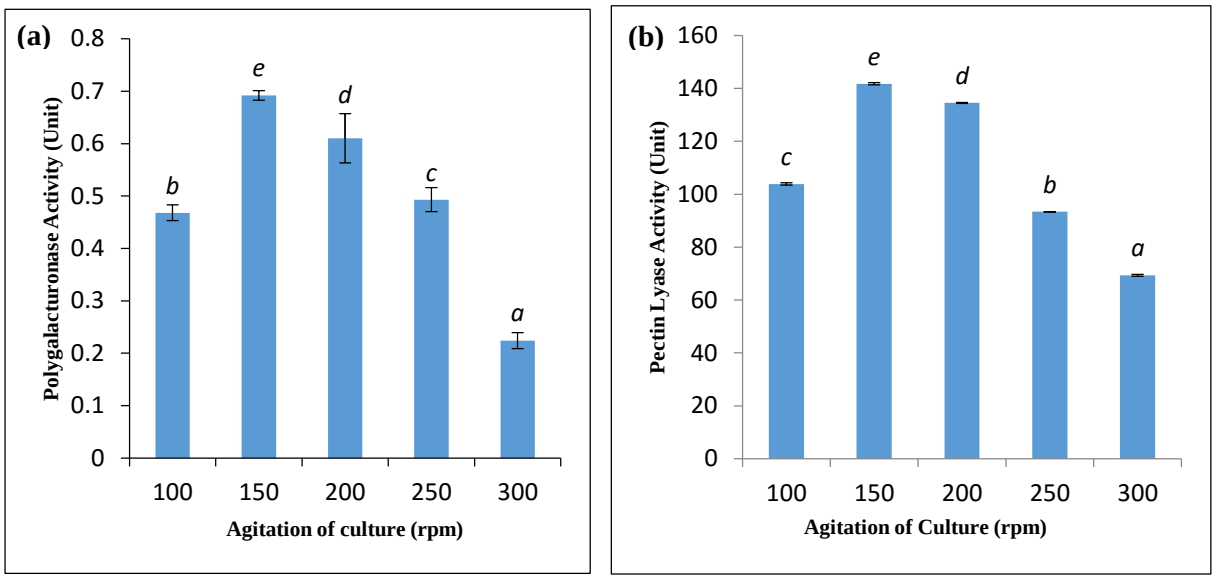
Figure 7. Effect of initial pH on pectinase production by *B. subtilis* IE7. (a) PG (b) PL

The production of PG increased to a maximum level of 1U/ml at pH9 with no significant increase ($p>0.05$) at pH10 (Figure 7(a)). The same trend was observed for PL production (Figure 7(b)) with maximum production of 131U/ml at an initial cultural pH of 9 and 10. The caeco-colic bacteria in rabbits pass through the whole gut in the process of caecotrophy hence the bacteria must have developed the ability to adjust to the pH ranges at different regions of the gut. Growing it *in vitro* made it to exhibit its alkalophilic trait but was still able to produce about 60% pectinase at pH 6 compared to pH9 (Figure 7(a) and (b)). Apoorvi and Vuppu, (2012) stated that most pectinase producing bacterial species have optimum pH of 7–10. Particularly, Ahlawat et al. (2009) reported optimum pectinase production by *B. subtilis* SS at pH 9.5. The present result is consistent with this observation and the *B. subtilis* 1E7 is an alkalophilic strain. Kashyap et al (2000) recorded an optimum pectin lyase production (15.7U/ml) by a strain of *Bacillus* sp DT7, a soil isolate, at a neutral pH (7.2). There are some exceptions in a number of pectinase producing bacteria. Jayani et al. (2010) reported optimum pectinase production by *B. sphaericus* (MTCC 7542) at pH 6.8 while Farooqi (2012) observed optimum production of pectinase in a culture of *B. subtilis* at pH 6.5. The bacterial strains in the two separate reports were isolated from soil. Literature (Kashyap et al., 2001) elucidated the use of alkaline pectinases in the pre-treatment of pectic wastewater and paper making, that these pectinases are most produced by bacteria especially, *Bacillus* spp. Wang et al. (2007) also reported details of the production of alkaline pectinase by *B. subtilis* WSH B04-02 and stressed its inherent ability to better degrade pectin in bio-scouring of cotton fabrics giving it greater application potential in textile processing.

3.3.1.3. Cultural agitation

The effects of agitation on PG production is as shown in Figure 8(a). Production increased as cultural agitation increased from 100 rpm and achieved maximum production (0.7U/ml) at the 150 rpm speed. In a similar manner, Figure 8(b) shows that PL production increased to a

maximum of 144U/ml at 150 rpm. There was significant ($p<0.05$) decrease in PL production at the agitation speed lower and higher than 150 rpm. This result is consistent with the report of Darah et al. (2013). Literature (Kao et al., 2006; Potumarthi et al., 2007; Darah et al., 2013) postulated that at the optimum agitation, cultural aeration assist bacterial pectinase production thereby leading to rapid metabolism.



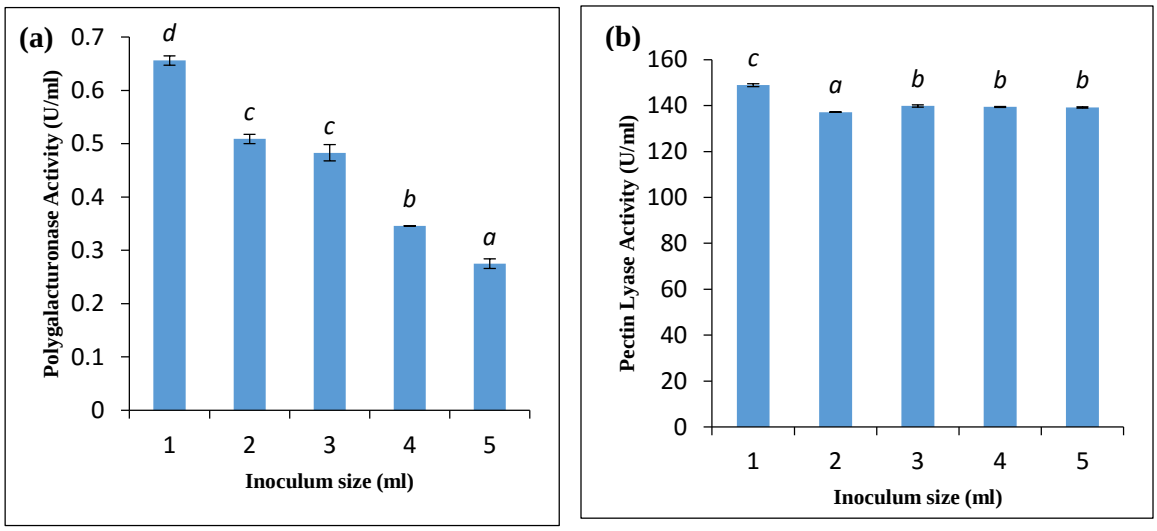
Letters *a-d* indicate significant difference ($p< 0.05$) in the activity of pectinases produced by *B. subtilis* in culture extract.

Figure 8. Effect of culture agitation on pectinase production by *B.subtilis* IE7 (a) PG, (b) PL

Darah et al. (2013) further stated that at agitations higher than optimum (200-300 rpm), decline in pectinase production could be ascribed to higher shear stress when the bacterial cells are involved in collisions that could damage them. It may as well generate heat, causing adverse conditions that may make some of the cells to remain dormant and stop production. A decline in pectinase production could also be due to oxidation of the enzyme through frothing.

3.3.2. Inoculum Size

The *B. subtilis* 1E7 inoculum standardised as OD~0.7 at 600nm wavelength gave a bacterial viable count of about 21.7±2 x 10^8cfu/ml. Figure 9(a) shows that 1ml inoculum per 20ml culture produced the highest PG activity of 0.66U/ml which decreased as inoculum size increased from 2ml to 5ml. The PL production (Figure 9(b)) also exhibited optimum production of 149U/ml with 1mL inoculum and yielded reduced production at higher inoculum volumes.



Letters *a-d* indicate significant difference ($p < 0.05$) in the activity of pectinases produced by *B. subtilis* in culture extract

Figure 9. Effect of inoculum size on pectinases production by *B. subtilis* IE7. (a) PG (b) PL.

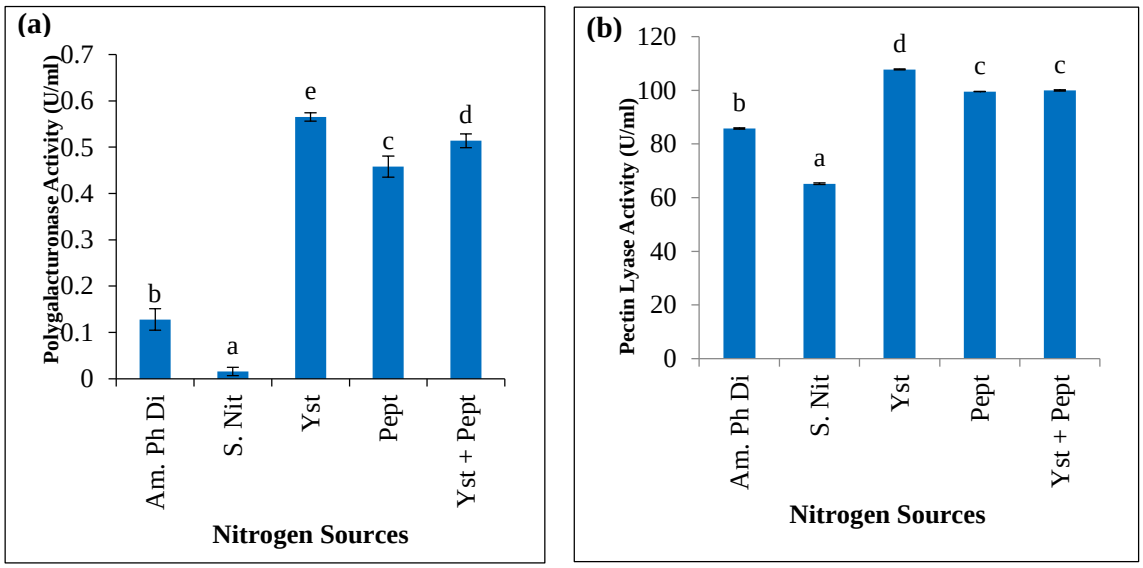
This observation was consistent with literature, particularly, Bayoumi et al. (2008) reported optimum pectinase production with 1ml bacterial (*B. firmus)* inoculum in 25ml (about 4%, v/v) medium. The inoculum they used contained 30 x 10^{15}CFU/ml. In another report, Darah et al. (2013) used 3% (v/v) inoculum containing 5.4 x 10^7 CFU/ml to achieve optimum pectinase production by *E. aerogenes* NB02 whereas Ahlawat et al. (2009) attained optimum pectinase production at 2.5% inoculum size of 18hour old *B. subtilis* SS. Actually, the effect of inoculum size on enzyme production is dependent on the bacterial cells concentration

/density and growth phase. In other words, cells at log phase will be more productive than those in stationary phase. After attaining the optimum production, higher inoculum levels decrease enzyme production and this could be due to increase in cells population that will compete for the decreasing nutrients in the growth medium. At optimum production, the available nutrients adequately support the inoculum size for highest productivity.

3.3.3. Nitrogen source

The nitrogen sources used in this experiment comprised of inorganic and organic sources that have been used and observed to have notable influence in previous studies (Kashyap et al., 2000; Bayoumi et al., 2008; Apoorvi and Vuppu, 2012; Darah et al., 2013). A combination of yeast extract:peptone (0.75%) was also tested as used by Ahlawat et al. (2009). In Figure 10(a) it can be seen that yeast extract resulted in the production of the highest activity of PG (0.6U/ml) followed by the mixture of yeast extract+peptone (0.75%) and peptone. Both ammonium phosphate dibasic and sodium nitrate produced very low PG of 0.12U/ml and 0.01U/ml respectively. Similarly, in Figure 10(b) it can be seen that PL production (108U/ml) was highest in yeast extract while peptone and combined yeast extract+peptone (0.75%) mixture attained the same PL production. Ammonium phosphate diabasic produced PL of 82U/ml while sodium nitrate produced 61U/ml which was the least. The organic nitrogen (yeast extract and peptone) sources were found to enhance pectinase production while the in-organic (ammonium phosphate diabasic and sodium nitrate) sources produced less. The present result is consistent with previous reports of Kashyap et al. (2000), which observed maximum pectinase yield by *Bacillus* sp DT7 particularly when used along with pectin in a medium. Darah et al. (2013) in their report observed that yeast extract best encouraged PG production by *E. aerogenes* NB02 followed by ammonium sulphate and peptone while sodium nitrate inhibited its production. Apoorvi and Vuppu (2012), in their work, described yeast extract as producing the highest pectinase activity among a range of nitrogen sources

while literature (Horikoshi, 1972; Kashyap et al., 2003; Ahlawat et al., 2009) generally concluded that yeast extract is the most widely used nitrogen source to enhance pectinase production by *Bacillus* spp. Yeast extract in a bacterial medium ordinarily will promote growth and productivity.



Letters *a-e* indicate significant difference ($p<0.05$) in the activity of pectinases produced by *B. subtilis* in culture extract.
Am. Ph Di= Ammonium phosphate dibasic; S. Nit= Sodium Nitrate; Yst Ex= Yeast Extract; Pept= Peptone

Figure 10. Effect of nitrogen sources on pectinases production by *B. subtilis* IE7 (a) PG (b) PL

3.3.4. Experimental design and optimisation of pectinase production using RSM

The main production parameters often evaluated for the optimisation of pectinase production are temperature, pH and cultural agitation (Bayoumi et al, 2008). These factors have been described as important and that they all exert great influence on pectinase productions (Malvessi and Silveira, 2004). In this context, the initial pH of the production medium, temperature and agitation of the culture were focused on in the present work examining optimisation of the pectinase production by RSM. From the above results, the pH of optimum

production was between 9 and 10 with no significant difference ($p>0.05$) (Figure 7(a) and (b)) which is similar to the work of Ahlawat et al. (2009) who reported an optimum pH of 9.5. The present report (Figure 6 and Figure 8) also show the temperature and agitation speed for optimum pectinase production was 40^{o}C and 150 rpm respectively, showing significantly decreased productions on either side of the 40^{o}C and 150rpm conditions. These two variables were identified as having greatest influence (showing a normal distribution) on the responses and were thus selected as variable factors for the optimization of pectinase production by the RSM.

Table 5: Analysis of variance (ANOVA) for the quadratic model of PG activity in the CCD

Source of variations	Sum of squares	df	Mean squares	F-value	P-value
Model	0.70	5	0.14	16.44	0.0010
X_1	0.030	1	0.030	3.56	0.1011
X_2	0.026	1	0.026	3.05	0.1244
X_1 X_2	2.890E-004	1	2.890E-004	0.034	0.8589
X_1^2	0.30	1	0.30	35.33	0.0006
X_2^2	0.11	1	0.11	12.96	0.008
Residual	0.059	7	8.494E-003		
Lack of fit	0.059	3	0.020		
Pure error	0.000	4	0.000		
Total	0.76	12			
R^2=0.9215					
Adjusted R^2=0.8655					

Table 6: Analysis of variance (ANOVA) for the quadratic model of PL activity in the CCD

Source of variations	Sum of squares	df	Mean squares	F-value	P-value
Model	9115.16	5	1823.03	53.82	< 0.0001
X_1	2625.46	1	2625.46	77.51	< 0.0001
X_2	1341.61	1	1341.61	39.61	0.0004
$X_1 X_2$	1311.53	1	1311.53	38.72	0.0004
X_1^2	490.24	1	490.24	14.47	0.0067
X_2^2	2029.56	1	2029.56	59.92	0.0001
Residual	237.11	7	33.87		
Lack of fit	237.11	3	79.04		
Pure error	0.000	4	0.000		
Total	9352.27	12			
R^2=0.9746					
Adjusted R^2=0.9565					

In the experimental design of the CCD, a three level combinations of the independent variables are as shown in Table 3 which led to 13 experimental runs. The experiments were performed as prescribed and the PG and PL activity (responses) were as shown in Table 4. The analysis of variance (ANOVA) in Table 5 and Table 6 indicated that the model is highly significant (p<0.05) with respect to PG and PL production responses. The R^2 value which is the measure of goodness of fit of the models were 0.9125 and 0.9746 (Table 5; Table 6) for PG and PL responses, respectively. This indicated that 91.25% and 97.46% of the total variation is explained by the model in PG and PL productions, respectively. In Table 5, the model shows that the linear effects (X_1; X_2) on PG production is non-significant (p>0.05) but

the quadratic effect of each of the cultural temperature and agitation (X_1^2; X_2^2) contributed significantly (p<0.05) to the model. Conversely in Table 6 the linear effect of both variables (X_1; X_2) were highly significant (p<0.05) with respect to PL production as well. The model therefore shows that either culture temperature and agitation or a combination of both conditions can be manipulated in order to optimize PL production (Moyo et al., 2003). The response surface plots showing the effect of the cultural agitation and temperature on PG and PL productions were generated and are shown in Figure 11 (a) and (b). The curvatures in both graphs were convex in nature which indicated that the optimum determined in both cases (PG and PL) were well defined. The elliptical nature of the contour plot shows that there were prominent interactions among the variable factors. The more circular, the less prominent the interactions among the variables. The contour plots in Figure 11(b) is more elliptical than in Figure 11(a) which means the quadratic interaction of the variable factors are more prominent in PL production compared to PG.

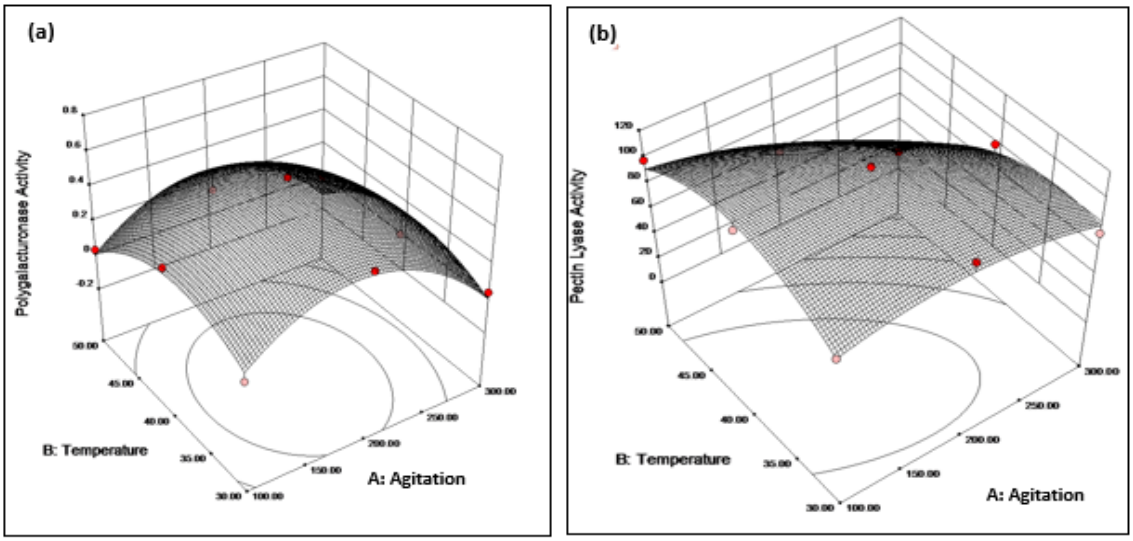


Figure 11. Response surface plot showing the quadratic effects of cultural agitation (rpm) and temperature (°C) on pectinase production by *B. subtilis* 1E7 strain isolated from the caeco-colic region of rabbit hind gut. (a) PG activity (b) PL activity.

The quadratic models are as indicated in the polynomial equations (3) and (4) representing responses y_1 and y_2 for PG and PL respectively. In this case, y_1 and y_2 were predicted pectinases production responses (PG and PL) while X_1 and X_2 represented cultural agitation and temperature terms respectively. Derived from equations (1) and (2), the model equations in terms of the coded terms, are as follows:

$$y_1 = 0.55 - 0.071X_1 – 0.66X_2 + 0.0085\ X_1\ X_2 – 0.33X_1^2 – 0.20X_2^2 \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

$$y_2 = 107.95 – 20.92X_1 – 14.95X_2 – 18.11X_1X_2 – 13.32\ X_1^2 – 27.11X_2^2 \ldots\ldots\ldots\ldots\ldots..(4)$$

(y_1 and y_2 are the predicted activity for PG and PL respectively). The models predicted an optimum PG production of 0.56U/ml and PL of 111.49U/ml under culture temperatures of 38.33^{o}C and 189.05 rpm agitation (Appendix V) with the PYB medium at an initial pH value of 10. To confirm these predictions, further pectinase production experiments were carried out under the predicted optimum conditions (38^{0}C; 190 rpm). In these experiments PG and PL activity were found to be 0.54±0.01U/ml and 110.64±0.04U/ml, respectively. These results indicated that the predicted model and experimental results were in agreement. In comparison, PG production is quite lower than PL (Moyo et al., 2003) but the PG activity 0.54U/ml produced in this study is higher than 0.30IU/ml observed in the crude filtrate of an *Aspergillus niger* strain by Khan et al. (2012). It is also notable that *B. subtilis* 1E7 in the present study also displays higher PL production (110.64 U/ml) than (53U/ml) from *A. flavus* strain isolated from soil by Yadav et al. (2008). Pilnik and Rombout (1981) reported that pectin lyase produced by fungi was not yet found in bacteria but subsequently Kashyap et al. (2000) was able to obtain purified pectin lyase from *Bacillus* sp DT7 which, after optimisation, had an activity of 53U/ml. It is interesting to note that the *B. subtilis* 1E7 used in our study produced an almost double PL activity of 110.64U/ml even in its crude state. Alana et al. (1990) reported that *Penicillium* spp. is the most important example of pectin

lyase producer in terms of quality and quantity. However, in their study, the highest PL activity (0.62U/ml) observed in *P. italium* was quite lower compared to the PL activity 110.64U/ml reported in the present study. These results confirm the value of our work and the potential of this strain for commercial production of alkaliphilic pectinases by the selected *B. subtilis* 1E7 strain under study.

3.4 Conclusion

In this study, attempts has been made to demonstrate the preliminary use of the conventional method to justify the parameters selected for the statistically designed response surface methodology to optimize the pectinase production by a strain of *B. subtilis* 1E7. These have enabled us to accurately determine the effects of certain nutritional and cultural conditions for the optimum production of both polygalacturonase and pectin lyase by *B. subtilis* 1E7 isolated from the ceaco-colic region of rabbit gut. The present results have shown that this bacterium previously identified as the best producer of pectinases from rabbit gut has optimised PG and PL production (0.54U/ml and 110.64U/ml respectively) at 38^{o}C, with 190rpm rotation and a pH of 10. This level of pectinase production especially the PL is considered to be of high activity compared to previous known producers in literature which makes this strain of *B. subtilis* 1E7 a potential alternative in production of commercial pectinases (PG and PL) for industrial uses.

CHAPTER 4

Purification and characterization of pectinases produced by *B. subtilis* 1E7 from the caeco-colic region of rabbit hind gut

The work presented in this chapter represents the following manuscript in preparation for submission to an appropriate journal

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of chapter 2, the nature and extent of my contributions to the work was the followings:

Nature of contribution	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Siow Lee Fong	SLF was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and the review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate' Signature	[redacted]	**Date 18/12/2015**

Main Supervisor' Signature	[redacted]	**Date 18/12/2015**

4.1 Introduction

The use of hydrolytic enzymes in industrial processes is rapidly replacing traditional methods such as the use of hot caustic soda to desize during textile processes which has been replaced with the use of pectinases and amylases (Hoondal et al., 2002). The use of biological catalysts have been found to be more efficient and eco-friendly. Macerating enzymes such as pectinases, cellulases, hemicellulases and other carbohydrases are widely produced and used (Hamdy, 2006). Pectinases are considered one of the most active hydrolytic enzymes and are extensively used in the textile, fruits and vegetable industries (Hamdy, 2005). Pectinases are a group of fibrolytic enzymes consisting of the esterases, hydrolases and lyases. Esterases demethylate pectin molecules while hydrolases depolymerize unesterified pectic acids. The lyases are the only pectinase enzymes capable of breaking down pectin with a high degree of esterification and can work on both methylated and un-methylated pectic substances (Yadav et al., 2008). For this reason this group of pectinases are important for use in industrial processes.

Pectinases are produced by many microbes such as bacteria, fungi and yeasts (Kashyap et al., 2000). Fungi are well known for the production of acidic pectinases whereas alkaline pectinases are produced by alkaliphilic bacteria and commonly *Bacillus* spp. (Kashyap et al., 2003). Currently virtually all commercial pectinases are produced by the moulds, *Aspergillus, Penicillium* and *Rhizopus* spp. (Jayani et al., 2010) while bacterial sources of pectinases are rarely available. Pectinases produced by species of bacteria, as compared to moulds, are more active at high pH ranges (Soares et al., 1999). Bacterial pectinases are therefore sought after for certain industrial processes such as bio-scouring in the textile industry and pectin laden waste water treatment (Wang et al. 2007). In order to bridge this gap, *B. subtilis* 1E7 isolated from rabbit gut was used to produce, partially purify pectinolytic enzyme and to characterize the PG and PL activity of the enzyme and its stability.

4.2 Materials and Methods

4.2.1 Source and maintenance of bacterial culture

The *B. subtilis* 1E7 used in this study was isolated and identified (accession number KT 713393) in chapter two and pectinase production from it optimized in chapter three. Pure cultures of the bacterium was preserved in 20% (v/v) glycerol and kept at -80^{0}C (Toit et al., 1998). When needed the culture was revived on nutrient agar and maintained with routine streaking as a working culture.

4.2.2 Inoculum preparation

The inoculum of the *B. subtilis* 1E7 was prepared using fresh stock seeded into a nutrient broth (Oxoid, England) and incubated 24 hours in a 37^{0}C rotary shaker incubator at 150 rpm. The culture suspension was standardized by adjusting the OD to 0.7 at 600nm wavelength in a Cole Parmer 1100RS Spectrophotometer.

4.2.3 Inoculation and culture conditions for pectinase production

The pectinase was produced by submerged fermentation in yeast pectin broth (YPB) medium consisting of KCl 0.5g, $MgS0_4.7H_2O$ 0.5g, K_2HPO_4 1g, $FeS0_4.7H_2O$ 0.01g, yeast extract (Difco, England) 2g, citrus pectin (Sigma-Aldrich, Denmark) 10g and 1000ml distilled water (Janani et al., 2011; Reddy et al., 2012). The medium was adjusted to initial pH of 10 with 0.1M NaOH (Ahlawat et al., 2009). The YPB with an initial pH of 10 was divided into five aliquots of 200ml each in 1000ml Schott bottles. Each aliquot was inoculated with 10ml standardized (OD ~ 0.7 at 600nm) overnight grown bacterial cells stock. The culture was grown for 48 hours in a shaker incubator at 38^{0}C and 190 rpm agitation based on the earlier (Chapter 3) predicted culture conditions for optimum pectinase production.

4.2.4 Preparation of extracellular crude pectinases

After 48 hours, incubation the culture was centrifuged at 10,000*xg* for 10 minutes at 4^0C. The supernatant was regarded as the crude pectinase which was used in the activity assay and further tests.

4.2.5 Assays for pectinases and proteins

The activity of the crude pectinases was established by reaction with appropriate solutions of citrus pectin (Sigma Aldrich) substrate and quantification of the reducing groups liberated in the process. The polygalacturonase (PG) pectinolytic activity was determined by measuring the reducing group produced using the dinitrosalicylic acid (DNS) reagent according to the method previously described by Soares et al., (1999). The chromogenic reaction of the pectin breakdown product with 0.04M thiobarbituric acid (TBA) and 0.2M dilute hydrochloric acid was the basis for PL activity assay as previously described by Nedjma et al., (2001) and Hayrunnisa et al., (2010). In both cases of activity assays galacturonic acid monohydrate was used as the standard. One unit of pectinase activity was expressed as the milligrams of galacturonic acid released per milliliter of the enzyme per minute under specific assay conditions (Jayani et al., 2010). Quantification of protein content was by the method of Bradford (1976), using bovine serum albumin (BSA) as standard.

4.2.6 Pectinase purification

A liter liquid culture of *B. subtilis* 1E7 in YPB medium yielded 1000ml crude pectinase, which was taken through a three-step purification processes. The purification steps involved the ammonium sulphate fractionation and two steps of column chromatography using AKTAprime plus (GE Healthcare, JCSoMHS-R-00472, Sweden.) machine. Detailed description of the purification processes are given below.

4.2.6.1 Ammonium Sulphate

The one liter cell free crude pectinase was saturated with crystals of ammonium sulphate (AS) to two cut offs (0-40%, 40%-90% saturation) in a cold room at 4^0C. Appropriate quantities of the ammonium sulphate crystals were weighed based on the volume of the crude pectinase. The quantity of the ammonium sulphate was calculated from the standard table (Rosenberg, 1996; Appendix VI). The ammonium sulphate crystals were slowly added with gentle stirring until it dissolved completely after which the cloudy suspension was allowed to settle overnight in cold room at 4^0C. At each stage of cut off, the precipitates were pelleted by centrifugation (15,000*x*g, 15 minutes in 4^0C) but later re-suspended in a minimum volume of 10mM Tris-HCl, pH 7.5 buffer (Kashyap et al., *2003*). The pooled protein concentrate was dialysed overnight (desalting) with a continuous flow of the same buffer in cold room (4^0C). The dialysed protein suspension was tested for pectinolysis qualitatively on pectin agar plates.

4.2.6.2 Gel Filtration chromatography

A dilute (10^{-1}) solution of the dialysed sample was used for gel filtration chromatography with a Sephadex G-50 resin packed into a glass column (1.6cm x 65cm), pre equilibrated with 10mM Tris-HCl, pH 7.5 buffer using Aktaprime plus (GE health) machine set at flow rate of 0.4ml per minute and eluting protein collections of 2ml per fraction. Each fraction was tested for pectinolysis on pectin plates and positive fractions were pooled together, freeze dried and preserved for further processes.

4.2.6.3 Ion exchange chromatography

Re-dissolved pectinase positive samples were loaded onto a DEAE-cellulose anion-exchanger which was packed into a glass column (1.5cm x 40cm) and pre-equilibrated with 10mM Tris-HCl pH 7.5 buffer. The sample was later eluted at 0.2ml per minute flow rate

with 1M NaCl prepared in the same buffer and 2ml fractions were collected (Kashyap et al., 2000; Yadav et al., 2008) using an AKTAprime plus machine (JCSoMHS-R-00472, Sweden). The peaks were tested for pectinolysis on pectin agar and the pectinase-positive fractions were pooled, freeze dried and preserved for further process. The dried sample was reconstituted in deionized water and was desalted using Sephadex C-25 packed into a glass column (1cm x 20cm). The sample solution was loaded onto gel bed pre-equilibrated with de-ionised water and eluted using the same de-ionised water at flow rate of 0.2ml/min and fractions of 2ml were collected. The pectinase–positive fractions were pooled and saved for further analysis. At each stage of purification step, pooled fractions were analysed for PG and PL activity as well as protein determination (Bradford, 1976), using bovine serum albumin (BSA) as standard.

4.2.7 Characterisation of partially purified pectinases

Partially purified pectinases were characterized by examining the effects of physico-chemical factors on its activity and stability.

4.2.7.1 Effect of temperature on activity of partially purified pectinases

Different incubation temperatures of the reaction mixture ranging from 30^0C – 100^0C at intervals of 10^0C were used for pectin hydrolysis with the incubation time fixed based on the protocol of assay (10 minutes for PG and 3 hours for PL). Enzyme activity was then quantified for each temperature of incubation.

4.2.7.2 Effect of pH on the activity of partially purified pectinase

The test for pH dependence of the pectinases was determined by preparing the reaction mixture with buffers of different pH's from pH 6 to pH 11. The pH 4-6 were prepared using 0.02M citrate-phosphate buffer while 0.2M Tris-HCl buffer was used for pH 8 and 9. For pH

10 and 11, 1M sodium bicarbonate-sodium hydroxide buffer was used. All buffers were prepared using standard conditions and methods. The reaction mixture was then incubated and assayed for activity using the protocols described earlier. Pectinase activity was then quantified for each pH of the reaction mixture.

4.2.7.3 Effect of substrate concentration on the activity of the partially purified pectinases

Effect of different concentrations of the citrus pectin substrate on the pectinase activity was studied by preparing the reaction mixture with a gradient of citrus pectin concentration from 4mg/ml to 16mg/ml (intervals of 2) for PG and 4mg/ml to 24mg/ml (intervals of 4) for PL. Activity assay was carried out for the reaction mixtures. The outcome was then used to determine the K_m and V_{max} of the partially purified pectinases.

4.2.7.4 Effect of heat on stability of the partially purified pectinases

The influence of heat on the stability of the enzyme was determined by incubating the aliquots of the pectinase sample at various temperatures for 40^0C – 100^0C (intervals of 10) for one hour in a Thermo-Block TDB-120-Biosan. The pectinases activity assay was carried out after exposure and the activity was plotted against the variable parameters.

4.2.7.5 Effect of pH on stability of partially purified pectinases

Enzyme stability to acid was tested by storing the enzyme in the buffers of different pHs (6-12) as described (4.2.7.2) above for 24 hours at 4^0C to reduce degradation of enzymes while sustaining its biological activity. Activity assay of the enzymes were determined after exposure and the units of activity plotted against the parameters.

4.2.7.6 Effect of metal ions and chemicals on stability of the partially purified pectinases

The partially purified pectinases were also tested for their response to selected metal ions (Ca^{2+}, Mg^{2+}, Hg^{2+}, Fe^{3+}, Co^{2+}) and chemicals (EDTA, Glycine, Mercapto-ethanol). Aqueous solutions of all the metal ions and chemicals were prepared and used at 1mM concentrations. All the metal ions and chemicals were selected based on previous literature reports in which each of them has been identified as either an inhibitor or enhancer of pectinolytic enzyme activities. Freeze dried enzyme was re-dissolved in the 1mM aqueous solutions of the various metal ions and the chemicals, which were incubated for 24hours at 4^0C after which the activity of the enzymes was assayed with a positive control (enzyme was dissolved in ddH_2O). The result was expressed as relative activity (%U) compared to the positive control.

4.2.8 Liquid Chromatography-Mass spectroscopy (LC-MS) analysis of the partially purified pectinases

Liquid chromatography-mass spectrometry (LC-MS) analysis of the pectinase positive enzyme sample (desalted) was carried out. The sample was treated with tryptic digestion of the sample solution and the product was analyzed using Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass LC/MS (Quadrupole-time-of-flight, QTOF) machine. The LC parameters comprised column of Agilent Large capacity Chip, 300Å, C18, 160µl enrichment column and 75µm x 150mm analytical column (P/N: G4240-62010), flow rate 4µl/min from Agilent 1200 series capillary pump and 0.5µl/min from the nano pump. Solvents used were A, 0.1% formic acid aqueous solution and B, 90% Acetonitrile in water with 0.1% formic acid. Auto sampler temperature was 8^0C while injection volume was 1µl. The MS analysis parameter comprised of positive ion polarity running with fragmentor voltage of 175V, gas temperature of 325^0C and drying gas flow rate

at 5 l/min. Collision energy was 3.7V/100Da with reference masses of 299 and 12212 positive polarity. Data analysis was processed with PEAKS 7 (Bioinformatics solution, Waterloo, Canada; 150901-150828-00059-DP) proteomics software. The data base search NCBInr.Bacillus.Aug2015.4693818.fasta (Q-TOF, CID fragmentation, trypsin enzyme and carbamidomethylation as fixed modification) was used.

4.3 Results and Discussion

B. subtilis 1E7 was previously determined to be the most potent producer of pectinase enzyme from a number of bacteria isolated from the caeco-colic region of rabbit hind gut (Chapter 2). A liter of crude pectinase produced based on previously determined optimum conditions had a total protein content of 163mg. The PG specific activity was 3.28U/mg while the PL was 613U/mg. This translates to 535U and 99,930U total activity for PG and PL, respectively (Table 7 and Table 8). The difference in the activity of these two enzymes is a confirmation of the important role PL plays, as compared with the PG, in the pectinolytic functions in the rabbit gut. Although pectinase production by alkaliphilic bacteria has not been commonly reported (Kashyap et al., 2000), the PG activity of 0.54U/ml reported in the present is higher than 0.4U/ml reported for *Bacillus* sp B1.3 and 0.3U/ml for *Bacillus* sp P4.3 in submerged fermentation by Soares et al. (1999). The specific activity of the PL (613.07U/mg; Table 7) in this report is higher than 0.32U/mg reported in *A. flavus* by Yadav et al., (2008) and also the 102.94U/mg reported in *R. oryzae* by Hamdy (2005). The lyases are important among the pectinase group because of the ability to degrade pectin having high esterification (Soriano et al., 2006). Pectin lyase is preferred in the fruit juice industry because it attacks pectin molecules without affecting the ester groups which preserves the

Table 7: Purification table of PG in the crude pectinase

Steps	Volume	Total Activity	Total Protein	Specific Activity	Yield	Purification Factor
	(mL)	(U)	(mg)	(U/mg)	(%)	
CP (1)	1000	535	163	3.28	100	1
AS (2)	48	422.4	112.8	3.74	78.95	1.14
GF (3)	900	229.5	27	8.5	42.90	2.59
IEC (4)	324	171.4	6.77	25.32	32.04	7.72

Steps: (1) Crude Pectinase; (2) Ammonium Sulphate fractionation; (3) Gel Filtration; (4) Ion Exchange Chromatography

Table 8: Purification table of PL in the crude pectinase

Steps	Volume	Total Activity	Total Protein	Specific Activity	Yield	Purification Factor
	(mL)	(U)	(mg)	(U/mg)	(%)	
CP (1)	1000	99930	163	613.07	100	1
AS (2)	48	75820.8	112.8	672.17	75.87	1.1
GF (3)	900	38826	27	1438	38.85	2.35
IEC (4)	324	26577.72	6.77	3925.81	26.6	6.40

Steps: (1) Crude Pectinase; (2) Ammonium Sulphate fractionation; (3) Gel Filtration; (4) Ion Exchange Chromatography

inherent aroma of the fruits in juices and more importantly, methanol is not formed (Apoorvi and Vuppu, 2012). Acidic pectinases from fungal sources, particularly *A. niger*, are often used in fruit juice and wine making industries. However there are certain conditions associated with industrial processes that require alkaline pectinases. Alkaline pectinases are particularly useful in the degumming and retting of fibers as well as in pectic waste water pre-treatment. It has been reported by Kashyap, et al. (2001) that alkaline pectinases are obtainable largely from bacteria and especially *Bacillus* spp. Kumar and Sharma (2012) also reported alkaline pectinase production with optimized activity of 13.96U/ml by a *Cocci* sp. Acidic pectinases have been well described but few alkaliphiles producing alkaline pectinases have been studied (Kashyap et al., 2000).

4.3.1 Partial purification of pectinases

The purification of enzyme proteins is essential in enzymology because it affords detailed understanding of the potential of such enzymes. The pectinase produced in the present study was taken through a 3-step purification processes and the purified enzyme was evaluated for catalytic performance and stability. Table 7 and Table 8 show the summary of the results of the enzyme purifications. All precipitates obtained from the two cut offs in AS fractionation showed appreciable pectinolysis (halo formation) on pectin agar plate (Appendix VII). The final volume of the precipitate was 48ml. The AS precipitation step is an effective method of separating the proteins and a volume reducing step which decreased a large volume (1000ml) of extract into a suitable protein concentrate (48ml total volume) for subsequent analysis. The protein precipitation is as a result of using the AS to achieve dehydration in the micro-environment of the protein molecules. The sulphate ions (SO_4^{2-}) in solution attract a large number of water molecules, thereby reducing the number of water molecules available to interact with the hydrophilic region of the protein molecules. At a particular concentration of the AS (cut off), the water molecules available to the protein species in the solution are so

low that the protein molecules precipitate overnight at 4^0C. The total protein content of the sample was 112.8 mg and the specific activity was 3.74U/mg for PG with a 78.95% yield while it attained 1.14 folds purification (Table 7). The specific activity for PL in the AS precipitate was 672.17U/mg representing 75.87% yield and a 1.1 fold purification factor (Table 8).

Figure 12 shows the proteins peaks A, B, C, D, as observed during gel filtration of the pectinase. Peak A (earliest peak) was pectinase-positive while peaks B, C, and D were completely negative (Appendix VIII). The present result illustrated in Figure 12 shows a well separated peak A as the earliest fractions among the other protein peaks (B, C D) which sufficiently indicated that the pectinase positive protein in peak A has the highest molecular weight protein. This observation is consistent with Kashyap et al. (2000) who reported a high molecular weight pectinase in *Bacillus* sp. DT7. The pooled fractions within peak A showed the total protein content of 27mg. The PG specific activity was 8.5U/mg showing 42.9%

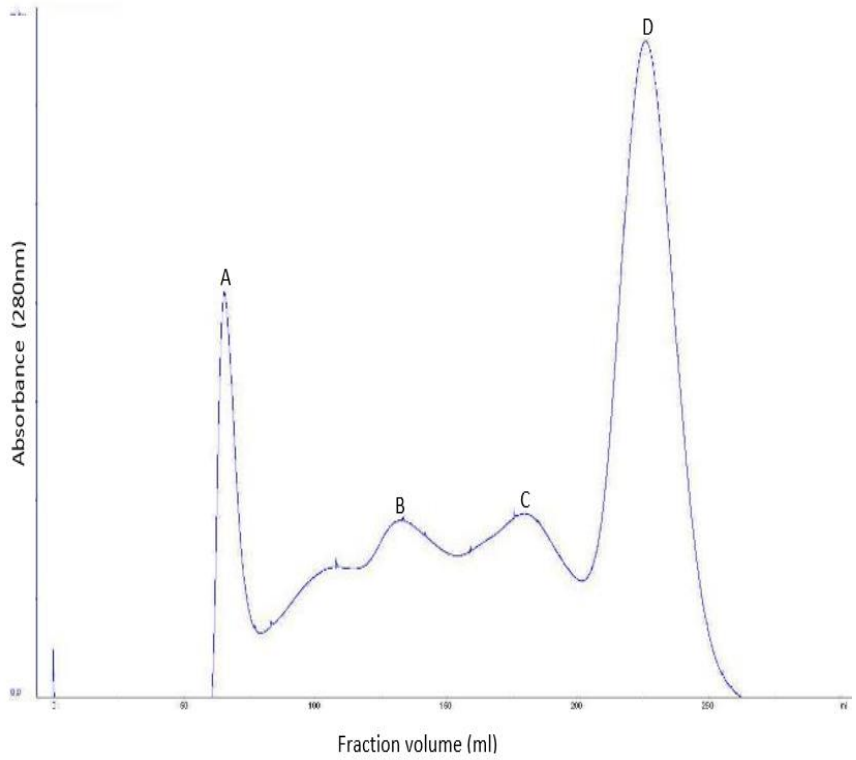


Figure 12. Gel (sephadex G-50) filtration chromatography of pectinase produced by *B. subtilis* 1E7.

yield while the purification factor was 2.59 folds (Table 7). The PL at this stage showed specific activity of 1438U/mg, representing 38.85% yield and purification factor of 2.35 folds over the activity of the crude pectinase (Table 8). The increases in specific activity observed are as a result of the efficiency of the purification method and were consistent with previous reports (Arotupin et al, 2008; Yadav et al., 2008; Hamdy, 2006) where specific activity of pectinase increased as purification progressed.

Figure 13 shows two major peaks I and II but only the fractions within peak I were positive for pectinolysis on pectin agar plate (Appendix IX). The fractions within peak I were pooled together and lyophilized. The lyophilized sample, which was white and crystalline, appeared salty. The high salt content was as a result of eluting the protein with 1M NaCl solution made with the buffer. At this level of the purification, characterizing a salty pectinase will not give reliable results because of the cations present can interfere with the activity. Desalting was carried out through sephadex G-25 column (gel filtration) which gave one major protein peak (Figure 14) that was pectinase-positive and which was pooled and regarded as the partially purified pectinase used for activity assay and characterization. As can be seen in Table 7, the total protein content was 6.77mg, the PG specific activity 25.32U/mg, a 32.04% yield and 7.72 folds purification. The PL on the other hand had a higher specific activity of 3925.81U/mg, 26.6% yield and 6.4 folds purification (Table 8). It is evident that the purification processes/steps confer improved specific activity on the pectinases which is

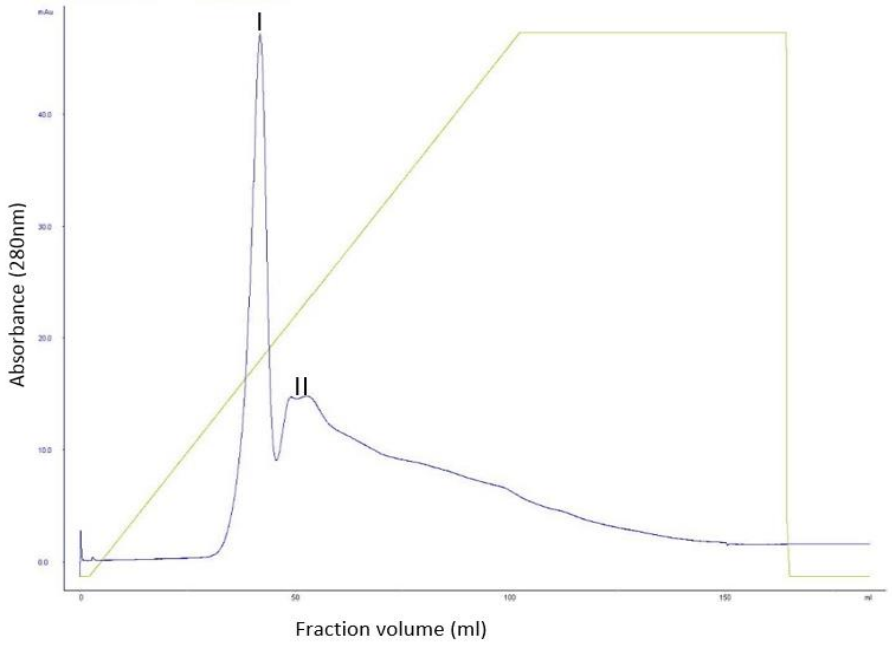


Figure 13. Anion-exchange chromatography through DEAE-cellulose column of the pectinase produced by *B. subtilis* (1E7)

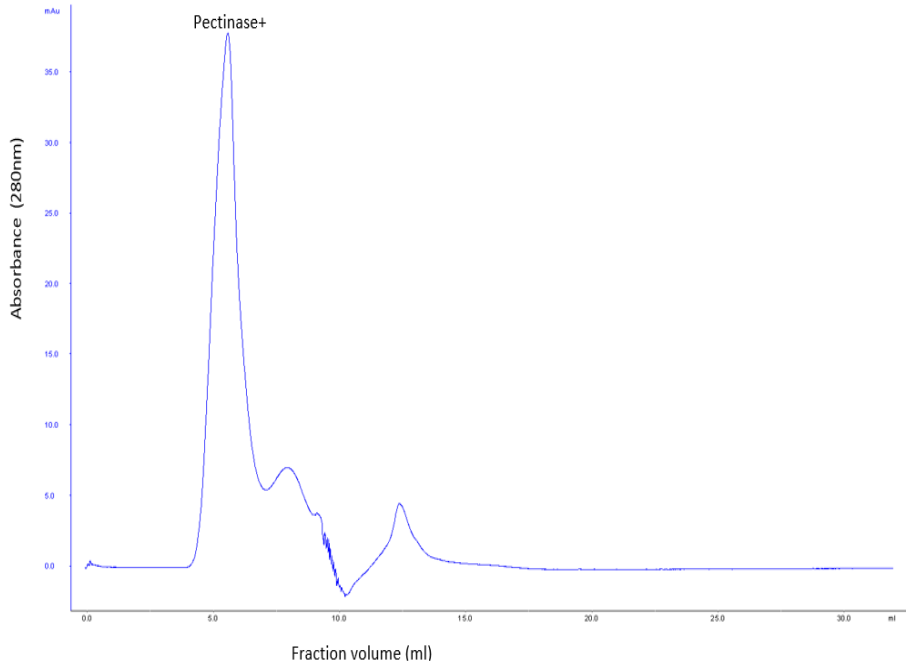


Figure 14. Desalting of partially purified pectinase with Sephadex C-25

consistent with literature. The present study is the first attempt made to partially purify pectinase produced by a bacterium from the rabbit gut. The PG specific activity was lower compared to that previously reported (18.33U/ml by a *Bacillus* sp, isolated from soil) by

Kobayashi et al. (2001). Conversely, the PL production in the present study was much higher than (53U/ml) previously reported in a *Bacillus* sp DT7 by Kashyap et al. (2000), 2313U/mg from *R. oryzae* by Hamdy, (2006) and 18U/mg from *A. flavus* by Yadav et al. (2008). Al Balaa et al. (2014) observed PL specific activity of 59U/mg in *B. subtilis* BPL SY1, another isolate from soil.

4.3.2 Characterisation of partially purified pectinase

4.3.2.1 Effect of temperature on activity of partially purified pectinases

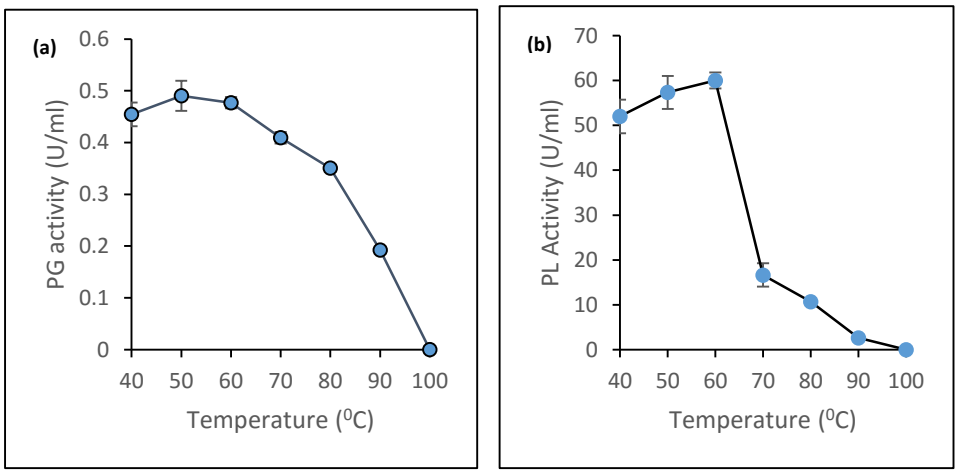


Figure 15. Optimum activity temperature of partially purified pectinase.
(a) PG (b) PL

The data represented in Figure 15 (a) shows that the PG activity of the partially purified pectinase was optimum at 50^0C and 60^0C (no significant difference, $p>0.05$) but at higher incubation temperatures, there was gradual but steady loss in pectinase activity. Similar optimum activity temperature (50^0C) was observed for PG activity in a previous report by Soares et al. (1999). The pectinase in the present study is more temperature tolerant compared to the PG purified from *A. niger* by Arotupin et al. (2012) in which 40^0C was reported as the optimum temperature. The PL activity in this study (Figure 15 (b)) increased steadily from incubation temperature of 40^0C and reached the highest activity (60U/ml) at 60^0C but which

decreased sharply as incubation temperature increased through to 100^0C. This result showed that the partially purified pectinase contains thermo-tolerant pectinases. This property is consistent with the report of Kashyap et al. (2000) where the activity of the PL produced by a strain of *Bacillus* DT7 was the highest at temperature of 60^0C. However, the PL activity in the present study is more thermo-tolerant than observed by Yadav et al. (2008) in a pectinase produced by *A. flavus* where the optimum temperature was 50^0C.

4.3.2.2 Effect of pH on activity of partially purified pectinases

Variations in the optimum temperatures of pectinase activities may be dependent on the nature, sources and differences in the physiological activities of the producing organism (Arotupin et al., 2012).

The data presented in Figure 16 (a) and (b) show that the PG activity of the partially purified pectinase was optimum at pH 8 while PL activity was optimum at pH 10. These indicated that the PG and PL in the present study are alkaliphilic and the PL appeared to be more alkaliphilic than PG. The optimum pH observed in this study agreed with previous studies where report showed that pectinases from bacteria, particularly *Bacillus* spp, generally act best in neutral to alkaline pH ranges (Arotupin et al., 2008). Kobayashi et al. (2001) reported that PG produced by *Bacillus* KSM-P40 optimised activity at pH 7.

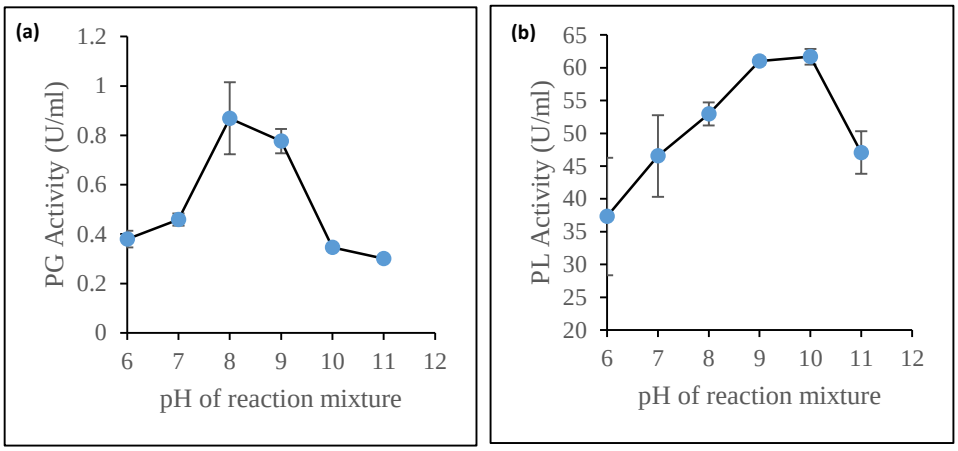


Figure 16. Optimum activity pH of partially purified pectinase.

(a) PG (b) PL

Kashyap et al. (2000) also reported pH 8 as optimum in a pectin lyase by *Bacillus* sp. DT7 while Jayani et al. (2005) cited a *Bacillus* sp. that showed maximum activity at pH 11 but vast majority of fungal pectinases act best in acidic pH ranges. Enzymes act maximally in a pH condition whereby the active site is catalytically active and that the prevailing pH condition at a time has an effect on the affinity of the enzyme for the substrate (Arotupin et al., 2012)

4.3.2.3 Thermal and pH stability of the partially purified pectinase

Enzyme deactivation and stability are major issues of concern in certain industrial processes. Stability study of enzymes is a reflection of the versatility of the enzyme and it's a great significance in biotechnological process. Pectinases stability is known to be affected chiefly by physical parameters as temperature and pH (Gummadi and Panda, 2003). The present partially purified enzyme, when subjected to heating at different temperatures (40 – 100^0C) in a Thermo block TAB-120-BIOSAN for a period of 1 hour Figure 17(a) shows PG relative activity in 40^0C with no significant difference from the same enzyme aliquot not placed under heating (positive control) and remained stable up to 60^0C. At 70^0C, PG still retained about 50% of the original activity.

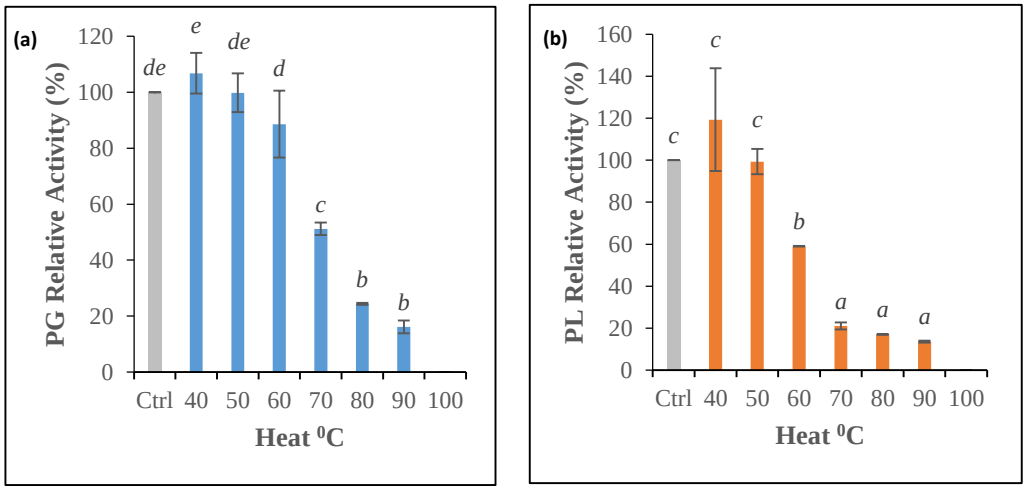


Figure 17. Thermal-stability of the partially purified pectinase. (a) PG (b) PL relative activity

In a similar manner (Figure 17b), the PL activity with the same treatment was also not significantly different from the original activity (positive control), while at 60^0C, PL still retained up to 60% of original activity. The enzyme showed considerable stability at wider range of heating temperatures between 40^0C - 60^0C. This indicated that the enzyme is able to maintain stability and activity even at 60^0C, which is adequate to prevent contamination by limiting the growth of mesophylic bacteria (Soares et al., 1999). The present finding is similar to the report of Yadav et al. (2008) in which pectin lyase produced by *A. flavus* retained 98% activity up to 50^0C heat treatment. In another similar report, Hamdy (2005) observed the pectinase from *R. oryzae* retained its original (100%) activity up to 50^0C. Soares et al. (1999) reported a PG produced by *Bacillus* P4.3, which remained stable up to 60^0C in a similar treatment while Kashyap et al. (2000) observed a pectin lyase produced by a strain of *Bacillus* sp DT7 that was stable at 60^0C. At high temperatures, there is thermal inactivation of enzymes and at the extreme; there will be hydrolysis of peptide bonds, deamination and destruction of di-sulphide bonds which will result to denaturation of the protein molecules.

Concerning the pH stability, the partially purified pectinase was stored in buffers at different pHs (6-12) for 24 hours at 4^0C. The data in Figure 18 a and b show that the relative activity of PG increased to its highest level at pH 9 after which activity declined but was still fully active even at pH 12. The pectin lyase also exhibited similar trend with significant activity up to pH 12. Figure 18 (a) and (b) showed that the present pectinase exhibits stability at wide range of pHs (7-12), which indicated that it is an alkaliphilic pectinase that can be applied under pHs of neutrality to alkaline.

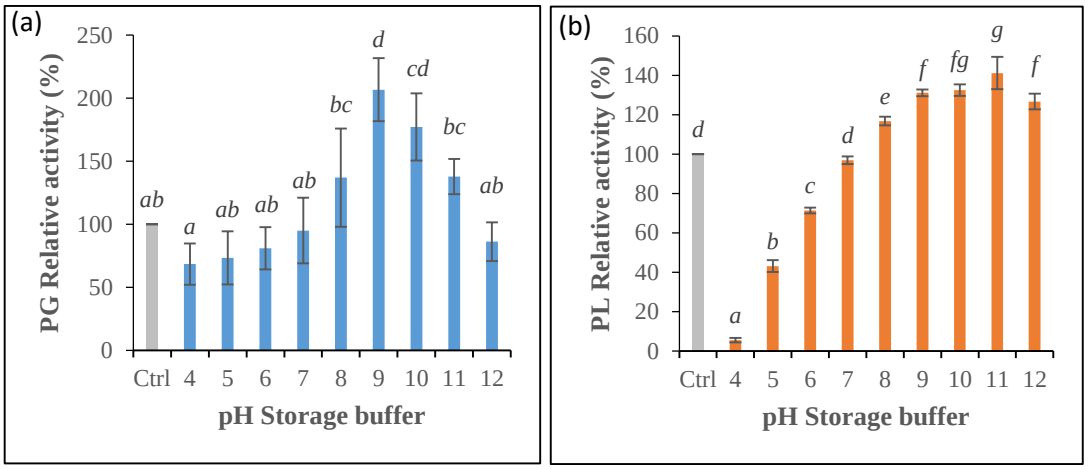


Figure 18. pH stability of the partially purified pectinase. (a) PG (b) PL relative activity

This result is consistent with the report of Singh et al. (1999) where pectinase produced by a strain of *B. licheniformis* demonstrated stability in a pH range of 7-11. In another work, by Yadav et al. (2008), a pectin lyase produced by *A. flavus* showed stability at pH 10 while Kashyap et al. (2000) observed stability at pH 9 for pectinase produced by *Bacillus* sp DT7.

4.3.2.4 Effect of substrate concentration on activity and determination of the kinetics of the partially purified pectinases

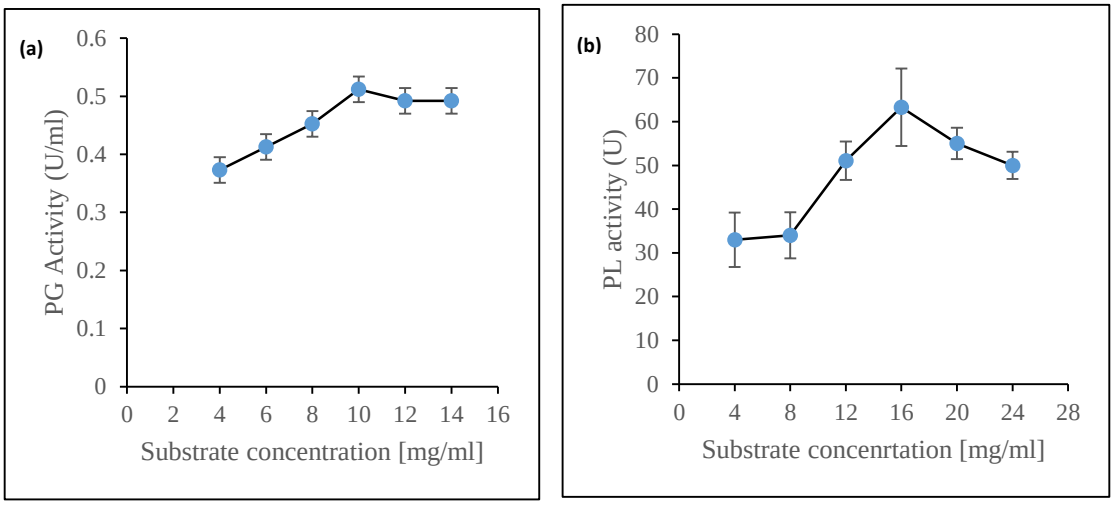


Figure 19. Effect of substrate concentration on activity of the partially purified enzyme. (a) PG (b) PL

Studies of the effects of different concentrations of pectin substrate on activities of the pectinases are presented in Figure 19 (a) (b). For both PG (a) and PL (b) activities, enzymatic reactions were first-order at the initial (low) substrate (citrus pectin) concentrations and at higher concentrations, the reactions became zero-order. The PG activity was highest at 10mg/ml after which further increase in the concentrations of the substrate did not result to further increase in PG activity. PL on the other hand attained its maximum activity at 16mg/ml after which further increase in substrate concentration resulted to no increase in the PL activity. This observation could be explained from the fact that effective binding of the substrate on the active site of the pectinases (Arotupin et al., 2008). Further explanation is made suggested in terms of the chemical union between the enzyme and the substrate to form the enzyme-substrate complex (a reversible reaction) which eventually breaks down to give the product in the forward reaction. This continues at a steady rate until optimum is attained. Activity decline after the optimum is as a result of saturation and over saturation of the active sites on the pectinases due to the presence of excess substrate, which will lead to the reaction

equilibrium shift and the substrate concentration becoming inhibitory to the reaction. The V_{max} and K_m for the pectinases were determined by double reciprocal plot using Lineweaver-Burk method shown in Figure 20(a), (b). The V_{max} is a parameter that determines the rate of an enzyme reaction at a stage when the active sites are saturated with substrate.

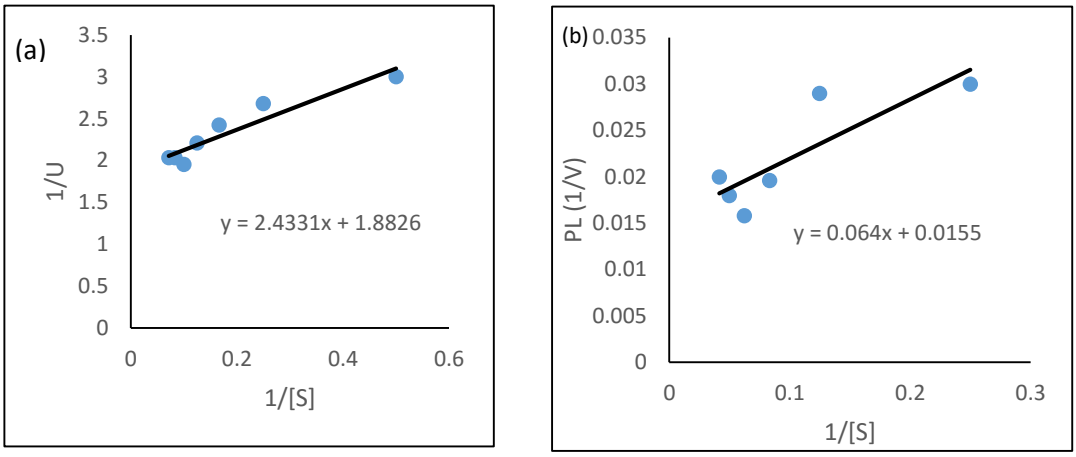


Figure 20. Lineweaver-Burk plot for the activity of the partially purified pectinases (a) PG activity (b) PL activity pectinase

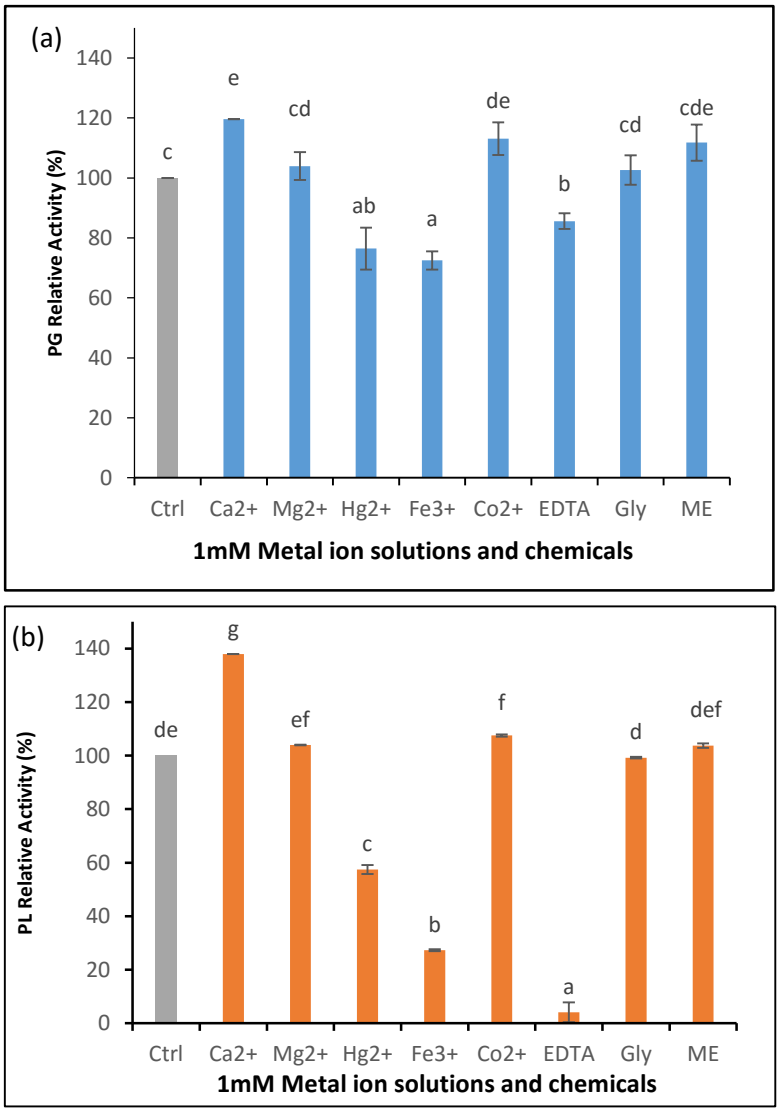
The V_{max} for the PG was calculated to be 0.53U/ml, which is low because its production was relatively low throughout this study. K_m on the other hand is defined as the substrate concentration at which the enzyme exhibits 50% of the *Vmax*, the maximum activity. It is also used as a measure of strength of binding between the enzyme and substrate. The lower the K_m value the stronger is the enzyme affinity for the substrate. This translates to, the lower the binding strength, the easier the product is formed. The K_m of the PG was calculated to be 1.29mg/ml, which is stronger than the PG produced by an *A. niger* (2.7mg/ml) enzyme in the report of Arotupin et al. (2012). The V_{max} of PL was 64.52U/ml while the K_m was calculated to be 4.13mg/ml. The K_m value is good and compares favourably with the K_m of the enzymes produced by some of the moulds commonly used for pectinase production. K_m determination for bacterial pectinases have been scarcely reported but available K_m values of fungal

pectinases include that of *R. oryzae* at 3.87mg/ml (Hamdy, 2005), *P. canescens* at 2.9mg/ml (Sinitsyna *et al.* 2007) and *P. oxalium* at 4.6mg/ml (Yadav and Shastri, 2007). This shows that the pectin lyase is very potent with strong affinity and can be a good alternative for the production of commercial pectinases especially the lyases.

4.3.2.5 Effects of metal ions and chemicals on activity of partially purified pectinases

The effects of metal ions and chemicals, which have been previously shown to be enhancers or inhibitors of pectinases, were tested on the purified pectinase. All chemicals and metal ion solutions were used at a concentration of 1mM according to Kashyap et al. (2000). The PG and PL relative activity compared to a positive control (enzyme aliquot without treatment) and the data are as presented in Figure 21(a) and (b). In this study, Ca^{2+} ion stimulated both PG and PL relative activity showing significant ($p<0.05$) increase with the highest value (120% and 140% in PG and PL, respectively). Bacterial pectinase characterization is scarcely reported. However, the present result is consistent with previous results available in literature. Kashyap et al. (2000), Afifi et al. (2002), Hamdy (2005), Hamdy (2006) and Arotupin et al. (2008), in separate reports, showed that the alkali-metal ions such as Ca^{2+} and K^{+} are capable of stimulating pectinases activity but the extent of stimulation depends largely on the nature and source of the enzyme producer. The high productivity of the alkali metal ions could be explained from the point of view of their ability to bind weakly to the enzymes, forming ternary complexes involving the enzyme (E), metal ions (M) and substrates (S), which is known to enhance the enzyme conformation and activity (Hamdy, 2006). This study also established that the pectinases depend largely upon presence of Ca^{2+} for activity. The alkali-metal cations can also act directly on substrate molecules by changing its conformation and total charge to suit the sensitivity of the pectinase (Sinistyna et al., 2007; Arotupin et al., 2012). Some enzymes have certain transition metals bound to their active sites. Such metals like cobalt (used in the present study) acts to polarize the enzyme to attract substrates for

catalysis (electrophilic catalysis). This assertion is in line with the result shown (Figure 21 a, b) where $CoCl_2$ significantly enhanced relative activity next to the Ca^{2+} by both PG and PL.



Control=Untreated enzyme; Ca^{2+}=$CaCl_2$; Mg^{2+}=$MgCl_2$; Hg^{2+}=$HgCl_2$; Fe^{3+}=$FeCl_3$; Co= $CoCl_2$ EDTA=Ethylene diethyl tetra-acetic acid; Gly=Glycine; ME=Mercapto-ethanol

Figure 21. Effect of chemicals and metal ions on partially purified pectinase (a) PG (b) PL relative activity

This result is consistent with the report of Yadav et al., (2008) who stated that Co^{2+} slightly activated pectinase relative activity. Conversely, Hamdy, (2006), in his report, noted a moderate inhibition, which may be due to difference in the enzyme sources.

Magnesium metal ion in the present pectinase showed no effect (100% relative activity) on PG and PL activities. This is consistent with the report of Kashyap et al. (2000) who reported that Mg^{2+} had no effect on the relative activity of a pectinase produced by *Bacillus* sp. It is likely that the Mg^{2+} is not an essential ions required in the catalytic process of the pectinase under study. Hg^{2+} significantly inhibited the productivity in both PG and PL (Figure 21a, b) which is in accordance with previous reports (Kashyap et al., 2000; Hamdy, 2006; Yadav et al., 2008; Arotupin et al., 2012). Mercuric ions in solution are able to form bonds with the sulfhydryl (SH) group on enzymes proteins. This changes the shape of the enzyme and blocks its activity. This shows that the SH group participates in the pectinase activity. In the report of Hamdy (2006), the effect of Hg^{2+} on the pectinase was more severe compared to the present pectinase. This shows that the pectinase under study is able to resist the inhibition to an extent.

Ferric ion ($FeCl_3$) showed inhibitions in PG and PL in the present pectinase (Figure. 21 a, b). The report is consistent with the observation of Kashyap et al. (2000) in a pectinase from *Bacillus* sp. DT7. Ferric ion when in contact with an enzyme, binds to the sulfur to form the iron-sulfur (Fe-S) clusters, which coils up the polypeptide chains thereby blocking the active site from activity (Moran et al., 2011) Of the chemicals tested, glycine (Gly) and mercapto-ethanol (ME) had no effect on the relative activity both for PG and PL. Contrary to previous report of Kashyap et al. (2000), pectinase activity is said to increase but the case is different in the present pectinases. Mercapto-ethanol disrupts the sulfide bonds of most enzymes and thereby affects activity of enzymes. The pectinase seem to have resistance for the disruptive tendencies of the ME which is a sign of its strong structural compactness.

EDTA in the present study had slight but statistically significant ($p<0.05$) inhibitory effects on PG while the effect on PL activity was highly significant ($p<0.05$). This is consistent with the report of Kashyap et al. (2000) who observed a decrease in pectinase activity after

incubation in EDTA for 24hrs at 4^0C. However, in the report of Hamdy, (2006), EDTA showed no effect on the catalytic property of his pectinase. The EDTA is a chelating agent that has the ability to adsorb metal ions in its environment. The Hamdy (2006) report stated that certain enzymes absolutely require Ca^{2+} and other metal ions for activity and the cations are present on the active sites. In the presence of EDTA, the required ions are adsorbed and the removal of the ions made the enzyme unable to be active. The severity in the inhibition in the PL (5% relative activity) suggests that the PL seems to have high requirement for Ca^{2+} and probably other ions too (which have now been removed) for its catalysis. This observation is typical of pectate lyase in particular (Soriano et al., 2006) which made us suspect that the lyase present is pectate lyase. PG has less requirement for Ca^{2+} hence the relative activity was higher.

4.3.2.6 Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis of partially purified pectinases

Analysis of the trypsin in-solution digestion of the partially purified pectinase as shown in Table 9 consists the list of the proteins in the sample. A total of 22 proteins were identified which shows that the sample is partially purified. Notable among the proteins present is the protein group 9, protein ID 3625 with accession gi/670919786 which is identified as a pectate lyase from *Bacillus subtilis* TO-A with a molecular weight of 45kDa. Also present is protein group 15, accession number gi/857328673 which is described as pectate lyase (*Bacillus subtilis)* with molecular weight of 38kDa. The protein group 12, accession number gi/670921674 with a description of a metal dependent hydrolase (another name for polygalacturonase) from *Bacillus subtilis* TO-A (molecular weight, 25kDa) is also found. The presence of these three proteins shows that our sample contains pectinolytic enzymes. The pectate lyases of groups 9 and 12 with molecular weights 45kDa and 38kDa respectively. These are two forms of pectate lyases occurring together in our pectinase. Such occurrence

seldom happens as was recorded by Parini et al., 1988 when two forms of pectin lyases having molecular weights, 89kDa and 55kDa, were identified in their pectinase produced from *Aureobasidium pollulans* LV10. These pectinase proteins are generally responsible for its pectinolytic activity both for the galacturonase and the lyase. The three proteins on the list have -10logP values of 182.49, 135.57 and 149.92 respectively, which are high enough because values greater than 20 is the lowest limit of confidence for the presence of a protein (personal communication). It can also be observed that the table shows that virtually all the proteins listed were traceable to *Bacillus subtilis* which also is an evidence that the sample was a product of a pure culture of the *B. subtilis* 1E7. The protein list (Table 9) coverage (%) showed 36%, 19% and 62% for protein groups 9, 12 and 15 respectively as well as the unique peptides of 17, 8 and 9 respectively which were sufficient for their identification.

Table 9: List of proteins detected by LC-MS in the pectinase sample

Protein List

Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	#Peptides	#Unique	PTM	Avg. Mass	Description
6	36	gi|825064261	275.12	24	23	23	N	154855	peptidase S8 [Bacillus subtilis]
5	3648	gi|505296354	253.03	64	25	25	N	14887	MULTISPECIES: hypothetical protein [Bacillus subtilis group]
11	3635	gi|906390573	210.69	77	17	17	N	22490	superoxide dismutase [Bacillus subtilis]
10	3619	gi|505065258	198.45	39	18	18	N	61478	MULTISPECIES: peptide ABC transporter substrate-binding protein [Bacillus subtilis group]
9	3625	gi|670919786	182.49	36	17	17	N	45498	pectate lyase [Bacillus subtilis TO-A]
14	3677	gi|857329720	174.27	38	11	11	Y	23965	hypothetical protein VL08_01850 [Bacillus subtilis]
13	3713	gi|899710071	155.42	57	7	7	N	16594	general stress protein [Bacillus sp. FJAT-27445]
12	3642	gi|670921674	149.92	62	9	9	N	24831	metal-dependent hydrolase [Bacillus subtilis TO-A]
15	3671	gi|857328673	135.57	19	8	8	N	38021	pectate lyase [Bacillus subtilis]
18	3658	gi|1709639	135.10	20	7	7	N	45509	RecName: Full=Peptidase T; AltName: Full=Aminotripeptidase; Short=Tripeptidase; AltName: Full=Tripeptide aminopeptidase
16	3665	gi|874361113	130.01	24	7	7	N	43476	oxalate decarboxylase [Bacillus subtilis]
20	3727	gi|857329553	118.50	19	5	5	N	32154	arginase [Bacillus subtilis]
19	3699	gi|825064500	116.90	26	5	5	N	25496	hypothetical protein ABA10_09625 [Bacillus subtilis]
21	3692	gi|825066671	113.46	21	6	6	N	51519	hypothetical protein ABA10_18250 [Bacillus subtilis]
17	3705	gi|899710641	112.52	27	8	8	N	28343	spore coat protein [Bacillus sp. FJAT-27445]
23	3757	gi|825064575	106.54	11	4	4	N	64123	gamma-glutamyltranspeptidase [Bacillus subtilis]
24	3762	gi|670920751	99.81	34	4	4	N	16177	deoxyuridine 5'-triphosphate nucleotidohydrolase yncF [Bacillus subtilis TO-A]
25	3733	gi|763058210	90.32	4	4	4	N	154444	MULTISPECIES: 2', 3'-cyclic nucleotide 2'-phosphodiesterase [Bacillus]
22	3700	gi|825063950	84.63	16	5	5	Y	54797	glucuronate isomerase [Bacillus subtilis]
27	3789	gi|855286025	79.34	9	3	3	N	47541	glucuronoxylanase [Bacillus sp. GO-13]
26	3778	gi|899704219	66.43	28	3	3	N	17682	spore coat protein [Bacillus sp. FJAT-27445]
31	3816	gi|907410700	57.13	9	2	2	N	33363	protein iolH [Bacillus licheniformis]
total 22 proteins									

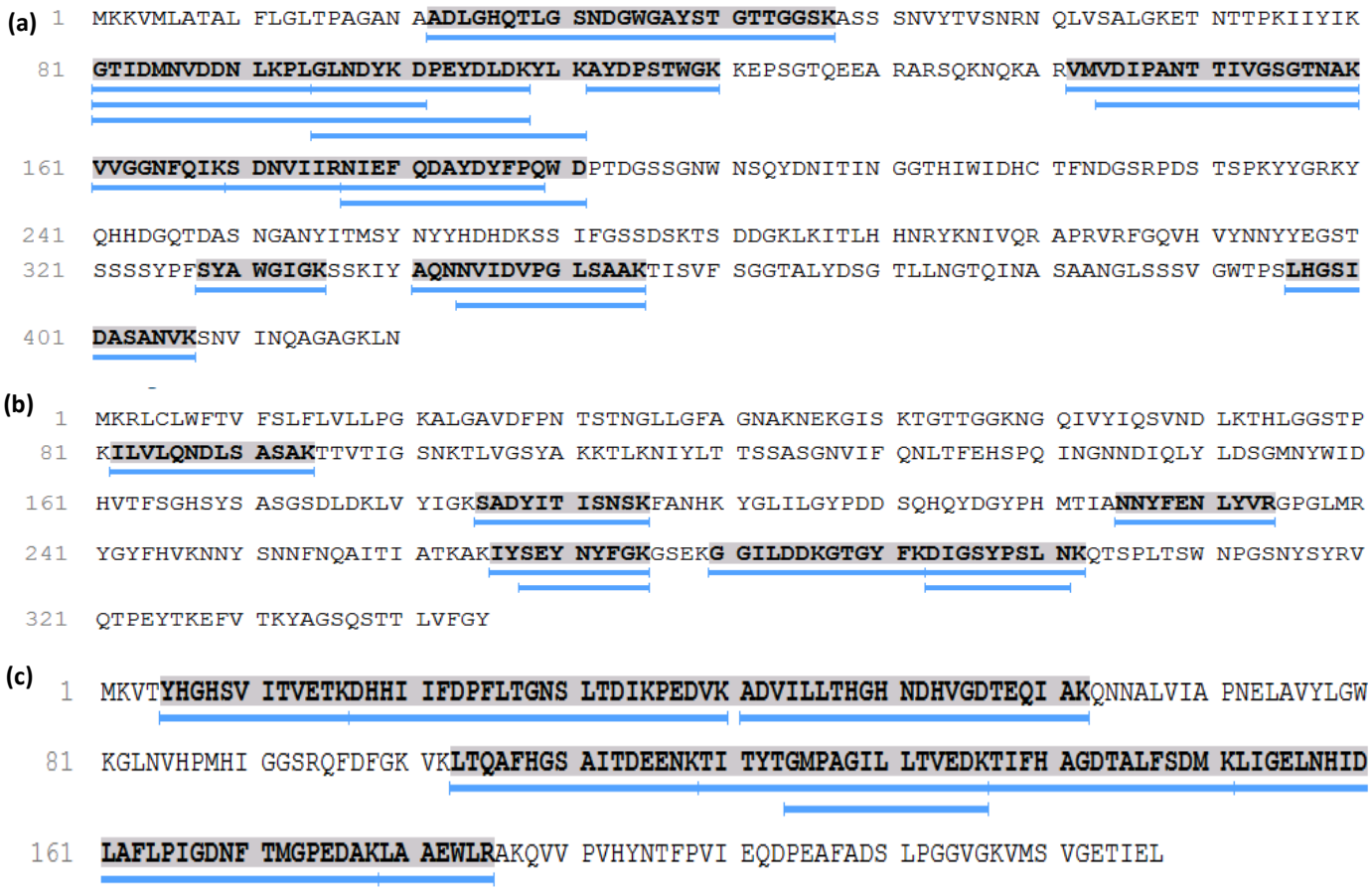


Figure 22. Homology of peptide sequence of the sample pectinases in alignment to ncbi database for (a) Pectate lyase, (*B. subtilis* TO-A) (b) Pectate lyase (*B. subtilis*) (c) Metal dependent Hydrolase (*B. subtilis* TO-A) proteins.

Figure 22 (a), (b), (c) shows the peptide coverage of the three pectinases described. The figures revealed the homology (bold letters) of the present pectinases against the ncbi data base for the polypeptide chains sequence of pectinases from *Bacillus* spp.

4.4 Conclusion

The *B. subtilis* 1E7 used in the present work appears to be a novel strain in pectinase production. This is the first attempt to produce, partially purify and characterize pectinase produced by bacteria from rabbit gut. The chromatographic purification process used

produced pectinase having improved activity with lyase showing far greater activity over the polygalacturonase. The Table A7 in Appendix X compared the present pectinase and was found to exhibit some outstanding properties. The partially purified enzyme showed high pectinolytic activity along with its alkaline nature and alkaliphilic activity as well as its thermo-stability and thermophilic activity. These properties suggest that this bacterial strain from rabbit gut is a better pectinase producer than the earlier reported microbes. Furthermore, other additional features such as enhancement of pectinolytic activity with Ca^{2+} and its resistive tendencies towards some known potent inhibitors of enzymes is indication of the suitability of the pectinases produced for industrial processes.

CHAPTER 5

General Discussion and Conclusions

5.1 Major findings and contributions of this study

The primary aims of this research work were to (i) isolate, and identify bacteria that produce pectinase in the caeco-colic contents of rabbit fed with high fibre meals, (ii) assess the potentials of the isolates for pectinases (PG, PL, PME) production, (iii) optimize (nutritional and growth conditions) pectinase production by the selected bacterial isolate for maximum pectinase yield, (iv) partially purify and characterize the pectinases obtained and test the effects of physico-chemical conditions on the purified enzyme and (v) determine stability of the pectinase to temperature (heat), acidity (pH) and chemicals. Based on the results obtained, a number of results can be deduced.

The caeco-colic region of rabbit hind gut is a reservoir for an array of symbiotic bacteria carrying on bioconversion of fibers and contributing to the growth and wellbeing of the animal. Initially (Chapter 2), twenty seven pectinolytic cultivable bacterial strains were isolated and identified from the gut of high fiber fed rabbits in this study. Molecular identification (16S rDNA) showed the presence of pectinolytic *B. subtilis, B. pumilus, B. ginsengihumi, B. megaterium, B. licheniformis and Paenibacillus* spp. All the bacterial strains were screened for the production of three important members of the pectinase group; polygalacturonase, the lyases and pectinmethyl esterase. It was found that the gut bacteria rarely produced the esterase, polygalacturonase was produced only seldomly low but lyase was produced substantially by the majority of the bacterial strains. This suggested that lyase is the main pectinase involved in pectin digestion in rabbit gut. Lyase is the only pectinase that can act without dependence on pre-action of any other enzyme and can hydrolyse highly esterified pectin molecules with no toxic methanol produced. The strain *B. subtilis,* 137, (accession number KT 713393, appendix 4) was found to be the most prolific or substantial producer of both the polygalacturonase (PG) and pectin lyase (PL). This work was the first to evaluate the profile of the three pectinases production by the caeco-colic bacterial species.

Apart from the selected *B. subtilis*, 1E7, there are other strains (Table 2) that can be studied (future) because of their good pectinolytic activities.

In maximising pectinase production by the selected *B. subtilis* 1E7, using RSM in the chapter 3 of this study, cultural temperature (38^0C) and agitation (190 rpm) conditions influenced the optimum production of both PG and PL in a pectin yeast broth (PYB) at initial pH 10. *B. subtilis* 1E7 appeared to be a novel strain because of its ability to substantially produce alkaline lyase pectinase considered to be an important industrial pectinase rarely produced by bacteria.

In the third approach (Chapter 4), the crude pectinase produced by the *B. subtilis* 1E7 was taken through three steps of purifications which included ammonium sulphate precipitation and subsequent dialysis, followed by gel filtration and anion exchange chromatography. The pectinase produced after chromatography purification had improved activity while the lyase still showed far greater activity than polygalacturonase. This is the first attempt to produce, purify and characterise pectinases sourced from rabbit gut bacterial species. However the table in Appendix X compared the present pectinase with those previously produced from other sources and was found to exhibit some outstanding properties. The LC-MS analysis revealed the enzyme produced was a partially purified sample and confirmed that it consisted of pectinases, particularly, the hydrolase (polygalacturonase) and lyases (pectate lyase; 2 types) from *B. subtilis.* The partially purified enzyme showed outstanding properties in high pectinolytic activity along with its alkaline nature and the alkaliphilic activity as well as its thermo-stability and thermophilic activity. Furthermore, other additional features like enhancement of pectinolytic activity in the presence of calcium ion and its resistive tendencies towards some known potent inhibitors of enzymes is indication of the structural strength and suitability of the present pectinases which makes us suggest that this bacterial strain is a better alternative to some of the microbial sources currently used as commercial

pectinase producers. In this research, we have been able to produce, optimize, partially purify and characterize alkaline pectinases (PG and PL) from *B. subtilis* 1E7 isolated from the caeco-colic region of rabbit hind gut.

5.2 Future work

This study has revealed the pectinolytic capability of certain bacterial strains in the caeco-colic region of rabbit hind gut. It is a breakthrough to a bacterial ecosystem where the majority are potential producers of the alkaline lyase pectinase which are scarcely available commercially. Listed below are possible areas of future research that could be conducted in order to produce a pure sample that will validate the potentiality of the *B. subtilis*, 1E7 strain for pectinase production.

5.2.1 Purification of pectinases to homogeneity

The LC-MS analysis result in Chapter 4 showed that the pectinase was partially purified. In future research, the purification should be further carried out by eliminating the contaminating proteins. This can be achieved by serial gel filtration using sephadex beads that will remove those proteins bigger than 45kDa in the void volume. This can be followed with both cation and anion exchange chromatograpy till homogeneity is achieved. In-between check with LC-MS should be done in order to know the level of purity.

5.2.2 Pectinase gene cloning and expression

The gene encoding the pectate lyase from the *B. subtilis*, 1E7 can be cloned into a vector and transform in *E. coli* to optimise the expression levels of the recombinant enzyme. A higher enzyme production will be observed with an efficient transformant when compared with the native strain. Expression of the recombinant gene may be confirmed by SDS-PAGE, zymography methods and Western blotting. Consecutive induction using Isopropyl-1-

thiogalacto-pyranoside (IPTG) concentration (1 mM) can be carried out in order to further improve the enzyme production. Purification of pectinase can be carried out using size exclusion chromatography, ion exchange chromatography IMAC and affinity chromatography. The pure pectinase can then be characterized for optimal activity and stability (Abuhammad et al., 2013).

5.2.3 Potential application of pectinases

The potential application of the pure pectinase can be tested under optimum conditions for desizing and bio-scouring of cotton as well as pulping of Crotalaria stem and also in clarification of juice. The activity can then be compared to commercial pectinases commonly used in industrial processes.

Apart from the *B. subtilis,* 1E7, other strains identified can be used independently in the same way as described above.

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Appendix I

Analysis of the feed and gut digesta of rabbits

Following appendix contains the analysis of the physical and chemical properties of the feed and caeco-colic contents of rabbits gut to ascertain how suitable the meal is for gut health and microbial activity.

Table A1: Physico-chemical composition of the feed and gut samples of the rabbits

SAMPLES	pH	Moisture Content	Dry Matter	Organic Matter	Ash	Nitrogen	Crude Protein	Crude Fiber
Feed	6.86 ± 0^{c}	10.35 ± 0.09^{a}	89.65 ± 0.09^{b}	83.73 ± 1.19^{a}	16.27 ± 1.19^{a}	2.68 ± 0.01^{a}	16.75 ± 0.05^{a}	23.2 ± 0.1^{b}
Caecum	6.06 ± 0.01^{a}	71.32 ± 1.16^{b}	28.68 ± 1.16^{a}	82.00 ± 0.22^{a}	18.00 ± 0.22^{a}	3.08 ± 0.02^{b}	19.26 ± 0.14^{b}	18.29 ± 0.01^{a}
Colon	6.15 ± 0.01^{b}	70.04 ± 0.94^{b}	29.96 ± 0.94^{a}	81.39 ± 0.93^{a}	18.61 ± 0.93^{a}	3.03 ± 0.03^{b}	18.9 ± 0.18^{b}	18.26 ± 0.01^{a}
P	0.000	0.000	0.000	0.065	0.065	0.000	0.000	0.004
F	4.165	4.283	4.283	4.979	4.979	305.705	254.693	20.951

Data are presented in mean ± standard deviation. Different letters in the same column indicate significant difference where $p < 0.05$.

Appendix II

Mineral composition of the pectinase producing medium

The following appendix contains the Composition of the Yeast Pectin (YPA and YPB), a Modification of Soares *et al.*(1999)used for the bacterial isolation and production of pectinases

Table A2: Chemical composition of the yeast pectin medium

$NaNO_3$…… 0.2 g
K_2HPO_4……... 0.3 g
$MgSO_4$…… 0.02 %
$FeSO_4.7H_2O$ …… 5mg/L
$MnSO_4$…… 1.6mg/L
$CaCl_2$..... 2mg/L
Pectin…… 1%, PH 7
YPA….Solid medium……..Agar…….. 1% (The liquid medium (YPB) lacked 1% Agar)

Appendix III

Ranking of pectinolytic capability among bacterial isolates

Following appendix contains the yardstick for ranking of qualitative pectinolytic screening (Diameter of halo) of bacterial isolates on pectin agar plates medium

Table A3: Ranking of pectinolysis

0 mm	Non Producer
<5mm	Poor Producer
6-15mm	Moderate Producer
>16mm	Strong Producer

Appendix IV

Identity of the bacterial isolates sequenced at First base

The following Appendix contains representative strain of bacterial isolates that were sent for accession and the blast.

Table A4: Similarity values of 16S rDNA for selected pectinolytic bacterial isolates.

Isolate	Similar Genus	Accession Number	Query Cover %	Identity %	Pectinase produced
1E7	*Bacillus subtilis*	KT 713393	99	100	PG, PL
1E10	*B. ginsenghumi*	KT 713394	100	99	PG, PL
2E2	*B. megaterium*	KT 713395	100	99	PL, PME
2E7	*B. licheniformis*	KT 713396	100	99	PL
3E10	*B. pumilus*	KT 713397	99	100	PG, PL

Appendix V

Design Expert summary outcome

Following contains table contains the prediction of optimum conditions for pectinase production and their model equations in the coded terms

Final Equation in Terms of Coded Factors: Polygalacturonase Activity

Coefficient values	Terms	Coefficient (coded)
+0.55	Offset	b_0
-0.071	*A	b_1
-0.066	*B	b_2
+8.500E-003	*A*B	b_{12}
-0.33	$*A^2$	b_{11}
-0.20	$*B^2$	b_{22}

Final Equation in Terms of Coded Factors: Pectin Lyase

Coefficient values	Terms	Coefficient (coded)
+107.95	Offset	b_0
-20.92	*A	b_1
-14.95	*B	b_2
-18.11	*A*B	b_{12}
-13.32	$*A^2$	b_{11}
-27.11	$*B^2$	b_{22}

Table A5: Design expert prediction

Solutions (Prediction)

Number	Agitation	Temp	PG (U/mL)	PL(U/mL)	Desirability	
1	189.05	38.33	0.556411	111.488	0.988	Selected

Appendix VI

Table of ammonium sulphate solute required for protein fractionation based on the initial and final concentration desired

Following appendix contains chart of the standard quantity of ammonium sulphate salts required to precipitate proteins at different percentage saturation with reference to both first and final concentrations.

Table A6: Amount of ammonium sulphate required for saturation of protein solutions- Reprinted from Rosenberg (1996)

		Final concentration of ammonium sulphate, % saturation																
Initial concentration of ammonium sulphate, % saturation		10	20	25	30	33	35	40	45	50	55	60	65	70	75	80	90	100
		Grams solid ammonium sulphate salt to be added to 1 liter of solution																
	0	56	114	114	176	196	209	243	277	313	351	390	430	472	516	561	662	767
	10		57	86	118	137	150	183	216	251	288	326	365	406	449	494	592	694
	20			29	59	78	91	123	155	189	225	262	300	340	382	424	520	619
	25				30	49	61	93	125	158	193	230	267	307	348	390	485	583
	30					19	30	62	94	127	162	198	235	273	314	356	449	546
	33						12	43	74	107	142	177	214	252	292	333	426	522
	35							31	63	94	129	164	200	238	278	319	411	506
	40								31	63	97	132	168	205	245	285	375	469
	45									32	65	99	134	171	210	250	339	431
	50										33	66	101	137	176	214	302	392
	55											33	67	103	141	179	264	353
	60												34	69	105	143	227	314
	65													34	70	107	190	275
	70														35	72	153	237
	75															36	115	198
	80																77	157
	90																	79

Key

	0-40% saturation
	40-90% saturation

Appendix VII

Analysis of pectinolytic activity

Testing for presence of pectinolytic activity when pectin agar is flooded with iodine solution

The qualitative testing of the precipitates (AS) for pectinolysis on pectin agar plate to determine the cut offs required for the complete precipitation of the proteins present in the crude pectinase.

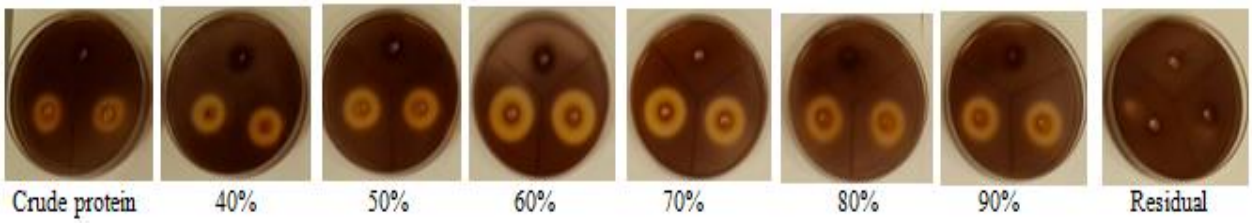

Figure A1: Profile of qualitative pectinolytic activity of $(NH_4)_2SO_4$ precipitates from crude pectinases (40% to 90% saturation)

Testing for presence of pectinolytic activity

when pectin agar is flooded

with iodine solution

The qualitative testing of the gel filtration eluent fractions on pectin agar plate to determine which of the peaks that could show pectinolysis.

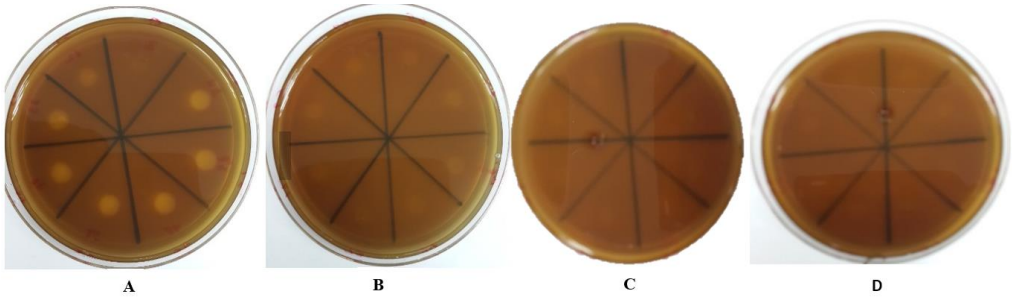

Figure A2: Qualitative pectinolytic activity tests of eluent fractions of gel filtration at different peaks.

The qualitative testing of the eluent fractions on pectin agar plate to determine which of the two peaks from anion exchange could show pectinolysis.

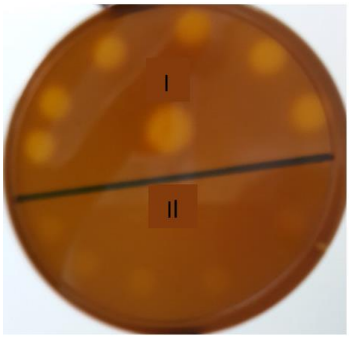

Figure A3: Qualitative pectinolytic activity tests of eluent fractions of anion-exchange chromatography for peaks I and II.

Appendix VIII

Comparison of the present pectinase

with other pectinase sources in literature

Following appendix contains a tabular comparison of the properties of our partially purified pectinase produced in chapter 4 with pectinases produced similarly from other sources

Table A7: Our pectinase compared with similar ones in literature

Bacteria	Source	Specific Activity	Optimum pH	Optimum Temperature	pH Stability	Thermal Stability	References
Bacillus subtilis (1E7)	Rabbit gut	3926	10	60	7-12	60	Present
*Bacillus*sp. KSM-P40	Soil	18	8	55	6-12	30	Kobayashi *et al*, 2001
Bacillus sp DT7	Soil	53	8	60	9	60	Kashyap *et al*. (2000)
A. flavus	Soil	18	8	50	9	50	Yadav *et al.* (2000)
R. oryzae	Soil	2313	7.5	50	7.5	50	Hamdy, (2006)