Exploitation of indigenous strain, *Bacillus subtilis* BKDS1 for augmented pectinase production using agro-waste

Thesis submitted to
the University of Calicut for the award of
the degree of

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

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Under the guidance of

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This is to certify that this thesis entitled **"Exploitation of indigenous strain, *Bacillus subtilis* BKDS1 for augmented pectinase production using agro-waste."** is a bonafide research work done by **Mr. Bijesh K.,** under my supervision and guidance in the Department of Life Sciences, University of Calicut, for the award of the degree of Doctor of Philosophy in Microbiology, under the faculty of Science of the University of Calicut. I also certify that the same has not been submitted for any other degree diploma or associateship in any other University.

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I also hereby certify that the corrections/suggestions from the adjudicators have been incorporated in the revised thesis. Content of the CD submitted and the hardcopy of the thesis is one and the same.

University of Calicut **Dr. Denoj Sebastian**

DECLARATION

I Bijesh K., hereby declare that this thesis entitled **"Exploitation of indigenous strain, *Bacillus subtilis* BKDS1 for augmented pectinase production using agro-waste"** is being submitted to the University of Calicut, in partial fulfillment of the requirement of the degree of Doctor of Philosophy in Microbiology under the faculty of Science. This thesis is the result of my work carried out in the Department of Life Sciences under the guidance and supervision of Dr. Denoj Sebastian, Assistant Professor in Microbiology, Department of Life Sciences, University of Calicut. This thesis or any part thereof has not been submitted for any other degree, diploma or any other similar title of any University.

University of Calicut **Bijesh K**
31. 10. 2018

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All those who supported me in this endeavor

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LIST OF ACRONYMS USED

Acronyms used		Expansion
AG	:	Apiogalacturonan
AGE	:	Agarose gel electrophoresis
ANN	:	Artificial neural network
ANOVA	:	Analysis of variance
AO	:	Acridine orange
AWEM	:	Agro-waste extract media
BATH	:	Bacterial Adherence to Hydrocarbons
BBD	:	Box-Behnken Design
BLAST	:	Basic Local Alignment Search Tool
BPE	:	Banana peduncle extract
BWD	:	Box–Wilson design
CCD	:	Central Composite Design
CTAB	:	Cetyl trimethylammonium bromide
DNA	:	Deoxyribonucleic acid
DNS	:	Dinitrosalicylic acid
EDTA	:	Ethylenediaminetetraacetic acid
EMS	:	Ethyl methanesulfonate
Etbr	:	Ethidium bromide
FDA	:	Food and Drug administration
GalA	:	Galacturonic acid
HG	:	Homogalacturonan
IMViC	:	IMViC: Indole, Methyl red, Voges-Proskauer, Citrate
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
LB	:	Luria Bertani
lpm	:	Liters per minute
MEGA	:	Molecular Evolutionary Genetics Analysis
MMS	:	Methyl methanesulfonate
NCBI	:	National Center for Biotechnology Information
NFW	:	Nuclease free water
NTG	:	Nitrosoguanidine

OFAT	:	one- factor- at- a –time
OG	:	Oligogalacturonase
OGL	:	Oligogalacturonide lyase
OVAT	:	one- variable- at- a –time
PAE	:	Polyascetylesterase
PAGE	:	Polyacrylamide gel electrophoresis
PBD	:	Plackett–Burman design
PCR	:	Polymerase chain reaction
PG	:	Polygalacturonase
PL	:	Pectate lyase
PME	:	Pectin Methyl Esterase
PME,	:	Polymethylesterase
PNL	:	Pectin lyase
Ppase	:	Protopectinase
PPE	:	Pineapple peel extract
PSE	:	Pineaple stem extract
RD	:	Restriction Digestion
rDNA	:	Ribosomal DNA
RGI	:	Rhamnogalacturonan
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
RSM	:	Response surface methodology
RMSD	:	Root-Mean-Square Deviation
SDS	:	Sodium Dodecyl Sulfate
SmF	:	Submerged fermentation
SSF	:	Solid-state fermentation
UV	:	Ultra Violet
WMRE	:	Watermelon rind extract
XG	:	Xylogalacturonan
YEP	:	Yeast extract pectin

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Enzymes are incredibly efficient and highly specific biocatalysts. Over last few decades, with the advancement in biotechnology, they have contributed momentously to the traditional and modern chemical industry by enhancing the existing processes. Enzymes have been exploited by humans for Centuries and they are one among the most important products obtained for human needs through microbial sources.

The history of industrial enzymes backdated to 1874 when Hansen produced chymosin from the stomach of calves for making of cheese. Jokichi Takamine was the first person to manufacture an enzyme (Taka-diastase) from a microbial source (*Aspergillus)* as a digestive enzyme in 1894. The modern era of industrial enzymology began in 1913 when Otto Rˆhm obtained a patent for the use of a crude protease mixture isolated from pancreases in laundry detergents (Rastall, 2007). Though the discovery of enzyme production from microorganisms backdated in the 20^{th} century, studies on their isolation, identification, characterization, properties, production on bench-scale to pilot-scale and their application in bio-industry have continuously progressed, and the knowledge has repeatedly been updated (Nigam, 2013). Thus, the properties of many enzymes appropriately been understood only in the recent era and research on enzymes has now entered a new phase with the fusion of ideas from protein chemistry, molecular biophysics, and molecular biology (Aehle *et al.,* 2007). The demand for industrial enzymes is increasing each year on a worldwide basis. As per the recent data, the industrial enzymes market was priced at $ 4.2 bn in 2014 and is estimated to

increase at a Compound Annual Growth Rate (CAGR) of 7.0 % from 2015 to 2020. The market for food & beverage projected to reach a value of $ 2.0 bn by 2020 (Rohan, 2017).

1.1. Pectinase

The enzyme pectinases comprise a group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. Primarily, there are two groups of pectic enzymes; the de-esterifying enzymes which catalyze the de-esterification of pectins and the depolymerizing enzymes which break the glycosidic α-(1- 4) bonds between GalA (galacturonic acid) residues either by hydrolysis (hydrolases) or by trans-elimination (lyases). Another group of the pectic enzyme is protopectinase (PPase) which convert insoluble native protopectin into soluble pectins (Alkorta *et al.,* 1998; Garg *et al.,* 2016; Hassan *et al.,* 2016; Kashyap *et al.,* 2001; Sharma *et al.,* 2013).

Pectinolytic enzymes having great industrial importance and are widely used in food and textile industries (Kashyap *et al.,* 2001). Among the different enzymes, pectinase is important in the food processing industry and it has been evaluated that microbial pectinases represent 25 % of the worldwide food enzymes sales (Jayani *et al.,* 2010). The commercial application of pectinase started since 1930 for the making of wines and fruit juices. Today, they are one of the emerging enzymes and have been used in diverse conventional industrial processes.

1.2. Pectin; the substrate for pectinase enzyme

The structural polysaccharide pectin or pectic substances are a heterogeneous group of high molecular weight, complex molecules composed of GalA residues joined by - (1-4) linkages (Figure: 1.1) (de Vries *et al.,* 2001; Voragen *et al.,* 2009). It is a structural polysaccharide contained in the primary cell wall and middle lamella of fruits and vegetables and perhaps the supreme complex macromolecule in nature. The plant cell walls comprise of three strata or layers namely middle lamella, primary cell wall and secondary cell wall as depicted in Figure: 1.2. The primary walls of enlarging plant cells are composed of approximately 30 % cellulose, 30 % hemicellulose and 35 % pectin with about 1-5 % structural protein (glycoprotein) on a dry weight basis (Cosgrove, 1997). The middle lamella is the layer which is rich in pectins. This outermost layer develops the interface between adjacent plant cells and sticks them together (Buchanan *et al.,* 2015). In plants, the pectins form a unified and continuous layer between adjacent cells. Normally, it is difficult to distinguish the middle lamella from the primary wall, particularly in cells that develop thick secondary walls. In such cases, the two adjacent primary walls and the middle lamella, and perhaps the first layer of the secondary wall of each cell, may be called a compound middle lamella (Raven *et al.,* 2001).

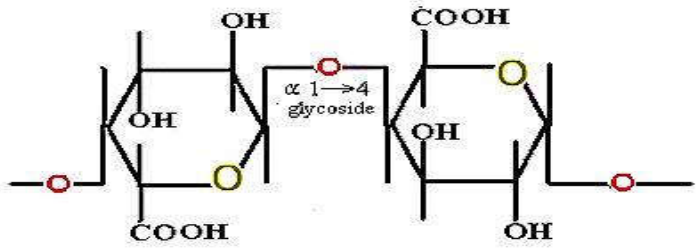


Figure: 1.1. Primary structure of pectin

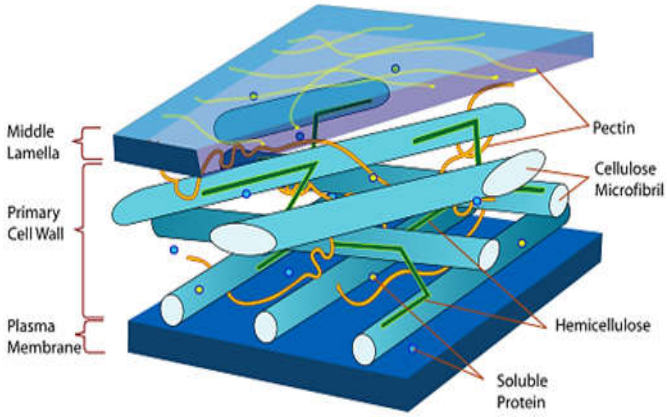


Figure: 1.2. Structure of the plant cell wall: Adapted from (foundation.wikimedia.org)

Lignified tissues have a low content of pectic substances compared with young, actively growing tissues. In higher plants, the content of pectic substances is extremely low (usually <1 %). But they are predominantly found in fruits, vegetables and a large part of some algal biomass (up to 30 %) (Kashyap *et al.,* 2001; Sakai *et al.,* 1993). Contrary to the proteins, lipids and nucleic acids, which are polysaccharides, pectic substances do not have defined molecular masses.

Pectin exhibits increasing applications in various fields; especially in food industry as a thickener, stabilizer, emulsifier, texturizer, water binder in jams and jellies, confections and bakery products, milk products etc. (Saha *et al.,* 2010), pharmaceutical industry as a matrix for the entrapment drugs, carrier for drug delivery and site-specific targeting, as dietary fibre etc. (Sriamornsak, 2003). In several foods, pectin is the solely allowable gelling agent and is definitely the foremost obvious selection as the fruits naturally contain pectin and added pectin supplementing this. Over a long period, encouraging public reference of pectin has proven helpful in its pervasive use, and this may be a contributing factor to the growing interest in investigating pectin for possible direct health benefits and thus applications in the regulated non-food segment as well as in functional foods and nutraceuticals. Pectin is also studied for its potential in drug delivery (Chambin *et al.,* 2006) and for making biodegradable films (Hoagland *et al.,* 1996).

1.3. Structural types of pectin

By definition, pectin is as a hetero-polysaccharide mostly comprising GalA residue, in which varying proportions of the acid groups are present as methoxyl esters, while a certain amount of neutral sugars might be present as side chains (Kertesz, 1951). It can be divided into two regions ‘‘smooth region’’ and ‘‘hairy region’’. De Vries recognized a pattern of “smooth” homo galacturonic regions and ramified “hairy” regions, in which most of the neutral sugars are located (de Vries *et al.,* 2001). Generally, it comprises of three

structurally well-characterized polysaccharide motifs; homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Prathyusha *et al.,* 2011; Voragen *et al.,* 2009). These three polysaccharides form a network, which has considerable potential for modulation of its structures by the action of cell wall degrading enzymes.

Homogalacturonan (HG): The primary component of pectic polysaccharides is HGA (constituting about 65 % of the pectin) and contains (1→4)-d-linked GalA which can be acetylated or methyl esterified, called smooth regions of pectin.

Rhamnogalacturonan I (RGI): RGI constitutes 20 % to 35 % of the pectin and is composed of a backbone of alternating rhamnose and GalA residues with side chains containing galactose and/or arabinose residues.

Rhamnogalacturonan II (RGII): RGII is also a homogalacturonan chain but with complex side chains attached to the GalA residue and constitute less than 10 %. Vincken *et al.,*(2003) suggested a pectin molecule structure model in which HG and RGII are long side chains of RGI backbone. The hairy regions of pectin molecule include both rhamnogalacturans (RG I and RG II).

Other substituted galacturonans (GS) have also been described in a small number of plants. Xylogalacturonan (XG) contains β-D-xylosyl (Xylp) linked in C3 of the main chain and is existent in reproductory tissues of plants such as carrot, apple and cotton. Apiogalacturonan

(AG) comprises monomers or dimers of β-D-apioduranosyl (Apif) attached in C-2 and C-3 of the main chain. Apiogalacturonan is found in some monocotyledons (Ridley *et al.,* 2001). The schematic representations of these structural domains are depicted in Figure: 1.3.

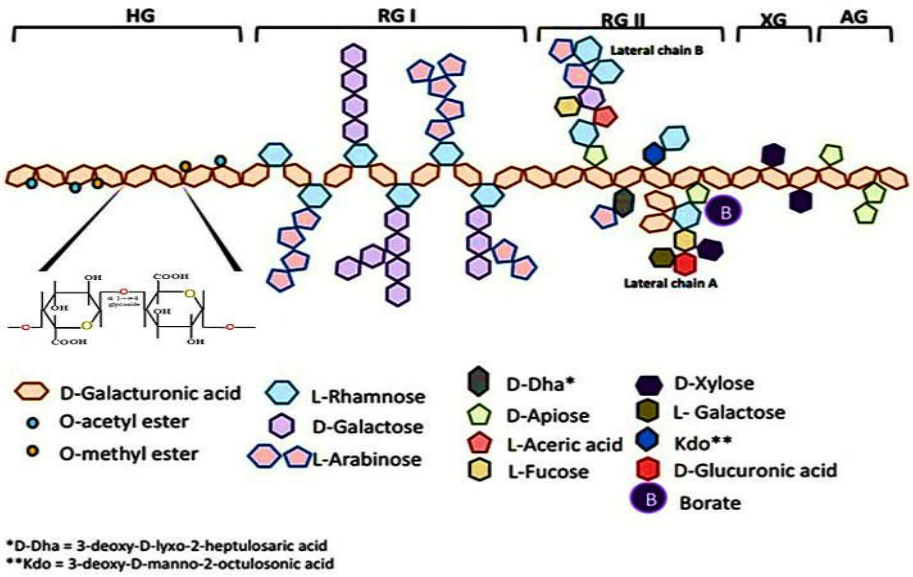


Figure: 1.3. Schematic representation of pectin structure. Data modified from (Leclere *et al.,* 2013)

1.4. Classification of pectic substances

Pectic substances are classified to four main types by the American Chemical Society as follows; (1) Protopectin, (2) Pectic acid (3) Pectinic acids and (4) Pectin (Alkorta *et al.,* 1998; Kashyap *et al.,* 2001; Kertesz, 1951). Protopectin is the water insoluble immature parent pectic substance and on hydrolysis, they yield pectin or pectic acid. Except for protopectin, the other three are either entirely or

partially water soluble. Pectin is the generic name for the mixture of widely differing compositions consists of pectinic acid as the major component. More description about these pectic substances are given in Table: 1.1.

Table: 1.1. Classification of pectic substances

Protopectin	• Precursor of pectin found in the unripen fruits • Pectic substance fixed in the plant tissue • Insoluble in water • It cannot form gels • Transformed to pectin by heating with water or acid • On restricted hydrolysis, protopectin yields pectin or pectic acid
Pectin	• Formed from protopectin during ripening of the fruits • Partly, 75 % of the carboxyl groups of the GalA residues are esterified with methanol • Soluble in water
Pectinic acid	• Partially de-esterified pectin • contains > 0 and < 75 % methylated galacturonate units • Soluble in water
Pectic acid	• Completely de-esterified pectin found in over ripen fruits • Contains an insignificant amount of methoxyl groups. • Soluble in water

1.5. Classification of pectinase

Generally, pectin degrading enzymes are classified as three categories based on;

a) Substrate preference – either pectin, pectic acid or oligo-D-galacturonate as the substrate used.

b) Pattern of action- if the cleavage is random (*endo*-enzymes) or terminal (*exo*- enzymes) and

c) Type of cleavage - whether pectinases act by hydrolysis (hydrolases) or by transelimination (lyases) (Blanco *et al.,* 1999; Tapre *et al.,* 2014).

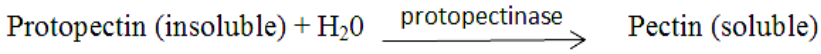
Protopectinases, polygalacturonases, lyases and pectin esterases are among the extensively studied pectinolytic enzymes. The extensive classification of pectinase acting on pectic substrates are shown in Table: 1.2 and in Figure: 1.4. Further, the action of these enzymes in the pectin backbone is shown in Figure: 1.5.

Table: 1.2. Extensive classification of pectinase enzymes

Enzyme	EC No.	Other names of the enzyme
De-esterifying enzymes		
Pectin methyl esterase (PME)	3.1.1.1	pectinesterase/pectin demethoxylase / pectin methoxylase/ pectase/ pectinoesterase
Pectin acetyl esterase (PAE)	3.1.1.6	
De-polymerizing enzymes		
a). Hydrolases		
Endopolygalacturonase (endo-PG)	3.2.1.15	poly (1,4- α -D-galacturonide) glycanohydrolase/ pectin depolymerase/ pectinase/mpectolase/ pectin hydrolase/ pectin polygalacturonase/ endo-D-galacturonase.
Exopolygalacturonase (exo-PG)	3.2.1.67	poly (1,4- α -D galacturonide) galacturonohydrolase/ poly(galacturonate) hydrolase/ exo-D-galacturonase/exo-D-galacturonanase/ exopoly-D-galacturonase
Exopolygalacturonan-digalacturono hydrolase	3.2.1.82	poly(1,4-α-D-galactosiduronate) digalacturonohydrolase Exopolygalacturonase-II/ exopolygalacturonosidase
Oligogalacturonate hydrolase (OGH)		
Endopolymethylgalacturonase (endo-PMG)		Pectin hydrolase
Exopolymethylgalacturonase (exo-PMG)		Pectin hydrolase
b. Lyases		
Endopolygalacturonate lyases (endo-PGL)	4.2.2.2	poly (1,4- α -D-galacturonide) lyase/ pectate lyase / polygalacturonic acid trans-eliminase endopectin / methyltranseliminase/ pectate transeliminase/pectic acid transeliminase endogalacturonate transeliminase/ pectic acid lyase/ Polygalacturonic acid trans-eliminase/ pectic lyase/ PL/ PGL/PGTE/PEL
Exopolygalacturonate lyases (exo-PGL)	4.2.2.9	poly(1,4- α -D-galacturonide) exolyase/ exopectate lyase (exo-PL)/ pectate disaccharide-lyase/ pectate exo-lyase/ exopectic acid transeliminase (PATE)/ exopolygalacturonic acid-trans-eliminase (exo-PGL)
Endopolymethylegalacturonate lyases (endo-PMGL)	4.2.2.10	poly(methoxygalacturonide) lyase, pectin transeliminase/ endopectin lyase/ polymethylgalacturonic transeliminase/ pectin methyltranseliminase/ pectolyase/ PNL, PMGL,PMTE
Exopolymethylgalacturonate lyase (Exo-PMGL)		Exopectinlyase
Protopectinase (PPase)		
Type -A PPase	acts on polygalacturonic acid moiety (inner site) in protopectin for solubilization	
Type -B PPase	acts on the remaining moieties (e.g., linkage site between a pectic substance and a cellulose molecule)	

1.5.1. Protopectinases (PPases)

The enzyme system PPases is known to convert insoluble native protopectin into soluble pectins and brings about a maceration of cells. It catalyzes the following reaction;



There are two types of protopetinases based on their reaction mechanism. A-type: A-type PPases react reacts with the polygalacturonic acid region of protopectin (inner site). B-type: they act on the outer site, ie., with the polysaccharide chains that may link the polygalacturonic acid chain and cell wall constituents (Sakai *et al.,* 1993). A-type PPases are again classified into PPase-F, -L and –S based on the organism they isolated and all these types are having an approximate molecular weight of 30 kDa. B-type PPases are also in different forms such as PPase- B, -C and –T. PPase-B, -C and -T have molecular weights of 45, 30, and55 kDa, respectively. PPase-B and -C have an isoelectric point (pI) of around 9.0 whereas PPase-T has a pI of 8.1.

1.5.2. Esterases

The group pectin esterases comprise pectin methylesterase (PME) and pectin acetyl esterase (PAE). These enzymes act before the action of PG and PL which need non-esterified substrates (Kashyap *et al.,* 2001). These are a well-studied group of enzymes, which belong to

carbohydrate esterase (CE) family 8 of CAZy database (Remoroza *et al.,* 2015).

1.5.2.1. Pectin methylesterase (PME): (EC 3.1.1.11)

The pectic enzyme PME hydrolyzes de-esterification of the methoxyl group of pectin releasing pectic acid and methanol (Stutzenberger, 1992). It is reported that the molecular weight of most microbial and plant PEs is in a range between 30-50 kDa (Christensen *et al.,* 1998; Gummadi *et al.,* 2007; Hadj-Taieb *et al.,* 2002). The optimum pH for activity is between 4.0 and 7.0. PE from *Erwinia* is an exception and having an optimum pH in the alkaline range. The temperature optimum varies between 40 – 60°C, and the pI between 4.0 and 8.0.

1.5.2.2. Pectin acetyl esterase (PAE): (EC 3.1.1.6)

In both HG and RG-I, the GalA residues can be acetylated at positions O-2 or O-3 and the degree of acetylation can be regulated by PAE (Philippe *et al.,* 2017). They hydrolyze the acetyl ester from the HG region of pectin forming pectic acid and acetate (Remoroza *et al.,* 2014).

1.5.3. Depolymerizing enzymes:

The depolymerizing enzymes/ depolimerases break the glycosidic α-(1- 4) bonds between GalA residues either by hydrolysis (polygalacturonases) or by trans-elimination (lyases).

1.5.3.1. Hydrolases: Depolymerizing enzymes break the glycosidic α-1- 4- bonds between GalA residues include;

1.5.3.1.A. Polymethylgalacturonases (PMG) - (EC 4.2.2.2): PMG attack pectins of high methoxyl content and catalyze the hydrolytic cleavage of α –1,4-glycosidic bonds forming 6-methyl-D-galacturonate. Based on the pattern of action, they may be;

i. **Endo-PMG:** act by random cleavage of α –1, 4-glycosidic linkage of pectin (mostly highly esterified pectin).

ii. **Exo-PMG:** make successive cleavage of α –1, 4-glycosidic linkage of pectin from the non-reducing end of the pectin chain.

1.5.3.1.B. Polygalacturonases (PG) – PG prefer pectic acid (polygalacturonic acid) as the substrate to catalyze the hydrolytic cleavage of α –1,4 -glycosidic linkages with endo and exo activities as in the above case;

i. **Endo-PG -** (EC 3.2.1.15): also known as poly (1,4-a-D-galacturonide) glycanohydrolase, pectin depolymerase/ pectinase/ pectin hydrolase/ pectin polygalacturonase/ endo-polygalacturonase/ endo-D-galacturonase. They act by hydrolysis of α - 1,4-glycosidic linkages of pectic acid in random mode.

ii. **Exo-PG -** (EC 3.2.1.67): also known as poly (1,4-a-D-galacturonide) galacturonohydrolase/ polygalacturonate hydrolase/ exo-D-galacturonase/ exopoly-D-galacturonase.

They act on α -1, 4-glycosidic linkage of pectic acid in a serial fashion.

Exo-PGases can be differentiated into two types: fungal exo-PGases and bacterial exo-PGases based on the GalA acid end product produced. The first one produces monogalacturonic acid and the later produces digalacturonic acid as the primary end product (Sakai *et al.,* 1993). Often, these enzymes present in different forms and the molecular weight varies between 30 - 80 kDa, and pI is in the range of 3.8 and 7.6. Their optimum pH is in the acidic range of 2.5 - 6.0 and the optimum temperature between 30 – 50 °C.(Singh *et al.,* 2002; Takao *et al.,* 2001).

1.5.3.2. Lyases: Lyases or trans-eliminases are depolymerizing enzymes which break α (1, 4)-glycosidic bond by eliminative cleavage and forms oligosaccharides with a unsaturation between C-4 and C-5 at the non-reducing end. They comprise**;** polymethylegalacturonate lyases (PMGL) and polygalacturonate lyases (PGL).

1.5.3.2.A. Polymethylegalacturonate lyases (**PMGL):** They act on the substrate pectin and break down the chain by trans-eliminative cleavage. They are;

i. **Endo-PMGL -** (EC 4.2.2.10): they catalyse the random cleavage of a-1,4-glycosidic linkages in pectin. Also known as pectin *trans*-eliminase (PTE)/ endo-pectin lyase (PNL), PL, poly (methoxygalacturonide) lyase, polymethylgalacturonic

transeliminase/ pectin methyltranseliminase/endo-pectin lyase/ pectolyase.

ii. **Exo-PMGL**: catalyzes the sequential breakdown of pectin by trans-eliminative cleavage.

1.5.3.2.B. Polygalacturonate lyases (PGL): These enzymes act on the substrate pectic acid and catalyze breakage of a-1,4-glycosidic linkage by trans-elimination. Also divided into endo and exo acting enzymes.

i. **Endo-PGL** - (EC 4.2.2.2): also known as pectate lyase (PEL)/ polygalacturonic transeliminase/pectate transeliminase/ pectic acid transeliminase/ endogalacturonate transeliminase/ pectic acid lyase/ α-1,4-D-endopolygalacturonic acid lyase/ endo-α-1,4-polygalacturonic acid lyase/ polygalacturonic acid lyase/ pectin *trans*-eliminase/ polygalacturonic acid *trans*-eliminase/ pectic lyase. They break down the α-1,4 glycosidic linkages in pectic acid by random cleavage.

ii. **Exo-PGL** - **(**EC 4.2.2.9): These enzymes catalyze the progressive breakage of α-1, 4-glycosidic linkages in pectic acid. Also having different names such as poly (1, 4-α-D-galacturonide) exolyase/ exopectate lyase/ exopolygalacturonic acid trans-eliminase/ pectate exo-lyase/ pectate disaccharide-lyase/exopectic acid transeliminase/ PATE.

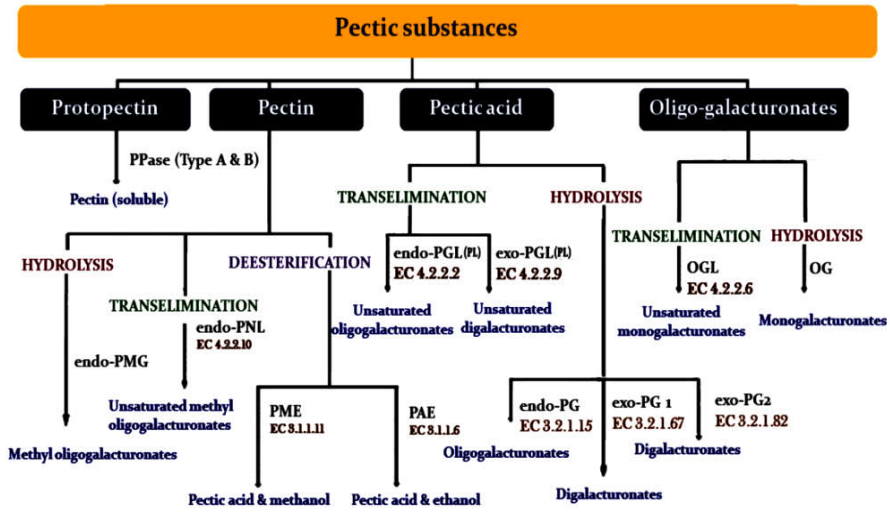


Figure:1.4. The action of pectinase enzymes on pectic substances: Data modified from (Alkorta *et al.,* 1998; Jayani *et al.,* 2005) PMG: Polymethyl galacturonase, PNL: Pectin lyase, PGL: Polygalacturonate lyase, PN: Pectate lyase PME: Pectin methylesterase, PAE: Pectin acetylesterase, OGL: Oligogalacturonide lyase, OG: Oligogalacturonase

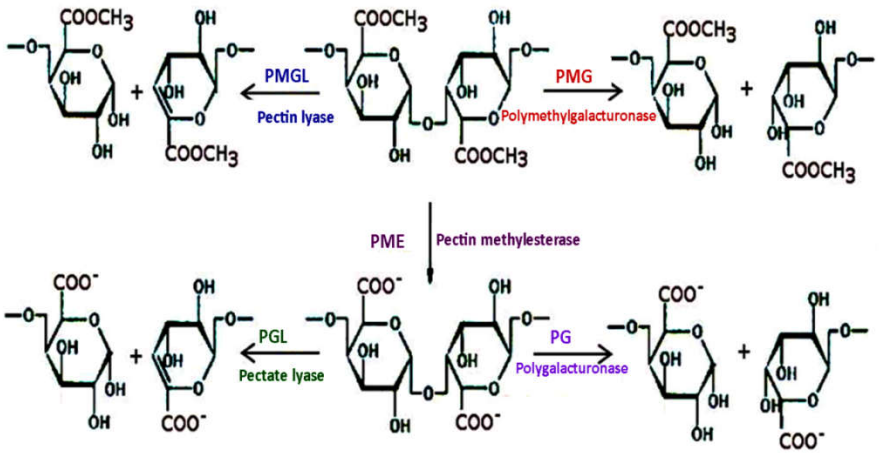


Figure:1.5. Enzymatic mode of action of PMGL, PMG, PGL and PG on the pectin molecule (Sharma *et al.,* 2013).

Based on the optimum pH for enzyme activity, pectic enzymes are also classified into two, acidic and alkaline pectinase. Acidic pectinases are used mainly in the fruit juice industry for extraction and clarification of fruit juices, improvement of chromaticity and stability of red wines etc. It also has application in maceration of plant tissue, liquefaction and saccharification of biomass, isolation of protoplasts. Whereas, alkaline pectinase are mostly used in the degumming and retting of fiber crops, textile processing and bioscouring of cotton fibers, pretreatment of pectic wastewater from fruit juice industries, paper making, coffee and tea fermentation, enzyme based oil extraction etc. (Kashyap *et al.,* 2001; Sharma *et al.,* 2013).

Deuel and Stutz classified PG into three types, although every type may contain PG of different specificities and properties. Type 1, the liquefying PG, which split the glycosidic linkages more or less at random. They preferentially attack pectins of low degree of esterification. Type 2, they preferentially attack pectins of high degree of esterification. Type 3, saccharifying enzyme, which hydrolyzes pectins only from one end of the chain molecule (Deuel *et al.,* 1958).

1.6. Source and industrial production of pectinase

It is a well-known fact that, microbes are the preeminent source of enzymes because they allow an economical technology with truncated resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Hoondal *et al.,* 2002). The production of pectin degrading enzymes has been extensively reported and meticulously studied in bacteria and

filamentous fungi because they play an essential role in the phytopathogenesis (Blanco *et al.,* 1999). Several reports are available for the pectinase enzymes by microorganism such as bacteria (Prathyusha *et al.,* 2011; Yu *et al.,* 2018), fungi (Alkorta *et al.,* 1998; Finkler *et al.,* 2017) yeast (Alimardani Theuil *et al.,* 2011), actinomycetes (Kuhad *et al.,* 2004) etc**.** They are also distributed in higher plants and some protozoa, nematodes and insects but they are not found in higher animals (Whitaker, 1990). Pectinolytic enzymes from microorganisms thus play a crucial role in nature by breaking down pectin polymer for nutritional purposes (Yadav *et al.,* 2009). It is reported that, dominant share (50 %) of the accessible enzymes are initiated from fungi and yeast followed by bacteria (35 %). The remaining 15 % are either of plant or animal origin (Soares *et al.,* 1999).

Microbes are chosen as a source of enzyme production compared to plants and animals because; (a) they produce a wide variety of enzymes and their enzyme contents are more predictable and controllable, (b) generally economical in bulk production and dependable provisions for raw material of constant composition, (c) high productivity rate and enzymes obtained via microbial source are higher in volume, (d) microbes are easy to manipulate to derive enzymes of desired nature and they can be cultured in large quantities in a relatively short period of time by the established method of fermentation using sophisticated tools, (e) they can be made to produce enzymes over wide range of environmental condition and (f) plant and animal tissues contain more potentially harmful materials than

microbes, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases (Chaplin *et al.,* 1990).

1.7. Microbial production of pectinase

1.7.1. Fungi

Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge, 1997). Many extracellular enzymes are produced by fungi which are capable of decomposing organic matter and one such enzyme is pectinolytic enzymes. The fungi are considered as one of the most potent producers of pectinases and they can be employed extensively in SSF for the economic production process. Various types of fungal species have been reported to be employed for the production of pectinases. *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Gummadi *et al.,* 2003). Other species of *Aspergillus* were also reported to produce pectinase including *A.oryzae A. fumigatus*, *A.terreus, A.soje, A.awamori* etc. (Garg *et al.,* 2016; Pedrolli *et al.,* 2009). Further, species of *Penicillium*, *Fusarium, Mucor*, *Neurospora crassa*, *Sclerotinia sclerotiorum,* etc. also have a role in pectinase production (Garg *et al.,* 2016; Pedrolli *et al.,* 2014; Pedrolli *et al.,* 2009).

1.7.2. Yeast

The pectinolytic enzyme production in yeasts has received less consideration and only a few yeast species show this ability (Blanco *et al.,* 1999). Yeast species reported for pectinase production includes;

Saccharomyces sp., Cryptococcus sp., Aureobasidium pullulans, *Rhodotorula dairenensis*, *Kluyveromyces marxianus*, *Geotrichum klebahnii*, *Wickerhanomyces anomalus* etc. (Alimardani Theuil *et al.,* 2011; Hassan *et al.,* 2016; Merin *et al.,* 2015; Naumov *et al.,* 2016).

1.7.3. Bacteria

A review of the currently available literature reveals little quantitative information about the diversity of bacterial genera having pectinolytic properties. Bacterial pectinase is produced mainly by bacteria belonging to genera *Bacillus* and *Erwinia.* Among Erwinia species, *E. carotovora* and *E. chrysanthemi* have been gained substantial importance in pectinase production. The important Bacillus sp.have been reported for pectinase production include; *B. subtilis, B. pumilus, B. licheniformis, B. Polymyxa,et.* A list of Bacillus spp. involved in pectinase production was reviewed by Kavuthodi & Sebastian, (2018). Other major bacterial genera reported to have pectinolytic properties include species of; *Pseudomonas* (Sohail *et al.,* 2016)*, Streptomyces* (Ramirez-Tapias *et al.,* 2015) *Lactobacillus* (Karam *et al.,* 1995), *Enterobacter* sp. (Reddy *et al.,* 2016) etc.

1.8. Importance of bacterial production of pectinase

Aspergillus niger, a generally recognized as safe (GRAS) microorganism is the major organism used for the industrial production of pectinase. However, this mould also secretes several other enzymes which may trigger collateral reactions such as the release of volatile phenols less desirable for the production of wine or fruit juices, for

instance, arabinofuranosidase, which can cause turbidity (Whitaker, 1990). Pectinases from fungal sources are produced best under acidic pH and low temperature conditions and can therefore not be used in various industrial bioprocesses which utilize neutral to alkaline pH with high temperatures exceeding 45 °C. It has been shown that bacteria produce pectinase that withstands high pH and temperature (Andrade *et al.,* 2011; Hoondal *et al.,* 2002). Also, it is easy to harvest pectinase than fungus as it is an extracellular product in bacterial culture (Sohail *et al.,* 2016). Bacterial strains producing commercial enzymes are always preferred over fungal strains because of ease of fermentation process (for production) and implementation of strain improvement techniques or any modern technology to increase the production yield (Prathyusha *et al.,* 2011). Moreover, bacterial pectinases with novel properties have the added advantage that enzyme production is achieved in less time as compared to fungal sources (Joshi *et al.,* 2015).

1.9. *Bacillus* species for industrial enzyme production

Among the diverse types of microorganisms inhabiting the soil, bacteria are the amplest and major organism. Considering the bacterial genera of soil, Bacilli are most abundant followed by Cocci and Spirilla. The genus Bacillus and Cocci comprises several varieties of industrially important species contributing approximately half of the existing commercial production of bulk enzymes (Aaisha *et al.,* 2016). *Bacillus* species have been the imperative industrial enzyme producers with roles in applied microbiology for over a millennium.

Because of several reasons *Bacillus* species continue to be the predominant bacterial workhorses in microbial fermentations (Satyanarayana *et al.,* 2005; Schallmey *et al.,* 2004). They produce more than two dozen biologically active molecules generating a high potential for biotechnological and biopharmaceutical applications (Stein, 2005). Also, it is estimated that enzymes from *Bacillus* spp. makeup about 50 % of the total enzyme market (Schallmey *et al.,* 2004). Another major feature that makes these groups predominant is that most of them are environmental friendly, don't have fastidious nutritional requirements and are easy to grow and handle (Sohail *et al.,* 2016). *Bacillus* species such as *B. subtilis* and *B. licheniformis* are on the Food and Drug Administration's GRAS status (Schallmey *et al.,* 2004). Moreover, the biochemistry, physiology, and genetics of *B. subtilis* and other species are well studied and the complete genome sequence of *B. subtilis* 168 comprising of 4100 protein-coding genes have been published in 1997 (Kunst *et al.,* 1997).

Various properties of *Bacillus* strains are reported which make them superior in industrial biotechnology, including their high growth rates leading to short fermentation cycle times, ability to secrete proteins into the extracellular medium (Acton, 2012; Barros *et al.,* 2013) and their ability to adapt with changing environmental and nutritional conditions (Nicolas *et al.,* 2012). The capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/l) of extracellular enzymes has placed them among the most important industrial enzyme producers (Satyanarayana *et al.,* 2005). Moreover, many researchers used strains belonging to *Bacillus,* because this

genus includes strains (such as *B. subtilis)* that can grow on cheap substrates such as agro-wastes (Sakai *et al.,* 1989).

1.10. Pectinase production by *Bacillus* spp.

Even though, the chief source of acidic pectinases is fungi but alkaline pectinases are produced from alkalophilic bacteria, primarily *Bacillus* sp. Over the past few years, pectinolytic properties have been described in several *Bacillus* species. It is evident from many research works that, among different bacterial isolates screened for pectinolytic properties *Bacillus* strains were selected as the most potent enzyme producers (Jayani *et al.,* 2010; Kavuthodi *et al.,* 2015; Rehman *et al.,* 2012; Soares *et al.,* 1999; Sohail *et al.,* 2016). Aside from the principal fact (*Bacillus* spp. are the chief producers of alkaline pectinase), there are also some other reasons for researchers to focus on pectinase from *Bacillus* spp. These include; (i) they produce all class of pectic enzymes, (ii) have short fermentation period for enzyme production, (iii) can produce enzymes very economically by using different agro-wastes as cheap substrates, (iv) fermentation can be attained by either SSF, SmF and (v) genetic information regarding pectinase genes of many *Bacillus* spp. are available in various nucleotide sequence databases. Thus it supports successful cloning and expression of pectinase gene in other organisms (Kavuthodi *et al.,* 2018).

1.11. Industrial application of pectinase

Pectinase enzymes have a varied range of applications and are widely used in an industrial sector chiefly in the food industry. They

also have major application in textile, paper and pulp industries, waste-water treatment. Recently, application of pectinase along with cellulase in bioethanol production from lignocellulosic biomass also reported. The important applications of pectinase are shown in Figure: 1.6 and discussed below.

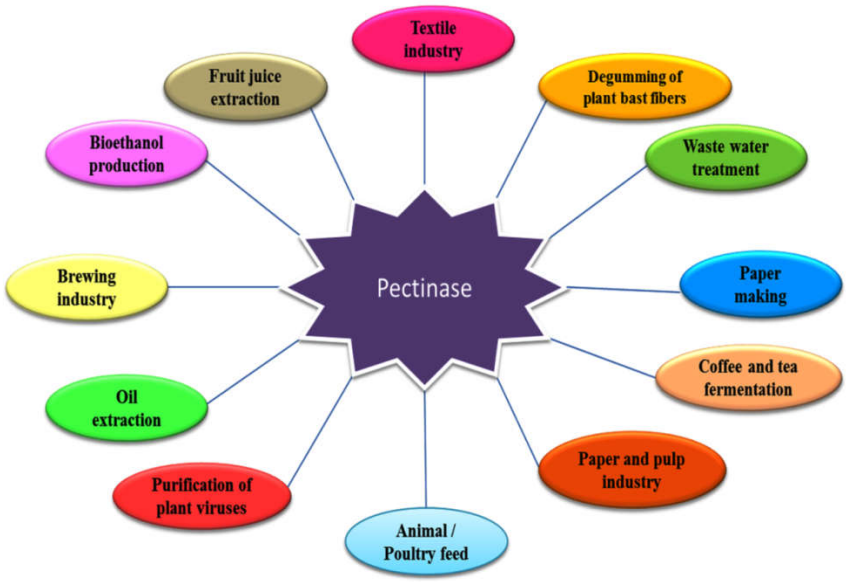


Figure: 1.6. Applications of pectinases in various industries

1.11.1. Fruit juice extraction

The fresh fruits and vegetables are classified as highly perishable commodities as they contain more than 80 % moisture content. The major component of fruits is water (75–90 %) mainly, sited in vacuole causing turgor to the fruit tissue. The cell wall of fruit consists of crystalline cellulose microfibrils embedded in an

amorphous matrix of pectin and hemicelluloses (Kumar, 2015). Generally, fruit juice is prepared by mechanical squeezing or macerating fresh fruits without the application of heat or solvents. Mechanical crushing of the pectin rich fruits results in a highly viscous fruit puree. It is very difficult to extract juice simply by direct pressing because, the mechanical crushing of the tissues gives juice that remains bound to the pulp to form a jellified mass. The pectin divides itself between the liquid phase and the pulp particles which in turn results in an upsurge in the viscosity of the juice and facilitating water retention (Bayindirli, 2010). The main advantages of using enzymes in fruit juice industry are; to increase extraction of juice from raw material, to increase processing efficiency (pressing, solid settling or removal), and generate a final product that is clear and visually attractive (Sharma *et al.,* 2015).

Acceleration in juice extraction and yield is a vital goal for juice manufacturing industries. The modern processes for fruit and vegetable juice manufacture exploit enzymes as key processing aids to obtain higher yields and clarity (Wang *et al.,* 2009). Pectinase has a major role in the juice industry for extraction and clarification of fruit juice. Enzymatic extraction of juices results in higher yield. Mainly the enzymes pectinase, cellulase, hemicellulase, arabinases, xylanases proteases, amylases and combination of these enzymes have been used to improve the pressing efficiency in fruits juice extraction process (Gailing *et al.,* 2000). Pectinase alone or mixture of pectinase with other carbohydratases like cellulase and amylase are used to clarify fruit juices (Sharma *et al.,* 2015). An increase in mango juice yield and

sensory quality was reported by Reddy *et al.,* (2009). In many studies, use of pectinases and amylases has been reported as an efficient substitute for depectinisation and thereby reducing turbidity.

1.11.2. Textile processing and bioscouring of cotton fibers

The application of enzymes in textile manufacturing has a long tradition and is an excellent example in the development of eco-friendly technologies in fibre processing and strategies to improve the final product quality (Araujo *et al.,* 2008). By replacing harmful chemicals such as caustic soda, pectinase in conjunction with amylases, lipases, cellulases and hemicellulases has been used to remove sizing agents from cotton in an environmental friendly and safe manner (Hoondal *et al.,* 2002). Bioscouring is an innovative process in the textile industry, for removal of noncellulosic impurities from the fiber with the aid of specific enzymes. Pectinases have been used for the bioscouring process without harming the cellulosic fibres. In bioscouring, pectinase is selected based on their pH and temperature compatibility concerning the time of treatment, end-product quality, water absorbency, whiteness and residual pectin (Hoondal *et al.,* 2002).

1.11.3. Degumming of plant bast fibers

Bast fibre is plant fibre obtained from the phloem or bast surrounding the stem of certain dicotyledonous plants. Commercially useful bast fibres include flax, hemp, jute, kenaf, ramie, roselle, sunn, and urena. These fibers are soft and have higher tensile strength than other fibers. Degumming of plant fibers such as ramie, sunn hemp, jute, flax and hemp is one of the most upcoming applications of

pectinolytic enzymes (Bruhlmann *et al.,* 1994; Cao *et al.,* 1992). Usually, these fibre is composed of overlapping cellulose fibres and a cohesive gum, or pectin thus before its use in textile making, the gum contained should be removed. Biological degumming using enzymes such as pectinase in combination with xylanases offer a profitable and eco-friendly alternative compared to the chemical process which is a non eco-friendly process (Kapoor *et al.,* 2001).

1.11.4. Wastewater treatment

Fruit and vegetable processing industries discharge wastewater containing pectin as a by-product that is difficult for microbial degradation during the activated-sludge treatment. Pretreatment with pectinolytic enzymes facilitates removal of pectinaceous material from these wastewaters and make it easier for further treatment process (Hoondal *et al.,* 2002).

1.11.5. Papermaking

In papermaking, pulping is the step in which cellulose fibers are broken apart and most of the lignin is removed. The residual lignin is then removed by a multistep bleaching process. Substitution of the chemical bleaching with biotechnological processes comprising microorganisms and enzymes such as pectinases (Reid *et al.,* 2000), cellulase and xylanases (Buzała *et al.,* 2016) have increased demand as they are both economical and eco-friendly. Presence of pectins in the pulp declines dewatering during sheet formation because of their high cationic demand and cause yellowness of paper. Treatment with

the pectinase has been reported to lower the cationic demand of thermomechanical pulp bleached with alkaline peroxide in the laboratory (Ricard *et al.,* 2004).

1.11.6. Coffee and tea fermentation

Application of pectinase in tea fermentation not only accelerates the fermentation process but also revoke the froth forming property of instant tea powders by breaking pectins (Wood, 2012). Also, they are used in the coffee fermentation to remove the mucilaginous coat from the coffee beans (Murthy *et al.,* 2011).

1.11.7. Animal / Poultry feed

Pectinase in combination with other enzymes is used for the making of animal feeds. The use of pectinases in ruminant feed production induces viscosity reduction, which in turn enhances absorption and liberation of nutrients 'either by the breakdown of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and so decreases the fecal matter' (Hoondal *et al.,* 2002).

1.11.8. Purification of plant viruses

Isolation and purification of viruses from the infected plant are essential to study their physiochemical and morphological characteristics. If the virus is localized in specialized plant cells such as phloem, then it is very difficult to isolate and it has been a concern of virologists for many years. The virus can be isolated from these

tissues with the aid of enzymatic treatments by pectinases and cellulases (Salazar *et al.,* 1999).

1.11.9. Oil extraction

Enzyme-based oilseed processing technologies emerge as one of the most environmental safe processing methods. Enzymes including cellulase, hemicellulase, pectinase and even proteases are the most promising enzymes for degrading the cell wall in oilseeds to loosen oil sacs embedded in the structures (Kalia *et al.,* 2001). Pectinase enzymes enhance the oil extraction by destroying the emulsifying properties of pectin that affects with the extraction of oil from citrus peel extracts (Kohli *et al.,* 2015; Pedrolli *et al.,* 2009). Application of pectinase was remarkably useful in the extraction of tocopherol from different genotypes of sunflower (Perez *et al.,* 2013). Pectic enzyme treatment resulted in increased calamansi oil yield (Espino *et al.,* 2005).

1.11.10. Improvement of chromaticity and stability of red wines

In the process of winemaking, improved visual characteristics (color and turbidity) was observed by the addition of pectinolytic enzymes in macerated fruits prior to the addition of wine yeast (Revilla *et al.,* 2003). Compared to the control wines, the pectinase treated wines exhibited more stability and easily filterable. The higher level of alcohol production and improvement in wine sensory quality was reported by (Reddy *et al.,* 2009) in pectinase treated mango wines.

1.12. Fermentative production of pectinase

Like the industrial production of every microbial enzyme, pectinase also produced by both submerged fermentation (SmF) and solid state fermentation (SSF) (Taragano *et al.,* 1999). In SmF, microorganisms are cultivated in large fermenters or bioreactors and the growth of microorganism takes place in liquid media (broth) and requires agitation, aeration, automatic pH, temperature and dissolved oxygen control. Whereas in SSF, the fermentation progression occur in the lack or very little amount of free liquid and microorganisms grown on a solid support selected for this purpose. Even though, SmF is widely used for enzyme productions like alkane pectinase, the major factor hindering the process include high production costs because of the overpriced reagents in synthetic media and low productivity because of the very long fermentation time. Therefore, microbial strains that grow quickly on cheap substrates and capable of tolerating SmF-based process for the production of pectinase needs to be developed to reduce the production cost (Zou *et al.,* 2014). Usually, SmF is applied in case of enzyme production by bacteria because of the requirement of higher water potential (Chahal, 1983). Researchers all over the world used various agro-residues for the microbial production of pectinase and other enzymes. But most of the works were based on SSF production. Compared to SmF, the great advantage of SSF is the lesser capital and operating costs due to the utilization of low cost agricultural and agro-industrial wastes as substrates (Mussatto *et al.,* 2012). This drawback of SmF can be overcome by the formulation of cheap media composed of agro-residues.

1.13. Pectinase enzyme production using agro-waste

Biowastes are highly perishable materials and their dumping often is a problem in processing industries. Presently, agricultural waste in India is generally burnt at the field itself or dumbed as soil fills this will create various environmental problems. An abundant amount of waste materials are produced by agricultural and fruit processing industries, which pose considerable disposal problems and ultimately leads to pollution. Vast varieties of microorganisms are present in the environment which can be exploited for the utilization of waste material. This will, in turn, can deliver a lot towards the proper management of agro-wastes and also ensure a better cleaner environment. Pectinase production from Bacillus spp. can be achieved economically by utilizing different agro-waste as substrates including, orange peel wheat bran, apple pomace, sugar beet, sugar cane bagasse and wheat straw etc. (Bibi *et al.,* 2016; Embaby *et al.,* 2014; Jahan *et al.,* 2017; Kaur *et al.,* 2017; Kuvvet *et al.,* 2017). This study tested the efficiency of various agro-wastes such as banana peduncle, watermelon rind, pineapple stem and pineapple peel for SmF production of pectinase.

Research objectives

In this contemporary biotechnological era, pectinolytic enzymes are of significant importance with their all-embracing applications mainly in food and textile industries. Although microbial pectinase is widely used in food processing and other industries in developed countries, it is still in its immaturity in developing countries

like India mainly because of the high costs involved. If economically viable technologies for production are available, it will promote the applications in our country also. Therefore, the priority of pectinase enzyme has elevated considerably and a lot of research is taking place for the effective and economical production of pectinase enzymes using inexpensive substrates generated from agro-residues. The proposed research also aims for the utilization of locally available agro-wastes for pectinase production by indigenous microbes and their improvement for enhanced enzyme production. The present work deals with the following main objectives;

- Isolation, screening and identification of pectinolytic bacteria.
- Application of strain improvement and media optimization (Response Surface Methodology-RSM) strategies for augmented pectinase production.
- The production of pectinase as a value added product from various agro-wastes, media formulation, RSM optimization and submerged fermentation production using laboratory scale fermenter.
- Purification and characterization of pectinase produced by the strain and cloning, sequencing and *in-silico* analysis of its pectate lyase (*pel*) gene.

DETAILS OF PUBLICATION

I. Research articles

1. **Bijesh K** and Denoj S. 'Biotechnological valorisation of pineapple stem for pectinaseproduction by *Bacillus subtilis* BKDS1: Media formulation and statistical optimization for submerged fermentation'. Biocatalysis and Agricultural Biotechnology. 2018, Corrected proof in Press, DOI: 10.1016/j.bcab.2018.05.003.

2. **Bijesh K** and Denoj S. 'Response surface methodological approach to optimize the critical medium components for augmented pectinase production by *Bacillus subtilis* BKDS1' Journal of Pure and Applied Microbiology, Vol. **12**(2), p. 981-991. DOI: 10.22207/JPAM.12.2.62.

3. **Bijesh K**, Steni K.T and Denoj S. 'Co-production of Pectinase and Biosurfactant by the Newly Isolated Strain *Bacillus subtilis* BKDS1'. British Microbiology Research Journal. 2015; 10 (2):1-12. DOI: 10.9734/BMRJ/2015/19627.

II. Review articles

1. **Bijesh K** and Denoj S. **'**Review on bacterial production of alkaline pectinase with special emphasis on Bacillus species'. Bioscience Biotechnology Research Communication. 2018*;* 11(1): 18-30. DOI: 10.21786/bbrc/11.1/4.

III. Abstract/ Proceedings/ Presentations

1. **Bijesh K** and Denoj S. 'Pineapple stem extract media; a new methodology for cost-effective production of pectinase using *Bacillus subtilis* BKDS1. 'Proceedings of two day international

seminar on Molecular Biology-an Underpinning to life Sciences; Jan 8-9,2018. Organized by Department of Zoology, Farook College, Calicut, Kerala.

2. **Bijesh K,** Ansha A, Malavika N.J and Denoj S. 'Exploitation of Agro-waste as a cost Effective Media for Exo-pectinase Production by submerged fermentation using *B. subtilis* BKDS1. 28th Kerala Science Congress (Jan 2016) Extended Abstracts: Agriculture & Food Sciences; 85-86.

3. **Bijesh K**, Malavika NJ, Ansha A and Denoj S. 'Biotechnological Prospective of Watermelon Rind for Economical Production of Exo-Pectinase using *Bacillus subtilis* BKDS1 by Submerged Fermentation.' Proceedings of the International Conference on Biodiversity andEvaluation: Perspectives and Paradigm shifts. Organized by Sree Sankara College, Kalady in association with the department of Marine Biology, Microbiology & Biochemistry, School of Marine Science, CUSAT, Cochin. 2015; 223-226 (ISBN 978-93-80095-70-7).

4. **Bijesh K**, Vinitha P and Denoj Sebastian. 'Isolation, Screening and Biological characterization of Pectinolytic bacteria from soil samples of Malabar area'. Southern Regional Conference of association of Clinical biochemist of India. National Conference on Advances in Laboratory Practice held at MES Medical College, Perinthalmanna, Malappuram, Kerala, on 13th and 14th of June 2015.

5. Vincy K, **Bijesh K** and Denoj Sebastian. 'Probiotic efficiency of *Bacillus subtilis* BKDS1, a potent pectinolytic strain isolated from dump yards of vegetable wastes'. BIOSPARK '16, UGC Sponsored National Seminar on Nanotechnology meets Microbiology. Organized by EMEA College of Arts & Science Kondotty, Malappuram-Kerala.

6. Vincy K, **Bijesh K** and Denoj Sebastian ‘Probiotic properties of *Bacillus subtilis* BKDS1, a potent bacteriocin producing stain isolated from dump yards of vegetable and fruit wastes’. Recent Trends in Microbiology, Twodays’ National seminar organized by Dept. of Life sciences, university of Calicut

CHAPTER 2

ISOLATION, SCREENING AND BIOCHEMICAL CHARACTERIZATION OF PECTINOLYTIC BACTERIA: MOLECULAR IDENTIFICATION OF THE SELECTED PECTINOLYTIC STRAIN AND ITS INDUSTRIALLY IMPORTANT PROPERTIES

2.1. Introduction

In the current biotechnological era, pectinases are one of the forthcoming enzymes showing a progressive increase in their market. They maintained the average annual growth rate of 2.86 % from 27.6 million $ in 2013 to 30.0 million $ in 2016, and it is estimated that by 2021, the market size of the pectinase will reach 35.5 million (Global Pectinase Market Research Report, 2017)

Naturally, pectinases are produced by the diverse range of microorganisms comprising fungi, bacteria, yeast and actinomycetes. Pectinases from fungal sources produce best under acidic pH and low temperature conditions and can therefore not be used in various industrial bioprocesses which utilize neutral to alkaline pH with high temperatures exceeding 45 °C. It has been shown that bacteria produce pectinase that withstands high pH and temperature (Andrade *et al.,* 2011; Hoondal *et al.,* 2002). Also, it is easy to harvest pectinase than fungus as it is an extracellular product in bacterial culture (Sohail *et al.,* 2016). So, considering these entire factors novel bacterial strains have to be identified for better production of pectinase.

Literature review reviled the fact that bacterial pectinase is produced mainly by bacteria belonging to genera *Bacillus.* Various properties of *Bacillus* strains are reported which make them superior in industrial biotechnology, including their high growth rates leading to short fermentation cycle times, ability to secrete proteins into the extracellular medium (Acton, 2012; Barros *et al.,* 2013) and their ability to adapt with changing environmental and nutritional conditions

(Nicolas *et al.,* 2012). Also, the capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/l) of extracellular enzymes has placed them among the most important industrial enzyme producers (Satyanarayana *et al.,* 2005). Moreover, many researchers used strains belonging to *Bacillus,* because this genus includes strains (such as *B. subtilis)* that can grow on cheap substrates such as agro-wastes (Sakai *et al.,* 1989).

In view of the potential industrial applications of pectinases, it is essential to identify the new source of microorganisms capable of producing the enzyme at a cheap rate. Therefore, in the present study, we aimed to isolate pectinase producing bacterial strains. Soil samples from different fruit and vegetable waste dumping sites of Malabar region-Kerala were taken for isolation of pectinase producers. A summary of the chapter is depicted in the graphical abstract Figure: 2.1.

2.2. Aim and objectives of the study

- Isolation, screening and identification of pectinolytic microbes from different sources.
- Pectinolytic activity confirmation by both qualitative (agar plate assay) and quantitative (DNS assay) methods.
- Selection of the potent pectinolytic strain and its identification by biochemical and molecular methods.
- Selection of the best culture media for pectinase production.

- Study of various industrially important properties of the selected strain.

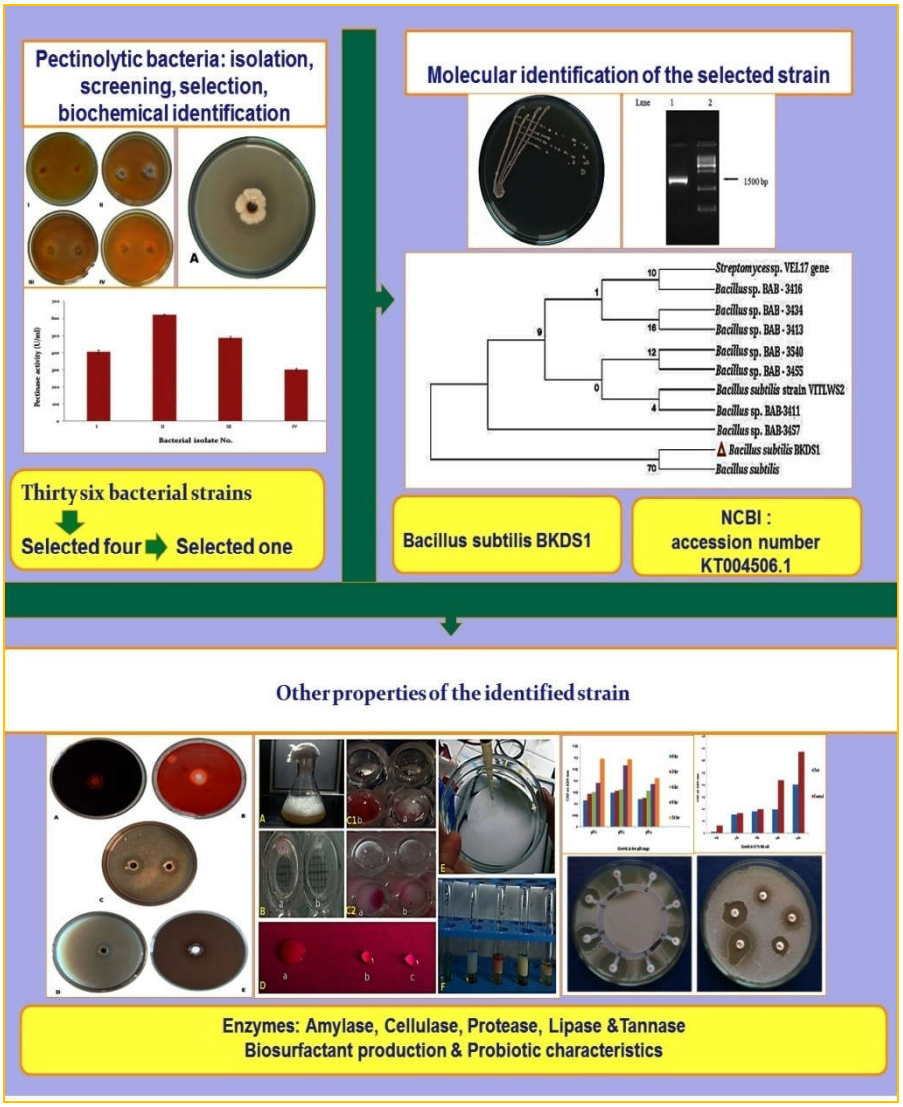


Figure: 2.1. Graphical abstract of the study

2.3. Review of literature

The potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Aehle, 2007). According to Jayani *et al.,* (2005), pectinase constitutes 25 % of the global food enzyme market. Information regarding pectinase types, microbial production, industrial applications, structure, substrates, sources, important characteristics etc. has been documented and reviewed by various authors.

2.3.1. Isolation and identification of microorganisms

The discovery of various industrially important microorganisms from the soil maximized the need of scientists for the isolation and their characterization for diverse application including the production of various primary and secondary metabolites. Though different methods are available to isolate and enumerate microorganisms from assorted samples, the serial dilution-agar plating method or viable plate count method is one of the commonly used procedures for the isolation and enumeration (Aneja, 2003). Several methods were designed for the identification and classifications of microorganisms. Bergey's Manual of Systematic Bacteriology, published subsequent to the Bergey's Manual of Determinative Bacteriology is the central resource for determining the identity of prokaryotic organisms, emphasizing bacterial species, using every characterizing aspect (McClung, 1985). Molecular identification or

genotypic identification of microorganisms have many significant advantages over conventional identification methods and is evolving as an substitute or complement to traditional phenotypic methods (Spratt, 2004; Tang *et al.,* 1998). A number of molecular markers that aids identification of specific microbial taxa and their phylogenetic classification have been reported over the past several spans (Clarridge, 2004; Liu *et al.,* 2012; Perrin *et al.,* 2015; Srinivasan *et al.,* 2015). It is widely accepted that, among these molecular markers, 16S rRNA, a 1500 base pair gene coding for a catalytic RNA that is part of the 30S ribosomal subunit is an important molecular marker to study bacterial phylogeny and taxonomy. Several features such as its essential function, ubiquity, and evolutionary properties make 16S rRNA the most common housekeeping genetic marker (Patwardhan *et al.,* 2014; Srinivasan *et al.,* 2015). The universal16S rDNA primer 8F and1492R was primarily used for PCR amplification of genomic DNA (Turner *et al.,* 1999).

2.3.2. Microbial production of pectinase

It is a well-known fact that microbes are the preeminent source of enzymes because they allow an economical technology with truncated resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Hoondal *et al.,* 2002). The production of pectin degrading enzymes has been reported and studied in bacteria and filamentous fungi because they play an essential role in the phytopathogenesis (Blanco *et al.,* 1999). Several reports are available for the pectinase enzymes by

microorganism such as bacteria (Prathyusha *et al.,* 2011; Yu *et al.,* 2018), fungi (Finkler *et al.,* 2017) yeast (Alimardani Theuil *et al.,* 2011), actinomycetes (Kuhad *et al.,* 2004) etc. *Aspergillus niger* is the major fungal species used for industrial production of pectinase (Gummadi *et al.,* 2003). Findings on the isolation, characterization, selection, properties and fermentation of *A. niger* strains for the production of pectinolytic enzymes using different substrates was made by various workers (Finkler *et al.,* 2017). Other species of *Aspergillus* were also reported for pectinase production including *A.oryzae* (Meneghel *et al.,* 2014), *A. fumigatus* (Wang *et al.,* 2015), A.terreus (Sethi *et al.,* 2016), *A.soje* (Demir *et al.,* 2016), *A. awamori (*Anuradha *et al.,* 2016), *A. giganteus* (Pedrolli *et al.,* 2014). Production of endo-PGL was firstly reported in *A. giganteus* (Pedrolli *et al.,* 2014). Further, species of *Penicillium* (Li *et al.,* 2015), Fusarium (Reddy *et al.,* 2015), Mucor (Thakur *et al.,* 2010) etc. The fungus produces these enzymes to extract nutrients from the plant's middle lamella (Rodrigues, 2016).

According to Blanco *et al.,* (1999), the pectinolytic enzyme production in yeasts has received less consideration and only a few yeast species show this ability and these yeasts mostly belonged to the genera of *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces.* The first reports of pectinolytic production by yeasts were described by Luh and Phaff in (1951) in *Saccharomyces fragilis*. Among different yeast species representing all yeast genera, they found that only six (*S. fragilis*, *S. fragilis* var. no. 351, *S. thermantitonum*, *Torulopsis kefyr*, *Candida pseudotropicalis* var. *lactosa*, and *Candida pseudotropicalis*)

were capable of causing a noticeable change in pectin (Luh *et al.,* 1954). Continuous pectinase production by the immobilized packed bed system was demonstrated using the yeast *Kluyveromyces* marxianus CCT 3172 (Almeida *et al.,* 2003**).** Importance of pectinolytic yeast in the winemaking process and their characteristics were studied by Merin *et al.,* (2015).

Bacterial pectinase is produced mainly by bacteria belonging to genera *Bacillus* and *Erwinia.* Elrod (1942), reported that the bacterium *Erwinia* sp. can degrade pectin with the aid of pectin degrading enzymes. Further, the pectinase production was comprehensively studied in soft-rotting *Erwinia* species such as *E. carotovora* and *E. chrysanthemi* which have been reported to produce pectinolytic enzymes such as PL, PG, PME, and PAE (Matsumoto *et al.,* 2003). The ability of *Streptomyces halstedii* ATCC 10897 for PG production was studied by Ramirez-Tapias *et al.,* (2015). Analysis of pectinolytic properties of *Pseudomonas* strains isolated from rotten fruits and vegetables reviled that *P. aeruginosa* isolated from oranges was the most competent PG producer equivalent to *B. vallismortis* (Sohail *et al.,* 2016). The ability of *Enterobacter* sp. PSTB-1for pectinase production and effect of media optimization was reported by Reddy *et al.,* (2016).

2.3.3. Pectinase production by *Bacillus* spp.

Bacteria in the genus *Bacillus* are the source of several enzymes of current industrial interest (Acton, 2012; Barros *et al.,* 2013). Pectic enzymes are of functional relevance in the retting process

and evidence regarding pectinolytic properties of *Bacillus* spp. was recorded years ago. Different species of the genus *Bacillus* have been reported to be retting agents and active against pectic materials (Potter *et al.,* 1955). The first *in vitro* fermentation studies of pectin and pectic acid was reported in 1955 using *B. Polymyxa*strain 30 (Potter *et al.,* 1955). Over the past few years, pectinolytic properties have been described in several *Bacillus* species. Studies conducted by various researchers reviled the fact that, *Bacillus* species were the predominant bacterial strain selected for pectinase production (Jayani *et al.,* 2010; Kavuthodi *et al.,* 2015; Rehman *et al.,* 2012; Soares *et al.,* 1999; Sohail *et al.,* 2016). In a recent study, it is reported that out of ninety five pectinolytic isolates from coffee pulp, it is observed by molecular identifications that, 70 % of the isolates are under genus Bacillus (Oumer *et al.,* 2018).

Bacillus spp. are reported to produce all classes of pectinases including hydrolases, lyases, esterases and protopectinases (Soriano *et al.,* 2005). *Bacillus* strains are inferred to be the potent sources of PG (Jahan *et al.,* 2017; Kobayashi *et al.,* 2001; Nagel *et al.,* 1961). Reda A *et al.,* (2008) reported PG production from *B. firmus*-I-4071. He used Czapek'sDox pectin medium for the growth and maintenance of this strain. Jayani *et al.,* (2010) screened different bacterial strains for pectinase production and the prominent strain was identified as *B. sphaericus* (MTCC 7542). This was the first report of PG production from *B. sphaericus* and for this study, they used production media containing 1 % citrus pectin. Among several bacterial strains isolated from soil and rotten vegetables, the strain which produced maximum

PG was identified as *B. licheniformis* KIBGE IB-21 (Rehman *et al.,* 2012). Pectinolytic properties of a new soil isolate B. *subtilis* C4 was reported in (2014) by Kusuma and reddy. PMG activity from *Bacillus* sp. strain BR1390, a novel environmental isolate was presented in the same year (Karbalaei-Heidari *et al.,* 2014). *Bacillus* spp. were also reported for endo PG production. Endo-PG, exo-PG and PNL activities of five *Bacillus* strains isolated from decaying vegetable material was studied by Soares *et al.,* (2001) and these enzymatic solutions resulted in a maximal reduction of the solution of citrus pectin viscosity, between 80 and 97 %.

Details regarding a bacterial strain (*Bacillus* sp. PN33) producing large amounts of extracellular PNL was revealed in 1998 where, the maximum activity was found at acidic pH of 6 and is an unusual example for bacterial PNL (Kim *et al.,* 1998). Another high yielding pectinase strain, *Bacillus* sp. DT7 producing alkalothermophilic PNL with a shorter incubation period of 24 hr was reported in (2000) by Kashyap *et al*. They used YEP medium for the isolation of this bacterium. A thermophilic bacterial strain of *Bacillus* sp. with endo-PL activity has been isolated by Tako *et al.,* (2000) and noted that it had PPase activity, besides PL activity on lemon protopectin and cotton fibers. A novel alkalophilic strain of *B. pumilus* BK2 producing a new type of extracellular endo- PL with high pI and a high pH optimum was reported in 2006 (Klug-Santner *et al.,* 2006). The PL producing ability tested from a group of six *Bacillus* species (*B. subtilis*, *B. pumilus*, *B. sphaericus*, *B. cereus*, *B. thuringiensis*, and *B. fusiformis*) isolated from cocoa fermentation, it was revealed that *B. fusiformis, B. subtilis*, and *B. pumilus* species

were the best PL producers compared to other species (Ouattara *et al.,* 2011). The major strains of *Bacillus* spp. that has been reported for pectinase production and its characteristic features are shown in Table: 2.1.

2.3.4. Present scenario

The reports on pectinase production by *Bacillus* sp. are continuing with the latest publications. Aaisha *et al.,* (2016) reported that among different microbial species screened for pectinase production, most prominent pectinase producing isolates were *Bacillus* sp. identified as *B. firmus*, *B. coagulans*, *B. endophyticus* and *B. vietnamensis*. Extracellular pectinase production and its purification from a new strain of isolated *B. subtilis* was published by Mercimek Takcı *et al.,* (2016). The results of a study conducted by Nawawi *et al.,* (2017) indicated that out of 20 isolates screened for xylanopectinolytic enzyme activity, the most preeminent stain was identified as *B. subtilis* ADI1. Kaur *et al.,* (2017) reported pectinolytic enzyme production in SSF by *B. subtilis* SAV-21 isolated from fruit and vegetable market waste soil. Thermo acidic pectinase production from *Bacillus* sp. ZJ1407 has an excellent acidic and thermal stability within a pH range of 3.0-5.0 and at 80–90 °C (Yu *et al.,* 2018). The first report on pectinase production by *B. sonorensis* was published in 2018 by Sindhu *et al.,* (2018).

2.3.5. Screening and assay methods for pectinase

Agar plate assay is used for primary screening of pectinase (Singh *et al.,* 2015). The pectinolytic microorganisms utilize the substrate (pectin) present in the agar media and form a clear zone of

substrate utilization surrounding to their growth. Various reagents that react with polysaccharides are used to detect the substrate utilization zone. In most of the work, Cetyl trimethylammonium bromide (CTAB), the quaternary ammonium surfactant is used for visualization of pectin degradation zone (Namasivayam *et al.,* 2011; Tewari *et al.,* 2005). Many researchers used iodine potassium iodide (IKI) solution for this purpose (Oumer *et al.,* 2018; Qureshi *et al.,* 2012; Soares *et al.,* 1999; Sohail *et al.,* 2016). Ruthenium Red solution is also used to detect the pectin depolymerization zone (Strauss *et al.,* 2001). Ghazala *et al.,* (2016) screened the pectinase producers by flooding the culture plates with Congo red followed by NaCl wash.

Specific enzyme assays were used to detect different pectinolytic enzyme group. Generally, for quantitative assay of pectinase, Dinitrosalicylic acid (DNS) assay attributed to Miller (1959) using 3,5-dinitrosalicyclic acid (DNS) is widely used to measure the free reducing sugar (GalA) formed. When an alkaline solution of 3,5-dinitrosalicylic acid reacts with reducing sugar (GalA), it is converted into 3-amino-5-nitrosalicylic acid with orange color and is measured at 540 nm (Wang, 2013).

2.3.6. *Bacillus* spp. for production of other industrially important enzymes and biologically active molecules

The *Bacillus* spp. produce more than two dozen biologically active molecules generating a high potential for biotechnological and biopharmaceutical applications (Stein, 2005). Also, it is estimated that enzymes from *Bacillus* spp. makeup about 50 % of the total enzyme market (Schallmey *et al.,* 2004). Another major feature that makes

these groups predominant is that most of them are environmentally friendly, don't have fastidious nutritional requirements and are easy to grow and handle (Sohail *et al.,* 2016). Production of industrially important enzymes such as amylase, cellulase, protease, tannase, lipase etc. from *B. subtilis* was reported by various authors. A multi enzyme complex from *B. licheniformis* SVD1 having endoglucanases, xylanases, pectinases and mannanases activities was published in 2010 (van Dyk *et al.,* 2010).

Use of biosurfactants in the commercial application has been getting major attention due to varied advantages of biosurfactants over chemically synthesized surfactants. Biosurfactants produced by different strains of *Bacillus* for various applications has been intensively reported (Gagelidze *et al.,* 2016; Montagnolli *et al.,* 2015). Primary screening methods such as foam formation, drop collapse assay, oil displacement test, emulsification activity, microplate assay, penetration assay and BATH assay etc. were used to screen the presence of biosurfactants (Chakraborty *et al.,* 2014; Montagnolli *et al.,* 2015; Volchenko *et al.,* 2007; Youssef *et al.,* 2004). *Bacillus* species have a long past of use in biotechnology and allied field as the food supplement for humans and animals of agricultural importance (Liu *et al.,* 2013). Many researchers have studied on *Bacillus* spp. for probiotic potential and safety properties and also reported their possible applications in healthcare formulations, personal care products, food and animal feed etc. (AlGburi *et al.,* 2016; Duc *et al.,* 2004; Lee *et al.,* 2017).

Table: 2.1. Bacillus spp. reported to produce different types of pectinase with their unique characteristics

No	Bacterial species	Characteristics				Reference
		Pectinase Type	pH	Temp (°C)	Special Features	
		Pectinase	6.-11	45	No function in softening of cucumbers or olives	(Nortje *et al.,* 1953)
		PL	8.5	60-65	Mol wt : 33000 Da, PL and PE activities	(Chesson *et al.,* 1978)
		PAE	8		Protein was named as 'YxiM'	(Bolvig *et al.,* 2003)
		Pectinase	8.0	50	Isolated from agro-waste	(Torimiro *et al.,* 2013)
		Endo- PG	5	60	Mol wt : 67 kda V max : 1.21 mg/ml & Km : 2423 mol/min/mg	(Munir *et al.,* 2015).
1.	*B. subtilis* IFO 12113	PPase	5-9	50	PPase-B, Mol wt :30000 Da	(Sakai *et al.,* 1989)
2.	*B. subtilis* IFO 3134	PPase	5-9	37-60	PPase-C, Mol wt :30000 Da	(Sakai *et al.,* 1990)
		PL	8	60	PPase-NMol wt : 43000 Da	(Sakamoto *et al.,* 1994)
		PNL	8	60	PPase-R, Mol wt : 35000: Da	
3.	*B. subtilis* SO113	PL	8.4	40	Mol wt : 42kDa, Km : 0.862 g/l & V max : 1.475 µmol	(Nasser *et al.,* 1990)
4.	*B. subtilis* WSHB04-02	Pectinase	9.2	57	Can be used in bioscouring of cotton	(Wang *et al.,* 2007)
5.	*B. subtilis* RCK	Exo-PG	10.5	35	The enzyme was produced by SSF	(Gupta *et al.,* 2008)
6.	*B. subtilis* SS	Pectinase	9.5	65	Alkaline & thermostable	(Ahlawat *et al.,* 2009)
7.	*B. subtilis* CM5	Exo-PG	7	50	Incubation period: 6 days	(Swain *et al.,* 2009)
8.	*B. subtilis* KSM-P358	Exo –PG	8	55	Mol wt: 105 kDa The gene was cloned and characterized	(Sawada *et al.,* 2001)
9.	*B. subtilis* EFRL 01	PG	8	45	Yielded a pectinase titer of~2700 U/ml	(Qureshi *et al.,* 2012)
10.	*B. subtilis* (TCCC11286)	PL	9	50	The gene was cloned in *B. subtilis* WB600, Mol wt: 45497.9 Da	(Liu *et al.,* 2012)

No	Bacterial species	Characteristics				Reference
		Pectinase Type	pH	Temp (°C)	Special Features	
11.	*B. subtilis*168	PL	9.5	50	stable & efficient for degumming ramie fiber	(Zhang *et al.,* 2013)
12.	*B. subtilis* C4	PG	9	60	Mol wt: 43-66 kDa	(Kusuma *et al.,* 2014)
13.	*B. subtilis* 7-3-3	PGL	6.5	34	Used Fed-Batch Fermentation for enzyme production.	(Zou *et al.,* 2014)
		pectinase	8.42	30	Xylanopectinolytic activity detected	(Nawawi *et al.,* 2017)
14.	*B. subtilis* SAV-21	pectinase PNL			Agro-waste can be utilized for enzyme production	(Kaur *et al.,* 2017)
15.	*B. subtilis* PB1		9.5	50	A novel PL K m :0.312 mg/ml & V max : 1248 U/ml	(Zhou *et al.,* 2017)
16.	*Bacillus* No. P-4-N	PG	10-10.5	65	Type 1 PG	(Horikoshi, 1972)
17.	*Bacillus* sp. NT-33	PG	10.5	70	Excellent capacity for degumming ramie fibers	(Cao *et al.,* 1992)
18.	*Bacillus* sp. YA-14	Endo-PL			More active on low methyl esterified pectin	(Han *et al.,* 1992)
19.	*Bacillus* sp. PN33. J	Endo-PNL	6	40	Mol wt: 52 kDa	(Kim *et al.,* 1998)
20.	*Bacillus* sp. KSM-P15	PL	10.5	50-55	Mol wt : 20300 Da, PL with PG activity	(Kobayashi *et al.,* 1999)
21.	*Bacillus* sp. KSM-P103	PL	8	50	Gene was cloned, & sequenced (1038bp)	(Hatada *et al.,* 1999)
22.	*Bacillus* sp.TS 47	PL	8.0	70	PLwith PPase activity	(Takao *et al.,* 2000)
23.	*Bacillus* sp.BP-23	PL	10	50	Gene cloned in *E.coli* (1214 bp)	(Soriano *et al.,* 2000)
24.	*Bacillus* sp. MG-cp-2	PG	10	60	Thermo-alkali stable	(Kapoor *et al.,* 2000)
25.	*Bacillus* sp. DT 7	PNL	8	60	Mol wt: 106 kDa	(Kashyap *et al.,* 2000)
26.	*Bacillus* sp. KSM-P576	Exo -PG	8	55	Mol wt : 115 kDa	(Kobayashi *et al.,* 2001)

No	Bacterial species	Characteristics				Reference
		Pectinase Type	pH	Temp (°C)	Special Features	
27.	*Bacillus* sp. BP7	PL, PEL, PG			Zymograms showed 4 bands	(Soriano *et al.,* 2005)
28.	*Bacillus* sp. RN1	PL	10	90	Mol wt: 33 kDa, Cloned & expressed in *E.coli* BL21	(Sukhumsiirchart *et al.,* 2009)
29.	*Bacillus* sp. N16-5	PL	11.5	50	Mol wt:42 kDa, Cloned & expressed in *E.coli*	(Li *et al.,* 2010)
30.	*Bacillus* sp. SMIA-2		10	60 - 70	Retains 82 % of activity at 70 °C after 2 h of incubation & stable over the pH range 8-10	(Andrade *et al.,* 2011)
31.	*Bacillus* sp. strain BR1390	PMG	6	60	Acidophilic, thermal & detergent tolerant	(Karbalaei-Heidari *et al.,* 2014)
32.	*Bacillus* sp. ZGL14	pectinase	8.6	50	Mol wt: 65 kDa	(Yu *et al.,* 2017)
33.	*Bacillus* sp ZJ1407	pectinase	5	37	Have good acidic & thermal stability	(Yu *et al.,* 2018)
34.	*B. pmilus*	Pectinase	6 - 11	45	Having no function in softening of cucumbers or olives	(Nortje *et al.,* 1953)
35.	*B. pumilus*	Endo-PATE	8-8.5	60	Neither PE nor PG was detected	(Dave *et al.,* 1971)
36.	*B. pumilus* dcsr1	pectinase	10.5	50	Alkalo thermostable	(Sharma *et al.,* 2006)
37.	*B. pumilus* BK2	endo PL	8.5	70	Mol wt: 37.3 kDa, Did not require Ca^{2+} ions for activity	(Klug-Santner *et al.,* 2006)
38.	*B. pumilus*ASH	Pectinase	6-10	60	Xylanopectinolytic activity detected	(Ahlawat *et al.,* 2007)
39.	*B. pumilus* DKS1	PL	7	30-40	Can be used for degumming of ramie fibre	(Basu *et al.,* 2009)
40.	*B. pumilus* (NRRL B-212)	Exo-pectinase	8	30	wheat bran & sugar beet pulp utilized for enzyme production	(Tepe *et al.,* 2014)
41.	*B. pumilus*	PL	8	65	Thermostability enhanced by cloning (75	(Liang *et al.,* 2015)

No	Bacterial species	Characteristics				Reference
		Pectinase Type	pH	Temp (°C)	Special Features	
	(ATCC 7061)				°C)	
42.	*B. licheniformis* 14A	Exo-PGL	11	69	Mol wt: 38 kDa	(Singh *et al.,* 1999)
43.	*B. licheniformis* SVD1	PL	7		produce a multi-enzyme complex	(van Dyk *et al.,* 2010)
44.	*B. licheniformis* KIBGE IB-21	PG	7	37	Max. production at 37 °C & 48 h of incubation	(Rehman *et al.,* 2012)
			8-10	45	Mol wt : 153 kDa, Km : 1.017 mg/ml & Vmax : 23800 μM/min	(Rehman *et al.,* 2015)
45.	*B. licheniformis* SHG10	PG	8	37.8	Utilized orange peel waste to produce PG	(Embaby *et al.,* 2014)
46.	*B. licheniformis* DSM-13	PAE	8	50	Mol wt: 26.7 kDa	(Remoroza *et al.,* 2014)
		PME	8	50	Mol wt: 35.1 kDa	(Remoroza *et al.,* 2015)
47.	*B. licheniformis* KIBGE IB-3	PG	7	37	PG production was achieved by utilizing different agro-residues	(Jahan *et al.,* 2017)
48.	*B. licheniformis*	Exo-PG	6.5	60	Mol wt : 54 kDa Vmax : 4.18 μM/s & Km : 3.25 mg/ml	(Evangelista *et al.,* 2018)
49.	*B. Polymyxa* strain 30	PG			Pectic acid fermentation was very rapid in shake flasks	(Potter *et al.,* 1955)
50.	*B. polymyxa*	PG	8.4-9.4	45	Maximal cell yield and PG production in medium with biotin and 3 % pectin	(Nagel *et al.,* 1961)
51.	*B. polymyxa*	PNL	8.5	30	Can produce protease, amylases, and cellulases	(Rajabi *et al.,* 1999)
		PL	9	30		
52.	*B. stearothermophilus*	Endo-PATE	9	70	Mol wt: 24000 Da	(Karbassi *et al.,* 1980)
53.	*B. stearothermophilus*	pectinase	7.5	60	Isolated from agro-waste & used pectin as	(Torimiro *et al.,* 2013)

No	Bacterial species	Characteristics				Reference
		Pectinase Type	pH	Temp (°C)	Special Features	
					the substrate	
54.	*B. cereus*	Pectinase	8.5	37	Max. enzyme production: 44U/ml	(Namasivayam *et al.,* 2011)
55.	*B. cereus*	Pectinase	8	50	used pectin as the substrate	(Torimiro *et al.,* 2013)
56.	*B. macerans*	Endo-PL	9	60	Mol wt : 35000	(Miyazaki, 1991)
57.	*B. gibsonii* S-2	PG	10.5	60	Used dry sugar beet pulp max. prouction	(Li *et al.,* 2005)
58.	B.coagulans	PG			Mol wt: 6.5 kDa	(Odeniyi *et al.,* 2009)
59.	*B. sphaericus* (MTCC 7542)	PG	6.8	30	Incubation time:72 h	(Jayani *et al.,* 2010)
60.	*B. megaterium* AK2	PL	8.5	50	Nanoparticle supplementation can enhance thermostebility	(Mukhopadhyay *et al.,* 2012)
61.	*B. clausii*	PNL	10	60	Mol wt: 35 kDa	(Li *et al.,* 2012)
62.	*B. firmus* -I-10104	PG	6	37	SSF production of pectinase using agro-waste	(Reda A *et al.,* 2008)
63.	*B. firmus*	PG	7	50	Km : 0.27 % & Vmax : 90.090 U/ml	(Roosdiana *et al.,* 2013)
64.	*B. halodurans* M29	pectinase	10	80	Mol wt : 39 kDa Km : 4.1 g/l & Vmax : 351 U/mg	(Mei *et al.,* 2013)
65.	*B. tequilensis* SV11	PL	9	60	Two subunits with mol masses of 135 & 43 kDa. V max : 1.220 mg/ml & Km :1773U/ml	(Chiliveri *et al.,* 2014)
66.	*B. mojavensis* I4	Pectinase	8	60	Used Carrot peel as a cheap substrate for enzyme production	(Ghazala *et al.,* 2015)
67.	*B. vallismortis* (JQ990307)	PG			Isolated from plant waste material	(Sohail *et al.,* 2016)
68.	*B. sonorensis* MPTD1	Pectinase			first report on pectinase production by this organism	(Sindhu *et al.,* 2018)

2.4. Materials and Methods

2.4.1. Sample collection

Samples including soil (from dump yards of market vegetables and fruits), rotten fruits and vegetables were collected from various sites of Malabar region-Kerala and stored in an airtight polyethylene bags at 4 °C.

2.4.2. Isolation and screening of pectinase producing strains

From the collected sample, one gram was pooled and homogenized in sterile distilled water and ten-fold serial dilutions were prepared. Aliquots (1 ml) from each dilution were inoculated by spread plate method on yeast extract pectin (YEP) agar medium with pH 7. The samples were incubated at room temperature (30 °C) for 24 h. Pure cultures were subcultured onto slant media and maintained for identification and further studies.

2.4.3. Primary screening for pectinolytic bacteria: Qualitative assay

For the primary detection of pectinolytic producers, agar plate assay was used to detect the depolymerized pectin around the colonies. The substrate utilization zone was identified using iodine and confirmed by CTAB.

2.4.3.1. Iodine assay

The YEP medium with 2 % agar was inoculated with the test organism and incubated for 24 h at 30 °C. After incubation, the plates

were poured over with KI solution (1.0 g iodine, 5.0 g potassium iodide and 330 ml H_2O). The colonies were selected based on the surrounding clear zone formed by substrate utilization (Tariq *et al.,* 2012).

2.4.3.2. CTAB assay

In this method, 24 h cultures of the test organism in YEP medium with 2 % agar was overlaid with the CTAB solution (3.3 %) and kept for 10 min to detect clearance zones (Tewari *et al.,* 2005).

2.4.3.3. Agar well diffusion

The pectinolytic activity was also confirmed by well diffusion method. A 10 mm diameter well was made aseptically on a YEP agar plate using a cork borer. The wells were poured with 100 µl of culture filtrate and incubated at 30 °C overnight. Pectinolytic activity was detected by observing the substrate utilization zone around the wells with the help of KI solution (Tariq *et al.,* 2012) and also confirmed by the CTAB solution.

2.4.4. Quantitative assay for pectinase activity: Colorimetric assay by DNS method

The selected strains were grown in YEP medium (24 h, 30 °C and 150 rpm). After incubation, the cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. The pectinase activity was measured in the culture supernatant using a method described by (Miller, 1959). For this, 1 ml of the cell free supernatant was mixed with an equal volume

of 1 % citric pectin (Sigma) in 0.02M Tris-HCl buffer (pH 8) as the substrate. The mixture was incubated at 40 °C for 15 min. Dinitrosalicylic acid reagent (3 ml) was then added and the reaction mixture was boiled in a water bath for 15 min. Immediately after boiling, 1 ml of Rochelle Salt (sodium potassium tartrate, 40 %) was added to the mixture for colour stability. The mixture was cooled to room temperature in a water bath and its absorbance was read at 540 nm against a blank.

The standard curve was established using α, D-galacturonic acid as reducing sugar. One unit (U) of polygalacturonase activity is defined as the amount of enzyme that releases 1 μmol of galacturonic acid per min under the assay conditions. The enzyme activity was calculated by the formula;

$$\text{Enzyme Activity} = \frac{\text{D} - \text{galacturonic acid } (\mu\text{mol/ml}) \text{ x Total solution volume } (\text{ml})}{\text{Enzyme added } (\text{ml}) \text{ x Reaction time } (\text{min})}$$

2.4.5. Identification of pectinolytic bacteria

2.4.5.1. Microscopic observation and biochemical characterization

The pectinolytic bacterial isolates showing maximum enzyme activity was morphologically, microbiologically and biochemically characterized. These characterization tests include; colony morphology, Grams reaction, spore formation, motility, catalase, oxidase, urease, starch and gelatin hydrolysis, nitrate reduction, carbohydrate fermentation tests, IMVIC tests and tolerance to sodium chloride (Cappuccino *et al.,* 2005). Based on the results the isolates

were identified up to the genus level according to Bergey's Manual of Determinative Bacteriology (Bergey et al. 1974).

2.4.6. Testing of different culture media for pectinase activity

Different media previously used by various researchers (Table: 2.2) were tested with the selected bacterial strain for pectinase activity.

Table: 2.2. Different media used for the production comparison of pectinase

Media 1 - YEP (Kashyap *et al.*, 2000)		**Media 2 - Czapek'sDox pectin medium (Reda A *et al.*, 2008)**	
Citrus pectin	0.25 %	Pectin	1 %
Yeast extract	1 %	$NaNO_3$	0.2 %
pH	7	$KH_2 PO_4$	0.1 %
		KCl	0.05 %
		$MgSO_4 7H_2 O$	0.05 %
		$FeSO_4 .7H_2O$	0.001 %
		$CaCl_2$	0.001 %
Media 3 (Soares *et al.*, 1999)		**Media 4 (Jayani *et al.*, 2010)**	
Citrus pectin	1 %	Citrus pectin	1 %
$(NH_4)_2SO_4$	0.14 %,	Tri-sodium citrate dihydrate	0.1 %,
K_2HPO_4	0.6 %,	Citric acid	0.1 %,
KH_2PO_4	0.20 %	Yeast extract	0.1 %,
$MgSO_47H_2O$	0.01 %	Casein	0.1 %
Nutrient solution	0.10 %	$MgSO_47H_2O$	0.1 %
pH	6	KCl	0.05 %
		pH	6.8

2.4.7. Molecular identification of the best pectinolytic microbial culture using 16S rDNA based technique

2.4.7.1. Isolation of bacterial Genomic DNA (gDNA)

DNA was isolated from the pure culture using the XcelGen bacterial gDNA kit (Cat # XG2411-01) in accordance with the protocol suggested by the manufacturer. For this, the bacterial culture (1-3 ml) was pelleted by centrifugation at 10000 rpm for 5 min at RT. Completely discarded the supernatant and the pellet was resuspended in 180 µl TE Buffer. The cell suspension was added with lysozyme solution (18 µl, 50 mg/ml) and RNase A (5 µl), incubated at 30 °C for 15-30 min and centrifuged (8000 rpm, 5 min, RT). The pelleted cell was resuspended in 10 µl residual liquid by vortexing followed by lysis buffer (buffer TL) was added and vortexed for 5min. Proteinase K (25 µl) was added and vortexed again 10 sec. The mixture was incubated at 55 °C in a shaking water bath for 30 min followed by 220 µl of buffer BL was added and mixed by vortexing.

The samples were incubated (65 °C, 10 min) and added absolute ethanol (220 µl), mixed by vortexing (20 sec). The whole sample was transferred to a DNA mini-column and centrifuged (10000 rpm, 1 min) for binding of DNA to the column. The collection tube and flow-through were discarded and the mini column (with bound DNA) was inserted to a sterile Eppendorf tube. Added 500 µl Buffer KB to the mini column and centrifuged (10000 rpm, 1 min). Emptied the collection tube, re-inserted the mini column to the same tube, washed the DNA by adding 650 µl wash Buffer (diluted with ethanol) and centrifuged (10000 rpm, 1 min). To remove the contaminants, the

washing step was repeated. The mini column was removed from the collection tube and placed into a new sterile tube after a mini spin for 1min at 10000 rpm. To the centre of the mini column, 50 µl of pre-warmed elution buffer was added and centrifuged (10000 rpm, 1 min). The eluted DNA was collected and diluted after checking the purity.

2.4.7.2. Fragment 16S region amplification by PCR

The universal 16S rDNA primer 8F and 1492R (Table: 2.3) were used for amplification of genomic DNA by polymerase chain reaction (PCR).

Table: 2.3. Primers used for amplification of genomic DNA

Primer	Sequence (5'-3')	bp	Target group	Reference
8F	AGAGTTTGATCCTGGCTCAG	20	Universal	(Turner *et al.,* 1999)
1492R	ACGGCTACCTTGTTACGACTT	21	Universal	

2.4.7.2.A. Reagents for PCR reaction

PCR amplification was done in 5 system of 25 µl of reaction volume containing MBI Fermentas PCR master mix 12.5 µl, Forward & Reverse primer 0.5 µl each and template DNA 1 µl.

2.4.7.2.B. PCR temperature profile

The PCR reaction was run for 30 cycles in a Thermal Cycler (Eppendorf) with the following pattern as shown in Table: 2.4.

Table: 2.4. PCR temperature profile

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
95 °C	94 °C	52.0 °C	72 °C	72 °C
2 min	30 sec	30 sec	90 sec	5 min
	30 cycles			

2.4.7.3. Agarose gel electrophoresis

PCR product of 5 µl from each tube was mixed with 1 µl of DNA loading dye, and this mixture was subjected to electrophoresis on 1.2 % agarose gel to confirm the targeted PCR amplification. From the agarose gel, the amplified product was excised and purified using QIAamp DNA Purification Kit (Qiagen).

2.4.7.4. Gel extraction & purification

The desired band from the Gel was excised with a scalpel and extracted using purelink quick gel extraction kit (PureLink® Quick Gel Extraction and PCR Purification Combo Kit Catalog number K2200-01 Publication Part Number 7015020). After gel extraction, the DNA was purified using centrifuge as described in the manual.

By using NanoDrop (NanoDrop 200C –Thermo Scientific), the concentration of the purified DNA was determined, and the 16S PCR product of the isolate was sequenced in both directions. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BigDye Terminator v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser (Applied Biosystems, USA) following manufacturer's instructions mentioned in the kit.

2.4.8. Sequence analysis and phylogenetic tree construction

The consensus sequence of 1427 bp of the 16S region was generated from forward and reverse sequence data using aligner software. The 16S region sequence was used to carry out BLAST with the nr database of NCBI GenBank. Multiple sequence alignment was performed by using CLUSTALW (Thompson *et al.,* 1994). Subsequently, an evolutionary distance matrix was then generated from these nucleotide sequences in the dataset. These alignment results of the first ten high similarity sequences were selected along with the unknown sequence to construct the phylogenetic tree using MEGA 5.0 software tool (Tamura *et al.,* 2007). Kimura two-parameter method (Kimura, 1980) was used to compute the evolutionary distances and the codon positions included were 1st+2nd+ 3rd+Noncoding. The phylogenetic tree was inferred using the neighbor-joining methods (Saitou *et al.,* 1987). Bootstrap analysis was based on 500 resamplings.

2.4.9. Ability to produce other enzymes

The ability of the selected stain to produce different other industrially important enzymes were screened by primary agar diffusion test (Cappuccino *et al.,* 2005).

2.4.9. 1. Amylase

Starch agar plate was used to detect the amylase activity using well diffusion method. Wells (10 mm diameter) were cut on the starch agar plate (using cork borer) and poured with 100 µl of culture filtrate and incubated for overnight. The substrate utilized zone was observed

around the wells for production of amylase with iodine-potassium iodide solution.

2.4.9.2. Cellulase

For the detection cellulolytic activity of the strain, CMC agar plate was prepared and the substrate utilization zone around the well was observed with the help of Congo red.

2.4.9.3. Protease

For the detection of protease, casein-agar plate assay was used and the enzyme production was detected by the presence of clear zone formed by substrate utilization.

2.4.9.4. Tannase

Tannic acid agar plate assay was used for the detection tannase production.

2.4.9.5. Lipase

Lipase producing capability of the strain was tested using tributyrin agar plates. A positive result was observed by the formation of clear zones after incubation.

2.4.10. The ability of the stain for biosurfactant production

Different tests were performed to analyze the biosurfactant production capability of the selected strain. These include;

2.4.10.1. Foam formation activity

The bacterial strain was cultured separately in 100 ml Erlenmeyer flask containing 20 ml modified YEP broth. The flask was

incubated at 30 °C on a shaker incubator (150 rpm) for 72 h. Foam activity was detected based on the duration of foam stability, foam height and shape in the graduated cylinder (Dehghan-Noudeh *et al.,* 2003).

2.4.10.2. Microplate assay

For microplate assay, 100 µl of culture supernatant was taken in a well of a 96 – microwell plate. The well with supernatant was watched using a backing sheet of paper with a grid. If biosurfactant is present, the concave surface distorts the image of the grid below and it provides a qualitative assay for the presence of surfactants (Chakraborty *et al.,* 2014).

2.4.10.3. Drop collapse and oil displacement test

The drop collapse qualitative test according to Youssef *et al.,* (2004) was done. To a solid surface 2 µl mineral oil was added and equilibrated for 1 h at room temperature. To this oil surface, 5 µl culture supernatant was added and inspected after 1 min. In the oil displacement test, 15 µl of crude oil was placed as a film on the surface of the distilled water (40 µl) in a Petri dish. Over the oil layer, 10 µl of culture supernatant was poured and the clear halo under visible light visualized was noticed (Morikawa *et al.,* 1993).

2.4.10.4. Penetration assay

The cavities of a 96-microwell plate were filled with 150 µl of a hydrophobic paste consisting of oil and silica gel. The paste was

covered with 10 µl of oil. Then 90 µl supernatant was coloured with 10 µl of a red staining solution (safranin). The coloured supernatant was then placed on the surface of the paste. If biosurfactant is present, the hydrophilic liquid will break through the oil film barrier into the paste (Walter *et al.,* 2010).

2.4.10.5. Emulsification activity (E24)

To measure emulsification capacity, 2 ml of different oils (kerosene, engine oil, diesel and petrol) were added to equal volume of culture supernatant and the mixture was vortexed at high speed for 2 min. After 24 h, the height of the stable emulsion layer was measured. The uninoculated medium was used as the control. The emulsion index was calculated as the ratio of the height of the emulsion layer and the total height of the liquid (Cooper *et al.,* 1987).

2.4.10.6. Bacterial adhesion to hydrocarbon assay (BATH)

A turbid aqueous suspension (2 ml) of washed microbial cells was mixed with 2 ml of hydrocarbon (kerosene). After mixing for 2 min, the two phases were allowed to separate. Hydrophobic cells become bound to hydrocarbon droplets and rise with the hydrocarbon. They were removed from the aqueous phase. The turbidity of the aqueous phase was measured at 600 nm. The decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cell. The percentage of the cells bound to the hydrophobic phase (H) was calculated by the following equation;

$$H = \left(1 - \frac{A}{A_0}\right) \times 100$$

where, A_0 and A were A_{600} before and after mixing with hydrocarbon, respectively (Vegt *et al.,* 1991).

2.4.11. Probiotic characteristics of the strain and bacteriocin production

Survival of *B. subtilis* BKDS1 under conditions stimulating human GI tract was tested as major criteria. These tests include resistance to low pH, tolerance to bile salt and sensitivity to the antimicrobial drug were tested (Bassyouni *et al.,* 2012).

2.4.11.1. Resistance to low pH

Two ml of overnight culture of the test organism was centrifuged for 20 min at 5000 rpm and the pellets were resuspended in the nutrient broth of pH values as 2, 3, 4 and 7. The cultures were incubated at 37 °C for 24 h. The bacterial growth was periodically checked by measuring OD_{620} at 0, 3, 6, 9, 24 h intervals. (Khochamit *et al.,* 2015).

2.4.11.2. Bile tolerance

The tolerance of the test organism to bile salts (BS) was evaluated in nutrient broth supplemented with 0.3 % bile salt. For this, 2 ml each of the overnight grown culture of the test organism was centrifuged at 5000 rpm for 20 min. The culture pellet obtained in one tube was resuspended in nutrient broth containing 0.3 % bile salt (test)

and the culture pellet of the second tube was resuspended in plain nutrient broth (control). Both of the tubes were incubated at 37 °C and the growth was monitored in intervals of 0, 3, 6, 9, 24 h at OD_{620} (Khochamit *et al.,* 2015).

2.4.11.3. Resistance to antibiotics

Antibiotic resistance was determined by disc diffusion method using antibiotic discs. For evaluating antibiotic resistance, plates of nutrient agar were seeded with the isolated culture samples and discs of Chloramphenicol, Erythromycin, Gentamicin, Ampicillin, Amikacin, Novobiocin, Kanamycin, Cephalothin, Streptomycin, Vancomycin and Oxacillin, Clindamycin, Amoxicillin were carefully placed on the agar plates. Plates were incubated at 37 °C overnight, and the diameters of the clear zone of inhibition around the discs were measured (Sreekumar *et al.,* 2010).

2.5. Results

2.5.1. Isolation and screening of bacterial isolates for pectinolytic activity

This research succeeded to isolate thirty six bacterial isolates from the collected samples. Pure cultures of the isolate were made by streak plate method and subjected to quantitative screening by plate assay method. The preliminary screening was done by iodine assay and confirmed by CTAB.

2.5.2. Selection of best strains based on the zone diameter

Four isolates with pectinolytic activity were selected on the basis of zone size ranging from 20 mm to 28 mm after flooding the plate with iodine solution (Figure: 2.2) and by CTAB (Figure: 2.3). These isolates were designated tentatively as BKDS (1-4).

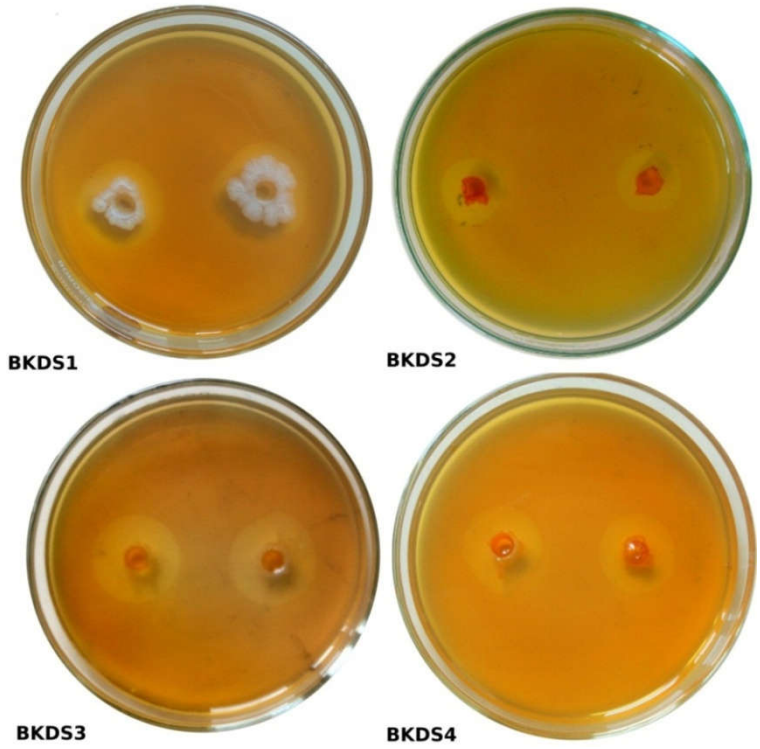


Figure: 2.2. Pectinolytic zones formed by bacterial isolates (BKDS 1-4) on YEP media (Screening by iodine solution)

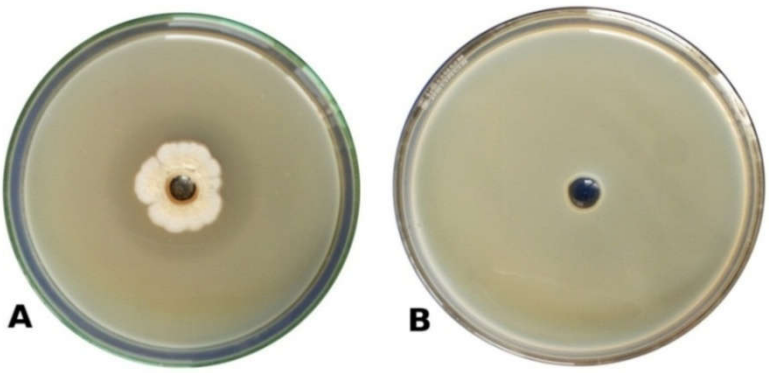


Figure: 2.3. Confirmation of zone formation of the selected strain (BKDS1) using CTAB
A. Test plate and **B**. Control plate

2.5.3. Colorimetric Assay for pectinase

By using these four isolates (BKDS1-4), pectinase enzyme production was assayed by DNS assay in YEP broth culture medium after 24 h of incubation at 30 °C and 150 rpm. The result of this DNS assay was shown in Figure: 2.4.

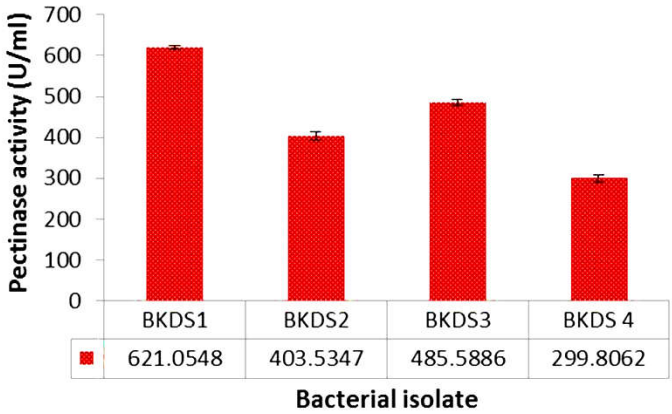


Figure: 2.4. Pectinase activity shown by isolated bacterial strains

2.5.4. Microbiological and biochemical identification of the isolated strains

The selected pectinolytic bacterial strains were characterized by microbiological and biochemical identification tests. The results of these tests were shown in **Table: 2.5 & 2.6.**

Table: 2.5. Microbiological characters of the selected strains

Strain No.	Zone diameter (mm)	Grams staining	Spore staining	Motility	Colony characteristics
BKDS1	26	+	+	+	Irregular, umbonate, undulate, cream coloured
BKDS2	22	+	+	+	Irregular, flat, undulate, large, light brown coloured
BKDS3	23	+	+	+	Irregular, umbonate, undulate, cream coloured
BKDS4	22	+	-	-	Irregular, umbonate, undulate, cream coloured

Table: 2.6. Biochemical characters of the selected strains

Biochemical tests	Isolate code			
	BKDS1	BKDS2	BKDS3	BKDS4
Nitrate reduction	+	+	-	-
Urease	-	-	-	-
H_2S production	-	-	-	-
Oxidase	+	+	+	+
Starch hydrolysis	+	+	+	+
6.5 % NaCl Growth	+	-	-	-
Indole production	-	-	-	-
Methyl red	-	+	-	-
Voges Proskauer	+	+	-	-
Citrate utilization	+	+	-	-
Glucose	+	+	-	-
Lactose	-	-	-	-
Sucrose	+	+	+	-
Mannitol	-	-	-	-
Arabinose	-	-	-	-

2.5.5. Effect of different production media on pectinase activity by the selected isolate

Four different production media used by various researchers for pectinase production was tested to choose the best pectinase production media for the selected organism (BKDS1). The result of this test was given in Figure: 2.5.

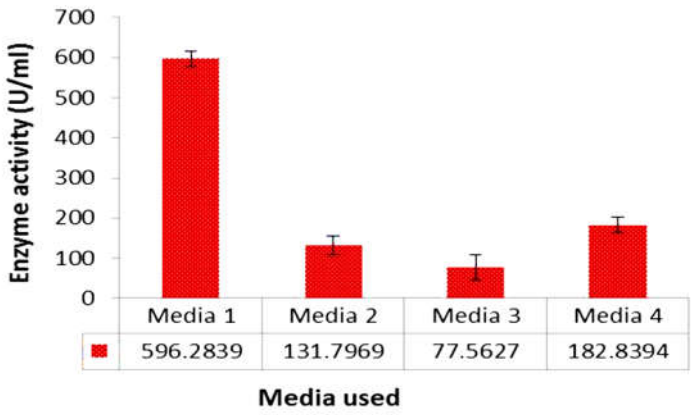


Figure: 2.5. Pectinase production by isolate BKDS1with different production media

2.5.6. Molecular identification of the best pectinolytic bacterial isolate (BKDS1) using 16S rDNA based technique

2.5.6.1. Genomic DNA isolation, PCR and agarose gel electrophoresis

The best pectinolytic strain (BKDS1-Figure: 2.6) was further identified by 16S rDNA based molecular technique. Bacterial DNA isolation kit was used to isolate the genomic DNA. PCR reactions were carried out using this isolated DNA as the template. The optimum

annealing temperature was found to be 52.0 °C. The agarose gel image of the PCR product along with DNA marker was given in Figure: 2.7.

Figure: 2.6. Bacterial isolate BKDS1

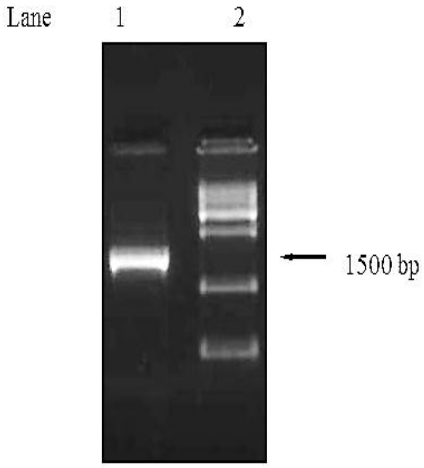


Figure: 2.7. PCR amplicon band of 1500 bp visualized on agarose gel

Lane 1. 16S rDNA amplicon band Lane **2.** DNA marker

2.5.7. Sequence analysis and phylogenetic tree construction

These PCR amplicons were purified and subjected to automated DNA sequencing using BDT V3.1 cycle sequencing kit on ABI 3730 XL genetic analyzer from both forward and reverse directions using the same primers. Figure: 2.8 represents the DNA sequencing result generated using aligner software. Using the BLAST search program, the consensus sequence of 1427 bp was compared with the NCBI gene bank and the BLAST search result with identity score was depicted in Table: 2.7. The first ten sequences were selected based on the maximum identity score and the phylogenetic tree was constructed using MEGA 5. The constructed phylogenetic tree was shown in Figure: 2.9.

CATGCACGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGA
GTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA
CCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTAC
AGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTAC
CGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGG
GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCA
TTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTGGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGC
TACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAAT
CCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAA
TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAGGAGC
CAGCCGCCGAAGGT

Figure: 2. 8. Consensus sequence of PCR product of 16S rRNA gene sequence of BKDS1

Table: 2.7. BLAST search identity score of rRNA gene sequence of isolate BKDS1

Accession	Description	Query coverage	Max identity
AB042061.1	***Bacillus subtilis***	**100 %**	**99 %**
AB914463.2	Streptomyces sp. VEL17 gene	100 %	99 %
KJ210578.1	*Bacillus* sp. BAB-3540	100 %	99 %
KF929418.1	*Bacillus subtilis* strain VITLWS2	100 %	99 %
KF917187.1	*Bacillus* sp. BAB-3457	100 %	99 %
KF917185.1	*Bacillus* sp. BAB-3455	100 %	99 %
KF917164.1	*Bacillus* sp. BAB-3434	100 %	99 %
KF917147.1	*Bacillus* sp. BAB-3416	100 %	99 %
KF917144.1	*Bacillus* sp. BAB-3413	100 %	99 %
KF917142.1	*Bacillus* sp. BAB-3411	100 %	99 %

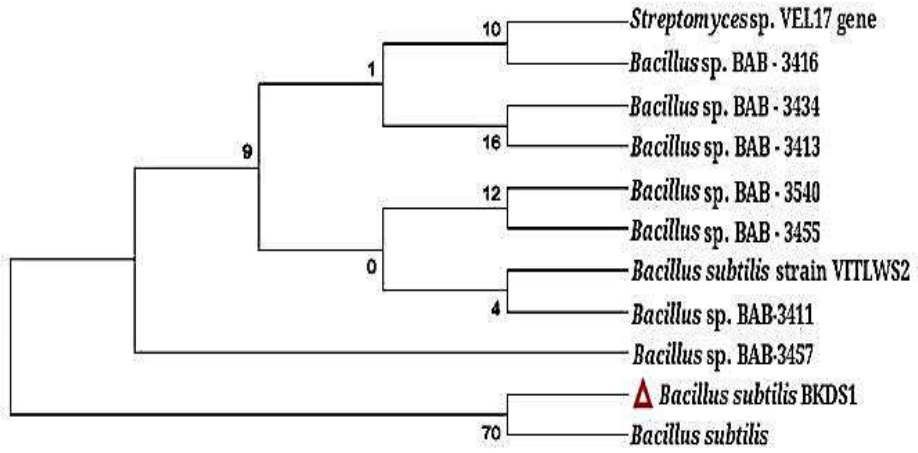


Figure: 2.9. Phylogenetic tree of the isolate *B. subtilis*BKDS1 with the selected best homologous known bacterial strains

2.5.8. Other properties of the identified strain

Various other properties of the identified strain which make them suitable for industrial application were also tested. These include

the capability to produce metabolites such as hydrolytic enzymes, biosurfactant, bacteriocin and also examined the probiotic properties.

2.5.8.1. Ability to produce other enzymes

The ability of *B. subtilis* BKDS1 for producing various hydrolytic enzymes was tested by agar well diffusion method and the combined result was given in Figure: 2.10.

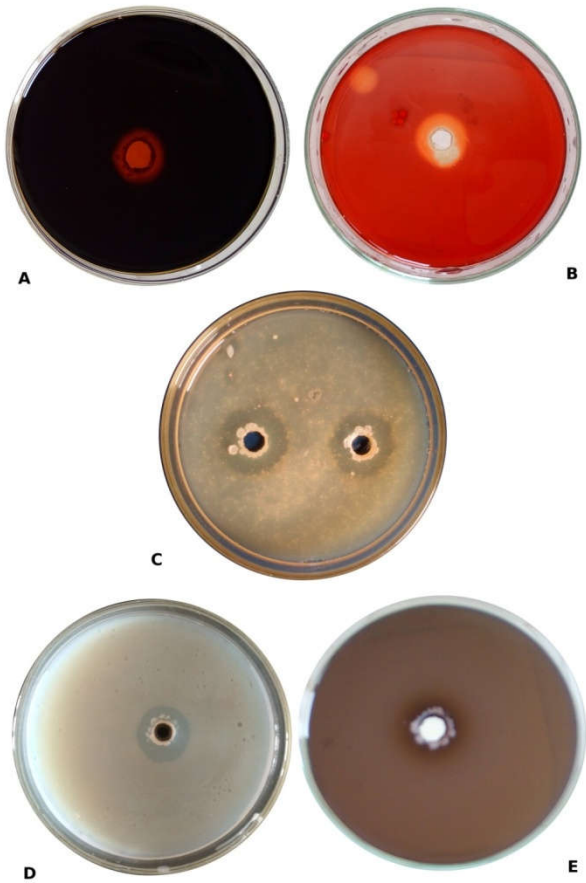


Figure: 2.10. Agar diffusion test for detection of various enzyme production capabilities of *B. subtilis* BKDS1: **A.** Amylase **B.** Cellulase **C.** Protease **D.** Lipase **E.** Tannase

2.5.8.2. Detection of biosurfactant activity

Biosurfactant detection in the culture supernatant was carried out by various primary screening methods such as foam formation, drop collapse assay, oil displacement test, emulsification activity, microplate assay, penetration assay (Figure: 2.11). Bacterial adhesion to hydrocarbon assay (BATH) was also carried out to check the biosurfactant production capability which gives a result of 52.7 %.

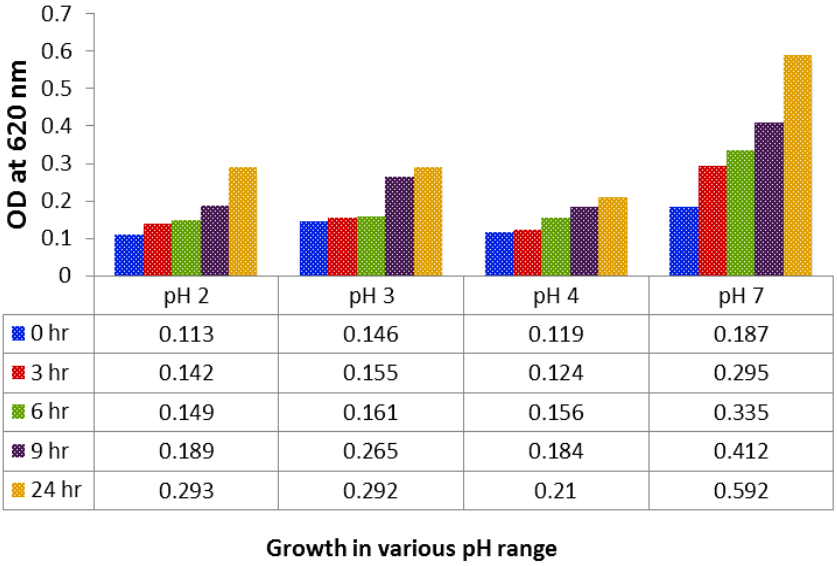
Figure: 2.11. A. Culture showing Foam formation **B**. Microplate assay (**a**. Test - culture supernatant b. Control – distilled water). **C**. Penetration assay - **C1** Upper phase & **C2**. Bottom phase (**a**. Test **b**. Control) **D**. Drop collapse Test (**a**. Test - culture supernatant **b**. Control – uninoculated medium **c**. Distilled water) **E**. Oil displacement test F. Estimation of emulsification activity

2.5.8.3. Probiotic characteristics of *B. subtilis* BKDS1

Probiotic properties of *B. subtilis* BKDS1 was tested by the basic assay methods such as; survival in low pH, bile salt tolerance, sensitivity to antimicrobial drugs etc.

2.5.8.3.A. Survival in low pH

The growth status of the isolates in nutrient broth having different pH is shown in Figure: 2.12.



	pH 2	pH 3	pH 4	pH 7
0 hr	0.113	0.146	0.119	0.187
3 hr	0.142	0.155	0.124	0.295
6 hr	0.149	0.161	0.156	0.335
9 hr	0.189	0.265	0.184	0.412
24 hr	0.293	0.292	0.21	0.592

Figure: 2.12. The growth of *B. subtilis* BKDS1 in low pH

2.5.8.3.B. Bile salt tolerance

The tolerance of the isolate to the bile salt was studied; the isolate tolerated a level of 0.3 % bile salt. The result of bile salt tolerance test is shown in Figure: 2.13.

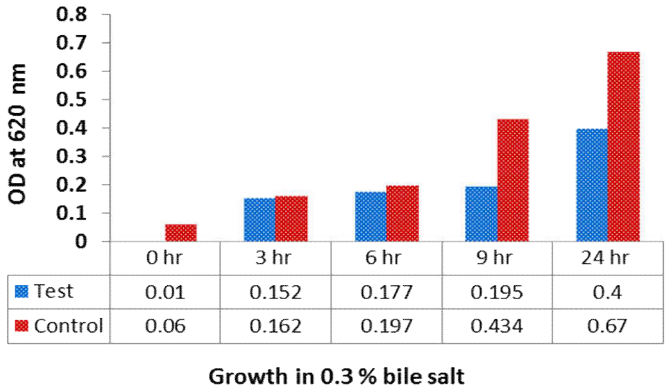


Figure: 2.13. Bile salt tolerance showed by *B. subtilis* BKDS1

2.5.8.3.C. Sensitivity to antibiotics

Sensitivity study of the isolate against various common drugs was conducted through the standard technique, *i.e.,* disc diffusion method by using antibiotic discs, such as Chloramphenicol, Erythromycin, Gentamicin, Ampicillin, Amikacin, Novobiocin, Kanamycin, Cephalothin, Streptomycin, Vancomycin and Oxacillin, Clindamycin, Amoxicillin. The result obtained was showed in **Figure: 2.14.**

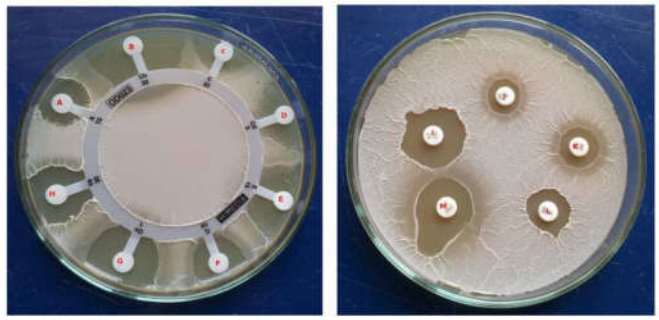


Figure:2.14. Antibiotic sensitivity test - **A**: Ampicillin, **B**: Cephalothin, **C**: Chloramphenicol, **D**: Clindamycin, **E**: Erythromycin, **F**: Gentamycin, **G**: Oxacillin, **H**: Vancomycin, **I**: Novobiocin, **J**: Kanamycin, **K**: Amikacin, **L**: Streptomycin, **M**: Amoxicillin

2.6. Discussion

Soil samples from various places of Malabar were collected and transported to the laboratory in a clean sterile container. Immediately after reaching the laboratory, the samples were serially diluted and plated in YEP plates for isolating pectinolytic bacterial strains. A total of thirty six bacterial isolates were isolated from the collected samples. These isolated strains were subjected to pectinolytic screening assay in YEP agar medium. From these, four isolates were selected based on the substrate utilization zones exhibited on pectin agar plates. Among these four isolates, the bacterial isolate BKDS1 showed highest zone diameter of 26 mm followed by isolate BKDS3 (23 nm). Bacterial isolate BKDS2 and BKDS4 produced substrate utilization zone of 22 nm (Figure: 2.2). Quantitative enzyme assay (pectinase assay by DNS method) was then employed to test the extent of enzyme produced by these isolates grown in YEP media. From this data of enzyme activity assay (Figure: 2.4), the bacterial isolate BKDS1 showed maximum enzyme activity (621.054 U/ml) followed by isolate BKDS3 (485.588 U/ml) and BKDS2 (403.534 U/ml). Bacterial isolate IV showed the lowest enzyme activity (299.80 U/ml). So the result of quantitative assay was very much correlating to the result of qualitative test (plate assay).

All the four isolates in this study are gram positive bacilli that are subsequently identified as; *B. subtilis* (BKDS1), *B. coagulans* (BKDS2), *B. pantotheticus* (BKDS3), *and Corynebacterium sp.(C. kutsceri)* (BKDS4) using biochemical methods. Out of four isolates selected for study, three were from the genus *Bacillus*. In many previous studies, *Bacillus* sp. were reported as prominent pectinolytic

enzyme producers (Jayani *et al.,* 2010; Rehman *et al.,* 2012; Soares *et al.,* 1999).

In summary, the results of both the qualitative (plate assay) and quantitative (DNS) assay revealed that, isolate BKDS1 is the most efficient strain compared to other three isolates. So this strain is chosen for further studies and was identified by 16S rDNA sequencing using the universal primers 27F and 1429R. The phylogenetic tree generated using 16S rDNA gene sequences of the bacterial isolate BKDS1 showed that the bacterium has the highest homology (99 %) with *B. subtilis* (GenBank Accession Number: AB042061.1). The partial 16S rRNA gene sequences of the isolate *B. subtilis* BKDS1 have been deposited in the NCBI nucleotide sequence database under the accession number KT004506.1(Kavuthodi *et al.,* 2015). In most of the recent publications, the characteristic bacterial isolate selected for showing pectinolytic activities were of strains of *Bacillus* species with a majority share of *B. subtilis.* Works of (Kaur *et al.,* 2017; Mercimek Takcı *et al.,* 2016; Nawawi *et al.,* 2017) support our identification results.

Pectinase production by the selected strain BKDS1 was then tested in various media containing pectin as the sole carbon source. From the result of this test (Figure: 2.4), it is clear that, YEP (Media 1) is the best prominent media for pectinase production. Media 2 showed the second highest enzyme production. The least enzyme production was found in Media 3. The enzyme production in various production media was found to be in the order Media 1 > Media 4 > Media 2 > Media 3. So for further enzyme studies, Media 1 (YEP) was used.

2.6.1. Other properties of the identified strain

Various other properties of the identified strain which make them suitable for industrial application were also tested. These include the capability to produce metabolites such as hydrolytic enzymes, production of biosurfactant and bacteriocin, probiotic properties of the strain etc. The bacterium, *B. subtilis* BKD1 is capable of producing hydrolytic enzymes such as amylase, cellulase, protease, lipase and tannase in addition to the pectinolytic enzyme (Figure: 2.11). Many reports were available that indicates the efficiency of *Bacillus* spp. for the production of industrially important enzymes (Schallmey *et al.,* 2004).

Biosurfactant detection in the culture supernatant was carried out by various primary screening methods such as foam formation, drop collapse assay, oil displacement test, emulsification activity, microplate assay, penetration assay and BATH assay. All of these methods showed positive results. The foam produced in the culture broth by the organism after 72 h of incubation (Figure: 2.11A) was the indication for the presence of biosurfactant. The height of the foam was 2.5 cm and the round shaped foam was stable for 1- 2 days. Foam formation by the culture is considered as the primary indication for biosurfactant production. The culture supernatant showed optical distortion in the microplate assay. The concave surface distorted the image of the grid below indicated the presence of surfactants **(**Figure: 2.11B). In penetration assay, the test sample was broken through the oil film barrier into the paste (Figure: 2.11C1). The silica gel entered into the hydrophilic phase and the upper phase changed from red to cloudy white (Figure: 2.11C2) which resulted in the mixing of the two

distinct phases within 15 min. This assay relies on the phenomenon that silica gel is entering the hydrophilic phase from hydrophobic paste much more quickly if biosurfactants are present. Recent work is done on *B. subtilis* SJ301 and *B. vallismortis* JB2011 supported this study (Chakraborty *et al.,* 2014).

Drop collapse test and oil displacement test were highly positive for culture supernatant of tested bacterial strain. In drop collapse test, the culture supernatant gave flat drops over oil coated solid surface very quickly (Figure: 2.11D). The drops spread or collapsed because the force or interfacial tension between the liquid drop and the hydrophobic surface was reduced. In the oil displacement test, culture supernatant placed on the center of the oil layer displaced the oil and a clear zone of approximate size 38 - 40 mm was formed (Figure: 2.11E). This was supported by Youssef et al. (Youssef *et al.,* 2004). Emulsification assay is an indirect method used to screen biosurfactant production. It was assumed that if the cell free culture broth used in this assay contains biosurfactant, then it will emulsify the hydrocarbons present in the test solution. In this assay (Figure: 2.11F) the maximum emulsification index was showed with kerosene (75 %), followed by diesel (60 %) and engine oil (42.5 %). Petrol showed least emulsification index (33.5 %). In a recent work on biosurfactant production by *B. subtilis,* the highest emulsification was obtained with kerosene (46.90 %) (Montagnolli *et al.,* 2015). The hydrophobicity of *B. subtilis* obtained in this BATH assay was 52.7 %. This is an indication for the affinity of the bacterial cells towards the hydrophobic substrate. In some studies, BATH assay was considered

as the principal method for screening of biosurfactant production (Volchenko *et al.,* 2007).

Probiotic properties of the strain were tested by various tests such as survival in low pH, bile salt tolerance, sensitivity to antimicrobial drugs etc. The isolate was able to grow well at low pH of 2 (Figure: 2.12). Being resistant to low pH is one of the major selection criteria for probiotic strains, because to reach small intestine they have to pass through the stressful condition of the stomach. The tolerance of the isolate to the bile salt was another test performed to test the probiotic property. The result of this test (Figure: 2.13) indicates that, the isolate tolerated a level of 0.3 % bile salt. The relative percentage of tolerance was 16.6 %, 93.8, 89.8 %, 44.3 % and 59.7 % with time interval 0, 3, 6, 9 and *2*4 h respectively. These two tests were considered as one of the essential requirement for probiotic cultures to establish in the intestines (Khochamit *et al.,* 2015).

Sensitivity study of the isolate against various common drugs was conducted through the standard disc diffusion method using various antibiotic discs, such as Chloramphenicol, Erythromycin, Gentamicin, Ampicillin, Amikacin, Novobiocin, Kanamycin, Cephalothin, Streptomycin, Vancomycin and Oxacillin, Clindamycin, Amoxicillin. The result obtained was showed in Figure: 2.14. From the sensitivity test, it is evident that *B. subtilis* BKDS1 was sensitive to most of the common antibiotics such as Chloramphenicol, Erythromycin, Gentamicin, Ampicillin, Amikacin, Novobiocin, Cephalothin, Streptomycin, Vancomycin, Oxacillin, Clindamycin and Amoxicillin. The three preliminary tests carried out here to check the probiotic efficiency of *B. subtilis* BKDS1 showed a positive result.

Moreover, the strain showed a positive result for the production of hydrolytic enzymes such as protease (Figure: 2.10). The expression of these enzymes enriches the probiotic characteristics of the strain by; enhancement in protein digestion, reduction in allergenicity and can able to involve in the host defense mechanism by cleaving the receptor sites of toxins in the epithelial cells of the intestine (Lee *et al.,* 2017). The ability of the strain to adhere to the epithelial cells and intestinal mucosa is an important criterion to consider a culture as probiotic. In this study, the hydrophobicity of *B. subtilis* obtained in this BATH assay was 52.7 %. All these results supported the probiotic efficiency of the isolate, *B. subtilis* BKDS1. Probiotic properties of *Bacillus* species including *B. subtilis* was reported earlier by various researchers (AlGburi *et al.,* 2016; Duc *et al.,* 2004; Lee *et al.,* 2017) supported these findings.

2.7. Conclusion

Over the years, the possibilities of exploiting microorganisms as sources of industrially pertinent enzymes have inspired upsurge in the search of extracellular microbial enzymes. In this study, thirty-six pectinolytic bacterial strains were isolated from different soil samples. The pectinase production by these selected strains were analyzed both by qualitative and quantitative assays and the isolate designated as BKDS1 is found to be the most efficient strain compared to other isolates. Hence this isolate, later identified as *Bacillus subtilis* by biochemical and molecular characterization was selected for further studies. The 16S rRNA gene sequene of this strain was deposited in Genbank database with accession number KT004506.1. Among,

various production media tested, YEP media showed higher enzyme activity and was used for further pectinase enzyme analysis.

In addition to pectinolytic activity, some other properties of the selected strain were also tested. This include; capability of producing other hydrolytic enzymes such as amylase, cellulase, protease, lipase and tannase. The organism showed a positive result in all these enzyme production capabilities. Another property of the strain tested was the biosurfactant production capability. The results of these tests revealed that the isolated strain *B. subtilis* BKDS1 is suitable for biosurfactant production. Tolerance to low pH values (2-3), high bile concentrations (0.3%) and susceptibility to major antibiotics proved the probiotic properties of the strain.

In conclusion, the isolated strain *B. subtilis* BKDS1 is a potent pectinolytic strain showing various other important characteristics such as the capability of producing assorted hydrolytic enzymes, biosurfactant and having some probiotic properties. So this strain can be used in industries as a pectinase producer after production and process optimization.

CHAPTER 3

AN APPROACH TO ENHANCE PECTINASE PRODUCTION BY *BACILLUS SUBTILIS* BKDS1 USING STATISTICAL MEDIA OPTIMIZATION AND STRAIN IMPROVEMENT METHODS

3.1. Introduction

The drastic growth in the application of microbial enzymes in various industries in the last few decades demands an extension in both qualitative improvement and quantitative enhancement. As the quantities produced by wild strains are usually too low, strain improvement and medium optimization were the prime requirements for quantitative enhancement leading to over production of the enzymes. For industrial use, the enzyme must be produced at a low cost. Strain improvement is usually done by mutating the microorganism by techniques such as classical mutagenesis or molecular genetic methods. If the genes engaged in the production of a metabolite or enzyme of interest are unknown, then the production yield is improved by introducing random mutations into the chromosomes of the synthesizing microbe by physical irradiation or treatment with mutagens (Adrio *et al.,* 2006). On the other hand, molecular genetic methods such as gene disruption and overexpression are employed if the genes involved in the production of particular metabolite are known (Stephanopoulos *et al.,* 1998).

The conventional method of strain improvement involves exposing the microbe to physical or chemical mutagens. Physical mutagens include radiations such as X-rays, γ-rays, UV rays etc., whereas Ethyl methanesulfonate (EMS), Methyl methanesulfonate (MMS), Nitrous acid, Acridine orange, Ethidium bromide (EtBr), N-methyl-N'-nitro-N-nitrosoguanidine (NTG), 4-Nitroquinoline 1-oxide (4-NQO), Acridine orange (AO) etc., are chemical mutagens. In

molecular genetic methods, an increase in enzyme production is often achieved by overexpressing the genes encoding the enzymes with the help of promoters of constitutively expressed genes or inducible genes in the presence of specific inducer molecules (e.g., IPTG).

Another method for achieving enhanced microbial production is process development (optimization) and scale-up of a target product. It is a well-known fact that medium components play a very important role in enhancing cell growth and increase the target product accumulation and culture media development continues to be an effective area that offers the potential to dramatically improve the productivity of microbial fermentation process. Factors like carbon and nitrogen sources and their concentrations have always been of great interest to the researchers in the industry for the low cost media design. Also, it is estimated that approximately 30-40 % of the production cost of industrial enzymes is expected to be the cost of growth medium. Therefore, it is of immense significance to optimizing the conditions for cost-efficient enzyme production (Palaniyappan *et al.,* 2009).

There are two options of approach to research designs; one is the change of one variable at a time (Classical method), the second approach is to change one or more variables from one test to the next (statistical design). The conventional system for optimizing enzyme production by *One- factor- at- a –time* (OFAT) method is arduous, cumbersome and prolonged procedure entailing a large number of trials when several variables are to be considered and does not reflect interactions among variables. Further, there are so many additional

experiments to conduct the research which lead to an increase the time and expenses as well as an increase in the consumption of reagents and materials (Hanrahan *et al.,* 2006).

To overcome this problem, a substitute and more resourceful method is the use of statistical methods like RSM. It is useful for a small number of variables (up to five), but impractical for a large number of variables, due to a high number of experimental runs required (Sharma *et al.,* 2006). Therefore, for screening more than five factors, Plackett–Burman Design (PBD) is recommended (Plackett *et al.,* 1946). The PBD is principally useful for initial screening as it is used for the estimation of only the main effects. The selected significant factors obtained from the initial screening experiments could be further optimized by with the help of RSM that enables the study of interaction effects among different variables. It usually involves an experimental design such as Central Composite Design (CCD) to fit a second-order polynomial by the least squares technique. An equation is used to describe the test variables and describe the combined effect of all the test variables in the response. Nowadays, RSM is used in a wide range of scientific fields including production media optimization (Ghaffari-Moghaddam *et al.,* 2014).

In view of the potential industrial applications of pectinases, the present investigation was carried out to boost the pectinase enzyme production from *B. subtilis* BKDS1. Two approaches were tried to enhance the enzyme production. First, strain improvement strategy by using conventional methods such as UV mutation and mutation with

chemicals (EtBr and AO). The second method used for production augmentation is media optimization. PBD and CCD of RSM were applied for finding the significant variables and its optimization for maximizing the enzyme production. The graphical abstract of the study was depicted in Figure: 3.1.

3.2. Aim and objectives of the study

- Application of strain improvement strategies for enhanced pectinase production
- Statistical based media optimization for augmented pectinase production
- Comparison of the optimized media with various other pectinase production media
- Optimum temperature and incubation time standardization for maximal enzyme production by SmF.

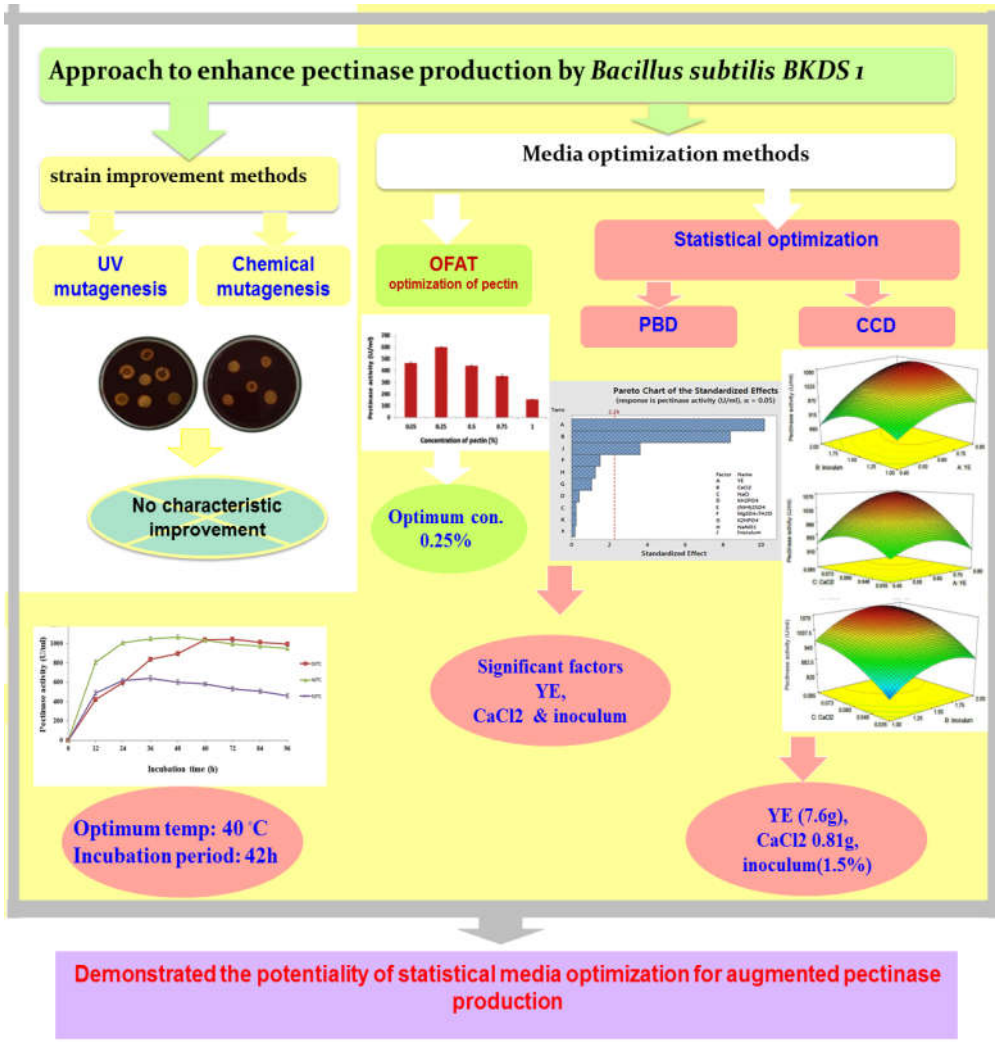


Figure: 3.1. Graphical representation of the study

3.3. Review of Literature

An increase in productivity reduces the overall cost of the product, as well as the production cost; hence, it is one of the important topics for the research. Usually, enhanced productivity can be achieved either by strain improvement or by optimizing the process parameters.

Singh *et al.,* (2016) reviewed that strain improvement and optimization are "*Catch-22*" situation. It was unable to choose a lead strain until having the best medium and cannot propose a finest medium until having the lead strain. Strain improvement and optimization studies for enhanced production of microbial products were discussed previously by many authors (Savergave *et al.,* 2011; Subathra Devi *et al.,* 2015).

3.3.1. Strain improvement for enhanced production of industrial enzymes

The potential productivity of the organisms is controlled by its genes so their genome is of prime importance to be altered for the increased production of biomolecules including enzymes. Strain improvement methods were employed on bacteria, fungus and yeast to enhance particular properties or to improve the production of primary or secondary metabolites. According to Winston, (2008), the mutagenesis can increase the frequency of mutation up to 100-fold per gene without excessive killing of the cells and without a significant frequency of double mutants. Different techniques are employed for this which includes; classical methods and molecular genetic methods. The literature review showed that enzyme production including pectinase was enhanced by both classical and molecular methods.

If the genes responsible in the synthesis of a metabolite or enzyme of interest are unknown, the production yield is boosted by introducing random mutations into the chromosomes of the synthesizing microbe by UV irradiation or treatment with mutagens

(Adrio *et al.,* 2006). Thermostable amylase yield was increased 40 times through mutagenesis with EMS (Sidhu *et al.,* 1997). A pooled mutagenesis method using UV, N-methyl-N′-nitro-N-nitrosoguanidine and Co60−γ-ray was adopted by Wang *et al.,* (2007) for the successful creation of a strain of *B. pumilus* that can produce high levels of alkaline proteases having application in dehairing of cowhides and goatskins in the tannery. Sudi *et al.,* (2008) used UV and EtBr mutagenesis to enrich the potentials of *L. bulgaricus* and *S.thermophilus* as a starter culture in yogurt.

3.3.2. Strain improvement for enhancement of pectinase

Ihuaku *et al.,* (2009) conducted UV-mutagenesis of a strain of *A. niger* through the use of a selective culture medium containing 2 deoxy D glucose (2dg). Among the selected mutagenic strains, the best strain showed 465 % and 230 % higher pectinase activity than the wild strain in SSF and SmF respectively. In a recent work, (Muzzamal *et al.,* (2016) noted a three-fold increase in the PG production by *Bacillus* strains through mutation with UV light and AO for different periods of time. While, Yin *et al.,* (2016) observed 73.6 % increase in the pectinase production after mutating the *Fusarium oxysporum* strain BM-201 with UV radiation followed by diethyl sulfate. And the mutant strain showed stability even after ten generations.

3.3.3. Media optimization for better microbial production

Production medium optimization is a vital part of microbial fermentation process development. Commonly, this includes the addition of several supplements to an existing basal medium

formulation. Having a well-defined media formulation optimized for maximum product formation can significantly improve product titers, thereby reducing costs and improving efficiency. Jerums *et al.,* (2005) stated that, common fermentation medium consisting of carbon sources, nitrogen sources, mineral salts, trace elements, peptides, amino acids, vitamins and/or other growth factors determines the chemical and nutritional environment of cells in a reactor. The concentrations of these factors along with the physical factors such as temperature, pH, agitation, aeration, etc. are very important for the efficient manufacturing of products accumulated in the cells or secreted into the medium. Screening experimental designs with these variables at low (-1) and high (+1) levels are useful for determining which components have the significant effect on cell growth, viability and productivity. Panda *et al.,* (2007) reviewed that optimization techniques such as such as borrowing, component swapping, biological mimicry, OFAT, factorial design, PBD, Box–Wilson design (BWD), Box-Behnken Design (BBD), CCD, RSM, evolutionary operation, evolutionary operation factorial design, fuzzy logic and genetic algorithms, artificial neural network (ANN) etc. are extensively used for optimization of microbial production media.

3.3.4. *One- factor- at- a –time* (OFAT) Optimization

The monothetic analysis (OFAT /OVAT) is an experiment designing method connecting the testing of factors or variables one at a time rather than simultaneous testing of the factors. Even though OFAT is a non-statistical optimization method, it is used in some cases by various researchers for microbiological media optimization even in recent times. The OFAT method was used by Qureshi *et al.,* (2012) to

test the effect of carbon, nitrogen, pH and temperature on pectinase production by *B. subtilis* EFRL 01 in a date syrup medium. Similarly, the effect of pectin concentration in production media for pectinase production using *A. niger* was studied by Islam *et al.,* (2013) and found that addition of 2 g pectin to the production media resulted in marked increase in pectinolytic activity while higher concentrations of pectin decrease pectinase activity. OFAT optimization of xylanase production from *T.viride*-IR05 in SSF was conducted by Irfan *et al.,* (2014) and selected sugarcane bagasse to be best for enzyme synthesis. (Suhaimi *et al.,* (2016) used OFAT approach to develop a cost-effective industrial medium for pectinase production using a newly isolated *A. niger* strain. Similarly, (Sethi *et al.,* (2016) also used OVAT approach to optimize process parameters for increased pectinase production by *A. terreus* NCFT 4269.10 using banana peels as substrate.

3.3.5. Response surface methodology (RSM)

RSM is a medley of mathematical and statistical techniques for empirical model building primarily established and defined by Box and Wilson (Box *et al.,* 1951). By using RSM, it can able to identify and optimize significant factors/ variables with the purpose of determining what levels of factors maximize the response (Sayyad *et al.,* 2007). RSM optimization is done with the use of experimental designs such as PBD, CCD (Colla *et al.,* 2016; Kai *et al.,* 2016)**,** BBD (Sayyad *et al.,* 2007) etc. to develop empirical models that relate a response and mathematically describes the relationships existing between the independent and dependent variables of the process under consideration (Panda *et al.,* 2007). RSM is empirical statistical techniques that are based on second-order (quadratic) polynomial models

used for multiple regression analysis. Many commercial tools have recently come to the market (e.g., MINITAB, Design Expert, E-Chip, Statistica, SAS etc.) that are generally used for the analysis. The graphical representation of these equations are called as response surfaces, could be used to designate the single and cumulative effect of the test variables on the response and to determine the mutual interaction between the test variables and their subsequent effect on the response (Cornell *et al.,* 1987; Montgomery, 1991).

The RSM is a powerful and efficient mathematical approach widely applied in the optimization of fermentation process including media components on enzyme production (Bibi *et al.,* 2016; Reddy *et al.,* 2016; Rekha *et al.,* 2013) production of other metabolites like vitamins (Mahdinia *et al.,* 2018) antibiotics (Wang *et al.,* 2011), ethanol (Arora *et al.,* 2015), bioplastic (Yadav *et al.,* 2017), acid metabolites (Hujanen *et al.,* 2001), biosurfactants (Almeida *et al.,* 2017) spore production & inactivation (Almeida *et al.,* 2017; Yu *et al.,* 1997) and biomass production optimization (Banerjee *et al.,* 2017). It can give information about the interaction between variables, provide information necessary for design and process optimization, and give multiple responses at the same time. The significance and theoretical concepts behind RSM based experimental design and optimization approach in research and development efforts has been perfectly discussed in a number of informative articles (Dinarvand *et al.,* 2017; Hanrahan *et al.,* 2006; Nwabueze, 2010; Yadav *et al.,* 2015).

3.3.6. Application of RSM in the optimization of media components on enzymes production

RSM is the most popular optimization method used in recent years for enzyme production and process optimization. Lots of reports and reviews are published based on the application of RSM in enzyme production (Chen *et al.,* 2010; Dinarvand *et al.,* 2017; Ghaffari-Moghaddam *et al.,* 2014; Jeong *et al.,* 2006). By analyzing the data published in various research reports, it is found that there is an effective fold increase in the enzyme production after media optimization by RSM compared to un-optimized medium (Ghaffari-Moghaddam *et al.,* 2014).

In the case of amylase enzyme production, many published data are available to prove the effectiveness of RSM. Recently, Ait K.E.E *et al.,* (2016) reported that, the use of statistical experimental designs would be a reliable and effective alternative for the optimization of the fermentation processes of thermostable α-amylase production by a newly isolated *R.oryzae* FSIS4. Similarly Mouna imen *et al.,* (2015) also reported the effectiveness of RSM in α-amylase production by halophilic *Streptomyces sp*. In a statistical approach study conducted by Gangadharan *et al.,* (2008) showed significant results for optimizing the process parameters for increased α-amylase production under SmF using *B. amyloliquefaciens* ATCC 23842 and allowed rapid screening of a large number of variables. The RSM based optimization using *A. oryzae* CBS 819.72 revealed an enhanced α-amylase yield of 72.7 % (Kammoun *et al.,* 2008).

Statistical analysis proved to be a useful and powerful tool in developing optimum fermentation conditions for amylase production by *A.oryzae* and found to be 20 % increase in enzyme yield in optimized SSF process (Francis *et al.,* 2003).

In connection with lipase production, latest reports by Colla *et al.,* (2016) used PBD and CCD for media optimization for lipase production under submerged fermentation by filamentous fungi. Kai *et al.,* (2016) also used the similar methodology for optimization of lipase production from a novel strain *Thalassospira permensis* M35-15 Kishan *et al.,* (2013) reported that maximum enzyme production of (9.40 U/ ml) was obtained under optimal condition, where the production media is optimized with PBD and CCD. It is reported a 3.14-fold increase in lipase activity after fermentation media optimization by RSM using *R. delemar* (Acikel *et al.,* 2010).

Production of other enzymes such as cellulase, protease, chitinase, keratinase, xylanase, etc. also exploits media optimization by RSM. Singh *et al.,* (2014) used RSM mediated media optimization for visualizing the combined interactive effects of different variables for the production of cellulase by marine *Bacillus* VITRKHB. In case of protease production, (Shabbiri *et al.,* (2012) recently reported that rate of protease production by *Brevibacterium linens* DSM 20158 was found to be two-fold higher in the statistically optimized medium as compared to the unoptimized reference medium. Optimization of Chitinase production by *B. pumilus* with the help of PBD and RSM was reported by Tasharrofi *et al.,* (2011). For the optimization of

keratinase from poultry feather by *Streptomyces* sp7, Tatineni *et al.,* (2007) conducted a two-step response RSM experiment and found to be very effective. The results of RSM based optimization studies by (Ramanjaneyulu *et al.,* 2016) indicate that the model developed for the optimal production of xylanase by the isolate *Fusarium* sp. is reliable and accurate. RSM based optimization for maximizes growth and production of inulinase and invertase by *A.niger* ATCC 20611 was conducted by (Dinarvand *et al.,* 2017).

3.3.7. RSM optimization in pectinase enzyme production

The production of extracellular pectinase in microorganisms is significantly influenced by a number of factors. The relationships between these variables have a marked effect on the ultimate production of the pectinase. There are reports on the influents of various fermentation parameters on pectinase production by different bacteria and fungi. Many studies were reported regarding media optimization for pectinase production focused on RSM - statistical optimization technology and found very effective. Sharma and Satyanarayana (2006) used PBD and RSM for the production of a highly alkaline and thermostable pectinase of *B. pumilus* in SmF. Three fermentation variables (C: N ratio, K_2HPO4, and pH) were selected by PBD and were further optimized using CCD. By optimizing the media by RSM, they achieved a very high increase (41-fold) in alkaline pectinase production. Gummadi *et al.,* (2006) used PBD for optimization of PNL and PL by *Debaryomyces nepalensis* in SmF and noted that four of the eleven fermentation

variables (yeast extract, galactose, lemon peel and temperature) showed significant effect on both PNL and PL production and RSM optimization it was found that the enzyme productivities increased by 2.5 and 2.9 fold for PNL and PGL, respectively.

Rekha *et al.,* (2013) conducted a study to optimize the parameters influencing the PG production by the sequential statistical approach using Taguchi and CCD of RSM. They concluded that these statistical approaches were effective for the enhanced production of cold-active PG by *Thalassospira frigidphilosprofundus*. The results of a study conducted by Amin *et al.,* (2013) also indicated that RSM could be used to get enhanced activities of exo-PG under SSF using *P. notatum*. According to Yannam *et al.,* (2014), RSM proved to be an effective method for optimization of fermentation conditions for PGase production by SmF using *A. foetidus*. Where, the average PG production (36.5 U/ml) was enhanced by 4.1-fold after optimization of critical parameters using RSM. Tepe *et al.,* (2014) studied the effects of parameters such as concentrations of solid substrates, ammonium sulphate and yeast extract on the production of exo-pectinase by *B. pumilus* with the help of RSM and it was determined that exo-pectinase activity increased when relatively low concentrations of ammonium sulphate (0.12–0.21 %w/v) and yeast extract (0.12–0.3 %w/v) and relatively high wheat bran (~5–6 %w/v) were used.

According to Bibi *et al.,* (2016), RSM is a good and reliable optimization method by which it can increase pectinase production from 39 U/ ml to 219 U/ml in SmF with B. *licheniformis*. Bennamoun

et al., (2016) noted that, among seven factors tested in PBD, three factors (lactose, $CaCl_2$ and pH) were selected that significantly effecting exo-PG production by *Aureobasidium pullulans* and the optimum composition of these factors was determined by applying CCD. After optimization, they attained a five-fold increase in enzyme production. The effect of fermentation condition on PG production by *T. harzianum* was studied using RSM design by Daoud *et al.,* (2016) and revealed that the highest production of PG reached a maximum of 145.6 U/ml at a temperature of 30 °C, pH 6, incubation time 5 days, orange peels concentration 3 % and ventilation speed 150 rpm. Handa *et al.,* (2016) used RSM to optimize various environmental parameters such as temperature, moisture and incubation days for pectinase production by *Rhizopus* sp. C4 under SSF. These factors were studied statistically for a total of 20 runs using CCD. They obtained the highest yield of the enzyme of 11.63 IU/ml and concluded that optimization through RSM could improve the enzymatic characteristics and yield of the enzyme. The results of a study conducted by Reddy & Saritha, (2016) indicated that RSM is suitable for predicting accurate quantities of media components for enhanced pectinase production by *Enterobacter* sp. PSTB-1. They used CCD to find the best concentration of media components and noted that the result obtained by experiment is in close agreement with the predicted value. Similarly, the RSM with BBD was used to optimize the pectinase production media for *B. subtilis* ZGL14 and observed that factors such as starch, peptone, KH_2PO_4 and $K_2HPO_4 \cdot 3H_2O$ significantly affect the activity of alkaline pectinase (Yu *et al.,* 2017). Recently Sindhu *et al.,*

(2018) used PBD and BBD for pectinase production media optimization for *B. sonorensis*.

3.4. Materials and Methods

3.4.1. Inoculum preparation

The YEP medium was inoculated with *B. subtilis* BKDS1 culture and incubated overnight at 150rpm and 30 °C. Generally, 1 % concentration of the seed culture was used as inoculum in the optimization studies.

3.4.2. Identification of optimum conditions for pectinase activity

3.4.2.1. Optimum pH

Pectinase activity in the cell free supernatant was assayed in varying pH ranges (5.0 – 10) by preparing substrate (Citrus pectin, 1.0 %,w/v) in 0.02 M of different buffers, such as citrate phosphate buffer, pH 5.0 – 5.5; phosphate buffer, pH 6.0 – 7.9; Tris – HCl buffer, pH 7.5 – 8.5; and glycine NaOH buffer, pH 9.0 – 10.0 (Tewari *et al.,* 2005). DNS assay was performed with substrates dissolved in these pH ranges.

3.4.2.2. Optimum temperature

For analyzing optimum temperature activity for the enzyme, cell free supernatant was incubated at different temperature (30-60) at optimized pH with 1 % citrus pectin for 15 min and DNS assay was performed.

3.4.3. Strain improvement for enhanced pectinase production

3.4.3.1. Mutagenesis by UV radiation

UV mutagenesis of the selected bacterial strain was done as per the methodology followed by Sudi *et al.,* (2008) with minor modifications. The bacterial strains were grown overnight at 37 °C in 5 ml of LB-broth. Next day, the culture was inoculated into fresh media and the OD was adjusted to reach around 0.1-0.2 in 600 nm. The cells were then harvested by centrifugation (5000 rpm for 10 min), the pellet was washed twice with cold saline (sterile) and re-suspended. From the re-suspended suspension, 1.5 ml aliquots were transferred to sterile Petri dishes and exposed wider UV-light (254 nm) for different time phases (5, 10, 15,20,25 and 30 min). Each irradiated sample was centrifuged at 5000 rpm for 15 min, re-suspended in 5 ml YEP broth and incubated for 6 h at 37 °C. After incubation, centrifuged the tubes at 5000 rpm for 10 min and discarded the supernatant. Re-suspended the pellet in sterile saline and were spread plated after desired serial dilution.

3.4.3.2. Mutagenesis by chemical mutagens

The mutagens used for the test were, ethidium bromide (EtBr), acridine orange (AO) and their combination (EtBr + AO). The strain *B. subtilis* BKDS1 grown in LB broth with adjusted OD 0.1-0.2 (at 600 nm) was used for the study. A different set of LB broth (2.5 ml each) was prepared and the mutagens were added to get a final concentration of 0.5, 1.0, 1.5 and 2 mg/ml and one tube was kept as a control without any mutagen. To these tubes, 25 µl of test bacterium was added and in incubated for 1h at 37 °C. After incubation, the culture tubes

centrifuged and the pellet was washed twice with sterile LB broth to remove the mutagen. The pellet was re-suspended in 1ml sterile LB broth and 100 µl from each tube was spread pated on YEP agar plates and incubated. After overnight incubation at 37 °C, morphologically different colonies expected to be mutated were selected for further studies

3.4.4. Optimization of growth medium for maximal pectinase production by *B. subtilis* BKDS1

3.4.4.1. Optimization of pectin -as the sole source of carbon

Addition of different amount of citrus pectin (0.05 – 1 %) in the YEP media on the pectinase production was studied by the conventional *OFAT* method. YEP broth media was prepared with varying the concentration of pectin. Media was sterilized and added with 1 % inoculum. Incubated at 40 °C in a shaker for 48 h and DNS assay was performed.

3.4.4.2. Response Surface Methodology (RSM) optimization of media compounds using statistical softwares

3.4.4.2.A. Plackett-Burman Design (PBD)

PBD (Plackett *et al.,* 1946) was used to screen the major factors for pectinase production by *B. subtilis* BKDS1 using the statistical Software Minitab (Release 16, PA, USA). It allows the evaluation of N variables in the N+ 1 experiment. Each variable is examined at two levels, i.e., low (-1) and high (+). Ten factors were chosen such as, yeast extract (YE), NaCl, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $CaCl_2$, $MgSO_4 \cdot 7H_2O$, $NaNO_3$ and pH. The levels of the factors in the design

are given in Table: 3.1. PBD is based on the first order polynomial model;

$$Y = \beta_0 + \Sigma\beta_i x_i \left(i = 1, \ldots, k\right)$$

Where, Y is the response the response measured as enzyme activity. β_0 and β_i are the model intercept and the linear coefficient respectively, x_i is the level of the independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. All experiments were carried out in duplicate and the averages of the pectinase activity were taken as the response. From the regression analysis, the variables which were significant at or above 95 % level ($P < 0.05$), were considered to have a greater impact on pectinase activity and were further optimized by CCD.

Table: 3. 1. Levels of the factors tested in the PBD

No	Code	Factors (g/L)	Min. value (-1)	Max. value (+1)
1	A	Yeast extract (YE)	0.20	1.0
2	B	Calcium chloride ($CaCl_2$)	0.01	0.11
3	C	Sodium Chloride (NaCl)	0.02	0.1
4	D	Ammonium sulphate ($(NH_4)_2SO_4$)	0.05	0.15
5	E	Potassium dihydrogen phosphate (KH_2PO_4)	0.03	0.15
6	F	Dipotassium phosphate (K_2HPO_4)	0.01	0.05
7	G	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.01	0.11
8	H	Sodium Nitrate ($NaNO_3$)	0.01	0.07
9	J	Inoculum size (%)	0.5	2.5
10	K	pH	6	8

3.4.4.2.B. Central composite design (CCD)

The CCD approach based on RSM was used for determining optimum levels of critical variables (identified by PBD) for enhanced enzyme production. CCD has been widely used as a statistical method based on the multivariate nonlinear model for the optimization of process and production variables. The statistical software 'Design Expert 6.0' was used to generate and analyze the experimental design. The CCD was used for fitting a second-order model which requires only a minimum number of experiments for modelling. Each significant parameter was assessed at five levels (-2, -1, 0, +1, +2), with six replicates at the centre points. Experimental range and levels of independent process variables are shown in Table: 3.2

Table: 3.2: Ranges of variables used in RSM

Sl No.	Variables	code	-2	-1	0	1	+2
1	YE	A	0.20	0.4	0.60	0.8	1.0
2	$Cacl_2$	B	0.01	0.035	0.06	0.085	0.11
9	Inoculum	C	0.05	1.0	1.5	2.0	2.5

A total of 20 experimental runs were conducted, and the results (response) were analysed by ANOVA. A second order polynomial equation (as shown below) can be used to represent the function in the range of interest.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Where Y is the predicted response, β_0 model constant; A, B and C are independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12},

β_{13} and β_{23} are cross product coefficients; β_{11} β_{22} and β_{33} are the quadratic coefficients. With the help of Design Expert Software, the 3-dimensional surface plots were generated. The quality of the fit of the polynomial model was expressed by the value of correlation coefficient (R^2). The model *F*-value (Fisher variation ratio), the probability value (Prob > *F*) and adequate precision are the main indicators showing the significance and adequacy of the employed model.

3.4.4.3. Validation of the experimental model

To compare the predicted result with the practical value, experimental validation was performed using the optimized conditions.

3.4.5. Comparison of enzyme production in optimized medium with various other pectinase production media

The pectinase production attained in the optimized medium was compared with culture media previously used by various researchers as described in chapter 2 (Section 2.4.6), such as (i). YEP (Kashyap *et al.,* 2000), (ii). Czapek'sDox pectin medium (Reda A *et al.,* 2008), and (iii). Pectinase production media used by (Soares *et al.,* 1999) and (iv) (Jayani *et al.,* 2010).

3.4.6. Pectinase enzyme production

Further enzyme production studies by the isolate were done in modified yeast extract pectin (YEP) broth containing (g/l) 2.5g Citrus pectin, 7.6 g yeast extract, 0.81g $CaCl_2$ and 1.5 % inoculum volume

with pH 7. Batch mode shake flask experiments were conducted at 40 °C and 150 rpm in 250 ml Erlenmeyer flasks containing 50 ml of the media. After 48 h of incubation, centrifuged (10000 rpm, 10 min) the fermentation broth and collected the cell free supernatant for further analysis.

3.4.7. Effect of incubation time and temperature on enzyme production

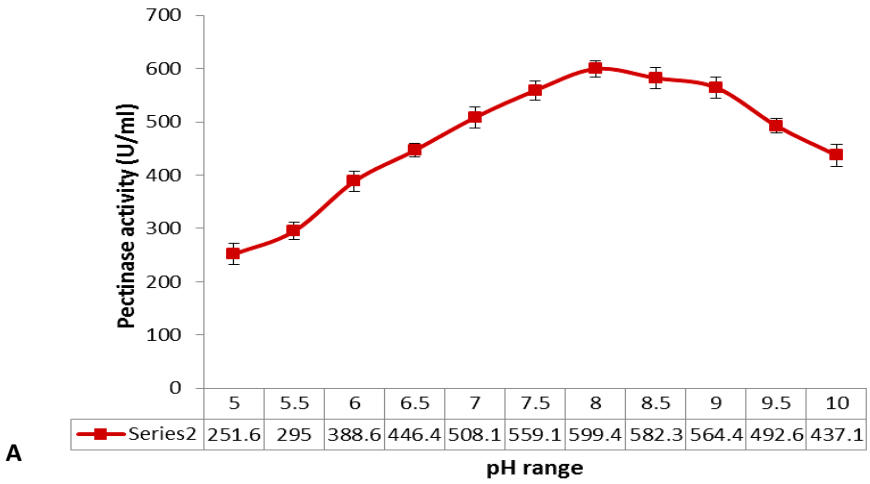
The optimized medium was used for analyzing the effect of incubation time and temperature on enzyme activity. The inoculum was prepared as mentioned above section and incubated in a rotary shaker at 150 rpm in different temperatures (30 °C, 40 °C and 50 °C). The enzyme assay was performed at every 12 h incubation period.

3.5. Results

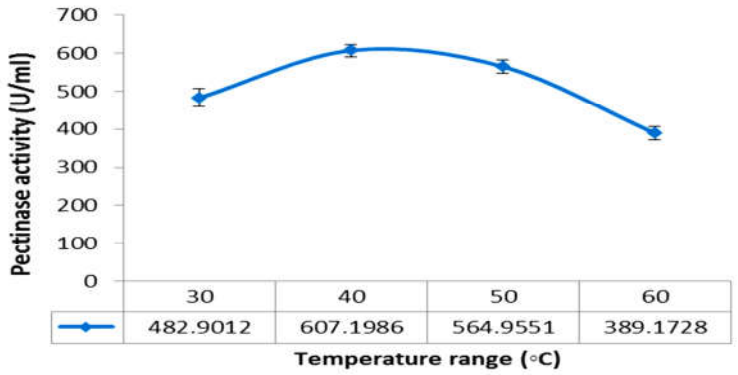
3.5.1. Identification of optimum condition (pH and temperature) for pectinase activity

The substrate (citrus pectin) was dissolved in buffers of varying pH (5-10) and DNS assay was performed to test the optimum pH for pectinase assay. Among different pH tested, it is found that substrate dissolved in Tris- HCl buffer of pH 8 gives higher activity (599.380 U/ml). Similarly, the favorable temperature for pectinase activity was also optimized by performing DNS assay at different temperatures ranging 30 °C -70 °C and the optimum temperature for maximum pectinase activity (607.199 U/ml) was found at a temperature of 40 °C.

So this optimized pH (8) and temperature (40 °C) was employed for further enzyme assay studies.



A



B

Figure: 3.2 (A&B). Effect of pH & temperature on enzyme activity

3.5.2. Strain improvement for enhanced pectinase production

3.5.2.1. UV-mutagenesis

The number of colonies developed in culture plates exposed to UV radiation of different time intervals was noted and counted. The results were shown in Table: 3.3.

Table: 3.3. Number of colonies observed in UV treated samples

Time of exposure (min.)	Colony forming units
5	$6x10^2$
10	$4 x10^2$
15	No growth
20	No growth
25	No growth
30	No growth

The colonies developed on the culture plates were finely observed for any morphological change from the control organism. But none of the colonies showed a visual morphological difference. Selected colonies were taken for plate assay and zone diameter is measured after incubation. The zone diameters showed by the selected colonies were shown in Figure 3.3.

Figure: 3.3. Pectin utilization by UV irradiated colonies with control colony at center

3.5.2.2. Mutagenesis with chemicals

Chemical mutagens such as EtBr, AO and their combination were tested for chemical mutagenesis. The number of colonies

developed on culture plates after treatment with mutagens was shown in Table: 3.4.

Table: 3.4. CFU observed in different concentrations of the mutagen

Mutagens	Concentrations of the mutagen			
	0.5 mg/ml	**1.0 mg/ml**	**1.5 mg/ml**	**2 mg/ml**
EtBr	12 $x10^1$	9 $x10^1$	8 $x10^1$	4 $x10^1$
AO	16 $x10^1$	11 $x10^1$	9 $x10^1$	6 $x10^1$
EtBr + AO	9 $x10^1$	7 $x10^1$	6 $x10^1$	2 $x10^1$
Control	78 $x10^2$			

3.5.3. Optimization of Growth Medium for maximal pectinase production by *B. subtilis* BKDS1

3.5.3.1. Concentration of pectin (as the sole source of carbon) optima for the enzyme

With culture supernatant of YEP broth prepared from the different concentration of pectin, enzyme activity was calculated by DNS method and the result was showed in Figure: 3.4.

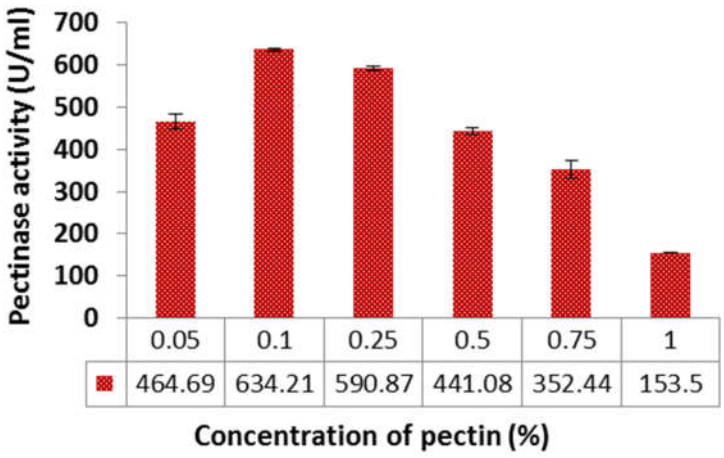


Figure: 3.4. Pectinase production at different concentrations of pectin

3.5.3.2. Response Surface Methodology (RSM) optimization of media compounds using statistical softwares

3.5.3.2.A. Screening of the most significant medium components by PBD

PBD was used to analyze the effect of 10 variables on pectinase production by *B. subtilis* BKDS1. Twenty runs were carried out to screen the effect of the variables on pectinase production. The data obtained after PBD analysis was given in Table: 3.5. The analysis of regression coefficients and t-value of nine ingredients are shown in Table: 3.6 and the corresponding Pareto chart is shown in Figure: 3.5.

Table: 3.5. The PBD experimental result for nine variables

Run order	Coded Factors										Pectinase activity (U/ml)
	A	B	C	D	E	F	G	H	J	K	
1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	137.247
2	-1	-1	-1	1	-1	1	-1	1	1	1	231.686
3	1	-1	-1	1	1	-1	1	1	-1	-1	295.162
4	1	-1	1	1	-1	-1	-1	-1	1	-1	487.911
5	1	1	-1	-1	1	1	-1	1	1	-1	812.256
6	-1	-1	1	1	-1	1	1	-1	-1	-1	191.433
7	1	-1	-1	-1	-1	1	-1	1	-1	1	377.990
8	-1	1	1	-1	-1	-1	-1	1	-1	1	285.873
9	-1	1	1	1	1	-1	-1	1	1	-1	440.552
10	1	-1	1	1	1	1	-1	-1	1	1	519.333
11	-1	-1	-1	-1	1	-1	1	-1	1	1	284.324
12	-1	1	-1	1	1	1	1	-1	-1	1	424.435
13	-1	1	-1	1	-1	1	1	1	1	-1	439.917
14	-1	1	1	-1	1	1	-1	-1	-1	-1	375.667
15	1	1	-1	1	1	-1	-1	-1	-1	1	616.410
16	1	1	1	1	-1	-1	1	1	-1	1	688.401
17	1	1	-1	-1	-1	-1	1	-1	1	-1	679.112
18	1	-1	1	-1	1	1	1	1	-1	-1	439.285
19	1	1	1	-1	-1	1	1	-1	1	1	886.569
20	-1	-1	1	-1	1	-1	1	1	1	1	206.915

Table: 3.6. Regression analysis of PBD

Term	Factors	Effect	Coef	SE Coef	*T*-Value	*P*-Value
A	YE	282.4	141.2	13.8	10.20	0.000
B	$CaCl_2$	231.8	115.9	13.8	8.37	0.000
C	NaCl	6.3	3.2	13.8	0.23	0.824
D	KH_2PO_4	-11.0	-5.5	13.8	-0.40	0.700
E	$(NH_4)_2SO_4$	4.8	2.4	13.8	0.17	0.866
F	$MgSO_4.7H_2O$	41.7	20.8	13.8	1.50	0.167
G	K_2HPO_4	29.1	14.5	13.8	1.05	0.321
H	$NaNO_3$	-34.4	-17.2	13.8	-1.24	0.245
J	Inoculum (%)	99.7	49.8	13.8	3.60	0.006
K	pH	6.3	3.2	13.8	0.23	0.824

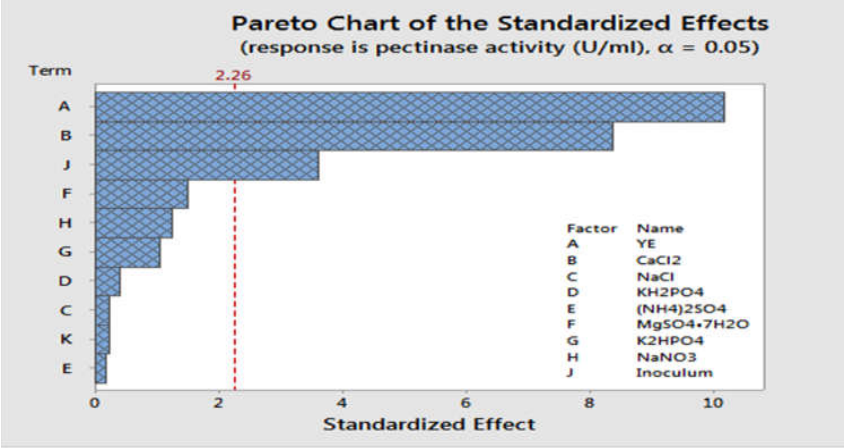


Figure: 3.5. Pareto chart showing the effect of media components on pectinase activity

The PBD analysis of ten factors indicated a marginal variation in pectinase activity from 191.43 U/ml to 886.569 U/ml in twenty trials. This variation ensured the impact of all the factors on enzyme activity. Among the ten factors tested, three factors namely YE, $CaCl_2$ and inoculum size (%) found to play a significant role in pectinase production.

3.5.3.2.B. Optimization of significant variables using CCD

The optimal levels of the three most significant factors were determined by CCD of RSM. The three independent variables were studied at five different levels (−2, −1, 0, +1,+2) as shown in Table:3.2 and a set of 20 experiments with a different combination of the selected variables were carried out. The actual yield of the enzyme, pectinase and the yield predicted by the model equation are given in Table: 3.7. The ANOVA analysis of the optimization study is given in Table: 3.8. The three-dimensional response surface plots for pectinase production showing the interactive effects between the selected media components were shown in Figure 3.6 (i-iii).

Table: 3.7. CCD matrixes of four variables with experimental and predicted response

Run	Yeast extract (A)		Inoculum (B)		$CaCl_2$ (C)		Pectinase activity (U/ml)	
	Coded	Actual	Coded	Actual	Coded	Actual	Observed	Predicted
1	0	0.6	+2	2.5	0	0.06	798.2293	1055.02
2	0	0.6	0	1.5	0	0.06	1065.229	1027.42
3	0	0.6	0	1.5	0	0.06	972.3383	1027.42
4	0	0.6	0	1.5	0	0.06	1026.525	1027.42
5	+2	1	0	1.5	0	0.06	948.4962	1085.66
6	+1	0.8	-1	1	-1	0.035	782.0662	935.7
7	0	0.6	0	1.5	0	0.06	1044.484	1027.42
8	0	0.6	0	1.5	+2	0.11	972.6479	1109.7
9	-1	0.4	+1	2	-1	0.035	853.7472	973.72
10	0	0.6	0	1.5	0	0.06	1026.525	1027.42
11	-1	0.4	-1	1	+1	0.085	860.8689	1018.02
12	-2	0.2	0	1.5	0	0.06	831.763	969.18
13	0	0.6	0	1.5	-2	0.01	800.7992	945.14
14	-1	0.4	-1	1	-1	0.035	865.2038	1014.12
15	+1	0.8	+1	2	+1	0.085	1058.495	1182.24
16	0	0.6	-2	0.5	0	0.06	810.0884	999.82
17	+1	0.8	-1	1	+1	0.085	933.9432	1086.64
18	0	0.6	0	1.5	0	0.06	1042.007	1027.42
19	+1	0.8	+1	2	-1	0.035	906.0759	1021.58
20	-1	0.4	+1	2	+1	0.085	868.3002	987.34

Table: 3.8. Analysis of variance (ANOVA table for response surface quadratic model-CCD)

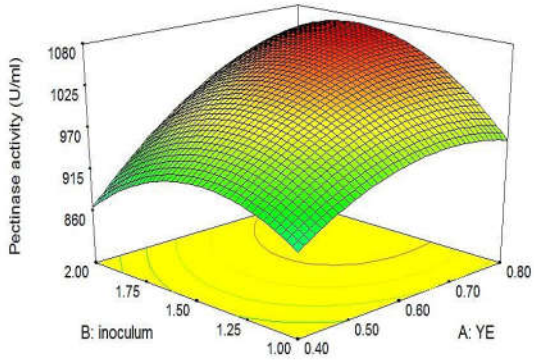
Source	Sum of Squares	df	Mean Square	*F*- Value	*p*-value Prob > F	
Model	171203.9	9	19022.655	18.802	< 0.0001	Significant
A-YE	13567.97	1	13567.975	13.411	0.0044	
B-inoculum	3047.531	1	3047.531	3.012	0.1133	
C-$CaCl_2$	27077.63	1	27077.633	26.764	0.0004	
AB	7974.996	1	7974.996	7.883	0.0186	
AC	10810.23	1	10810.230	10.685	0.0084	
BC	47.18933	1	47.189	0.047	0.8334	
A^2	32398.28	1	32398.281	32.023	0.0002	
B^2	82808.86	1	82808.863	81.850	< 0.0001	
C^2	33953.55	1	33953.547	33.560	0.0002	
Residual	10117.18	10	1011.718			
Lack of Fit	5174.495	5	1034.899	1.05	0.4806	Not significant
Pure Error	4942.686	5	988.537			
Cor Total	181321.1	19				

From the ANOVA analysis **(**Table: 3.8), the model *F*-value is 18.8 which indicates the model is significant. If the Prob>*F*-value is very small (< 0.05) designate model terms are significant. Here, A, C, AB, AC, A^2, B^2, C^2 are significant model terms. The regression equation coefficients were calculated and the data was fitted to a second order polynomial equation. Thus the response (Y), (in terms of coded factors) *i.e.* pectinase production by *B. subtilis* BKDS1 can be expressed in terms of the following regression equation;

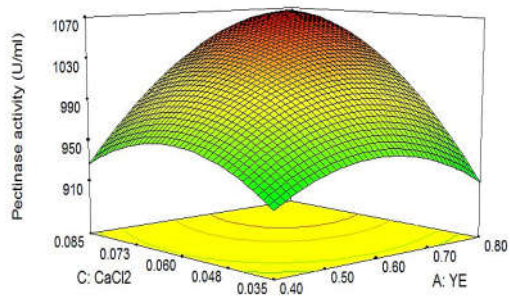
$$\text{Pectinase activity (U/ml)} = +1027.42 + 29.12A + 13.80B + 41.14C + 31.57AB + 36.76AC + 2.43BC - 35.90A^2 - 57.39B^2 - 36.75C^2$$

Where A is yeast extract, B is $CaCl_2$, and C is inoculum. The lack of fit *F*-value of 1.05 implies the lack of fit is not significant relative to the pure error. The *p*-value of lack of fit in in this model is 0.4806 (>0.05) means the model fits well. The design predicted an R-squared value of 0.7241, which is in reasonable agreement with the adjacent R-squared value of 0.8940. For a good statistical model, the R^2 value should be in the range of 0 – 1.0, and the value as obtained in the data analysis indicates that the model is good.

i



ii



iii

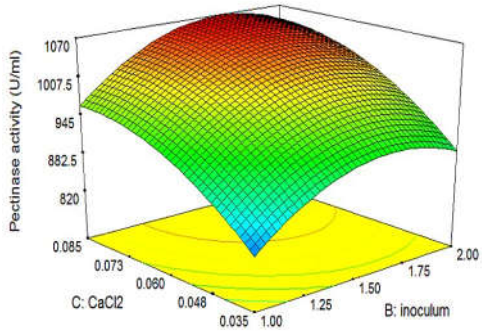


Figure: 3.6. Response surface plot for pectinase production showing the interactive effects of; i). A: YE and B: inoculum size ii). A: YE and C: $CaCl_2$ and iii). B: inoculum size C: $CaCl_2$

3.5.4. Validation of the Experiment

The acceptability of the experimental model was validated by performing the combinations of independent variables predicted for maximum response (Table: 3.9).

Table: 3.9. Experimental sets for model validation

Run	YE (g/l)	Inoculum (%)	$CaCl_2$ (g/l)	Pectinase activity (U/ml)	
				Observed	Predicted
1	7.6	1.5	0.81	1065.95	1069.84
2	8.0	1.8	0.84	1060.94	1068.43
3	7.7	1.64	0.79	1056.23	1067.53

Among these set of experiments, the optimized conditions: (g/l) yeast extract 7.6g, $CaCl_2$ 0.81g and inoculum size of 1.5 % + Citrus pectin 2.5g & pH 7 established by the regression model gave maximum activity (1065.95U/ml). So, the experimental result of pectinase activity (1065.95U/ml) was proximate to the actual response (1069.84U/ml) predicted by the regression model, which proved the validity of the model.

3.5.5. Comparison of enzyme production in optimized medium with various other pectinase production media

Pectinase production attained in the optimized medium was compared with culture media previously used by various researchers to analyze the fold in increase. The data obtained were plotted in Figure: 3.7.

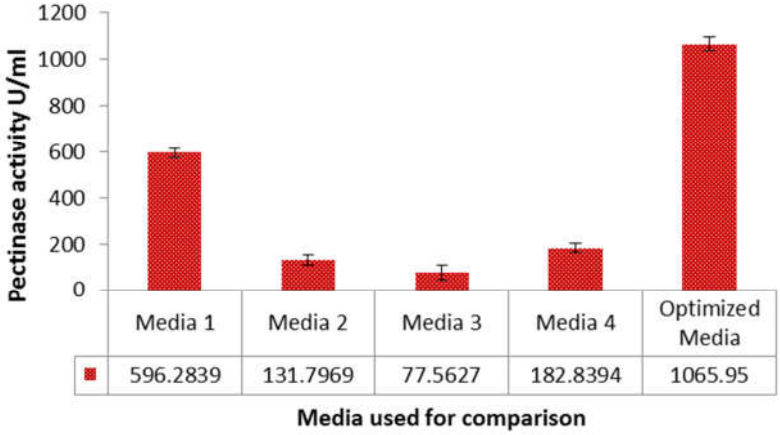


Figure: 3. 7. Comparison of pectinase production in unoptimized/ optimized media

3.5.6. Optimum temperature and incubation time for maximum enzyme activity

Effect of incubation time and temperature on enzyme production was studied in 12 h of time interval at a temperature range of 30 – 50 °C at150 rpm. The enzyme assay was performed at every 12 h incubation period and the result obtained was illustrated in Figure: 3.8.

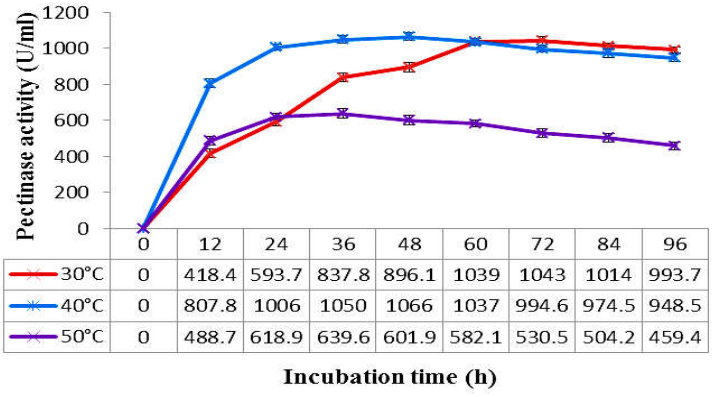


Figure: 3.8. Effect of Incubation temperature on pectinase activity

3.6. Discussion

It was obvious from the initial studies (Chapter 2), that the isolate *B. subtilis* BKDS1 is a powerful pectinolytic strain with additional industrially important characteristics. In this section, we aimed at the enhancement of pectinase production capability of this strain by strain improvement and media optimization strategies. In order to test the optimum pH for pectinase activity, the substrate (1 % citrus pectin) was dissolved in different buffers of pH ranging from 5-10 and the enzyme activity was tested. It was found that pH 8 (Tris-HCl buffer) gave the best result (599.380 U/ml) compared to substrate dissolved in other buffers (Figure: 3.2.A) and the enzyme activity is more in the alkaline condition than in the acidic. Similarly, the optimum temperature for maximum pectinase activity (607.199 U/ml) was found at a temperature of 40 °C (Figure: 3.2.B). The result also indicates that the enzyme can withstand a temperature range of 30-60 °C. So this optimized pH (8) and temperature (40 °C) was employed for further enzyme assay studies. The result indicates that the enzyme produced by *B. subtilis* BKDS1 is alkaline pectinase with thermostability. In previous studies majority of the *Bacillus* sp. showed optimum pH range in an alkaline region (pH 8–9) (Kashyap *et al.,* 2000; Nawawi *et al.,* 2017; Qureshi *et al.,* 2012; Torimiro *et al.,* 2013; Yu *et al.,* 2017). So the pectinase produced by *B. subtilis* BKDS1 is alkaline pectinase.

Strain improvement and media optimization methods were applied for augmenting the enzyme production by *B. subtilis* BKDS1.

This is achieved by classical methods of mutagenesis by UV radiation and chemical mutagens. But none of the mutagenesis methods showed characteristic improvement in the pectinolytic activity. Considering the effect of UV radiation, an exposure time of 15 min was found to be lethal. But none of the exposed colonies showed improvement in characteristic pectinolytic activities. The result was also confirmed by DNS assay. In the case of chemical mutagenesis, there was a gradual decrease in the number of colonies with the increasing concentration of mutagen. The combined effect of EtBr and AO was found to more lethal than their individual effect. But here also the pectinolytic property was not improved by any of the mutagen treated bacterial colony.

Media optimization approaches were the next strategy employed to enhance the enzyme production capacity of the strain. Here we adopted OFAT method to optimize the substrate concentration and rest of the media components were optimized by statistical RSM (PBD and CCD) method. In order to optimize the pectin concentration, the YEP broth was prepared with varying concentrations (0.05-1 %) of pectin and the enzyme activity is calculated from the culture supernatant. Maximum enzyme activity (599.38 U/ml) was found in medium containing 0.25 % of pectin (Figure: 3.4). This result clearly indicates that enzyme activity is decreased with increasing concentration of pectin. The similar type of observations were previously noted in *B. subtilis* (Kashyap *et al.,* 2000) and *Streptomyces* sp. RCK-SC (Kuhad *et al.,* 2004) where maximum pectinase activity was in 0.25 % of pectin. Decreased

enzyme production in a higher concentration of pectin can be accredited to the phenomenon of catabolite repression, where galacturonic acid or one of the metabolites produced is undergoing self catabolite repression (Joshi *et al.,* 2013; Tsuyumu S., 1979) and also because of viscosity increase in the culture broth (Palaniyappan *et al.,* 2009).

Application of statistical method in media optimization for pectinase production was reported previously by many researchers (Bibi *et al.,* 2016; Reddy *et al.,* 2016). PBD confirmed that pectinase production was significantly influenced by factors such as yeast extract, $CaCl_2$ and inoculum size. The analysis of regression coefficients and t-value of ten ingredients are depicted in Table: 3.6. Generally, a large t-value associated with a low *p*-value of a variable indicates a high significance of the corresponding model term. From Table: 3.6 and the corresponding Pareto chart in Figure: 3.5, it is clear that, variables YE, $CaCl_2$, and inoculum displayed a high positive significant effect for enzyme production with '0' *p*-value whereas NaCl, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, K_2HPO_4 and pH showed non-significant positive effects. Factors such as KH_2PO_4 and $NaNO_3$ displayed a non-significant negative effect. None of the tested factors showed a significant negative effect.

From the PBD assay, it is clear that yeast extract and the metal ion $CaCl_2$ showed the lowest *p*-value (Table: 3.6 and Figure: 3.5). Yeast extract, the complex nitrogen source used for optimization presented major impact on pectinase production. Yeast extract is

proved to be the chief nitrogen source possibly because it provided other stimulatory components such as vitamins (Qureshi *et al.,* 2012). Some previous reports are available to ratify this result; from various nitrogen sources tested, yeast extract (7.5 g/L) is proved to be the most effective in pectinase production by *B. subtilis* EFRL01(Qureshi *et al.,* 2012). Supplementation of the fermentative medium with yeast extract presents a positive effect on pectinase production by marine *B. subtilis* (Joshi *et al.,* 2013). Similarly, PG production by *B. shaericus* MTCC 7542 is maximum when grown on mineral medium containing yeast extract as sole nitrogen source (Jayani *et al.,* 2010).

The metal ion $CaCl_2$ is another factor selected on PBD (with *p*-value 0.000), that significantly affect pectinase production. Prior reports are available that indicate the significance of $CaCl_2$ in pectinase production; the maximum activity of endopolygalacturonase production by *B. subtillus* was observed whenever there was high concentration of calcium chloride (Munir *et al.,* 2015). Recent report indicates that exo-pectinase production by *Aureobasidium pullulans* is influenced by the presence of $CaCl_2$ in the production medium at a concentration of 0.09 g/l (Bennamoun *et al.,* 2016). Pectinases such as PGL has an absolute requirement of Ca^{2+} ions whereas PMGL also requires Ca^{2+} and other cations for its stimulation (Jayani *et al.,* 2005). This also implies the importance of $CaCl_2$ in pectinase activity.

Inoculum size is another factor selected by PBD that significantly affect the enzyme production (with *p*-value 0.003). The high inoculum density causes lesser enzyme production because of

competition for available nutrients. So the optimization of inoculum size was a well-accepted criterion in microbial fermentation. From previous studies, it is clear that inoculum size is an important factor that significantly effects enzyme production in various microorganisms (Gupta *et al.,* 2010; Reddy *et al.,* 2008; Shabbiri *et al.,* 2012). Among different factors tested for pectinase production using *B. mojavensis*, inoculum selected as one of the major factors that have a significant effect on enzyme production and an inoculum level of 3 % gave the best result (Ghazala *et al.,* 2015). The fermentation media inoculated with 7.5 % v/v inoculum of *B. shaericus* MTCC 7542 showed the best PG activity (Jayani *et al.,* 2010).

The interaction effects and optimal levels of the factors were determined by plotting the response surface curves. Figure: 3.6(i) depicts the interactive effects between factors yeast extract and inoculum and this is a significant interaction as the *p*-value is 0.0186 (Table: 3.6). From the figure, it is evident that the pectinase activity is maximum when the concentration of yeast extract reaches 7.6 g/l at inoculum volume 1.5 %. The enzyme activity tends to decrease above and below this range. The significant interactive effect between yeast extract and $CaCl_2$ is presented in Figure: 3.6(ii). The enzyme activity rises with increasing concentration of $CaCl_2$ and reaches the maximum at 0.81g/l of $CaCl_2$ at this point the concentration of yeast extract is 7.6 g/l. From Table: 3.6, it is visible that the interaction between inoculum and $CaCl_2$ is a non-significant interaction as the *p*-value is 0.8334

which is <0.05. The response surface plot of this interaction is shown in 3.6(iii).

The enzyme production achieved in the optimized medium is then compared with various other previously reported pectinase production media. From the comparison result (Figure: 3.7), it is clear that the optimized medium showed many fold increase compared to other pectinase production media tested. The corresponding fold of increase was; 1.78, 8.08, 13.74, 5.82 folds in media1, 2, 3 and 4 respectively. This implies a good optimization result.

Effect of incubation time and temperature on enzyme production was studied in 12 h time interval at a temperature range of 30 - 50°C at150 rpm. The enzyme assay was performed at every 12 h incubation period and the results obtained were illustrated in Figure: 3.8. From this figure, it is clear that incubation temperature and time had an impact on enzyme yield. The optimal incubation time for maximal pectinase activity in 30 °C was found to be 72 h. At the point when the temperature is increased from 30 °C to 40 °C, the optimum incubation period diminishes from 72 h to 48 h. The incubation period again decreased to 24- 36 h at a temperature of 50 °C but the level of enzyme production was very low compared to other temperature ranges. So 40 °C is taken as the optimum temperature for maximal enzyme production. Despite the fact that, the enzyme production achieved its peak at 48 h (1066.255 U/ml), the level of enzyme production increases even from 24 h (1006.398 U/ml) of the incubation period. Reports have shown that many Bacillus species produce pectinase maximally at an incubation time of 72 h and above (Jayani *et al.,* 2010; Kumar *et al.,* 2012; Paudel *et al.,* 2015).

3.7. Conclusion

Medium optimization is one of the most critically investigated processes that is carried out before any large-scale metabolite production. As a preferred statistical experimental method, RSM is suitable for describing a near optimum region and thus identifying the exact criterion for a multifactorial optimize the growth-promoting factors for the enhanced production of pectinase by the isolated strain *B. subtilis* BKDS1 using statistical methods. The study system, which reduces the number of experiments without neglecting the interaction among the parameters. The present study has been attempted to begin with optimizing the substrate pectin by OFAT method. Then, PBD was used to determine the relative importance of ten variables on pectinase production and found that yeast extract, $CaCl_2$ and inoculum size were the major factors. The optimal concentration ranges of the three factors were optimized successively by CCD. In the optimized fermentation broth that contains yeast extract (7.6 g/l), $CaCl_2$ (0.81 g/l) and pectin (2.5 g/l) at an inoculum size of 1.5 %, the pectinase activity reached 1065.95 U/ml compared with the predicted value of 1069.84 U/ml. Further, the incubation temperature and incubation period were also optimized and found to be 40 °C and 48 h respectively. The optimized media showed many-fold increase in enzyme production compared to various other production media tested. So, this study demonstrates the prospects of the new strain *B. subtilis* BKDS1 for pectinase production and applicability of statistical media optimization for augmented enzyme production.

CHAPTER 4

ENHANCED PECTINASE PRODUCTION BY SUBMERGED FERMENTATION (SMF) USING AGRO-WASTE: MEDIA FORMULATION AND STATISTICAL OPTIMIZATION

4.1. Introduction

Agro-wastes or biowastes are highly perishable materials and their disposal often is a problem in processing industries and household setter. Copious amount of waste materials is produced by agricultural and fruit processing industries, which pose considerable disposal problems and ultimately leads to pollution. In a developing country like India, dumping of solid wastes on land is the common methods of waste disposal which in turn creates various environmental and health related problems. Thus ecological matters and anxieties aimed at minimizing the ambient pollution have boosted the search for "clean Technologies" or "Green Technologies" to be used in the production of commodities of importance to chemical, energy and food industries. The agro-wastes with high amount of organic matter comprises of 50-60 % of total solid wastes which are being used as an substituting source for production of important compounds as these are valuable raw materials; rich in sources like energy and other nutrients (lignocelluloses, proteins, carbohydrates, lipids etc.) which would be lost if they are discarded in the open dump yards and landfills (Kandaiah *et al.,* 2015).

In our earth, agro-residues are present in abundance as biomass resources for the effective bioconversion into various bioproducts. These may comprise of whole plants, plant parts (comprising seeds, stalks, stems), fruits, plant constituents like polysaccharides (starch, cellulose, pectin), lipids, protein and fibre), processing byproducts (distiller's grains, corn solubles) etc. Agro-residues such as wheat bran,

rice bran, bagasse, and fruit peel (citrus, orange, apple, lemon, banana, mango etc.) have been extensively studied for their efficiency for fermentative production of various microbial metabolites (Mohamed *et al.,* 2013; Ruiz *et al.,* 2012; Sethi *et al.,* 2016). The pineapple (*Ananas comosus*) is one of the most important fruits in the world and is the leading edible member of the family *Bromeliaceae*. 'India is the fifth largest producer of pineapple with an annual output of about 1.2 million tonnes'. Generally, the pineapple by-products consist of the residual pulp, peels, stem and leaves. The food industries in the country used this fruit for the development of different products and the resulting wastes may cause various environmental problems. Researchers have focused on the utilization of pineapple wastes as low-cost substrates for the production of industrially important outcome (Upadhyay *et al.,* 2013). Watermelon (*Citrullus lanatus*) is an important cucurbitaceous vegetable grown in various regions of India and it is a common summer season vegetable crop. The watermelon rind constitutes approximately 30 % weight of a watermelon and it contains 13 % of pectin (Hartati *et al.,* 2015). Banana (*Musa* sp.) is the second most important fruit crop in India. Approximately, 13 % by weight of the harvested banana bunch is peduncle waste. Usually, all these agro-wastes are discarded or composted. In this study, an attempt is made to utilize these wastes for media constituent for submerged fermentation production of the enzyme pectinase.

In the current biotechnological era, pectinases have wide-ranging of applications mainly in food and textile industries. Although

microbial pectinase is widely used in food processing industries in other countries, it is still in its immaturity in developing countries like India mainly because of the high costs involved. If economically viable technologies for production are available, it will promote the food processing industries in this country. Agro-wastes including fruit rinds, peels, stalks, straw, bagasse, stems, etc. are a major part of wastes generated daily by households, agricultural sector and food processing industries. In most cases, these waste materials are dumped in landfills which lead to unhygienic conditions. Vast varieties of micro-organisms are present in the environment which can be exploited for the utilization of waste material.

Both SmF and SSF have been effectively applied in pectinase production by fungi and bacteria. Even though, SmF is widely used for enzyme productions like alkane pectinase, the major factor hindering the process include high production costs because of the overpriced reagents in synthetic media and low yield because of the prolonged fermentation time. Therefore, microbial strains that grow quickly on cheap substrates and capable of tolerating SmF-based process for the production of pectinase needs to be developed to reduce the production cost (Zou *et al.,* 2014).

Optimization of process and production parameters is one of the important precarious phases in the progression of an efficient and economic bioprocess. As discussed in the earlier chapter (Chapter 3) the classical approach of optimization OFAT is effective only in few cases but for improved optimization, studying the combined effect of

all the parameters is useful to consider. So, statistical approach of optimization RSM consists of PBD and CCD is widely used as this method has some advantage over the other terms of rapid and reliable short listing of process and production conditions (Bibi *et al.,* 2016).

In view of the above, the present study was to explore the best agro- waste for maximum production of pectinase with the aim of cleaning environment by utilizing solid waste in a productive way. The study also focused statistical screening of the most influencing media components by PBD and optimization of enriched medium by CCD and exploitation of the optimized agro-waste media for pectinase production using SmF with Lab scale fermenter of 1L capacity. The graphical abstract of the study is depicted in Figure: 4.1.

4.2. Objectives of the Study

- Selection of a suitable agro-waste for pectinase production by the strain *B. subtilis* BKDS1
- Determination of best agro-waste concentration for the maximum pectinase activity.
- Estimate the concentration of pectin in the selected agro-wastes.
- Media formulation for submerged fermentation (SmF)
- Optimization of agro-waste media components by RSM.
- Production of pectinase in lab scale bioreactor (of 1L capacity) using the optimized media.

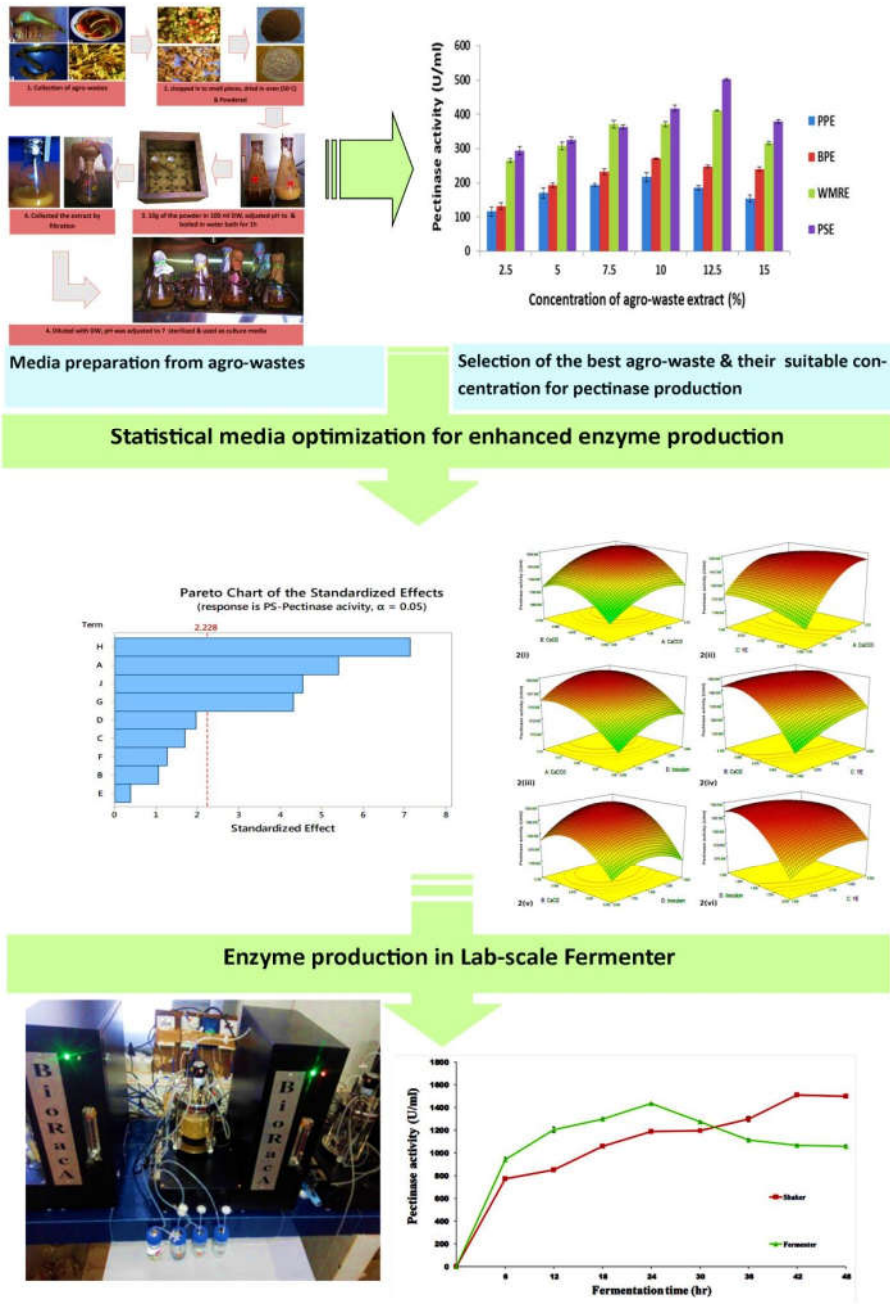


Figure: 4.1. Graphical representation of the chapter

4.3. Review of Literature

Agro-industrial residues are mainly composed of complex polysaccharides (lignocellulosic materials) that strengthen microbial growth for the production of industrially important enzymes. Lignocellulose-a major component of biomass available on earth and consists of cellulose, hemicellulose, and lignin. Other than these three components, natural lignocellulosic materials contain a small amount of pectin, nitrogenous compounds, and the secret ash (Chen, 2014). These are the most gifted feedstock as a natural and renewable resource essential to the functioning of modern industrial societies and lavishly accessible with high potential for bioconversion to value-added bio-products.

4.3.1. Application of agricultural residues/ biomass for enzyme production

Biomass can be demarcated as the mass of organic material from any biological material, and by extension, any large mass of biological matter. As discussed by Howard *et al.,* (2003) and Smith *et al.,* (1987), lots of biomass resources are available on our earth for bioconversion into various bioproducts. These may comprise of whole plants, plant parts, plant constituents, processing byproducts, materials of marine origin and animal byproducts, municipal and industrial wastes.

The term agro-residue or agro-waste is used to describe all the organic materials which are produced as by-products from harvesting

and processing of crops and fruits. Every year huge quantities of agricultural residues are accumulated in nature which creates environmental and other health problems. Further, the agro-residues are composed of starch, lignin, xylan and pectin would be lost if they are discarded in the open dump yards and landfills. These energy rich compounds can be used by variety microorganisms as carbon and energy sources producing enzymes and other products with high commercial values such as organic acids, biofuel, cheap energy sources for fermentation single cell proteins, antibiotics and other primary and secondary metabolites (Da Silva *et al.,* 1997).

There are some excellent and comprehensive works of literature which describe the use of various agro-residues in the production of various enzymes such as pectinase, amylase, carboxymethyl cellulose, cellulose, inulinase, lipase, protease, xylanase and so on (Mohamed *et al.,* 2013; Sethi *et al.,* 2016).

4.3.2. Pectinase enzyme production using agro-waste

Reports are available regarding the production of pectinase enzyme in SSF and SmF using varied agro-industrial wastes such as rice bran (Izzat *et al.,* 2011), wheat bran (Tepe *et al.,* 2014), banana peel (Barman *et al.,* 2015), watermelon rind (Mohamed *et al.,* 2013), sugarcane bagasse (Biz *et al.,* 2016), lemon peel, orange peel and citrus waste (Ruiz *et al.,* 2012) (Ahmed *et al.,* 2016; Biz *et al.,* 2016), apple pomace (Joshi *et al.,* 2006), mango peel (Joshi *et al.,* 2006), pineapple peel (Okafor *et al.,* 2010; Singh *et al.,* 2012). Table: 4.1

shows important agro-wastes used for pectinase production by a range of fungi and bacteria.

Remarkable pectinolytic enzyme production was detected by Ahmed *et al.,* (2016) in Czapeck media supplemented with orange waste peel as a cheap carbon source under SmF process using *A. niger*. A study conducted by Sethi *et al.,* (2016) exposed the potentials of effective utilization of various agro-waste residues such as mustard oil cake, neem oil cake, groundnut oil cake, black gram peels, green gram peels, chickling vetch peels, wheat bran, pearl millet residues, finger millet waste, broken rice, banana peels, apple pomace, and orange peels in fermentation processes as potential substrates where they can act as carbon, nitrogen sources and ultimately produced industrially pertinent enzymes. They also portrayed the capability of banana peel as a cheap substrate for enhanced pectinase production by *A. terreus* NCFT 4269.10. Exo-pectinase production by *B. pumilus* using different agricultural wastes such as wheat bran, sugar beet pulp, sunflower plate, orange peel, banana peel, apple pomace and grape pomace as substrate was studied by Tepe *et al.,* (2014) and demonstrated that wheat bran and sugar beet pulp could be successfully utilized in enzyme production.

Barman *et al.,* (2015) reported the effectiveness of banana peels as a cheap and efficient carbon source substrate for pectinase production by *A.niger* (Barman *et al.,* 2015). In a study conducted by Mohamed *et al.,* (2013) tells that watermelon and cantaloupe rinds can be used as cheap substrates compared to other agro- wastes like wheat, corn, rice, sugar cane and beet, banana waste, potato, tea, apple, and citrus fruits, which are used for production of xylanase and

polygalacturonase using *Trichoderma* species. The feasibility of using agro-industrial wastes such as orange bagasse, corn tegument, wheat bran and mango and banana peels as carbon sources for the production of polygalacturonase and pectin lyase by using *Penicillium viridicatum* strain Rfc3 was examined by Silva *et al.,* (2002).

Table: 4.1. Agro-wastes used for pectinase production:

A. Bacterial pectinase production using agro-waste				
No	**Microbe used**	**Agro-waste(s) used**	**Special features**	**Reference**
1	*Bacillus* sp. DT7	Wheat bran	Maximum activity at 36 h in 37 °C	**(**Kashyap *et al.,* 2000)
2	*B. subtilis*	Citrus limetta peels	Maximum activity at pH 5.0 & 40 °C	(Joshi *et al.,* 2013)
3	*Bacillus subtilis* SAV-21	Orange peel & coconut fiber	The combinational ratio (4:1) of the agro-waste recorded maximum activity	(Kaur *et al.,* 2017)
4	*B. licheniformis* SHG10	Orange peel	Production of PG was enhanced by RSM optimization	*(Embaby et al., 2014)*
5	*B. licheniformis* KIBGE IB-3	Peels of apple, orange, lemon, potato &wheat bran	wheat bran was selected as the best substrate for PG production	(Ruiz *et al.,* 2012)
6	*B. licheniformis*	Orange peel & wheat bran	fivefold increase after RSM optimization	(Bibi *et al.,* 2016)
7	*B.pumilus* dcsr1	Sesame oilseed cake & wheat bran	In comparison with SmF, 14.2 fold increase in SSF	(Sharma *et al.,* 2006)
8	*B. pumilus* NRRL B-212	Wheat bran, sugar beet pulp, sunflower plate, orange & banana peel, apple & grape pomace	wheat bran and sugar beet were the most efficient substrates for SSF production of exo-pectinase	(Tepe *et al.,* 2014)
9	*B.mojavensis* I_4	Carrot peel	Optimum activity at pH 6 and temp. 60 °C	(Ghazala *et al.,* 2015)
10	*Pseudozyma* sp. SPJ	Citrus peel	Achieved 18.5 fold increase by SSF	(Sharma *et al.,* 2012)
11	*Enterobacter* sp. PSTB-1	Mango fruit processing industrial waste	RSM was used for media optimization	(Reddy *et al.,* 2016)

B. Fungal pectinase production using agro-waste				
12	*Aspergillus niger* DMF 27 & DMF 45	Sunflower head	Produced both endo and exo pectinase by SSF & SmF	(Patil *et al.,* 2006)
13	*A. niger* ATCC 16404	Rice Bran	Optimum incubation period-3 days for SSF	(Izzat *et al.,* 2011)
14	*A. niger* Aa-20	Lemon peel pomace	high levels of pectinase activities were obtained by SSF	
15	*A. sojae*	Orange peel, wheat bran, and corn meal	Produced high exo-PG at low optimal pH	(Buyukkileci *et al.,* 2011)
16	*A.niger* MTCC 281	Banana peel	The partially purified enzyme showed more than 3 times of PG activity as compared to the crude enzyme	(Barman *et al.,* 2015)
17	*A. niger* NAIMCCF-02958	Mango peel	By RSM, maximum PG was attained in pH 4.0, 30 °C & 2 % inoculum	(Yadav *et al.,* 2015)
18	*A.oryzae* CPQBA 394–12 DRM 01	Citrus pulp, and sugarcane bagasse	Pilot-scale packed-bed SSF was successfully used for pectinase production	(Biz *et al.,* 2016)
19	*Rhizopus* sp. C4	Orange peels	Pectinase production was enriched by RSM	(Handa *et al.,* 2016)
20	*P.viridicatum* strain Rfc3	Orange bagasse, corn tegument, wheat bran, mango & banana peels	Found to produce PG & PL. PG was stable in neutral pH range & at 40 °C whereas PL was stable in acidic pH & at 35 °C for 1 h	(Silva *et al.,* 2002)
21	*Trichoderma harzianum* and *T. virens*	Watermelon and cantaloupe rinds	can be used as optional substrates for production of xylanase & PG	(Mohamed *et al.,* 2013)
22	*T.viridi*	Orange peel	Active at optimum pH 5 & temp. 60 °C.	(Irshad *et al.,* 2014)
23	*Botryosphaeria rhodina*	Orange bagasse	produced pectinase and laccase by both SSF & SmF	(Giese *et al.,* 2008)
24	*Schizophyllum commune*	Citrus waste	Optimized by RSM	(Mehmood *et al.,* 2018)

4.3.3. Role of *Bacillus* spp. in economical enzyme production utilizing agro-wastes as substrates

Bacillus spp. are the most predominant bacterial genera exploited for the economical production of pectinases by using different agro-waste as substrate. Many researchers used strains belonging to *Bacillus,* because this genus includes strains (such as *B. subtilis)* that can grow on cheap substrates such as agro-wastes (Sakai *et al.,* 1989). It is proved that orange peel waste can be used as a sole carbon source for pectinase production by various strains of *Bacillus* (Embaby *et al.,* 2014; Kapoor *et al.,* 2000; Kaur *et al.,* 2017; Tepe *et al.,* 2014). Wheat bran is another substrate, proven as a cheap and easily available source throughout the year for higher pectinase production and many researchers used wheat bran as an economical carbon source for pectinase production by *Bacillus* spp. Among various agro-byproducts studied for PG production by *Bacillus* sp. MG-cp-2, it is found that PG production level was boosted significantly by using wheat bran and ramie fibre in the production media (Kapoor *et al.,* 2000). *Bacillus* strains cultivated on wheat bran produced endo-PG, exo-PG and PNL in the crude enzymatic solution (Soares *et al.,* 2001). A high yield of pectinase (PG) was attained from *B. licheniformis* KIBGE IB-21(Rehman *et al.,* 2012) and *B. licheniformis* KIBE-IB3 using wheat bran as substrate (Jahan *et al.,* 2017). Various other agro-industrial wastes such as; rice bran, cassava bagasse, sugar beet pulp, carrot peels etc. are also exploited for pectinase production by *Bacillus* spp. (Ghazala *et al.,* 2015; Li *et al.,* 2005; Nawawi *et al.,* 2017; Swain *et al.,* 2009).

4.3.4. Agro-residues in other enzyme production

Suitability of using cheap and abundantly available banana fruit stalk (peduncle) waste for cellulase production by the bacterial strain *B. subtilis* (CBTK 106) in SSF system was reported in 1999 (Krishna, 1999). Use of cassava wastewater as a substrate for production of various hydrolytic enzymes such as amylases, proteases, and lipases by various strains of *B. subtilis* was reported by Barros *et al.,* (2013). In a study to investigate the possibility of using cost-effective agricultural residues like distillers dried grain with soluble, palm kernel meal, wheat bran or copra meal in producing cellulolytic and hemicellulolytic enzymes, Seo *et al.,* (2014) concluded that copra meal is an ideal substrate for cellulolytic and hemicellulolytic enzyme production using bacterial culture *B. licheniformis* (Seo *et al.,* 2014). Agro-waste cocktail including rice straw, sugarcane bagasse, rice husk, and empty fruit bunch, etc. were used by for cellulase production by an isolated strain of *B. licheniformis* 2D55 (Kazeem *et al.,* 2017). Among different agro-wastes tested, potato peel was selected as the best source for the production of Laccase (thermo-alkali stable) from *Pseudomonas* sp.S2 (Chauhan *et al.,* 2018).

4.3.5. Pectin extraction from agro-wastes

To extract pectin from pineapple peel, Karim *et al.,* (2014) used acid extraction followed by precipitation by ethanol. It was reported that extraction pH was the central criteria influencing the yield of pectin (Liew *et al.,* 2014). For the optimization of pectin extraction process, Prakash Maran *et al.,* (2014) applied RSM including BBD

coupled with desirability function methodology and the results indicated that, all the process variables have a significant effect on the yield of pectin extraction. After optimization, the yield of pectin was increased to 13.781 %. Tartaric acid was found to be the best extracting agent for microwave induced extraction of pectin from passion fruit peels. The maximum yield of pectin extracted was 9.1-13 %. (Seixas *et al.,* 2014). Similarly, Hartati *et al.,* (2015) used sulfuric acid solution (0.5 M) for microwave assisted extraction of pectin from watermelon rind. They achieved an extractive yield of 11.25 %. Extraction of pectin from saba banana showed that highest yield of pectin (17.05 %) was obtained using HCl (Castillo-Israel *et al.,* 2015). In a recent study, (Roy *et al.,* (2018) used HCl (0.1 N) at 90 °C for 120 min at pH 1.5 and 2.0 for extraction of pectin from pomelo peel and the yield was found to be 16.74 %.

4.3.6. RSM optimization of agro-waste media for enhanced enzyme production

RSM has already been effectively applied for the optimization of agro-waste based media for the economical production of various primary and secondary metabolites. Exo-pectinase production by *B. pumilus* using different agricultural wastes such as wheat bran, sugar beet pulp, sunflower plate, orange peel, banana peel, apple pomace and grape pomace as substrate was studied by Tepe *et al.,* (2014) and demonstrated that wheat bran and sugar beet pulp could be successfully utilized in enzyme production. Further, RSM was successfully applied to optimize medium components and noted that

supplementation of the solid agro-waste substrates with ammonium sulphate and YE were found to increase the exo-pectinase enzyme production. The PBD and CCD were effectively applied by Mahesh *et al.,* (2014) to optimize the enriched pectin extract medium (for enhanced pectinase production using *A. niger*) prepared from extracted pectin from orange peel. Similarly, PG production by *A. niger* NAIMCCF-02958 using the substrate mango peel was optimized by RSM Yadav *et al.,* (2015). Taguchi design followed by BBD was used to optimize the pectinase production from *B. mojavensis* I_4 using carrot peels powder as the substrate and noted the optimum conditions as; carrot peels powder 6.5 %, NH4Cl 0.3 %, inoculum level 3 % and cultivation time 32 h (Ghazala *et al.,* 2015).

Handa *et al.,* (2016) reported that RSM not only helps to analyze the optimum conditions of the process variables for the enhancement of pectinase production by *Rhizopus* sp.C4 using orange peel, but also proves to be well suited to assess the main effects and interaction effects of the process variables on enzyme production. Whereas, RSM was effectively applied for SmF optimization of pectinase production using powdered mango industrial waste as cheap substrate. Reddy *et al.,* (2016) used CCD of RSM for the enhanced pectinase production by Enterobacter sp. PSTB-1. They used mango fruit processing industrial waste as the carbon source for the submerged fermentation production of pectinase. The PL production through SSF from *Schizophyllum commune* using citrus waste was optimized by CCD of RSM and indicated maximum PL activity 480.45 U/ml (Mehmood *et al.,* 2018).

4.3.7. Fermentation methods

Submerged fermentation (SmF) and solid state fermentation (SSF) have been successfully used in pectinase production by fungi and by bacteria (Pedrolli *et al.,* 2009). Large varieties of microbial metabolites are produced by the well- developed SmF system and are technically easier than SSF. Usually, SmF is applied in case of enzyme production by bacteria because of the requirement of higher water potential (Chahal, 1983) whereas, SSF is favored when enzymes have to be extracted from fungi, which need lesser water potential (Troller, 1978). Higher fungi and their enzymes, as well as spores or metabolites, are well adjusted to growth on solid wet substrates and fungal spores produced by SSF show predominant characteristics in stability, resistant to drying and germination rates for extended periods of time after freeze-drying compared to SmF. In spite of the advantages, the technology is still in the developmental stage and the industrial application of SSF is waiting for advancements in heat and mass transfer, biomass separation and process control etc. Also, SSF showing certain technical drawbacks in control of temperature, pH, and nutrient gradients in the bioreactor, which complicate process monitoring, control and scale-up. So, more than 75 % of the industrial enzymes are produced using SmF (Thomas *et al.,* 2017).

In order to make the fermentation process more cost-effective in both SmF and SSF using agro industrial wastes, Kumar *et al.,* (2011) successfully applied RSM methodology to optimize the pectinase and cellulase production by *A. niger*. Improved PG from

Bacillus sp.MG-cp-2 under SmF and SSF and effect of amino acids and their analogs, vitamins and surfactants in fermentation was reported in 2002 (Kapoor *et al.,* 2002). Ezugwu *et al.,* (2013) used extracted pectin from dried mango peel as the sole carbon source for SmF production of pectinase by *A. fumigatus*. Medium optimization was found to effective in both SSF and SmF for pectinase production using *Bacillus* strains (Bibi *et al.,* 2016; Ghazala *et al.,* 2015; Kaur *et al.,* 2017). Zou *et al.,* (2014) developed a new Fed-Batch fermentation for enhanced production of alkaline PGL using *B. subtilis* 7-3-3. The process combines the enzymatic pretreatment of the carbon source with controlled pH of the fermentative broth to enhance enzyme production in a cheap manner.

4.4. Materials and methods

4.4.1. Agro-waste collection and pre-treatment

Different agro-wastes that are commonly available in Malabar regions of Kerala such as pineapple stem, pineapple peel, banana peduncle, watermelon rind were collected (Figure: 4.2**)**. The collected agro-wastes were washed with distilled water and sliced into small pieces and oven dried in 50 °C. The dried agro-wastes were then powdered with mixer grinder.

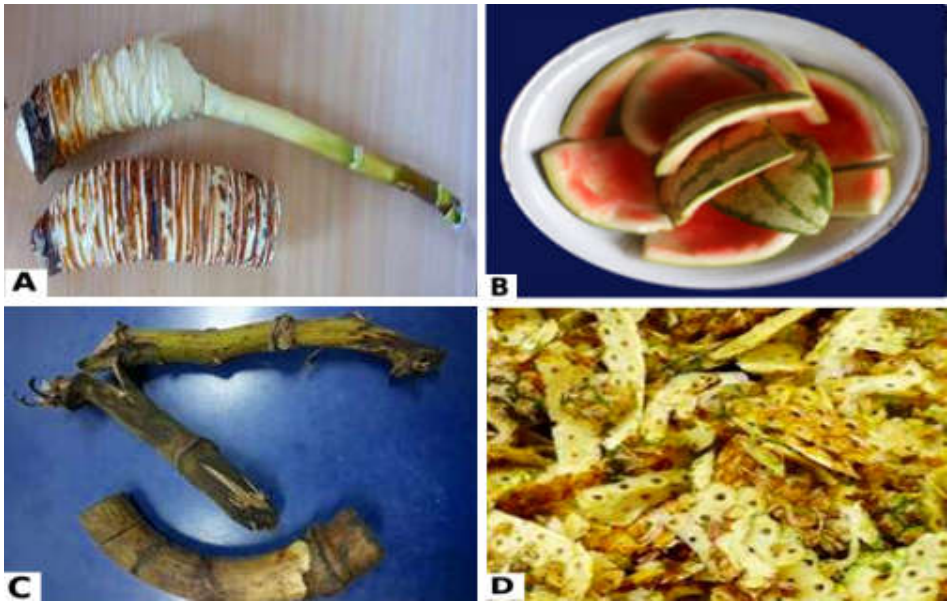


Figure: 4.2. Different agro –wastes selected for the study (**A.** Pineapple stem, **B.** Water melon rind, **C.** Banana peduncle and **D.** Pineapple peel)

4.4.2. Agro-waste extract (AWE) preparation for media formulation

4.4.2.1. Extract preparation

The extract was prepared from powdered agro-wastes according to the acid extraction method of pectin with some modifications (Castillo-Israel *et al.,* 2015, Campbell, 2006; Liew *et al.,* 2014). The schematic procedure for extract preparation is given in Figure: 4.3. Weighed 10 g of agro-waste powder and suspended in 100 ml distilled water in 250 ml Erlenmeyer flask and mixed well. The final pH of the mixture is adjusted to 2 using HCl (5N). The flask is then kept in a boiling water bath for about 1 h. After 1 h, the flask is taken from the water bath and kept in a shaker until it cools. The

extract is then filtered with a double layered muslin cloth and the filtrate is used for further analysis and media preparation.

Figure: 4.3. Extract preparation procedure-a schematic representation

4.4.2.2. Selection of best agro-waste for media formulation

For media preparation using the AWE, the pH of the extract is adjusted to 7 with NaOH (pH adjustment is crucial. Because above pH

8, precipitates will form) and various dilutions of this extract were prepared viz. 2.5%, 5%, 7.5%, 10%, 12.5%, and 15% by diluting the AWE with distilled water. These dilutions were used as culture media for the production of pectinase. Pectinase assay was carried out using the 24 h culture grown in the AWE media. By analyzing the enzyme activities, the best agro-waste and its most suitable concentration were selected for further studies.

4.4.3. Characteristics of the selected agro-waste (PS and WMR)

4.4.3.1. Product yield

The product yield of dried AWP obtained from the raw agro-wastes was calculated by taking weight before and after the drying process

4.4.3.2. Determination of pectin (Yapo, 2009)

The aqueous extract containing pectin was coagulated by using 95 % ethanol (twice the volume) at 4 °C and was left for 5 h for precipitating pectin. The precipitate obtained was recovered through centrifugation and filtration. The precipitate is then washed, dried and pectin yield was calculated using the formula;

$$\text{Yield of pectin from the AW extract} = \frac{(\text{amount of pectin})}{(\text{total amount of AW powder taken})} \times 100$$

4.4.3.1. C. Estimation of pectin as calcium pectate (Ranganna, 1986)

Dried pectin (200 mg) was weighed into a 1liter beaker and was wetted with 2 or 3 ml of absolute alcohol. The solution was boiled

after addition of 400 ml of distilled water with stirring. After cooling, volume was made up to 500 ml. Pipetted 200 ml aliquot into a conical flask followed by 250 ml distilled water and the solution was neutralized with NaOH (1N) using phenolphthalein as an indicator. Subsequently, 10 ml of NaOH (1N) in excess was added with constant stirring and kept overnight. Further, 50 ml acetic acid (1N) and 25 ml of calcium chloride (1N) was added. The solution was allowed to stand for 1 h, boiled for 2 min and filtered through previously prepared filter paper (filter paper was made wet in hot water, dried in an oven at 102 °C for 2 h, cooled in a desiccator and weighed in a covered dish). The precipitate obtained was washed thoroughly with boiling water and dried in a desiccator. This step was repeated once again before weighing. Pectin percentage was calculated as calcium pectate using the formula;

$$\% \text{ of pectin as calcium pectate} = \frac{(\text{weight of precipitates x } 500 \text{ x } 100)}{(\text{ml of filtrate x weight of sample taken for estimation})} \text{ x } 100$$

4.4.4. Optimization of selected AWE media by RSM for enhanced pectinase production

The optimization of media for enzyme production was carried out using the statistical design of experiments in two steps. In the first step, the screening of variables was done by PBD (Plackett *et al.,* 1946) (Using Minitab- Release 17, PA, USA). The second step involves the optimization of significant variables by RSM employing the CCD using Design Expert® 8.0.2.0 (Stat-Ease, Inc; Minneapolis, MN, USA).

4.4.4.1. Screening of the most significant medium components by PBD

Plackett-Burman Design (Plackett *et al.,* 1946) was used to screen the major factors for pectinase production by *B. subtilis* BKDS1. The methodology was the same as discussed in chapter 3 (Section 3.4.4.2.A). Nine factors were chosen such as, $CaCO_3$, yeast extract (YE), NaCl, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, $CaCl_2$, $MgSO_4 \cdot 7H_2O$ and inoculum volume. All experiments were carried out in duplicate and the averages of the pectinase activity were taken as the response. The concentration of AWE was kept as constant. The factors and levels were shown in Table 4.2.

Table 4.2: Levels of factors used for PBD screening

No.	Factor code	Factors (g/l)	Low (-1)	High (+1)
1	A	$CaCO_3$	0.30	1.50
2	B	NaCl	0.25	1.50
3	C	$(NH_4)_2SO_4$	0.50	1.50
4	D	KH_2PO_4	0.50	1.50
5	E	Na_2HPO_4	0.50	2.50
6	F	$MgSO_4.7H_2O$	0.10	0.50
7	G	$CaCl_2$	0.25	1.25
8	H	Yeast extract	2.0	10.0
9	J	Inoculum	1 %	3 %

4.4.4.2. Central composite design (CCD)

CCD approach was used for determining optimum levels of critical variables (identified by PBD) for enhanced enzyme production. The effect of each variable on enzyme production was studied at five

levels (Table: 4.3) with the help of Design Expert Software. Thirty experiments were carried out each at five levels. Pectinase production was analyzed using a second-order polynomial equation and the data were fitted to the equation by multiple regressions using the model equation.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD$$

Where β_0 is the model constant, β_1, β_2, β_3 and β_4 are linear coefficients, β_{11}, β_{22}, β_{33} andβ_{44} are quadratic coefficients, and β_{12},β_{13}, β_{14}, β_{23},β_{24} and β_{34} are interactive coefficients. A, B, C and D are the levels of the factors. Analysis of variance (ANOVA), and regression analysis were done using design expert software and contour plots were created.

Table 4.3: Concentration ranges for the four factors used in RSM

Factors	Code	Levels				
		-2	**-1**	**0**	**1**	**2**
$CaCO_3$	A	0.30	0.60	0.90	1.20	1.5
$CaCl_2$	B	0.25	0.50	0.75	0.10	1.25
Yeast extract (YE)	C	2.0	4.0	6.0	8.0	10.0
Inoculum volume (%)	D	1.0	1.50	2.0	2.50	3.0

4.4.4.3. Validation of experiments

The accuracy of the predicted statistical model for pectinase production was validated by comparing the predicted result with the practical value. The optimal concentrations of the critical variables were obtained by analyzing the three-dimensional plots.

4.4.5. Comparison of the enzyme production in optimized AWE medium

The enzyme production achieved in optimized AWSE media was compared with various other pectinase production media as described in chapter 2, Section 2.4.6.

4.4.6. Submerged fermentation of pectinase

Lab scale Fermenter –BioRacA (Figure: 4.3) of 1L capacity was used to test the efficiency of the optimized enriched AWE medium for large scale production by SmF. For this, 700 ml sterile medium (pH 7) was inoculated with 1.5 % of overnight culture and incubated till it gives its maximum activity. Throughout the incubation, agitation, aeration and temperature was set constant at 500 rpm, 0.49 lpm and 40 °C respectively. Coconut oil was used as the antifoam agent. The pH of the medium was monitored throughout the incubation period. At each interval of time, the sample was collected aseptically and assayed.

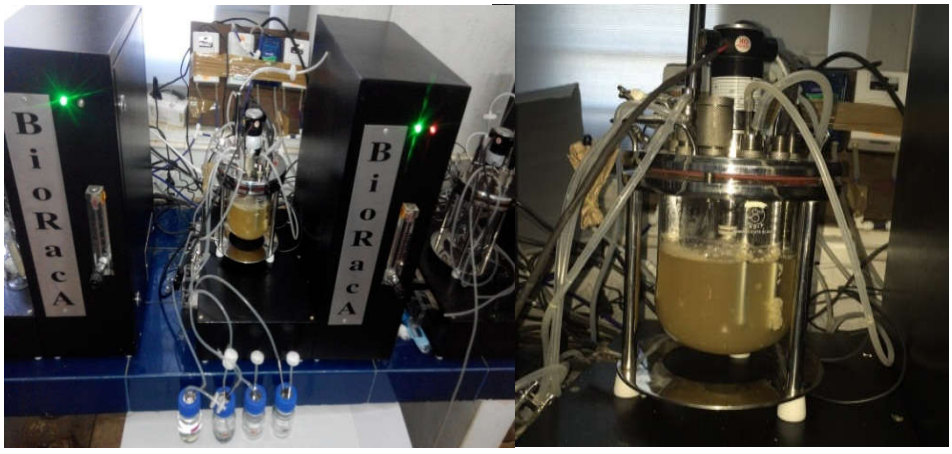
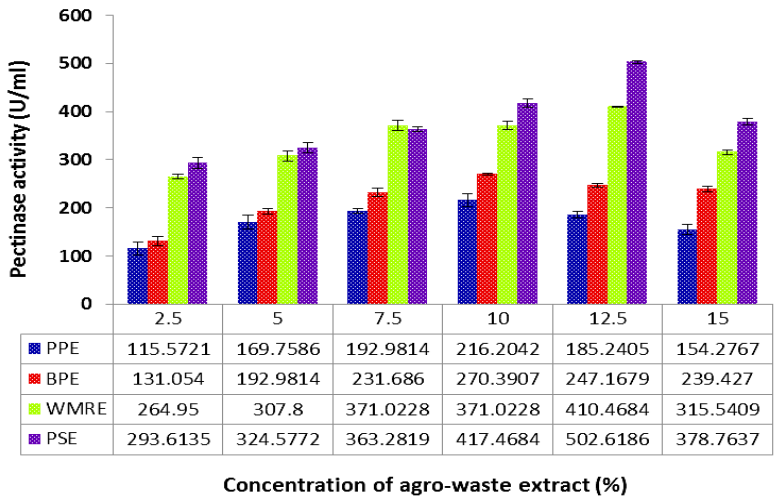


Figure: 4.4. BioRacA: Parallel bioreactor used for the study

4.5. Results

4.5.1. Selection of best agro-waste for media formulation

The prepared agro-waste extracts were diluted with distilled water in various concentrations and used as AWEM to find out the best agro-waste and most suitable concentration of the extract for pectinase production. The results of enzyme production in these AWEM are shown in Figure: 4.5.



	2.5	5	7.5	10	12.5	15
PPE	115.5721	169.7586	192.9814	216.2042	185.2405	154.2767
BPE	131.054	192.9814	231.686	270.3907	247.1679	239.427
WMRE	264.95	307.8	371.0228	371.0228	410.4684	315.5409
PSE	293.6135	324.5772	363.2819	417.4684	502.6186	378.7637

Figure: 4.5. Agro-waste extracts and their various concentrations used for pectinase production **(**PPE: pineapple peel extract, BPE: banana peduncle extract, WMRE: watermelon rind extract, and PSE: pineapple stem extract)

4.5.2. Characteristics of the selected agro-waste (PS and WMR)

The results obtained for the analysis of (i) productive yield of agro-waste, (ii) the crude yield of pectin (iii) and estimation of pectin as calcium pectate were presented in Table: 4.4.

Table: 4.4. Characteristics of selected agro-wastes for media preparation

Agro-waste	Dry powder obtained from 1kg of raw agro-waste	Yield of pectin from 100g of agro-waste	% of pectin as calcium pectate
Pineapple stem	140g	14.8 g	13 %
watermelon rind	70.5g	5.8g	14.12 %

4.5.3. Selection of the best AWE concentration as media for pectinase production

In this step, the prepared PS extract was diluted with distilled water in various concentrations and used as PSE media to find out the most suitable concentration of the extract for pectinase production. The results of enzyme production in these media are shown in Figure: 4.5.

4.5.4. Optimization of selected AWE media by RSM for enhanced pectinase production

4.5.4.1. Screening of the most significant medium components by PBD

Considering the commercial importance of pectinase, studies have been carried out to assess the optimum conditions for enhanced enzyme production and activity. This step was initialized with PBD to screen some vital factors that have an immense role in the pectinase production by *B. subtilis* BKDS1. The result of PBD studies is given in Table: 4.5 and 4.6 for PSEM optimization and in Table 4.7 and 4.8 for WMREM optimization.

Table: 4.5. PBD for PSEM optimization

Run order	Factor Code									Pectinase activity (U/ml)
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**J**	
1	1	1	-1	1	1	-1	-1	-1	-1	460.04
2	-1	1	1	-1	1	1	-1	-1	-1	421.34
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	586.99
4	1	-1	1	1	-1	-1	-1	-1	1	724.01
5	-1	-1	1	1	-1	1	1	-1	-1	610.99
6	1	-1	1	1	1	1	-1	-1	1	747.23
7	-1	-1	1	-1	1	-1	1	1	1	947.72
8	1	-1	1	-1	1	1	1	1	-1	1009.65
9	1	-1	-1	1	1	-1	1	1	-1	1025.13
10	1	1	-1	-1	-1	-1	1	-1	1	973.27
11	1	1	1	1	-1	-1	1	1	-1	933.79
12	-1	1	-1	1	-1	1	1	1	1	897.41
13	-1	1	1	-1	-1	-1	-1	1	-1	628.02
14	-1	-1	-1	-1	1	-1	1	-1	1	740.27
15	-1	1	-1	1	1	1	1	-1	-1	654.34
16	1	-1	-1	-1	-1	1	-1	1	-1	939.98
17	-1	1	1	1	1	-1	-1	1	1	786.71
18	-1	-1	-1	1	-1	1	-1	1	1	853.28
19	1	1	1	-1	-1	1	1	-1	1	926.82
20	1	1	-1	-1	1	1	-1	1	1	1158.28

Table: 4.6. Statistical analysis of PBD showing coefficient value, standard error coefficient value, t and *p*-value for each variable (for PSEM optimization)

Term	code	Effect	Coef	SE Coef	T-Value	*P*-Value
CaCo3	A	177.1	88.6	16.3	5.42	0.000
NaCl	B	-34.5	-17.3	16.3	-1.06	0.316
$(NH_4)_2SO_4$,	C	-55.3	-27.6	16.3	-1.69	0.122
KH_2PO_4	D	-63.9	-32.0	16.3	-1.96	0.079
Na_2HPO_4	E	-12.4	-6.2	16.3	-0.38	0.713
$MgSO4.7H_2O$	F	41.3	20.7	16.3	1.27	0.235
CaC_2	G	141.3	70.7	16.3	4.33	0.001
YE	H	233.5	116.7	16.3	7.15	0.000
Inoculum	J	148.5	74.2	16.3	4.54	0.001

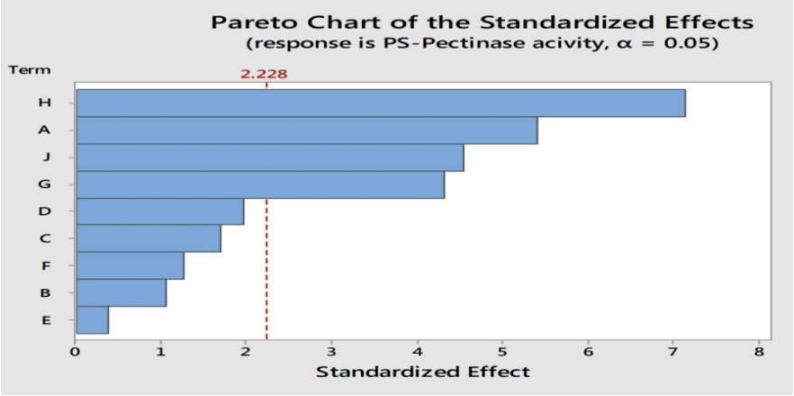


Figure: 4.6. Pareto chart (for PSEM optimization)

Table: 4.7. PBD for WMREM optimization

Run order	Factor Code									Pectinase activity (U/ml)
	A	B	C	D	E	F	G	H	J	
1	1	1	-1	1	1	-1	-1	-1	-1	735.6206
2	-1	1	1	-1	1	1	-1	-1	-1	665.1781
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	638.859
4	1	-1	1	1	-1	-1	-1	-1	1	772.003
5	-1	-1	1	1	-1	1	1	-1	-1	667.2682
6	1	-1	1	1	1	1	-1	-1	1	773.5512
7	-1	-1	1	-1	1	-1	1	1	1	873.4092
8	1	-1	1	-1	1	1	1	1	-1	949.4123
9	1	-1	-1	1	1	-1	1	1	-1	967.8485
10	1	1	-1	-1	-1	-1	1	-1	1	920.6288
11	1	1	1	1	-1	-1	1	1	-1	852.5087
12	-1	1	-1	1	-1	1	1	1	1	854.0568
13	-1	1	1	-1	-1	-1	-1	1	-1	760.3916
14	-1	-1	-1	-1	1	-1	1	-1	1	758.0693
15	-1	1	-1	1	1	1	1	-1	-1	768.9066
16	1	-1	-1	-1	-1	1	-1	1	-1	857.1532
17	-1	1	1	1	1	-1	-1	1	1	813.804
18	-1	-1	-1	1	-1	1	-1	1	1	824.6413
19	1	1	1	-1	-1	1	1	-1	1	1109.508
20	1	1	-1	-1	1	1	-1	1	1	916.7584

Table: 4.8. Statistical analysis of PBD showing coefficient value, standard error coefficient value, t and p-value for each variable (for WMREM optimization)

Term	code	Effect	Coef	SE Coef	T-value	*p*-value
$CaCO_3$	A	123	61.5	14.9	4.14	0.002
NaCl	B	31.5	15.8	14.9	1.06	0.314
$(NH_4)_2SO_4$,	C	-0.6	-0.3	14.9	-0.02	0.986
KH_2PO_4	D	-41.9	-21	14.9	-1.41	0.189
Na_2HPO_4	E	-3.4	-1.7	14.9	-0.12	0.91
$MgSO4.7H_2O$	F	29.3	14.7	14.9	0.99	0.347
Cacl2	G	96.4	48.2	14.9	3.24	0.009
YE	H	86	43	14.9	2.89	0.016
Inoculum	J	75.3	37.7	14.9	2.53	0.03

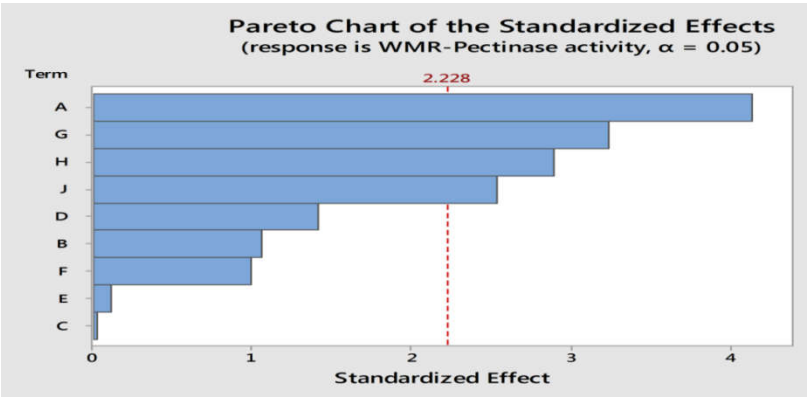


Figure: 4.7. Pareto chart (for WMREM optimization)

4.5.4.1.A. PBD analysis for PSEM optimization: The data presented in Table: 4.5 indicated a marginal variation in pectinase activity from 421.34 U/ml to 1158.28 U/ml in twenty trials. This variation ensured the impact of all the factors on enzyme activity. The analysis of regression coefficients and *t*-value of nine ingredients are depicted in Table: 4.6. From this table, it is clear that, variables $CaCO_3$, $MgSO_4$, $CaCl_2$, yeast extract and inoculum displayed a positive effect for enzyme production, whereas NaCl, $(NH_4)_2SO_4$, KH_2PO_4 and Na_2HPO_4 had a negative effect on enzyme activity. $CaCO_3$, $CaCl_2$, yeast extract

and inoculum had the significant positive effect. None of the components had a significant negative effect on enzyme activity. On the basis of the calculated *p*-values, yeast extract (*p*-value=0.00), $CaCO_3$ (*p*-value=0.00), inoculum (*p*-value=0.001) and $CaCl_2$ (*p*-value=0.001) were chosen for further optimization, since these factors had significant effect on the pectinase activity.

4.5.4.1.B. PBD analysis for WMREM optimization: Similar type of observation was showed by PBD analysis with WMRE. From Table: 4.8 it is clear that, factors $CaCO_3$, NaCl, $MgSO_4$, $CaCl_2$, yeast extract and inoculum showed positive effects. Whereas $(NH_4)_2SO_4$, KH_2PO_4 and Na_2HPO_4 displayed negative effects. Here also the same four variables ($CaCO_3$, $CaCl_2$, yeast extract and inoculum) were selected as the significant factors that influence the enzyme production.

4.5.4.2. Further optimization of the screened variables using CCD

At the end of the screening experiment by PBD, in both cases, four factors were found to play the significant role in pectinase production. The optimal levels of these four significant factors were determined by CCD, one of the commonly used analytical methods in RSM. For this, a set of 30 experimental runs were carried out with different combinations of the critical factors. The actual yield of the enzyme, pectinase and the yield predicted by the model equation are given in Table: 4.9 (for PSEM optimization) and Table: 4.10 (for WMREM optimization). The statistical significance of the second order polynomial equation was evaluated by the *F*-test and the result of ANOVA was given in Table: 4.11 (for PSEM optimization) and Table: 4.12 (for WMREM optimization).

Table: 4.9. CCD matrix of four variables with experimental and predicted response (For PSEM optimization)

Run No.	$CaCO_3$	$CaCl_2$	YE	Inoculum	Enzyme activity (U/ml)	
					Observed	Predicted
1	-1	1	-1	1	1011.972	1016.8
2	-1	-1	-1	1	1046.806	1023.1
3	0	0	0	0	1449.334	1465.19
4	-1	1	-1	-1	1097.122	1112.76
5	1	1	-1	1	1333.22	1316.62
6	0	2	0	0	1174.531	1174.05
7	0	0	0	0	1484.169	1465.19
8	-1	1	1	-1	1317.739	1294.2
9	1	1	-1	-1	1414.5	1401.94
10	-1	-1	1	1	864.894	879.42
11	0	0	-2	0	1360.314	1369.51
12	0	0	0	-2	1251.941	1236.61
13	0	0	0	2	1058.417	1059.21
14	-1	-1	-1	-1	984.879	978.74
15	2	0	0	0	1290.645	1275.99
16	1	1	1	1	1213.236	1221.3
17	-1	1	1	1	1058.417	1061.8
18	0	0	0	0	1468.687	1465.19
19	1	-1	1	1	957.785	954.72
20	0	-2	0	0	787.485	773.45
21	-2	0	0	0	911.34	911.51
22	1	1	1	-1	1406.759	1443.06
23	0	0	0	0	1437.723	1465.19
24	-1	-1	1	-1	942.303	971.5
25	1	-1	-1	1	1213.236	1238.72
26	0	0	0	0	1453.205	1465.19
27	1	-1	-1	-1	1174.531	1183.72
28	0	0	0	0	1498.039	1465.19
29	1	-1	1	-1	1039.065	1036.16
30	0	0	2	0	1290.645	1266.95

Table: 4.10. ANOVA table for the response surface quadratic model- CCD (of PSEM optimization)

Source	**Sum of Squares**	**df**	**Mean Square**	***F*- Value**	***p*-value Prob > F**	
Model	1.26E+06	14	89642.38	150.09	< 0.0001	Significant
A-$CaCO_3$	1.99E+05	1	1.99E+05	333.61	< 0.0001	
B-$CaCl_2$	2.41E+05	1	2.41E+05	403.02	< 0.0001	
C-YE	15780.08	1	15780.08	26.42	< 0.0001	
D-Inoculum	47204.18	1	47204.18	79.03	< 0.0001	
AB	7086.71	1	7086.71	11.87	0.0036	
AC	19685.31	1	19685.31	32.96	< 0.0001	
AD	113.29	1	113.29	0.19	0.6694	
BC	35602.1	1	35602.1	59.61	< 0.0001	
BD	19685.31	1	19685.31	32.96	< 0.0001	
CD	18614.21	1	18614.21	31.17	< 0.0001	
A^2	2.37E+05	1	2.37E+05	396.04	< 0.0001	
B^2	4.14E+05	1	4.14E+05	693.2	< 0.0001	
C^2	37029.15	1	37029.15	62	< 0.0001	
D^2	1.73E+05	1	1.73E+05	288.92	< 0.0001	
Residual	8958.99	15	597.27			
Lack of Fit	6357.97	10	635.8	1.22	0.4365	Not significant
Pure Error	2601.02	5	520.2			
Cor Total	1.26E+06	29				

4.5.4.2.A. CCD analysis for PSEM optimization: From the ANOVA analysis, the Model *F*-value of 150.09 implies the model is significant. Values of "Prob> F" less than 0.05 indicate model terms are significant. In this case A, B, C, D, AB, AC, BC, BD, CD, A^2, B^2, C^2, D^2 are significant model terms. The regression equation coefficients were calculated and the data was fitted to a second order polynomial equation. Thus the response (Y), (in terms of coded factors), i.e., pectinase production by the selected strain *B. subtilis* BKDS1 can be expressed in terms of the following regression equation;

$$\text{Pectinase activity}(U/ml) = +1465.19 + 91.12A + 100.15B - 25.64\,C - 44.35D + 21.05AB - 35.08AC + 2.66AD + 47.17BC - 35.08BD - 34.11CD - 92.86A^2 - 122.86B^2 - 36.74C^2 - 79.32D^2$$

Where A is $CaCO_3$, B is $CaCl_2$, C is yeast extract and D is inoculum. The mean squares values were calculated by dividing the sum of the squares of each variation source by their degrees of freedom, and a 95 % confidence level ($\alpha = 0.05$) was used to determine the statistical significance in all analyses. The "lack of fit *F*-value" of 1.22 implies the lack of fit is not significant relative to the pure error. The *p*-value of lack of fit in RSM >0.05 (not significant) means that the model fits well. The regression equation obtained from ANOVA showed that the R^2 (Multiple Correlation Coefficient) for pectinase production was 0.9929. The "predicted R-Squared" of 0.9681 is in reasonable agreement with the "adjacent R-Squared" of 0.9863. The R^2 value gives a measure of how much variability in the observed response values can be explained by the experimental parameters and their interactions. For a good statistical model, the R^2 value should be in the range of 0 – 1.0, and the value as obtained in the data analysis indicates that the model is good. The adequate precision value of the present model was 40.02 which indicates an adequate signal and that the model can be used to navigate the design space. Three-dimensional response surface plots for pectinase production showing the interactive effects between the selected media components were shown in Figure: 4.8 (i-vi).

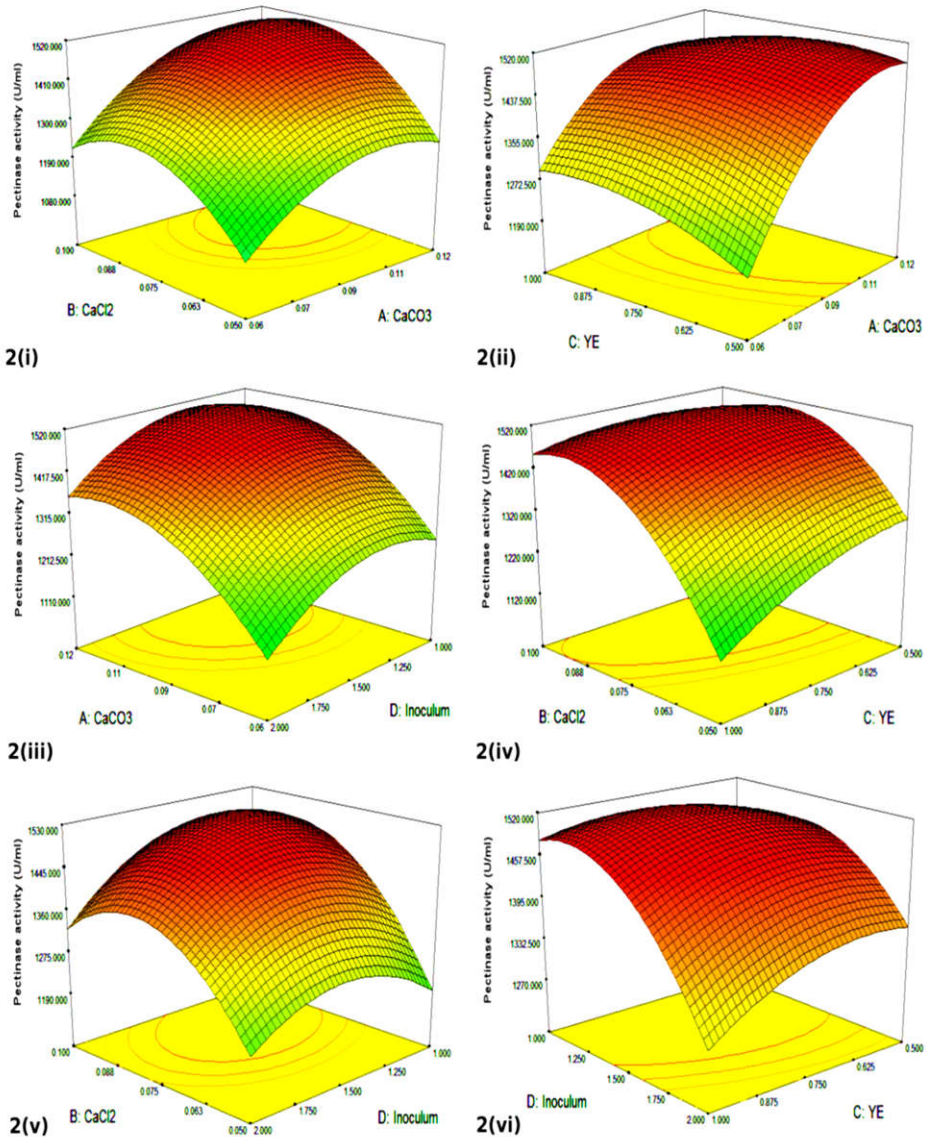


Figure: 4.8. Three dimensional response surface plots for pectinase production showing the interactive effects between: **(i)** $CaCO_3$ and $CaCl_2$ **(ii)** yeast extract and $CaCO_3$ **(iii)** $CaCO_3$ and inoculum % **(iv)** $CaCl_2$ and yeast extract **(v)** $CaCl_2$ and inoculum (vi) inoculum and yeast extract (for PSEM optimization).

Table: 4.11. CCD matrix of four variables with experimental and predicted response (for WMREM optimization)

Run No.	$CaCO_3$	$CaCl_2$	YE	Inoculum	Enzyme activity (U/ml)	
					Observed	Predicted
1	-1	1	-1	1	880.9179	917.99
2	-1	-1	-1	1	767.1262	730.03
3	0	0	0	0	1230.58	1176.45
4	-1	1	-1	-1	810.4754	846.53
5	1	1	-1	1	1102.308	1068.55
6	0	2	0	0	1139.465	1178.17
7	0	0	0	0	1191.329	1176.45
8	-1	1	1	-1	1099.212	1042.91
9	1	1	-1	-1	1088.375	1028.81
10	-1	-1	1	1	720.6806	756.09
11	0	0	-2	0	742.3552	723.75
12	0	0	0	-2	1081.408	1100.23
13	0	0	0	2	1080.634	1100.75
14	-1	-1	-1	-1	713.7138	716.61
15	2	0	0	0	713.7138	773.05
16	1	1	1	1	1136.369	1109.33
17	-1	1	1	1	1096.89	1061.73
18	0	0	0	0	1206.811	1176.45
19	1	-1	1	1	693.5874	642.69
20	0	-2	0	0	581.3439	581.61
21	-2	0	0	0	724.5511	704.17
22	1	1	1	-1	1099.986	1122.23
23	0	0	0	0	1197.522	1176.45
24	-1	-1	1	-1	776.4153	795.31
25	1	-1	-1	1	687.3946	719.59
26	0	0	0	0	1090.697	1176.45
27	1	-1	-1	-1	717.5842	737.89
28	0	0	0	0	1141.787	1176.45
29	1	-1	1	-1	774.8671	713.63
30	0	0	2	0	785.7044	843.23

Table: 4.12. ANOVA table for the response surface quadratic model- CCD) (for WMREM optimization)

Source	**Sum of Squares**	**df**	**Mean Square**	***F*- Value**	***p*-value Prob > F**	
Model	1209958	14	86425.6	27.76006	< 0.0001	Significant
A-$CaCO_3$	7119.627	1	7119.627	2.286837	0.1513	
B-$CaCl_2$	533839.6	1	533839.6	171.4703	< 0.0001	
C-YE	21409.07	1	21409.07	6.876631	0.0192	
D-Inoculum	0.399616	1	0.399616	0.000128	0.9911	
AB	25924.65	1	25924.65	8.327044	0.0113	
AC	10599.57	1	10599.57	3.404602	0.0848	
AD	1007.293	1	1007.293	0.323544	0.5779	
BC	13844.41	1	13844.41	4.44685	0.0522	
BD	3370.618	1	3370.618	1.082649	0.3146	
CD	2770.751	1	2770.751	0.88997	0.3604	
A^2	328636	1	328636	105.5585	< 0.0001	
B^2	150776.4	1	150776.4	48.42966	< 0.0001	
C^2	264693.2	1	264693.2	85.01995	< 0.0001	
D^2	9889.214	1	9889.214	3.176434	0.095	
Residual	46699.61	15	3113.307			
Lack of Fit	33627.25	10	3362.725	1.286196	0.4123	Not significant
Pure Error	13072.36	5	2614.472			
Cor Total	1256658	29				

4.5.4.2.A. CCD analysis for WMREM optimization:

The Model *F*-value of 27.76 implies the model is significant. Here, the values of Prob > F is < 0.0001, which is less than 0.0500 indicate model terms are significant. In this case, B, C, AB, A^2, B^2, C^2 are significant model terms. The regression equation coefficients were calculated and the data was fitted to a second order polynomial equation. Thus the response (Y), (in terms of coded factors), i.e., pectinase production by the selected strain *B. subtilis* BKDS1 can be expressed in terms of the following regression equation;

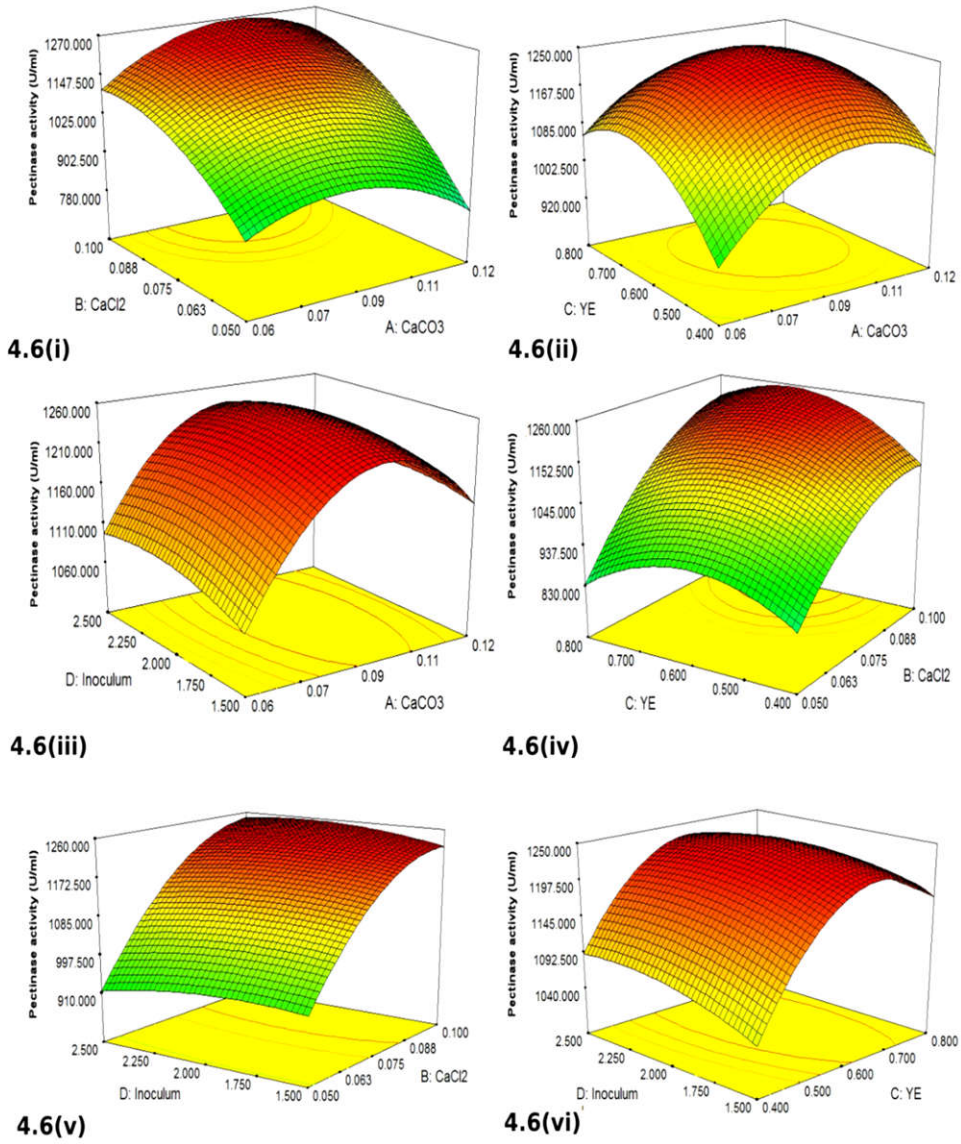


Figure: 4.9. Three dimensional response surface plots for pectinase production showing the interactive effects between: **(i)** $CaCO_3$ and $CaCl_2$ **(ii)** yeast extract and $CaCO_3$ **(iii)** $CaCO_3$ and inoculum % **(iv)** $CaCl_2$ and yeast extract **(v)** $CaCl_2$ and inoculum **(vi)** inoculum and yeast extract (for WMREM optimization)

$$\text{Pectinase activity } (\text{U/ml}) = +1176.45+17.22\,A+149.14\,B+29.87C+0.13D+40.25AB -25.74AC-7.93AD+29.42\,BC+14.51BD-13.16\,CD-109.46A^2-74.14B^2-98.24C^2-18.99D^2$$

Values greater than 0.1000 indicate the model terms are not significant. The lack of fit *F*-value of 1.29 implies the lack of fit is not significant relative to the pure error. There is a 41.23 % chance that a lack of fit *F*-value this large could occur due to noise. Non-significant lack of fit is good. The "Pred R-Squared" of 0.8309 is in reasonable agreement with the "Adj R-Squared" of 0.9282. The adeq precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, a ratio of 15.120 indicates an adequate signal. This model can be used to navigate the design space. Three-dimensional response surface plots for pectinase production showing the interactive effects between the selected media components were shown in Figure: 4.9 (i-vi).

4.5.5. Validation of the experiment

4.5.5.1. PSEM optimization: Validation of the experimental model was tested by carrying out the experiments under optimized conditions established by the regression model: yeast extract 6.40 g/l, $CaCO_3$ 1.10 g/l, $CaCl_2$ 0.92 g/l, inoculum 1.5 % and PSE (12.5 %) at pH 7. The experiments were performed in triplicates and the results were compared. The pectinase activity (1508.5 U/ml) obtained from experiments was very close to the actual response (1512.986 U/ml)

predicted by the regression model, which proved the validity of the model.

4.5.5.2. WMREM optimization: Here, the validation of the model was tested in optimized media containing yeast extract 6.82 g/l, $CaCO_3$ 0.9.0 g/l, $CaCl_2$ 0.92 g/l, inoculum 2.3 % and WMRE (12.5 %) at pH 7. The experiments were performed in triplicates and the results were compared. The pectinase activity (1230.02 U/ml) obtained from experiments was very close to the actual response (1240.677U/ml) predicted by the regression model, which proved the validity of the model.

4.5.6. Comparison of the enzyme production achieved in optimized AWEM

On comparison with the raw AWEM (Figure: 4.5), the pectinase production in optimized PSE media was considerably high. Both the PSEM and WMREM showed a 3 fold increase in enzyme production. The pectinase production in optimized PSE media was also compared with four other pectinase production media used by various other researchers and the result obtained was illustrated in Figure: 4.10.

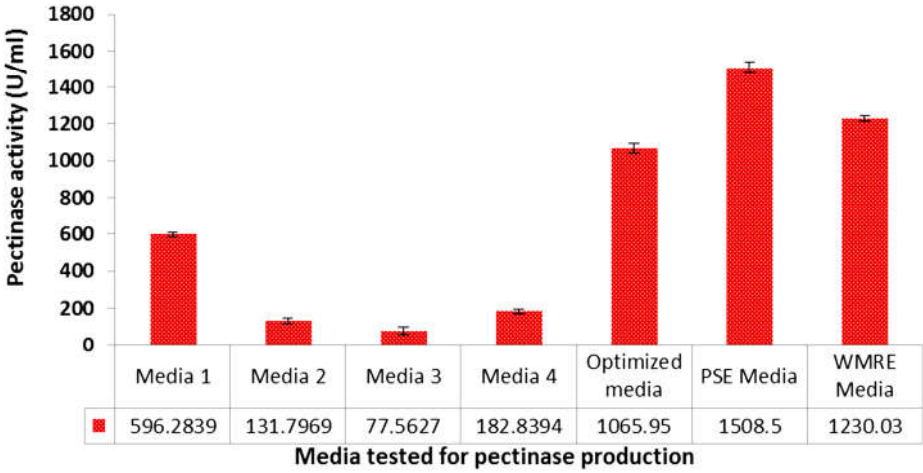


Figure: 4.10. Comparison of the enzyme production in optimized PSE medium V/S other pectinase production media

The results clearly indicate that the production of pectinase is very high in formulated PSE media compared to all other media tested. The folds of increase in enzyme production in these tested media were 2.5, 11.4, 19.4, 8.2 and 1.41 folds respectively and this suggests a good optimization outcome. In the case of optimized WMREM, the respective folds of increase were, 2.06, 9.33, 15.85, 6.72 and 1.153 respectively. Compared to optimized WMREM, the PSEM displayed a better result. The optimized WMRE media also showed good fold of increase compared to other media.

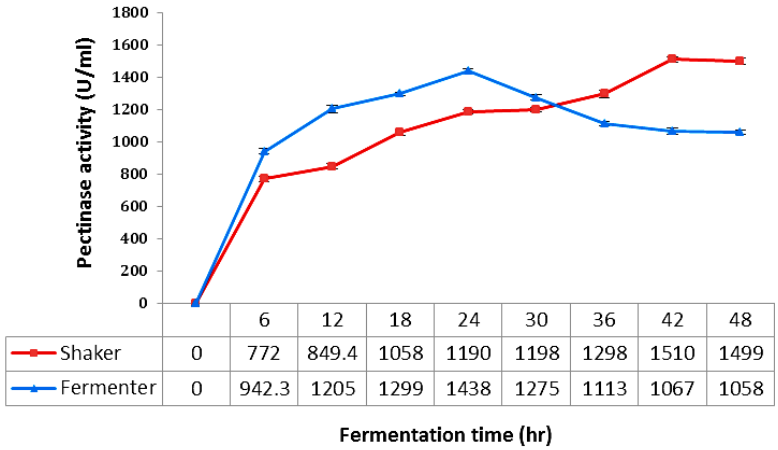
4.5.7. Selection of the best optimized media

Optimization of both PSEM and WMREM was done successfully by RSM methodology and presented promising result for pectinase production. But compared to modified PSEM, the enzyme

production attained in optimized WMREM is less (1230.02 U/ml), which is 1.2 times lesser than the optimized PSEM. So for further fermentation studies and enzyme characterization, only PSEM is considered.

4.5.8. Fermentation studies of pectinase in shaker v/s lab scale fermenter

The efficiency of the formulated PSE medium for pilot scale enzyme production by SmF using *B. subtilis* BKDS1 was studied with lab scale fermenter (BioRacA) of 1 L capacity. The study was done by comparing the effect of incubation time for maximal pectinase production in fermenter v/s shaker. The pectinase activity was determined all through the fermentation time of 48 h in time interim of 6 h and the data was recorded in Figure: 4.11.



		6	12	18	24	30	36	42	48
Shaker	0	772	849.4	1058	1190	1198	1298	1510	1499
Fermenter	0	942.3	1205	1299	1438	1275	1113	1067	1058

Figure: 4.11. Comparison of enzyme activity shown by *B. subtilis* BKDS1 in shaker v/s fermenter in different fermentation time

4.6. Discussion

Pectinase production from Bacillus spp. can be achieved economically by utilizing different agro-waste as substrates including, orange peel wheat bran, apple pomace, sugar beet, sugar cane bagasse and wheat straw etc. (Bibi *et al.,* 2016; Embaby *et al.,* 2014; Jahan *et al.,* 2017; Kaur *et al.,* 2017; Kuvvet *et al.,* 2017). In this study, different agro-wastes such as pineapple peel, pine apple stem, banana peduncle and watermelon rind were tested for economical media preparation for pectinase production. Extracts from these agro-wastes were prepared and diluted with distilled water in various concentrations and used as AWEM to find out the best agro-waste and most suitable concentration of the extract for pectinase production. From the result (Figure: 5) it is observed that pectinase production is achieved in all the tested AWEM in some extend. The enzyme production attained maximum in media prepared with PSE whereas least enzyme production was observed in PPEM. The order of AWEM for their efficiency in production was found to be PSEM>WMREM>BPEM>PPEM. PSE showed the best result (502.61 U/ml) at a concentration of 12.5 %. Media prepared with WMRE also displayed maximum enzyme production (410.46 U/ml) at the concentration of 12.5 %. As PS and WMR were presented best result among the tested ago-wastes, only these two were selected for further optimization studies.

The production of pectinase by the bacterial culture in the tested media showed that, pectinase production could attain in all the

tested agro-wastes in various extends. So, for further optimization studies, the AWE exhibited maximum enzyme production was chosen (*i.e*., PSE and WMR). So, for both cases, the AWE concentration of 12.5 % has been subjected to further optimization studies for prospecting the increase in pectinase production. The methodology adopted here to prepare the AWE medium was new. A similar type of pectin extract medium has been used by Mahesh *et al.,* (2014). They used Soxhlet method for extraction of pectin from orange peel and optimal dilution of 80 % v/v has been chosen for the medium optimization studies. However, in the present study, the maximum enzyme activity has been noticed at 12.5 % concentration. This indicated the requirement of a lesser amount of pineapple stem from which the bulk amount of PSE medium can be prepared. Similarly, Ezugwu *et al.,* (2013) also used extracted pectin from dried mango peels as the only carbon source for pectinase production by *A. fumigatus*.

The % yield of pectin extracted by using HCl from the PS extract was found to be 14.8 % for PS and 5.8 % for WMR. No past data were accessible to look at the yield of pectin from PS. The obtained pectin yield in PS was similar to those observed with pomelo peel pectin (16.740 %) (Roy *et al.,* 2018) and passion fruit peel pectin (9.1-13 %) (Seixas *et al.,* 2014) but lower than pectin from *Citrullus lanatus* fruit rinds (25.79 %) (Prakash Maran *et al.,* 2014). Further studies on extraction methods may increase the extraction yield of pectin from PS. Previous reports indicated that watermelon rind pectin extracted by conventional acid extraction was in the range of (17.4–

20.2 %) (Campbell, 2006; Jiang *et al.,* 2012). Recently Petkowicz *et al.,* (2017) reported 14.2 % of pectin from lyophilized watermelon rinds. Even though, the yield of crude pectin is less in WMR, the amount of pectin obtained as calcium pectate is more in WMR (14.12 %) compared to PSE *(*13 %).

PBD was applied to determine the main factors influencing pectinase production and from the PBD analysis (Table: 4.6 and 4.8). In both cases, it was found that among the nine variables tested, four factors *viz*. yeast extract, $CaCl_2$, $CaCO_3$ and inoculum volume were found to have a significant effect on the pectinase production by the strain *B. subtilis* BKDS1 and the result was very much correlating to the initial media optimization studies (Chapter 3).

The nitrogen sources, such as yeast extract have been reported to significantly affect the pectinase production by various microorganisms. Yeast extract is proved to be the chief nitrogen source possibly because it provided additional stimulatory components such as vitamins, nucleic acid, lipid and other substances (Qureshi *et al.,* 2012). Some previous reports are available to ratify this result; supplementation of the fermentative medium with yeast extract presents a positive effect on pectinase production by various *Bacillus* sp. (Joshi *et al.,* 2013; Kaur *et al.,* 2017; Qureshi *et al.,* 2012). Similarly, PG production by *B. shaericus* MTCC 7542 is maximum when grown on mineral medium containing yeast extract as sole nitrogen source (Jayani *et al.,* 2010).

The metal ion $CaCl_2$ is another factor selected on PBD (with *p*-value 0.001), that significantly affect pectinase production. Prior reports are available that indicate the significance of $CaCl_2$ in pectinase production in many microbial genera (Bennamoun *et al.,* 2016; Munir *et al.,* 2015; Ortiz *et al.,* 2017), Metal ions like Ca^{2+} might play a vital role in maintaining the active conformation of pectinases to stimulate the activity (Jayani *et al.,* 2005; Oumer *et al.,* 2017). This also implies the importance of $CaCl_2$ in pectinase activity.

PBD also selected factors such as $CaCO_3$ and inoculum concentration that significantly affects pectinase production. The impact of $CaCO_3$ on the production of pectinase has been well studied and reported by (Zou *et al.,* 2014). In the medium $CaCO_3$ may act as a pH regulator, the enzyme production could be suppressed under acidic pH. The $CaCO_3$ neutralizes the acid and buffer them from a sudden drop in pH of the medium (Spanos *et al.,* 1998). With respect to inoculum concentration, a high inoculum density cause lesser enzyme production because of competition for available nutrients. So the optimization of inoculum size is a well-accepted criterion in microbial fermentation. From previous studies, it has been understood that inoculum size is an important factor that significantly effects enzyme production in various microorganisms (Ghazala *et al.,* 2015; Jayani *et al.,* 2010; Reddy *et al.,* 2008; Shabbiri *et al.,* 2012). The results obtained were very much correlating to the results of the optimization study done in chapter 3.

RSM plays a vital role in recognizing the optimum values of the independent variables efficiently, under which dependent variable could reach the maximum response. In the response surface plot, the response (pectinase activity) was obtained along with two continuous variables, while the other two variables were fixed constant at their respective zero level (centre value of the testing ranges). So these plots are more helpful in understanding the interaction effects of these two factors. Interactions among the variables can be easily explicated by observing the shape of contour plots because, as a rule, the more elliptical the shape of the contour, the better is the interaction between the two variables (Bibi *et al.,* 2016).

In the case of PSEM optimization, the 3D response surface plot depicted in Figure: 4.8(i) describes the interaction between $CaCO_3$ and $CaCl_2$. The pectinase activity increases with increasing concentration of both the factors, though when the concentrations reach beyond the middle value ($CaCO_3$ 1.10 g/l and $CaCl_2$ 0.92 g/l), the activity tends to decline. The plot displays moderate interaction between the factors and which is confirmed in terms of *p*-value 0.0036 (Table: 4.6). Figure: 4.8(ii) represents the interaction between the factors $CaCO_3$ and yeast extract. In the case of $CaCO_3$, the surface curvature is rising indicating an increase in activity, but beyond about 1.1 g/l the activity decreases. The interaction between $CaCO_3$ and inoculum concentration was given in Figure: 4.8(iii). This response plot exhibits a non-significant interaction between the factors and which is confirmed in terms of the *p*-value of 0.6694 (Table: 4.6). Figure: 4.8(iv) denotes the significant interaction between the factors $CaCl_2$ and yeast extract on pectinase

production. It has been seen from the graphics that enzyme production increased with increasing concentration of $CaCl_2$ and reaches a maximum at 0.92 g/l, but the pectinase production decreases with increasing concentration of yeast extract above the level of 6.40 g/l. Figure: 4.8(v) illustrates the interaction between $CaCl_2$ and inoculum volume. Here, the pectinase activity increases with increasing concentration of both the factors, but the pectinase production diminishes when the concentrations reach above certain range ($CaCl_2$ 0.92 g/l and inoculum 1.5 %). Finally, the interaction between yeast extract and inoculum was shown in Figure: 4.8(vi). Here, the significant interaction is fairly similar to the interaction between $CaCl_2$ and yeast extract as depicted in Figure: 4.8(iv).

Similar types of response surface plots were obtained in case of WMREM optimization also. Figure: 4.9(i) illustrates the interaction between $CaCO_3$ and $CaCl_2$, which is the only significant interaction. The enzyme activity increases with increasing concentration of $CaCl_2$ and reaches the maximum when the concentration attains 0.92g/l. But in the case of $CaCO_3$, the pectinase activity increases with increasing concentration of and declines when the concentration reaches beyond the middle value (0.90 g/l). In case of the interaction between yeast extract and $CaCO_3$ (Figure: 4.9(ii)), the pectinase activity increases with increasing concentration of both the factors, but when the concentrations exceed the middle value ($CaCO_3$ 0.90 g/l and YE 6.82 g/l), the activity decreases. Figure: 4.9(iii) show the response surface plot of interaction between $CaCO_3$ and inoculum volume. Here the pectinase activity is at peak when the concentrations $CaCO_3$ 0.90 g/l

and inoculum volume 2.3 %. The pectinase activity increases with increasing the concentration of both $CaCl_2$ and inoculum volume (Figure: 4.9(v)). But in case of the interaction between inoculum and YE (Figure: 4.9(v)), it is fairly similar to the response surface plot shown by interactive effect between $CaCO_3$ and inoculum where the maximum activity is in around the middle value of the factors. The concentrations of the factors predicted by the model for WMREM optimization were somewhat similar to the predicted result of PSEM optimization except for the concentration of inoculum volume. In the case of WMRM optimization, the predicted concentration of inoculum volume is 2.3 % which is slightly higher than that of PSEM optimization (1.5 %). Generally, the three-dimensional surface plots are used to create visualizations of the effects of process variables on product responses and these graphical representations help us to absorb clear indication of the relationships that exist between the processing variables (Nwabueze, 2010).

Both PSEM and WMREM showed good fold of increase compared to other production media tested (Figure: 4.10). So the optimization resulted in a augmentation in pectinase production. While comparing the pectinase activity achieved in PSEM with WMREM, PSEM showed a better result. So for further studies, the formulated PSEM is used. The efficiency of formulated PSE medium for large-scale SmF production of pectinase was confirmed in Laboratory fermenter (BioRacA). It is evident (Figure: 4.11) that enzyme production was achieved at a faster rate in the fermenter compared to shaker. The bacterium grown in the fermenter gave maximum activity

(1437.723 U/ml) at 24 h and in shaker, the maximum reading (1510.391 U/ml) was at 42 h. Mostly, for the production of industrially important products with high commercial value and for the study of worthy biochemical and physiological aspects of the microbial metabolites formation, SmF system is very useful.

Moreover, the usage of SmF is technically easier than solid state fermentation (SSF) (Hansen *et al.,* 2015). Usually, SmF is applied in case of enzyme production by bacteria because of the requirement of higher water potential (Kamal *et al.,* 2017). Researchers all over the world used various agro-residues for the microbial production of pectinase and other enzymes. But most of the works were based on SSF production. Compared to SmF, the great advantage of SSF is the lesser capital and operating costs due to the utilization of low cost agricultural and agro-industrial wastes as substrates (Mussatto *et al.,* 2012). This drawback of SmF can be overcome by the formulation of cheap media composed of agro-residues. In this study, pectinase production using PSE was proved in laboratory scale fermenter. Also, the enzyme activities were compared in SmF of shake flask (Erlenmeyer flask - 100 ml) and fermenter across the 48 h fermentation period. The result showed that fermenter was more economic than in shaker because the pectinase activity was at the peak at 24 h in fermenter compared to the maximum activity in the shaker (42 h).

The overall results showed that, agro-residues such as pineapple stem and watermelon rind were a good source for the preparation of pectinase production media and PSE can be used for

large scale production of pectinase by *B. subtilis* BKDS1. Strategic exploitation of the above fact may be a breakthrough for so many pectinase utilizing industries.

4.7. Conclusion

The present study has been attempted to utilize the commonly available agricultural waste of Kerala like PS, PP, WMR, and BP for the production of pectinase. From the initial assay, it is observed that PS at a concentration of 12.5 % gave the best production media for economical pectinase production. So, the work as a whole proved that pineapple stem extract (PSE) could be used as a cheap media for the production of pectinase by submerged fermentation using the indigenous strain, *B. subtilis* BKDS1. RSM based statistical media optimization has been applied towards assessing the prospects of augmented pectinase production. A maximum pectinase production of 1508.5 U /ml was achieved with the following optimized factors; yeast extract 6.40 g/l, $CaCO_3$ 1.10 g/l, $CaCl_2$ 0.92 g/l, inoculum 1.5 % and PSE (12.5 %) at pH 7. Validation experiments were also carried out to verify the adequacy of the model, and results showed that the predicted value agreed with the experimental values well, and the optimized medium presented a 3 fold of increase in enzyme production compared to other pectinase production media tested. The fermentation studies in fermenter proved a shorter incubation period (24 h) compared to shaker experiment (48 h).

The overall study highlights an economically feasible method for the laboratory as well as the large scale commercial production of pectinase enzyme from agro-waste substrates such as pineapple stem. No earlier studies were reported related to pectinase production using pineapple stem and the methodology adopted in the present study for media preparation is novel and the results are very much promising. Also, the study ensured the management of agro-residues in a more reliable and strategic manner towards the continuance of a cleaner environment.

CHAPTER 5

CHARACTERISTICS OF PURIFIED PECTINASE PRODUCED BY *BACILLUS SUBTILIS* BKDS1; CLONING, SEQUENCING AND INSILICO ANALYSIS OF ITS PECTATE LYASE (*PEL*) GENE

5.1. Introduction

Purification of the enzyme is an important process to obtain information about structural and functional properties and thus to predict its applications. It is a well-known fact that, microbes are the prominent source of enzymes because they allow an economical technology with truncated resource consumption and low emission involving no social and political issues, as in the case of animal and plant sources (Chaplin *et al.,* 1990) . Even though, the enzyme purification from microbial genera experiences significant issues from contaminating substances contained in the culture broth and media components. Also, simultaneous production of unwanted enzymes and other metabolites may obstruct the purification of pectinolytic enzymes from the microbe. Generally, the enzymes produced by microorganisms are present either within the cell (intracellular), and may be situated in specific subcellular compartments, whereas others are secreted into the adjoining environment or to the cultivation media (extracellular). So, the purification method is based on the occurrence of enzyme ie., intracellular or extracellular. The majority of industrially used enzymes are extracellular enzymes produced either from fungi or bacteria (Robinson, 2015).

An enzyme can be purified into many folds by the application of various downstream processing techniques but the yield of the enzymes may be very poor. Because of this, industrial enzymes are purified as little as possible *i.e*. mainly for the elimination of intrusive materials. As further purification processes are overpriced in terms of

equipment, manpower and loss of enzyme activity additional purification procedures are avoided in many of the commercial enzyme preparations. In these preparations usually consist of concentrated fermentation broth with additives to stabilize the enzyme's activity (Chaplin *et al.,* 1990). Retaining the enzyme's maximum activity is the important criterion during purification. Because, many factors such as, temperature, proteolysis, pH, oxidation, denaturation, irreversible inhibitors and loss of co-factors and co-enzymes etc. will cause enzyme denaturation.

The precise application and end use of the enzyme depends on its level of purity. Majority of the purification process applied in laboratory research can be easily scaled to industrial production processes through a number of operations including centrifugation, filtration, cell disruption, precipitation, flocculation and various chromatographic methods. Usually, in the initial stage of extracellular enzyme purification, the large solid particles and microbial biomass are separated by centrifugation or filtration. After the removal of microbial cells, the liquid broth containing enzymes are concentrated with various procedures including salt and solvent precipitation, two aqueous-phase separations or ultrafiltration (Poletto *et al.,* 2015). In the final stage of the downstream process, various chromatographic and crystallization techniques are applied to achieve highly concentrated and pure fermentation products from the microorganisms (Stanbury *et al.,* 2013).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was usually performed to confirm the molecular

weight (by comparing with the molecular weight of marker proteins) of the subunit and the purity of enzyme. Zymography is a commonly performed technique in connection with PAGE to measure the enzyme activity and is very much helpful for characterizing the proteinase composition of complex biological samples. Determination of kinetic properties such as the Michaelis Menten constant (K_m), maximum catalytic velocity (V_{max}), thermal stability and pH stability of the enzyme is very essential for complete characterization of the enzyme (Poondla., 2016).

Advanced techniques in proteomics such as matrix-assisted laser desorption/ionization (MALDI) and the analyzer of the time of flight (TOF), Mass Spectrometry (MS), *i.e*., MALDI-TOF MS (Szerszunowicz *et al.,* 2017). Techniques like Isotope-coded affinity tags (ICAT) , Isobaric Tags for Relative and Absolute Quantification (iTRAQ), Absolute Quantification (AQUA), Micro electrospray ionization (ESI)-Quadrupole ion trap (QIT) Time of flight (TOF) mass spectrometer (MS), Surface-enhanced laser desorption/ionization (SELDI) , Fluorescence Spectroscopy (FS), Circular Dichroism (CD), Protein microarrays or chips etc are also utilized in various capacities for different research settings (Aslam *et al.,* 2017; Chandrasekhar *et al.,* 2014). By the establishment of bioinformatics databases and tools, it is able to handle various protein characteristics such as 3D structure prediction, protein domain and motif analysis, rapid analysis of protein-protein interaction and data analysis of MS, etc very easily (Aslam *et al.,* 2017).

Pectate lyase (PL or PEL) represents an important member of the pectinase group of enzymes responsible for eliminative cleavage of

the α-1,4-d galacturonic linkages, producing a Δ-4,5 unsaturated oligosaccharide at the non-reducing end of the product. The PL could act according to an *endo* (EC 4.2.2.2) or as an *exo* (EC 4.2.2.9) mode of action. They are also known in other names such as; poly (1,4- α -D-galacturonide) lyase or polygalacturonate lyase (PGL) or polygalacturonic acid trans-eliminase (PGTE) or pectate transeliminase or pectic acid transeliminase or pectic acid lyase or pectic lyase. The activity of PL depends on calcium (Ca^{2+}) ions and is specific for unmethylated polygalacturonate (pectate) also the degradation cannot take place in the presence of chelating agents such as EDTA. Mainly they are alkaline enzymes and can also be active on pectins with a low degree of methyl esterification (Pedrolli *et al.,* 2014; Soriano *et al.,* 2006). These enzymes have pronounced commercial importance in industrial applications. Mostly these are used in the degumming and retting of fiber crops, textile processing and bio-scouring of cotton fibers, pretreatment of pectic wastewater from fruit juice industries, paper making, coffee and tea fermentation, enzyme based oil extraction, etc (Pedrolli *et al.,* 2009). It is a well-known fact that using PL in degumming or scouring process has more advantages over conventional chemical scouring in terms of generating high-quality fibers, energy efficiency in the process, and eco-friendly environment (Jegannathan *et al.,* 2013).

Pectate lyase is widely distributed in diverse families of microorganisms and plants. The *pel* genes from numerous organisms consisting, bacteria, fungi, yeast, nematode and plants have been

cloned, sequenced, and characterized (Dubey *et al.,* 2016; Dubey *et al.,* 2010). Based on the primary amino acid sequence, PL is classified into different families of isozymes that share 29–91 % amino acid sequence similarity (Heffron *et al.,* 1995; Ouattara *et al.,* 2010). These enzymes are classified into the polysaccharide lyase families 1, 2, 3, 9 and 10 of CAZy database (cazy.org/Polysaccharide-Lyases). Even though, PL from family 1 has been extensively studied (Zheng *et al.,* 2012).The present study describes the purification and characterization of pectinase produced by *B. subtilis* BKDS1. The work also focused on cloning, and sequencing of *pel* followed by its *in-silico* studies. The graphical abstract of the study was presented in Figure: 5.1.

5.2. Objectives of the study

- Purification and characterization of pectinase.
- Assay of different pectinase activity.
- Cloning of *pel* gene in *E.coli.*
- Gene sequencing.
- *In-silico* studies of *B. subtilis* BKDS1 PL (BKDS1 PL) protein sequence
- 3D structure prediction by homology modeling.
- Docking of modeled protein with different ligands for the determination of the catalytic centre residues.

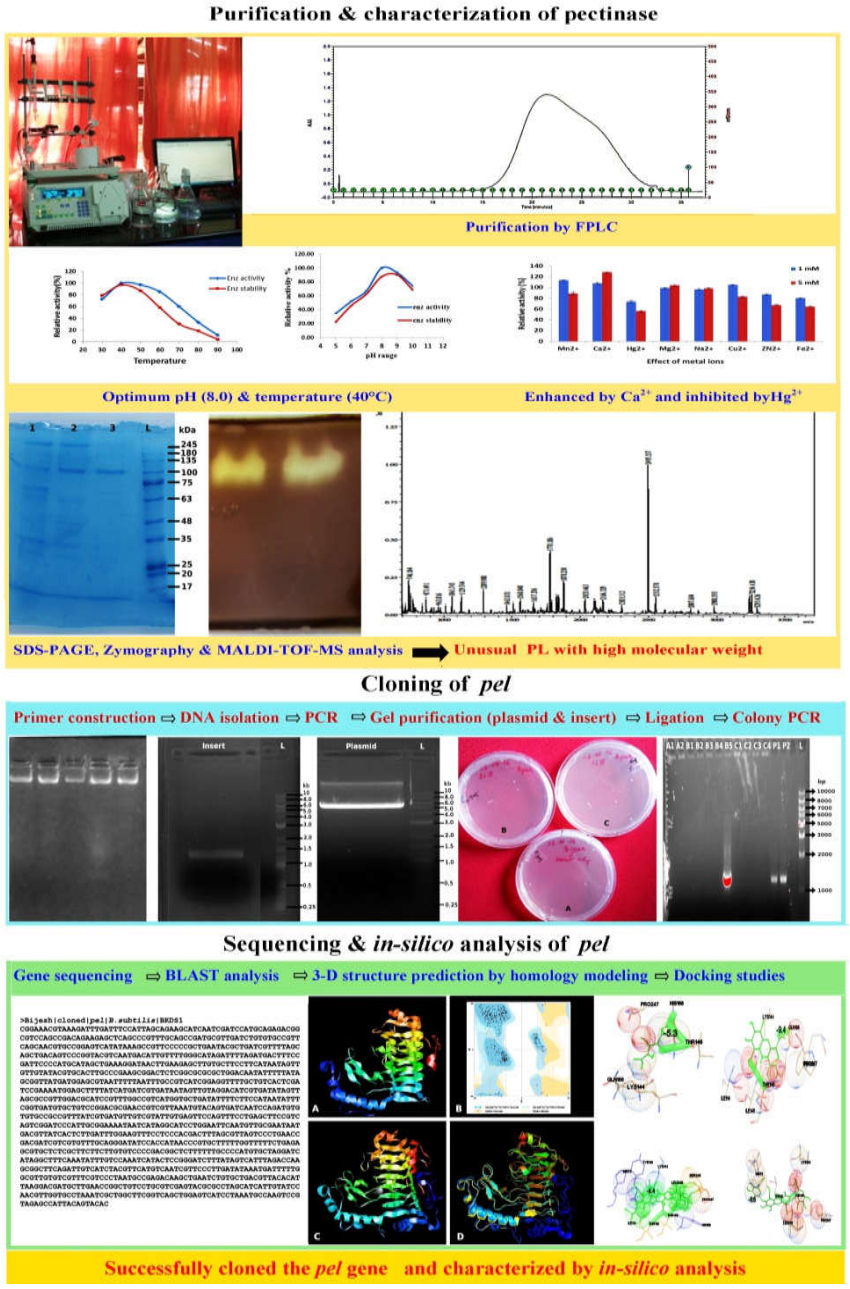


Figure: 5.1. Graphical abstract of the study

5.3. Review of literature

Pectinases are one of the major enzymes having a wide assortment of modern significances with high commercial value and it is essential to understand the nature and properties of these enzymes for efficient and effective usage. Along these lines, different research has been carried out on isolation and characterization of pectinases and its cloning, sequencing and *in-silico* studies to improve the catalytic efficiency (Gummadi *et al.,* 2003).

5.3.1. Purification and characteristics of pectinase

A low-molecular-weight (20,300 Da), high-alkaline PL was found in an alkaline culture of *Bacillus* sp. strain KSM-P15 by Kobayashi *et al.,* (1999) and suggested that this may be a novel enzyme that belongs to a new family. Later, they it was also reported that the strain KSM-P15 produces high alkaline PL with high molecular weight (70,000 Da) (Ogawa *et al.,* 2000). Kobayashi *et al.,* (2001) also reported another pectinolytic strain, *Bacillus* sp. strain KSM-P576 which produces alkaline exo-PG with high molecular weight (115000 Da). Ammonium sulphate precipitation and followed by dialysis is the most common method used for initial purification of proteins (Berg *et al.,* 2002). Various proteins precipitate at varying levels of ionic strength because of their difference in secondary and tertiary structure and '% saturation' is the unit used to represent ionic strength. Kashyap *et al.,* (2000) achieved the partial purification of pectinase from Bacillus sp. DT7 using 40-100 % $(NH_4)_2SO_4$

precipitation followed by final purification with ion exchange and column chromatography. The molecular mass of purified pectinase was observed to be 106 kDa. Whereas, (Kapoor *et al.,* 2000) used 90 % $(NH_4)_2SO_4$ saturation followed by dialysis for partial purification and further characterization of PG from *Bacillus* sp. MG-cp-2. The optimum activity of this enzyme was found at 60 °C at pH 10.0 and detergents such as Tweens, Triton X-100 and SDS stimulated the PG activity up to 41 %. While, metal ions, such as Ag^{+}, Li^{+}, Ca^{2+}, Ba^{2+} and Ni^{2+} stimulated the PG up to 28 %, but complete inhibition of PG activity was showed by Mn^{2+}, iodoacetamide and iodoacetic acid. The purified recombinant PG from Bacillus sp. KSM-P358 had a mol. mass of 105 kDa and 8.0 as the optimum pH (Sawada *et al.,* 2001). The purified PG from *Bacillus* sp. strain KSM-P576 was showed maximum activity toward polygalacturonic acid (PGA) at 55 °C and pH 8.0 in 100 mM Tris-HCl buffer. Also, it was observed that the PG was quite stable in various pH buffers between pH 6 and 12 when incubated at 30 °C for 1 h (Kobayashi *et al.,* 2001). According to Jayani *et al.,* (2005) lyases have the optimal pH well as isoelectric points in the alkaline range (7 -11) and the optimal temperature varies from 40-70 °C. In the case of PG, the majority have an acidic range (3.5-5.5) for optimum pH and 30–50 °C for optimum temperature.

Li *et al.,* (2012) purified PNL from *B. clausii* using ultrafiltration, ammonium sulfate fractionation, DEAE Sepharose Fast Flow, and Sephadex G-75 gel filtration. Karbalaei-Heidari, (2014) reported an acidic pectinase producing strain, *Bacillus* sp. BR1390 is

secreting acidophilic, thermal and detergent tolerant PMG. The molecular weight of the purified protein found to be 104 kDa with a unique dimeric structure. The purified protein showed a molecular weight of 35 kDa, and optimal activity with K_m of 0.87 mg/ml at pH 10.0 and 60 °C. Kusuma *et al.,* (2014) got the PG activity in the 30-40 % salt saturation fraction of *B. subtilis* cell free supernatant. The homogeneity of the PG produced by *B. subtilis* C4 was judged by SDS-PAGE. It was found that PG had a molecular weight ranging between 43 kDa and 66 kDa. The catalytic properties of partially purified PG from *B. licheniformis* KIBGE-IB21 indicate highly alkaline nature with apparent K_m 1.017 mg/ml and V_{max} 23,800 μM/min and showed an approximate molecular weight of 153 kDa (Rehman *et al.,* 2015). Anand *et al.,* (2016) used acetone precipitation (0–60 % saturation) for partial purification process of PG from A. *fumigatus* MTCC 2584. While, Mercimek Takcı *et al.,* (2016) reported maximum pectinase activity from *B. subtilis* in ethanol precipitation fraction (90 %) and the SDS-PAGE analysis showed two protein bands located approximately at 60 and 64 kDa.

The pectinolytic enzyme from B. *subtilis* PB1 was purified by ammonium sulfate precipitation (30-40 %), ion exchange and gel filtration chromatography and the molecular weight were found to be 43.1 kDa. The MALDI-TOF-MS analysis showed that the purified enzyme shared homology with PEL. Further, the optimum temperature and pH was determined as 50 °C and 9.5 respectively and it presented a K_m of 0.312 mg/ml and V_{max} of 1248 U/ml (Zhou *et al.,* 2017). The

pectinase production from *B. subtilis* Btk 27 showed maximum activity at pH 7.5 and temperature 50 °C. Also, cations Mg^{2+} and Ca^{2+} stimulated the pectinase activity and the enzyme's K_m and V_{max} values were noted as 1.879 mg/ml and 149.6 U, respectively (Oumer *et al.,* 2017). Alkaline pectinase from *B. subtilis* ZGL14 was purified by ammonium sulphate precipitation, ion-exchange) and size exclusion (Sephadex G-100) chromatography and the purified protein presented a molecular weight of 65 kDa, the optimal temperature of 50 °C and optimum pH of 8.6 (Yu *et al.,* 2017). Similarly, the same author, purified a heat-tolerant acidic pectinase from *Bacillus* sp. ZJ1407 and the purified pectinase showed a molecular weight of about 23 kDa, optimal temperature of 37 °C and pH 5.0. The enzyme also exhibited high thermo-stability (80 – 90 °C) and the pectinase activity was enhanced with Ba^{2+} while inhibited by Mn^{2+} (Yu *et al.,* 2018). The exo-PG from *B. licheniformis* demonstrated characteristic features such as optimum pH of 6.5, the optimal temperature of 60 °C, Molecular weight 54 kDa, V_{max} of 4.18 µM/S and K_m of 3.25 mg/ml (Evangelista *et al.,* 2018). *Bacillus* spp. That has been reported for pectinase production and its characteristic features are shown in Table: 2.1 of Chapter 2.

5.3.2. Types of pectinase activity and its assays

Bacillus spp. are renowned producers of all classes of pectinases including hydrolases, lyases, esterases and protopectinases (Kavuthodi *et al.,* 2018). Pectinases such as PPases, PL and PNL were reported from *B. subtilis* IFO 3134 (Sakai *et al.,* 1990; Sakamoto *et*

al., 1994). Endo-PG, exo-PG and PNL activities of five *Bacillus* strains isolated from decaying vegetable material was studied by Soares *et al.,* (2001) and these enzymatic solutions resulted in the maximal reduction of the solution of citrus pectin viscosity, between 80 and 97 %. Pectinesterases such as PME and PAE were also reported from Bacillus sp. (Remoroza *et al.,* 2015; Remoroza *et al.,* 2014).

DNS assay described by Miller (Miller, 1959) is widely used to measure the free reducing sugar formed because of the action of hydrolases such as PG &PMG. They are also determined by measuring the reduction in the viscosity of the reaction mixture using a viscometer. Usually, an assay for release of reducing sugars is performed for exo-pectinase and viscosity reduction assay for endo-pectinase activity (Solis *et al.,* 1990). Gusakov*et al.,* (2002) developed an improved viscometric method for pectinase (endodepolymerase) activity analysis. But according to Jayani *et al.,* (2005), the viscometric reduction analysis of pectinase have limited success as there is no direct correlation between viscosity reduction and number of glycosidic bonds hydrolyzed. Continuous spectrophotometric rate determination method (Albersheim, 1966) and Thiobarbituric acid (TBA) assay and (Nedjma *et al.,* 2001) were used for the quantification of the PL/PNL activity. The pectin esterase (PME) activity is generally measured by titration method-with a pH stat or pH meter (Kertesz, 1951) or continues spectrometric assay (Hagerman *et al.,* 1986).

5.3.3. Pectinase zymography

Zymography is an electrophoretic approach, usually connected with PAGE, which contains a substrate copolymerized within the polyacrylamide gel matrix, for the detection of hydrolyzing activity of the enzyme. Any biological samples can be analyzed by this technique based on the substrate provided (Lantz *et al.,* 1994). Different methodologies have been suggested for the detection of pectic enzymes in electrophoretic gels. In some of the studies, the activity of pectic enzymes is detected by the introduction of pectic polymers into the gel (Jaramillo *et al.,* 2015). While on some other studies, the pectinolytic activities were detected by an agar-overlay technique based on the contact between the electrophoresis gel and the pectin–agarose gel and the pectinolytic activity was detected mainly by ruthenium red and CTAB, which provides a rapid, effective, and determined method for the analysis of a complex mixture of pectinases (Hadj-Taieb *et al.,* 2011). Balali *et al.,* (2002) used pectic zymography techniques in the identification and genetic variation of *Fusarium* species. Soriano *et al.,* (2005) reported different pectinase zymographic pattern by *Paenibacillus* sp.BP and *Bacillus* sp.BP-7 in different production media. They also noted that five bioactive bands with the molecular weight (25, 32, 38, 44, and 53) were produced in case of pectinase zymographic analysis with Paenibacillus sp.BP. Whereas Bacillus sp.BP-7 showed four bioactive bands with molecular weight. (28, 42, 56, and 63 kDa) in the same pectinase production media. The pectin zymogram analysis with enzyme preparations of *B.*

licheniformis SVD1 showed a single clear zone (one pectinase – a PL) at 70–72 kDa (van Dyk *et al.,* 2010). But the zymography analysis of the crude pectinase from *B. mojavensis* I4 indicated the presence of three pectinases as evident by three clear zones at different positions (Ghazala *et al.,* 2015).

5.3.4. Pectate lyase gene cloning, sequencing and characterization

The most cloned pectinase gene from *Bacillus* sp. is PL gene '*pel*'. The first cloning and characterization of a *pel* gene from the *Bacillus* genus was reported in *B. subtilis.* The cloned gene indicated a 1,260 bp open reading frame (ORF) encoding a 420 amino acid polypeptide which includes a 21 amino acid (aa) signal sequence. Molecular weight is found to be 45,605 Da and the purified enzyme had similar properties to the PL isolated from extracellular media of the organism (Nasser *et al.,* 1993). Kunst *et al.,* (1997) published the complete genome sequence of *B. subtilis*. Since then many researchers reported the successful cloning, expression, sequencing and characterization of pectinase genes (especially PL) from several *Bacillus* strains (Dubey *et al.,* 2016; Li *et al.,* 2010). A review on molecular biology and list of cloned pectate lyase genes from bacterial and fungi given by Dubey *et al.,* (2016). The *pel*A gene from Alkaliphilic *Bacillus* sp. N16-5 was cloned in *E. coli* BL21 (DE3) using the vector pUC18. The gene contained an ORF of 1,089 bp, encoding a 36- amino acids signal peptide and a mature protein of 326 amino acids and the deduced amino acid sequence displayed considerable homology to those of known PL in polysaccharide lyase family 1(Li *et al.,* 2010). Similarly, the amino acid sequences deduced

from the cloned genes of three *Bacillus* strains (*B. pumilus* BS22, *B. subtilis* BS66, and *B. fusiformis* BS90) showed the characteristics of *Pel*s belonging to Family 1(Ouattara *et al.,* 2010). In order to utilize new genetic resources, Xiao *et al.,* (2012) cloned the the *pel* gene from *B. subtilis*521 in to in *E.coli* DH5α and expressed in *E.coli* BL21 using pET-22b (+) vector. Further sequence analysis showed that it is having 26-82 % homology with other strains in GenBank. Liang *et al.,* (2015) cloned and expressed the *pel* gene from *B. pumilus* (ATCC 7061) in *E. coli* using the vector pET-28a. The recombinant enzyme showed significant increases in thermo activity and thermostability and having noteworthy application in textile industry for ramie degumming.

5.3.5. *In-silico* computational studies of pectate lyase

The altering of the microbial enzymes has become a trend in the field of protein engineering to defeat the constraints of characteristic biocatalysts and to create process- specific enzymes. Researchers all over the world have been endeavoring to create enzymes which can withstand harsh and unfavorable conditions prevailing in industrial processes (Joshi *et al.,* 2015). NCBI GenBank provides the deposited sequences of a number of enzymes including pectic enzymes (PNL, PL, PG, and PE) isolated from various organisms. *In-silico* analysis can be carried out with the available full length protein sequences. Pickersgill *et al.,* (1994) solved the structure of the *B. subtilis* PL in complex with calcium. Yadav *et al.,*(2009) performed some *in-silico* studies using 48 full-length PL sequences of different organisms retrieved from NCBI and reported the sequence-based similarity existing among different pectinases and nature of the

mechanism of enzymatic activity. Similarly, Kumar *et al.,* (2014) retrieved a total of 28 PL sequences representing different species of *Aspergillus* from NCBI and the translated amino acids sequences were characterized by various *in-silico* studies. They concluded that the *in-silico* studies could provide an apposite understanding of the various targets for genetic manipulation as desired for the industrial application. The sequence analysis for PL from *B. subtilis* 521revield that it is having 26-82 % homology with other strains in GenBank.

5.3.6. Homology modeling, structure prediction and docking studies

In the field of computational biology, structural bioinformatics is one of the important research areas. This perspective, not only to enhance structures with biological information but also to link disparate sources of information and to put the structures in a broader biological environment (Dorn *et al.,* 2014; Gutmanas *et al.,* 2013). Various bioinformatics tools are available for modeling, structure prediction, model validation, superimposition, docking etc. which are helpful for innumerable *in-silico* studies in the field of biology (Krieger *et al.,* 2009; O'Boyle *et al.,* 2011; Rueda *et al.,* 2013; Trott *et al.,* 2010; Webb *et al.,* 2014). The review by (Herron *et al.,* 2000) explains how structural information of pectic enzymes (*pel*-C) can contribute to an understanding of the complicated steps of pathogenesis. In order to model the 3-D structure of *pel* from from *B. subtilis* 521, Xiao *et al.,* (2012) used ExPASy proteomics server and 3D-JIGSAW protein comparative modeling server and found that, the modeled structure had high homology to those from other bacteria. Chakraborty *et al.,* (2015) carried out some computational studies

including modeling, substrate binding and stability studies of PL (PL1B). The modeled protein structure was right handed parallel β-helix, in which three parallel β-sheets connected by loops coils around to form the β-helix core. Further, by docking studies, they determined the catalytic centre and active site residues of PL1B and reported that amino acid residues such as Asp151, Arg209, Asn234, Arg236, Tyr271 and Ser272 have major role catalysis.

5.4. Materials and Methods

5.4.1. Partial purification of pectinase

5.4.1.1. Preparation of cell-free culture centrifugate

The strain *B. subtilis* BKDS1 was grown in formulated PSEM and cell-free supernatant was prepared by centrifugation (10000 rpm for 15 min, 4 °C). The supernatant was analyzed for enzyme activity and protein content.

5.4.1.2. Ammonium sulphate precipitation

The cell free supernatant was saturated with $(NH4)_2SO_4$ to different cut-offs (0-20, 20-40, 40-60, 60-80 and 80-100). The precipitation calculations were done with the help of online ammonium sulfate precipitation calculator by (EnCor Biotechnology Inc.). The precipitations were done at 4 °C. After overnight precipitation, the precipitates were collected by centrifugation (13,000 rpm, 30 min, 4 °C) and were dissolved in the least amount of Tris-HCl buffer (0.02M, pH 8) and dialyzed (using dialysis membrane-110, Hi-Media) against the same buffer. Enzyme activity was analyzed in each fraction. It was

observed that fractions from 40-100 showed good result. So only two cut-offs (0-40 %, 40-100 % saturation) were made for further studies (Kashyap *et al.,* 2000).

5.4.1.3. Purification by size exclusion chromatography

The protein purification by size exclusion chromatography was done in Fast protein liquid chromatography (FPLC) system -Biologic LP; Bio-Rad. Before starting the procedures, all the buffers were degassed properly and the slurry was prepared as per the manufacturer's instruction. For this, weighed 2.0 g of Sephadex G-100 (Pharmacia-Sweeden) powder and was swollen in 30 ml of 0.02M Tris-HCl buffer (pH 8) and mixed well. After proper mixing, the slurry was packed in Econo-column (Bio-Rad). The column was equilibrated with the same buffer to wash down any impurity. The partially purified protein sample as prepared in the above section (5.4.1.2) was loaded on to the sample injection port and the flow rate was adjusted to 1.0 ml/m. All the fractions were collected using fraction collector linked to the instrument. The instrument measured the absorbance (280 nm) of individual fractions and the formed peak was noted. Fractions collected from the corresponding peaks were assayed for pectinase activity and the fractions of the single peak were pooled and used for further analysis.

5.4.4. Estimation of protein content by (Lowry *et al.,* 1951)

For estimation of protein content in the sample, Lowry's method was used. Bovine Serum Albumin (BSA) was used as the standard.

5.4.5. Characteristics of pectinase

5.4.5.1. Effect of temperature on activity and stability

For the evaluation of optimum temperature for pectinase activity, enzyme assay was performed at various temperatures ranging (30 - 90 °C). Relative enzyme activity in these temperature ranges was calculated by considering the maximum activity as 100 %. For assessing the thermostability, the enzyme was incubated at different temperature ranges (30 – 90 °C) for 1hr. After incubation, the pectinase assay was done (at optimum pH & temperature).

5.4.5.2. Effect of pH on pectinase activity and stability

The pectinase activity assay was performed at optimum temperature (40 °C) and varying pH ranges (5.0 – 10) as described by (Kashyap, 2003). The substrate (Citrus pectin, 1.0 %, w/v) was prepared in 0.02 M of various buffers, such as citrate phosphate (pH 5.0); phosphate (pH 6.0 and 7.0); Tris – HCl (pH 8 and 8.5); and glycine NaOH (pH 9.0 and 10.0). Pectinase assay was performed with substrates dissolved in these pH ranges. The highest activity obtained was considered as 100 %. To study the pH stability, the enzyme was incubated with the above mentioned buffers for a time period of 1 h. After incubation, activity assay was performed under optimum conditions of pH and temperature (8.0 and 40 °C).

5.4.5.3. Effect of various metal ions on pectinase activity

The effect of different metal ions of final concentration (1mM and 5mM) in the assay mixture was examined separately for their

effect on pectinase activity at optimized condition (40 °C and pH 8.0). Pectinase activity without any metal ion was taken as control (100 %). The divalent metal ions tested for their impact were Mn^{2+}, Ca^{2+}, Hg^{2+}, Mg^{2+}, Na^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+}.

5.4.5.4. Kinetics of pectinase -Determination of Michaelis-Menten constant (K_m) and V_{max} values

In order to study the effect of substrate concentration on reaction velocity, different concentrations (2.5-17.5 mg/ml) of the substrate (Citrus pectin) was prepared in 20 mM Tris-HCl buffer (pH 8.0) and the pectinase assay was performed at 40 °C for 15 min. The scientific graphing software GraphPad Prism 7.04 was used to calculate the K_m and V_{max}.

5.4.6. Molecular weight determination by SDS PAGE

In order to determine the protein profile, SDS –PAGE (10 %) was carried out using Vertical slab Mini Gel Electrophoresis as per the procedure described by Laemmli, (1970). The composition for the preparation of separating and stacking gel is given in Table: 5.1.

Plate assembly and gel casting: Slab gel mini apparatus was used to cast and run the SDS-PAGE. The gel plates and spacers were washed thoroughly and dried. The two plates were then separated using 1.0 mm thickness spacers and tightened using bulldog chips. The assembly was then positioned in an upright position and sealed by molten agar. Separating gel (10 %) was poured between two plates and allowed to polymerize for 30 min at room temperature. Then, 5 % stacking gel

was layered over the separating gel, a comb was inserted in the stacking gel and the gel was allowed to get polymerized.

Sample preparation: The protein samples were mixed with sample buffer (Table: 5.2) in 3:1 ratio, boiled for 3-5 min in a water bath and analyzed on SDS-PAGE.

Electrophoresis: The whole gel unit was placed in a buffer tank filled with running buffer (Table: 5.2) and samples (30 µl) were loaded into the individual wells of the gel. Wide range protein marker (Mol Bio, Himedia) ranging from 10 to 245 kDa was used for determination of molecular weight and it was loaded on one of the wells. The gel was run at a constant current of 80 followed by 100 V for stacking and separating gel respectively. After the complete run, the gel was separated from the glass plates, transferred into a suitable container with a staining solution (Table: 5.2) and incubated overnight in shaking condition. After staining, the staining solution was removed and the gel was rinsed with Milli-Q water (MQH_2O) to remove excess stain. The gel was then placed in destaining solution (Table: 5.2) and kept in shaking condition (rocker). The destaining solution was replaced at suitable intervals and agitates until the proper level of destaining is achieved. The protein bands observed in the destained gel was analyzed for molecular weight calculation using Vision-Capt software (Vilber lourmat).

Table: 5.1. Composition of separating (10 %) and stacking (5.0 %) gel

Components	volume	
	10 %	**5 %**
MQH_2O	4.0 ml	3.4 ml
Acrylamide-bisacrylamide solution (30 %)	3.3 ml	0.83 ml
1 M Tris -buffer (pH 8.8)	2.5 ml	-
1 M Tri- buffer (pH 6.8)	-	0.63 ml
SDS (10 %)	100 µl	50 µl
APS (10 %)	100 µl	50 µl
TEMED	6.0 µl	3.0 µl
Total volume	10 ml	5.0 ml

Table: 5.2. Composition of SDS loading dye (4X), Tank buffer (4X), Staining and destaining solution

Components	**Quantity**	
SDS loading dye (4X)		
1 M Tris- buffer (pH 6.8)	:	2 ml
SDS	:	0.8 g
Glycerol	:	4 ml
β-mercaptoethanol	:	3 ml
Bromophenol blue	:	40 mg
Total volume (made up with MQH_2O)	:	10 ml
Tank buffer (1X)		
Tris-base		1.5 g
Glycine		7.4 g
SDS		0.5 g
MQH_2O		500 ml
Staining solution (100 ml)		
Coomassie Brilliant Blue (CBB) R-250	:	0.25 g
Methanol	:	45 ml
Acetic acid	:	10 ml
MQH_2O	:	45 ml
Destaining solution (100 ml)		
Methanol	:	45 ml
Acetic acid	:	10 ml
MQH_2O	:	45 ml

5.4.7. Pectinase zymography

A semi denaturation PAGE was used for the zymogram analysis. The test sample was mixed with loading buffer (prepared separately without adding any denaturing agents β-mercaptoethanol and SDS) in 3:1dilution and loaded on to the gel without any prior heating. The gel was run at a constant current of 80 V in cold condition (4 °C). After electrophoresis, the gel was washed with washing buffer (2.0 % triton X-100 in 20 mM Tris-HCl buffer, pH 8.0) for 30 min in shaking condition to remove SDS. Then the washing buffer was discarded and washed with 20 mM Tris-HCl buffer, pH 8.0 for 10 min in shaking condition. The gel was again washed with the same buffer and carefully placed over a previously prepared pectin-agarose plate (1 % pectin and 1 % agarose in 20 mM Tris-HCl buffer, pH 8.0) and incubated for 30 min at 40 °C. After incubation, the gel was detached from the pectin - agarose plate and stained with KI solution to observe the substrate utilization clear zone against the brown background.

5.4.8. Peptide mass fingerprinting (PMF) analysis by MALDI-TOF MS

The specific band of interest was excised with a clean scalpel, washed with MQW and subjected to PMF analysis using MALDI-TOF-MS (UltrafleXtreme, Bruker Daltonics Germany) as described by (Karthik *et al.,* 2012). The MALDI-TOF-MS analysis was done from Proteomics facility, IISc., Bangalore.

5.4.9. Types of pectinase activity

Different methods as described by Tewari *et al.,* (2005) were used to detect the types of pectinase activity.

5.4.9.1. Polygalacturonase (PG)

PGase activity was assayed by the DNS method (as discussed in section 2.4.4 of Chapter-2). The substrate used here was 0.5 % (w/v) polygalacturonic acid (Himedia).

5.4.9.2. Pectin lyase (PNL)

The PNL activity of the strain was assayed by the thiobarbituric acid (TBA) method according to Pitt, (1988). The cell free supernatant (0.1 ml) was added to 0.5 ml of the pectin solution (1 % w/v). Test sample volume was adjusted to 1.0 ml with Tris- HCl buffer (0.02 M, pH 8) and incubated (40 °C, 1 h). To this, $ZnSO_4$ (0.06 ml, 9.0 % w/v) and NaOH (0.06 ml, 0.5 M) were added and centrifuged (3000 rpm, 10 min). Transferred 0.5 ml clear supernatant to a clean test tube followed by added TBA (0.3 ml, 0.04 M), HCl (0.25 ml, 0.1 M) and MQH_2O (0.05 ml). Heated the samples for 30 min in a boiling water bath. The absorbance of pink colour formed was measured after cooling at 550 nm against a reference cuvette which contained the same reagents as that of the experimental cuvette but for which the $ZnSO_4$ and NaOH were added before adding the enzyme and substrate. The amount of enzyme that caused a change in absorbance (OD_{550}) of 0.01 under the conditions of the assay was defined as 1 unit (U) of PNL activity.

5.4.9.3. Pectate lyase (PL)

PL assay and enzyme activity calculation was also done using the same method as discussed in the above section (2.7.2) for PNL. The substrate used here was 0.5 % (w/v) polygalacturonic acid instead

of pectin. The occurrence of a peak at 550 nm was due to the hydrolytic products formed by PL.

5.4.9.4. Viscosity-based assay for pectinase activity

Assay for pectinase activity (endo-pectinase) was done by the viscometric method using the Ostwald's viscometer (Gusakov *et al.,* 2002). In order to perform the assay, the Ostwald's viscometer was cleaned properly and dried in an oven. 10 ml of pectin solution (1 %) prepared in Tris- HCl buffer (0.02 M, pH 8) was taken in taken in the viscometer and preincubated at 40 °C for 15 min for stabilization. To this added 2 ml of enzyme solution and immediately took the efflux time reading (V_0) with the help of a stopwatch. The apparatus containing the enzyme substrate mixture was then incubated in a water bath at 40 °C for 30 min and viscometric reading (efflux time) was measured (V_t). The percent loss of viscosity was calculated by using the formula;

$$A = \frac{V_0 - V_t}{V_0 - V_s} \times 100$$

Where, $\mathbf{V_0}$ = Flow time in seconds at zero min, $\mathbf{V_t}$ = Flow time of the reaction mixture at time T (30 min) and $\mathbf{V_s}$ = Flow time of buffer alone.

5.4.10. Cloning of *pel* gene from *B. subtilis* BKDS1to *E.coli*

5.4.10.1. Bacterial strain and plasmid

The cloning was performed by using the *E.coli* DH5α and plasmid pET-32a(+) (Figure: 5.2) obtained from Novagen and Invitrogen respectively.

5.4.10.2. Primer design

Primers for amplification of pectate lyase (*pel*) gene without signal sequence were designed using a reference *pel* gene sequence embX74880.1 from *B. subtilis* (ncbi.nlm.nih.gov/nuccore/X74880). The cloning primers (Table:5.3) for the vector pET-32a(+) (Figure:5.2) with restriction sequences for *Xba* I and *Xho* I in forward and reverse primer respectively were obtained from Xcelris Labs Limited, Ahmadabad.

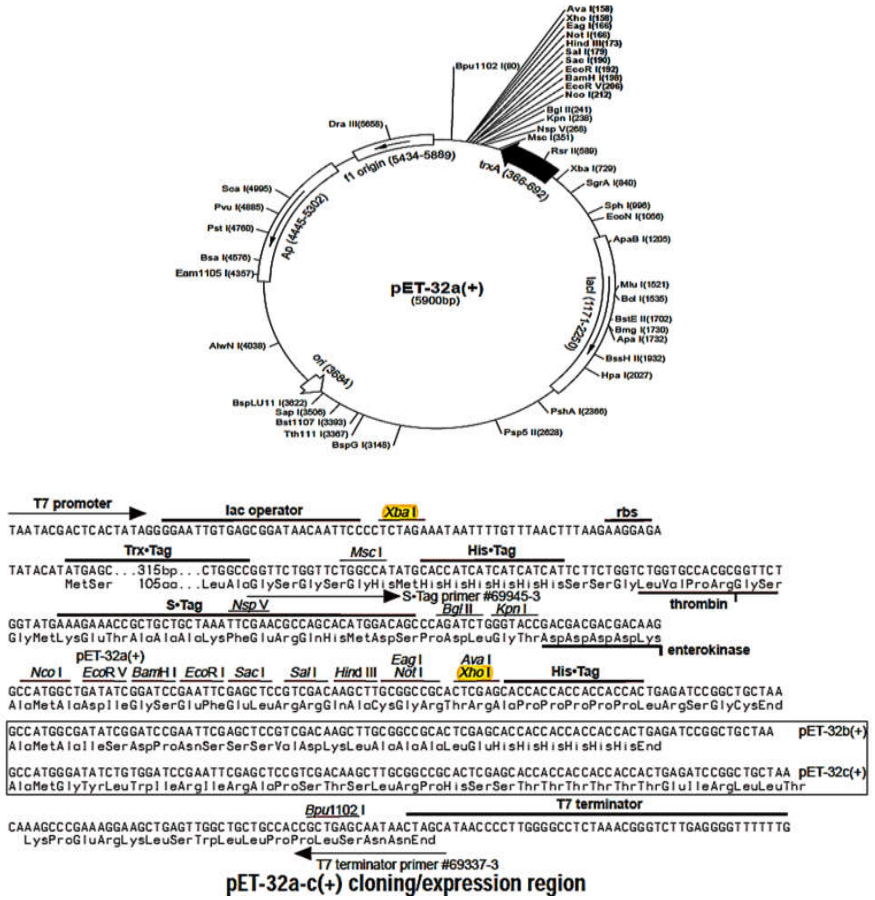


Figure:5.2. Vector diagram and sequence of expression vector pET-32a(+)

Table: **5.3.** Sequences of the designed primers

Primer	Restriction site	Sequence	Bases	Tm	GC %
Forward	*Xba* I	GCCTCTAGAATGAAAAAAGTGATGTTAGCT	30	57.2	36.7
Reverse	*Xho* I	CTCGAGATTTAATTTACCCGCACC	24	55.9	45.8

5.4.10.3. Isolation of genomic DNA

The total gDNA was isolated from *B. subtilis* BKDS1 by the modified method described by (Narayan *et al.,* 2016). An overnight grown culture of the strain in LB broth was pelleted by centrifugation (5000 rpm, 5 min at 4 °C). The cell pellets were gently mixed in freshly prepared lysozyme (50 µl, 100 mg/ml) and incubated at 37 °C for 45 min with shaking. After incubation, proteinase K (20 µl, 20 mg/ml) was added and incubated at 55 °C for 45 min. Further cell lysis was achieved with SDS treatment (50 µl, 20 %; w/v) at 65 °C and incubated for 45 min with intermittent mixing. The cell lysate was then centrifuged (13,000 rpm, 3 min) and subjected to RNAse treatment (10 µl, 10 mg /ml) and incubated for 15 min at 37 °C. To the lysate, added 0.35^{th} volume potassium acetate (2.5 M, pH 8.0) for precipitation of cellular proteins and other cell debris and centrifuged (7,000 rpm, 3 min). The supernatant was transferred into a new sterile Eppendorf tube and added an equal volume of isopropanol for the precipitation of genomic DNA and the precipitated DNA was pelleted by centrifugation (13,000 rpm, 20 min). Discarded the isopropanol, added 500 µl of 70 % ice-cold ethanol and centrifuged (13,000 rpm, 2 min). Excess ethanol was evaporated and the dried pellet was dissolved in 100 µl NFW. Using NanoDrop, the concentration and purity of the

extracted gDNA was measured and was also observed in 1 % agarose gel for qualitative examination.

5.4.10.4. Polymerase chain reaction (PCR)

A gradient PCR in the range of 50 – 60 °C was performed with 50 ng of the DNA to validate the primers and for the best annealing temperature and fixed at 52.3 °C. The DNA was diluted into three different concentrations (1:5, 1:10 and 1:100) to check the best concentration for PCR reaction. After standardizing the appropriate PCR condition (**Table**:**5.4**), the PCR amplification was done in 5 system of 25 µl of reaction volume containing Origin Taq PCR master mix 12.5 µl, Forward & Reverse primer 0.5 µl each and template DNA 1 µl (from 1:10 dilution).

Table:5.4. PCR temperature profile

Initial denaturation	Denaturation	Annealing	Extension	Final extension
94°C	94 °C	52.3 °C	72 °C	72 °C
2 min	30 sec	30 sec	1:30 min	5 min
	40 cycles			

5.4.10.5. Purification of PCR product

The five PCR product systems were pooled and purified using Gel PCR cleaning up system- Promega. To start the purification, an equal volume of membrane binding solution was added to the pooled PCR amplification product and transferred the whole volume into SV minicolumn positioned over the collection tube, incubated for 1 min (RT) and centrifuged (3000 rpm, 1 min). Decanted the flow-through and placed the minicolumn again into the collection tube

followed by added 700 µl membrane wash solution and centrifuged (13000 rpm, 1 min), decanted the flow-through, reinserted the minicolumn into the collection tube and the step was repeated with 500 µl membrane wash solution. The contents of the collection tube were discarded and the column assembly was re-centrifuged and allowed evaporate of any residual ethanol present. The DNA bound on the minicolumn was eluted with 50 µl of NFW by centrifugation (13000 rpm, 1 min). The purity of the product was checked by NanoDrop and was stored at -20 °C till use.

5.4.10.6. Plasmid isolation

The pET-32a(+) plasmid transformed on *E.coli* cell was isolated by QIAGEN- plasmid isolation kit quick start protocol (Midi preparation). The overnight bacterial culture (grown in the presence of carbenicillin-final concentration 0.1 mg/ml) was centrifuged (10000 rpm, 10 min, 4 °C) for cell pelleting. Resuspended the cell pellet in buffer P1 (4 ml) followed by added buffer P2 (4 ml), mixed properly by inverting 4–6 times, and incubated at RT for 5 min. To the cell lysate, prechilled buffer P3 (4 ml) was added and mixed well by inverting 4–6 times. The tube was incubated for 15 min on ice and pelleted by centrifugation (18000 rpm, 30 min, 4 °C). The supernatant was loaded on QIAGEN-tip 100 column (pre-equilibrated with 4 ml buffer QBT) and permitted to enter the resin by gravity flow. Washed the QIAGEN-tip twice with buffer QC (10 ml). The DNA was eluted into a sterile falcon tube (15 ml) using buffer QF (5 ml) and precipitated by adding isopropanol (0.7 volumes). The precipitated

DNA was collected by centrifugation (18000 rpm, 30 min, 4 °C) and washed with ethanol (70 %, 2 ml). The ethanol was decanted after centrifugation (13000 rpm, 10 min) and the plasmid DNA pellet was dried and redissolved in NFW (100 µl). Purity was further checked on NanoDrop and agarose gel.

5.4.10.7. Restriction digestion (RD) of Vector & Insert

The concentration of purified vector and insert was found to be 1456.3 (ng/ µl) and 83.8 ng/ µl respectively. For the RD, 800 ng of each vector and insert DNA is needed. So, 0.6 µl of plasmid DNA and 10 µl of insert is used for RD. Xba1 and Xho1 were the restriction enzyme used. 4 systems of (20 µl) were prepared as follows (Table: 5.5.A and B). The RD was done at 37 °C (for 1 h) followed by enzyme inactivation at 65 °C (for 20 min).

Table: 5.5.A. Components for RD of vector

Components (µl)	**Uncut**	**Single cut**		**Double digestion**
		Xba1	**Xho1**	
Vector	0.6	0.6	0.6	0.6
Buffer	2	2	2	2
Enzyme	-	1	1	1+1
NFW	17.4	16.4	16.4	15.4

Table: 5.5.B. Components for RD of insert

Components (µl)		**Uncut**
Insert	:	10
Buffer	:	2
Enzyme	:	1+1
NFW	:	6

5.4.10.8. Gel extraction

The EtBr stained 1 % agarose gel containing the restricted products were observed under Gel Documentation system. The gel containing the region of interest was sliced with a clean blade and purified using the pure link quick gel extraction kit (Invitrogen). For this, the portion containing the DNA was weighed into a clean tube and added 3 volumes of gel solubilization buffer (L3) to 1 volume of the gel (3:1). The tube was incubated in a pre-equilibrated water bath at 50 °C for 10 min and in every 3 min, inverted the tube for proper gel dissolution. The tubes were re-incubated (at 50 °C) for an additional 5 min for complete gel digestion and added 1 gel volume of isopropanol and mixed well. The sample was loaded on to the quick gel extraction column placed in a wash tube and spinned (13,000 rpm, 1 min). The flow-through liquid was emptied and placed the column into the wash tube. To this, added 500 µl wash buffer (W1) containing ethanol and spun (13000 rpm, 1 min). Decanted the flow-through liquid and inserted the column back into the wash tube and centrifuged at maximum speed for 1–2 min. The column was placed into the recovery tube and added 50 µl elution buffer (E5) to the center of the column and incubated for 1mim at RT. Centrifuged the column at 13000 rpm for 1 min, collected the purified DNA and purity was checked.

5.4.10.9. Ligation of insert and vector

The ligation mix was calculated using the online ligation tool (insilico.uni-duesseldorf.de/Lig_Input). As per the tool, vector and

insert were used in two ratios (1:3 and 1:5) and the ligation was performed in a total volume of 20 µl (Table: 5.6) with 200 ng of vector. A sample containing all the ligation components except insert was kept as control. For ligation, the mixture was incubated overnight at 16 °C.

Table: 5.6. Components of ligation mixture (20 µl)

Components	**Vector only (µl)**	**Vector + Insert (µl)**	
		1:3	**1:5**
Vector	2.79	2.79	2.79
Enzyme	1	1	1
Buffer	2	2	2
Insert	-	9.2	15.33
NFW	14.	5.01	-
Total volume	20		

5.4.10.10. Ligation conformation

Confirmation of the ligated product (plasmid+insert) was done by single digestion with the restriction enzyme (Xba1) and observing the resulted bands on 1 % agarose gel. The restriction was done at 37 °C for 1 h followed by enzyme inactivation at 65 °C for 20 min. The components of this reaction mixture are given in Table: 5.7.

Table: 5.7. Restriction digestion of ligated product

Components	**Volume (µl)**
DNA	: 3
Enzyme (X ba1)	: 1
Buffer	: 2
NFW	: 14

5.4.10.11. Competent cell preparation using *E.coli* DH5α

The $CaCl_2$ method is used to prepare the competent cells using *E.coli* DH5α. An overnight culture of DH5α is prepared in 5 ml LB broth. From this, an inoculum volume of 1000 µl was inoculated to 100 ml of LB broth and incubated (37 °C, 200rpm). Optical density was checked at A_{600} during different intervals until the OD reached about 0.35-0.80 (Never let it cross 0.4). After reaching the specific OD, chilled the culture flask on ice for 20 min. For harvesting the bacterial cell, the cultures were centrifuged (6000 rpm, 5 min, 4 °C) in two pre-chilled falcon tubes. Decanted the supernatant and gently resuspended the pellet in 2 ml of 0.1M $MgCl_2$ solution without vortexing and the tubes were incubated for 30 min on ice. Centrifuged (6000 rpm, 10 min, 4 °C) the tubes again and the supernatant was discarded carefully. Keeping the tubes in ice, the cell pellet was smoothly resuspended in 2 ml of ice-cold 0.1M $CaCl_2$ solution and incubated further on ice for 30 min. The tubes were again spun (6000 rpm, 10 min, 4 °C) and the collected cell pellet was gently resuspended in 0.5 ml of 0.1 M $CaCl_2$ and 0.5 ml of 80 % sterile glycerol. Dispensed aliquots (100 µl) to prechilled microtubes and kept at -80 °C till use.

5.4.10.12. Transformation

The prepared competent cells were thawed on ice and added with 10 µl of ligated product, mixed gently by tapping and incubated on ice for 30 min without any disturbance. Heat shock was given to the

ligation and competent cell mixture at 42 °C for 2 min. The tubes were transferred quickly onto the ice and incubated for 4- 5 min. To the mix, 900 µl of plain LB broth (without antibiotic) was added and the tubes were placed in an orbital shaker at 37 °C for 1.30 hour with agitation of ~200 rpm. During the incubation period, 50 ml of LB agar was melted and allowed to cool to 40 °C. To the 50 ml of molten LB agar, 50 µl of carbenicillin (100 mg/ml) was added. The molten agar was mixed properly without forming air bubbles and poured on to the sterile Petri plates. The plates were allowed to solidify for 10-15 min and were incubated at 37°C until plating. After 1.30 h incubation in an orbital shaker, the tubes containing cells were centrifuged (5000 rpm, 10 min, 4 °C) and 900 µl of supernatant was discarded. The pellet was resuspended in remaining 100 µl broth and spread plated on the antibiotic containing LB agar plate. The plates were incubated overnight at 37°C.

5.4.10.13. Colony PCR

The colonies observed on LB agar plates were noted down and the presence of plasmid insert was screened by colony PCR. With the help of a loop, a portion of the selected colonies were aseptically suspended in 10 µl of 1X PBS. The plates were again kept in incubator (37 °C) for further growth from the picked colonies. PCR was carried out with *pel* primers and the suspended colony as template (1 µl) by following the same procedure described above (Table: 5.4) with an initial denaturation of 7 min. Isolated gDNA from *B. subtilis* BKDS1 was kept as the positive control. Positive colonies were streaked on

antibiotic containing LB agar plates and also in LB broth for further studies.

5.4.10.14. Plasmid isolation

The plasmid containing the ligated gene of interest transformed on *E. coli* DH5α was isolated by QIAGEN- plasmid isolation kit quick start protocol as described in section 5.4.10.6.

5.4.10.15. Confirmation by restriction digestion (RD)

Confirmation of the positive clone was carried out by RD of the recombinant plasmid isolated from *E. coli* DH5α. Using the restriction enzymes, *Xba* I and *Xho* I double digestion (Table 5.8) was carried out for 1 hour at 37 °C followed by enzyme inactivation at 65 °C for 20 min. Uncut vector was used as the control. The restriction digested products were examined on 1 % agarose gel.

Table: 5.8. Restriction digestion of cloned plasmid

Components	**Volume (µl)**
Plasmid DNA	: 3
Enzyme (X ba1+ Xho1)	: 1+1
Buffer	: 2
NFW	: 13

5.4.11. Gene Sequencing

10 µl of the isolated plasmid DNA sample was sent for sequencing at (SciGenom Labs Private Ltd., Cochin, Kerala-682037,

India). The reverse primer used was gene reverse primer and forward primer was T7 primer (T7 promoter in vector backbone).

5.4.12. *In-silico* characterization of pectate lyase (*pel*)

The nucleotide sequence obtained were analyzed for similarity search using the nBLAST tool of NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi) and also for multiple sequence alignment. From the nucleotide sequence, the amino acid sequence was deduced using the online translate tool, ExPASy (web.expasy.org/translate) also, the physiochemical properties of the deduced protein were studied with Expasy's ProtParam Proteomics server. The secondary structure was analyzed using protein secondary structure prediction tools PsiPred (bioinf.cs.ucl.ac.uk/psipred) and GOR (npsa-prabi.ibcp.fr). The deducted aminoacid sequence was also searched in Pfam (pfam.xfam.org) for conforming the protein family.

5.4.13. 3D- structure prediction by Homology modeling

The 3D-structure of BKDS PL was modeled using Modeller 9.19 (2017 Version) (Webb *et al.,* 2014). The template of the PL protein was modeled on the basis of BLASTp search result (chain A of *B. subtilis* pectate lyase-Accession No. 1BN8 A). The models were visualized using RasMol. The best modeled structure was chosen based on the assessment of the Ramachandran Plot (mordred.bioc.cam.ac.uk/rapper/rampage.php). Then YASARA (Krieger *et al.,* 2009) and Mod Refiner software were used to minimize the protein energy. The refined model was again analyzed by

Ramachandran Plot – RAMPAGE. The quality of the modelled protein was evaluated by analyzing the stereochemical parameters using PROCHECK, Verfiy3D and ERRAT at SAVES server (servicesn.mbi.ucla.edu/SAVES). Superimposition of the predicted protein model with the template (1BN8 A) was done using the bioinformatics software PyMOL and its root-mean-square deviation (RMSD) value was calculated.

5.4.14. Docking of modeled protein with different ligands

The 3D structure of the modelled BKDS1 PL was docked with different ligands in order to determine the catalytic centre and active site residues as discussed by (Chakraborty *et al.,* 2015). The docking studies were performed using AutoDock 4.2.6 (Trott *et al.,* 2010) along with the MGLTools 1.5.6 (mgltools.scripps.edu). For docking, the ligands were selected and downloaded in 3D SDF from PubChem (pubchem.ncbi.nlm.nih.gov). The ligands used were given in Table: 5.9. By using OpenBabel 2.4.0 (O'Boyle *et al.,* 2011), the downloaded SDF files of the ligands were converted into corresponding PDB format.

Table: 5.9. Ligands used for docking

No.	PubChem CID	Chemical Name	Molecular Formula
1.	84740	D-galacturonic acid	$C_6H_{10}O_7$
2.	193487	2-amino-2-deoxy-d-galacturonic acid	$C_6H_{11}NO_6$
3.	439694	Digalacturonic acid	C12H18O13
4.	24892720	Trigalacturonicacid	$C_{18}H_{26}O_{19}$
5.	5459352	Tetragalacturonic acid	$C_{24}H_{34}O_{25}$

5.5. Results

5.5.1. Partial purification of pectinase

Ammonium sulphate precipitation followed by dialysis was done for the partial purification of pectinase. Dialysis membrane-110 (Hi-Media) was used for the dialysis and this tubing will retain most proteins of molecular weight 12000 or greater. The pectinase activity was observed in the 40 - 100 % salt saturation fraction. The ammonium sulphate purification fraction showed 2.33 fold increases in pectinase activity (Table: 5.1).

5.5.2. Purification by size exclusion chromatography

The partially purified protein sample was purified in FPLC by size exclusion chromatography with to Sephadex G-100 column. The protein sample was loaded on the sample injection port and elution was done with 0.02 M Tris-HCl buffer (pH 8). The elution profile of extracted protein was detected using the software–Bio-Rad and depicted in Figure: 5.3. Highest pectinase activity was detected in fraction numbers 21-25. These fractions were pooled and used for further analysis. Through this purification, the increase in pectinase activity was achieved to 5.43 fold (Table: 5.10).

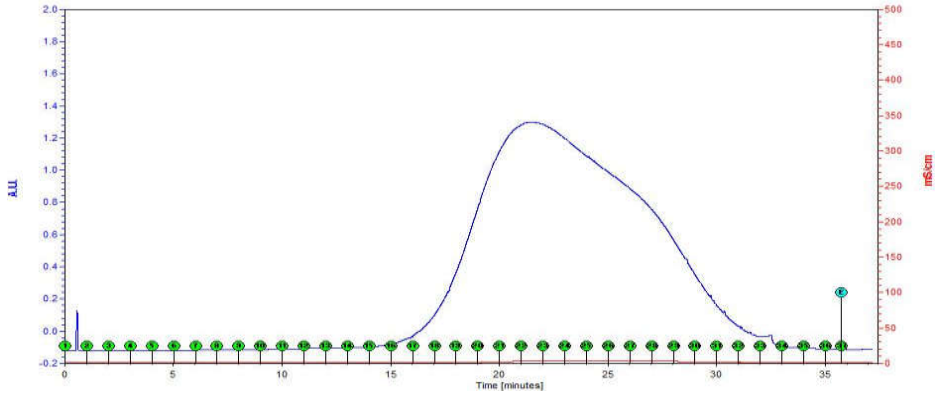


Figure: 5.3. Protein elution profile on Sephadex G-100

Table: 5.10. Pectinase purification by ammonium sulphate and Sephadex G-100.

Enzyme Preparations	Total activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification fold
Crude	75400	365.5	206.29	1
Ammonium sulphate precipitated	12960	26.9	481.78	2.33
Purified enzyme	5405	4.825	1121.3	5.43

5.5.3. Effect of temperature on activity and stability

The pectinase activity was assayed in various temperatures and its effect on enzyme activity and stability was presented in Figure: 5.4. From the figure, it is clear that the enzyme showed nearly similar enzyme activity in temperature ranges of 40-60 °C. Though, 40 °C was considered as the optimum temperature for pectinase activity. The enzyme activity gradually decreases from 60 °C. In case of enzyme stability, even after 1 h incubation, the enzyme retained its original activity in 40 °C. The enzyme stability gradually declines above 40 °C,

but even after 1 h incubation at 80 °C, the enzyme retained 18.08 % activity.

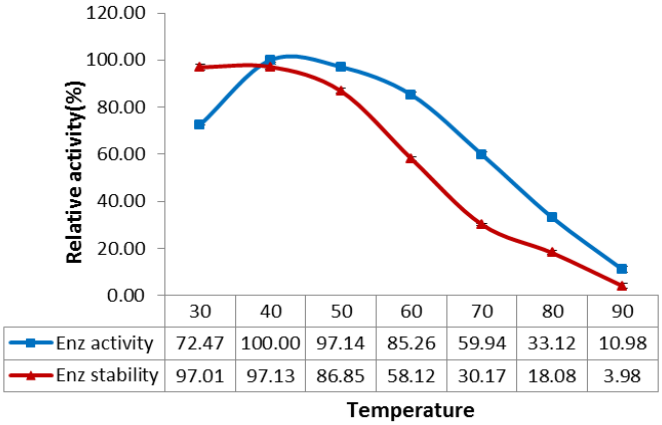
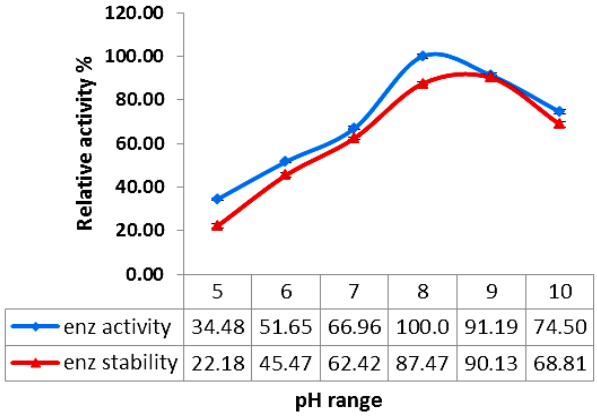


Figure: 5.4. Effect of temperature on activity and stability

5.5.4. Effect of pH on activity and stability

The pH is an important factor that determines enzyme activity and stability. The profile showing the effect of pH on pectinase activity and stability were depicted in Figure: 5.5. The maximum pectinase activity was detected at pH 8.0 followed by pH 9.0. Considering the enzyme stability, the enzyme showed maximum stability in alkaline pH range (8-9).



	5	6	7	8	9	10
enz activity	34.48	51.65	66.96	100.0	91.19	74.50
enz stability	22.18	45.47	62.42	87.47	90.13	68.81

Figure: 5.5. Effect of pH on activity and stability

5.5.5. Effect of metal ions on pectinolytic enzyme activity

Effect of various metal ions on enzyme activity was studied by incubating the purified protein with the presence of metal ions and the obtained result was presented in Figure: 5.6. Two concentrations (1mM and 5 mM) were tested. Among the metal ions tested, the enzyme activity was significantly enhanced by Ca^{2+} at 1 mM and 5 mM. Mn^{2+} stimulated activity at 1 mM but inhibited activity at 5 mM concentration. There is a minor increase in the enzyme activity with Mn^{2+} and CU^{2+} at 1 mM but decreases at 5 mM concentration. Compared to other metal ions, incubation with Hg^{2+} inhibited pectinase activity considerably.

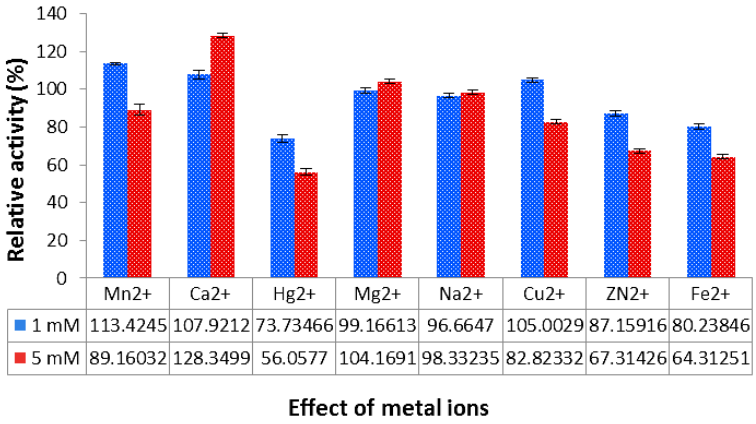


Figure: 5.6. Effect of metal ions on activity

5.5.6. Kinetic studies of pectinase

The kinetic parameters of purified pectinase were studied using the software Graph Pad Prism 6.0. The Michaelis-Menten (Figure: 5.7) and Line-Weaver Burk (Figure: 5.8) plots were plotted using this software and K_m and V_{max} of the enzyme were computed as 0.2202 mg/ml and 1343.0 U/ml respectively.

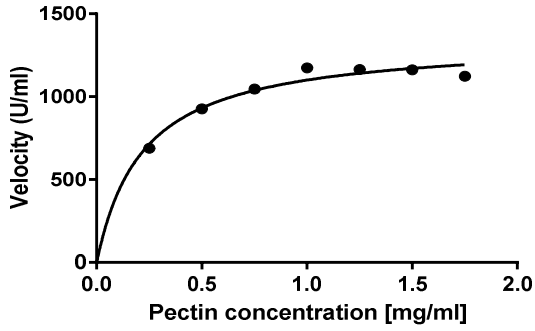


Figure:5.7. Michaelis-Menten plot of purified pectinase

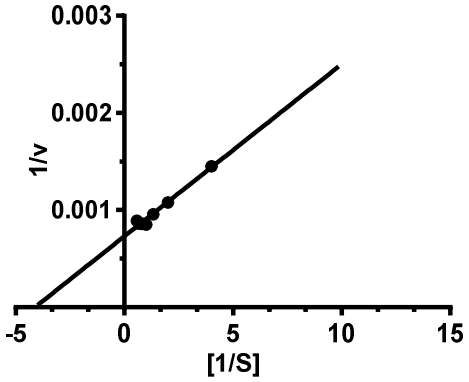


Figure:5.8. Lineweaver–Burk double reciprocal plots of purified pectinase

5.5.7. SDS-PAGE and Zymography

The protein profile was created using SDS–PAGE (10 %) with CBB staining. The photograph of CBB stained gel was shown in Figure: 5.9.A. The lane 1,2 and 3 represents; the crude sample, the partially purified ammonium sulphate precipitated sample and Sephadex G-100 purified sample respectively. Different protein bands are observed in crude samples at positions 30, 35, 60, 70, 104, 222 and 274 kDa. The most prominent band was observed in all samples at around 104kDa (±5kDA). Semi denaturation PAGE is carried out for zymogram analysis by sandwich method (Figure: 5.9.B). In zymogram, a clear area was observed around the protein band corresponding to 104kDa.

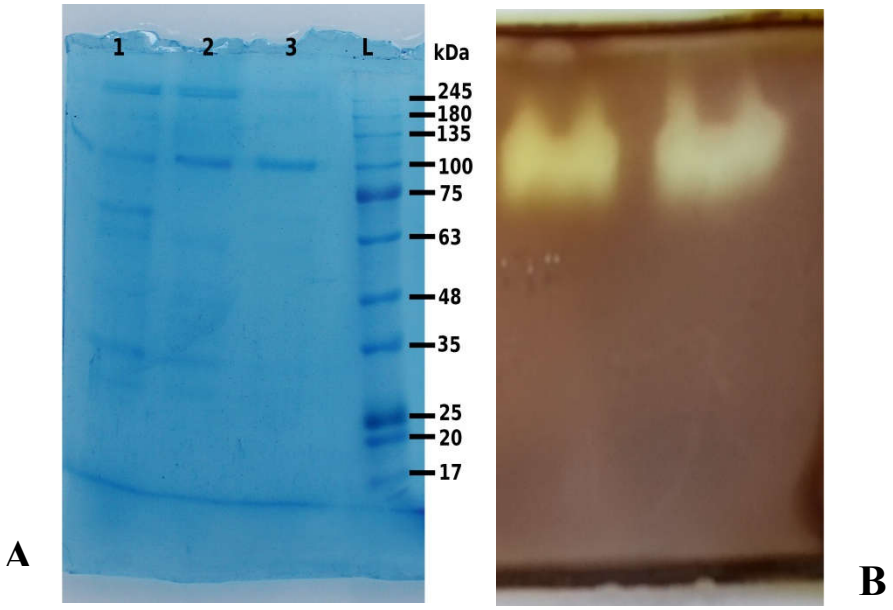


Figure: 5.9.A. SDS PAGE of pectinase samples**:**
lane 1- crude sample, **lane 2**- ammonium sulphate precipitated sample, **lane 3**- purified sample, **lane L**- protein marker and **Figure: B.** zymogram analysis of pectinase

5.5.8. Peptide mass fingerprinting (PMF) analysis by MALDI-TOF MS

The spectra obtained after PMF analysis by MALDI-TOF-MS was presented in Figure: 5.10. With the obtained PMF (m/z) values, protein identification was performed by searching for *B. subtilis* proteins in the latest version of the NCBInr database using the Mascot search engine. Since we didn't see any pectinase related hit in the search result, we searched the obtained PMF with the unreviewed database of *B. subtilis* and got the result proto pectinase-N (Figure: 5.11).

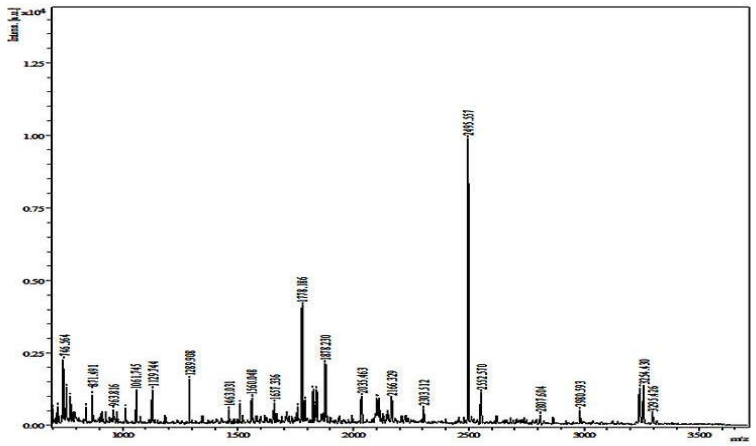


Figure: 5.10. Peptide mass fingerprint obtained by MALDI-TOF-MS from trypsin digest of the eluted protein band

Database : UP_B.subtilis_pec B.subtilis_pec (1 sequences; 18 residues)
Timestamp : 23 Mar 2017 at 02:25:47 GMT
Top Score : 3 for Q9R501_BACIU, PROTOPECTINASE-N (Fragment) OS=Bacillus subtilis PE=1 SV=1

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 13 are significant (p<0.05).

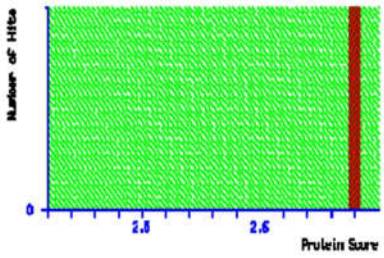


Figure: 5.11. MASCOT database search results of MALDI spectrum

5.5.9. Types of pectinase activity

The purified pectinase was tested for various pectinolytic enzyme activity such as PG, PNL, PL and endo pectinase activity. The PG assay was done by DNS method using the substrate 0.5 %

polygalacturonic acid. The result indicated that it is having PG activity of 1234.598 U/ml. The PNL and PL activity of the sample was assayed by the TBA method by using the substrate pectin (1 %) and 0.5 % polygalacturonic acid. The PNL and PL activity was found to be 166 U and 259 U respectively under assay condition. The endo pectinase activity was measured by the viscometric method using an Ostwald viscometer and a reduction of 94.28 % viscosity under assay condition at 30 min.

5.5.10. Cloning and sequencing of *pel* gene

5.5.10.1. Isolation of genomic DNA

The genomic DNA from *B. subtilis* BKDS1 was extracted and analyzed for purity by the spectrometric method and also by loading on 1 % agarose gel. On agarose gel, a single high molecular weight DNA was observed (Figure 5.12).

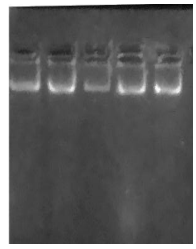


Figure: 5.12. Extracted genomic DNA from *B. subtilis* BKDS1

5.5.10.2. Primer construction and PCR standardization

The primers were incorporated with the restriction sites present in the expression vector. Here it was chosen to add XbaI and XhoI restrictions sites to the 5' end of the Forward and Reverse primer

respectively. For standardization of annealing temperature, a gradient PCR was conducted with annealing temperature at ranges of 51–59 °C. Individual bright bands at position of 1300 bp were obtained at a temperature of 51.7 °C, 52.3 °C and 53.3 °C. From these, 52.3 °C was chosen as the annealing temperature for further PCR reaction.

5.5.10.3. Plasmid isolation, restriction digestion, gel extraction, ligation and its confirmation

The plasmid was isolated by QIAGEN-plasmid isolation kit quick start protocol and the concentration was found to be 1456.3 ng/ µl. The plasmid and purified PCR product were treated for double restriction digestion with XbaI and XhoI and loaded on to agarose gel (Figure: 5.13). The gel containing the region of interest was sliced out and purified. The concentration of gel eluted insert and plasmid was found to be 65.35 ng/ µl and 71.6 ng/ µl respectively. A 20 µl ligation reaction was set up in 1:3 and 1:5 ratios of eluted vector DNA and eluted insert and incubated at 16 °C overnight. Ligation confirmation was done by single digestion with the restriction enzyme (Xba1) and observing the resulted bands on 1 % agarose gel (Figure: 5.14.). In this figure, a new band was observed at 7538 bp region which is formed because of the ligation of the plasmid vector (5900 bp) with insert DNA (1263 bp). So the observation of particular band at 7538 bp region confirmed the ligation step.

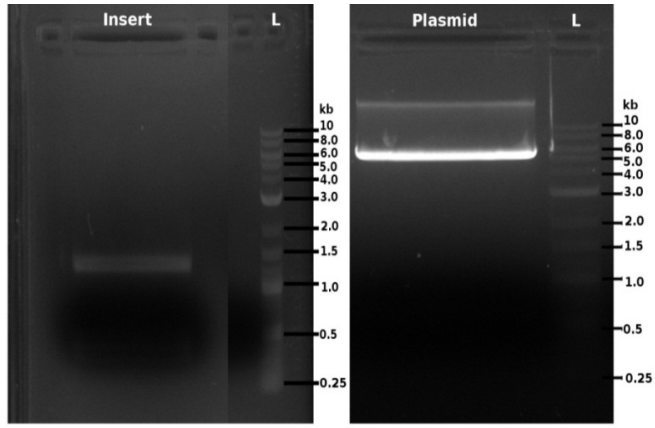


Figure: 5.13. PCR product (insert) and plasmid vector (pET-32a(+)) on agarose gel for gel elution

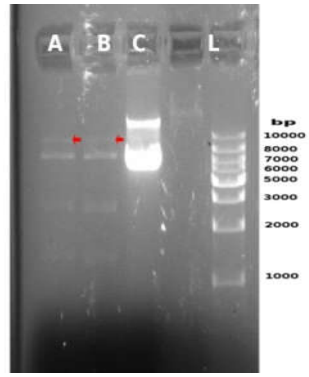


Figure: 5.14. Ligation confirmation (**A**.1:3 dilution, **B**.1:5 dilution, **C**. intact plasmid and **L**. DNA Ladder)

5.5.10.4. Transformation

The ligated product was transformed into prepared competent cells of *E.coli* DH5α cells and plated on antibiotic containing LB agar with a control containing only vector.

5.5.10.5. Colony PCR

All colonies observed in LB plates were analyzed by colony PCR with the *pel* forward and reverse primer to check the presence of insert DNA. One colony on B5 Lane showed a positive result (Figure: 5.15). This colony was cultured and preserved for further studies.

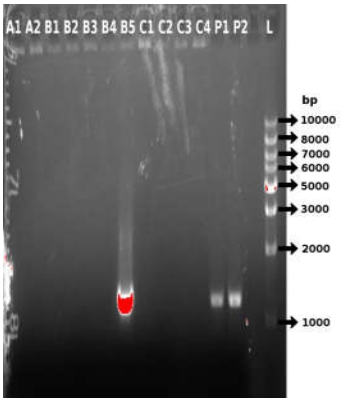


Figure: 5.15.: Colony PCR

5.5.10.6. Plasmid isolation and clone confirmation

The plasmid DNA from clone B5 was isolated and the presence of the *pel* gene was confirmed by PCR amplification and restriction digestion. The isolated plasmid was subjected to restriction digestion (double digestion) and the products were analyzed by agarose gel. Two bands were observed on the gel (Figure: 5.16.); one band at 1263 bp position indicates the release of *pel* gene from recombinant plasmid and another at 5900 bp region indicates the vector thus confirming recombination. The undigested plasmid DNA was observed with a band size of 7538 bp (1263 bp+5900 bp).

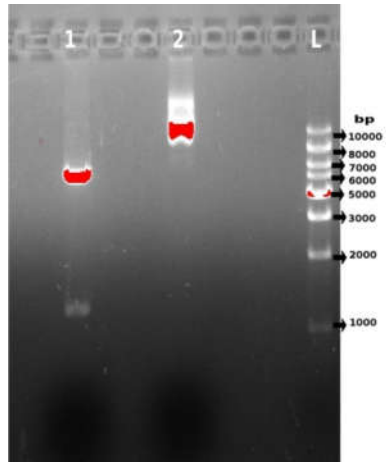


Figure: 5.16. Restriction digestion of the recombinant plasmid
1. Double digestion, **2.** Uncut and **L.** DNA ladder

5.5.10.7. Gene Sequencing

The *pel* gene cloned in pET-32a(+) was sequenced using the *pel* reverse primer and T7 forward primer (T7 promoter in vector backbone) the nucleotide sequence of 1211 bp was obtained (Figure: 5.17). The partial *pel* gene sequences of the isolate *B. subtilis* BKDS1 have been deposited in the NCBI nucleotide sequence database under the accession number **MK030142.1**

>BKDS1

TCCATTAGCAGAAGCATCAATCGATCCATGCAGAGACGGCGTCCAGCCGACAGAAGAGCTCAGCCCG
TTTGCAGCCGATGCGTTGATCTGTGTGCCGTTCAGCAACGTGCCGGAGTCATATAAAGCCGTTCCCCCG
CTGAATACGCTGATCGTTTTAGCAGCTGACAGTCCCGGTACGTCAATGACATTGTTTTGGGCATAGATT
TTAGATGACTTTCCGATTCCCCATGCATAGCTGAAAGGATAACTTGAAGAGCTTGTGCTTCCTTCATAA
TAGTTGTTGTATACGTGCACTTGCCCGAAACGGACTCTCGGCGCGCGCTGGACAATATTTTTATAGCGG
TTATGATGGAGCGTAATTTTTAATTTGCCGTCATCGGAGGTTTTGCTGTCACTTGATCCGAAAATGGAG
CTTTTATCATGATCGTGATAATAGTTGTAGGACATCGTGATATAGTTAGCGCCGTTGGACGCATCCGTT
TGGCCGTCATGGTGCTGATATTTTCTTCCATAATATTTCGGTGATGTGCTGTCCGGACGCGAACCGTCG
TTAAATGTACAGTGATCAATCCAGATGTGTGTGCCGCCGTTTATCGTGATGTTGTCGTATTGTGAGTTC
CAGTTTCCTGAGCTTCCGTCAGTCGGATCCCATTGCGGAAAATAATCATAGGCATCCTGGAATTCAATG
TTGCGAATAATGACGTTATCACTCTTGATTTGGAAGTTTCCTCCCACGACTTTAGCGTTAGTCCCTGAA
CCGACGATCGTCGTGTTTGCAGGGATATCCACCATAACCCGTGCTTTTTGGTTTTTCTGAGAGCGTGCT
CTCGCTTCTTCTTGTGTTCCCGACGGCTCTTTTTTGCCCCATGTGCTAGGATCATAGGCTTTCAAATATT
TGTCCAAATCATACTCCGGATCTTTATAGTCATTTAGACCAAGCGGCTTCAGATTGTCATCTACGTTCA
TGTCAATCGTTCCCTTGATATAAATGATTTTTGGCGTTGTGTTCGTTTCCTTCCCTAATGCCGAGACAAG
CTGGTTTCTGTTGCTGACGGTATACACATTTGAGGACGATGCTTTTGCCCCGCCTGTCGTGCCGGTCGA
GTACGCGCCCCAGCCATCATTGGATCCCAACGTCTGGTGGCCTAAATCAGCTGCGTTCGCGCCAGCTG
GAGTCAATCCTAAAAACAAAGCCGTAGCTAACATCACTT

Figure: 5.17. DNA sequencing result of cloned *pel* gene from *B. subtilis* BKDS1

5.5.11. *In-silico* characterization of *B. subtilis* BKDS1 *pel*

Pairwise alignment of the sequence with the reference sequence (>emb|X74880.1) showed 96 % similarity. BLASTn analysis showed 97 % identity with *B. subtilis* strain RCK pectate lyase gene, complete cds (Accession No. JQ347520.1). Standard protein BLAST was carried out using the deduced amino acid sequence and showed a 99 % similarity to the chain A, pectate lyase of *B. subtilis*. The secondary structure analysis showed Alpha helix (Hh): 11.67 %, Extended strand (Ee): 24.61 %, Random coil (Cc): 63.72 %.

5.5.12. 3D structure prediction by homology modelling

The 3D structure of *BKDS1* PLwas modelled using Modeller software and the structure represented the conventional parallel β-helix structure. The Ramachandran plot of the protein model was depicted in Figure: 5.18.A. The quality of the modelled protein was evaluated by analyzing the stereochemical parameters using PROCHECK, Verfiy3D and ERRAT at SAVES server and the result was depicted in Table:5.11. in comparison with the template -1BN8 A. Superimposition of the modelled structure with the template (Figure:5.18.C) was performed using PyMOL. The output of structural alignment is shown in Figure: 5.18.D and obtained a RMSD value of 0.47.

Table: 5.11. Assessment plot statistics results of the modelled protein and template by PROCHECK, VERIFY3D and ERRAT.

Proteins	**PROCHECK**				**Verify 3D** (3D-ID Score)	**ERRAT** (Quality Factor)
	Most favored regions	Additional Allowed regions	Generally allowed regions	Disallowed regions		
Modelled	86.5 %	12.8 %	0.4 %	0.4 %	91.48 %	74.747
Template	84.3 %	14.9 %	0.3 %	0.6 %	99.75 %	87.0466

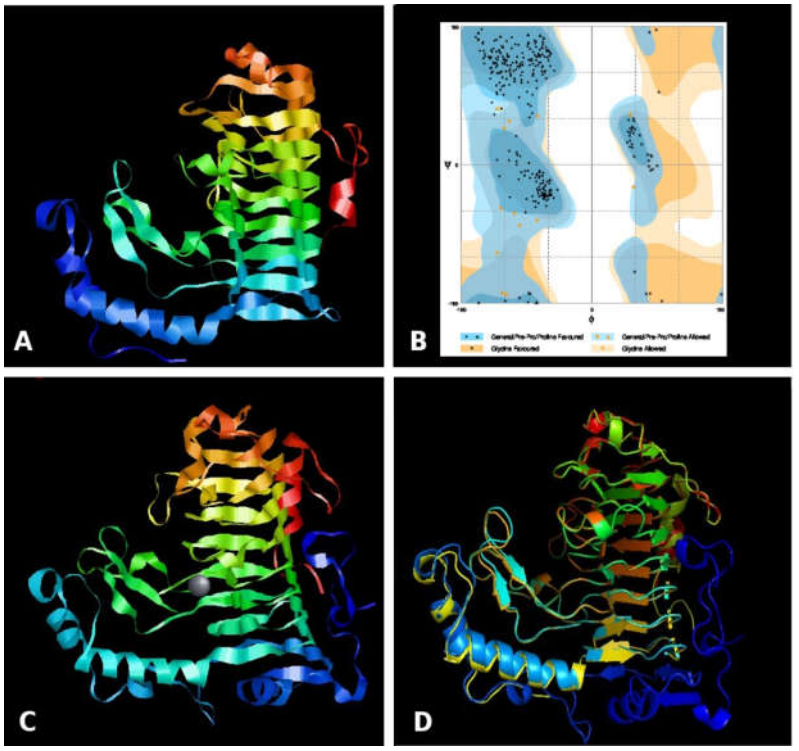


Figure:5.18 (A-D). Figure: Homology model of PL from *B. subtilis* BKDS1 (A) Modelled 3D structure (B) Ramachandran plot (C) Template used for modeling (1BN8 A) and (D) Superimposed structure of PL with template-1BN8 A

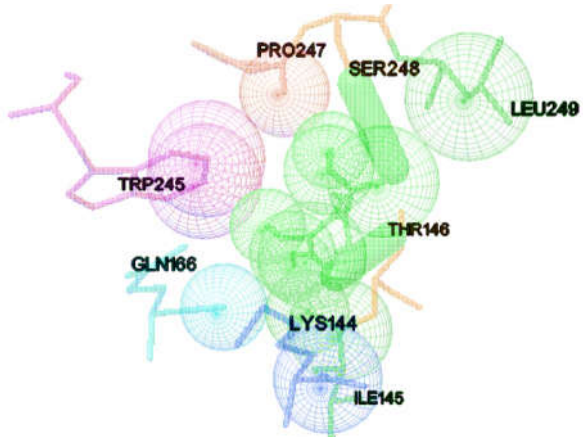
5.5.13. Docking studies of modeled protein with different ligands

The structures of different ligands such as d-GalA, 2-amino-2-deoxy- GalA, di-GalA, tri-GalA, tetra-GalA were docked with the binding sites of modelled protein (BKDS1 PL) by using AutoDock 4.2.6. The results of molecular docking and their corresponding binding energies, amino acids present in the binding cavity and no. of hydrogen bonds formed are listed in Table: 5.12.

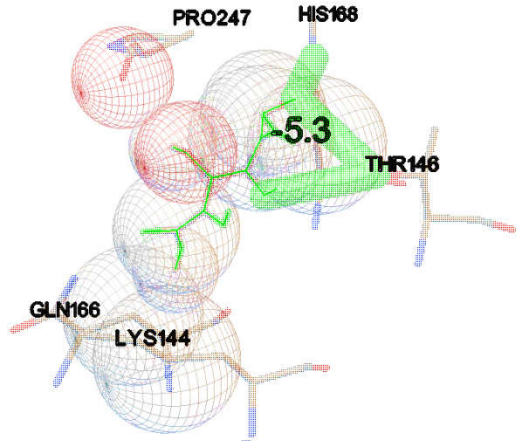
Table: 5.12. Results of the receptor (modelled protein, BKDS1 PL) ligand interactions studied by molecular docking

Ligands	Binding energy	Amino acids present in the binding cavity of modeled BKDS1- PEL									No. of H-bonds	Best binding force
d-GalA	-5.4	Thr 146	Pro 247	Lys 144	Gln 166	Trp 245	Leu 249	Ile 145	Ser 248		2	Fig:5.19.A
2-amino-2 -deoxy-GalA	-5.3	Thr 146	Pro 247	Lys 144	Gln 166	His 168					3	Fig:5.19.B
di-GalA	-9.4	Thr 146	Pro 247	Lys 144	Gln 166	Trp 245	Ile 144	Ile 145			2	Fig:5.19.C
tri-GalA	-8.4	Thr 146	Pro 247	Lys 144	His 72	His 168	Ile 114	Thr 115	Leu 249	Ser 248	3	Fig:5.19.D
Tetra- GalA	-9.6	Thr 146	Pro 247	Lys 144	His 72	Thr 115	Leu 249				2	Fig:5.19.E

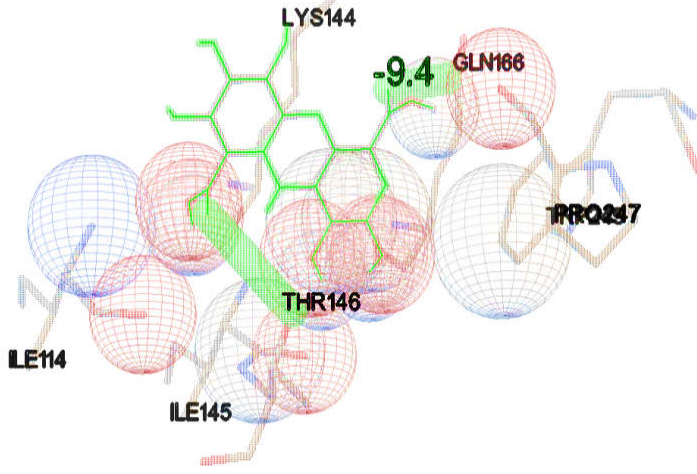
A.

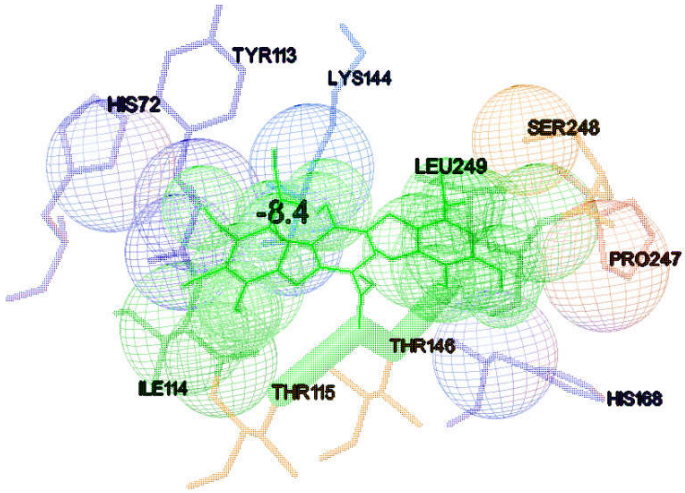

PRO247
SER248
LEU249
TRP245
THR146
GLN166
LYS144
ILE145

B.

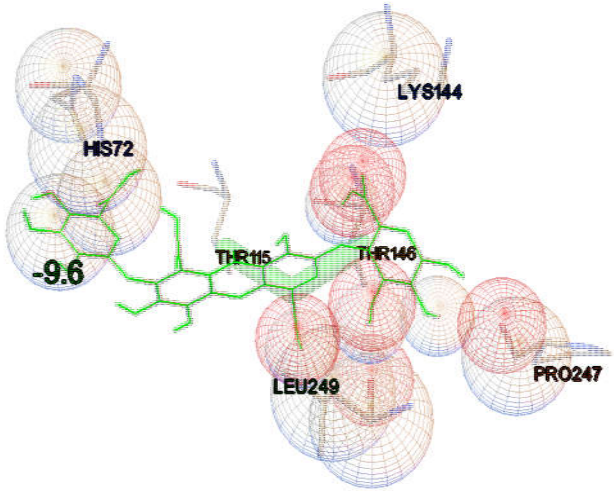

PRO247
HIS168
-5.3
THR146
GLN166
LYS144

C.


LYS144
-9.4
GLN166
THR146
ILE114
ILE145



D.



E.

Figure: 5.19 (A-E). The best binding pose between BKDS1 PEL and LIGANDS (**A**. GalA, **B**. 2-amino-2-deoxy GalA, **C**. DiGalA, D. TriGgalA, and E.TetraGalA) performed by molecular docking using AutoDock Vina

5.6. Discussion

The partial purification of pectinase from *B. subtilis* BKDS1 was done by ammonium sulphate precipitation and the active fraction was concentrated by dialysis. The enzyme activity was found in the 40 - 100 % salt saturation fraction. Previous work on *Bacillus sp*. DT7 by Kashyap *et al.,* (2000) also reported similar result. For further purification of the protein, size exclusion chromatography with Sephadex G-100 column was used. A single large peak is observed in the elution profile spectra. The fractions showing highest enzyme activity were pooled and used for further analysis. The purified enzyme showed a 5.43 fold increase in the specific as compared to the crude enzyme. This indicated a good purification result.

The enzyme showed nearly similar enzyme activity in temperature ranges of 40-60 °C. Though, 40 °C was considered as the optimum temperature for pectinase activity. In case of enzyme stability, even after 1 h incubation, the enzyme retained its original activity in 40 °C (Figure:5.4). Many previous studies on Bacillus spp. presented this temperature range (37-50 °C) as the optimum (Li *et al.,* 2010; Rehman *et al.,* 2015; Yu *et al.,* 2018). Considering the optimum pH, the purified enzyme showed pH 8.0 as the optimum pH for its activity and is having stability in wide alkaline range (8-10) (Figure:5.5). In most of the studies related to Bacillus sp., this pH range was selected as the optimum (Kashyap *et al.,* 2000; Kobayashi *et al.,* 2001; Liang *et al.,* 2015; Sawada *et al.,* 2001). This result indicated

that the pectinase produced by *B. subtilis* BKDS1 is an alkaline pectinase.

The pectinase activity was strongly stimulated by metal ion Ca^{2+} and slightly by Mg^{2+}. The metal ion Mn^{2+} stimulated the enzyme activity in its lower concentration (1mM) but inhibited in higher concentration (5 mM). The enzyme activity was strongly inhibited by metal ions such as $Hg2^{+}$ and Fe^{2+} and Zn^{2+}. It is also noted that, the metal ion Na^{2+} is not having any major role in enzyme activity (Figure: 5.6). Similar type effect by metal ions on PMG activity by *Bacillus* sp. BR1390 was reported by Karbalaei-Heidari & Rastegari (2014) where the pectinase activity was greatly enhanced by metal ions such as Ca^{2+} and Mg^{2+} while, Hg^{2+} and Fe^{3+} have an inhibitory effect. Similarly Oumer & Abate (2017) also observed a stimulatory effect of Ca^{2+} and Mg^{2+} on pectinase activity by *B. subtilis* Btk 27.

The kinetic parameters are important elements that regulate the catalytic efficiency of an enzyme. The K_m of an enzyme is an inverse measure of affinity towards its substrate. Lower the value of K_m, higher is the affinity. V_{max} signifies the maximum velocity of a reaction, indicates how fast the enzyme can catalyze the reaction. The K_m and V_{max} values of BKDS1 pectinase were estimated to be 0.2202 mg/ml and 1343.0 U/ml respectively (Figure: 5.7 and 5.8). Recently, Zhou *et al.,* (2017) observed similar type of result in PL production by *B. subtilis* PB1 with K_m and V_{max} of 0.312 mg/ml and 1248 U/ml, respectively.

The purified pectinase was tested for various pectinolytic enzyme activity such as PG, PNL, PL and endo pectinase activity. The assay result revealed significant positive result for all these tested assays. The PG activity was assayed by the quantification of reducing sugars using the DNS method and the unit enzyme activity was found to be 1036.743 U/ml. Pectin and pectate lyase activity was assayed by the TBA method and the enzyme activity obtained was 166 U and 259 U for pectin and pectate lyase respectively. The endo pectinase activity was assayed by viscometric method and is observed to be 94.28 % reduction in viscosity under assay condition at 30 min. As discussed in the review part, *Bacillus* spp. are renowned producers of all classes of pectinases including hydrolases, lyases, esterases and protopectinases. Previous reports of Kashyap *et al.,* (2000) and Soares *et al.,* (2001) supported this result.

In polyacrylamide gel electrophoretic analysis to determine the mol. mass, different protein bands were observed in crude samples at positions 30, 35, 60, 70, 104, 222 and 274 kDa (approx.) and the number of bands reduced in each purification step (Figure: 5.9). One band at position 104 kDa (±5kDa) noticeable in each sample was the characteristic observation. In zymogram, a single clear area was observed around the protein band corresponding to 104kDa. So the BKDS1pectinase confirmed to have an approx. mol. weight of 104kDa.

The specific band of interest was subjected to PMF analysis by MALDI-TOF-MS (Figure: 5.10) and subsequent Mascot search

indicated the presence of Ppase-N**,** which is actually PL (Sakamoto *et al.,* 1994). Bacillus sp. have been reported for pectinolytic enzymes with molecular mass was ranging from 25-160 kDa including unusual PL with high molecular weight. Kashyap *et al.,* (2000) recorded 106 kDa as the molecular weight of purified pectinase from Bacillus sp. DT7. Similarly, the recombinant PL from Bacillus sp. strain KSM-P358 had a molecular mass of 160 kDa (Kobayashi *et al.,* 2003) and also the recombinant PG from the same organism showed a molecular mass of 105 kDa (Sawada *et al.,* 2001). An unusual high-alkaline PL with high molecular weight (70 kDa) was also reported in 2000 (Ogawa *et al.,* 2000). So the pectinase produced by *B. subtilis* BKDS1 is may be an unusual PL with PG, PMG and PNL activity.

As the pectinase produced by BKDS1 have different pectinase activates, specific primers for various pectinase genes of *B. subtilis* were designed and tried to amplify the responsible genes. Primers designed for pectate lyase gene (*pel)* gave the significant result. Even though, different pectinase gene from Bacillus sp. were cloned and sequenced previously, majority of them were PL (Kavuthodi *et al.,* 2018). Besides, *pel* is the only pectinolytic gene present as *B. subtilis* protein-coding genes in their complete genome sequence (Kunst *et al.,* 1997). So this gene is selected for cloning, sequencing and *insilico* studies. The PCR amplification of BKDS1 *pel* gene produced a product of ~1,260 bp as observed after agarose gel electrophoresis gel (Figure: 5.13). Observation of single band on the gel specified exact amplification of *pel* gene by end-specific primers. This good quality

purified PCR product was taken for restriction digestion with restriction enzymes (XbaII and XhoI). The recombinant vector ((pET-32a(+) BKDS1-*pel* gene) was then transformed into *E. coli*DH5*α*. Colony PCR followed by gene sequencing was performed to confirm the presence of the gene. Thus, in this work, we were successful in cloning the *pel* gene from strain BKDS1 using pET-32a(+) vector in *E.coli* DH5α.

A sequence of 1211 bp was obtained (Figure: 5.17) and on BLASTn analysis, the sequence showed 97 % similarity with *B. subtilis* strain RCK pectate lyase gene, complete cds. The result of BLASTp revealed 99 % similarity to the chain A, pectate lyase of *B. subtilis (*Accession No. 1BN8). The Pfam search revealed the presence of a catalytic domain pectate lyase C (*pel*C). Li *et al.,* (2010) discerned a similar finding in cloned *pel* gene of alkaliphilic *Bacillus* sp. N16-5.

The predicted secondary structure of the protein displayed the presence of Alpha helices (11.67 %) , extended strands (24.61 %) and random coils (63.72 %), which are somewhat similar in the structures of PL and visible from the previous characterizations (Chakraborty *et al.,* 2015; Pickersgill *et al.,* 1994). In biology, the functional depiction of a protein sequence is one of the most common problems and this burden is usually expedited by the accurate 3-D structure of the studied protein (Webb *et al.,* 2014). In this work, the modeled 3D structure of BKDS1 PL represented the conventional parallel β-helix structure. For modeling the 3-D structure of the protein, suitable template was identified (by BLASTp) as *B. subtilis* PL, chain A (Accession No.

1BN8 A) which showed 99 % identity and 96 % query cover. The PDB structure of 1BN8 A was downloaded and used as a template for modeling the protein using Modeller 9.1.16. The modeled structure of the protein denoted the conventional parallel β-helix structure as noticed by Chakraborty *et al.,* (2015). After the enerqy minimization, the quality of the modelled protein structure was evaluated on the SAVES server and for Ramachandran plot analysis (Table: 5.11). The analysis report revealed that 86.5 % residues were in the favored region, 12.8 % and 0.4 % residues were in the additional and generously allowed region, respectively. In the disallowed region have only 0.4 % residues. This implies a very good modeling result. In further Verfiy3D and ERRAT analysis of the server showed that 91.48 % score and 74.747 as the quality factor. ERRAT measure the overall quality in which higher scores indicating higher quality. Usually, a range >50 is accepted for a high quality model. In this case, the value is 74.747 which confirm the quality of the predicted model.

Superimposition of the modelled protein 3D structures with the template (1BN8 A) was done to identify the similarities of protein folds (Figure: 5.18). Using PyMOL, the RMS deviation value was calculated as 0.47Å. The low RMD value is an indication of better superimposition of the target with the template as two identical structures display zero RMSD and dissimilar ones will show values proportional to their dissimilarity (Rueda *et al.,* 2013).

The results of molecular docking showed that, the ligand tetraGalA (CID 5459352) showed the least binding energy of -9.6

Kcal/mol, followed by diGalA acid (-9.4 Kcal/mol) and triGalA **(**-8.4 Kcal/mol) (Table: 5.12). GalA and 2-amino-2-deoxy-GalA presented the highest binding energy of -5.4 Kcal/mol and -5.3 Kcal/mol respectively. Comparing the amino acid residues involved in the binding cavity of BKDS1 PL, it is clear that Thr146 enhance the binding of all the five ligands by the formation of H bonds. It is also, evident that amino acid residues Pro247 and Lys144 were also involved in interactions with all the ligands. These observations suggest that amino acid residues such as Lys144, Thr146 and Pro247 might play an essential role in the enzymatic degradation pathway of the substrate GalA. In a similar docking study Chakraborty *et al.,* (2015) observed that amino acids such as Asp151, Arg209, Asn234, Arg236, Ser272 and Tyr271 were the key residues engaged in the enzyme catalytic process of endo PL of *C. thermocellum***.** Thus these observations may contribute valuable hint in designing improved hydrolyzing enzymes with enhanced productivity. Further studies are required to test these result *in vivo* by site directed mutagenesis.

5.7. Conclusion

Purification of protein is very essential for the study of its characterization, function, structure, substrate specificities, interactions, etc. In this study, pectinase from *B. subtilis* BKDS1 was purified using ammonium sulphate precipitation, dialysis and size exclusion chromatography. The purified enzyme showed a 5.43 fold increase in the specific activity as compared to the crude enzyme. Though, 40 °C was considered as the optimum temperature for pectinase activity, the enzyme showed nearly similar enzyme activity in temperature ranges of 40-60 °C and is having high stability at 40 °C.

In case of pH, the purified enzyme showed pH 8.0 as the optimum pH for its activity and is having stability in wide alkaline range (8-10). Thus, the pectinase produced by *B. subtilis* BKDS1 is an alkaline pectinase.

Considering the effect of metal ions on enzyme activity, the pectinase activity was strongly stimulated by metal ion Ca^{2+} and slightly by Mg^{2+}. While, the enzyme activity was strongly inhibited by metal ions such as $Hg2^{+}$ and Fe^{2+} and Zn^{2+}. The low K_m value (0.2202 mg/ml) of the enzyme indicates high affinity towards its substrate and the V_{max} value was found to be 1248 U/ml. On PAGE, the molecular mass of the protein was observed as 104 kDa (±5kDa) and a single zone of substrate utilization area is observed in zymography. The purified enzyme showed significant positive result for various enzyme activities such as PG, PNL, PL and endo pectinase activity. In addition, the MALDI-TOF-MS analysis and corresponding MASCOT search of the purified protein band revealed the presence of an unusual PL.

The pectate lyase gene (*pel)* gene was successfully cloned and sequenced. Further, with the help of various bioinformatics tools, various *insilico* studies were conducted and the predicted secondary structure of the protein displayed the presence of Alpha helices (11.67 %), extended strands (24.61 %) and random coils (63.72 %). The 3-D structure of the protein was modelled and denoted the conventional parallel β-helix structure. Acceptance of the modelled protein structure was validated by Ramachandran plot, Verfiy3D and ERRAT analysis and all the analysis displayed a good result. The low RMD value (0.47Å) is a clear indication of better superimposition of the target with the template PL.

The substrate specificity of the BKDS1 PL was determined by docking studies with various ligands and tetraGalA showed least binding energy of -9.6 Kcal/mol, followed by diGalA acid (-9.4 Kcal/mol) and triGalA **(**-8.4 Kcal/mol). GalA and 2-amino-2-deoxy-GalA presented highest binding energy of -5.4 Kcal/mol and -5.3 Kcal/mol respectively. It is observed from the docking analysis is that the binding of the substrates were most probably emphasized by amino acid residues such as Thr146, Pro247 and Lys144. Among this, Thr146 enhance the binding of all the five ligands by the formation of H bonds. The results of this study may boost out new ideas to outline an efficient enzymatic hydrolysis process with higher yields and lower production cost

CHAPTER 6

SUMMARY AND CONCLUSIONS

Pectinases consist of an exclusive group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls. Today, pectinases are the major group of upcoming enzymes that hold a leading position among the commercially produced industrial enzymes. Microorganisms including yeast, bacteria, actinomycetes and a large number of filamentous fungi are commonly recognized as the best natural sources for the production of pectinase enzyme. The chief source of acidic pectinases is fungi but alkaline pectinases are produced from alkalophilic bacteria, primarily *Bacillus* spp. The alkaline pectinase has developed as important commercial enzymes with far-flung applications mainly in textile processing, bio-scouring of cotton fibers, degumming and retting of fiber crops, pretreatment of pectic wastewater etc. The main aim of the study was to isolate an efficient pectinolytic bacterium and its exploitation for augmented pectinase production using agro-wastes.

The major finding of the study can be summarized as follows:

- A total of thirty-six bacterial isolates were isolated from the collected samples and among this, four isolates (BKDS1-4) were selected based on their zone of clearance on YEP plates. These isolates were culturally, morphologically and biochemically identified.

- The results of pectinase production analysis by both qualitative and quantitative assay were very much correlating and revealed that, isolate BKDS1 is the most efficient strain as compared to other three isolates.

- The isolate BKDS1 has been chosen for further studies and was identified by 16S rRNA gene sequencing. Based on BLAST analysis and phylogenetic tree results, the strain was found to belong to *Bacillus subtilis* and deposited in Genbank with accession number KT004506.1.

- Among, various production media tested, YEP media showed higher enzyme activity and was used for further pectinase enzyme analysis.

- The production of extracellular enzymes such as amylase, cellulase, protease, lipase and tannase by the strain BKDS1 was detected by plate assay. Biosurfactant production by the strain was studied through various primary screening methods and displayed satisfactory result. The strain also showed significant positive results on tests such as growth in low pH, bile salt tolerance and susceptibility to major antibiotics to prove probiotic properties.

- Strain improvement using classical methods like UV and chemical mutagenesis was applied for augmenting the enzyme production by *B. subtilis* BKDS1. But none of the mutagenesis methods showed characteristic improvement in the pectinolytic activity.

- The pectinase production media was optimized by a combination of OFAT and RSM methods. The ideal concentration of the substrate pectin was determined as 0.25 %

using OFAT and it is kept as constant throughout the optimization. Three variables (yeast extract, $CaCl_2$ and inoculum size) that have significant effect on pectinase production was selected by PBD. Further, optimum levels of these variables were determined using CCD. The incubation temperature and incubation period were also optimized and found to be 40 °C and 48 h respectively.

- The optimized media showed many-fold increase in enzyme production compared to various other production media tested and the study validated the applicability of statistical media optimization for augmented enzyme production.

- A new methodology was adopted to prepare agro-waste extract media (AWEM) for submerged fermentation production of pectinase and found that enzyme production was achieved in all the tested AWEM to some extent. The order of AWEM for their efficiency in production was found to be PSEM (pineapple stem extract media) >WMREM (watermelon rind extract media) >BPEM (banana peduncle extract media) >PPEM (pineapple peel extract media). Media prepared with PSE and WMRE showed highest enzyme production at a concentration of 12.5 % and were selected for further optimization.

- The % yield of pectin extracted by using HCl from the PS extract was found to be 14.8 % for PS and 5.8 % for WMR. Even though, the yield of crude pectin is less in WMR, the amount of pectin obtained as calcium pectate is more in WMR

- RSM optimization was applied to enhance the enzyme production using the selected AWEM. In both case, the PBD selected four variables *viz*. yeast extract, $CaCl_2$, $CaCO_3$ and inoculum volume were found to be significant factors for pectinase production. The CCD was also applied to determine the optimum levels of the significant variable selected by PBD.

- The validation of the experimental model was tested by carrying out the experiments under optimized conditions established by the regression model. The pectinase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model.

- While comparing the pectinase activity achieved in PSEM with WMREM, PSEM showed a better result. So for further studies, the formulated PSEM is used. The efficiency of formulated PSE medium for large-scale SmF production of pectinase was confirmed in Laboratory fermenter (BioRacA) and revealed that enzyme production was achieved at a faster rate in the fermenter compared to shaker. The bacterium grown in the fermenter gave maximum activity (1437.723 U/ml) at 24 h and in shaker, the maximum reading (1510.391 U/ml) was at 42 h.

- The purification of pectinase was achieved through various purification processes such as ammonium sulphate precipitation, dialysis, and size exclusion chromatography. The

purified enzyme showed 5.43 fold increase in the specific activity as compared to the crude enzyme and thus indicated a good purification result.

- Even though 40 °C was considered as the optimum temperature for pectinase activity, the enzyme showed nearly similar activity in temperature ranges of 40-60 °C. In case of enzyme stability, even after 1 h incubation, the enzyme retained its original activity in 40 °C. Considering the optimum pH, the purified enzyme showed pH 8.0 as the optimum pH for its activity and is having stability in wide alkaline range (8-10). The pectinase activity was strongly stimulated by metal ion Ca^{2+} and slightly by Mg^{2+}. While, the enzyme activity was strongly inhibited by as Hg^{2+} and Fe^{2+} and Zn^{2+}. The K_m and V_{max} values of BKDS1 pectinase were estimated to be 0.2202 mg/ml and 1343.0 U/ml respectively.

- The purified pectinase showed significant positive result for various pectinolytic enzyme activity such as PG, PNL, PL and endo-pectinase activity.

- In poly acrylamide gel electrophoretic analysis to determine the mol. Mass and the zymogram analysis a protein band corresponding to 104kDa was obtained.

- The specific band of interest was subjected to PMF analysis by MALDI-TOF-MS and subsequent Mascot search indicated the presence of Ppase-N**,** which is actually PL.

- As the pectinase produced by BKDS1 have different pectinase activates, specific primers for various pectinase genes of *B. subtilis* were designed and tried to amplify the responsible genes. Primers designed for pectate lyase gene (*pel*) gave significant result.

- Further, the gene encoding pel was successfully cloned in *E.coli* DH5α using the vector pET-32a (+). On gene sequencing, a sequence of 1211 bp was obtained and on BLASTn analysis, the sequence showed 97 % similarity with *B. subtilis* strain RCK pectate lyase gene, complete cds. With the help of various bioinformatics tool, the amino acid sequence, secondary and tertiary structures of PL were predicted.

- The 3-D structure of the protein was modelled and denoted the conventional parallel β-helix structure. Acceptance of the modelled protein structure was validated by Ramachandran plot, Verfiy3D and ERRAT analysis and all the analysis displayed good results.

- The substrate specificity of the BKDS1 PL was determined by docking studies with various ligands and tetra-GalA showed least binding energy of -9.6 Kcal/mol, followed by di-GalA acid (-9.4 Kcal/mol) and tri-GalA (-8.4 Kcal/mol). d-GalA and 2-amino-2-deoxy-GalA presented highest binding energy of -5.4 Kcal/mol and -5.3 Kcal/mol respectively. It is observed

from the docking analysis that, the binding of the substrates were most probably emphasized by amino acid residues such as Thr146, Pro247 and Lys144.

Conclusion

Although microbial pectinases are widely used in food processing industries in other countries, it is still in incipient stage in developing countries like India mainly because of the high production costs involved. If economically viable technologies for production are available, it will promote the food processing industries in this country. Agro-wastes are being used as a substituting source for production of important compounds as they are valuable raw materials with rich sources of energy and other nutrients (lignocelluloses, proteins, carbohydrates, lipids etc.) which would be lost if they are discarded in the open dump yards and landfills. In this study we were successful in isolating an efficient native pectinolytic enzyme producers and the preeminent bacterial strain was identified and designated as *Bacillus subtilis* BKDS1. The approach of RSM media optimization found very effective in enhancing the pectinase production by the strain *B. subtilis* BKDS1. However, a more economical strategy is needed for the industrial level production of the enzyme. In this regard, different agro-wastes were tested with a new methodology for SmF production of pectinase and have found that, PS (Pineapple Stem) was very promising for formulating the media. Further RSM was fruitfully employed for augmented production and the optimized media was tested in Lab-scale fermenter. Thus, the study highlights an

economically feasible method for the laboratory as well as large scale commercial production of pectinase enzyme from agro-waste substrates such as pineapple stem. The study also ensued the management of agro-residues in a more reliable and strategic manner towards the continuance of a cleaner environment. Further, the cloning and *in-silico* studies boosted out new ideas to outline an efficient enzymatic hydrolysis process with higher yields and lower production cost. The overall findings may aid in designing and developing improved hydrolyzing enzymes with enhanced productivity.

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