Purification of pectin lyase from *Byssochlamys fulva* MTCC-505: its application in tea leaves and wine fermentation

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ABBREVIATIONS

PNL	Pectin lyase
PL	Pectate lyase
PG	Polygalacturonase
PME	Pectin methyl esterase
µg	Microgram
µl	Microlitre
°C	Degree centigrade
Fig.	Figure
et al.	et alia
%	Percent
E. C.	Enzyme Commission
g	Gram
h	Hour
U	Units
L	Litre
min	Minute
sp.	Species
ml	Millilitre
M	Molar
mM	Millimolar
mg	Milligram
pH	Hydrogen ion concentration
w/v	Weight per volume
N	Normal
BSA	Bovine serum albumin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
DEAE	Diethyl amino ethyl
rpm	Rotation per minute

TEMED	N, N, N, N Tetramethylethylenediamine
kDa	Kilo dalton
TF	Theaflavin
TR	Thearubigin
HPS	Highly Polymerized Substances
TLC	Total Liquor Colour

CONTENTS

Chapter No.	Title	Page No.
Chapter 1	INTRODUCTION	6-7
Chapter 2	REVIEW OF LITERATURE	8-21
Chapter 3	MATERIALS AND METHODS	22-35
Chapter 4	RESULTS AND DISCUSSION	36-45
Chapter 5	SUMMARY	46
Chapter 6	REFERENCES	47-55
Chapter 7	ANNEXURE	56-59

CHAPTER 1 INTRODUCTION

Pectinase is a general term of enzymes such as pectolyase, pectozyme and polygalacturonase, which hydrolyses the pectin, a polysaccharide substrate that is found in cell walls of plants. Pectin is a major component of the plant primary cell wall and its biological function is to cross-link cellulose and hemicellulose fibers, providing rigidity to cell wall (Zhao *et al*., 2008; Lagaert *et al*., 2009; Wolf *el al*., 2009). Pectinases are widely distributed in nature. They mainly occur in fungi (Ramanujam *et al*., 2008; Yadav *et al*., 2008; Pedrolli and Carmona, 2009), bacteria (Kapoor *et al*., 2000; Kashyap *et al*., 2001; Nadaroglu *et al*., 2010; Jayani *et al*.,2010), yeasts (Blanco *et al*., 1999). These enzymes are also produced by many insects, nematodes, protozoa and plants (Jayani *et al*., 2005). Pectinases are inducible extracellular enzymes secreted by microorganisms such as pectate lyase (EC 4.2.2.2), pectin lyase (EC 4.2.2.10), polygalacturonase (EC 3.2.1.15) and pectin methylesterase (EC 3.1.1.11) (Gummadi and Kumar 2005). Pectinases are one of the upcoming enzymes of the commercial sector. The peel of citrus fruits contains a large percentage of pectin which can be a good substrate for pectinolytic microorganisms. These microbes secrete large amount of extracellular enzymes to degrade the cell wall of substrates (Batool *et al*., 2013).

Pectin lyases degrade pectin polymer by β-elimination mechanism so as to form 4, 5 unsaturated oligogalacturonides. Pectate lyases (PELs), which cleave glycosidic bonds of pectate or low methylated pectin by a trans-eliminative mechanism to yield unsaturated products, are widely distributed among microorganisms (Barras *et al*., 1994; Herron *et al*., 2000).

Pectinases are synthesized by all microbial groups, but commercially moulds are the preferred ones since more than 90% of the enzyme can be extracted in the culture medium itself. The increasing energy demands have focused worldwide attention on the utilization of renewable resources, particularly agricultural and forest residues, the major components of which are cellulose, starch, lignin, xylan and pectin. These materials have attracted considerable attention as an alternative feed stock and energy source, since they are abundantly available. Several microbes are capable of using these substances as carbon and energy sources by producing a vast array of enzymes in different environmental niches (Antranikian, 1992; Kaur *et al*., 2004).

Pectinases have number of application in different industries such as textile industry, paper industry, poultry industry (Raghuwanshi *et al*., 2013). Pectinases are extensively used in the feed and drink industries (Gupta and Singh, 2004). These enzymes included in the

clarification of fruit juices, grape musts, maceration of vegetables or fruit and the reduction of cloudiness in soft drinks (Fogarty and Kelly, 1982). Enzymatic degradation of fruit pectin can clarify juices and improve juice yields (Ishii and Yokotsuka, 1975). With the addition of pectinases the viscosity of fruit juice drops, the jelly structure disintegrates and the clear fruit juice is obtained (Alkorta *et al.,* 1998; Kashyap *et al.*, 2001).

Tea is natural beverage brewed from the leaves of evergreen plant called *Camellia sinensis*. It has a large number of health benefits and its pharmaceutical and industrial applications are also in development (Wang *et al*., 2008). In Himachal Pradesh tea gardens are present in Kangra valley. Application of external enzymes that degrade the cells of tea leaves result in complete maceration of cells of tea leaves hence better fermentation and better tea quality (Samaraweera, 1989). The manufacturing of high quality black tea is very important to increase the profit and maintain the economic feasibility of our state. This research work can assist the tea industry in improving the quality of tea which can be determined on the basis of its flavor and appearance. Besides that, they also have other application including curing of coffee and cocoa beans, refinement of vegetable fibers and manufacturing of pectin-free starch.

Wine production and consumption is quite popular all over the world and also one of the ancient practices. Although grapes are the main raw material used for the wine production, there is an increasing interest in the search of other fruits, such as apricot, apple, plum, strawberry (Joshi and Attri, 2005), kiwi (Vaidya *et al.*, 2009) and palm sap, suitable for wine making. Wine is one of the most recognizable high value-added products from fruits. It can also be used as a substrate for the manufacture of vinegar, a by-product of wine manufacture.

In fact, in the textile industry, pectinases are sometimes used for improving the scouring process as part of the pretreatment of cotton fabric (Arne *et al.*, 2004).

In the present study, pectin lyase producing organism *Byssochlamys fulva* was procured from MTCC, IMTECH, Chandigarh. Keeping in view the immense potential and the extensive applications of microbial pectinases, the present study was undertaken with following objectives:

a) Purification of pectin lyase produced from *Byssochlamys fulva* using Ion-exchange & Gel-permeation chromatography.
b) Biochemical characterization of the enzyme including molecular weight determination.
c) Use of purified enzyme in tea leaves and wine fermentation.

CHAPTER 2 REVIEW OF LITERATURE

Pectin

Pectic substance is the generic name used for the compounds that are acted upon by the pectinolytic enzymes. They are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate (Jayani *et al*., 2005).

Pectin is a complex structure that has pectic acids as main component. When interlined with other structural polysaccharides and proteins, these pectic acids form an insoluble compound known as protopectin. It is also described in other structure namely xylogalacturonan (XGA) (Round *et al.,* 2010). In an unripe fruit, pectin is bound to cellulose micro fibrils in the cell wall. Such pectin is insoluble and hence confers rigidity on cell walls. However, during ripening the structure of pectin is altered by naturally occurring enzymes in the fruits. These alterations involve the breakdown of the pectin chain or of side chains attached to the units, which make up the main chain. In either case, the result is that the pectin becomes more soluble and its grip on the surrounding cell walls is loosened and the plant tissue softens.

The pectic substances account for about 0.5±4% of the weight of fresh material. When the tissue is ground, the pectin is found in the liquid phase (soluble pectin) causing an increase in viscosity and the pulp particles, whereas other pectin molecules remain bound to cellulose fibrils by means of side chains of hemicellulose and thus facilitate water retention (Piteri *et al*., 1989). Pectin is rich in galaturonic acids (GalA) and three polysaccharide distinct domains are evident: homogalacturonan (HGA), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) as shown in Figure 1 (Willats *et al*., 2006).

Sources of pectin

Pectin is abundantly present in citrus fruits such as oranges, lemons and grapefruits. Fruits like apples, guavas, quince, plums and gooseberries contain high levels of pectin, while soft fruits like cherries, grapes and strawberries contain lower amounts (Srivastava and Malviya, 2011). Pectic substances occur in the middle lamella and the primary cell wall of higher plants and prominent in parenchymatous tissue. These are abundantly present in apple, lemon, orange, mango, tomato, beet, and carrots (Pilnik and Voragen, 1993; Lal *et al*, 1998) as shown in Table 1.

STRUCTURE OF PECTIN

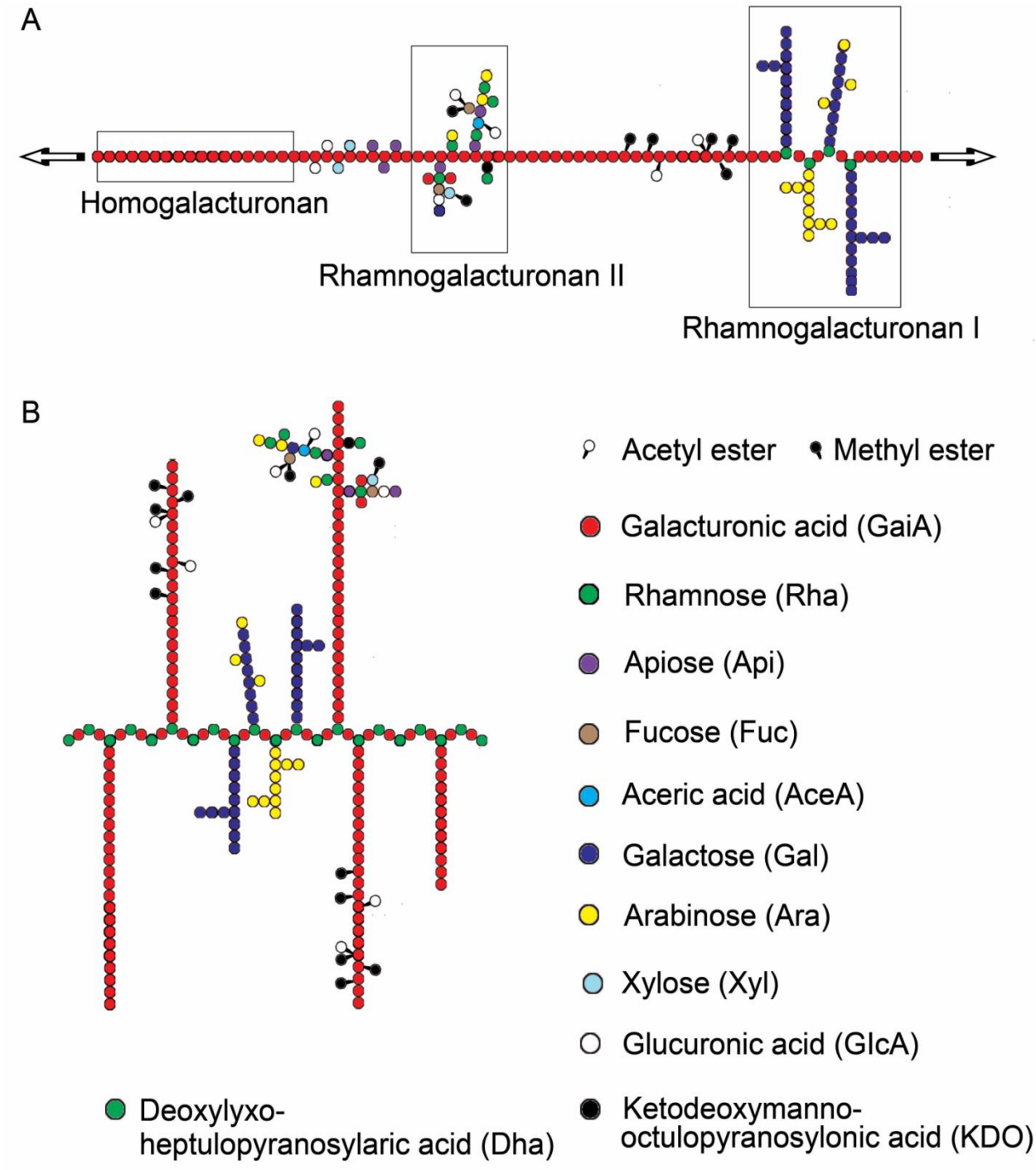


Fig. 1 The basic structure of pectin. Schematic representation of the conventional (A) and recently proposed alternative (B) structure of pectin (Willats *et al.*, 2006)

Table 1 Sources of pectin (Pilnik and Voragen, 1993; Lal *et al*, 1998).

Material	% pectin in fresh material	% pectin in dry weight basis
Apple Pomace	1.5 - 2.5	15-18
Lemon Pulp	2.5 - 4.0	30-35
Orange Pulp	3.5 – 5.5	30-40
Beet Pulp	1.0	25-30

Pectin yield, methoxylated pectin and degree of esterification (DE) in different sources of pectin as observed by Rao and Maini (1999) is given below in Table 2.

Table 2 Methoxylated (Meo) pectin and degree of esterification (DE) in different sources of pectin (Rao and Maini, 1999).

Source	Yield	Meo (%)	DE (%)
Apple pomace	17.0	8.9	74.9
Lime peels	32.0	8.6	63.2
Lemon peels	27.7	9.2	73.4
Sweet orange peels	17.8	7.7	57.0
Mandarin orange	18.4	9.5	64.0

Pectinases

Enzymes hydrolysing pectic substances are called as pectinolytic enzymes or pectinases (Saad *et al.*, 2007). Food processing enzymes including pectinases account for 45% of enzyme usage. Pectinases are phytopathogenic substances (Yadav *et al*., 2005). Microbially derived pectinases find more use due to their advantage over plant and animal derived pectinases. The reasons being cheap production, easier gene manipulations, faster product recovery, further microbial enzymes are usually free of harmful substances. Pectinases based on the mechanism used to attack the galacturonan backbone are classified as polysacchride hydrolases, polysaccharidelysase and carbohydrate esterases. These include endo-polygalacturonases (EC 3.2.1.1.5), exo-polygalacturonases (EC 3.2.1.67), pectate lyases (EC 4.2.2.2), pectin lyases (EC 4.2.2.10) and pectin methyl esterases (EC 3.1.11). Endo-polygalacturonases catalyses random hydrolysis of α-1, 4 glycosidic linkage between twonon methylated acid residues. Endo-polygalacturonases are produced by bacteria, fungi, yeast and come under glycosyl hydrolase family (Carpita and Gibeaut, 1993). Exo-polygalacturonases are glycoproteinas and degrade in a terminal fashion. They are of two types: exo-polygalacturonases EC (3.2.1.67) and exo-polygalacturonases EC (3.2.1.82), which cleave α-

1,4 glycosidic bonds of GAlA residues (Saleh *et al*., 2006). Pectinlyases degrade pectin polymer by β-elimination mechanism so as to form 4,5 unsaturated oligogalacturonides (Yadav *et al*., 2005). Pectin methyl esterases catalyse the esterification of pectin which is methylated polygalacturonic acid resulting in the formation of de esterified pectin which releases hydrogen ions and methanol (Laratta *et al*., 2008). Pectin lyases catalyze the trans-eliminative cleavage of the galacturonic acid polymer (Singh *et al*., 2005). Pectin methyl esterase is found in peel and core of tissue prints, cellular subdivision of juice vesicles and in stigma cells. Orange and lime has highest level of pectin methyl esterase but lowest levels in lemon. Pectin methyl esterase has isoforms PME I, PME II, PME III as a result of their elution order in a heparin sepharose column (John *et al.*, 2002).

Pectinases are naturally produced by many organisms, including bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. A quarter of the global food enzymes sale is met with microbial pectinases (Jayani *et al*., 2005). Although fungal pectinases are being industrially used, pectinase production from actinomycetes has also been reported earlier (Beg *et al*., 2000; Kar and Ray, 2011). Actinomycetes belong to a distinct class of gram-positive bacteria which are of great biotechnological significance due totheir ability to synthesize chemically diverse, commercially important bioactive compounds like antibiotics, enzymes, pigments etc (Dharmaraj, 2010). They constitute numerous unique species which are capable of growing in extreme, hostile and polluted environments (Ballav *et al*., 2012). Among the actinobacterial population, *Streptomyces* species have cosmopolitan distribution due to dispersion of their spores (Antony *et al*., 2008). About 80% of the metabolites produced by actinomycetes originate from *Streptomyces* sp. (Watve *et al*., 2001). Pectinases from streptomycetes have good thermal stability (Jacob and Prema, 2008) and activity over a broad range of pH (Kuhad *et al*., 2004).

Classification

The group of enzymes which are involved in the degradation of ‘‘smooth region’’ (homogalacturonan) include deesterifying enzymes i.e. pectin methyl esterases (PME, E.C. 3.1.1.11) and pectin acetyl esterase (PAE, E.C. 3.1.1.6). Pectinases, the pectolytic enzymes include one esterase, six polygalacturonases and four Lyases, which removes methoxyl and acetyl residues of pectin producing polygalacturonic acid (Whittaker, 1991). The other subclass of homogalacturonan degrading group are broadly termed as depolymerases which break the α-1, 4-linkages either by hydrolysis i.e. polygalacturonases (PG, E.C. 3.2.1.15) or via transelimination mechanism namely pectate lyases (PL, E.C.4.2.2.2) and pectin lyases (PNL, E.C. 4.2.2.10). The main role of these enzymes (pectinases) is basically to degrade pectin.

However, there are other accessory enzymes involved in the degradation of side chains of pectins which include α-arabinofuranosidase (E.C. 3.2.1.55), β-galactosidase (E.C. 3.2.1.23), endogalactanase (E.C. 3.2.1.89) and feruloyl and p-coumaroyl esterase. An extensive classification of pectinolytic enzymes is given in Table 3 (Jayani *et al*., 2005).

Table 3 Classification of pectinases (Jayani *et al*., 2005)

Enzyme	E.C. No.	Action Mechanism	Substrate	Product
Esterase				
1) PME	3.1.1.11	Hydrolysis	Pectin	Pectic acid+methanol
Depolymerizing enzymes				
A) Hydrolase				
1.Protopectinases	3.2.1.15	Hydrolysis	Protopectin	Pectin
2.endopolygalacturonase	3.2.1.67	Hydrolysis	Pectic acid	Oligogalacturonates
3.exopolygalacturonase	3.2.1.82	Hydrolysis	Pectic acid	Monogalacturonates
4.exopolygalacturonan-digalacturonohydrolase		Hydrolysis	Pectic acid	Digalacturonates
5.oligogalacturonate hydrolase		Hydrolysis	Trigalacturonate	Monogalacturonates
6.endopolymethylgalacturonases		Hydrolysis	Highly esterified pectin	Oligomethylgalacturonates
7.endopolymethylgalacturonase			Highly esterified pectin	Oligogalacturonates
B) lyases				
1.endopolygalacturonase lyase	4.2.2.2	Transeliminat on		Unsaturated oligogalacturonates
2.exopolygalacturonates lyase	4.2.2.9		Pectic acid	Unsaturated digalacturonates
3.oligo-D-galactosiduronate lyase	4.2.2.6	Transeliminat on	Pectic acid	Unsaturated monogalacturonate
4.endopolymethyl-D-galactosiduronate lyase	4.2.2.10	Transeliminat on	Unsaturated digalacturonate	Unsaturated methyl oligogalacturonates
		Transeliminat on	Unsaturated poly-(methyl-D-digalacturonate)	

Pectin lyase

Commercial pectinase is a mixture composed of three different enzymatic activities:- polygalacturonase, pectin esterase and pectin lyase (E.C. 4.2.2.10), polymethylgalacturonate lyase) (Sunnotel and Nigam, 2002). Commercial preparations with pectin lyase are preferable as the major component in juices and wine processing because it avoids the production of methanol, the precipitation of pectin partially de-esterified with endogenous calcium, and the damage of volatile ester content responsible for the specific aroma of various fruits (Taragano

and Pilosof, 1999). Furthermore, pectin lyase is the only enzyme which is able to cleave the 1, 4 glycosidic bond of highly esterified pectins such as fruit pectin without the prior action of other enzymes (Sharma, 1987; Cao *et al.,* 1992).

Sources of pectin lyase

Most of pectin lyases studied so far have been reported from microorganisms but there are scanty reports of their presence in plants and animals also. Pectin lyase is mainly produced by fungal genera but there are a few reports on bacterial and yeast pectin lyase, given as in Table 4 (Whittaker, 1991).

Table 4 Sources of pectin lyase

Name of the organism(s)	Reference(s)
Aspergillus niger	Ishii and Yokotsuka, 1975
Erwinia aroideae	Kamimiya *et al*., 1974
A. niger	Taragano *et al*., 1999
A. japonicas	Ishii and Yokotsuka, 1975; Dinnella *et al.*, 1995
Alternaria mali	Hasui *et al*., 1976
Penicillium paxilli	Szajer and Szajer, 1982
A. oryzae	Lim *et al*., 1983
Colletotrichum	Wijesundera *et al*., 1984
Pseudomonas marginalis	Sone *et al*., 1988
Rhizoctonia solani	Bugbee, 1990
Rhizopus oryzae	Hamdy *et al*., 2005
A. flavus	Yadav *et al*., 2009
A. ficuum	Yadav *et al*., 2009
P. italicum	Alana *et al*., 1991
Pseudomonas fluorescens	Schlemmer *et al*., 1987

Mechanism of action

Pectin lyases degrade pectin polymers directly by β-elimination mechanism that results in the formation of 4, 5-unsaturated oligogalacturonides shown in Figure 2. While other pectinases act sequentially to degrade pectin molecule completely. There are three ways by which enzymic degradation of α-1, 4 linkages can occur in pectin these are:-

- Combined action of pectin esterase and polygalacturonases.

- Hydrolytic polymethyl galacturonase action.
- Lyases or pectin transeliminase action.

Demethylation action progressively slows down because it cannot cleave on either side of demethylated residue, if the residue at the other end of cis-bond is methylated. Pectinases are a complex heterogeneous group of different enzymes that act specifically on pectic substances. Pectinases act on and decrease the intracellular adhesivity and tissue rigidity (Tatiana *et al.*, 2005).

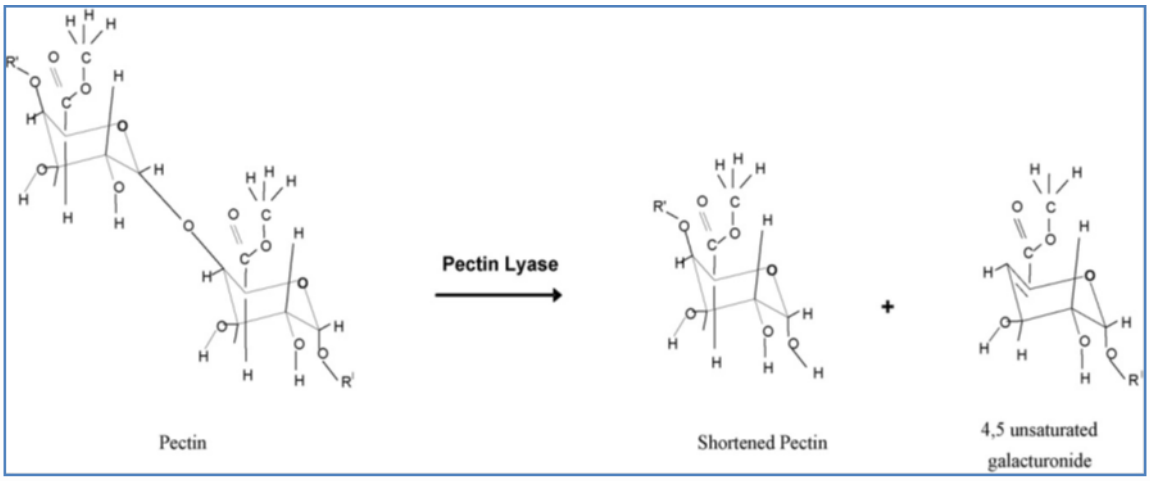


Fig. 2 Mechanism of action of pectin lyase

Occurrence of pectic enzymes:

Pectinases are produced by microorganisms as well as plants, which produce PE, PG, PAL and PL as well as shown in Table 5 and Table 6 (Rombouts and Pilnik, 1986).

Pectin lyases (PNLs) also known as pectin transeliminase is involved in the degradation of pectic substances (Gummadi and Kumar, 2005). The enzyme has a virulent factor, which

degrade the pectic components of plant cell wall (Mayans *et al*., 1997). Pectin lyase does not produce methanol which is toxic, on the other hand, PGs and PEs act together to degrade the pectin molecule completely and liberate methanol as a byproduct of PE action (Taragano and Pilosof, 1999).

Therefore, the use of PNLs as a major component of commercial preparations is preferred in fruit juices and wine industries as it decreases the viscosity without damaging the volatile ester content responsible for specific aroma of various fruits (Alana *et al*., 1990). Pectin lyases plays a role in tissue maceration by plant pathogenic bacteria has been demonstrated using mutants that lack pectate lyase activity (Beaulieu *et al*., 1993). Recently, several reports have been suggested that PNL may be a pathogenicity factor in fungal plant interactions (Wijesundera *et al*., 1984; Crawford and Kolatlukudy, 1987; Wattad *et al*., 1994; Wattad *et al*., 1995). Microorganisms have been reported to produce pectin lyase during plant and fruit infection (Schink *et al*., 1981; Sakiyama *et al*., 2001). It has been reported that PNL is mostly produced by fungi while pectate lyase exclusively produced by pathogenic fungi (Gummadi and Kumar, 2006). The major source of PNL on industrial scale is fungi belonging to genera *Aspergillus, Penicillium and Fusarium* although few bacterial PNL are also reported (Sathyanarayana *et al*., 2003).

Table 5 Occurrence of pectinolytic enzymes in microorganism (Rombouts and Pilnik, 1986)

Organism	**PE**	**PG**		**PAL**		**PL**
		Endo	**Exo**	**Endo**	**Exo**	

Aspergillus	++	++	++	+	-	+
Penicillium	+	+	-	-	-	+
Fusarium	+	+	-	-	-	-
Rhizopus	+	++	-	-	-	-
Sclerotina	+	+	-	-	-	+
Kluyveromyces	++	+	-	-	-	-
Bacillus	-	-	-	+++	-	-
Clostridium	+	+	-	-	+	-
Erwinia	-	-	+	+++	+	+
Pseudomonas	-	-	-	+++	-	-
Arthrobacter	-	-	-	-	-	-

Table 6 The presence of PE and PG in higher plants (Rombouts and Pilnik, 1986)

Plants	**PE**	**PG**	
		ENDO	**EXO**
Apple	+	_	+
Apricot	+	_	_
Banana	+	+	_
Berries	+	_	_
Lime	+	_	_
Mandarin	+	_	_

Orange	+	_	_
Grapes	_	_	_
Mango	+	+	_
Peach	_	+	+
Pear	+	_	+
Plums	+	_	_
Carrots	+	_	_
Leek	+	_	+
Pea	+	_	+
Potato	+	_	+

Applications

Over the years, pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, oil extraction, treatment of industrial waste containing pectinacious material etc. They have also been reported to work on purification of viruses (Salazar and Jayasinghe, 1999) and in paper making (Viikari *et al*., 2001; Reid and Richard, 2004).

Fruit juice manufacturing and clarification

Pectinases are endowed with potent biotechnological applications in fruit juice manufacturing industry. Using byproducts for pectinase extraction is still more profitable (Aravantinos *et al*., 1994). During extraction pectin passes into juice and makes the juice cloudy. Cloudiness in pulp can be removed by enzymatic hydrolysis. Practically different steps notably; washing, sorting and crushing followed by pressing and maceration are utilized in fruit juice processing for production of fruit juices (Kashyap *et al*., 2001). Literature emphasizes that application of mixture of commercial cellulases and pectinases solubilises almost 90% of orange peel solids. Pectinases find their way in extraction and clarification processes. Pectinases are the one among the group of macerating enzymes used in extraction, clarification and stabilization phases in the industry. They are also applicable in increasing the volume of the juice and to soften the peels for their easy removal (Bhatt, 2000).India is

the major citrus fruits producer particularly in states of Maharashtra, Tamilnadu, Andhra Pradesh, Himachal Pradesh, Punjab and Haryana. In citrus juice processing pectic enzymes contribute to remove the cloudiness and for juice stabilization (Roberts, 1981).

Paper and pulp industry

During papermaking, pectinase can depolymerize and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Viikari *et al*., 2001; Reid and Richard, 2004).

Animal feed

Pectinases are used in the enzyme cocktail, used for the production of animal feed. This reduces the viscosity, which increases absorption of nutrients, liberates nutrients either by hydrolysis of non-biodegradable fiber or by liberating nutrients blocked by these fibers and reduces the amount of faeces (Hoondal *et al*., 2002).

Oil extraction

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

Purification of plant viruses

In cases where plant virus particle is restricted to phloem, alkaline pectinases and cellulase can be used to liberate the virus from the tissue to give very pure preparations of the virus.

Waste water treatment

Vegetable food processing industries release pectin containing waste waters as by product, pre-treatment of this waste water with pectinolytic enzymes facilitates removal of pectinaceous material and renders it suitable for decomposition by activated sludge treatment (Hoondal *et al*., 2002).

Degumming of plant bast fibers

Bast fibers are the soft fibers formed in groups outside the xylem, phloem or pericycle e.g. ramie and sunn hemp. The fibers contain gum, which must be removed before its use in textile making (Hoondal *et al.,* 2002). The chemical degumming treatment is polluting, toxic and nonbiodegradable. Biotechnological degumming using pectinases in combination with xylanases present is ecofriendly and economic alternative to the above problem (Kapoor *et al*., 2002).

Retting of plant fibers

Pectinases have been used in retting of flax to separate the fibers and eliminate pectins

(Hoondal *et al*., 2002). Flex fibers develop from the cells located within the phloem of the flax stem (Sumere, 1992). During maturation, the fiber cells differentiate from the phloem parenchyma by synthesizing successive layers of cellulose and pectins. With enzyme-retting, flax stems are incubated in the presence of specific plant cell wall-degrading enzymes. Ideally enzyme selection and treatment would result in hydrolysis of flax pectins and hemicelluloses without damage to the cellulose fibers. In addition, by treating with specific enzyme mixture, retted fibers would result (Sumere, 1992).

Textile processing and bioscouring of cotton fibers

Pectinases have been used in conjuction with amylases, lipases, cellulase and hemicellulase to remove the sizing agents from cotton in a safe and eco-friendly manner replacing toxic caustic soda used for the purpose earlier (Hoondal *et al*., 2002). Bioscouring is a novel process for removal of non-cellulosic impurities from the fiber with specific enzymes. Pectinases have been used for this purpose without any negative side effect on cellulose degradation (Guebitz *et al*., 2006). Bioscouring of fabrics with pectinase resulted in enhancement of various physical properties of fabrics viz whiteness, tensile strength and tearness over conventional alkaline scoured fabrics (Ahlawat *et al*., 2009).

Tea fermentation

The role of enzymes in tea processing started more than ten years ago (Sanderson and Cuggon, 1977; Jain and Takeo, 1984), its application is still at a nascent stage. Conversion of fresh shoots into consumable tea is based on the action of enzymes present in the raw material (Sanderson, 1972). Tea is classified based on the "processing methods" of which the "degrees of fermentation" are among one of the most important characteristics. According to the various levels of fermentation, teas can be divided into three major categories: unfermented green teas, semi-fermented oolong teas and fully fermented black teas (Wang *et al*., 2008). The essential difference in the processing is that the black tea is allowed to ferment before firing, while green tea is rapidly dried. During fermentation process, the tea catechins are oxidized to orthoquinones which condense to form Theaflavin (TF).These TF acts as oxidizing agents for the substances like gallic acid and condense with TF to produce the polymeric Thearubigin (TR) (Roberts, 1962). These TR are responsible for colour, body and taste while TF content in tea determines the briskness, brightness and quality of the liquor (Hilton and Ellis, 1972). Among the various components estimated and evaluated for the quality of black tea, only TF content was reported to play a major role in the tea quality (Hilton and Ellis, 1972; Cloughley, 1980; Owuor, 1982; Owuor *et al*., 1986). Catechins, TR and Caffeine have been treated for inhibiting the cancer formation in animal models. The TF

in black tea are found to inhibit lung and oesophaeal carcinogenesis (Morse, 1997). Hence any increase in tea phenolic compounds could improve the quality and therapeutic value of tea. During fermentation, the colour change associated with the development of characteristic aroma takes place.

The rate of fermentation will depend upon the degree of contact between enzymes and substrate since they are not in homogenous system (Angayarkanni *et al*., 2002). It has been reported that endotypes of pectinases both hydrolyse and lyase, are the major factors responsible for plant tissue maceration (Tanabe and Kobayashi, 1986; Bhattacharya *et al*. 2007) studied that aeration during the early stage of fermentation favors the formation of TF route which is responsible for quality attributes of black tea, viz., color and strength of liquor. The original purpose of tea fermentation is to enhance the flavor of tea. Flavor, described as taste aroma, is the most important element for tea evaluation (Wang *et al*., 2004). For the quantification of TF and TR, the spectrophotomeric method is still practically the major method of analysis (Angayarkanni *et al.,* 2002; Yaho *et al.,* 2006). In another report the pectinase isolated from *Aspegillus* spp., *Aspergillus indicus*, *Aspergillus flavus* and *Aspergillus niveus* were used for fermentation of tea leaves. The effect of both crude enzyme preparation and purified pectinases enzymes on the improvement of tea leaf fermentation was determined in terms of TF, TR, Highly Polymerzied Substances (HPS), Total Liquor Color (TLC), dry matter content and total soluble solids of tea produced (Angayarkanni *et al.,* 2002).

Improvement of chromaticity and stability of wines

Wine makers often supplement naturally occurring fruit enzymes with commercial enzymes to increase production capacity of clear and stable wines with enhanced body, flavor and bouquet (Ugliano, 2008). The most widely used enzymes available for commercial use are: pectinases, hemicellulases, glucanases and glycosidase. The latter three types are generally sold as blends with pectinases. With the exception of glucanase all the enzymes are produced by *Aspergillus niger*, whereas glucanase is produced by *Trichoderma harzianium.*

Pectinolytic enzymes added to macerate fruits before the addition of wine yeast in the process of producing red wine resulted in improved visual characteristics (color and turbidity) as compared to the untreated wines. Enzymatically treated red wines presented chromatic characteristics, which are considered better than the control wines. These wines also showed greater stability as compared to the control.

Pectinase enzymes break the pectin molecules into smaller components thereby exposing

some of the positively charged particles underneath the protective layer. After crushing, negatively charged pectin molecules form a protective layer around positively charged particles. This keeps the particles in suspension. These positive charges bind to the negative charges of the pectin. When particles become too big, they settle out. Settling enzymes are the most basic commercial enzymes with regards to their composition and mode of action. These are pectin lyase (PL), pectin methyl esterase (PME) and polygalacturonase (PG). Settling enzymes work mainly on the soluble pectins (mainly homogalacturonans) of the pulp of fruit.

In red winemaking, pectinase preparations, often in combination with cellulose and hemicellulase, are often used to increase the degradation of skin cell walls, in order to obtain increased pressing yield and improved extraction of colour and aroma precursors during maturation (Ugliano, 2008). Pectinases can also be used to prevent filter clogging prior to bottling.

CHAPTER 3 MATERIALS AND METHODS

MATERIALS

Collection of sample

The pectin lyase producing fungus *Byssochlamys fulva* was procured from MTCC, IMTECH, Chandigarh. The apple pulp used for this study was procured from Department of Horticulture, Navbahar, Shimla. Tea leaves were plucked from *Camellia sinensis* bushes from Palampur tea gardens

Chemicals

All the chemicals used in the present investigation were either procured from Sigma Aldrich (U.S.A.) or HIMEDIA (Mumbai, India) and were of high quality analytical grade. Polygalacturonic acid from Citrus Fruit (Sigma) was used as substrate.

METHODS

Maintenance of *Byssochlamys fulva*

The fungal culture was stored and maintained on potato dextrose agar (PDA) slants. The slants were prepared by suspending 41.0g of PDA/1000 ml of distilled water. The medium was heated to boil so that it dissolved completely. The medium was autoclaved, poured in sterile tubes and allowed to cool in slanting position. The fungal culture was streaked on PDA slants and incubated at 30˚C for 3-4 days. Subculturing was done after every 30 days.

Growth medium

Growth medium for *Byssochlamys fulva* was potato dextrose agar medium. The growth medium used had following composition.

Components	(w/v)%
Potato	20
Dextrose	2.0
Agar	2.0
pH	4.0

Production medium

Production medium used for *Byssochlamys fulva* was inoculated by taking three loops full of slant culture in 1 ml of sterile normal saline and from this 100 µl was taken to inoculate 50 ml of production medium and incubated for 48 hrs at 30˚C. After 48 hr of incubation the production medium was harvested by filtration with Whattman's filter paper. Production medium had following composition:

Components	(w/v) %
KCl	0.05
$MgSO_4$. $7H_2O$	0.1
Casein Enzyme Hydrolysate	0.1
Yeast extract	0.1
Na_2HPO_4	0.1
KH_2PO_4	0.1
Pectin	0.5

The pH of the medium was adjusted to 4.0 using HCl prior to sterilization (15 psi, 20 min).

Enzyme Assay (Dygert *et al.*, 1965; Sozgen *et al.*, 2006).

Reagents

- 1 M Citric acid/ Phosphate Buffer (Assay Buffer), pH 4.0
- Colour Reagent A: Dissolved 40 g anhydrous Na_2CO_3 in 600 ml reagent grade water. Added 16 g glycine and stirred untill dissolved. Then added 0.45 g $CuSO_4$ in 100 ml distilled water and again stirred untill dissolved. Made the volume to 1 litre with reagent grade water.
- Color Reagent B: Dissolved 1.2 g Neocuproine HCl in 1 litre reagent grade water.
- D-galacturonic acid standard, 1 mg/ml.
- 0.5% Polygalacturonic acid (Substrate): Heated 500 ml Assay Buffer on a hot plate. While heating and stirring, slowly added 2.5 g polygalacturonic acid. Heated again and stirred untill dissolved. It was slightly viscous and opaque.

Assay procedure

- Prepared three test tubes, each containing 6 ml of substrate and 1 ml of diluted enzyme (pectin lyase).
- Incubated at 25°C for 60 minutes.
- Kept the tubes in ice water to stop reaction for 5 minutes.
- Aliquot from each test tube (100 µl) was put in new washed and dried test tube (also on ice).
- To each reaction tube, added 2 ml of colour reagent A and 2 ml of color reagent B.
- Mixed well by inversion.
- Incubated in boiling water bath for 13 min.
- Cooled the test tubes and added 2 ml of distilled water.

- Mixed well by inversion.
- Read the absorbance at 450 nm against a blank.
- Calculated mean absorbance for each set of triplicate.
- Calculated activity from standard curve of D-galacturonic acid.

Unit of enzyme activity

One unit of enzyme activity is defined as the amount of enzyme required to release one micromole of galacturonic acid per ml per minute under standard assay conditions.

Protein Estimation (Lowry *et al.*, 1951)

Reagents

- Alkaline Copper reagent

Components

a) Copper sulphate 1%
b) Sodium Potassium Tartarate 2%
c) Sodium carbonate in 0.1 N NaOH 2%

Prepared by mixing 1 ml (a) 1 ml (b) and 98 ml (c). The solution was always made fresh when required.

- Folin Ciocalteau's reagent: Diluted 1:1 with distilled water just before use.
- Bovine Serum Albumin standard solution by taking 1 mg/ml stock.

Procedure

- The samples were appropriately diluted with water to 1 ml and a blank was taken which had no enzyme.
- 3 ml of alkaline reagent was added and left at room temperature for 15 minutes.
- 0.3 ml of Folin Ciocalteau's reagent was added and the tubes incubated at 37˚C for 30 minutes. The absorbance was measured at 660 nm.
- The protein concentration was calculated from the BSA standard graph.

Purification of pectin lyase

Precipitation of pectin lyase

The crude enzyme was precipitated with 70% of ammonium sulphate. Enzyme and

ammonium sulphate mixture was allowed to precipitate over night at 4°C and next day it was centrifuged at 10,000 rpm for 20 minutes in a single run and precipitate were separated and the supernatant was centrifuged again. The precipitates were reconstituted in minimum amount of 0.05 M sodium-citrate buffer (pH 4.0).

Ion exchange chromatography DEAE-Cellulose chromatography

- Already swollen matrix of DEAE-Cellulose was washed and suspended in boiled distilled water and allowed to soak for 2-3 hrs. A glass column of size (20 x 1.6 cm) was packed properly.
- The column was washed and equilibrated with 0.1 M NaOH and then by 0.1 M NaCl.
- Then washed with 40 ml of 50 mM sodium-citrate buffer (pH 4.0).
- The precipitated enzyme (5 ml) was loaded in this column.
- Initial 10 fractions were collected with 50 mM sodium-citrate buffer (pH 4.0).
- 50 fractions (2 ml each) were collected with different molarity of NaCl.
- Fractions having high specific activity were pooled.

Gel permeation chromatography

Sephacryl S-100 chromatography

The gel permeation chromatography was performed by Sephacryl S-100 matrix packed in a column of 22×1.25 cm. Sephacryl is a cross linked copolymer of allyl dextran and N, N-methylene bis-acrylamide. The cross linking gives matrix good rigidity and chemical stability. It was performed as follows:

- The pre-swollen matrix of Sephacryl S-100 was packed in a column after making its slurry and poured in the column with help of a glass rod. The matrix was allowed to settle overnight and next day it was equilibrated.
- Void volume of the column was determined with the help of dextran blue. 1% Dextran blue (2.0 ml) was added over the column. 15 fractions (3.0 ml each) were collected at the flow rate of 1 ml/min. Absorbance of each fraction was taken at 660 nm.
- After ensuring an appropriate packing, equilibration of column was done by passing filtered distilled water through the column at a flow rate of 0.5 ml/min. After that 50 mM sodium citrate buffer were made to pass through the column for washing.

- The pooled sample from DEAE-Cellulose column was loaded onto the column.
- 50 fractions, each of 2.0 ml volume at a flow rate of 0.5 ml/min were collected.
- The absorbance of fractions was measured at 280nm (UV 1700 Plasma Spec UV Vis Spectrophotometer Shimadzu).

Polyacrylamide gel electrophoresis (Laemmli, 1970**)**

Reagents

- Acrylamide (Electrophoresis grade)
- Bis Acrylamide (N, N-methylenebisacrylamide)
- Tris (2-hydroxymethyl-2-methyl-1, 3-propane diol)
- SDS (Sodium dodecyl sulphate or Sodium lauryl sulphate)
- TEMED (N, N, N, N-tetramethleneethylenediamine)
- Ammonium persulphate
- 2-mercaptoethanol
- Glycerol
- Bromophenol blue
- Glycine
- HCl

Stock solutions

1) 2 M Tris-HCl (pH 8.8), 100 ml

- weighed out 24.2 g Tris
- added to 50 ml distilled water
- added concentrated HCl slowly to pH 8.8 (about 4 ml)
- allowed solution to cool to room temperature
- added distilled water and made the total volume of 100 ml

2) 1 M Tris-HCl (pH 6.8), 100 ml

- weighed out 12.1 g Tris

- added to 50 ml distilled water
- added concentrated HCl slowly to adjust pH 6.8 (about 8 ml)
- allowed solution to cool to room temperature
- added distilled water and made the total volume of 100 ml

3) 10% (w/v) SDS, 100 ml, stored at room temperature

- weighed out 10 g SDS
- added distilled water to a total volume of 100 ml

4) 50% (v/v) glycerol, 100 ml

- poured 50 ml 100% glycerol
- added 50 ml distilled water

5) 1% (w/v) bromophenol blue, 10 ml

- Weighed out 100 mg bromophenol blue.
- Brought to 10 ml distilled water and stirred until dissolved. Filtration would remove aggregated dye.

Working solutions

- **Solution A**: (Acrylamide stock solution), 100 ml

30% (w/v) Acrylamide, 0.8% (w/v) bis-acrylamide

A. 29.2 g acrylamide

B. 0.8 g bis-acrylamide

Added distilled water to make 100 ml and stirred until completely dissolved. Stored in the refrigerator.

- **Solution B**: (4X Separating Gel buffer), 100 ml

a.) 75 ml 2M Tris-HCl (pH 8.8) 1.5 M

b.) 4 ml 10% SDS 0.4%

c.) 21 ml H_2O

Stored in the refrigerator.

- **10% Ammonium persulfate**

a. 0.5 g ammonium persulfate

b. 5 ml H_2O

Stored in the refrigerator.

- **Electrophoresis buffer** (1 litre)

3 g Tris	25 mM
14.4g Glycine	192 mM
1 g SDS	0.1%

Added H_2O to make 1 litre, pH 8.0. Stored at room temperature.

- **5X SDS Sample Loading Buffer, 10 ml**

0.6 ml 1M Tris-HCl (pH 6.8)	60 mM
5 ml 50% glycerol	25%
2 ml 10% SDS	2%
0.5 ml 2-mercaptoethanol	14.4 mM
1 ml 1% bromophenol blue	0.1%

0.9 ml H_2O and stored at 20^oC.

Separating Gel

Ingredients	**X%**	**10%**	**12%**
Solution A	X/3 ml	2.33 ml	2.8 ml
Solution B	2.5 ml	1.75 ml	1.75 ml
H_2O	(7.5-X/3) ml	2.92 ml	2.45ml
10% APS	50µl	35µl	35µl
TEMED	10µl	20µl	20µl

Stacking gel

Ingredients	**5%**	**4%**
Solution A	0.502 ml	0.400 ml
Solution C	0.750 ml	0.750 ml
H_2O	1.725 ml	1.850 ml
10% APS	30 µl	40 µl

TEMED	20 µl	20 µl

Fixing and staining solution (1litre).

Methanol	450 ml
Glacial acetic acid	100 ml
Coomassie brilliant blue	1 g
Distilled water	450 ml

Destaining solution

Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

Pouring the separating gel

- The gel forming plates were properly washed and cleaned with ethanol.
- The gel plates were properly sealed and assembled according to the manufacturer instructions.
- Resolving gel (10%) was prepared by mixing Solution A, Solution B, Distilled water, APS and TEMED in appropriate amounts in a small test tube.
- The resolving gel solution was carefully introduced into the gel sandwich using a pipette to fill 4/5 of the cast. It was overlaid with 500 µl of distilled water and allowed to polymerize.

Pouring the stacking gel

- The water of separating gel was poured off.
- The stacking gel solution (5%) was prepared by mixing Solution A, Solution C, APS and TEMED in appropriate amount in a small test tube.
- The stacking gel solution was poured gently onto the separating gel using a pipette.
- The comb was inserted carefully by avoiding the entrapment of any air bubbles and the gel was allowed to polymerize.
- When the polymerization has been completed the comb was removed carefully, the gel slab was inserted in the electrophoresis unit and the inner and outer reservoirs were filled with 1X chilled running buffer.

Sample preparation

- 5 µl of 5X SDS sample loading buffer was mixed with 20 µl of the sample in an eppendorf tube.
- The sample was kept in a boiling water bath for exactly 5 minutes and instantly cooled.
- 5 µl of Bangalore Genei medium molecular weight marker (range 14.3 kDa to 97.4 kDa) and 15 µl of the denatured sample were loaded onto the gel.
- The samples were run at a constant voltage of 50 V in the stacking gel and then at a constant voltage of 100 V in the resolving gel at 150 mA for 50 minutes by keeping it at 4°C.
- The gel was run till the dye front reached the bottom of the gel.

Staining of gel

- The gel was removed from the apparatus and gently placed in the staining tray containing distilled water to remove any particles which may hamper proper staining.
- Then threw this water and added fixing and staining solution in the tray and allowed the gel to stain for 20 min by placing tray onto a staining plate shaker**.**
- Then the staining solution was removed and it was replaced with destaining solution (without dye) and again placed on staining plate shaker**.**
- The destaining solution was changed many a times and destaining was performed for overnight.

Application of pectinase in wine fermentation

Apple juice studied for fermentation was obtained in crude state from the Department of Horticulture, Naubahar, Shimla, Government of Himachal Pradesh.

Microorganism for fermentation

The ethanol producing yeast *Saccharomyces cerevisiae* was obtained from Department of Biotechnology, Himachal Pradesh University, Shimla and used in the experiment. The culture was maintained on yeast potato dextrose agar slants.

Fermentation

During batch fermentation 5 U of enzyme was added to 250 ml of apple juice containing sugar (100 mg/1000 ml) and was inoculated with *Saccharomyces cerevisiae*. The mixture was then incubated at 20°C for 30 days (pH 4.5). The sample of 3 ml was withdrawn at a regular interval of 5 days, centrifuged at 10,000 g for 10 min to pellet down the yeast. The supernatant was further used for estimation of the amount of carbohydrates, phenolics and ethanol (Figure 3).

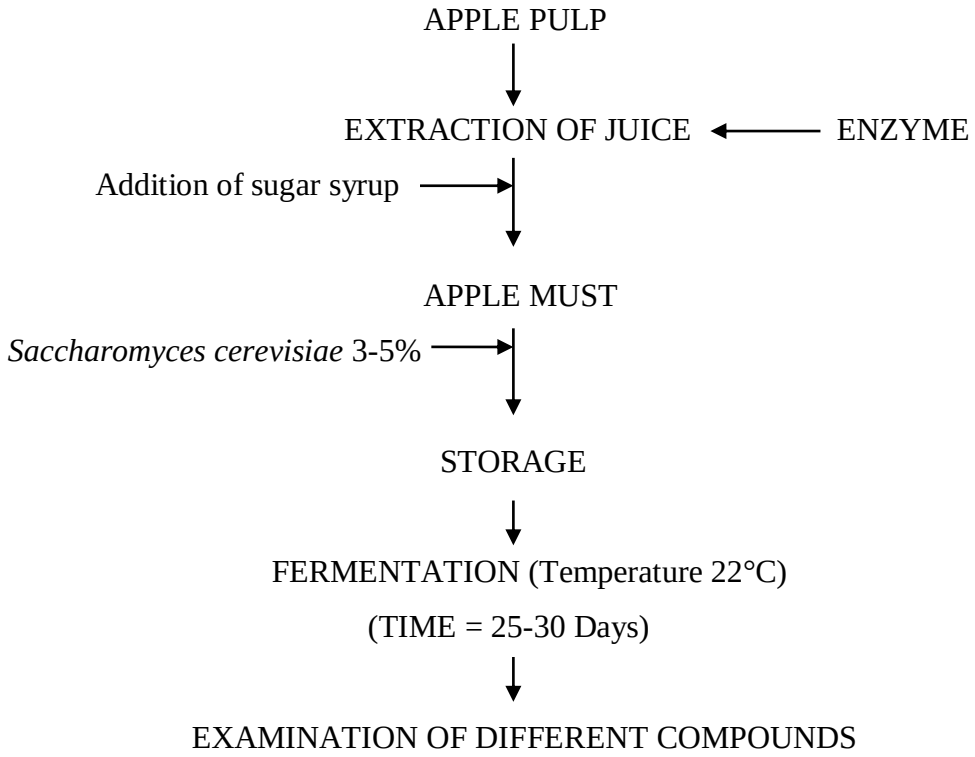


Fig. 3 Flow chart of apple juice fermentation

Analytical method

The amount of carbohydrates and phenolics were estimated both before and during the course of wine fermentation.

a) Estimation of carbohydrates: Carbohydrates were assayed by the phenol sulphur method **(**Duboisz *et al.*, 1956*;* Sadasivam and Manickam, 1996).

Reagents:

Phenol	l5 %
Sulphuric acid	96 %
Standard glucose	Stock- 100 mg in 100 ml of distilled water
Working standard	10 ml of stock diluted to 100 ml with distilled water

Procedure

- Pipetted 0.2, 0.4, 0.6, 0.8 and1ml of the working standard into a series of test tubes.
- Took 0.1 and 0.2 ml of the sample solution in two separate test tubes.
- Total volume in each test tube was made 1 ml with distilled water.

- Set a blank with 1 ml of distilled water.
- Added 1 ml of phenol solution to each test tube.
- Added 5 ml of 96% sulphuric acid to each test tube and shook well and kept as it is for 10 min.
- After 10 min shook the contents in the tubes and placed in a water bath at 25°C for 15 min.
- Absorbance was read at 490 nm.

b) Estimation of phenolics: The phenolics were estimated by Folin-ciocalteau method (Duboisz *et al.,* 1956*;* Sadasivam and Manickam, 1996).

Reagents:

Folin-Ciocalteau reagent	
Na_2CO_3	20%
Standard solution	Stock of 100 mg catechol in 100 ml distilled water
Working solution	Stock is diluted to 10 times

Procedure

- Diluted the sample with distilled water (5 ml)
- Pipetted out different aliquots (0.2 to 2 ml) into test tubes.
- Made up the volume in each tube to 3 ml with distilled water.
- Added 0.5 ml of Folin-Ciocalteau to each test tube.
- After 3 min, added 2 ml of 20% Na_2CO_3 solution to each test tube.
- Mixed thoroughly, tubes were placed in boiling water bath for one min.
- After cooling the absorbance was read at 650 nm.

c) Ethanol Detection by Spectrophotometric Method with potassium dichromate (Caputi e*t al.,* 1968).

Reagent:-

Potassium dichromate ($K_2Cr_2O_7$)	33.768 g
Sulphuric acid	325 ml

Make final volume 1 litre with distilled water.

Procedure

- Pipetted 200, 400, 600, 800 and 1000 µl of pure ethanol (standard) into a series of test tubes.
- Took 200 and 400 µl of the sample solution in two separate test tubes.
- Final volume in each tube was made 1200 µl with distilled water.
- Set a blank with 1200 µl of dis. water.
- Added 1 ml of $K_2Cr_2O_7$ to each tube.

- Placed the test tubes in water bath at 62°C for 20 min.
- Absorbance was read at 600 nm

Application of pectinase in tea leaves fermentation

Steps involved in tea processing

- **Plucking:** The apical bud and two young leaves (20 g) were plucked from *Camellia sinensis*.
- **Wilting or Withering:** The plucked apical buds and leaves were withered for 18 hrs at 26^{o}C till they became flaccid, wilting removed excess water from the leaves and allowed very light amount of oxidation.
- **Bruising (Rolling):** In order to promote and quicken oxidation, withered leaves werebruised by rolling.
- **Oxidation:** The leaves were left at room temperature for 2 hr, where they turned dark brown. In this process the chlorophyll in the leaves was enzymatically broken down, and their tannins were released. This process is termed as fermentation.

Treatment of tea leaves with pectin lyase enzyme

4g of oxidized tea leaves (cut dhool) were taken in three different plastic trays and sprayed with 1 ml Mellvaine's buffer (0.2 M, pH 5.0). Then one of the trays was sprayed with crude pectin lyase from *Byssochlamys fulva* (5 U/ml) and other tray was sprayed with purified pectin lyase (5 U/ml) and third one was placed without enzyme. Treated dhool was allowed to ferment at room temperature for 1 hr.

Drying or firing

After an hour the tea samples were heated at high temperature (97°C-105°C) for 5-10 min to stop oxidation. The fired tea was shifted and the biochemical constituents of the tea leaves, Theaflavin (TF), Thearubigin (TR), Highly Polymerized Substances (HPS) and Total Liquor Color (TLC) were estimated by Solvent Extraction Method of Angayarkanni *et al*. (2002) as shown in Figure 4 and briefly described as below:

- 4 g of dry tea was extracted in 200 ml of boiling water for 10 min. 1 ml of filtered infusion was eluted with 9 ml of distilled water and its absorbance at λ=460 nm was measured with a Perkin Elmer Lambda 3A UV/VIS spectrophotometer and was considered to be as A.
- 25 ml of infused tea was added to 25 ml of iso-butyl methyl ketone (IBMK) and after sufficient mixing, two phases were allowed to be separated. 1 ml of organic phase was

eluted with 9 ml of ethanol (45%) and its absorbance at 380 nm was considered to be as B.

- 10 ml of organic phase and 10 ml of Na_2HPO_4 (2.5%) were mixed together and allowed to be separated into two phases. The aqueous layer was discarded and 1 ml of organic layer was diluted with 9 ml of ethanol (45%). Its absorbance at 380 nm was considered as C.
- 10 ml of the first aqueous phase was mixed with 10 ml of n-butanol and allowed to be separated into two phases. 1 ml of this organic layer was mixed with 9 ml of ethanol and its absorbance at λ=380 nm was considered as D.
- 1 ml of the aqueous layer was mixed with 9 ml of ethanol and its absorbance at 380 nm was considered as parameter E.

Amounts of quality parameters of tea samples TF, TR, HPS and TLC were determined using the following formula, which was proposed by Angayarkanni *et al*., (2002).

$$TF\ (\%) = 4.313 \times C$$

$$TR\ (\%) = 13.643\ (B + D - C)$$

$$HPS\ (\%\ as\ TR) = 13.643 \times E$$

$$TLC\ (\%) = 10 \times A$$

The multiplication factors mentioned in the equations were derived from molar extinction co-efficient of pure compounds (Duncan, 1955) and dilution factors. In case of TLC, the dilution factor was 10. The accuracy of the contents was tested by conducting the analysis in triplicates.

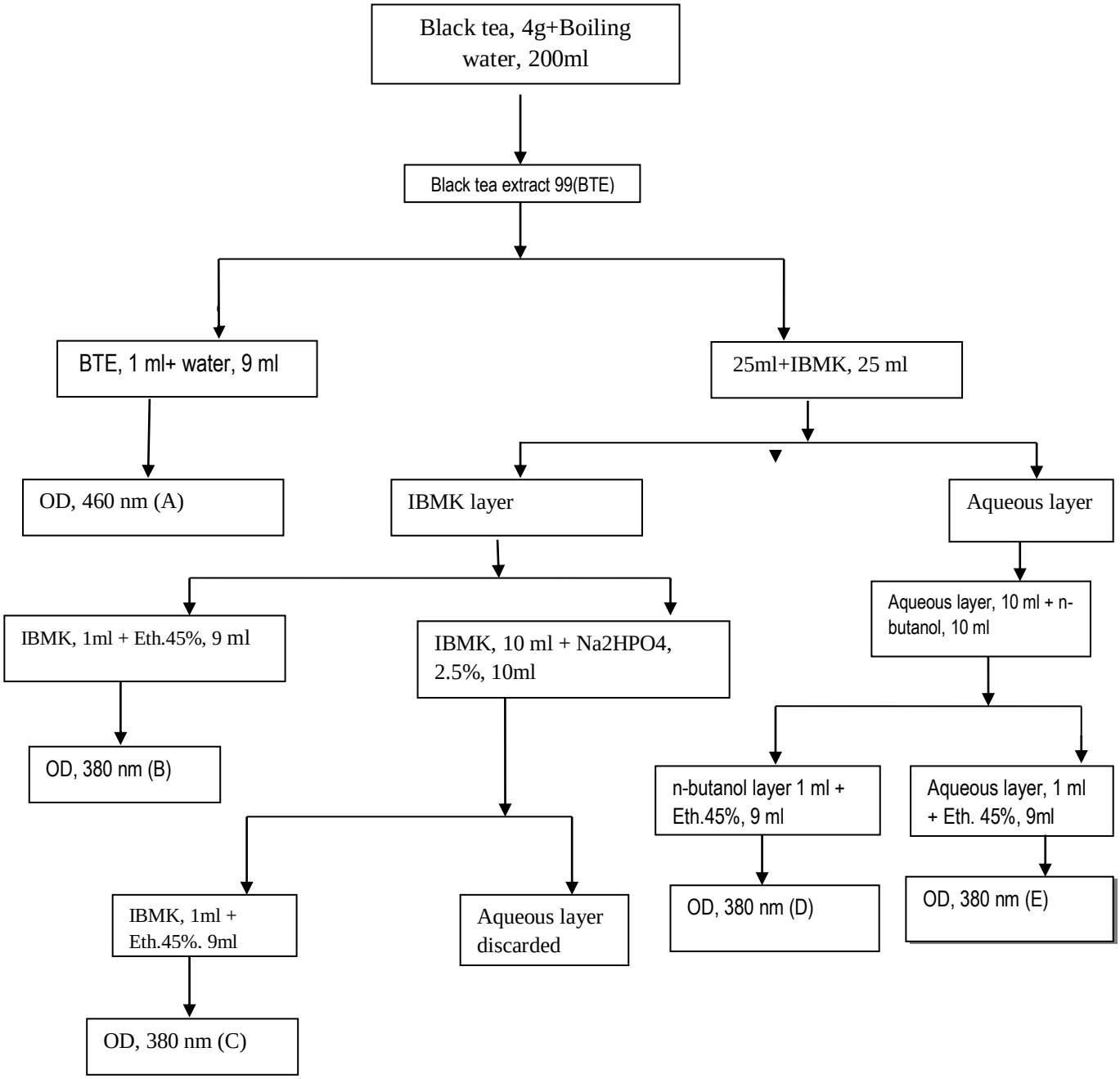


Fig.4 Solvent Extraction Method for estimating phenolic compounds of tea

IBMK- Isobutyl methyl ketone, Eth- Ethanol.

CHAPTER 4 RESULTS AND DISCUSSION

Crude enzyme

The initial activity of crude enzyme pectin lyase produced by *Byssochlamys fulva* was 0.98 U/ml and protein content 0.65 mg/ml. The specific activity was calculated to be 1.5 U/mg.

Ammonium sulphate precipitation

At 70 percent saturation of ammonium sulphate, enzyme activity was 1.73 U/ml, protein content was 0.72 mg/ml and specific activity 2.4 U/mg. Partial purification of *A. niger* at 60% salting out showed the activity of 382.45 U/ml/min (Batool *et al*., 2013).

Purification of pectin lyase using DEAE-cellulose column chromatography

The ion exchange chromatography of enzyme was done through DEAE-Cellulose column. As many as 60 fractions were collected (2 ml each). The absorbance of different fractions was recorded at 280 nm. The elution profile of pectin lyase on DEAE-Cellulose column chromatography is depicted in Figure 5. All 60 collected fractions were assayed for enzyme activity and protein content. The purified fraction 27, 28, 29, 30, 31 showed specific activity of 7.21 U/mg, 7.42 U/mg, 8.24 U/mg, 8.61 U/mg and 8.91 U/mg respectively. The total pectin lyase activity in the 10 ml of the pooled fractions was found to be 48 U and total protein content was 5.9 mg, specific activity was 8.13 U/mg indicating ~ 5-fold purification of enzyme. In another study the extracellular PNL produced by *A. flavus* MTCC 10938 purified on DEAE cellulose column and gel filtration Sephadex G–100 column showed hundred-fold purification (Yadav *et al*., 2013). Purification of pectin lyase from *Bacillus pumilus (P9)* was 3.39-fold after DEAE-cellulose chromatography and 36.36 after Sephadex G-150 (Nadaroglu *et al*., 2010). Pectin lyase from *Pleurotus ostreatus* grown on lemon pulp waste was purified 15.6-fold after DEAE-cellulose column chromatography (Rashad *et al.*, 2011). Pectin lyase was produced by *G. stearothermophilus* Ah22 by using DEAE cellulose anion exchange chromatography and was purified 40.7-fold.

Purification of pectin lyase using Sephacryl S-100 column chromatography

The gel filtration chromatography was done using Sephacryl S-100 column chromatography with the pooled fractions from the DEAE column. As many as 50 fractions (2.0 ml each) were collected. The absorbance of these fractions was taken at 280 nm. The elution profile of pectin lyase on Sephacryl S-100 is depicted in Figure 6. The total pectin lyase activity in the 11^{th} (2 ml) fraction was found to be 10.4 U and total protein content was found to be 0.92 mg.

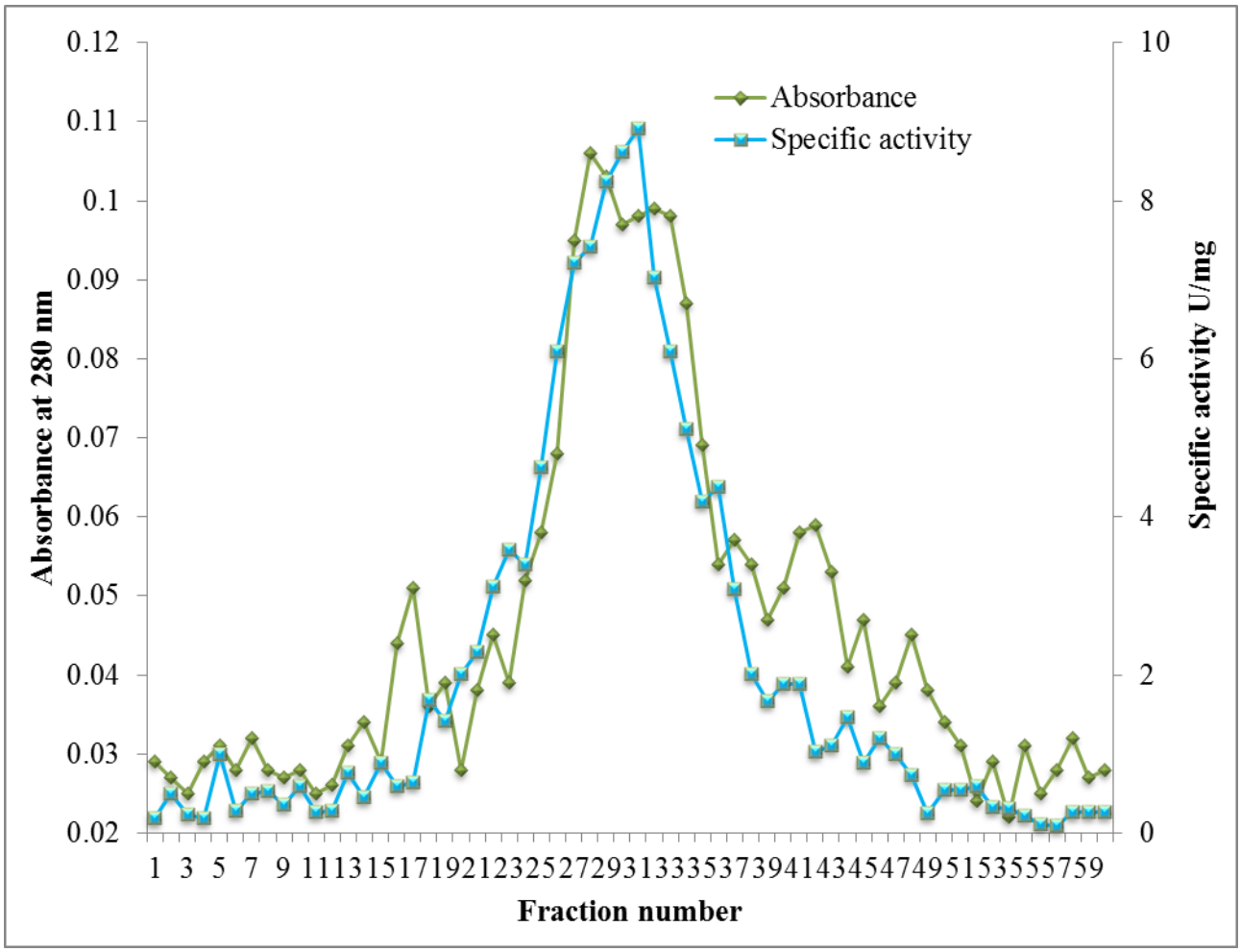


Fig. 5 Elution profile of pectin lyase by DEAE cellulose column chromatography

The specific activity of fraction was found to be 11.3 U/mg of protein, indicating ~7-fold purification. These results have been summarized in Table 7. Purification of PG from *Aspergillus carbonarius* using polymeric membranes resulted in 10-fold purification (Nakkeeran *et al.*, 2008). Purification of PG from *Thermoascus aurianticus* CBMAI-756 using Sephadex G-75 column resulted in 13.7 fold purification and in SP-Sepharose column resulted in 21.0 fold purification (Martins *et al*., 2007). Purification of PL from *Aspergillus ficuum* by ion exchange and gel filtration chromatography resulted in 86 fold purification (Yadav *et al*., 2009). Purification of pectin lyase from *Aspergillus terricola* was 35.17 fold by using Sephadex-G100 (Yadav *et al*., 2009). Purification of pectin lyase from *Bacillus pumilus* was 36.36 fold by using Sephadex G-150 chromatography (Hayrunnisa *et al*., 2010). The polygalacturonase from *Aspergillus niger* MTCC 3323 was purified about 6.52-fold with an increase in specific activity to 54.3 U/mg giving a yield of 5.01% (Kant *et al.*, 2013).

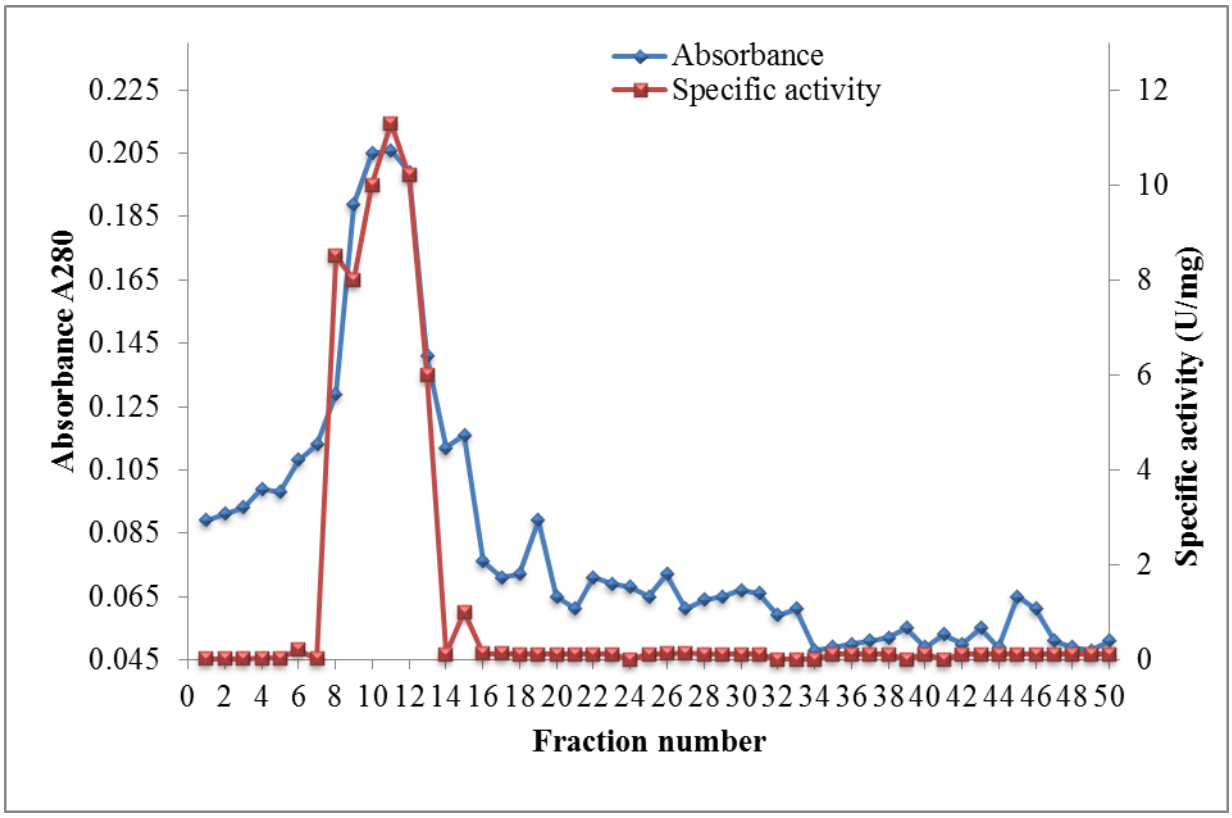


Fig. 6 Elution profile of pectin lyase by Sephacryl S-100 column chromatography

Table 7 Purification of pectin lyase produced extracellularly by *Byssochlamys fulva*

Step	Volume (ml)	Total enzyme activity (U)	Total protein (mg)	Specific activity(U/mg)	Fold purification	Yield (%)
Crude Enzyme	300	294	195	1.5	1	100
Precipitated enzyme (70%$(NH_4)_2SO_4$)	150	259.5	108	2.4	1.6	88.26
DEAE cellulose column chromatography	10	48	5.9	8.13	5.42	16.33
Sephacryl S-100 column chromatography	2	10.4	0.92	11.3	7.53	3.53

Molecular weight determination

SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme to determine the molecular weight of enzyme. It was carried out in 12% and 4% acrylamide concentrations for the running and the stacking gel respectively. The SDS-PAGE gave a single band and the molecular weight of purified enzyme was estimated to be 29 kDa. The electrophoretic pattern was recorded as shown in Figure 7.

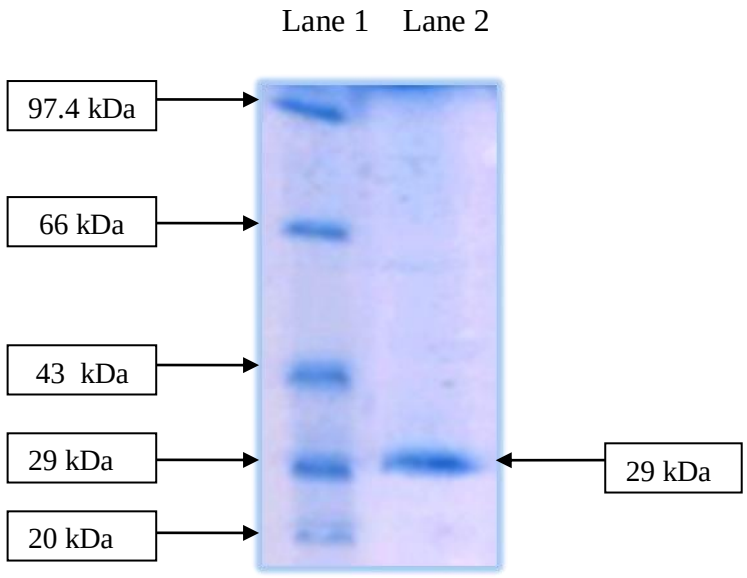


Fig. 7 SDS-PAGE of purified pectin lyase from *Byssochlamys fulva*

Lane 1. Bangalore Genei protein marker (medium molecular weight)

Protein	**Approximate MW**
Phosphorylase b	97,400
Bovine Serum Albumin	66,000
Ovalbumin	43,000
Carbonic Anhydrase	29,000
Soyabean Trypsin Inhibitor	20,000

Lane 2. Purified pectin lyase

Molecular weight of purified pectin lyase from *Byssochlamys fulva* after SDS-PAGE was found to be 29 kDa. The literature survey has indicated that the relative molecular mass of PNLs varied from 22 to 90 kDa (Yadav *et al.*, 2009).

It was revealed that PNL produced by *Aspergillus japonicus* and *Bacillus* PN–33 had relative molecular mass of 50 and 52 kDa, respectively (Semenova *et al*., 2003). Molecular weight of pectin lyase produced by *Pleurotus ostreatus* grown on lemon pulp waste was 23 kDa (Rashad *et al*., 2011). The molecular weight of pectate lyase from *Erwinia chrysanthemii* was 38 kDa (Doan *et al*., 2000). In another study molecular weight of pectin lyase from *Bacillus pumilus* has been found to be 25 kDa (Nadaroglu *et al*., 2010).

Role of pectinase in apple juice fermentation for wine formation

The present study has been carried out using apple juice. The reason behind choosing the apple juice was that it has large amount of undesirable polysaccharides or carbohydrates

along with useful sugars, which are used in preparation of some types of wines. In this study, the apple juice was inoculated with pectinase, sugar (100 g/1000 ml) and *Saccharomyces cerevisiae* and kept for 30 days for fermentation. Different components (carbohydrates, phenolics and ethanol) were measured at a regular interval of 5 days.

Effect on amount of carbohydrates

In this study, the amount of carbohydrates decreased from 160 to 142.28 µg/ml after 30 days of fermentation in presence of enzyme (Figure 8). The suspended particles were degraded by pectinase and debris settled down at the bottom of container (Figure 9).

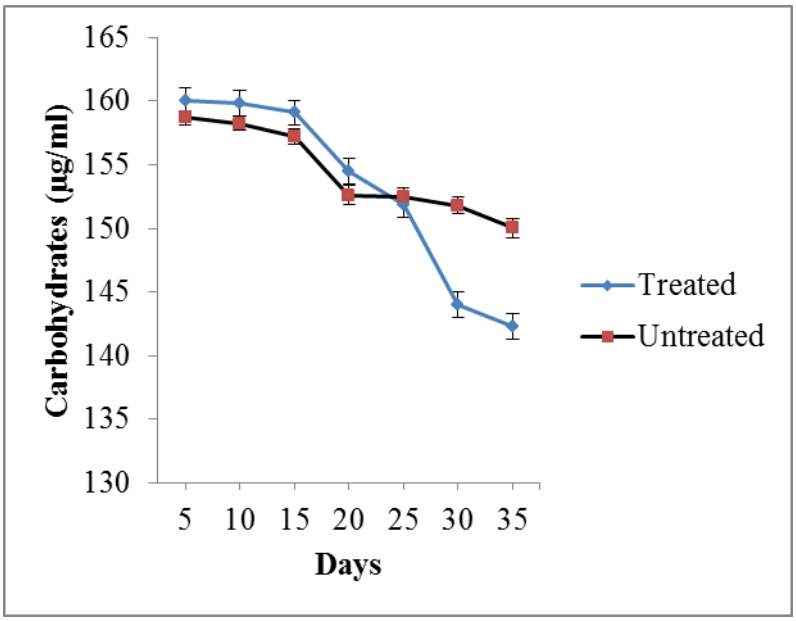
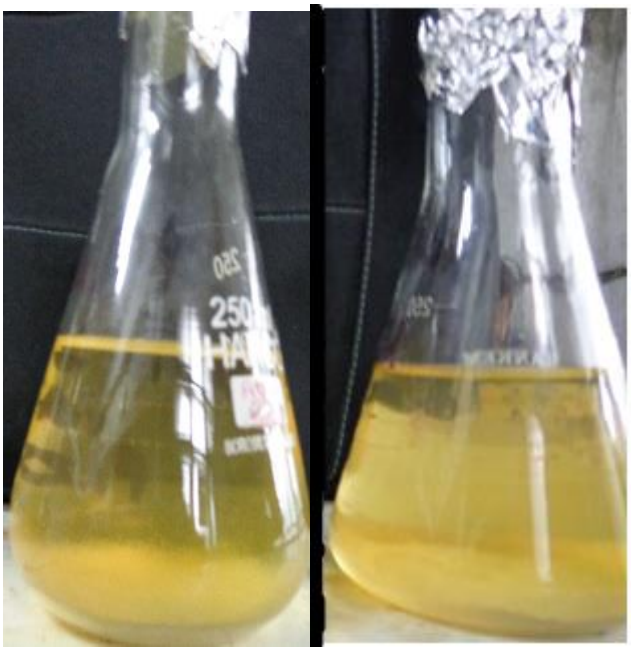


Fig. 8 Effect of pectinase treatment on carbohydrates

In another study, pectinase from *Mucor circinelloides* used in apple wine fermentation showed the amount of carbohydrates decreased from 102 to 61 µg/ml (Sharma *et al.*, 2013). Stepanova *et al.*, (2006) also found decrease in the contents of the carbohydrates in case of cherry plum wine, while using immobilized preparations of different commercially available polygalacturonases.



TEST CONTROL

Fig. 9 Sedimentation of suspended particles degraded by pectin lyase from *Byssochlamys fulva.*

Control: Apple juice fermented with *Saccharomyces cerevisiae* in absence of enzyme.

Test: Apple juice fermented with *Saccharomyces cerevisiae* in presence of enzyme.

Effect on phenolics

The amount of phenolics increased from 0.42 to 1.02 mg/ml in the presence of the enzyme after 30 days of fermentation (Figure 10). In another study phenolics increased from 0.29 mg/ml to 0.48 mg/ml in case of apple wine and from 0.77 mg/ml to 1.2 mg/ml in case of plum wine on addition of pectinase from *Mucor circinelloides* (Sharma *et al.*, 2013). The amount of phenolics also increased in case of cherry plum wine after treatment with pectinex immobilized on Aminosilochrome 10 and plum wine was having higher concentration of phenolics as compared to apple wine (Stepanova *et al.*, 2006).

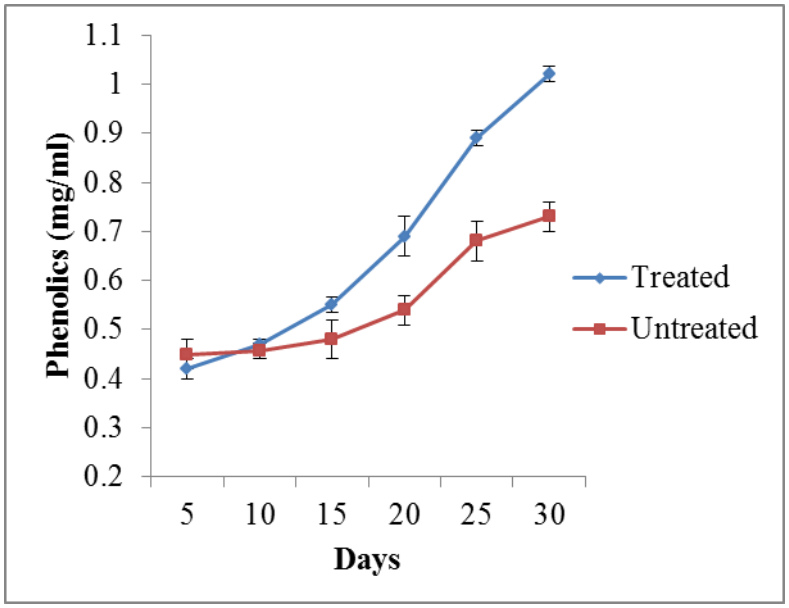


Fig. 10 Effect of pectinase treatment on phenolic compounds

Effect on ethanol production

The ethanol production increased from 6.6% to 9.01% in the presence of enzyme after 30 days of fermentation (Figure 11). In another study, pectinase from *Mucor circinelloides* used in apple wine fermentation showed ethanol level increased from 10.02% v/v to 14.2% v/v in case of apple wine and from 4.62% v/v to 6.09% v/v in case of plum wine after pectinase treatment during fermentation (Sharma *et al*., 2013).

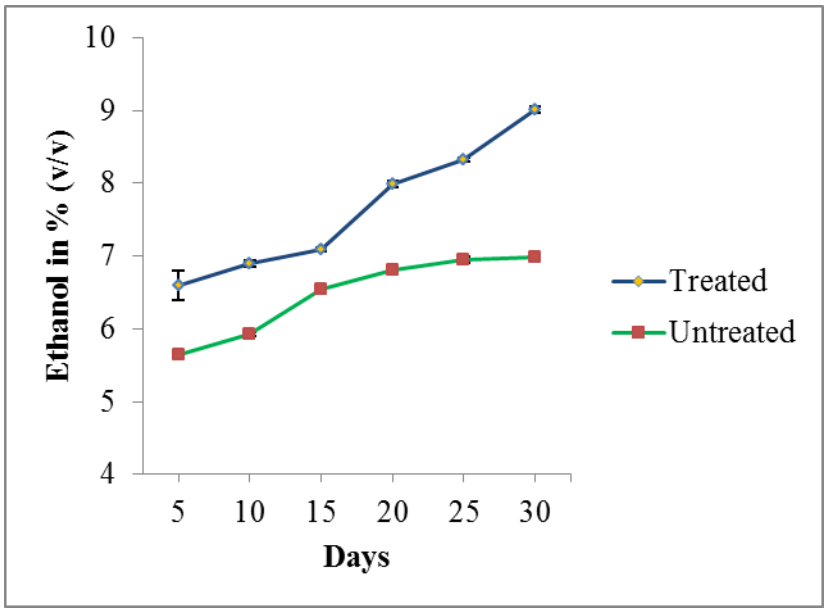


Fig. 11 Production of ethanol

Tea leaves fermentation by pectin lyase

In the present study, tea leaves fermentation was done with the help of pectin lyase enzyme and its effects on different phenolic compounds of tea were analyzed. All contents TF, TR, HPS and TLC increased more on addition of crude enzyme but less with purified enzyme. The crude enzyme extract from fungi comprised of all enzymes, cellulase, hemicellulase (xylanase), proteinase, pectinase, whereas the purified enzyme solution contained only pectinase. The tea leaf is composed of cellulose, hemicelluloses and pectin. When the crude enzyme extract was sprayed on tea leaves during fermentation, all the polymeric compounds, cellulose, pectin and hemicelluloses were hydrolyzed by the complex action of all enzymes in the extract, hence resulted in higher maceration of tea leaves and in turn fermentation. Purified enzyme hydrolyzed only pectin in the tea leaves and less maceration was achieved. Thus, the addition of external enzymes during tea leaves fermentation significantly increased the tea quality parameters.

Effect of added enzyme on tea quality

Effect on Theaflavin (TF) content

The use of crude pectin lyase from *Byssochlamys fulva* resulted in significant ($p< 0.005$) increase in TF. TF content of tea leaves was 2.52%. It was increased by 22.91% when treated with crude enzyme and by 10.99% increase in TF content using purified enzyme (Table 8 and Figure 12). The use of partially purified enzymes from *A. indicus, A. flavus* and *A. niveus* resulted in the increase of TF content by 43.81%, 62.86% and 59.05% respectively. Whereas the purified enzymes from *A. indicus, A. flavus* and *A. niveus* increased the TF content by 38.1%, 40% and 34.29% respectively and the commercial enzyme increased the TF content by 30.48% as compared to control (Angayarkanni *et al.,* 2002).

Effect on Thearubigin (TR) content

The use of pectin lyase from *Byssochlamys fulva* resulted in significant ($p< 0.005$) increase in TR. TR content of tea leaves was 3.25% and it was increased by 44.79% when treated with crude enzyme and by 22.04% when treated with purified enzyme as shown in (Table 8 and Figure 12). In studies by Angayarkanni *et al.,* (2002), use of crude enzyme preparation from *A. indicus, A. flavus* and *A. niveus* resulted in the maximum increase in TR content by 12%, 12.44%, and 11.78% respectively. The purified enzymes from *A.indicus, A. flaus* and *A. niveus* increased the TR content by 7.11%, 9.44%, and 6.78% respectively and the commercial enzyme by only 4% as compared to control.

Effect on Highly Polymerzied Substances (HPS) content

The use of pectin lyase from *Byssochlamys fulva* resulted in significant ($p< 0.005$) increase in HPS content. HPS content of tea leaves not treated with any enzyme was 17.86%. Crude pectin lyase increased HPS content by 20.37%. The purified enzyme exhibited 14.5% increase over control (Table 8 and Figure 12). HPS content was increased by 50.9% by crude pectin lyase and 63.3% by purified pectin lyase (Angayarkanni *et al*., 2002).

Effect on Total Liquor Color (TLC)

The crude and purified enzyme from *Byssochlamys fulva* resulted in significant ($p< 0.005$ and $p< 0.025$ respectively) increase in TLC content. Initial TLC content was 3.48%. The crude enzyme resulted in an increase by 23.36% and the purified enzyme increased the TLC by 16.26% as compared to control (Table 8 and Figure 12). In previous studies, the partially purified enzymes from A. *indicus, A. flavus and A. niveus* enhanced the TLC to maximum of 18.91%, 14.74% and 14.10% respectively over control. The purified enzymes from these fungi resulted in an increase of 12.18%, 11.54% and 11.22% respectively and the commercial enzyme increased the TLC content by only 11.54% as compared to control (Angayarkanni *et al*., 2002).

Table 8 Effect of pectin lyase treatment on TF, TR, HPS and TLC content during fermentation of tea leaves.

Content	Control	Crude	PNL treated
TF (%)	2.52±0.179	3.10±0.162*	2.80±0.185
TR (%)	3.25±0.0723.253±0.0723	4.71±0.154*	3.97±0.191*
HPS (%)	17.86±0.308	21.49±0.127*	20.45±0.216*
TLC (%)	3.48±0.185	4.29±0.281*	4.05±0.157***

Values are mean ±SD of 3 replicates.*$p<0.005$and ***$p<0.025$ as compared to control. Control means content measured when tea leaves were not treated with any enzyme.

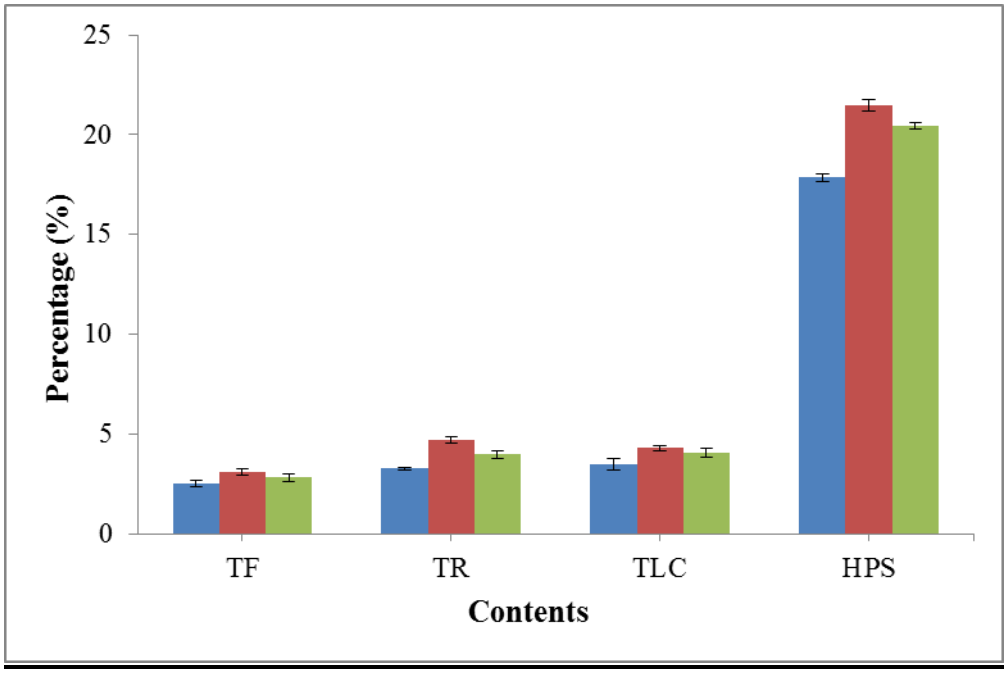


Fig.12 Effect of crude and purified pectin lyase on TF, TR, HPS, TLC contents during tea leaves fermentation.

CHAPTER 5 SUMMARY

In the present study, pectin lyase was produced extracellularly by *Byssochlamys fulva* when grown in production medium containing pectin as sole carbon source. Pectin lyase was purified ~5-fold by DEAE-Cellulose column chromatography (anion exchange) and 7.53-fold by Sephacryl S-100 column chromatography. The purified pectin lyase exhibited single band of molecular mass 29 kDa using SDS-PAGE. Purified enzyme was used for apple juice fermentation and fermentation of tea leaves. Apple juice fermentation was carried out for 30 days. The amount of carbohydrates decreased from 160 to 142.28 μg/ml. The amount of phenolics and ethanol increased from 0.42 to 1.02 mg/ml and 6.6 to 9.01% respectively during fermentation of juice treated with pectinase. Improvement of tea leaves fermentation was determined in terms of phenolic compounds i.e. Theaflavin, Thearubigin, Highly Polymerized Substances and Total Liquor Color of the tea. The TF, TR, HPS and TLC content was increased by 22.91%, 44.79%, 20.37% and 23.36% respectively when treated with crude enzyme and 10.99%, 22.04%, 14.50% and 16.26% respectively when treated with purified pectin lyase. Maximum increase in the phenolic content was found in tea leaves treated with crude enzyme hence crude enzyme improved the tea quality more in comparison to purified enzyme.

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Table 9 Absorbance of purified enzyme fractions at 280 nm after DEAE-cellulose column chromatography

Fraction No.	A_{280}	Fraction No.	A_{280}	Fraction No.	A_{280}
1	0.029	21	0.038	41	0.058
2	0.027	22	0.045	42	0.059
3	0.025	23	0.039	43	0.053
4	0.029	24	0.052	44	0.041
5	0.031	25	0.058	45	0.047
6	0.028	26	0.068	46	0.036
7	0.032	27	0.095	47	0.039
8	0.028	28	0.106	48	0.045
9	0.027	29	0.103	49	0.038
10	0.028	30	0.097	50	0.034
11	0.025	31	0.098	51	0.031
12	0.026	32	0.099	52	0.024
13	0.031	33	0.098	53	0.029
14	0.034	34	0.087	54	0.022
15	0.029	35	0.069	55	0.031
16	0.044	36	0.054	56	0.025
17	0.051	37	0.057	57	0.028
18	0.036	38	0.054	58	0.032
19	0.039	39	0.047	59	0.027
20	0.028	40	0.051	60	0.028

Table 10 Absorbance of purified enzyme fractions at 280 nm after Sephacryl S-100 column chromatography

Fraction No.	A_{280}	Fraction No.	A_{280}
1	0.089	26	0.072
2	0.091	27	0.061
3	0.093	28	0.064
4	0.099	29	0.065
5	0.098	30	0.067
6	0.108	31	0.066
7	0.113	32	0.059
8	0.129	33	0.061
9	0.189	34	0.048
10	0.205	35	0.049
11	0.206	36	0.05
12	0.199	37	0.051
13	0.141	38	0.052
14	0.112	39	0.055
15	0.116	40	0.049
16	0.076	41	0.053
17	0.071	42	0.05
18	0.072	43	0.055
19	0.089	44	0.049
20	0.065	45	0.065
21	0.061	46	0.061
22	0.071	47	0.051
23	0.069	48	0.049
24	0.068	49	0.048
25	0.065	50	0.051

Table 11 Standard curve of Galacturonic Acid

S.No.	Concentration (µg/ml)	A_{450}
1	20	0.029
2	40	0.038
3	60	0.056
4	80	0.074
5	100	0.101
6	120	0.109
7	140	0.121
8	160	0.148
9	180	0.165
10	200	0.179

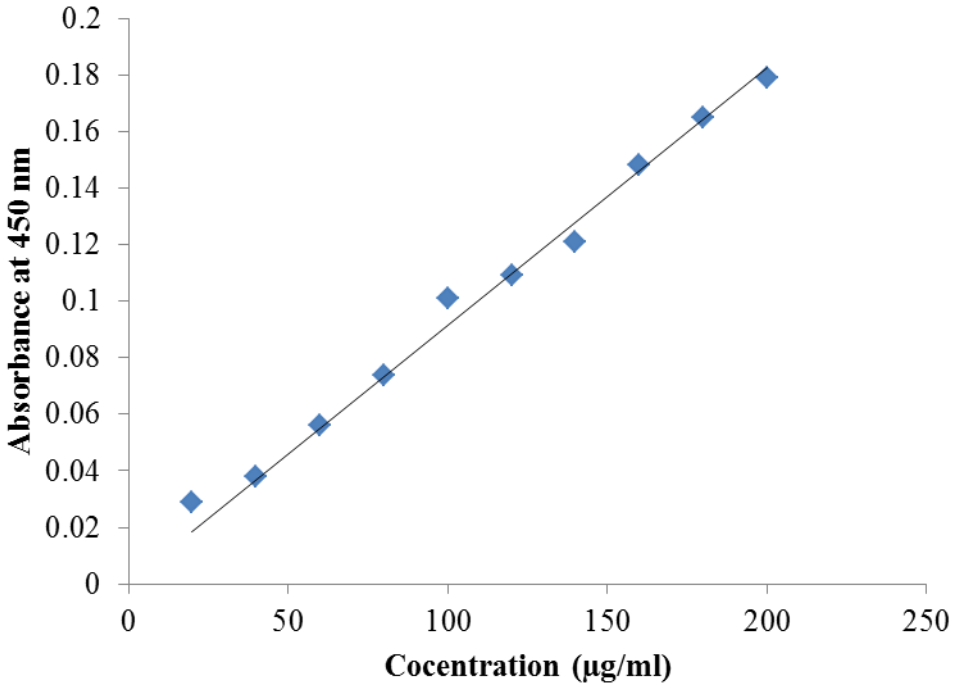

Fig. 13 Standard curve of Galacturonic Acid

Table 12 Standard curve of BSA

S.No.	Concentration (µg/ml)	A_{660}
1	100	0.132
2	150	0.251
3	200	0.369
4	250	0.589
5	300	0.641
6	350	0.721
7	400	0.849
8	450	0.969
9	500	0.996

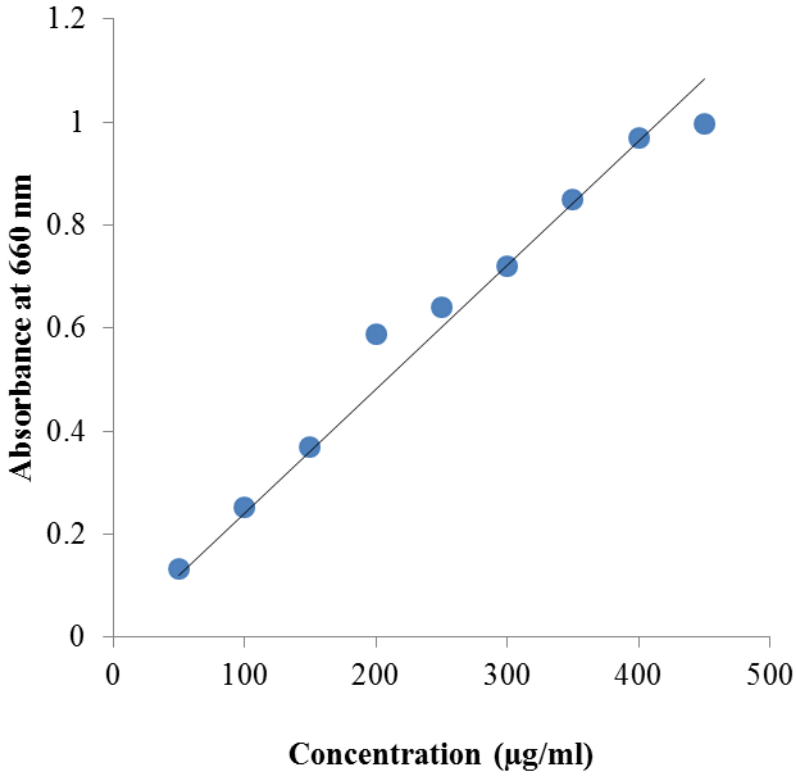

Fig. 14 Standard curve of BSA