PECTINASE PRODUCTION USING APPLE POMACE AS CARBON SOURCE BY MIXED CULTURE FERMENTATION

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PECTINASE PRODUCTION USING APPLE POMACE AS CARBON SOURCE BY MIXED CULTURE FERMENTATION

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

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There is a growing interest in enzyme production from food and agricultural wastes to reduce the cost of production. Pectinases are one of the important group of enzymes used in fruit processing, alcoholic beverages, coffee and tea fermentation and textile industries. Pectinases can be produced by both solid state and submerged fermentation from various plant and microorganisms; however fungal microorganisms are the largest sources of bulk commercial enzymes and there are numerous studies on pectinase production from fungal sources. Therefore, in this study; bacterial mix culture (*Bacillus subtilis* and *Bacillus pumilus*) was used to produce pectinase by submerged fermentation using apple pomace as carbon source.

Firstly, growth curves of both microorganisms (*Bacillus subtilis* and *Bacillus pumilus*) were constructed to determine the exponential and stationary phases. The cultures were used at the beginning of stationary phase during fermentation. Pre-treatment of apple pomace was carried out using one step and two step acid hydrolysis methods. Initial screening was done to fix the time variable of fermentation. A set of 15 experiments was constructed by Box-Behnken design to optimize fermentation conditions with respect to pH (7, 8 and 9), apple pomace (AP) concentration (5, 10 and 15 %) and mix culture ratio-*B.subtilis/B.pumilus* (1/4, 3.4/1.6 and 4/1). Further analyses at optimum condition were carried out to investigate any correlation between reducing sugar content, biomass, protein content and pectinase production.

Initial screening experiments revealed that maximum pectinase production was achieved after 24 h fermentation time. One-step acid hydrolysis with 4% H_2SO_4 was found the best pre-treatment method. The highest pectinase activity was 11.48±0.51 IU/mL measured at 15 % AP; pH 9 and mix ratio(*B.subtilis/B.pumilus*) 2.125. The determination coefficient (R^2=97.98) and insignificant lack of fit (p=0.124>0.05) proved that the model fitted well to the experimental data.

Pectinase production kinetics was evaluated by Leudeking and Piret model. Pectinase production was found growth-related whose coefficient (α) was 8.0431.

In conclusion, this study shows that apple pomace is an effective carbon source for the pectinase production and mix culture fermentation has enhanced pectinase production.

Keywords: Apple pomace, pectinase production, submerged fermentation, *Bacillus subtilis, Bacillus pumilus*

ÖZ

KARBON KAYNAĞI OLARAK ELMA POSASI KULLANILARAK KARIŞIK KÜLTÜR FERMENTASYONU İLE PEKTİNAZ ÜRETİMİ

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Üretim maliyetini azaltmak için tarımsal ve gıda atıklarından enzim üretimine karşı artan bir ilgi vardır. Pektinaz; meyve işleme, alkollü içecek, kahve ve çay fermantasyonu ve tekstil endüstrisi uygulamalarında kullanılan başlıca enzimlerden biridir. Pektinaz; çeşitli bitki ve mikroorganizmalardan katı kültür ve derin fermantasyon yöntemleri kullanılarak üretilir, ama ticari enzim üretiminde en yoğun olarak küfler kullanılmaktadır ve küflerden enzim üretimini inceleyen çalışmalar oldukça fazladır. Dolayısıyla; bu çalışmada karbon kaynağı olarak elma posası kullanılarak derin fermantasyon yöntemi ile pektinaz üretiminde karışık bakteri kültürü kullanılmıştır.

İlk olarak, logaritmik gelişim dönemi ve durma dönemlerinin saptanabilmesi için *B.subtilis* ve *B.pumilus* mikroorganizmalarının gelişme eğrileri oluşturulmuştur.

İki kültür de durma dönemlerinin giriş evrelerinde fermantasyonda kullanılmışlardır. Tek aşamalı ve çift aşamalı asit hidroliz yöntemleri kullanılarak ön-işlem çalışmaları yapılmıştır. Fermantasyon süresini sabit tutmak amacı ile ön çalışmalar yapılmıştır. Box-Behnken tasarımı ile 15 adet deney seti oluşturulmuş ve fermantasyon koşulları pH (7, 8 ve 9), elma posası(AP) konsantrasyonu (5, 10 ve 15 %) ve karışık kültür oranına göre (1/4, 3.4/1.6 ve 4/1) optimize edilmiştir. İndirgen şeker miktarı, biokütle, protein miktarı ve pektinaz aktivite arasındaki ilişkileri göstermek için optimum koşullarda ileri analizler yapılmıştır.

Ön değerlendirme analizleri; maksimum pektinaz aktivitesinin 24 saat fermantasyon süresi ile sağlanabildiğini ortaya çıkarmıştır. %4 H_2SO_4 ile uygulanan tek aşamalı asit hidrolizi en iyi ön-işlem yöntemi olarak bulunmuştur ve Box-Behnken YYY ile optimum koşullar 11.48±0.51 IU/mL'lik en yüksek aktiviteyle 15 % AP; pH 9 ve karışık kültür oranı (*B.subtilis/B.pumilus*) 2.125 olarak bulunmuştur. Modelin deneysel sonuçlara uyumu, hesaplanan varyasyon katsayısı (R^2=97.98) ve önemsiz uyum eksikliği (p=0.124>0.05) sonuçlarıyla doğrulanmıştır.

Pektinaz üretim kinetiği; Leudeking ve Piret modeline göre hesaplanmıştır. Pektinaz üretiminin gelişime dayalı olarak gerçekleştiği bulunmuştur. Gelişime dayalı faktör ise α=8.0431 olarak bulunmuştur

Sonuç olarak bu çalışma pektinaz üretimi için elma posasının etkin bir karbon kaynağı olduğunu ve karışık kültür fermantasyonunun pektinaz üretimini arttırdığını göstermiştir.

Anahtar sözcükler: Elma posası, pektinaz aktivitesi, derin fermantasyon, *Bacillus subtilis, Bacillus pumilus,* modelleme, karışık kültür

To all my beloved…

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CHAPTER 1

INTRODUCTION

Enzymes are bioactive compounds and responsible for regulation of many chemical reactions in living tissues and cells. The world enzyme market has a commercial annual of US$2 billon dollars and grows every year at a rate of 8-10% (Polizeli et al., 2014). Pectinases are a group of enzymes, which depolymerize different pectic substances in plant tissues (Suneetha V et al., 2014). Pectinases were launched in the food industry in 1930 (Nagodawithana et al., 1993) and became one of the important enzymes. Almost 75% of the estimated sale value among industrial enzymes belongs to pectinases in 1995 (Suneetha V et al., 2014). Pectinases have a wide range of applications such as; fruit juice extraction and clarification, wine clarification, degumming of plant fibers, treatment of aqueous residues, coffee and tea fermentation, animal feed production, paper and pulp industry (Pasha et al.,2013).

The main source of pectinases is microbial, out of which, 50% source from fungi and yeast, 35 % from bacteria, while the remaining 15 % from plant or animal origin (Anisa and Girish 2014). In this study, bacteria cultures (*B. pumilus and B. subtilis*) were used to produce pectinase (polygalacturonase) using apple pomace as carbon source.

Firstly, pre-treatment of apple pomace at various acid concentrations was carried out to identify the best one. Submerged fermentation was then used to optimize pectinase production according to concentration of substrate (apple pomace), pH and ratio of inocula. After implementing response optimization; further analysis at

optimum conditions were done to show any correlations between pectinase production, protein content, reducing sugar content and biomass. Finally; kinetic models were established to find out growth related and non-growth related factors during enzyme production.

Optimization study was conducted by Box-Behnken response surface methodology with 95 % confidence level.

The goal of this study was to enhance pectinase production using apple pomace as carbon source by mixed culture fermentation and to determine culture conditions for maximal pectinase production. The objectives supporting our goal are;

1. To analyze potential of apple pomace as substrate
2. To optimize culture conditions including pH, amount of solid load and inoculum ratio for pectinase production.
3. To discover importance of microbial interaction during enzyme synthesis
4. To show correlation between reducing sugar content, biomass, protein content and pectinase production at optimum conditions
5. To develop kinetic model on pectinase production

In chapter 2, literature review is given about apple pomace, enzymes, production of enzymes, inoculum type, pectinase production by *Bacillus* species to justify this work.

In chapter 3, materials and methods are presented to give detailed information about the experiments conducted.

In chapter 4, pectinase optimization results are discussed. Experimental results of pectinase production are analyzed and the optimum production conditions are reported and followed by kinetic model and growth and non-growth associated

factors were found by Leudeking-Piret equation. Thus, the reader will identify clearly the effect of each treatment with the help of quantitative values.

In chapter 5, the outcomes and recommendations are given to help take this study step further.

CHAPTER 2

LITERATURE REVIEW

2.1 Apple pomace

Food industry in general, generates a large quantity of waste (i.e peel, seed, pomace, rags, kernels etc.), which is biodegradable in nature (Joshi et al. 2006). Such wastes have great potential as a substrate in fermentation and involve production of beneficial products due to their rich composition in carbohydrates, dietary fibers and minerals.

Apple pomace is a residue after juice extraction containing peel, seeds and pulp. It represents about 25-35% of the weight of the fresh apple processed and consists of peel, core, seed, calyx, stem and soft issue (Cordoba, 2012). Apple pomace is mainly composed of water and insoluble carbohydrates like cellulose, hemicellulose and lignin (Cordoba, 2012). Although Apple pomace is rich in carbohydrates, acids, fibers, vitamin C and minerals; only a small amount of AP is used for deep processing, and the vast majority is not effectively utilized yet (Wang et al. 2009).

The total world apple production for 2012 was 76.378.700 tones according to FAOSTAT. Top ten countries by apple production are ranked in Table 2.1. Turkey is the third biggest apple producer with 2.889.000 tones of fruit.

Fig 2.1 shows that apple fruit is mainly used for fresh marketing and 30% by volume of apple fruit (around 22.9 million tones) is processed industrially for

production of apple juice concentrate, cider, apple puree, dried apple, apple juice NFC, apple wine, sauce, jelly, and pulp. Apple juice concentrate is the leader of the processed products with 65% share of industrial processed apple. (Around 14.9 million tones) Approximately 5.7-5.8 million tones apple pomace is generated during apple processing in the word.

Table 2.1 Top ten countries by apple production per year (FAOSTAT)

Rank	Country	Apple Production (tones)
1	China	37.001.601
2	United States	4.110.050
3	Turkey	2.889.000
4	Poland	2.877.340
5	India	2.203.400
6	Italy	1.991.310
7	Brazil	1.810.000
8	Chile	1.625.000
9	Russia	1.403.000
10	France	1.382.900

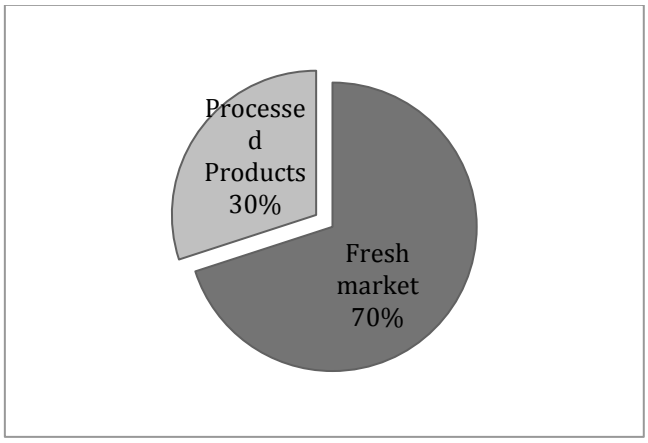


Figure 2.1 Worldwide apple fruit processing (Bhushan et al., 2008)

The waste apple pomace could be bioconverted into different value-added products such as pectin, polyphenols, fibers, enzymes, single cell protein, pigments, aroma compounds, alcohols, organic acids, polysaccharides, biohydrogen and antioxidant substances (Cordoba, 2012).

However, insufficient use of apple pomace causes important loss of biomass in worldwide.

2.1.1 Apple pomace composition

According to Carson et al. (1994), apple pomace has 2.2-3.3% seed, 0.4-0.9% stems and 70.0-75.7% apple flesh based on analysis of three apple cultivars (Cordoba, 2012).The composition of apple pomace can vary with variety, type of processing and number of press.

High amount of sugar content make apple pomace an excellent substrate for bioprocessesing. The other advantages of pomace are low cost and abundantly availability during harvesting season.

Average composition of apple pomace is given in Table 2.2 and Table 2.3 (Joshi et. al, 2006)

Table 2.2 Composition of 9 samples of apple pomace (M*: Moisture, A: Ash, Pr: Protein and minerals (P, K, Mn, Fe, Mg)

No	*M* (%w/w)*	*A (%w/w)*	*Pr (%w/w)*	*P (ppm)*	*K (ppm)*	*Mn (ppm)*	*Fe (ppm)*	*Mg (ppm)*
1	74.3	1.66	2.94	758	676	3.96	31.8	360
2	74.4	1.48	2.79	678	578	3.33	29.7	331
3	72.3	1.47	2.92	756	662	4.09	24.8	381
4	71.5	1.47	3.40	793	649	4.72	29.7	350
5	78.6	1.62	3.79	805	654	5.27	27.4	402
6	75.4	1.50	3.63	861	685	5.00	27.8	435
7	74.8	1.49	3.77	925	660	5.18	29.7	418
8	73.3	1.57	3.65	982	729	5.78	27.5	480
9	74.9	1.53	4.10	1090	678	5.49	33.3	489

*Data expressed directly from press

Table 2.3 Composition of extracts of 9 AP samples

Mass Fraction of extracts in AP samples *percent of oven dry apple pomace		**Mass fraction (percent of extract, oven dry basis)**			
No	***Mass fraction (%)***	***Saccharose***	***Glucose***	***Fructose***	***Xylose, mannose and galactose***
1	38.1	3.8	19.5	52.5	1.2
2	31.7	4.2	17.6	46.4	5.5
3	40.5	6.8	19.0	49.2	0.6
4	34.9	5.9	14.9	43.7	10.2
5	44.4	4.2	19.2	45.4	5.2
6	39.2	4.3	18.1	47.5	4.6
7	35.8	3.7	18.5	41.8	11.7
8	40.6	5.1	22.1	50.2	3.7
9	39.7	4.2	20.2	47.5	1.9

2.1.2 Apple pomace applications

Due to great potential of apple pomace for biotechnological processing, researchers' attention on this residue gradually increases. Since 1990, publications on apple pomace usage have increased continuously (Sudha et al, 2007). Main application areas with apple pomace are production of enzymes, organic acids, pectin, protein-enriched feeds, edible mushrooms, ethanol, butanol, natural antioxidants, aroma compounds, edible fibers, energy, phytochemicals and biofilms (Cordoba, 2012). Different purposes for apple pomace applications are given in Figure 2.2 and Figure 2.3.

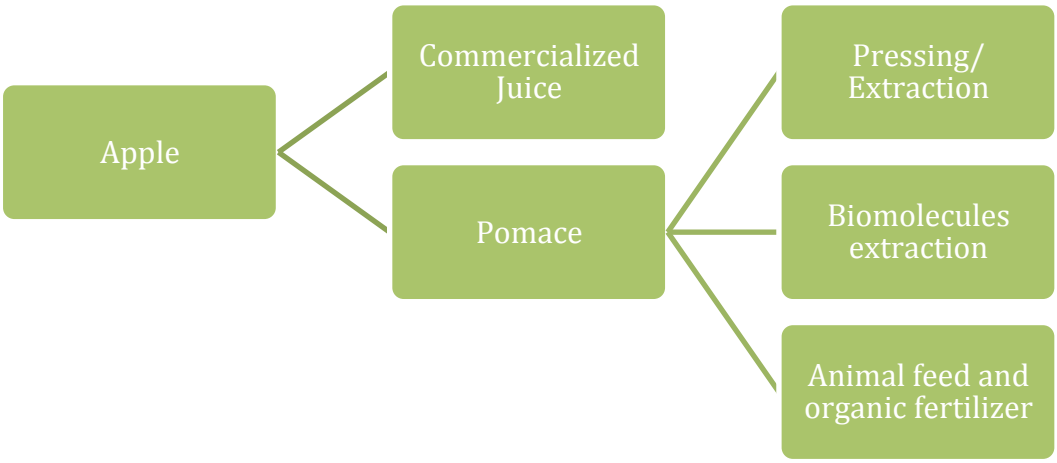
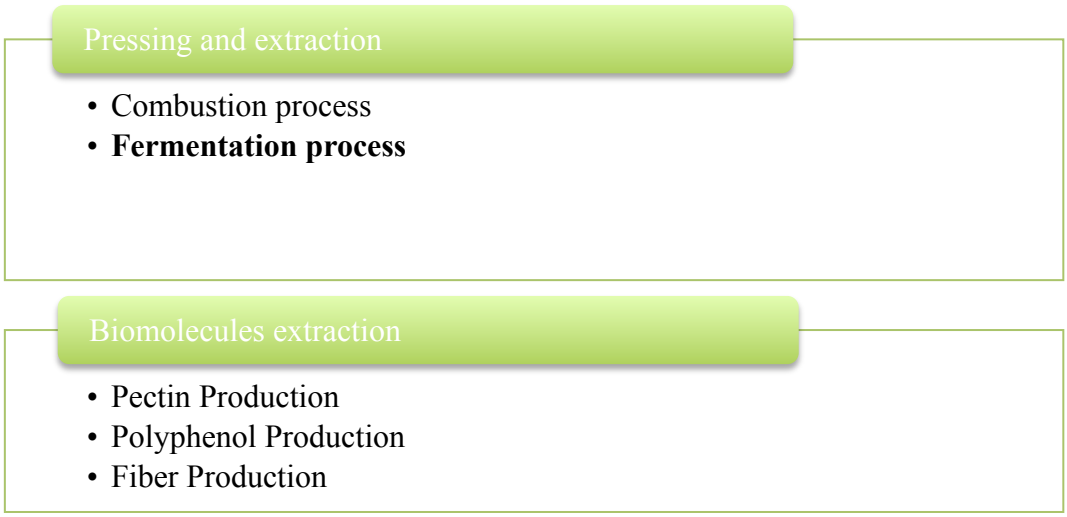


Figure 2.2 Apple pomace application-1



Fermentation Process
1. Production of Enzymes
2. Production of Aroma&Phenolic Compounds by Fermentation
3. Production of Alcohol (methyl, ethyl, propyl, butyl, and amyl alcohols)
4. Production of Hydrogen (Biofuels)
5. Production of Organic Acids (citric, lactic, acetic, linolenic acids)
6. Production of Polysaccharides (heteropolisaccharide-7-PS7-, xanthan)
7. Production of Biopolymers (chitosan)
8. Production of Edible Mushrooms
9. Production of pigments (carotenoids, melanins, flavins, quinones etc.)

Figure 2.3 Apple pomace application-2

There are numerous fermentation processes involving AP as seen from Figure 2.3. The most important area of AP utilization is the production of enzymes. Solid-state fermentation and submerged fermentation are used for enzyme production. For both methods; there are important process variables such as; temperature, pH, moisture content, and periodic mixing. Table 2.4 shows previous bioprocess studies using AP as the substrate

Table 2.4 Bioprocess studies using AP as substrate

Enzyme name	Organisms	Process	Reference
β-glucusidase	*Aspergillus foetidus*	SSF	Hang et al. (1994)
Polygalacturonase	*Aspergillus niger*	SSF	Hang et al. (1994)
Pectinase	*Polyporus squamosus*	SmF	Pericin et al. (1999)
Polygalacturonase	*Lentinus edodes*	SSF	Zheng and Shetty (2000)
Lignocellulolytic ezymes	*Candida utilis*	SSF	Villas-Boas et al. (2002)
Pectin metylesterase	*Aspergillus niger*	SSF/SmF	Joshi et al. (2006)
Pectinase	*Aspergillus niger*	SSF	Josh et al. (2008)
Xylanase	*Aspergillus niger*	SSF	Liu at al. (2008)
Cellulase	*Aspergillus niger*	SSF	Tao et al. (2009)
Pectinase	*Aspergillus niger*	SSF	Tao et al. (2009)
Proteinase	*Aspergillus niger*	SSF	Tao et al. (2009)
Ligninolytic enzymes	*Phanerocheate chrysosporium*	SSF	Gassara et al. (2010)
Cellulase	*Trichoderma sp.*	SSF	Sun et al. (2011)
Cellulase Hemicellulase	*Aspergillus niger*	SSF	Dhillon et al. (2012)

2.2 Enzymes

Enzymes are generally described as a protein with catalytic power (David L. Nelson, 2004). They are specific, versatile, and very effective biological catalysts, which increase the rate of reactions by factors 10^6 to 10^{12}; Enzymes can do this by reducing the 'activation energy' of the reactions. Activation energy is the total required energy to convert reactants into products.

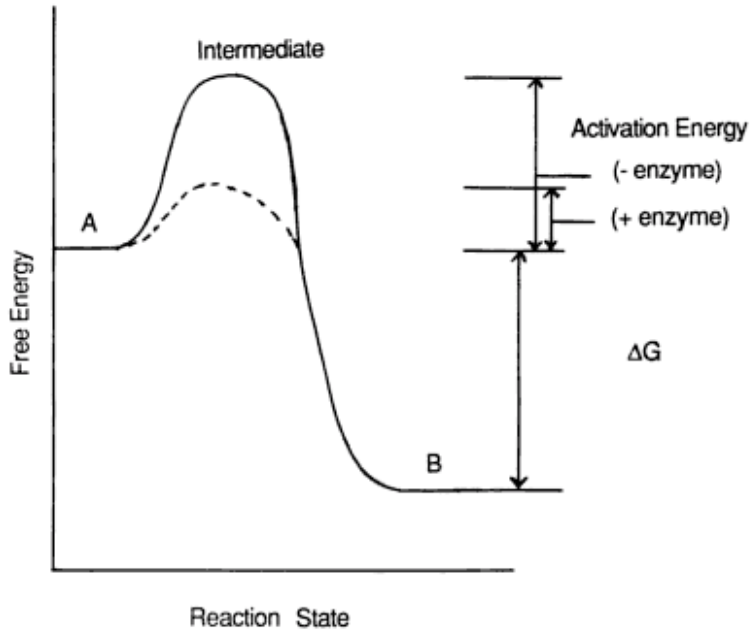


Figure 2.4 Activation energies of enzymatically catalyzed and uncatalyzed reactions (David L. Nelson, 2004)

Specificity is one of the important aspects of the enzymes. Lock and key mechanism can simply show this specificity of both substrates and enzyme. Fisher suggested the concept of an active site in 1984 to explain the specificity of enzymes. (Nelson, 2004) Active site of the enzyme has particular shape to perfectly match with its corresponding substrate

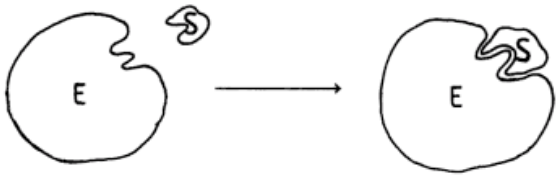


Figure 2.5 Schematic of the lock-and-key model of enzyme catalysis (David L. Nelson, 2004)

The molecular aspects of enzyme-substrate interaction have not been fully understood yet. Weak forces, such as van der Walls and hydrogen bonding are mainly responsible for this interaction.

2.2.1 Classification of enzymes

Enzymes are classified based on their mode of reaction. In Table 2.5, the broad classification of enzymes is listed based on the catalyzed reaction types.

Enzymes have a significant role in food industry. Food processing enzymes are included into several of these classes. In Table 2.6, important food enzymes and their role in processing are given with internal classification (Pedro Fernandes, 2010).

Table 2.5 International classifications of enzymes

CLASS	TYPE OF REACTION
OXIDOREDUCTASES	Oxidation-Reduction
TRANSFERASES	Transfer of functional groups
HYDROLASES	Hydrolysis
LYASES	Breaking of bonds
ISOMERASE	Isomerization
LIGASES	Joining of molecules

Table 2.6 An overview of enzymes used in food and feed processing

Class	Enzyme	Role
Oxidoreductases	Glucose oxidase	Dough strengthening
	Laccases	Clarification of juices, flavour enhancer (beer)
	Lipoxygenase	Dough strengthening, bread whitening
Transferases	Cyclodextrin Glycosyltransferase	Cyclodextrin production
	Fructosyltransferase	Synthesis of fructose oligomers
	Transglutaminase	Modification of viscoelastic properties
Hydrolases	Amylases	Starch liquefaction and sachcarification Increasing shelf life and improving quality Bread softness and volume,flour adjustment Juice treatment, low calorie beer
	Galactosidase	Viscosity reduction in lupins and grain legumes
	Glucanase	Viscosity reduction in barley and oats
	Glucoamylase	Saccharification
	Invertase	Sucrose hydrolysis,whey hydrolysis
	Lipase	Cheese flavor, in situ emulsification for dough conditioning
	Proteases	Protein hydrolysis,milk clotting,flavor enhancement in dairy products
	Pectinase	**Mash treatment, juice clarification**
	Peptidase	Hydrolysis of proteins
	Phospholipase	In-situ emulsification for dough conditioning
	Phytases	Release of phosphate from phytate,enhanced digestibility
	Pullulanase	Saccharification
	Xylanases	Viscosity reduction,enhanced digestibility,dough conditioning
Lyases	Acetolactate decarboxylase	Beer maturation
Isomerases	Xylose (Glucose) isomerase	Glucose isomerization to fructose

Food enzymes are mainly used in baking, beverages and brewing, dairy, dietary supplements, as well as fats and oils (Pedro Fernandes, 2010). Most commercial enzymes are hydrolytic with proteases (46%) and carbohydrase (47%) being the most common (Righelato and Rodgers, 1987). The most important carbohydrases are amylases (α-amylases and glucoamylases), invertases, galactosidases, glucosidases, fructosyltransferases, pectinases and glucosyltransferases (Contesini et al., 2013).

2.2.2 Pectin and pectolytic enzymes

Pectin is a complex structural polysaccharide and present in the middle lamella and primary cell wall of higher plants (J. Visser et al., 1996).

Pectin consists of three major polysaccharide groups, which can be seen in Figure 2.6. Homogalacturonan is a linear chain of α-(1–4) linked D-galacturonic acids which can be acetylated and/or methyl esterified. RG I consists of repeating disaccharide rhamnose- galacturonic acid. RG II is a homogalacturonan chain with complex side chains attached to the galacturonic residues. (Pedrolli et al.,2009)

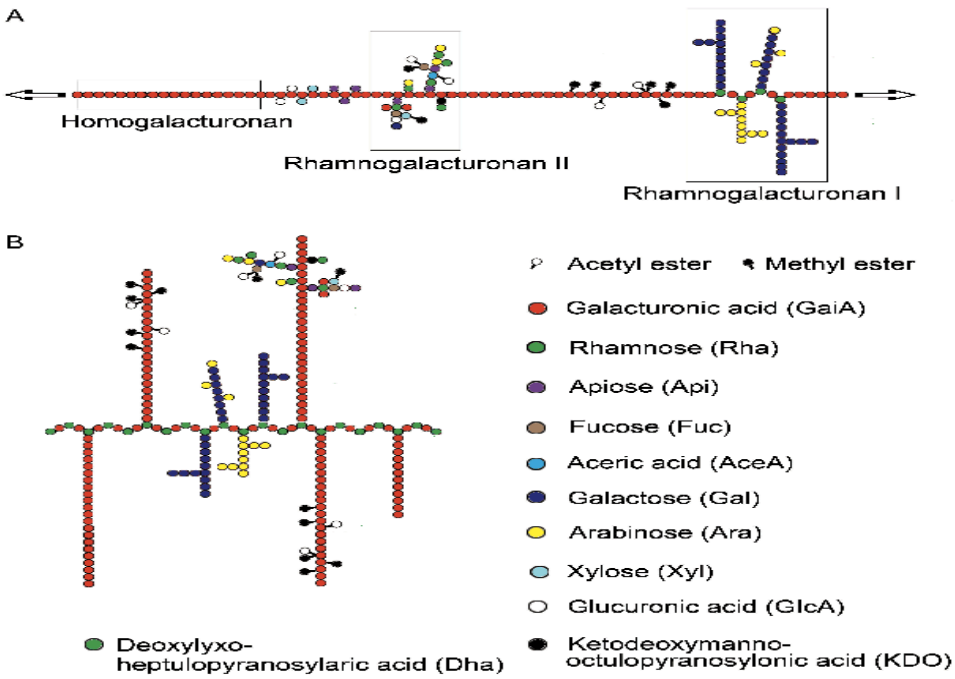


Figure 2.6 The basic structure of pectin as conventional (A) and recently proposed alternative (B) (Pedrolli et al.,2009)

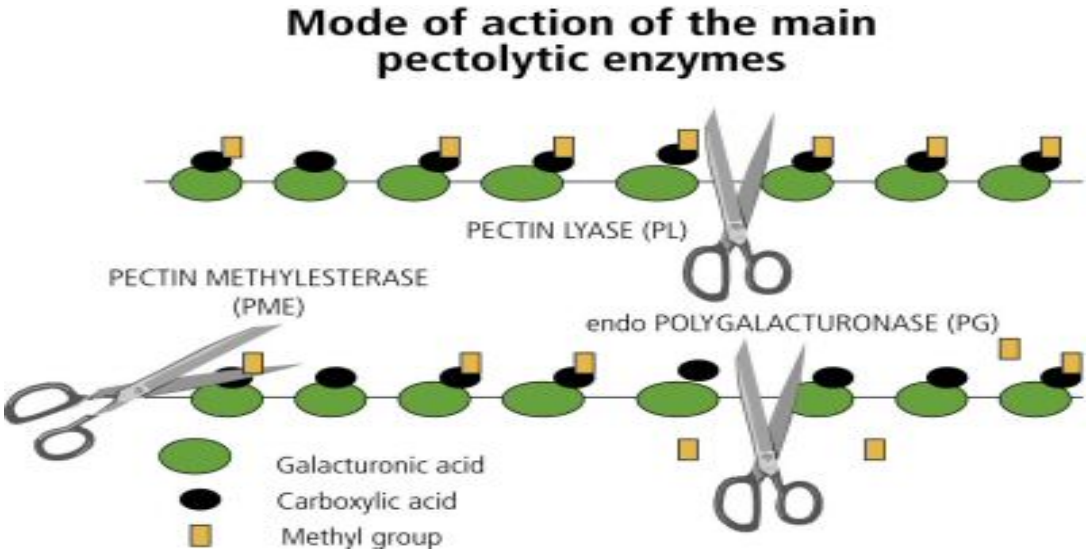


Figure 2.7 Mode of action of pectinases (Pedrolli et al.,2009)

Pectin can be used as gelling agents in the food industry; moreover enzymatic degradation or modification of pectin plays an important role in the processing steps of foods and beverages (J. Visser et al., 1996).

Pectinases are a large group of enzymes that break down pectin found in plant cell walls. Depolymerization (hydrolases and lyases) and deesterification (esterases) reactions are carried out during pectinase activity.

The well-known and mostly studied pectinase enzymes (*polygalacturonase, pectin methyl esterase and pectin lyase*) are shown in Fig 2.7 with their mode of action. *Pectin lyase* can work for cleavage of (1->4)-alpha-D-galacturonan methyl ester to give oligosaccharides. *Pectinesterases* also known as *pectinmethyl esterase,* catalyzes deesterification of the methoxyl group of pectin forming pectic acid. The enzyme acts preferentially on methyl ester group of galacturonate unit next to a non-esterified galacturonate units (Cosgrove, 1997). *Polygalacturonases* catalyze hydrolysis of α (1,4)-glycosidic linkages in pectic acid (polygalacturonic acid). There are two types of polygalacturonases as *Endo PG* and *Exo-PG*

Table 2.7 Extensive classification of pectinases (Kiran et al, 2007)

Name of Enzyme	Primary substrate	Product
Esterase		
1-Pectin methyl esterase	Pectin	Pectic acid+methanol
Depolymerizing enzymes		
a) Hydrolases		
1-Protopectinases	Protopectin	Pectin
2-Endopolygalacturonase	Pectic acid	Oligogalacturonates
3-Exopolygalacturonase	Pectic acid	Monogalacturonates
4-Exo polygalacturonan-digalacturono hyrolase	Pectic acid	Digalacturonates
5-Oligagalacturonate hydrolase	Trigalacturonate	Monogalacturonates
6- 4:5 unsaturated oliigogalacturonate hydrolases	4:5(Galacturonate)n	Unsaturated monogalacturonates & saturated (n-1)
7-Endopolymethyl-galacturonases	Highly esterified pectin	Oligomethyl galacturonates
8-Endopolymethyl-galacturonases	Highly esterified pectin	Oligogalacturonates
b) Lysases		
1-Endopolygalacturonase lyase	Pectic acid	Unsaturated oligogalacturonates
2-Exopolygalacturonase lyase	Pectic acid	Unsaturated digalacturonates
3-Oligo-D-galacturonase lyase	Unsaturated digalacturonates	Unsaturated monogalacturonates
4-Endopolymethyl-D-galactosiduronate lyase	Unsaturated poly-(methyl-D-digalacturones)	Unsaturated metyloligogalacturonates
5-Exopolymethyl-D-galactosiduronate lyase	Unsaturated poly-(methyl-D-digalacturones)	Unsaturated metylmonogalacturonates

Endo-PG catalyzes random hydrolysis of α (1,4)-glycosidic linkages in pectic acid,wherease; *exo-PG* catalyzes hydrolysis in a sequential fashion of α-1,4-glycosidic linkages on pectic acid (Tapre, A.R. et al.,2014).

2.2.3 Production of enzymes and enzyme assay

Commercial enzyme production has grown for more than a hundred years due to expanding markets and increasing demand for novel biocatalysts. Microorganisms can be considered as the major source of enzymes, however several enzymes are also obtained from animal or plant sources.

Since the early 1960s, microbial enzymes have gradually substituted those from animal and plant sources (Lambert and Meers, 1983). They might now represent approximately 90% of total market. Main reason is that microorganisms are excellent systems for enzyme production. They are quite versatile and easy to grow. Their nutritional requirements are easy to supply; also it is simple to make modification on their genetic identity.

Microbial enzyme production steps are shown in Figure 2.8. Actually the production process consist of 4 main stages:

- *Enzyme synthesis:* Fermentation step in the figure represents this stage
- *Enzyme recovery:* Involving solid- liquid separations, cell extraction or concentration steps to extract enzyme from producing cell system.
- *Enzyme purification:* Aiming to remove unwanted materials from crude enzyme.
- *Enzyme product formulation*: Involving final polishing operations, stabilization and standardization to give final product

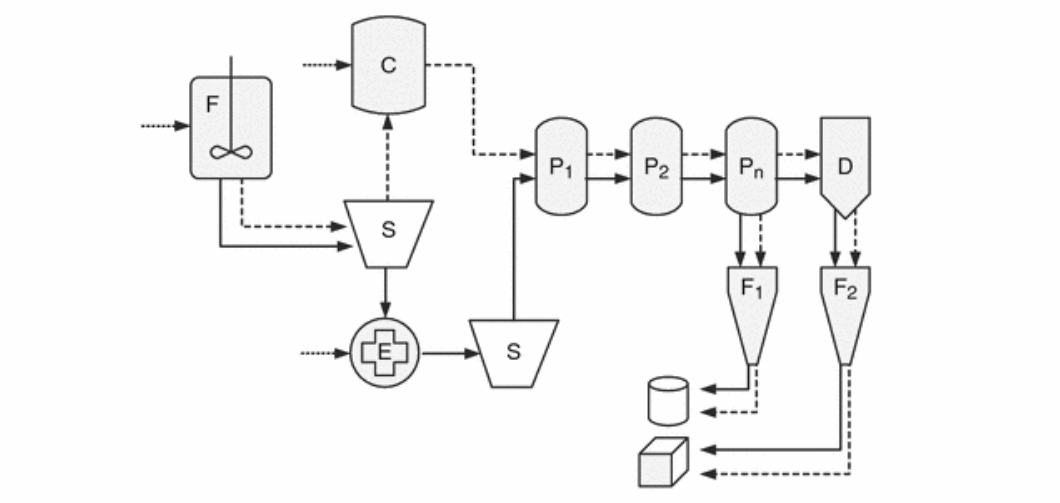


Figure 2.8 Scheme for the production of enzymes. F: fermentation; S: solid–liquid separation; E: cell extraction; C: concentration; Pi: operations of purification; D: drying; Fi: formulation

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and yeast. Common products of fermentation are carbon dioxide and alcohol. Moreover, several additional compounds, apart from these usual products; are produced during this metabolic breakdown. These additional compounds are called secondary metabolites. Enzymes are one of the secondary metabolites. There is a wide range of secondary metabolites from several antibiotics to growth factors (Balakrishnan and Pandey, 1996; Machado et al.,.2004; Robinson et al., 2001).

Microbial enzymes are mainly produced by fermentation under controlled conditions constituting now the most relevant option for enzyme synthesis. Bacteria and yeast can be used to produce a wide range of useful products. Fermentation performance is affected by the used culture and process conditions (Yang, 2006).

There are two methods of fermentation for enzyme production: Submerged fermentation (SmF) and solid-state fermentation(SSF). At the research level, both SSF and SmF have been used. Microbial enzymes are mainly produced by SmF under tightly controlled environmental conditions (Rose 1980). However, SSF has also a good potential for the production of enzymes (Raimbault 1998; Pandey et al. 1999).

Enzyme assays are laboratory methods for measuring activity of an enzyme. A knowledge of the activity of enzymes is required because of the below reasons:

- To search production and isolation of enzymes
- To determine and fix the properties of commercial preparations
- To establish the proper amount of enzyme to add for a particular commercial process (Yang, 2006).

2.2.3.1 Submerged fermentation (SmF)

Submerged fermentation (SmF) is defined as a fermentation process by the organism grown in a liquid medium in large tanks called fermenters. The bioactive compound like an enzyme is secreted into a liquid medium (nutrient broth).

Since the medium is at submerged state in a liquid form, heat and mass transfer is efficient and scale-up is relatively easy for SmF. It provides a better process control and analysis during medium optimization (P.Saranraj et al., 2014)

According to Bhargav (2007); production of polygalactouronase (PG) and pectin esterase (PE) was 6.4 times higher in SSF compared to submerged fermentation, however; SmF is more commonly applied for production of enzymes (Kumar et al. 2011) due to easier control at a large scale and shorter period (SSF requires

long fermentation periods (e.g. 5-6 days)) (Ustok et al., 2007). Most fermentation industries today use the submerged process for production of microbial products.

2.2.3.2 Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials like bran, bagasse and rice in the absence of free water (Subramaniyam et al. 2012).

Because of only solid material usage; nutrient-rich waste materials can be easily converted into substrates for SSF; which is main advantage of this technique. Other advantages can be listed as high volumetric productivity, low equipment cost, better yield of product and less waste generation (Bhargav et al, 2007). However, solid-state is limited on mass and heat transfers and also it is hard to set factors because of handling solids (Yang, 2006).

SSF can match perfectly with fermentation techniques involving fungi and other microorganisms that require less moisture content, since fungi can grow in nature on solid substrates. However, SSF cannot be used in fermentation processes involving organisms that require high water activity (a_w) (Babu and Satyanarayana, 1996).

Comparison between SSF and SmF is given in Table 2.8 (Dubasi Govardhana Rao, 2010).

Table 2.8 Comparison between SSF and SmF

Characteristic feature	SSF	SmF
Condition of microorganism and substrate	Static	Agitated
Status of substrate	Crude	Refined
Nature of microorganism	Fungal	-
Availability of water	Limited	High
Supply of oxygen	By diffusion	By bubbling/ sparging
Contact with oxygen	Direct	Dissolved oxygen
Requirement of fermentation medium	Small	Huge
Energy requirements	Low	High
Study of kinetics	Complex	Easy
Temperature and concentration gradients	Sharp	Smooth
Controlling of reaction	Difficult	Easy
Changes of bacterial contamination	Negligable	High

2.3 Inoculum type

Inoculum type is one of the important variables of fermentation. Fermentation may require only a single species of microorganism or mixed culture organism. For single culture studies; the substrate should be sterilized, to remove undesirable microorganisms prior to inoculation with the desired microorganisms. On the other hand, generally in food processing; mixed culture fermentation is applied to reach desirable final product. It requires participation of several microbial species. These species can act simultaneously or sequentially to give desirable product (Christi, 1999).

2.3.1 Mixed culture

Dynamics of mix cultures is important both in commercial fermentations (Shuler and Kargi, 2002) and in all natural fermentation processes (Shuler and Kargi, 2002). Numerous food fermentations, such as cheese manufacture, wine production etc. are applied by mixed culture to manufacture desirable product with respect to colour, flavor and aroma. It is important to evaluate interaction between two microorganisms in mixed culture to adjust effects on the fermentation. Scheme of classification of mixed culture interactions is shown in Table 2.9 (Smid EJ, 2013)

Two main advantages can also be mentioned for mix cultures: higher production yield and increased shelf –life of the product. One of the bacterial polymer, polyhydroxyalkanoates (PHA) was produced by using mix culture by Thomas Shalin et al. (2013); who discovered that mix culture had the potential to produce large amounts of PHA with seemingly lower costs due to lower sterility, equipment and control requirements. Antagonistic effect of mix culture can prevent the growth of undesirable microorganisms and by this way, longer shelf-life is achieved.

Table 2.9 Classification of mixed culture interactions

Effect of presence of B on growth rate of A	***Effect of presence of A on growth rate of B***	***Qualifying remarks***	***Name of interaction***
-	-	Negative effects caused by removal of resources	*COMPETITION*
-	0		
-	-	Negative effects caused by production of toxin or inhibitors	*ANTOGONISM*
-	0		*AMENSALISM*
-	+	Negative effects caused by production of lytic agent; positive effect caused by solubilization of biomass	*ECCRINOLYSIS*
+	0	Positive effects caused by production by B(host) of a stimulus for growth of A(commensal) or by removal by B of inhibitor for growth of A	*COMMENSALISM*
+	+	See remarks for commensalism. Also presence of both populations is not necessary for growth of both	*PROTO-COOPERATION*
+	+	See remarks for commensalism. Also, presence of both populations is necessary for growth of either	*MUTUALISM*
-	+	B feeds on A	*FEEDING*
-	+	The parasite (B) penetrates the body of its host(A) and converts the host's biomaterial or activities into its own	*PARASITISM*
+	0 or +	A and B are in physical contact; interaction highly specific	*SYMBIOSIS*
-	-	Competition for space	*CROWDING*

In another study; a positive effect of mixed culture fermentation on enzyme production was reported; Bertrand Tatsinkou Fossi et al. (2014) studied interactions occurring between *Saccharomyces cerevisiae* and two thermostable α-amylase producing strains (*Bacillus amyloliquefaciens 04BBA15* and *Lactobacillus fermentum 04BBA19)* They reported two types of interactions in mixed culture; commensalism between *S.cerevisiae* and *B.amyloliquefaciens 04BBA15* and mutualism between *S.cerevisiae* and *L. fermentum 04BBA19.*Also, they concluded that α-amylase production in mix culture was higher than monoculture. Therefore they recommended microbial interaction for use in the enhancement of industrial microbial enzyme production.

2.4 Pectinase production by *Bacillus* species

Pectinases are produced by a wide range of microorganisms including bacteria,yeast and fungi. Generally the enzymes used in the food industry are fungal origin; such as *Aspergillus niger,* because fungi has a high producing capacity of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3.0 to 5.5 (R.C Patil, 2012); however this acidic pectinases; produced from fungi, is not suitable for production of vegetable purees or other preparations in which pH values are close to neutral (R.C Patil, 2012). According to Torimiro and Okonji (2013), pectinases from fungal sources can only be used under acidic pH; however it avoids using these enzyme preparations in various industrial processes where neutral to alkaline pH. In the industrial sector; alkalophilic pectinases have immense use in the degumming of ramie fibers (Cao et al. 1992), retting of flax (Sharma 1987), plant protoplast formation and treatment of effluents discharged from fruit processing units (Tanabe et al. 1987). Alkaline pectinases are produced from alkalophilic bacteria, mainly Bacillus spp. (Kashyap et al. 2000)

Torimiro and Okanji reported pectinase production from *B.cereus, B.subtilis* and *B.stearothermophilus* and showed that three *Bacillus* species have the capacity to produce pectinase.

Soares et al. (1999) screened bacterial strains for pectinolytic activity and five *Bacillus* sp were reported to indicate an excellent activity when assayed by the plate method. Submerged medium prepared in 125ml Erlenmeyers flasks with 25 ml of medium and consisted of 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4$ $7H_2O$ at pH 6.0. Meanwhile, solid state medium was employed using a 250 ml Erlenmeyer flask containing 5g of wheat bran and 10 ml of 1% $(NH_4)_2SO_4$ and 0.02% $MgSO_4$ (67% of moisture). Both media were inoculated with a suspension containing 10^6 cells/ ml for SmF and 10^6 cells per gram for SSF. Both experiments were done at 30 °C. Fermentation periods were reported as 48 h for SmF and 72 h for SSF. The results of this study are given in Table 2.10

Table 2.10 Production of PG (U/mL) by *Bacillus* sp in solid state (SSF) and submerged (SmF) fermentation

Microorganisms	SSF	SmF
Bacillus sp Ar1.2	0.8	3.5
Bacillus sp B1.3	0.4	3.0
Bacillus sp M2.1	0.7	4.0
Bacillus sp P4.3	0.3	4.0
Bacillus sp P6.1	0.4	2.7

Another study carried out on optimization of polygalacturonase (PG) production using *Bacillus subtilis* in submerged fermentation by Plackett–Burman (PB) design and response surface methodology (RSM). The results showed that a

maximal PG activity of 5.60 U/mL was achieved by using hazelnut shell hydrolysate in the condition of 0.5%(w/v) yeast extract, pH: 7.0, 72 h of fermentation time, 30 °C of temperature and 0.02% (w/v) K_2HPO_4 (Uzuner and Cekmecelioglu, 2015).

Maximum activity is higher in Uzuner's study (2015) when we compare with Soares' study (1999). The reason can be considered as efficient hydrolysis techniques on hazelnut shell and using optimum blend of nutrients and physical parameters to evaluate hazelnut's potential of high PG activity.

Sharma and Satyanarayana (2005) carried out on optimization of polygalacturonase (PG) production using *Bacillus pumilus* in submerged fermentation by Plackett–Burman (PB) design and response surface methodology (RSM). In this study; it was confirmed that C:N ratio, K_2HPO_4 concentration and pH are significant parameters that affect pectinase production by using Plackett-Burman design. After that; the three significant factors were further optimized by RSM. Maximum pectinase activity was found as 20.69 U/ml by using micronutrient solution with 0.5% citrus pectin, 0.25% $MgSO_4$, 0.5% Na_2HPO_4, 0.1%(w/v) yeast extract, 0.3% (w/v) K_2HPO_4 and 0.163% casein at 40 °C, pH: 9.5 for 24 h.

Exo-pectinase production by marine *B.subtilis* and using citrus limetta peels was evaluated up to 40 h of submerged fermentation by Joshi et al.(2013).Maximum pectinase activity was found as 24.18 U/ml after 28 h of incubation. pH, temperature, concentration of citrus limetta and concentrate as yeast extract were found optimum as 5.00 and 40 °C, %3.0 and %2.5. Citrus limetta peels are very promising substrate for pectinase production (Joshi et al.,2013). The high pectinase production was provided by high amount of citrus limetta and yeast extract that can increase enzyme production rate.

Bacillus species are becoming popular producers in food industry. It is clear that *Bacillus* species are robust and efficient strains and are preferred hosts for a number of new and improved products (Schallmey et al., 2004).

2.5 Aim of the study

Pectinases are an enzyme group, which breaks down pectin, found in cell walls of plants. There are several applications of pectinases from different fields such as food, textile, waste water treatment and paper&pulp. Important examples of these applications are extraction and clarification of fruit and vegetable juices and wines, acceleration of the tea fermentation process, elimination of mucilaginous coat from coffee beans, vegetable oil extraction, manufacture of animal feeds, softening during the process of pickle fermentation (Suneetha V et al.,2014).

Apple pomace is considered as agricultural waste by most of the countries, it is produced in large amounts all around the world and either burnt in the field or used as animal feed. However apple pomace is lignocellulosic material, therefore it has a large potential to use in the production of industrial goods such as, enzymes.

It is critical to develop efficient methods to produce pectinase. Pectinases are commercially produced using *Aspergillus spp.*, however; the bacterial strains like *B. subtilis and B. pumilus* have excellent capacity to produce high quantities of microbial pectinases when optimal conditions are provided. There is no study on the pectinase production by *B. subtilis and B. pumilus* from apple pomace.

Most of the studies are focused on single culture to produce pectinase. To discover importance of microbial interaction during enzyme synthesis; mixed culture fermentation was carried out in our study.

The main objective of this work to examine potential of apple pomace as natural substrate for pectinase production. In considering sensitivity about the environment, using one of the biggest agricultural wastes in a production of industrial good can give distinct feature to this study. From enzyme industry's point of view; using apple pomace as a substrate is very productive and can overcome high enzyme production cost. It is also possible to prevent environmental pollution by using one of the industrial waste in the production of enzymes.

In this study, it was aimed to enhance alkaline pectinase production using apple pomace as carbon source by co-culturing of *B.subtilis and B.pumilus.* Cullture conditions including pH, amount of solid load and inoculum ratio in mix culture (*B.pumilus/B.subtilis*) were optimized by using response surface methodology (RSM).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw materials and chemicals

Apple pomace was obtained from Goknur Foodstuff Company in Niğde, Turkey. It is the residue from apple concentrate production. Apple varieties are Amasya and Starking.

Pre-treatment of apple pomace was initiated by drying at 70 °C in a laboratory scale tray dryer (Eksis Endustriyel Kurutma Sistemleri, Isparta, Turkey) for 4 h. It was ground by a laboratory type-grinding mill (Thomas-Wiley Laboratory Mill, Model 4, Arthur H.Thomas Company, Philadelphia, PA, USA) to pass 1 mm sieve to increase efficiency of acid hydrolysis. The ground apple pomace were stored inside plastic bags and kept at room temperature until use.

All the chemicals used in this study are listed in Appendix A

3.1.2 Microorganisms and medium preparations

Bacillus pumilus NRRL NRS-272 and *Bacillus subtilis* NRRL B-4219 were kindly provided by Northem Regional Research Laboratory

(NRRL),Peoria,Illinois, USA. Microorganisms were activated on nutrient agar at 35 °C and 120 rpm to prepare stock cultures.

Growth media were used to prepare seed cultures of each fermentation experiment, which was prepared in 250 ml Erlenmeyer flask. Incubation condition was constant at 35 °C,120 rpm and 24 h. Contamination was avoided using fresh inoculum, prepared every time for each experiment. Growth medium composition is given in Table3.1

Table 3.1 Growth medium (GM) composition

Components	G.M-Amount (g/100 mL)
Yeast Extract	0.1
Glucose	1
Pectin	0.2
$MgSO_4$	0.04
K_2HPO_4	0.04
KH_2PO_4	0.02

The submerged fermentation medium was prepared in 500 ml Erlenmeyer flask with 100 mL working-volume including ingredients and using apple pomace as carbon source. Fermentation medium consisted of apple pomace, yeast extract (5g/L), pectin (0.2 g/L), $MgSO_47H_2O$ (0.2 g/L), and K_2HPO_4 (0.2 g/L). Each flask

was inoculated with $5x10^8$ CFU/ 100 mL of inoculum and incubated under shaking (130 rpm) at 30 ^oC for 24 h.

3.1.3 Buffers and solutions

Buffers and solutions used in this study, are given in Appendix B.

3.2 Methods

3.2.1 Cell density

Optical density at 600 nm (OD_{600}) was measured to represent the cell density using a spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). The number of cells was determined by plate count method.

3.2.2 Acid pretreatment

Two different acid pretreatment methods were applied on apple pomace for comparison one-step acid and two-step acid hydrolysis methods (Sibel Uzuner, 2015).

One-step Acid Pretreatment: 1 g of dried and ground apple pomace was mixed with 20 ml of 4% H_2SO_4 and autoclaved at 121°C for 15 min to achieve *dilute acidic pretreatment.*

Two-step Acid Pretreatment: 1 g of dried and ground apple pomace was mixed with 10 ml of 72% H_2SO_4 solution and kept for 1 h at room temperature. Then, sample was diluted with 140 mL of distilled water and autoclaved at 121°C for 15 min. (Tomy SX-700E, Tomy Kogyo Co., Tokyo, Japan).

After pretreatment, vacuum filtration was applied to separate the supernatant and then pH of the supernatant was adjusted to required pH levels with %10 NaOH solution. Schematic of the pre-treatment is shown in Figure 3.1

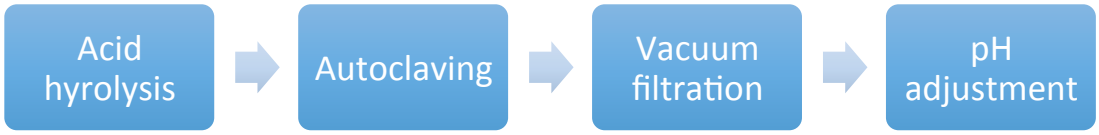


Figure 3.1 Schematic of one-step acid pretreatment

3.2.2.1 HPLC analysis of pre-treated samples

Sugar composition analysis were done using high performance liquid chromatograph (Perkin Elmer, Flexar LC, Ohio, USA) equipped with a refractive index detector for sugar. Waters Sugar Pak TM 1 6.5*300 mm column (Waters, MA, USA) was used. Samples (20 µL) were injected into mobile phase (HPLC grade water) at a flow rate 0.5 mL/min while the column temperature was kept at 75 °C. Retention times of standard solutions are given in Table 3.2

External standard stock solutions were composed of 1 g fructose 1 g glucose and 1 gr sorbitol in 100 mL water. Five different solution concentrations (0.10, 0.20, 0.30, 0.40, 0.50 g/L); in distilled water were used to prepare calibration curve.

Table 3.2 Retention time of standard stock solutions

Peak	Sugar	Retention time(min)
1	Sucrose	7.5
2	Glucose	9.4
3	Fructose	11.6
4	Sorbitol	15.8

3.2.3 Inoculum preparation

Growth media were prepared according to Table 3.1 and autoclaved at 121°C for 15 min. After cooling, 1 mL of stock cultures from each bacterium was inoculated into Erlenmeyer flasks and incubated at 35°C, pH: 7.0, 130 rpm in an incubator shaker for 20-22 h.

3.2.4 Enzyme production by submerged fermentation

Fermentation medium was prepared after acid hydrolysis pretreatment using 100 ml of supernatant mixed with the other ingredients after pH adjustment (between 7.0-9.0). Fermentation medium was then autoclaved at 121°C for 15 min. After cooling, it was incubated with pre-defined amount of culture (10^6 CFU/ml). To achieve this amount for both cultures, OD values of growth medium samples should be 0.6 for *B.pumilus* and 1.25 for *B.subtilis* (Uzuner & Cekmecelioglu, 2013) .

Fermentation medium was kept in an incubator shaker at 30°C, pH: 7.2 and 130 rpm for 24 h. The pectinase (polygalacturonase, PG) production steps aligned as shown in Figure 3.2

After fermentation, the biomass was separated by centrifugation at 2000xg for 20 min and the supernatant was used a crude enzyme in the pectinase assay.

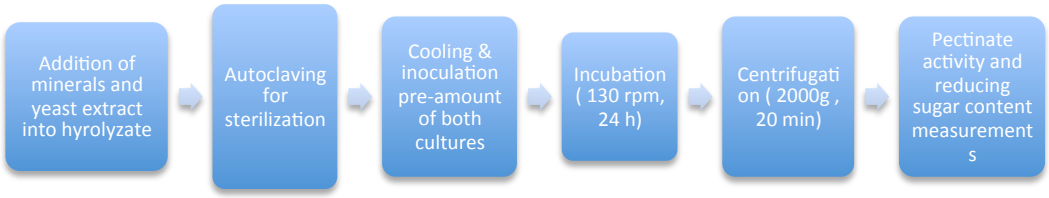


Figure 3.2 Schematic of pectinase production steps

In this experiment, three different fermentation variables were screened for the effects on the PG production. These variables are pH, substrate concentration and inoculum ratio of the bacterial strains.

3.2.5 Pectinase assay

The standard curve for enzyme assay was prepared using five different concentrations of D-galacturonic acid (0.1, 0.15, 0.20, 0,25, 0.30 g/L) in distilled water. Three ml of each D-galacturonic acid samples was mixed with 3 ml of DNS solution. Each tubes were mixed with vortex and put into 90 °C water bath for 15 min. After developing the red-brown colour, test tubes were taken out from water bath and 1 ml of 40% Rochelle salt was added to stop the reaction. The tubes were allowed to cool and finally absorbance was measured at 575 nm. Standard curve for enzyme activity is given in Appendix C.

The mechanism of polygalacturonase assay is based on measuring the release of reducing groups from polygalacturonic acid using 3.5 dinitrosalicylic acid (DNS) reagent (Miller, 1959). In this method; the number of reducing groups was expressed as galacturonic acid.

The polygalacturonic acid solution (%1) in phosphate buffer (pH: 7.0) was used as substrate in this assay. Half ml of cell free supernatant was incubated with 0.5 ml of polygalacturonic acid in 0.1 M phosphate buffer with pH: 7.0 and reaction mixture was incubated 50°C and 130 rpm for 30 min. After that, 60 µl reaction mixture was diluted with 2.940 µl distilled water to give %2 dilution rate and mixed with 3 ml DNS solution to stop the reaction. All tubes were placed in 90°C water bath for 15 minutes. 1 ml Rochelle salt was added finally and let the tubes for cooling. The absorbance was measured at 575 nm. The amount of enzyme, which release one µmol of galacturonic acid per minute under assay conditions, was defined as enzyme activity unit (U)

$$U/_{l} = Absorbance * F * \left(\frac{1}{incubation\ time}\right) * DF * \left(\frac{1}{212.12}\right) * R_v \qquad (1)$$

F*= a factor to convert absorbance to gr of galactronic acid by standard curve.*

Incubation time*= time of incubation of the enzyme with substrate (30 min)*

1/212.12*= conversion from grams of galacturonic acid to moles of galacturonic acid.*

DF*= Dilution factor*

R_v*=Ratio of total reaction volume to volume of crude enzyme*

3.2.6 Total reducing sugar

Total reducing sugar can act as nutrient source for mix culture fermentation. Since, total reducing sugar content is highly correlated with bacterial growth rate and pectinase production rate. Therefore, total reducing sugar content was determined in our study.

The DNS colorimetric method was used to determine total reducing sugar (Miller, 1959). This method measures the presence of free carbonyl group (C=O) as reducing sugar source.

DNS reagent was prepared each time for this analysis. It was described in Appendix B

To prepare standard curve of reducing sugar; glucose was used as external reducing sugar standard. Seven different glucose concentrations (0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40 g/l) ; in distilled water were used. 3 ml of sample was mixed with 3 ml of DNS reagent reduce 3,5- dinitrosalicylic acid (DNS) to 3-amino, 5-nitrosalicylic acid under alkaline condition. After developing the red-brown color, test tubes were taken out from water bath and %40 Rochelle salt was added as 1ml to stop reaction. The tubes were let to cool and finally absorbance was measured for each tube with spectrophotometer at 575 nm. Standard curve for DNS method is given in Appendix D. It was prepared based on absorbance level to calculate total reducing sugar content of fermentation medium by spectrophotometer.

3.2.7 Protein content

The 'Lowry Assay' was used to determine protein content in biological samples. (Lowry et al., 1951) Bovine Serum Albumine (BSA) was used as standard protein to prepare standard curve used for Lowry Method for the determination of total protein concentration. Appendix E.

The Lowry solution was prepared as described in Appendix F. Half ml of diluted sample was mixed with 0.7 ml Lowry solution in test tubes. After that, the test tubes were incubated at ambient temperature for 20 min in dark. Folin's reagent (0.1 ml) was added into each tube after incubation and mixed by vortex and

second incubation was carried out at ambient temperature for 30 min in dark. After this period, absorbance of each sample was measured by spectrophotometer at 750 nm against blank solution.

3.2.8 Biomass

Biomass is an amount of biological matter and in our study; represents the mix culture (*Bacillus pumilus* and *Bacillus subtilis*) amount in the medium.

During 24 h fermentation period; samples were taken from Erlenmeyer flasks for every 4 h. Analyses were duplicated for each replicate. The samples were centrifuged for 5 min and washed three times. After that, all tubes were put into drying oven at 70 °C for 24 h. By using below calculation, biomass of the samples was simply determined as below;

Net biomass (g/L) = wet biomass (g/L) – dry decrease biomass (g/L)

3.2.9 Preliminary test to fix fermentation period

Pectinase production by mixed culture (*Bacillus pumilus* and *Bacillus subtilis*) was carried out under constant temperature (30 °C), pH (7.2), agitation (130 rpm) inoculation volume (1ml of each culture) and substrate concentration as apple pomace (5 gr/100 mL) for 72 h. The samples were taken from Erlenmeyer flasks for every 6 h. Pectinase activity was calculated for each sample to determine the optimum fermentation time.

3.2.10 Optimization of pectinase production by response surface methodology (RSM)

RSM is a model with a collection of statistical techniques. Interactions between several variables can be identified with fewer experimental trials by the help of RSM (Tepe and Dursun, 2014). The series of experiments was designed using Minitab® 16.1.1 software (Minitab Inc., State Collage, PA, USA) for fermentation part of the study. The response surface methodology (RSM) with Box-Behnken design was constructed by 3 parameters (pH, substrate concentration and inoculum ratio- *Bacillus subtilis/Bacillus pumilus*-) with 3 levels (Table 3.3). The response was the enzyme activity as measured by polygalacturonase activity (PG)

A set of 15 experiments was constructed in two randomized replicates (Table 3.4) By this way, thirty experimental run were carried out with 6 center runs. The individual and combined effects of 3 variables were analyzed

Table 3.3 Range of variables for BB design for PG production

	Variable	Low level (-1)	Center (0)	High level (+1)
X_1	Solid concentration (g/100 mL)	5	10	15
X_2	Mix Ratio- B.subtilis/B.pumilus (% v/v)	1/4	3.4/1.6	4/1
X_3	pH	7	8	9

Table 3.4 Experimental design matrix of BB design for PG production

Run Order	pH	Solid load(%)	Mix ratio (*B.Subtilis / B.Pumilus*)
1	7	10	1/4
2	8	5	4/1
3	8	10	3.4/1.6
4	7	15	3.4/1.6
5	8	5	1/4
6	9	5	3.4/1.6
7	8	15	1/4
8	9	10	4/1
9	8	15	4/1
10	9	15	3.4/1.6
11	7	5	3.4/1.6
12	7	10	4/1
13	8	10	3.4/1.6
14	9	10	1/4
15	8	10	3.4/1.6
16	7	10	1/4
17	8	5	4/1
18	8	10	3.4/1.6
19	7	15	3.4/1.6
20	8	5	1/4
21	9	5	3.4/1.6
22	8	15	1/4
23	9	10	4/1
24	8	15	4/1
25	9	15	3.4/1.6
26	7	5	3.4/1.6
27	7	10	4/1
28	8	10	3.4/1.6
29	9	10	1/4
30	8	10	3.4/1.6

The results were checked whether normal or not. If not, data were normalized by Box-Cox method. Experimental design matrices with two replications were optimized by the response optimizer tool of the Minitab® 16.1.1.

95 % confidence level was used in all of the statistical calculations. ANOVA was performed.

Experimental data were fit to the quadratic equation (2).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

where Y is response (enzyme activity), b's are regression coefficients, and X_1,X_2,X_3 are amount of solid load, mix ratio, and pH respectively.

3.2.10.1 Model verification

The predicted models were verified by three verification experiments at optimum points. Performance of quadratic models was also evaluated by calculating coefficient of determination (R^2), root mean square error (RMSE) (Eqn. 3) and mean absolute error (MAE) values (Eqn. 4).

$$\mathrm{RMSE} = \left[\frac{1}{N}\sum_{i=1}^{N}\left(X_{pred,i} - X_{exp,i}\right)^2\right]^{0.5} \quad (3)$$

$$\mathrm{MAE} = \frac{1}{N}\sum_{i=1}^{N}\left|X_{pred,i} - X_{exp,i}\right| \quad (4)$$

where X_{exp} is the experimental value and X_{pred} is the predicted value of total reducing sugar concentration, N is the number of data.

3.2.11 Kinetic models of pectinase production from apple pomace by mix culture:

The kinetic study of Leudeking and Piret showed that the kinetics of product formation can be combined rate known as growth associated and non- growth associated (Bailey and Ollis, 1986). This model is given in Equation (5)

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \quad (5)$$

In this model, P represents pectinase and X represents biomass (amount of *B.subtilis and B.pumilus*), and α and β are growth and non-growth associated coefficients; respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth curve of *B.subtilis and B.pumilus*

Growth curves of both microorganisms were constructed under optimum growth conditions, determined in previous study (Sibel Uzuner, 2014). The standard growth media were inoculated with 1 mL *B.subtilis* and 1 mL *B.pumilus* separately and incubated at 35 °C and 130 rpm for 72 h. Fig 4.1 and Fig 4.2 show growth curves of *B.subtilis* and *B. pumilus,* respectively. The *B.subtilis* cells were observed to reach stationary phase after the 16^{th} hour of incubation whereas; *B.pumilus* cells reached the stationary phase after 28^{th} hour of incubation. When the growth curve of *B. subtilis* is examined in details, there is a small increase on the bacterial growth between 20^{th}-28^{th} hours of incubation.

There are opposing views on using cells at stationary phase or exponential phase. For example; amylase production by *B. subtilis* occurs mainly during the stationary phase. Another study on protease production, by submerged fermentation revealed that maximum level of enzyme production was during early stationary phase (Hindhumathi et al., 2011). However; maximum polygalacturonase production by *Bacillus sphaericus* (MTCC 7542) was found in the exponential phase of culture. Optimum length of exponential phase during fermentation lead to higher levels of enzyme production (Jayani et al., 2010). In

this study; the cultures were inoculated into fermentation medium at beginning of stationary phase between 22-24 h.

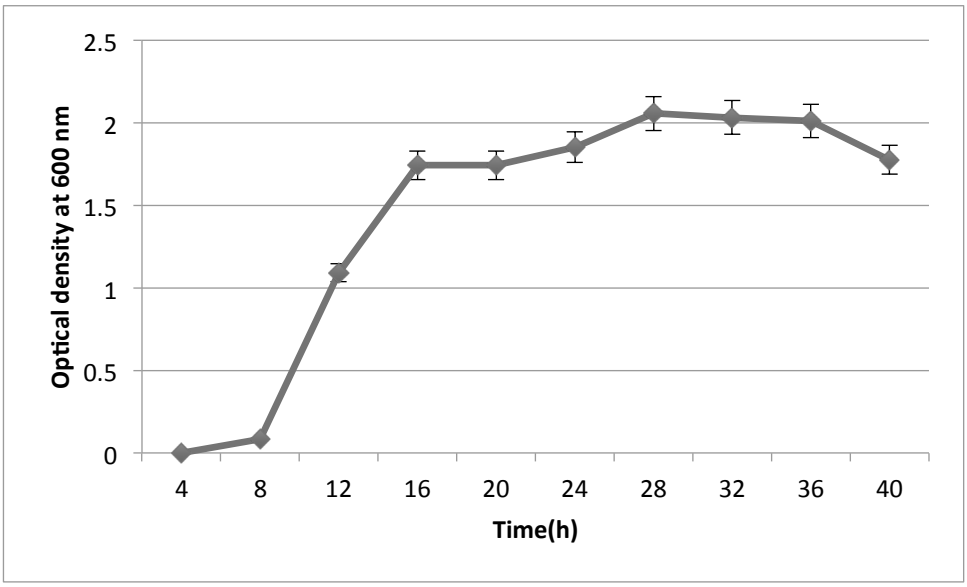


Figure 4.1 Growth curve of *B.subtilis*

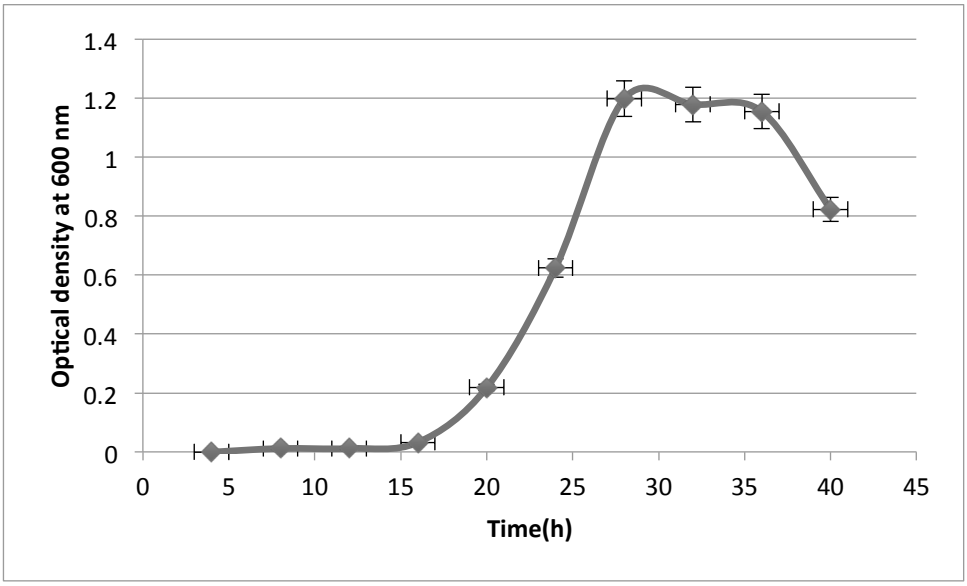


Figure 4.2 Growth curve of *B.pumilus*

4.2 Effect of pretreatment methods on fermentable sugar production

The sugar content released from Apple Pomace(AP) was analyzed after one-step and two-step acid pretreatment methods. One-step pretreatment was found more efficient based on DNS analysis results. Four different acid (H_2SO_4) concentrations (1%, 2%, 3% and 4%, w/w) were used at 121 °C for 15 min to assess the impact of acid concentration. The average results are shown in Fig. 4.3.Increasing the acid concentration from 1% to 4% (w/w) increased the reducing sugar concentration from 15.78 g/L to 20.25 g/L. Moreover, Table 4.1 shows that one-step acid hydrolysis is more efficient than two-step acid hydrolysis method.

Table 4.1 Reducing sugar concentration with respect to pretreatment method

Pretreatment method	Reducing Sugar Concentration(g/L)
One-step acid hydrolysis	20.26±0.53
Two-step acid hydrolysis	19.32±0.23

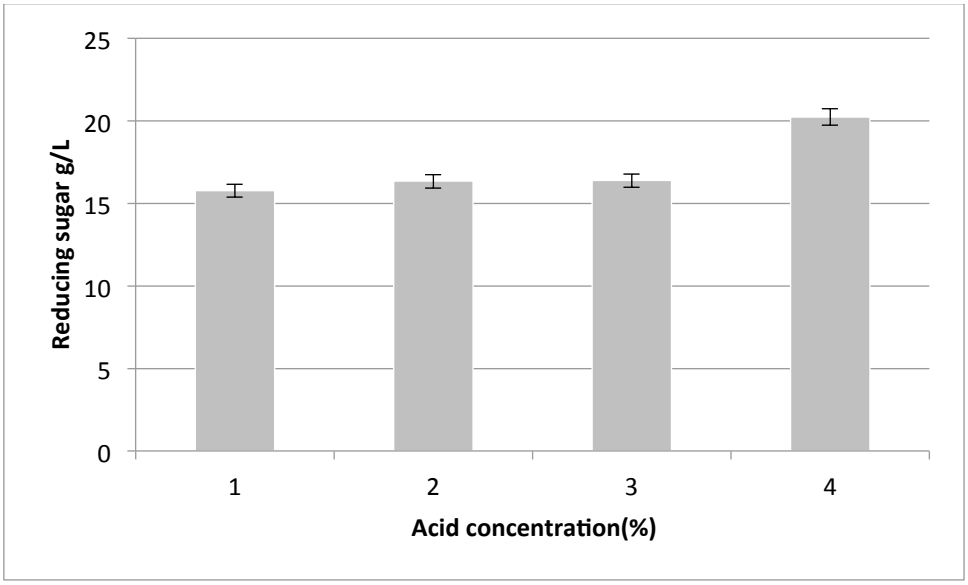


Figure 4.3 Pretreatment of apple pomace at various acid concentrations

Increasing the acid concentration leads to an increase in the concentration of hydrogen ions, by this way; the hydrolysis reaction rate increases and consequently high conversion of hemicellulose fraction into fermentable sugars was provided by the glycosidic bonds breakage (Kumar et al., 2009; Mosier et al.,2002).

4.2.1 Sugar composition analysis of pretreated AP by HPLC

HPLC chromatogram of pretreated apple pomace with %72 H_2SO_4 is given in Figure 4.4. Peaks were observed for glucose between 9^{th} and 10^{th} minutes; for fructose between 11^{th} and 12^{th}.

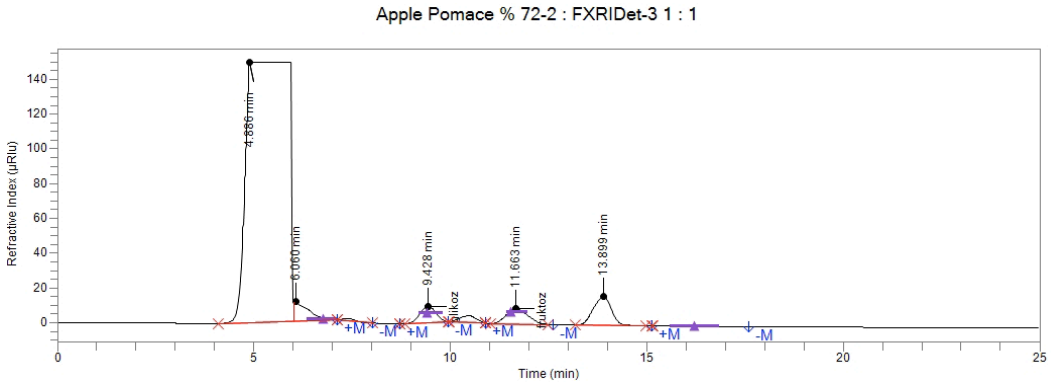


Figure 4.4 A sample of HPLC chromatogram of pretreated AP (%72 H_2SO_4)

HPLC chromatogram of pretreated apple pomace with %4 H_2SO_4 is given in Figure 4.5. Peaks were observed for glucose between 9th and 10th minutes; for fructose between 11th and 12th and for sorbitol between 16th and 17th minutes.

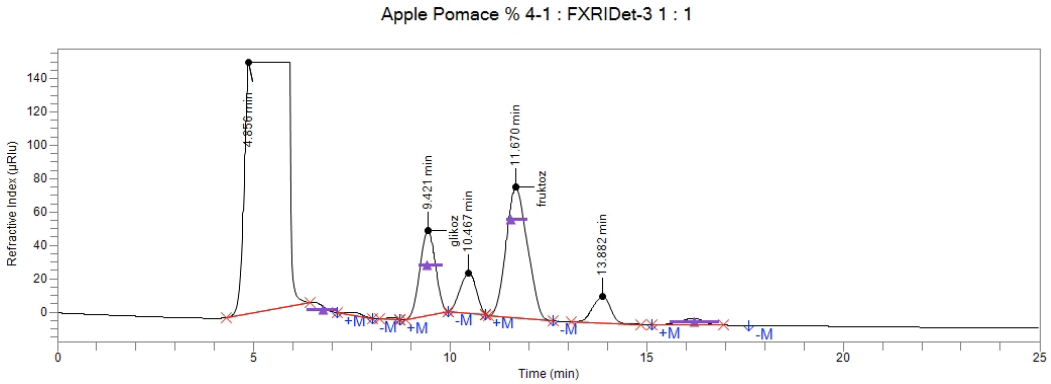


Figure 4.5 A sample of HPLC chromatogram of pretreated AP (%4 H_2SO_4)

Based on HPLC results, total reducing sugar amount of AP, which was pretreated with one-step acid hydrolysis, was more than AP, which was pretreated with two-step acid 49ydrolysis.

4.3 Initial screening experiments

Initial screening trials were carried out to fix the time variable by both synthetic and apple pomace media. During initial screening; all process variables were fixed i.e. temperature: 30 °C, pH: 7.2, agitation: 130 rpm, inoculation volume: 1 mL *B.subtilis* and 1 mL *B.pumilus*; solid load: 2 gr glucose/100 mL or 5 g apple pomace/100 mL Inıtial screening experiment results are given in Figure 4.6.

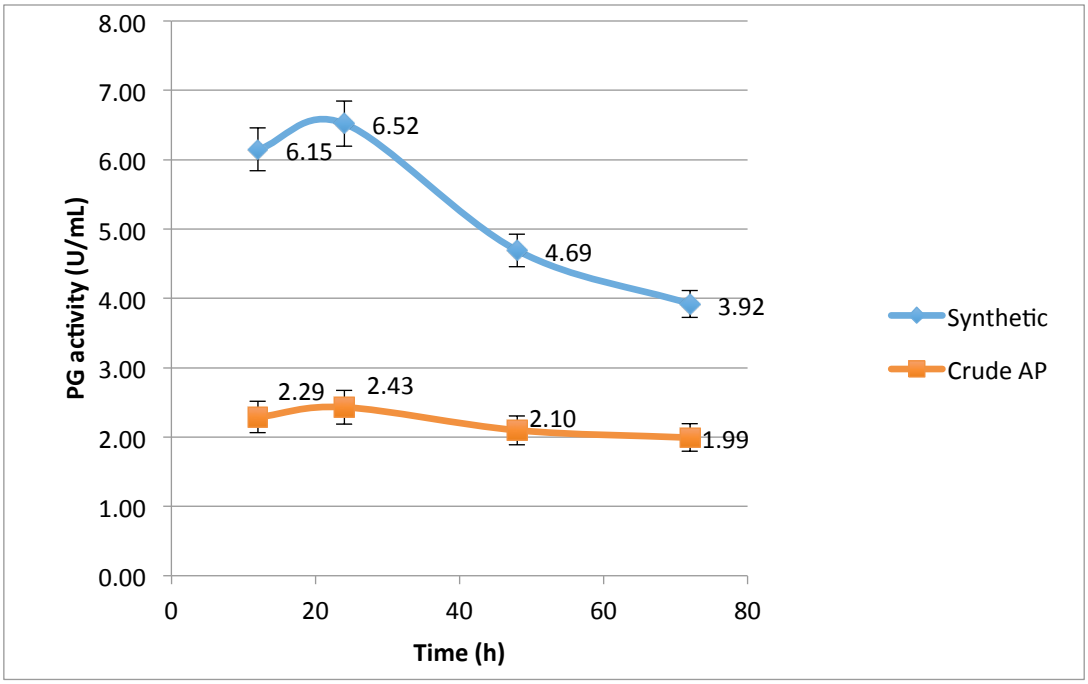


Figure 4.6 Synthetic medium (glucose) and Crude AP medium initial testing results

These results indicated that the highest enzyme production was achieved after 24 h fermentation time for both apple pomace and synthetic medium. Highest enzyme activity of synthetic medium was found as 6.521 U/mL, whereas highest enzyme activity of apple pomace was found as 2.430 U/mL. The main reason of

this difference is that no pre-treatment on apple pomace was applied before preliminary tests.

Different culture conditions such as pH, temperature and type of microorganism can affect the optimum time variable for pectinase enzyme production. However; we used the same culture conditions for both apple pomace medium and synthetic medium to minimize this effect and find the optimum fermentation time.

Sharma and Satyanarayana (2005) performed optimization analysis for PG production using *B. pumilus* in submerged fermentation and they also found maximum pectinase activity after 24 h fermentation.

4.4 Optimization by response surface method

Box-Behnken response surface optimization method was used as it offers advantages compared to CCC and CCF designs. Box-Behnken requires a fewer number of runs for three factors by utilizing center points and midpoints. (*e-Handbook of Statistical Methods*, 2013)

The experimental plan by Box-Behnken response surface method (RSM) and polygalacturonase(PG) activity values for various combinations are shown in Table 4.3. The highest PG activity was obtained as 11.48±0.51 IU/mL at pH:9, 15% (w/w) solid load, and mix ratio(*B.subtilis/B.pumilus*): 2.125 whereas the poorest polygalacturonase activity was found as 3.40±0.51 at pH:9, 5% (w/w) solid load, and mix ratio(*B.subtilis/B.pumilus*): 2.125. The conditions' significance will be further discussed in this chapter.

Table 4.2 Box-Behnken experimental design for optimization PG production

Run Order	pH	Solid load(%)	*B.subtilis*	*B.pumilus*	Activity (IU/mL)	
			(mL/100 mL)	*(mL/100 mL)*	*Experimental*	*Predicted*
1	7	10	1.00	4.00	5.87±0.50	6.11
2	8	5	4.00	1.00	3.89±0.50	3.45
3	8	10	3.40	1.60	5.30±0.57	5.66
4	7	15	3.40	1.60	11.46±0.50	11
5	8	5	1.00	4.00	4.22±0.51	3.45
6	9	5	3.40	1.60	3.40±0.51	3.9
7	8	15	1.40	4.00	10.42±0.51	10.55
8	9	10	4.00	1.00	6.39±0.54	6.11
9	8	15	4.00	1.00	10.26±0.55	10.55
10	9	15	3.40	1.60	11.45±0.51	11
11	7	5	3.40	1.60	3.45±0.55	3.9
12	7	10	4.00	1.00	6.18±0.51	6.11
13	8	10	3.40	1.60	5.69±0.52	5.66
14	9	10	1.00	4.00	6.60±0.57	6.11
15	8	10	3.40	1.60	5.88±0.50	5.66
16	7	10	1.00	4.00	6.33±0.64	6.11
17	8	5	4.00	1.00	3.91±0.51	3.45
18	8	10	3.40	1.60	5.82±0.51	5.66
19	7	15	3.40	1.60	11.13±0.52	11
20	8	5	1.00	4.00	4.13±0.51	3.45
21	9	5	3.40	1.60	3.76±0.52	3.9
22	8	15	1.00	4.00	10.36±0.56	10.55
23	9	10	4.00	1.00	6.34±0.51	6.11
24	8	15	4.00	1.00	10.38±0.54	10.55
25	**9**	**15**	**3.40**	**1.60**	**11.48±0.51**	**11**
26	7	5	3.40	1.60	3.44±0.51	3.9
27	7	10	4.00	1.00	6.16±0.52	6.11
28	8	10	3.40	1.60	5.81±0.51	5.66
29	9	10	1.00	4.00	6.55±0.51	6.11
30	8	10	3.40	1.60	5.55±0.51	5.66

Regression analysis and Analysis of Varience (ANOVA) evaluations performed in order to fix the experimental data to second order polynomial equation as

function of coded factors (solid load-pH-mix ratio) and their interactions. ANOVA results and estimated regression coefficients are shown in Table 4.4 and Table 4.6 respectively.

Table 4.3 Estimated Regression Coefficients for PG production

Term	*Coefficient*	*S.E Coefficient*	*T value*	*P value*
Constant	**5.655**	**0.159**	**35.490**	**0.000***
X_P	0.121	0.097	1.249	0.227
X_S	**3.546**	**0.097**	**36.344**	**0.000***
X_M	-0.06	0.097	-0.621	0.542
X_P*X_P	**0.448**	**0.143**	**3.124**	**0.006***
X_S*X_S	**1.342**	**0.143**	**9.347**	**0.000***
X_M*X_M	0.198	0.143	1.384	0.182
X_P*X_S	0.008	0.137	0.063	0.95
X_P*X_M	-0.07	0.137	-0.507	0.618
X_S*X_M	0.051	0.137	0.371	0.714

According to the Table 4.4 results, the factors which can significantly affect PG production are X_S, X_P*X_P and X_S*X_S which are solid load, square of pH and square of solid load respectively. ($P<0.05$) The insignificant terms were excluded and re-run on Minitab to find out proper equation. Revised table is shown in Table 4.5

Table 4.4 Estimated regression coefficients for PG production-revised Table

Term	*Coefficient*	*S.E Coefficient*	*T value*	*P value*
Constant	**5.790**	**0.112**	**46.941**	**0.000***
X_P	0.121	0.090	1.340	0.192
X_S	**3.546**	**0.090**	**36.055**	**0.000***
X_P*X_P	**0.425**	**0.133**	**3.187**	**0.004***
X_S*X_S	**1.318**	**0.133**	**9.890**	**0.000***

The final equation in coded values as:

$Y=5.79+ 3.55*X_S+0.42*X_P*X_P+ 1.32* X_S* X_S$ (6)

By looking at coefficients in Eqn. (6), solid load (%w/v) is the most effective variable on PG production among all three variables. pH and mix ratio were found as insignificant terms. Among interactions, the square of solid load and the square of pH are important factors in this equation respectively.

Table 4.5 ANOVA results for PG production

Source of Variation	Degrees of freedom	Sum of Squares	Mean Squares	F ratio	P value
Regression	9	215.805	23.978	157.41	0.000*
Linear	3	201.511	67.170	440.94	0.000*
Square	3	14.233	4.744	31.15	0.000*
Interactions	3	0.061	0.020	0.13	0.939
Residual Error	19	2.894	0.152		
Lack of Fit	**15**	**2.683**	**0.179**	**3.39**	**0.124***
Pure Error	4	0.211	0.053		
Total	29	218.710			

According to ANOVA results (Table 4.6), insignificant lack of fit (P=0.124>0.05) indicated that the model fit the experimental data well. The quality of fit was also evaluated by values adjusted R^2 and predicted R^2. These values are found as %97.98 and %96.35 meaning that around 97% of the response variable variation can be explained by the model in equation (1). Root mean square error (RMSE) and Mean absolute error (MAE) values were calculated as 0.36 and 0.30 respectively.

The response surface plots for the effect of mix ratio, solid load (v/v) and pH, and their interactions are indicated in Figure 4.7-4.9. In Fig 4.7 it can be seen that gradual increase in pectinase production with increase in solid load. Also; it can

be concluded that mix ratio has no significant effect on pectinase production and yielding no change in PG production for different values.

Joshi et al., (2006) examined pectinase production at different dilution levels with apple pomace. They took analysis with 25 g apple pomace and different dilution rates (1:3, 1:4, 1:5, 1:6). Results showed that pectinase production took place at all dilution levels but decreased subsequently with the increase in dilution level. A dilution rate of 1:3 gave the highest pectinase activity. Apple pomace amount can directly affect the enzyme activity because of its high importance during enzyme production (V.K.Joshi et al., 2006).

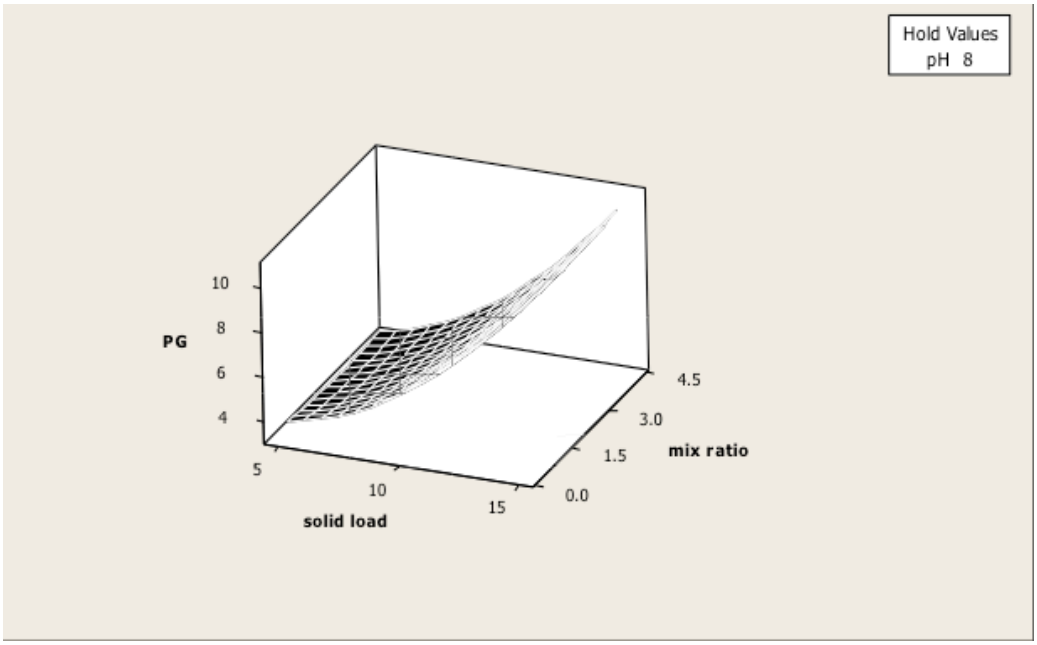


Figure 4.7 Surface plot showing the effect of mix ratio and solid load at constant pH: 8, 30 °C, 130 rpm for 24 h.

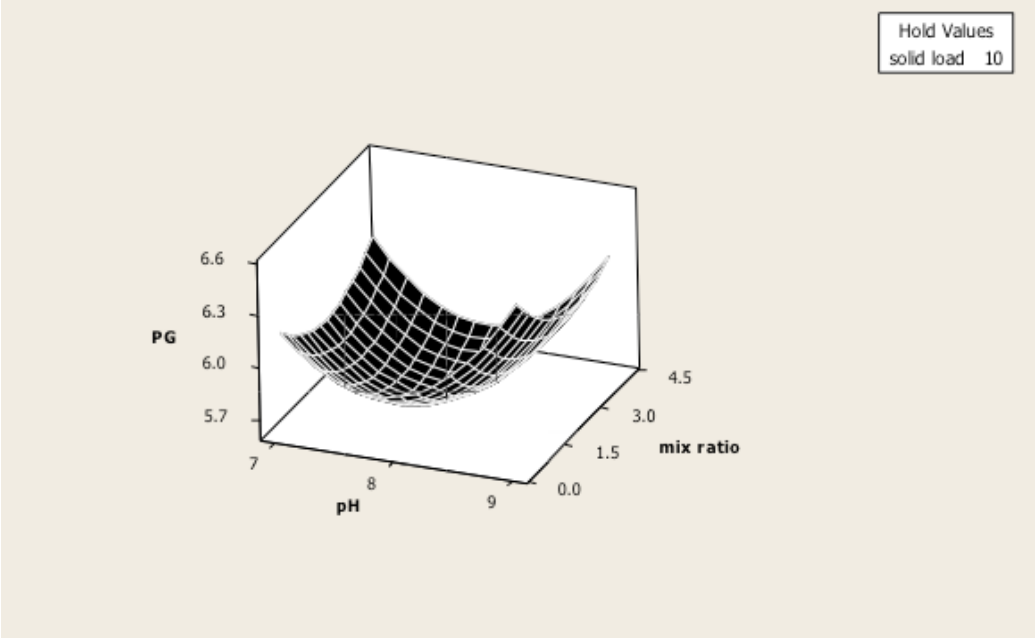


Figure 4.8 Surface plot showing the effect of mix ratio and pH at constant solid load: 10, 30 °C, 130 rpm for 24 h.

In Figure 4.8; concave up shape was observed with the effect of mix ratio and pH when solid load was kept constant at %10. Negative interaction between pH and mix ratio can explain this phenomena. There is not exact maximum point for this plot. A minimum was observed around pH:8 and mix ratio : 1.5-3.0. The effect of pH can be obviously seen from Figure 4.8 where the PG production was higher at the edges. For most of the bacteria, optimum pH for growth and pectinase production is in the range of 7.0 to 10 (Ahlawat et al., 2009). In Figure 4.8, a slight decrease occurs while increasing from pH 7.0 to about pH 8.0, and rapid increase in pectinase production was observed as pH was increased from 8.0 to about 9.0. Between the pH values 7.0-9.0; PG production show rapidly or slightly increase during enzyme production by *Bacillus* species (Torimiro and Okonji, 2013).

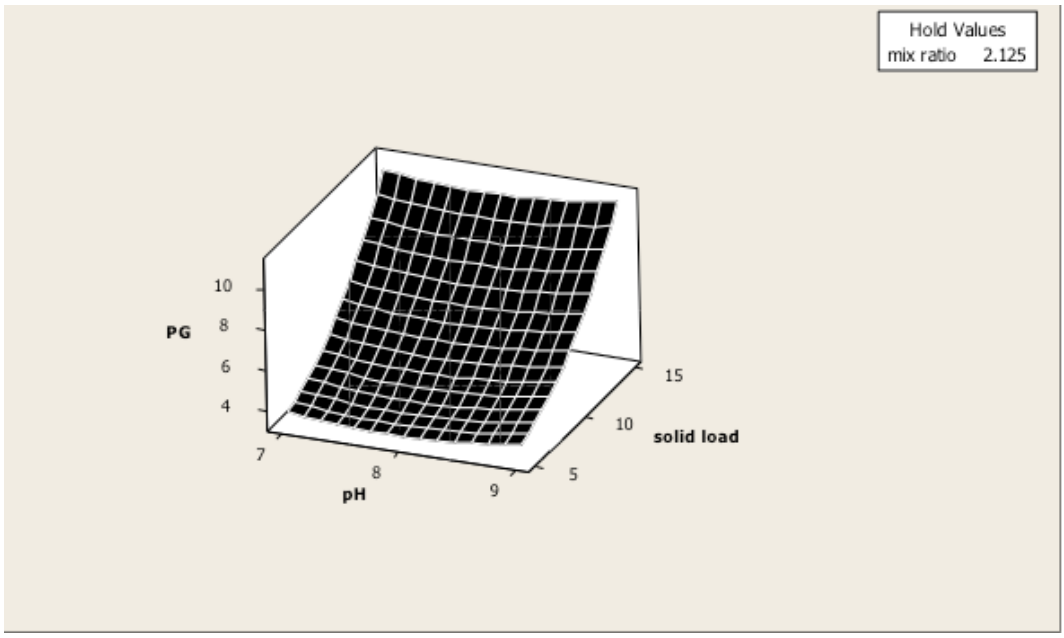


Figure 4.9 Surface plot showing the effect of pH and solid load at constant mix ratio: 2.125, 30 °C, 130 rpm for 24 h.

In Figure 4.9 surface plot of pH and solid load is presented at constant mix ratio at 2.125. Valley shape was observed with a minimum at around pH:8. Pectinase production increased until the maximum point pH: 9 and solid load 15 g/ 100 mL.

In our experiment, slight decrease of PG production by increasing of solid load. There should be further research and analysis to find the maximum solid load to reach optimum level of PG production.

Contour plots can also present the effect of process variables. Figure 4.10 shows the effect of solid load and mix ratio while pH 8 was kept as constant. It is clearly observed that PG production increases by increasing solid load. Mix ratio has no large impact on the PG production, however it can be recognized that mid values on the mix ratio slightly decrease PG production. Especially when the solid load is between 5-7.5; PG production decreases by increasing mix ratio up to 2.0. After

this point, PG production slightly increases by increasing mix ratio from 2.0 to 4.0.

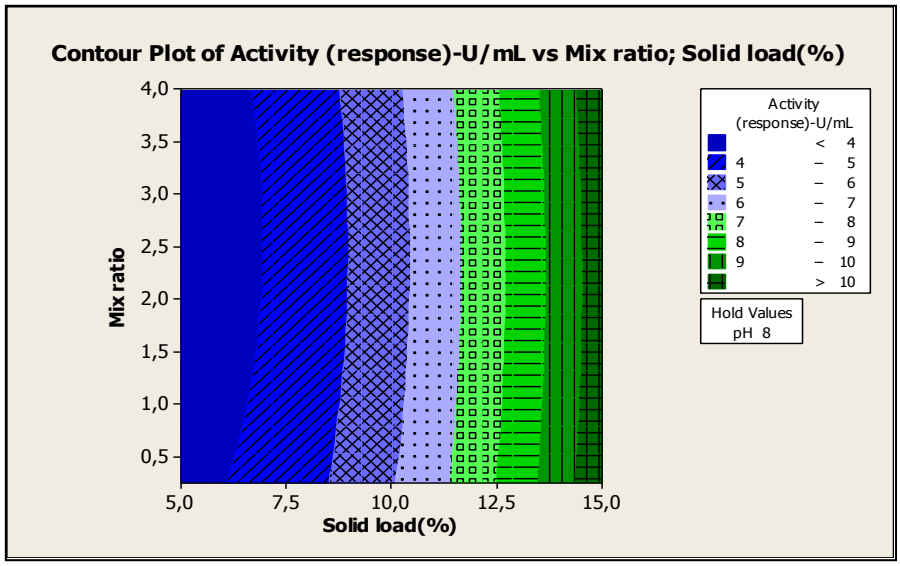


Figure 4.10 Contour plot displaying the effect of solid load (%w/v) and mix ratio on pectinase production at constant pH: 8, 30 °C, 130 rpm for 24 h.

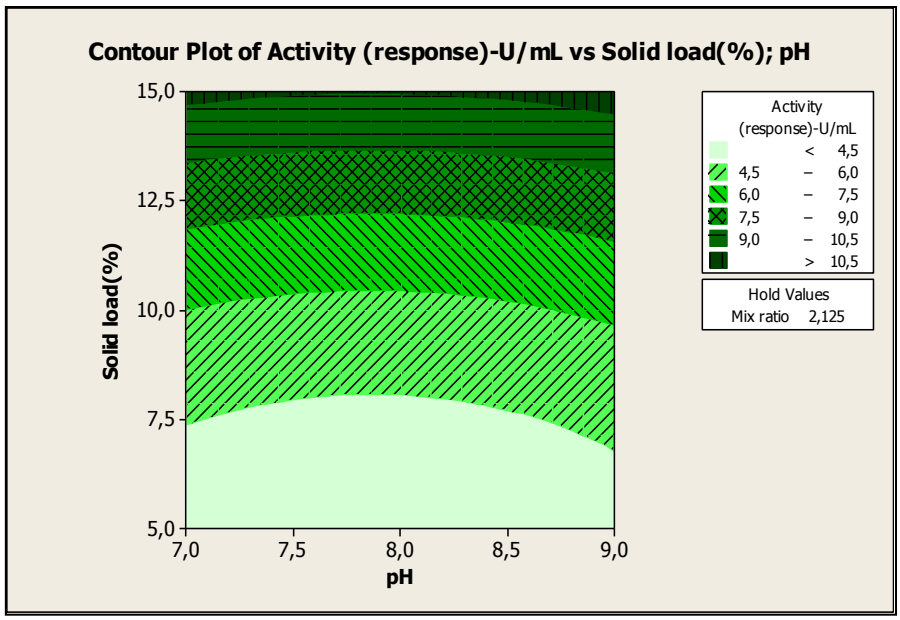


Figure 4.11 Contour plot displaying the effect of solid load (%w/v) and pH on pectinase production at constant mix ratio: 2.125, 30 °C, 130 rpm for 24 h.

Figure 4.11 shows the effect of pH and solid load while mix ratio:2.125 was kept constant. It is again clearly seen that PG production increases by increasing solid load. Mix ratio has no large impact on the PG production, however it can be stated that mid values on the pH slightly decrease PG production. Especially when the solid load is between 5-7.5; PG production increases by increasing mix ratio up to 8.0. After this point, PG production slightly increases by increasing pH from 8.0 to 9.0.

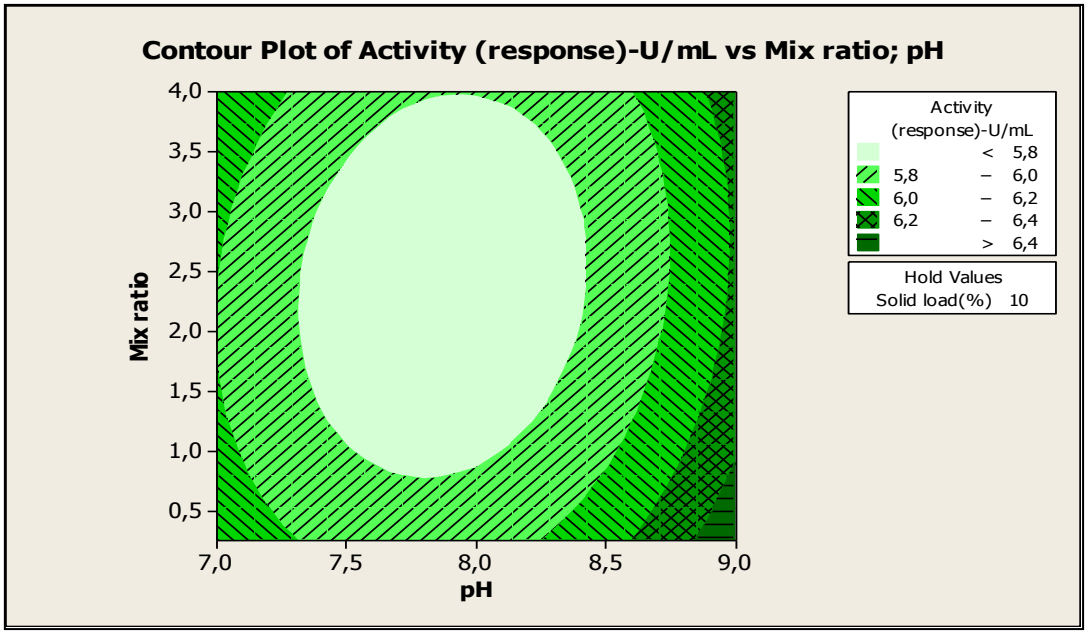
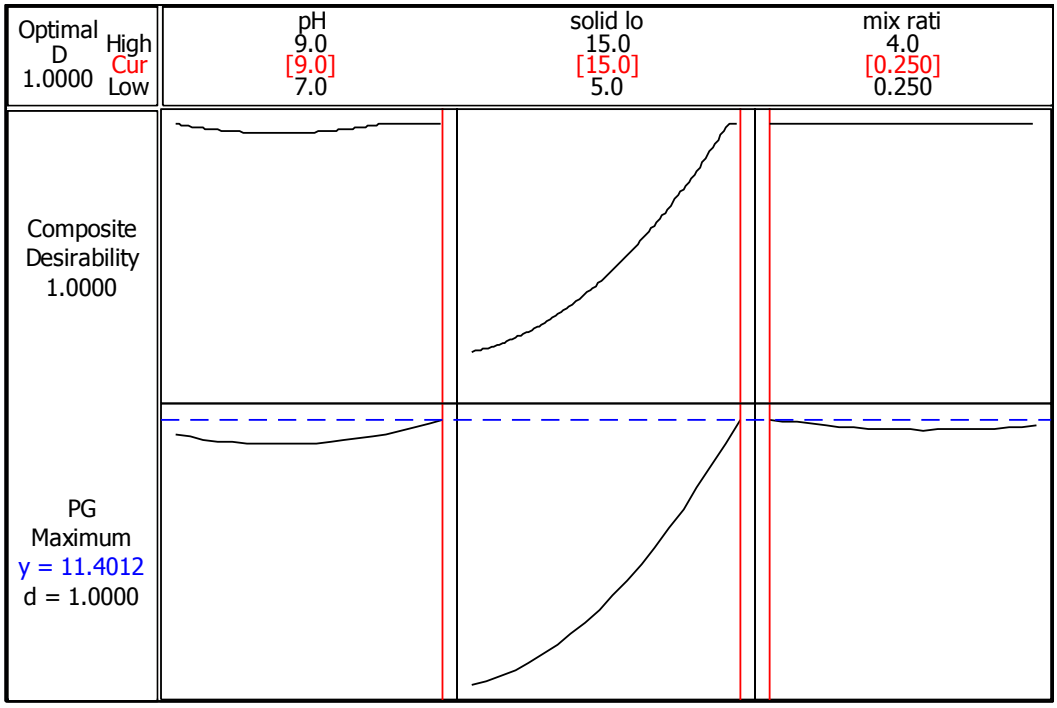


Figure 4.12 Contour plot displaying the effect of mix ratio and pH on pectinase production at constant solid load (w/v): 10, 30 °C, 130 rpm for 24 h.

Figure 4.12 shows the effect of mix ratio and pH on pectinase production during constant solid load as 10 g/100 mL. Elliptic formation in contour plots represents a perfect interaction between variables (Muralidhar et al., 2001). Slight elliptical shape was observed in contour plot of pH vs. mix ratio. It can be regarded as small interaction between these two variables. However contour plot of solid load vs pH (Figure 4.11) and solid load vs. mix ratio indicate that there should be less chance of interactions between these two variable sets.

Table 4.6 Response optimization



Optimal points were evaluated by using response optimizer tool of Minitab 16 based on equation (6). Optimum conditions were found as pH: 9, solid load: 15%(w/v) and mix ratio *(B.subtilis/ B.pumilus*): 1/4 as shown in Table 4.6. At optimal conditions, the predicted maximum PG activity was 11.40 IU/ml.

Verification runs were performed in triplicate to validate the predictive model at optimum condition and average pectinase activity was obtained as 11.25 IU/ml. Although actual activity (11.25 IU/mL) was slightly lower than the predicted maximum activity (11.40 IU/mL), it was still in comply with the predicted value in 95% confidence interval.

Comparison of optimum conditions for maximum PG activity is given in Table 4.7. PG activity was increased from 5.60 to 11.40 IU/mL with this study. Mix ratio, type of solid load and amount of solid load should directly and positively affected increasing of solid load.

Table 4.7 Comparison of optimum conditions for maximum PG activity

Variables (units)	**In our study**	**Uzuner, 2014**
Solid load %(w/v)	15	5
Culture	Mix culture *B.sub/ B.pum*	Single culture *B.subtilis*
pH	9	7
Yeast extract (w/v)	%0.5	%0.5
Time(h)	24	72
PG activity (IU/mL)	11.40	5.60

4.5 Further analysis at optimum conditions to show correlations

Based on RSM results optimum conditions were found as pH:9 , solid load:15% (w/v) and mix ratio (*B.subtilis/ B.pumilus*):0.25. The fermentation experiments were repeated three times in the same conditions and samples were collected every 4 hours to measure biomass, PG activity, protein content by Lowry method and reducing sugar content to assess the possible correlations between results. All results are given in Table 4.8.

Table 4.8 clearly shows how these parameters were affected by each other. During PG production by mix culture; the first 24 h was investigated based on total biomass, PG activity, reducing sugar content and protein content. Biomass rises up till 12 h. and after 12 h; it falls down sharply. PG production and protein content increased constantly till 24 h. This also supports our preliminary results that showed maximum PG production at 24 h. Protein content is directly related with PG activity; because of that enzymes' building stone is protein.

Table 4.8 Further analysis results at optimum conditions

Time(h)	Biomass(g/ L)	Activity (U/mL)	Reducing sugar (g/L)	Protein (mg/L)
4	1.0000	8.0178	4.1804	22.9801
8	1.2000	8.4344	3.6345	23.8960
12	1.3000	11.4042	2.4245	24.3038
16	0.9000	11.4137	2.3500	25.2480
24	0.8000	11.4518	2.2971	26.1483

Fig 4.13 shows the correlation between pectinase activity (U/mL) and biomass(g/L) during time course-24 h- of pectinase production. Positive correlation between *B.subtilis* and *B.pumilus* can be observed till 12 h since it results in an increase in the growth rate of group of organisms. It can also be stated that such interaction could be used to enhance the production of growth-associated enzymes (Bertrand Fossi et. al., 2014) After 12 h, the decrease of growth rate was observed because of exhaustion of nutrients in the culture medium.

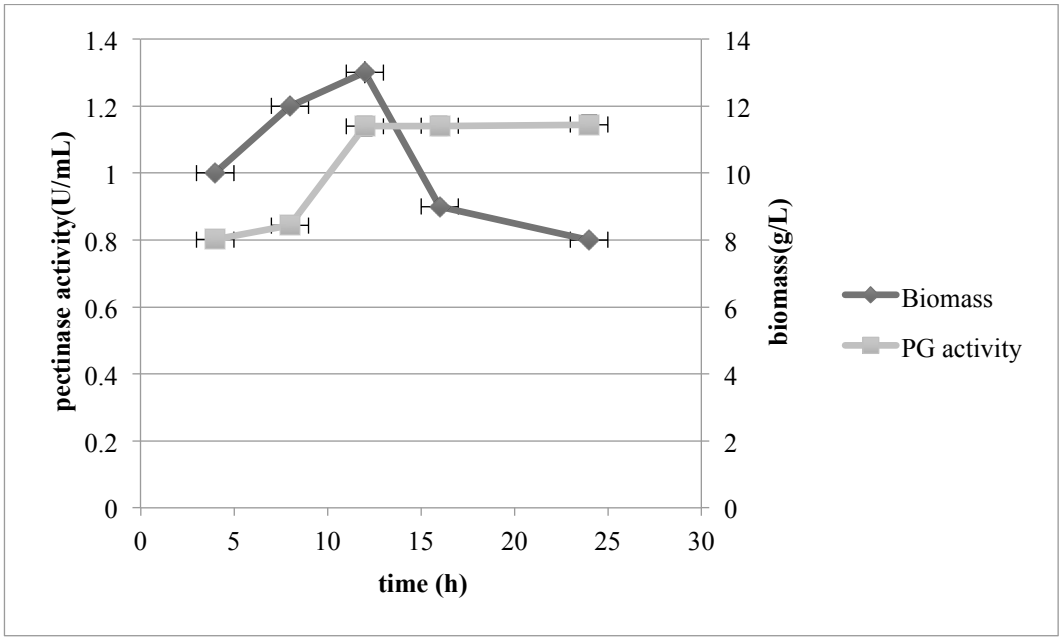


Figure 4.13 Correlation between pectinase activity (U/mL) and biomass(g/L)

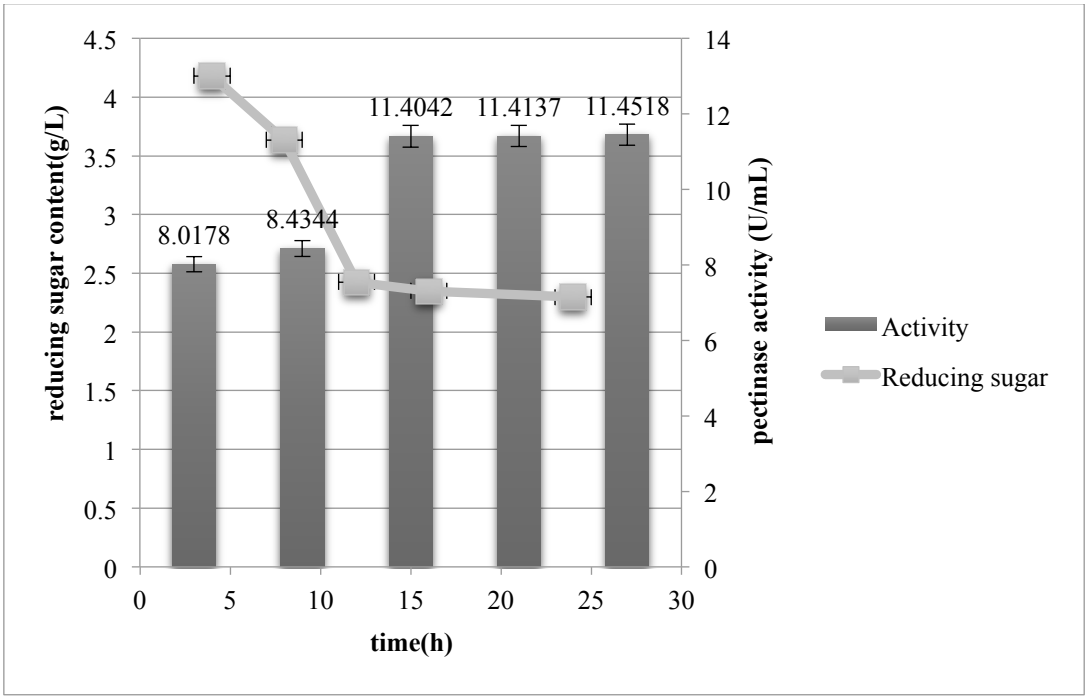


Figure 4.14 Correlation between pectinase activity (U/mL) and reducing sugar content (g/L)

Reducing sugar including glucose is main carbon source of the mix culture. Utilization of reducing sugar was carried out during fermentation process and decrease in reducing sugar content was observed with increasing enzyme production proportionally (Fig 4.14).

4.6 Kinetic model of pectinase production from apple pomace by mix culture

Leudeking and Piret carried out a kinetic study on lactic acid fermentation using *Lactobacillus delbruekii* (Bailey and Ollis, 1986) and developed an unstructured model combining growth associated and non-growth associated aspects toward product formation rate (Manikandan et al., 2007). The rate of product formation is expressed by Leudeking and Piret rate model as follows:

$$\frac{dP}{dt} = \alpha \frac{dx}{dt} + \beta X \quad (7)$$

The model employs rate equations for biomass (X) and pectinase (P) to allow a correlation between the cell mass and product (pectinase) concentration.

In this study, pectinase production can be assumed as growth related model. In Fig 4.13; it can be clearly observed the pectinase production increases by increasing biomass till 12 h.

For the growth associated product formation, the terms can be put as below:

$\alpha \neq 0$ $\beta = 0$ (Pazouki et al., 2008)

By this way, the equation can be simplified as follows:

$P = K + \alpha X$ (8)

The experimental data for pectinase production was fitted into equation (8). As we assumed growth associated product formation until 12 h., the experimental data of 12h fermentation was fitted in the equation; the results were found as; α=8.0431; K=0.0777 with correlation coefficient (R^2)=0.97.This relationship can indicate that pectinase production is strongly growth related until 12 h and growth associated coefficients is α=8.0431.

Sethi et al. (2015) carried out study on enhanced production of pectinase by *Aspergillus terreus* NCFT 4269.10 using banana peels as substrate and they also employed Luedeking-Piret model for microbial growth and enzyme biosynthesis. The kinetic evaluation results showed that extracellular biosynthesis of pectinase by *A. terreus* provided with a constant growth rate. The specific production rate and growth coefficient were calculated and revealed that there is growth associated product formation until 96 h. Similar results were also founded by Iftikhar et al. (2010) for production of lipase from *Rhizopus oligosporus* var. *microsporus*.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In this study, the potential of enhancing pectinase production by mixed culture of *Bacillus subtilis and Bacillus pumilus* using apple pomace as carbon source was evaluated.

First, apple pomace was investigated as the source of pectinase production due to economic and environmental benefits. One-step acid hydrolysis with %4 H_2SO_4 was selected the best pre-treatment method. Optimum pectinase production conditions were found as apple pomace (solid load) concentration: %15, pH: 9 and mix culture ratio (B.subtilis/B.pumilus): 1/4 by Box-Behnken response surface methodology.

The optimum pectinase activity result was found as 11.25 IU/ml under shaking (130 rpm) at 30 ^{o}C for 24 h.

Second, further analyses were carried out at optimum conditions to find out correlation between reducing sugar content, biomass, protein content and pectinase activity. Based on the analyses' results; growth associated product formation was assumed and growth associated coefficient was found as α=8.0431.

In conclusion, this study show that enhancement on the pectinase production can be provided by mixed culture; moreover, lignocellulosic food processing wastes can be utilized as carbon source for enzyme production to reduce the cost of the enzyme production and prevent environmental pollution

For future studies, microbial interactions can be focused on to enhance pectinase production. Moreover, other kinetic models can be established to underline mechanism of pectinase production by using mix culture submerged fermentation. Another important point; detailed investigations are required on apple pomace pre-treatment methods to increase utilization of this material by using in any industrial applications.

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APPENDIX A

CHEMICALS AND SUPPLIER INFORMATION

Table A.1 Chemicals and supplier information

Chemical	Supplier
Citrus Pectin	Sigma-Aldrich
D-glucose	Merck
Dipotassium hydrogen phosphate (K2HPO4)	Merck
3'-5'-Dinitrosalcylic acid	Merck
Magnesium sulphate (MgSO4.7H2O)	Merck
Nutrient Agar	Merck
Phenol	Sigma-Aldrich
Polygalacturonic acid	Sigma-Aldrich
Potassium di-hydrogen phosphate (KH2PO4)	Merck
Rochelle salt	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic	Merck
Sulphuric acid	Merck
Sodium hydroxide	Merck
Sodium sulfite	Merck
Yeast extract	Merck

APPENDIX B

COMPOSITIONS OF BUFFERS AND SOLUTIONS

1-Composition of DNS reagent:

1.00 g Dinitrosalicylic acid

0.2 g phenol

0.05 g sodium sulfite

1.00 g sodium hydroxide

Add water to: 100 ml

Rochelle salt: Potassium sodium tartrate solution, 40%

2-Composition of Phosphate Buffer (pH 7)

Stock solutions:

A: 0.1 M solution of sodium phosphate monobasic

B: 0.1 M solution of sodium phosphate dibasic

39 ml of solution A and 61 ml of solution B and 100 ml deionized water are mixed in 200 ml total volume to adjust the pH: 7

pH is checked by pH-meter after preparation of solution

APPENDIX C

STANDARD CURVE FOR ENZYME ACTIVITY

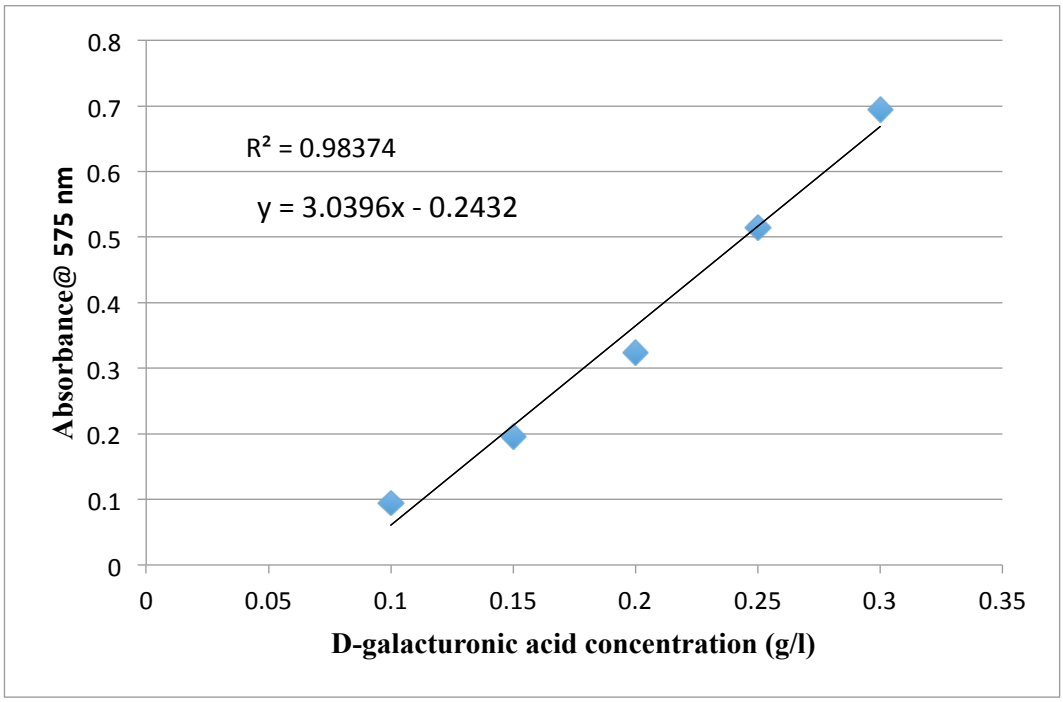

Figure C.1 The standard curve for enzyme activity

$$U/_l = Absorbance * F * \left(\frac{1}{incubation\ time}\right) * DF * \left(\frac{1}{212.12}\right) * R_v \qquad (1)$$

F= *a factor to convert absorbance to gr of galactronic acid by standard curve.*

Incubation time= *time of incubation of the enzyme with substrate (30 min)*

1/212.12= *conversion from grams of galacturonic acid to moles of galacturonic acid.*

DF= *Dilution factor*

$\boldsymbol{R_v}$= *Ratio of enzyme in the reaction mixture.*

APPENDIX D

STANDARD CURVE FOR TOTAL REDUCING SUGAR

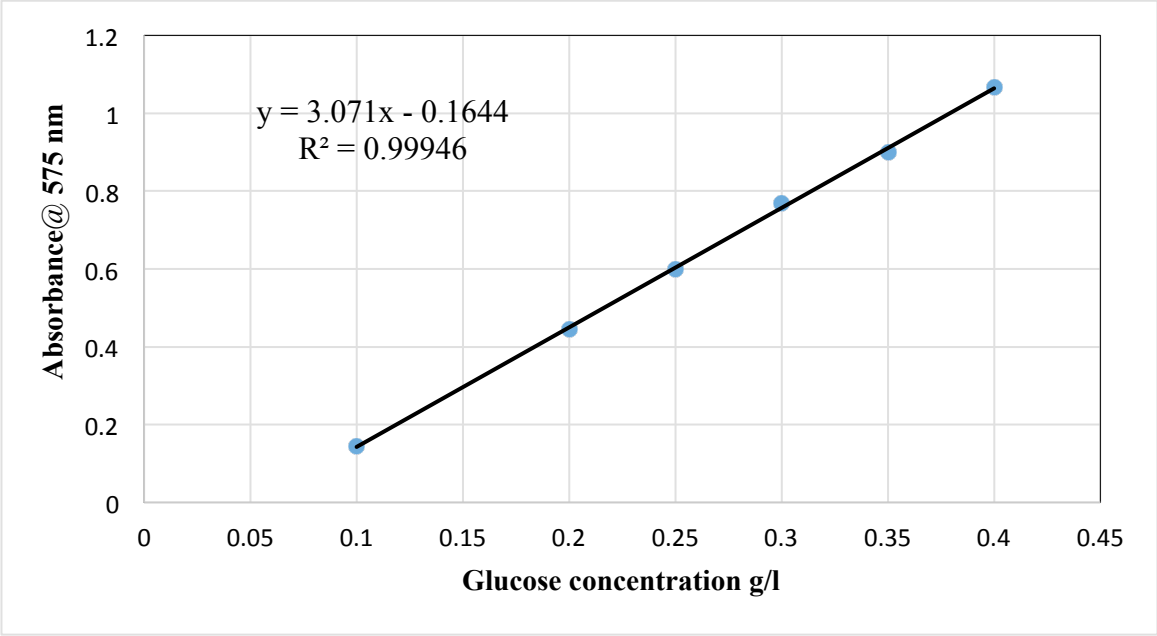

Figure D.1 The standard curve for DNS method

According to standard curve, total reducing sugar concentration was calculated as below:

$$\textbf{Total reducing sugar(g/l)} = \frac{\boldsymbol{Absorbance}+0.1644}{3.071} * \boldsymbol{dilution\ rate}$$

APPENDIX E

STANDARD CURVE FOR TOTAL PROTEIN CONCENTRATION

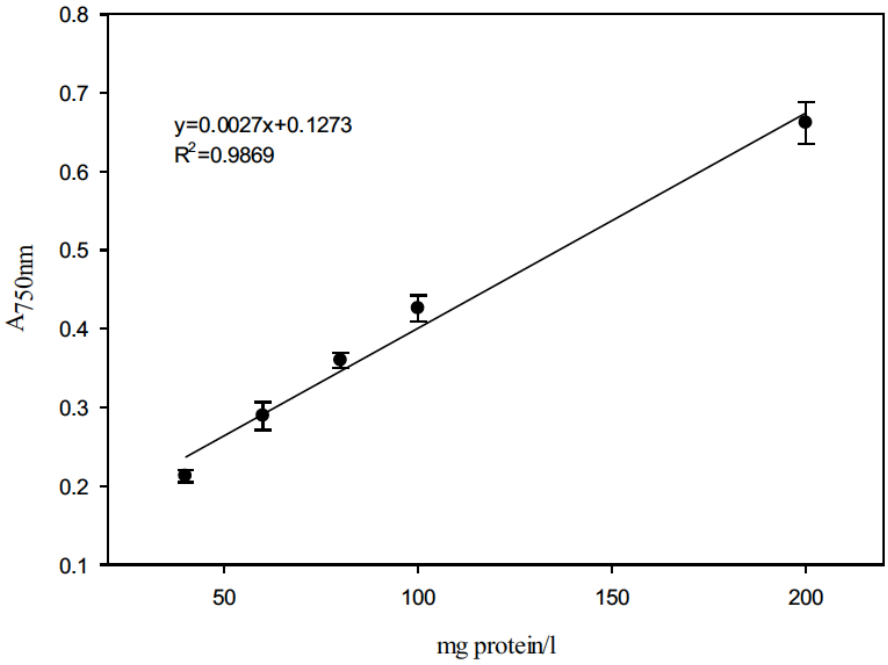

Figure E.1 BSA Standard curve for total protein concentration (Adapted from Uzuner and Cekmecelioglu, 2014)

APPENDIX F

LOWRY SOLUTIONS:

Solution A (alkaline solution, for 500 ml)

- 2,8598 g NaOH
- 14.3084 g Na_2CO_3

Solution B (for 100 ml)

- 1.4232 g $CuSO_45H_2O$

Solution C (for 100 ml)

- 2.85299 g Na_2Tartarate.2 (H_2O)

Lowry Solution (fresh: 0.7 ml/ sample)

- Solution A + Solution B + Solution C with a ratio (v/v) of (100/1/1)