UTILIZATION OF ORANGE PEEL EXTRACT FOR FUNGAL ENZYME PRODUCTION

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ABSTRACT

UTILIZATION OF ORANGE PEEL EXTRACT FOR FUNGAL ENZYME PRODUCTION

Pectinases are a group of hydrolytic enzymes that degrade pectic substances and produced by a variety of microorganisms including filamentous fungi.

In previous studies orange peel especially in solid state fermentations was utilized as a source of additional sugars and nutrients. In this study, orange peel, an agro industrial residue was used for exo-polygalacturonase (exo-PG) production in submerged fermentation (SmF) by *Aspergillus sojae* which have been shown to be a potential exo-polygalacturonase producer. Solubilization of carbohydrates within orange peel was provided by water extraction and treatment with dilute phosphoric (at 0.4% acid concentration and 120 ^{o}C in 20 min.) and sulfuric (at 0.5% acid concentration and 116 ^{o}C in 13 min.) acid hydrolysis. Phosphoric acid hydrolysis was optimized selecting temperature (^{o}C), time (min.) and acid concentration (v/v %) as variable factors, while reducing sugar concentration (gL^{-1}) was the response factor.

Two different dilute acid hydrolysis (at 0.4 % phosphoric acid concentration and 120 ^{0}C in 20 min and at 0.5 % sulfuric acid concentration and 116 ^{0}C in 13 min.) and water extraction were carried out to obtain media that were utilized for enzyme productions. *A.sojae ATCC 20235* and mutant *A.sojae* strains were used to produce exo-PG in these hydrolysates and water extract of orange peel. To enhance exo-PG production, trace element solutions added as well as macro nutrients. Pellet size, number, density, pH and spore inoculation of fermentation were the other parameters that were controlled during fermentations.

The highest exo-PG production was 18.4 UmL^{-1}, which was obtained by mutant *A.sojae* in phosphoric acid hydrolysate but on the other hand mutant *A.sojae* produced 19.7 UmL^{-1} exo-PG activities in water extract of orange peel.

ÖZET

FUNGAL ENZİM ÜRETİMİNDE PORTAKAL KABUĞU EKSTRAKTININ KULLANILMASI

Pektinazlar pektik bileşikleri parçalayan hidrolitik bir enzim grubudur ve filamentli fungusların da dâhil olduğu çok çeşitli organizmalar tarafından üretilmektedir.

Daha önceki çalışmalarda portakal kabuğu özellikle katı kültür fermantasyonlarda ilave şeker ve besin kaynağı olarak kullanıldı. Bu tez çalışması kapsamında, tarımsal bir atık olan portakal kabuğundan, pektinaz üreticisi olduğu kanıtlanmış olan *Aspergillus sojae* kullanılarak batık kültürde ekzo-poligalakturonaz (ekzo-PG) üretilmeye çalışıldı. Portakal kabuğu içerisindeki karbonhidratların çözünmesi su ekstraksiyonu ve seyreltik fosforik (% 0,4 asit derişimi, 120 ^{o}C ve 20 dak.) ve sülfürik asit (% 0,5 asit derişimi, 116 ^{o}C ve 13 dak.) hidrolizi ile sağlandı. Fosforik asit hidrolizi ayrıca optimize edildi ve farklı sıcaklık (^{o}C), zaman (dak.) ve asit derişimler (% v/v) değişken faktörler olarak belirlenirken indirgen şeker derişimi yanıt değişkeni oldu.

Enzim üretimlerinde kullanılacak olan ortamları elde etmek için belirlenen iki farklı seyreltik asit hidrolizi (120 ^{o}C, % 0,4 fosforik asit konsantrasyonu, 20 dak. ve 116 ^{o}C, % 0,5 sülfürik asit konsantrasyonu, 13 dak) ve su ekstraksiyonu gerçekleştirildi. Hem *A.sojae ATCC 20235* hem de mutant *A.sojae* 'nin bu asit hidrolizatları ile portakal kabuğu ekstraktında ekzo-PG üretmesi sağlandı. Enzim üretimini arttırmak için iz element solüsyonu kullanıldı. Pellet çapı, sayısı, yoğunluğu, pH ve fermantasyonun spor inokülasyonu fermantasyonlar boyunca takip edilen diğer parametrelerdendi.

En yüksek ekzo-PG üretimi 18,4 UmL^{-1} idi ve bu mutant *A.sojae* tarafından sülfürik asit hidrolizatında elde edildi ancak diğer taraftan mutant *A.sojae* portakal kabuğunun su ekstraktında 19,7 UmL^{-1} ekzo-PG aktivitesi üretti.

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CHAPTER 1

INTRODUCTION

Pectinases which are involved in the degradation of pectin, among enzymes that are commonly used in paper, textile, wastewater processes and especially in food industry. Price of enzymes that are used in industrial applications was 2 billion $ in 2004 and this value was estimated to increase to 2.4 billion $ in 2009 (Thakore 2004). Today, the world industrial market for pectinase is around 70 million $ by representing almost 5% of global enzyme sales (Alimardani-Theuil et al. 2011). Therefore, pectinases and other superior enzymes should be produced by using microorganisms and the processes should be optimized.

Citrus is one of the fruits which are mostly grown and consumed all over the world and large amounts of wastes are generated every year from the processing of citrus industries. Orange constitutes 55 % of citrus capacity (Oberoi et al. 2010) and accounts for 66 million tons annual production (Talebnia et al. 2008). Most of these wastes are used as animal feed or burned as alternative to avoid environmental pollutions. However, such wastes usually have a composition rich in sugars, minerals and proteins. The presence of carbon sources and nutrients in these wastes provides suitable conditions for the development of microorganisms, and this open up great possibilities for their reuse in fermentation processes.

Several fungal species are important degraders of pectic substances, being able to produce high amounts of pectinolytic enzymes. *Aspergillus species* are among these producers.

The aim of this study is production of exo-polygalacturonase (exo-PG) from *A. sojae* using orange peel extract in submerged fermentations. Previous studies in which orange peel was used directly have shown that fungal growth was in both pellet and mycelium form in submerged fermentations. Although pellet ratio was higher than mycelium ratio in fermentation medium, viscosity was high thus agitation was not proper. It was proposed in this study that orange peel extracts could overcome these problems and exo-PG production can be optimized by using orange peel extracts.

CHAPTER 2

LITERATURE REVIEW

2.1. Citrus Fruits

Citrus fruits residue which consists of oranges, lime, lemon, grapefruit, malta, sweet orange etc is a huge family among other processing residues so that it constitutes an important group of fruit crops produced all over the world. The main producers are Brazil, Spain, Italy, the Unites States especially Florida, Mexico and China and these are stand for approximately two-thirds of global citrus production (Figure 2.1).

The production capacity was between 600,000 and one million tons of dried citrus residues in 1980 and has grown continuously (Grohmann and Bothast 1994). This capacity was estimated more than 105 million tons annually between 2000 and 2004 (Rivas et al. 2008).

2.2. Orange Peel and Production Capacity

Orange peel, which constituted approximately half of the world production in 2004 (Rivas et al. 2008), is an important member of citrus fruits residues and had 66 million tons of annual production in 2007 (Talebnia et al. 2008). The estimation for 2010 was 66.4 million metric tons, which indicates more than 14% increasing that of 1990s (Mamma et al. 2008).

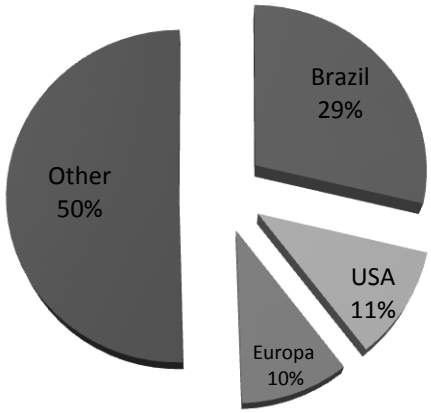


Figure 2.1.World orange production
(Source: López et al. 2010)

2.3. Composition of Orange Peel

Orange peel is composed of two main parts; soluble and insoluble carbohydrates. The soluble or in other words fermentable sugars are glucose, fructose and sucrose. The insoluble carbohydrates are pectin, cellulose and hemicelluloses and these compounds are located in the cell walls of orange peel (López et al. 2010). The composition of orange peel on dry basis is shown in Figure 2.2.

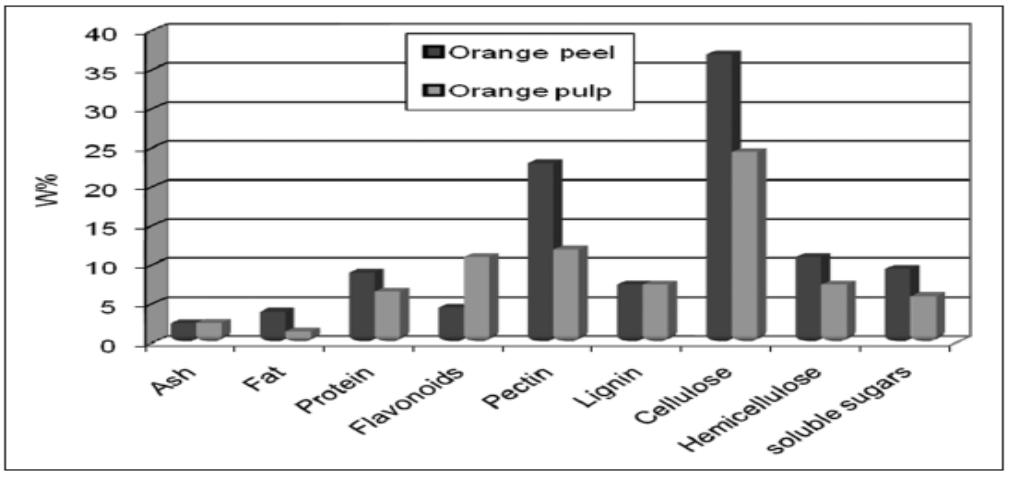


Figure 2.2.Composition of orange peel
(Source: Rivas-Cantu et al. 2013)

2.3.1. D-limonene

Fat content of orange peel is also called essential oil and it is one of the high value compounds such as pectin, cellulose and hemicelluloses of orange peel. These oil sacs are located irregularly in flavedo, the outer layer of orange fruit.

The essential oils are rich in terpenes, especially d-limonene (4-isopropenyl-1-methylcyclo hexene). D-limonene is a hydrocarbon. It is colorless and has a strong orange odor, because of this odor D-limonene is used in food, medicine, chemical, cosmetics and household product industries (López et al. 2010).

Terpenoids can be active against fungi as antimicrobial agents. Giese et al. (2008) faced with inhibition of fungal growth with increasing concentrations of orange oil extract suitably to expectation.

2.3.2. Cellulose

Cellulose which is the main constituent of plant cell walls is the most abundant organic compound. Unbranched structure of cellulose polymer is composed of D-glucose molecules linked by α-1, 4 bonds. Cellulose chains protect the linear form by internal hydrogen bonds. Cellulose chain is also polarized; there are reducing and no reducing groups at the two ends oppositely. Glucose residues are added to the no reducing end during polymerization. Parallel cellulose chains interact, through hydrogen bonds and van der Waals forces, resulting in micro fibrils, which are very extensive and crystalline aggregates.

The polymeric cellulose consists of up to 12000 residues of D-glucose molecules (Aro et al. 2005) and the length of a cellulose molecule is related to degree of polymerization that is number of glucose units in the polymer.

2.3.3. Hemicelluloses

Hemicelluloses represent the other largest fraction of the plant cell wall and agricultural residues. It is a heterogeneous polysaccharide and composed of mainly xylose units linked together by β-1,4 bonds, mannose, glucose, galactose, arabinose and small amounts of rhamnose and galacturonic acid (Taherzadeh and Karimi 2007).

Although hydrolysis of hemicelluloses is easier than cellulose's, its degradation is limited due to concentration of hemicelluloses in primary cell wall. Hemicelluloses are a physical barrier which is associated intimately with lignin, which exerts a strong negative influence on fiber digestion (Graminha et al. 2008, Taherzadeh and Karimi 2008).

2.4. Pectic Substances

Pectic substances are composed of acid polysaccharides with a backbone of galacturonic acid and branched hetero-polysaccharides. According to the modifications which are happened on the carboxyl groups of the backbone, pectic substances are classified into four main groups; protopectin, pectic acids, pectinic acids and pectin in Table 2.1. Normal or acid salts of pectic acid and pectinic acid are called pectates and pectinates (Kashyap et al. 2001).

Pectic substances have different functions such as lubricating in the cell wall of higher plants, interaction between plants and their pathogens and affecting the texture of fruits during growing, ripening and storage. They have gained new application fields like using gelling agent and nutritional fibers since 1990s (Alkorta et al. 1998).

Table 2.1.Classification of pectic Substances
(Source: Kashyap et al. 2001, Jayani et al. 2005)

Pectic substance	Composition	Solubility in water	DE*
Protopectin (Parent substance)	Pectin/Pectinic acid	insoluble	-
Pectic acid	Galacturonan	soluble	negligible
Pectinic acid	Polygalacturonan	-	0-75%
Pectin	Galacturonate	insoluble	>75%

*DE; Degree of Esterification

2.5. Pectin (Polymethyl Galacturonate)

Pectin is a generic name for the mixture of widely differing compositions containing pectinic acid as the major component (Kashyap et al. 2001). It is mainly used in jams and jellies as a gelling agent and thickener, also in drinks, sauces, syrups and to make a desirable texture in food and pharmaceutical industries (Mesbahi et al. 2001) and as nutritional fibers (Mamma et al. 2008).

2.5.1. Structure of Pectin

Pectin which is mainly in the middle lamella of plants cell walls forms about one-third of the cell wall of dicotyledons and some monocotyledons (Mesbahi et al. 2005). It may come together with other structural polysaccharides and proteins to form insoluble forms like binding to cellulose micro fibrils in the cell wall at the early stages of the growth. This interaction supports the resistance of the cell walls. After a while, pectin molecules become soluble due to the ripening by losing its molecules on main chain or side chains so the plant tissues softens (Kashyap et al. 2001).

Pectin is polymer of D-galacturonic acid molecules which link together end to end with α-1,4-glycosidic linkages, negatively charged and acidic (Jayani et al. 2001). Negatively charged group of galacturonic acid molecule can be seen on the left side of the chain in Figure 2.3 and it has acidic character due to the presence of carboxyl groups.

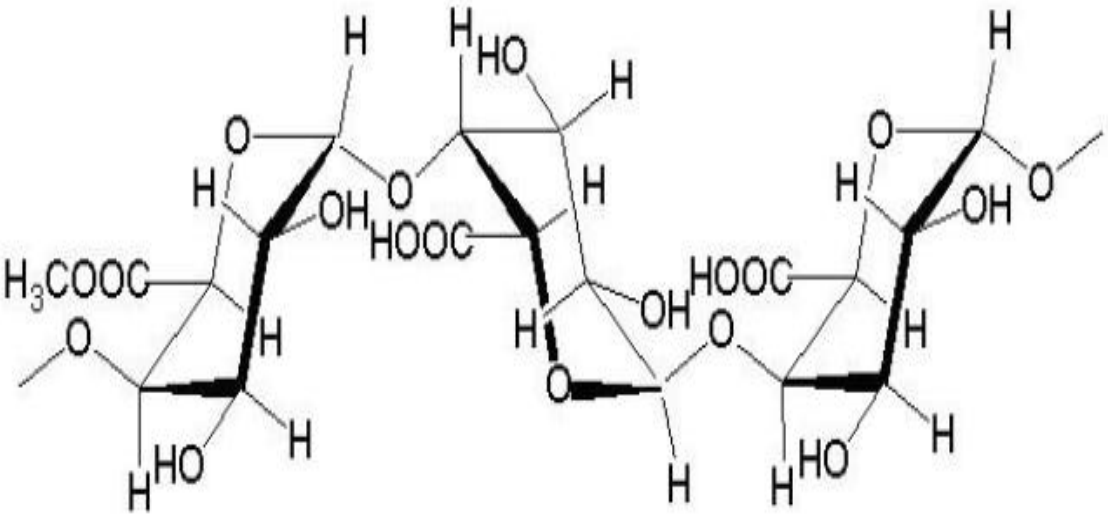


Figure 2.3.Structure of pectin molecule
(Source: Benhura and Chidewe 2011)

Galacturonic acid molecules contribute the backbone of the whole molecule. A few or hundreds of molecules which may be arabinose, galactose, arabinogalactose, glucose, mannose, and xylose also be located on the branches. Thus pectin has high molecular weight (Alkorta et al. 1998, Jayani et al. 2005, Mesbahi et al. 2005. Although the estimated molecular mass of pectin is 25-360 kDa (Jayani et al. 2005), there is no exact information because there are so many pectin types according to degree of esterification, type of neutral sugars on side chains etc. that the estimation of fine structure of pectin has not been possible.

Figure 2.4 represents the backbone and side chains of pectin molecule. Homogalacturonan and rhamnogalacturonan together constitutes the backbone of pectin molecule. Homogalacturonan, smooth region, is a linear polymer consisting of 1, 4-linked α-D-galacturonic acid residues which can be methyl-esterified at C-6 and carry acetyl group on O-2 and O-3 (Niture 2008). On the other hand rhamnogalacturonan (RG) is termed by branched or hairy region of pectin molecule. (Niture 2008) RG-I is composed of the repeating disaccharide rhamnose-galacturonic acid. The galacturonic residues can carry side chains of neutral sugars as galactose, arabinose and xylose. However, it is called rhamnogalacturonan; RG-II is a homogalacturonan chain with side chains attached to the galacturonic residues (Willats et al. 2006).

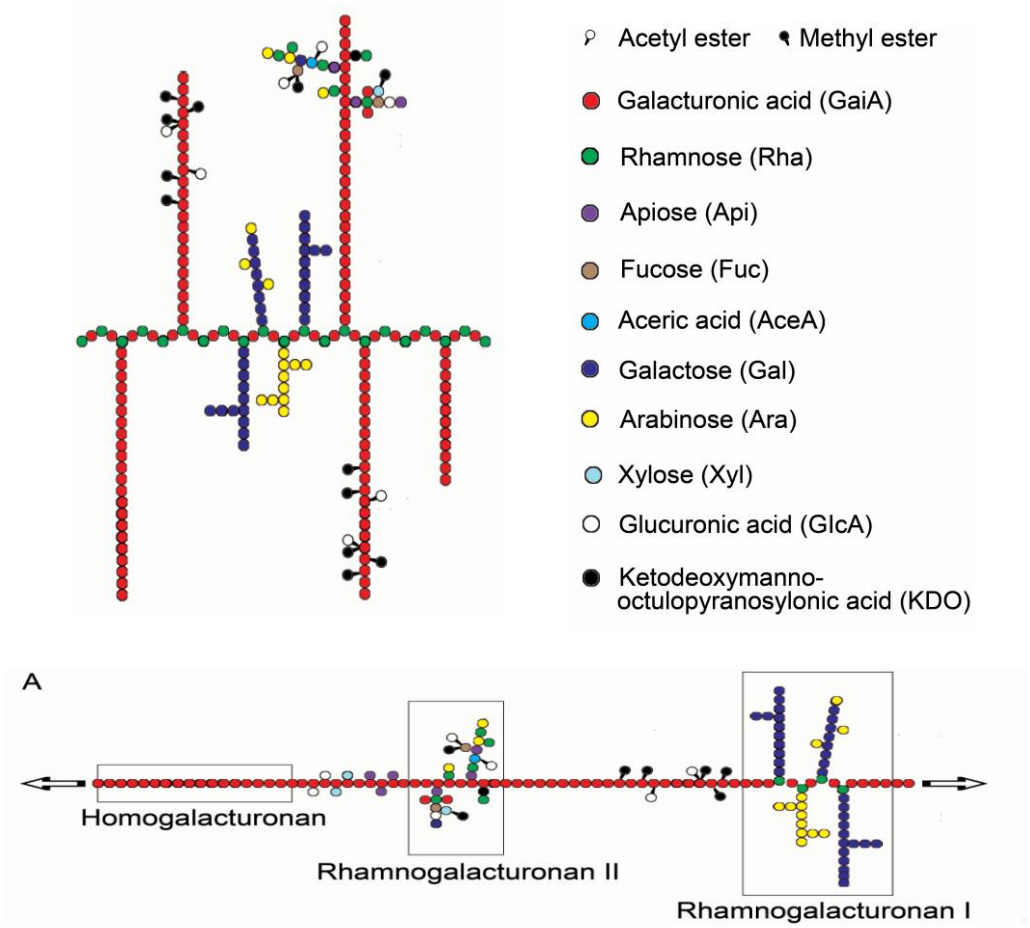


Figure 2.4.Illustration of different components of pectin molecule
(Source: Pedrolli et al. 2009)

2.5.2. Degree of Esterification

The carboxyl groups of galacturonic acid may be methyl esterified and neutralized partially or completely in the presence of sodium, potassium or ammonium ions. The percentage of these esterified groups is called as degree of esterification. Depending on the degree of esterification, pectin is divided into two major groups: high methoxyl pectin (HMP) with a DE higher than 50% and low methoxyl pectin (LMP) with a DE lower than 50% (Mesbahi et al. 2005). The smooth region (homogalacturonan) of pectin molecule classified according to its esterification level: pectin has at least 75 % of the carboxyl groups methylated; pectinic acid has less than 75 % of the carboxyl groups methylated; pectic acid or polygalacturonic acid has no methyl esterified carboxyl groups (Pedrolli et al. 2009).

2.5.3. Functions of Pectin

Pectin is mainly used in jams and jellies as a gelling agent and thickener, also in drinks, sauces, syrups and to make a desirable texture in food and pharmaceutical industries (Mesbahi et al. 2005) and as nutritional fibers (Mamma et al. 2008). Gelling tendency of pectic substances is originated from formation of three dimensional crystalline networks within homogalacturonan regions. This function is dependent on temperature, pectin type, esterification degree, acetylation degree, pH, sugar and other solutes, and mainly the interaction between calcium ions (Figure 2.5) and pectin un-esterified carboxyl groups (Pedrolli et al. 2009).

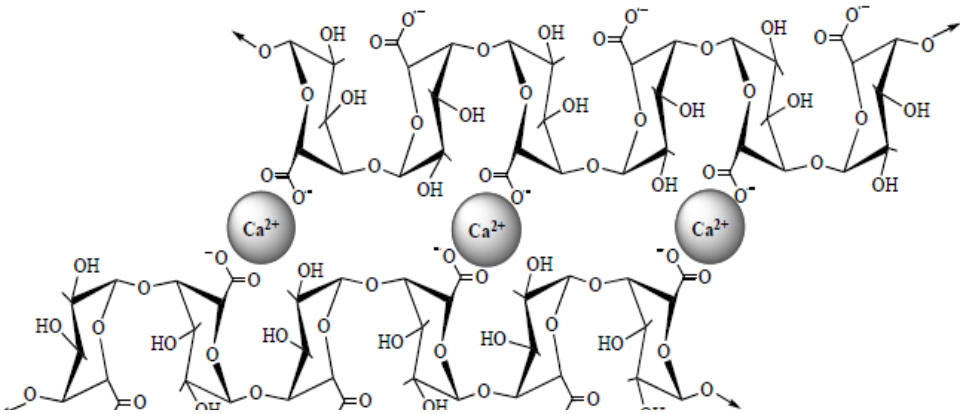


Figure 2.5.Insertion of calcium ions into homogalacturonan chains
(Source: Pedrolli et al. 2009)

2.6. Pectinases

Pectinolytic enzymes or pectinases are a heterogeneous group of related enzymes that hydrolyze the pectic substances that occur as structural polysaccharides in the middle lamella and the primary call walls of young plant cells.

2.6.1. Classification

An extensive classification of pectinolytic enzymes was made by (Mamma et al. 2008). They are classified into three groups according to whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate, pectinases act by transelimination or hydrolysis and the cleavage is random or terminal (Alkorta et al. 1998). The three major types of pectinases are summarized in Table 2.2.

2.6.2. Mode of Action

Acting patterns of pectic enzymes are illustrated in Figure 2.6. Depolymerizing enzymes are attached to α-(1, 4) glycoside bonds between galacturonic acid residues either by hydrolysis or β-elimination. These enzymes are classified into two groups according to these modes of actions; hydrolases and lyases (*trans*-eliminases) (Alkorta et al. 1998).

Among hydrolases, one of the subgroups which are called polygalacturonase prefers pectate and another subgroup whose substrate is pectin is called polymehylgalacturonase (Alkorta et al. 1998).

Although both endo- and exo-polygalacturonases are produced by so many organisms such as fungi, bacteria, yeasts, the main differences between endo- and exo-polygacaturonases besides their mode of action is presence of exo-polygalacturonase in fruits and vegetables (Alkorta et al. 1998).

Lyases are enzymes that are related to glycoside bond in pectate or pectin.

Protopectinases are also called as protopectin- solubilizing enzymes. A-type PPase is connected to the polygalacturonic acid region and B-type PPase is related to polysaccharide chain which is connected to polygalacturonic acid residue (Alkorta et al. 1998).

Table 2.2.Classification of pectic enzymes
(Source: Alkorta et al. 1998, Kashyap et al. 2001, Jayani et al. 2005)

	EC number	EC name	Common Name	Substrate	Cleavage	Action
Depolymerizing Enzymes						
Hydrolases						
	3.2.1.15	Endopolygalacturonase (Endo-PG)	Polygalacturonase	Pectate	Random	hydrolysis of α-1,4-glycosidic linkages in pectic acid
	3.2.1.67	Exopolygalacturonase 1 (Exo-PGl)	Polygalacturonase	Pectate	Terminal	hydrolysis of α-1,4-glycosidic linkages pectic
	3.2.1.82	Exopolygalacturonase 2 (Exo-PG2)	Polygalacturonase	Pectate	Terminal	
		Endopolymethylgalacturonase (Endo-PMG)	Pectin hydrolase	Pectin	Random	cleavage of α-1,4-glycosidic linkages of on highly esterified pectin
		Exopolymethylgalacturonase (Exo-PMG)	Pectin hydrolase	Pectin	Terminal	cleavage of α-1, 4-glycosidic linkage of pectin from the non-reducing end of the pectin chain.
Lyases						
	4.2.2.2	Endopolygalacturonate lyase (Endo-PGL)	Pectate lyase	Pectate	Random	cleavage of α-1,4-glycosidic linkages in pectic acid
	4.2.2.9	Exopolygalacturonate lyase (Exo-PGL)	Pectate lyase	Pectate	-	sequential cleavage of α-1,4-glycosidic linkages in pectic acid
	4.2.2.10	Endopolymethylgalacturonate lyase (Endo-PMGL)	Pectin lyase	Pectin	Random	cleavage of α-1,4-glycosidic linkages in pectin
		Exopolymethylgalacturonate lyase (Exo-PMGL)	Pectin lyase	Pectin	Terminal	catalyzes stepwise breakdown of pectin by trans-eliminative cleavage
De-esterifying Enzymes	3.1.1.11	Polymethylgalacturonate esterase (PMGE)	Pectinesterase	Pectin	Random	de-esterification of the methoxyl group of pectin forming pectic acid
Protopectinases (Pectinosinase)						
		A-type PPase	-	Protopectin	-	react with the inner site
		B-type PPase	-			react on the outer site

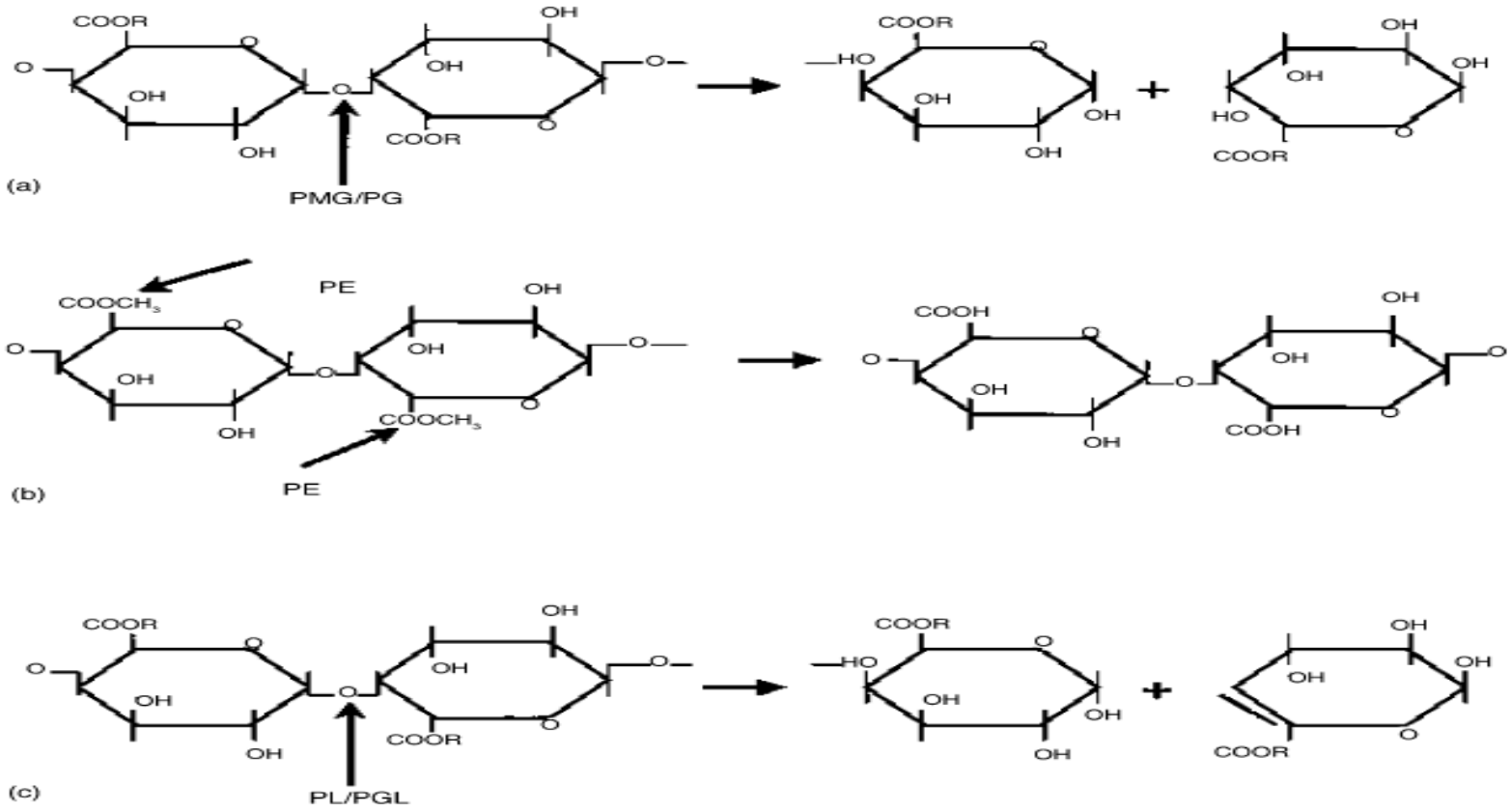


Figure 2.6.Mode of action of pectinases (a) R = H for PG and CH3 for PMG; (b) PE; and (c) R = H for PGL and CH3 for PL.
PG, polygalacturonases (EC 3.2.1.15); PE, pectinesterase (EC 3.1.1.11); PL, pectin lyase (EC-4.2.2.10)(Source: Jayani et al. 2005)

2.6.3. Applications of Pectinases

History of pectinases began with using in homes firstly and they were begun to use for preparation of wines and fruit juices commercially in 1930. On the other hand biochemical properties of plants and plant tissues have been recognized since 1960s. As a result, pectinases are today one of the upcoming enzymes of the commercial sector (Kashyap et al. 2001).

Fungal pectinases have used since 1940s and are of great importance nowadays with their wide application range as illustrated in Figure 2.7 (Patil and Dayanand 2006b).

Pectinases are used for clarifying in *fruit juice industry.* Pectin or pectic substances account for about 0.5 % of the weight of fresh fruits and most of the pectic substance is found in liquid phase after mechanical crushing. Due to the jelly structure and negative charge of pectin (Pilnik and Voragen 1993), pectin molecules repels one another and liquid phase gets more viscose by the process passes so it makes difficulties on extraction. The remained part of the pectic substances is bound to cellulose and hemicelluloses by causing water retention and this prevents the press ability of the pulp. However adding pectinases reduces the viscosity and decomposes the jelly structure with higher yields by reducing the filtration time up to 50% (Kashyap et al. 2001, Jayani et al. 2005). The commercially available pectinase preparations used in food processing are combinations of polygalacturonase, pectin lyase and pectin methyl esterase. These preparations are usually derived from mainly *Aspergillus* species (Pedrolli et al. 2009).

Degumming of plant bast fibers; Bast fibers are the soft fibers formed in groups outside the xylem and contain gum, which must be removed before use for textile making. The chemical degumming is toxic and non-biodegradable. Biotechnological degumming using pectinases in combination with xylanases presents an ecofriendly and economic alternative to the chemical degumming (Jayani et al. 2005).

Coffee and tea fermentation; Pectinase treatment accelerates tea fermentation and destroys the foam forming property of instant tea powders by destroying pectin. They are also used in coffee fermentation to remove mucilaginous coat from coffee beans (Jayani et al. 2005).

Paper and pulp industry; During papermaking, pectinase is used with cellulase and it can depolymerise pectin and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching (Jayani et al. 2005).

Animal feed; Pectinases are used in the enzyme cocktail, used for the production of animal feeds. This reduces the feed viscosity, which increases absorption of nutrients, liberates nutrients, either by hydrolysis of non-biodegradable fibers or by liberating nutrients blocked by these fibers, and reduces the amount of faeces (López et al. 2010).

Purification of plant viruses; In cases where the virus particle is restricted to phloem, alkaline pectinases and cellulases can be used to liberate the virus from the tissues to give very pure preparations of the virus (Jayani et al. 2005).

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 (Jayani et al. 2005).

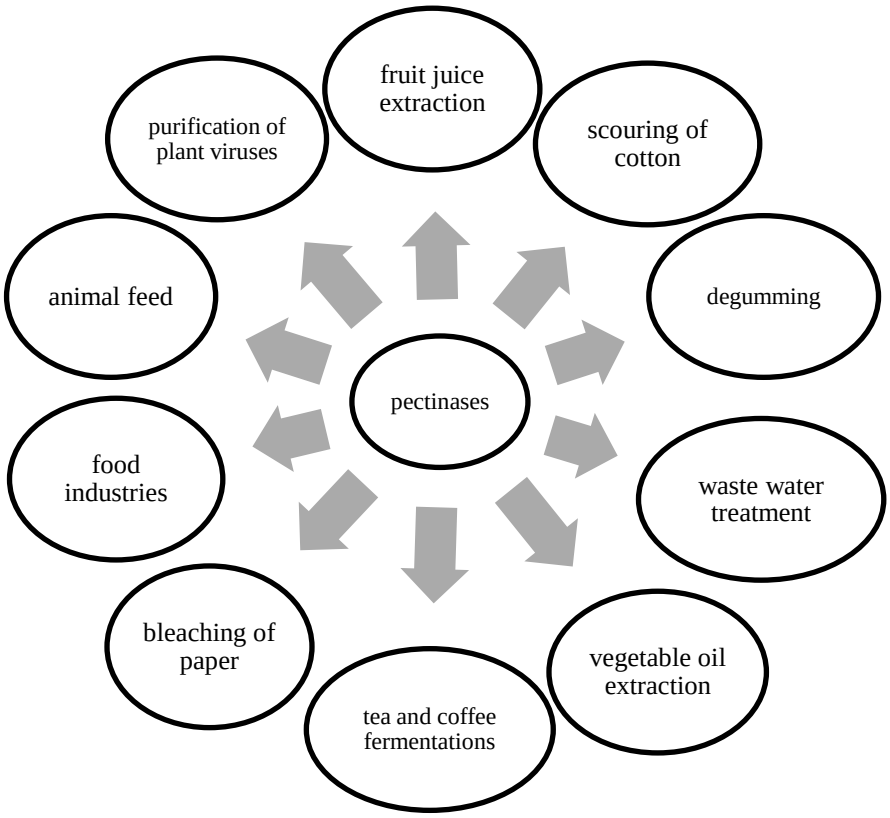


Figure 2.7.Application fields of pectinases
(Source: Jayani et al. 2005, Mamma et al. 2008)

2.6.4. Demand for Industrial Pectic Enzymes

Some of the important pectinase producers are Novozymes (Denmark), Novartis (Switzerland), Roche (Germany) and Biocon (India). For the market volume, it has been reported that microbial pectinases account for 25% of the global food enzymes sales. In 1995 the value of sales for all enzymes was 1$ billion but on the other hand the percentage of pectinases was 75$ million. By 2005, the whole world market for industrial enzymes is expected to be 1.7±2 $ billion (Godfrey and West 1996, Gummadi and Panda 2003). Today, the world industrial market for pectinases is around 70 million $ by representing almost 5% of global enzyme sales (Alimardani-Theuil et al. 2011).

2.7. Microbial Sources of Pectinases

Many microorganisms, viz., bacteria, yeast and fungi could produce pectinases. however commercial production depends on fungal sources (Jayani et al. 2005).

2.7.1. Fungal Sources

Filamentous fungi are among the most frequently employed organisms in industrial biotechnological processes. Most of them belong to the taxonomic group of *Ascomycota* found in diverse natural habitats, but taxonomic discussion has not concluded yet (Grimm et al. 2005).

There are so many fungal species which are reported as PG producers. These species vary according to isolating from soil, decomposed or infected plant parts and mangrove environments. Some of the fungal producers are listed in Table 2.3.

Aspergillus niger has been the most studied and commonly used fungal species for industrial production of pectinases because this strain possess GRAS (Generally Regarded As Safe) status so that metabolites produced by this strain can be safely used (Jayani et al. 2005, Niture 2008). This fungal strain produces various pectinases including polymethylgalacturonase (PMG), polygalacturonase (PG) and pectin esterase (PE). However, particular pectinases are used for specific purposes, for example only PG is used in baby food products (Gummadi and Panda 2003).

Table 2.3.Fungal producers of endo/exo PGs

Species	Reference
Aspergillus awamori *Aspergillus niger*	Kester and Visser 1990, Blandino et al. 2002
Botrytis cinerea	Cabanne and Donèche 2002
Colletorichum lindemuthianum	Herbert et al. 2004
Corticium rolfsii	-
Fusarium moniliforme	Niture et al. 2001
Neurospora crassa	Maria de Lourdes et al. 1991

Production of PGs by fungal sources depends on medium conditions that are presence of pectin source and nitrogen source, initial pH of medium, temperature and agitation (Niture 2008). According to Rombouts and Pilnik (1980) balanced concentrations of simple sugars and pectin is promoted PG production efficiently moreover the maximum production of PG activity (500 U mL^{-1}) was reported in *Aspergillus japonicus* when the fungus was grown of liquid medium containing pectin and glucose (Teixeira et al. 2000). The report from Niture et al. (2008) indicates the presence of 0.2 % glucose along with 1% pectin in fermentation medium is adequate to produce maximum concentration of PGs.

Agro industrial residues represent suitable fermentation media for fungus especially in solid-state fermentations. They promote PG production due to the presence of pectin in these agro industrial residues like wheat bran, sugar-cane bagasse, citrus peel and orange peel. Patil and Dayanand (2006) reported that they were increased PG production with addition of pure glucose (4%) and sucrose (6%) in solid-state fermentation of deseeded sunflower.

Although fungi are qualified PG producers, catabolic repression is seen frequently trough most species. PG production of *A. niger*, one of these species, is repressed when the glucose concentration is up to 10% but remaining species shows opposite. At the same glucose concentration PG regulation of *Kluyveromyces marxianus* was not affected by high glucose concentration (Niture et al. 2008).

pH of the medium is also an important parameter for PG production. *Fusarium roseum* (Perley and Page 1971) and *Aspergillus nidulans* (Dean and Timberlake 1989) had maximum PG activity when the pH of the medium was acidic and *Fusarium moniliforme* secreted more PGs while infecting tomato plants and the pH of the infected medium was approximately 6.8 (Niture et al. 2008). Researchers showed that PG production accelerated in acidic mediums.

2.7.2. Morphology

First phenomenological approaches to describe fungal morphology with microscopic pictures carried out in the 1970s. There were some limitations such as automation and measurement but these were solved by introducing automated digital image analysis in the late 1980s (Grimm et al. 2005).

Forms of fungi morphology are pellets and dispersed filaments which differ according to medium composition and shear, level of the inoculums and pH. Filamentous growth and pellets are required together or individually due to product and type of fungal fermentations. Pelleted growth is needed for citric acid production as an example; on the other hand filamentous growth is better for pectinase production in solid state fermentation (Pazouki and Panda 2000). However pellets are required for submerged fermentations.

Filamentous growth increases the viscosity of medium in submerged fermentations due to dispersed filaments. High viscosity makes difficulties for aeration and agitation thus the fermentation system requires more energy input. Although filamentous growth causes higher specific growth rates, pellet formations are required in submerged cultures because of pellets reduce viscosity of the medium. The reason of low specific growth rate with pellets is limitations of mass transfer. Due to the circular shape of pellet, inner sites are poor in the view of oxygen and these sites become anaerobic by reducing the specific growth rate (Phillips 1966).

Pellet numbers and especially pellet sizes cannot be fixed but they can be optimized due to requirements of fermentation products. Both small and large pellets can be in the same fermentation medium but if there is any nutrient limitation and product toxicity, pellets will get larger (Edelstein and Hadar 1983).

2.7.3. *Aspergillus sojae*

A. sojae is traditionally used in 'koji' which is a fermented Japanese food however *Aspergillus* species have been used as pectinase producers.

A. sojae ATCC 20235 is an important enzyme producer among *Aspergillus* species. Utilization of *A. sojae* for PG production has started within defined media by Gogus et al. (2006). They analyzed PG production, rheology and morphology of final fermentation medium in terms of different seed cultures with different medium formulations. Tari et al. (2007) investigated also mycelia growth (biomass) with PG production in defined media and determined the optimum regions for maximum PG production and biomass formation with a desired pellet morphology using low cost carbon and nitrogen sources. Tari et al. (2008) characterized the biochemical and thermodynamic properties of exo-PG in defined media. Later than, Buyukkileci et al. (2010) were able to use agro-industrial wastes for exo-PG production and orange peel gave the highest exo-PG activity (145.4 Uml^{-1}) among the agro-industrial wastes. Besides submerged fermentations, *A. sojae ATCC 20235* can produce exo-PG in solid-state cultures (Ustok et al. 2007) and also glycosidase, arabinofrunosidase and xylanase are some of the other enzymes (Kimura and Tajima 1998, Kimura et al. 1999, Kimura et al. 2000).

There is a contradiction on classification of *A.sojae ATCC 20235* because restriction fragment length polymorphism results (Heerikhuisen et al. 2005) indicates *A.sojae* is re-classified as *A. oryzae* because *A.sojae ATCC 20235* has no sufficient characteristics of *A.sojae*.

2.8. Pretreatments on Agro Industrial Residues

There are so many methods to hydrolyze macromolecules to their monomers and these methods can be classified into three main groups; physical, biological and chemical and physicochemical pretreatments (Taherzadeh and Karimi 2007). A summary of these pretreatments is given in Table 2.4.

Table 2.4.Classification of pretreatments
(Source: Taherzadeh and Karimi 2008)

Pretreatments	Mode of action	Advantages / Disadvantages
Physical	increasing of accessible surface area decreasing in cellulose crystalline decrease in degrees of polymerization	highly energy demanding methods cannot remove the lignin preferable not to use these methods for industrial applications no chemicals are required for these methods
Chemical & Physicochemical	increasing in accessible surface area partial or nearly complete delignification decrease in cellulose crystalline decrease in degrees of polymerization partial or complete hydrolysis of hemicelluloses	the most effective methods the most promising processes for industrial applications usually rapid treatment rate need harsh conditions there are chemical requirements
Biological	delignification reduction in degree of polymerization of cellulose partial hydrolysis of hemicelluloses	Low energy requirement No chemical requirement Mild environmental conditions Very low treatment rate not consider for commercial application

2.8.1. Chemical Hydrolysis

Chemical hydrolysis requires a defined period of time, a specific temperature and the presence of acid. Insoluble parts of orange peel that are cellulose, hemicelluloses and pectin are soluble with acid hydrolysis (Taherzadeh and Karimi 2007).

Chemical hydrolysis also can be classified into two groups according to acid concentration; concentrated (a low temperature and high acid concentration) and dilute (a high temperature and low acid concentration) acid hydrolysis (Taherzadeh and Karimi 2007, Taherzadeh and Karimi 2007).

2.8.2. Dilute Acid Hydrolysis

Dilute acid hydrolyses is both a chemical pretreatment and a direct method to decompose macromolecules besides it is the most common application. Hydrolysis with dilute mineral acids is also termed as *prehydrolysis* and solubilizes insoluble residues made up of cellulose, lignin and pectin mixed with soluble mono- and oligosaccharides from the orange peel (Rivas et al. 2008).

A comparison in terms of advantages and disadvantages of concentrated and dilute acid hydrolyses is given in Table 2.5. The most important disadvantage of dilute acid hydrolysis is formation of undesirable products. These by-products are originated by conversion of cellulose and hemicelluloses. Another disadvantage is the cost of reagents. These reagents are acid catalyst and neutralizing agent. Neutralization is an extra step for dilute acid hydrolysis and due to the concentration and type of diluted acid it causes labor and higher process costs (Rivas et al. 2008).

Table 2.5.Comparison of concentrated and dilute acid hydrolysis

Hydrolysis method	Advantages	Disadvantages
Concentrated acid	-operated at low temperatures - high sugar yield	-high acid consumption -equipment corrosion -high energy consumption for acid recovery -longer reaction time
Dilute acid	-Low acid consumption -short residence time	-operated at high temperature -low sugar yield -equipment corrosion -formation of undesirable by-products

The dilute acid hydrolysis can achieve high reaction rates at higher temperatures and lower concentration of acid. Moreover it can be performed either in short retention time at high temperature or in long retention time at lower temperatures.

Although concentrated acid hydrolysis has superior disadvantages, it has been continued to study for low overall costs. This study is also called 'biosulfurol' that is aimed to reduce process cost by using concentrated acid and in the following step it is diluted and hydrolyzed by adding water (Taherzadeh and Karimi 2008).

2.9. Inhibitors of Fungal PG

There can be so many undesirable product formations after dilute acid hydrolyses and these are furfural, 5- hydroxymethylfurfural (HMF), acetic acid, formic acid etc…Furfural and HMF (Figure 2.8) are only compounds produced at significant concentrations in hydrolysate. Furfural is more toxic to organisms then HMF because it effects important enzymatic reactions or enzymes itself like hexokinase, phosphofructokinase, alcohol dehydrogenase etc. Thus, HMF is less toxic for microbial growth because of it has four fold slower conversion rate then furfural.

Apart from by-products of dilute acid hydrolysis, there are also different undesirable products which prevent production of fungal PG. Fungal PGs are involved in plant pathogenesis and several studies have been carried out to cure. Nowadays these studies have shown that PGs are affected by metal ions such as Ba^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Mn^{2+} and Hg^{2+} (Mohamed et al. 2006). Interestingly, PG production is also inhibited by

its substrate and its end-products. Kester et al. 1996 reported galacturonic acid prevented exo-PG production of *Aspergillus tubingensis* and Niture et al. (2008) reported endo-PG produced by *Fusarium moniliforme* inhibited by pectin.

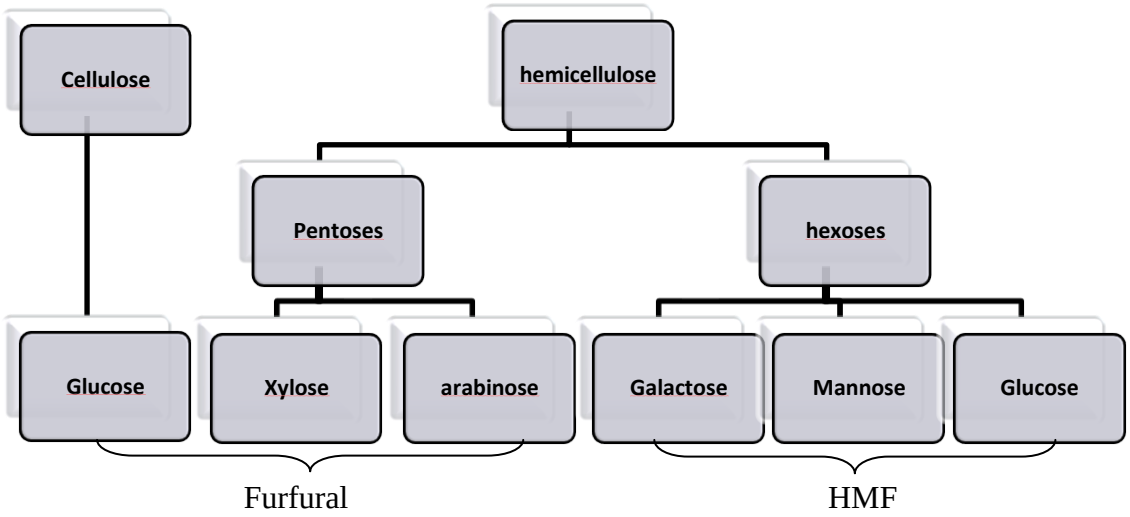


Figure 2.8.Formation of by-products

2.10. Water Extraction of Orange Peels

Water extraction of orange peel requires only water and the raw material, agro-industrial residue so that it does not cause decomposition of sugars such as furfural and HMF. Another interesting aspect of water extraction is it provides decomposition of hemicelluloses by via hydronium ions that are formed yet in the course of extraction (Rivas et al. 2008).

2.11. Fermentation Systems

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. The fermentation techniques such as solid-state fermentation (SSF) and submerged fermentation (SmF) have lead to industrial-level production of bioactive compounds such as enzymes.

2.11.1. Solid State Fermentation

Solid-state fermentation, which can be defined as 'the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water' (Pandey et al. 2000), is an attractive fermentation process, because it presents higher productivity due to simulate the living conditions of many higher filamentous fungi (Pedrolli et al. 2009), lower capital and operating costs, lower space requirements, simpler equipment and easier downstream processing compared to that of submerged fermentation (Pandey et al. 2000). It is also advantageous in the view of fast oxygenation (Viniegra-González et al. 2003).

Solid-state fermentation is widely used for the industrial production of microbial enzymes and metabolites. Studies on literature are interested in comparison of solid-state fermentation and submerged fermentation. As an example; solid state fermentation of *A. niger* produced more PG activities than submerged fermentation if the medium was supplemented with glucose, sucrose and galacturonic acid (Niture 2008). Moreover PG production rate of *A. niger* is six times higher in solid state fermentation according to Maldonado and de Saad (1998). And also Solis-Pereira et al. (1993) concluded that the overall productivity of the enzymes by SSF was 18.8 times higher than in SmF and they attributed the lower productivity in submerged fermentation to longer fermentation time and catabolic repression by free sugars released during fermentation.

The main problems associated with solid-state are difficulties in scale-up and control of process parameters such as pH, temperature, oxygen transfer and moisture. Besides, SSF process suffers from low mixing efficiency and higher impurity product increasing the product recovery costs. There is also evidence that some enzymes are less affected by catabolic repression, than those obtained by submerged fermentations (Mamma et al. 2008). However, SSF gained some attention in the last three decades due to the possibility of using cheap agro-industrial wastes as substrates.

2.11.2. Submerged Fermentation

Submerged fermentation has been strongly developed from the 1940s onwards and is a well-developed system used in industrial scale to produce a large variety of microbial metabolites especially antibiotics. Submerged fermentation using genetically manipulated microorganisms owing to several process advantages over SSF produces about 90% of all industrial enzymes.

Orange peels have utilized in submerged fermentations although they are preferred especially for solid-state fermentations (Pedrolli et al. 2009) and *A. sojae* has ability to utilize some agro-industrial products efficiently as substrates for exo-PG production in submerged culture (Buyukkileci et al. 2010).

Fungi exhibit different morphological forms ranging from dispersed mycelia growth to pellets of various sizes in submerged culture. Desired morphology depends on the fungus and the product. Generally, filamentous growth yields higher biomass but highly viscous broths with non-Newtonian behavior (Paul et al. 1999, Pazouki and Panda 2000). Due to the high viscosity, higher agitation rates, thus more power is required in order to attain desired mixing and oxygen transfer in stirred tank bioreactors (Amanullah et al. 2002). Pellet morphology has the advantage of exhibiting non-viscous broth with Newtonian behavior, so that better mixing and aeration can provided, and separation of biomass from the broth is simpler. However, the interior of the pellets are nutrient and oxygen limited, so that growth and product synthesis may be impaired (Papagianni 2004, Grimm et al. 2005, Fontana et al. 2009). To overcome this limitations, a significant study was achieved by Fontana et al. 2009. They compared solid state and submerged fermentation conditions for the same substrate and improved that it was possible to get same efficiency for both culture however exo-PG activity in solid state culture (18.6 UmL^{-1}) was higher than in submerged fermentation (16.1 UmL^{-1}).

2.12. Thesis Objective

The aim of this study was production of exo-PG by *A. sojae* using orange peel extract in submerged fermentation because orange peels are rich in pectin, cellulose, and hemicelluloses, which can be converted to fermentable substrates. *A. sojae* was able to use agro-industrial wastes directly (Buyukkileci et al. 2010). They obtained PG activity of 145.4 UmL^{-1} with both pellet and free mycelium formation. Besides high level of enzyme production, some rheological problems such as viscosity, unmixed regions and foaming in bioreactor studies were occurred. Applications of some pretreatments might prevent viscosity and undesirable problems instead of using orange peel directly. For this purpose optimization of phosphoric acid hydrolysis was carried out. Hydrolysis with sulfuric acid was also made to comprise the effect of acid type on exo-PG production and fungal growth. Observation of pellet production and pellet number is also considered during fermentations. As the other proposed objective of this study, water extraction of orange peel was performed to control the role of any acid for releasing the sugars and thus production of exo-PG.

CHAPTER 3

MATERIALS AND METHODS

3.1. Material

3.1.1. Chemicals

The chemicals used in the study are listed in Table 3.6. All the chemicals were reagent grade.

Table 3.6.Chemicals used in experiments

	CHEMICAL NAME	CODE
Hydrolysis & Neutralization	Calcium carbonate ($CaCO_3$)	Sigma 12010
	Phosphoric acid (85%) (H_3PO_4)	-
	Potassium hydroxide (KOH)	AppliChem A3871
	Sodium hydroxide (NaOH)	Panreac 141687
	Sulfuric acid 98% (H_2SO_4)	-
Reducing sugar & polygalacturonase assays	Acetic acid	-
	Ammonium heptamolybdate heptahydrate ($(NH_4)_6Mo_7O_{24}.\ 7H_2O$)	Sigma 31402
	Copper (II) sulfate pentahydrate	-
	Copper (II) sulphate-pentahydrate ($CuSO_4.\ 5H_2O$)	Sigma 12849
	D-(+)-Galacturonic acid	Fluka 48280
	D-(+)-Glucose monohydrate	Sigma 16301
	Disodium hydrogen arsenate heptahydrate ($AsHNa_2O_4.7H_2O$)	-
	Polygalacturonic acid	Sigma P3850
	Potassium sodium tartarate tetrahydrate ($C_4H_4KNaO_6.\ H_2O$)	Merck 1.08087
	Potassium sodium tartarate tetrahydrate ($C_4H_4KNaO_6.4H_2O$)	
	Sodium acetate trihydrate	Sigma 25022
	Sodium arsenate dibasic heptahydrate	Sigma A6756
	Sodium bicarbonate ($NaHCO_3$)	Sigma 31437
	Sodium carbonate (Na_2CO_3)	Sigma 13418
	Sodium sulfate (Na_2SO_4)	Sigma 13464
	Sulfuric acid 98% (H_2SO_4)	-

(Cont. on next page)

Table 3.6.(Cont.)

	CHEMICAL NAME	CODE
Spore propagation	Bacteriological Agar	BD 214010
	Copper (II) sulfate pentahydrate	Sigma 12849
	D-(+)-Glucose monohydrate	Sigma 16301
	Glycerol	Sigma G5516
	Iron (II) sulfate heptahydrate	Riedel-De Haën 12354
	Magnesium sulfate (MgSO4)	-
	Malt extract	BD 218630
	Manganese (II) Sulfate Monohydrate	Riedel-De Haën 13255
	Molasses	Pakmaya, Kemalpaşa
	Peptone	BD 211677
	Potassium phosphate (KH_2PO_4)	Sigma 04243
	Sodium chloride	Riedel-De Haën 13423
	Tween 80	-
	Yeast extract	Merck 1.03753
Fermentation and Enzyme production	Ammonium molybdate hydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$)	Sigma 31402
	Ammonium sulfate ($(NH_4)_2SO_4$)	Sigma 31119
	Calcium Chloride Dihydrate ($cacl_2.2H_2O$)	-
	Cobalt (II) chloride hexahydrate	Riedel-De Haën 12914
	Copper (II) sulphate pentahydrate	Sigma 12849
	Disodium ethylene diamine tetra acetic acid (Na-EDTA)	-
	Iron (II) sulfate heptahydrate	Riedel-De Haën 12354
	Magnesium sulfate (mgso4)	Sigma 63140
	Manganese(II) Chloride Tetra hydrate ($mncl_2.4H_2O$)	-
	Manganese(II)sulfate monohydrate	-
	Potassium phosphate (KH_2PO_4)	Sigma 04243
	Zinc sulphate	-
	Zinc sulphate heptahydrate	-

3.1.2. Orange Peel

Residual orange peels (O.P) were randomly taken from a canteen in Kucuk Park, Izmir. They were mixed to obtain a homogenous bulk. The inside segments of orange peels were separated from the outermost layer and peels were cut into small pieces with hand. Orange peel powder and orange peel pieces were tested as preliminary works. Orange peel powder and orange peel pieces at different sizes were suspended with adequate distilled water and subjected to dilute acid hydrolysis to determine suitable form and particle size.

3.1.3. Microorganisms

A. sojae ATCC 20235 was purchased in the lyophilized form, from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. *A. sojae ATCC 20235* was randomly mutated to obtain mutant *A.sojae* in Jacobs University gGmbH, Bremen by the group of Professor Marcello Fernandez-Lahore.

3.2. Methods

3.2.1. Determination of Dry Matter Content

The orange peel pieces were dried in incubator at 65 ^{o}C until constant weight was achieved. Dried orange peel pieces were stored in tapped dishes and boxes in room temperature**.**

3.2.2. Determination of Moisture Content

The moisture content of approximately 15 g of dried orange peel pieces was determined with Precisa Moisture Analyzer XM 60 (Precisa Instruments, Diekinton, Germany) by drying the samples at 105 ^{o}C until a stable weight was reached.

3.2.3. Spore Propagation

The propagation of the cultures was done on YME agar slant medium containing, malt extract (10 gL^{-1}), yeast extract (4 gL^{-1}), glucose (4 gL^{-1}) and agar (20 gL^{-1}), incubated at 30 ^{o}C until well sporulation (1 week). Stock cultures of these strains were prepared with 20 % glycerol water and stored at -80 ^{o}C. The spore suspensions used as inoculums were obtained on molasses agar slants containing glycerol (45 g L^{-1}), peptone (18 gL^{-1}), molasses (45 gL^{-1}), NaCl (5 gL^{-1}), $FeSO_4.7H_2O$ (15 mgL^{-1}), KH_2PO_4 (60 mgL^{-1}), $MgSO_4$ (50 mgL^{-1}), $CuSO_4.5H_2O$ (12 mgL^{-1}), $MnSO_4.H_2O$ (15 mgL^{-1}) and agar (20 gL^{-1}), after the pre-activation step performed on YME agar using the stock cultures. The incubation temperature and time for each of the steps were 30 ^{o}C and one week, respectively. The harvesting the spores from the slants was done using 5 ml of Tween 80-water (0.02%) solution. The spore suspensions were filtered through a glass funnel packed with cotton wool to get rid of agar and mycelia residues. The spore suspension was collected in a sterile glass tube and stored at 4 ^{o}C at most five days.

3.2.4. Spore Counting

The initial spore counts were recorded with Neubauer Haemocytometer after spore collections. Inoculation volume was determined according to the defined spore concentrations.

3.2.5. Water Extraction of Orange Peel

Extraction of orange peels was carried out at 80 ^{o}C in 60 minutes. The extraction with 10% solid loading (on dry basis) was carried out by using water bath and extracts were separated from solid part by cheese cloth. The aim was showing up the role of presence of acid on releasing reducing sugar.

3.2.6. Hydrolysis Procedure

Hydrolysis was carried out in Hirayama Autoclave Sterilizer HVP-50 by homogenization of orange peel pieces at 10% solid loading with aqueous part. The aqueous part consisted of diluted acid (20% v/v phosphoric acid-water and sulfuric acid-water) solution and distilled water. To achieve precise conditions, volumetric calculations were used. Volume of 10 % solid loading in distilled water was measured thus volume of acid solution and remaining distilled water volume were calculated. The hydrolysate was separated from solid part with cheese cloth after hydrolysis then total and individual sugar contents were determined with reducing sugar method (Nelson Somogyi) and HPLC analysis.

3.2.7. Optimization of Dilute Phosphoric Acid Hydrolysis

Optimization studies were carried out by using Design-Expert 7.0.0 Software (trial version) and central composite design (CCRD) was selected among response surface methodology. All experiments were done in triplicates.

Temperature (^{o}C), time (min) and phosphoric acid (H_3PO_4) concentrations (% v/v) were selected as variable factors to determine the most suitable hydrolysis conditions and response was amount of reducing sugar (gL^{-1}). Some preliminary studies were done (data not shown) with different ranges for each factor but a significant model could not obtain so that factor ranges were extended as shown Table 3.7 and Table 3.8.

Table 3.7.Factor ranges of optimization design

	Temperature (^{0}C)	Time (min)	Acid concentration (%)
-α	100	5	0.0
-1	107	15	1.2
0	117	30	3.0
1	127	45	4.8
+α	134	55	6.0

Orange peel solid loading could be a factor but it had been decided to hold solid loading at a high value due to high sugar concentrations were aimed after dilute acid hydrolysis. Orange peel solid loading was constant at 10% (dry mater) during all experiments.

Table 3.8.Summary of optimization study

Study type		Response surface			**Runs**	34			
Initial design		Central composite			**Blocks**	no blocks			
Design model		quadratic							
Factor	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	**Loc Coded**	**High Coded**	**Mean**	**Std. Dev.**
A	Temp.	^{o}C	numeric	107.0	127.0	-1.0	1.0	117.0	8.963
B	Time	min.	numeric	15.0	45.0	-1.0	1.0	30.0	13.444
C	Acid con.	%	numeric	1.2	4.8	-1.0	1.0	3.002	1.610
Response	**Name**	**Units**	**Obs**	**Analysis**	**Minimum**	**Maximum**	**Mean**	**Std. Dev**	**Ratio**
Y1	reducing sugar	(gL^{-1})	34	polynomial	26.700	42.400	35.909	3.994	1.588

3.2.8. Comparison of Phosphoric Acid and Sulfuric Acid Hydrolysis

There are so many studies in literature that use sulfuric acid in dilute acid hydrolysis. Talebnia, Pourbafrani et al. (2008) studied the optimization of sulfuric acid hydrolysis and they determined the optimum condition at 0.5% sulfuric acid concentration and 116 ^{o}C in 13 min with lower HMF formation. The optimum condition of that study was used as a reference to show differences between phosphoric and sulfuric acids at optimum points in terms of reducing sugar concentrations and enzyme productions.

3.2.9. Neutralization of Hydrolysates

Neutralization procedure was needed due to the high acidity of hydrolysates after dilute acid hydrolysis. Approximately 50% loss of hydrolysate volume was observed in preliminary studies when the hydrolysates were neutralized with alkaline

solutions. Thus calcium carbonate ($CaCO_3$), sodium hydroxide (NaOH) and potassium hydroxide (KOH) were tested as neutralizing agent to overcome this loss. Hydrolysates that were adjusted to defined pH value were centrifugated and supernatants were collected. Reducing sugar amounts were determined before and after centrifugation (9500 rpm for 10 min) of hydrolysates.

3.2.10. Enzyme Production

3.2.10.1. Composition of Fermentation Medium

pH-adjusted hydrolysates were prepared to be used as fermentation medium which was fixed at 70 ml for each 250 ml flasks. At this preparation step, some nutrients to which fungi could be needed were added and these nutrients are shown in Table 3.9.

Table 3.9.Additional nutrients for fungal growth

Composition	Concentration (gL^{-1})
$(NH_4)_2SO_4$	5
KH_2PO_4	2.5
$MgSO_4$	0,5
$FeSO_4.7H_2O$	$6,6x10^{-4}$
$ZnSO_4$	$6,2x10^{-4}$
$MnSO_4$	$1x10^{-5}$

Vishniac and Santer (1957) were studied the effects of different inorganic mediums on microbial growth. Microorganisms were used inorganic medium as basic energy source and they supported fermentation medium with trace element solutions. Due to this conclusion, trace element solution (0.1% v/v) was also used to test either it could affect enzyme production, fungal biomass or not (Table 3.10).

Table 3.10.Composition of trace element solution

Composition	Concentration (gL^{-1})
Na_2-EDTA	50
$ZnSO_4$ x 7 H_2O	22
$CaCl_2$ x 2 H_2O	5.54
$MnCl_2$ x 4 H_2O	5.06
$FeSO_4$ x 7 H_2O	5.00
$(NH_4)_6Mo_7O_{24}$ x 4 H_2O	1.10
$CuSO_4$ x 5 H_2O	1.57
$CoCl_2$ x 6 H_2O	1.61

3.2.10.2. Fermentation Parameters

Fermentations were carried out in 250 ml flasks with shaking incubator. The operation conditions were 200 rpm speed and 30 ^oC measured temperature (Jayani et al. 2005). All experiments were done in triplicates.

3.2.11. Analytical Methods

3.2.11.1. Determination of Reducing Sugar

The total reducing sugar amount was determined according to the Nelson Somogyi method (Somogyi 1952) calibrated with glucose (Appendix B, Figure B.38). In the first step of this method; glucose (or a reducing sugar) was oxidized using a solution of Cu (II) ion which in the process is reduced to Cu (I). Latter, the Cu (I) ions were then oxidized back to Cu (II) using a colorless hetero-polymolybdate complex, which was reduced to give the characteristic color. Hetero-polymolybdate complex was arsenomolybdate which was formed by the reaction of ammonium molybdate, $(NH_4)_6Mo_7O_{24}$, with sodium arsenate, Na_2HAsO_7 for this work. Finally the absorption of the arsenomolybdate was measured against water at 500 nm on Varian Cary Bio 100

UV-Visible spectrophotometer and compared to standards prepared from glucose solutions of known concentration, to determine the amount of reducing-sugar present.

3.2.11.2. Enzyme Assay

Exo-PG activity was carried out according to the procedure given by Panda et al. (1999) with minor modifications. Enzyme activity was determined using 2.4 gL^{-1} of polygalacturonic acid solution as substrate and 100 mM sodium acetate buffer at pH 4.8. The amount of substrate and enzymes used were 0.4 and 0.1 mL, respectively. The absorbance was read against water on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. In this study, one unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time (one minute) at standard assay conditions.

Galacturonic acid was used as standard for the calibration curve (Appendix B, Figure B.39) of PG activity. Calibration curve was prepared using 5, 10, 20, 40, 60, 80 μL volume of the 10 mM stock galacturonic acid solution and all experiments were done in triplicates.

Activity (U/mL) = (μg galacturonic acid/212.12)*(1/time)*(1/amount of enzyme) (3.1)

where; 212.12 is the molecular weight of galacturonic acid (g/mole). Time is expressed as minute and amount of enzyme is in mL.

3.2.11.3. High Performance Liquid Chromatography (HPLC) Analysis

The Perkin Elmer Series 200 HPLC system was used for determination of sugar profiles of hydrolysates which was equipped with auto-injector (20 μL), Perkin Elmer Series 200 column oven, and Perkin Elmer Series 200a refractive index detector. Aminex HPX-87H (1300 x 7.8 mm, 9μm) column was used and column temperature was kept at 65 ^{o}C. The system was isocratic at a flow rate of 0.6 mL min^{-1} and 5 mM sulfuric acid solution (H_2SO_4) was used as mobile phase, which was filtered through 0.2 μm filter with vacuum filtration system and degassed.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Dry Matter and Moisture Contents of Orange Peel

Randomly taken orange peels were minced and dry matter content of the peel was determined. Rangarajan, Rajasekharan et al. (2010) reported the dry matter content of the orange peel articles as approximately 30% and in this study orange peel pieces had 25(±0.01)% dry matter content. Solid content might change according to growth region, seasonal differences etc. Moisture were also analyzed on dried orange peel particles and the moisture content of the dried orange peel pieces was measured as 1.35 (±0.2) %.

4.2. Optimization of Dilute Phosphoric Acid Hydrolysis

The design was composed of 34 runs. The factor codes of each level and the amount of reducing sugar measured in hydrolysates are shown in Table 4.11 and the runs were sorted by standard order.

All of these runs were carried out in 100 mL hydrolysis volume and the solid loadings for all runs were 10 g dried orange peel particles (10% solid loading).

Response ranges were from 27 to 42 gL^{-1} and the ratio of maximum to minimum was 1.59. Ratio greater than 10 requires transformation for analysis of given data thus none transformation was selected for analysis.

Table 4.11.RSM table of coded and actual levels in the design of dilute phosphoric acid hydrolysis by Central Composite Rotatable Design (CCRD)

Std.	Run No.	Coded level of variables			Actual level of variables			Reducing Sugar (gL^{-1})	Reducing Sugar Yield (%)
		A	B	C	Temp. (^oC)	Time (min.)	Acid Conc. (%)		
1	11	-1	-1	-1	107	15	1.2	30.7	31
2	4	-1	-1	-1	107	15	1.2	31.9	32
3	6	1	-1	-1	127	15	1.2	34.3	34
4	7	1	-1	-1	127	15	1.2	33.1	33
5	20	-1	1	-1	107	45	1.2	31.9	32
6	13	-1	1	-1	107	45	1.2	29.6	30
7	34	1	1	-1	127	45	1.2	34.5	35
8	2	1	1	-1	127	45	1.2	34.4	34
9	17	-1	-1	1	107	15	4.8	36.9	37
10	33	-1	-1	1	107	15	4.8	36.2	36
11	25	1	-1	1	127	15	4.8	38.7	39
12	23	1	-1	1	127	15	4.8	38.1	38
13	12	-1	1	1	107	45	4.8	37.4	37
14	15	-1	1	1	107	45	4.8	39.6	40
15	8	1	1	1	127	45	4.8	40.8	41
16	10	1	1	1	127	45	4.8	37.1	37
17	1	-1.7	0	0	100	30	3	32.6	33
18	30	-1.7	0	0	100	30	3	30.4	30
19	22	1.7	0	0	134	30	3	38.8	39
20	5	1.7	0	0	134	30	3	34.5	35
21	19	0	-1.7	0	117	5	3	36.2	36
22	3	0	-1.7	0	117	5	3	36.9	37
23	31	0	1.7	0	117	55	3	35.3	35
24	16	0	1.7	0	117	55	3	39.8	40
25	14	0	0	-1.7	117	30	0	26.7	27
26	24	0	0	-1.7	117	30	0	27.1	27
27	21	0	0	1.7	117	30	6	38.9	39
28	27	0	0	1.7	117	30	6	39.7	40
29	29	0	0	0	117	30	3	42.3	42
30	9	0	0	0	117	30	3	38.0	38
31	32	0	0	0	117	30	3	39.1	39
32	26	0	0	0	117	30	3	40.6	41
33	18	0	0	0	117	30	3	36.4	36
34	28	0	0	0	117	30	3	42.4	42

Lack of fit test and model statistics of the design was shown in Table 4.12 and Table 4.13. Quadratic model was significant due to insignificant lack of fit and both high adjusted and predicted R-squared values.

Table 4.12.Lack of fit test of the design

Source	Sum of Squares	Degree of Freedom	Mean Square	*f* value	*p* value
linear	161	11	14.64	4.27	0.0028
2FI	156	8	19.51	5.69	0.0009
quadratic	14.8	5	2.97	0.87	0.52222
cubic	0	0			
pure error	65.15	19	3.43		

Table 4.13.Model statistics of the design

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared
linear	2.75	0.583	0.5413	0.5038
2FI	2.86	0.5921	0.5014	0.4783
quadratic	1.83	0.8525	0.7972	0.7216
cubic	1.85	0.8799	0.7914	0.6561

As shown in ANOVA table (Table 4.14), *p* value of the model was less than 0.05 (α-value) thus the model was significant. Temperature (A), acid concentrations (C) and their quadratic terms (A^2 and C^2) were significant (Figure 4.9 and Figure 4.10) however time did not affect the amount of reducing sugar significantly. The lack of fit for the design was not significant because the value was higher than 0.05 (α- value) so that it means there was also no interaction between factors.

Table 4.14.ANOVA table for optimization design

Source of variation	Sum of squares	df	Mean square	F-value	probability	
Model	462.4045	9	51.37828	15.41401	< 0.0001	significant
A-Temperature	42.22016	1	42.22016	12.66648	0.0016	
B-Time	2.828237	1	2.828237	0.8485	0.3661	
C-Acid Conc.	271.9397	1	271.9397	81.5847	< 0.0001	
AB	0.000597	1	0.000597	0.000179	0.9894	
AC	3.590781	1	3.590781	1.077271	0.3097	
BC	1.211417	1	1.211417	0.363437	0.5523	
A^2	74.2547	1	74.2547	22.27717	< 0.0001	
B^2	12.87191	1	12.87191	3.861706	0.0611	
C^2	105.9136	1	105.9136	31.77518	< 0.0001	
Residuals	79.99726	24	3.333219			
Lack of fit	14.69957	5	2.939914	0.855442	0.5283	Not significant
Pure Error	65.2977	19	3.436721			
Corr Total	542.4017	33				

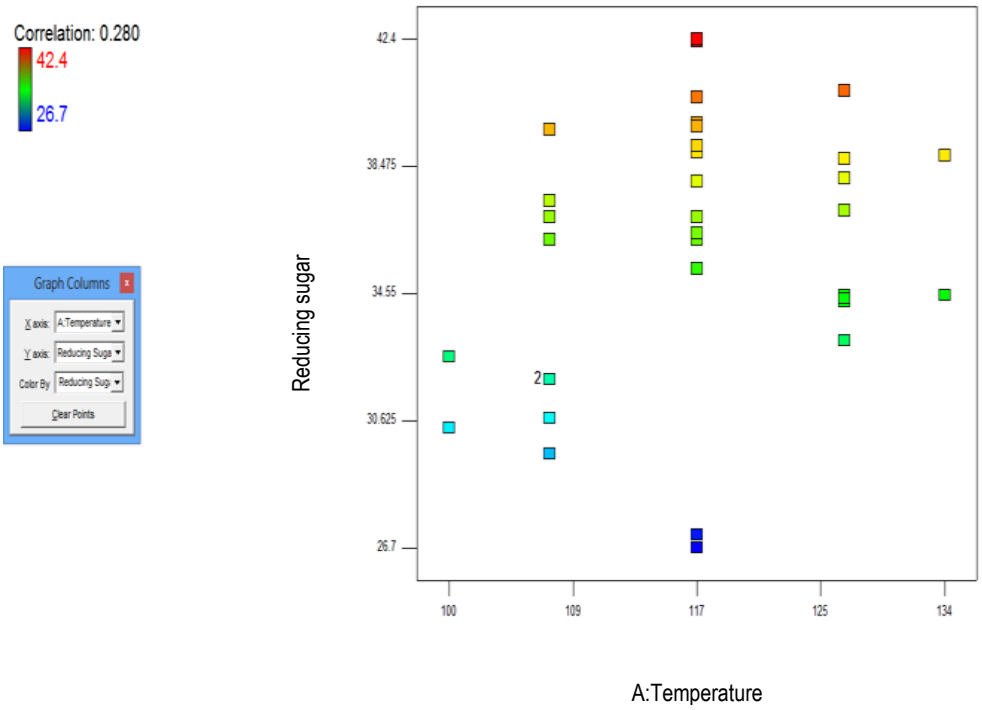


Figure 4.9.Effect of temperature on reducing sugar concentration

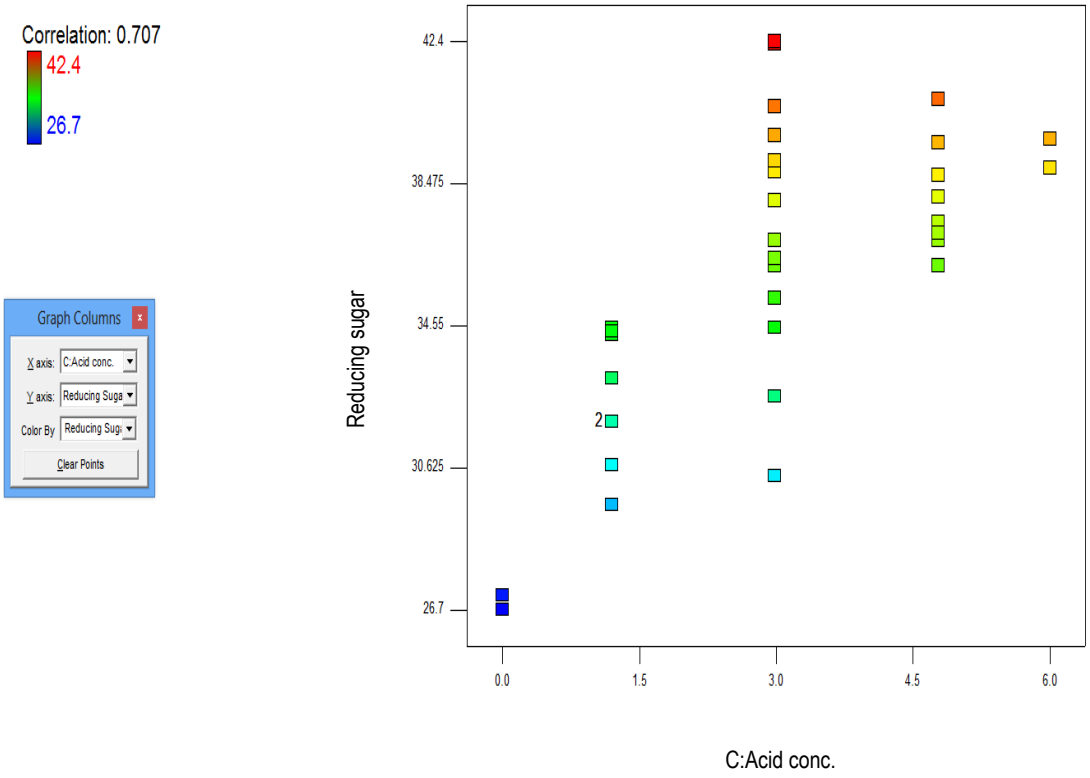


Figure 4.10.Effect of acid concentration on reducing sugar concentration

The model equation which involves all factors is given in Equation 4.1 with coded levels;

$$\textit{Reducing Sugar } (gL^{-1}) = +39.72 + 1.24*A + 0.32*B + 3.17*C + 6.111E - 003*A*B - 0.47*A*C + 0.28*B*C - 1.78*A^2 - 0.77*B^2 - 2.20*C^2 \quad (4.1)$$

where A, B and C are temperature, time and acid concentration and AB, AC and BC are their dual interactions

ANOVA analysis showed that model was significant and to control the model validation analyses were carried. Three points that were suggested by software were selected and hydrolysis experiments were repeated at these points (Table 4.15). After validation hydrolysis, reducing sugar contents of hydrolysates were measured.

Table 4.15.Total reducing sugar concentrations of validation analysis

Temperature (^{0}C)	Time (min.)	Acid concentration (%)	Measured Reducing Sugar (gL^{-1})	Model's suggestion (gL^{-1})	Measured / Model's suggestion
120	35	4.3	37.5	41.1	0.91
110	41	1.7	32.3	34.0	0.95
107	45	1.2	31.8	30.1	1.06

Reducing sugar results after validation analysis were compared with model's suggestions at Table 4.15. The ratios between experimental results and model's suggestions were calculated between 0.91 and 1.06 (values were close to 1) thus model was confirmed by validation analysis. According to the significant model, the highest sugar concentration could be obtained using 4.3% phosphoric acid at 120 ^{o}C in 35 min. but acid concentration was so high that volumetric losses would be increased at neutralization step and higher values of acid concentration may have caused undesirable by products (Taherzadeh and Karimi 2007). To overcome these limitations, temperature was fixed at the optimum point but lower acid concentrations were tried (Table 4.16). The reducing sugar concentration at 120^{o}C and 0.4 % phosphoric acid concentration in 20 min. was 34.4 gL^{-1} however it was 27 gL^{-1} at 117^{o}C in 30 min. So hydrolysis condition at 120^{o}C and 0.4 % phosphoric acid concentration in 20 min. was used to obtain fermentation media for exo-PG productions.

Table 4.16.Sugar amounts at lower phosphoric acid concentrations

Temperature (^{0}C)	Time (min.)	Acid concentration (%)	Reducing Sugar (gL^{-1})
117	30	0	27.0
120	20	0.4	34.4
120	20	1.2	36.0

Apart from given factor ranges in Table 3.7, hydrolysis experiments which had no phosphoric acid solution were also conducted. The reducing sugar concentration at 117 ^{o}C in 30 min was 27 gL^{-1} (Table 4.11 - 25th and 26th standards) however the maximum sugar releasing at 3% phosphoric acid concentration at 117 ^{o}C in 30 min was 40 gL^{-1} (Table 4.11) so that in the presence of high phosphoric acid concentration, 48% increase in reducing sugar concentration was observed. And also the optimum condition according to model suggested by validation analysis resulted 37.5 gL^{-1} reducing sugar amount Thus, a 39% increase in reducing sugar concentration could be observed at optimum conditions.

4.3. Optimization of Particle Size

Orange peel powder and orange peel pieces were hydrolyzed to determine the suitable sample form. Orange peel powder form caused high viscosity so that it made harder to separate hydrolysate from solid part and hydrolysate volume after filtration was very low. Due to these results, orange peels were minced with home type food homogenizer to approximately 3-5 mm particle size.

4.4. Releasing Reducing Sugar with Water Extraction

In the optimization study, the extraction in the absence of acid was performed at 117 ^{o}C in 30 min. and reducing sugar concentration after extraction was 27 gL^{-1} as shown in Table 4.11. However in the following study temperature of 80 ^{o}C and time of 60 min was used. Because some apparatus like autoclave was needed to achieve higher temperatures for extractions. Using extra steps or instruments would cause loss of labor and time. Moreover, a lower temperature with longer time periods for extraction was preferable if final total sugar concentrations were nearly close.

The reducing sugar concentration obtained at 80 ^{o}C in 60 min was measured as 18 gL^{-1} while it was 27 gL^{-1} in the extraction at 117 ^{o}C in 30 min so that extraction at 80 ^{o}C was preferable however it released lower concentration of reducing sugar and it can be advantageous due to longer process time instead of the higher temperature compared to the extraction at 117 ^{o}C. Also galacturonic acid concentration which was accepted as inducer (Patil and Dayanand 2006) of exo-PG production was measured as 4.5 gL^{-1} and

it constituted 13% of released reducing sugar during water extraction on the other hand Talebnia et al. (2008) reported the galacturonic acid content as 20 % of dried orange peels.

Sterilization has an interesting effect on sugar concentration because sugar concentration was increased at 35 gL^{-1}. This increase might be caused hydrolysis of polymeric sugars which could have remained in extracts or evaporation might have an effect by reducing the water ratio. Rivas et al. (2008) reported 38.2 gL^{-1} sugar releasing during extraction at 130 ^{o}C, so extraction at 80 ^{o}C was advantageous in the view of sugar releasing, lower extraction temperatures and lower operation costs.

Released sugar concentration at the optimum point after dilute phosphoric acid hydrolysis was 37.5 gL^{-1}; however water extraction caused approximately same release of sugar concentration (Taherzadeh and Karimi 2007). This contradiction could be explained by ratios of pentose and hexose. Acid hydrolysis of orange peel released mainly pentose (xylose, arabinose etc.) from the hemicelluloses part (Yáñez et al. 2004) on the other hand water based extraction solubilised mainly the simple hexose (galactose, mannose, glucose etc.) contained in the orange peel. Amount of hexose were higher than pentose thus; released sugar concentrations of acid hydrolysis and water extraction were closer.

4.5. Comparison of Sulfuric and Phosphoric Acid Hydrolysis

The highest reducing sugar concentration was obtained at 4.3 % phosphoric acid at 120 ^{o}C in 35 min. with optimization experiments. But this acid concentration was high to be used as fermentation medium because concentration of acid was important at neutralization step. At higher acid concentrations, volumetric losses were increased due to the amount of gypsum. To prevent this lost, lower acid concentrations were tried and according to these experiments hydrolysate of phosphoric acid which would be used as fermentation medium was obtained by 0.4 % phosphoric acid concentration at120 ^{o}C, in 20 min. and the contradictory results are shown in Table 4.17.

Table 4.17.Results after sulfuric and phosphoric acid hydrolysis

Temperature (^oC)	Time (min.)	Acid concentration (%)	Reducing Sugar (gL^{-1})
120	35	4.3 (H_3PO_4)	37.5
120	20	0.4 (H_3PO_4)	34.4
116	13	0.5 (H_2SO_4)	47.6

Table 4.17 shows reducing sugar concentrations (measured by Nelson Somogyi method) of hydrolysates that were carried out at 0.4 % phosphoric acid concentration and 120 ^oC in 20 min. and at 0.5 % sulfuric acid concentration and 116 ^oC, in13 min. Galacturonic acid concentration was measured as 11 gL^{-1} however it was twofold higher than galacturonic acid concentration of water extraction. This result showed the importance of acid hydrolysis which was able to release more sugars from insoluble carbohydrates of orange peel.

4.6. Neutralization of Hydrolysates

After acid hydrolysis completed, hydrolysis medium was filtered through cheesecloth and the liquid part which has already been called ‘hydrolysate’ was neutralized, forming salt that called 'gypsum'. Generally, these salts have low solubility and due to type of acid, residual of neutralization (gypsum) was different.

Gypsum ($CaS0_4.2H_20$) is almost always the calcium sulfate mineral (Nelson 1982) and when sulfuric acid was used the dilute acid hydrolysis produces large amounts of gypsum at neutralization step.

Neutralization step was required after hydrolysis because the high acidic characteristic of hydrolysate cannot allow fungal growth. The other important aspect of neutralization was detoxification after dilute acid hydrolysis. Formation of furan derivative HMF was inevitable after dilute acid hydrolysis and toxic compounds in hydrolysate were separated after neutralization to use this hydrolysate as fermentation medium (Cardona et al. 2010).

Amount of gypsum was very high when $CaCO_3$ was used as neutralizing agent alone. Combinations of KOH and NaOH with $CaCO_3$ were tested as an alternative. Higher amount of KOH was needed compared to NaOH although the two alkaline solutions were at same normality. This originates from higher alkali characteristic of NaOH than of KOH. Considering the large scale applications, $CaCO_3$ and/or NaOH were selected to be used as neutralizing agent. Hydrolsates were centrifugated after neutralization and pH values were checked again after the supernatants were collected and no changes were observed.

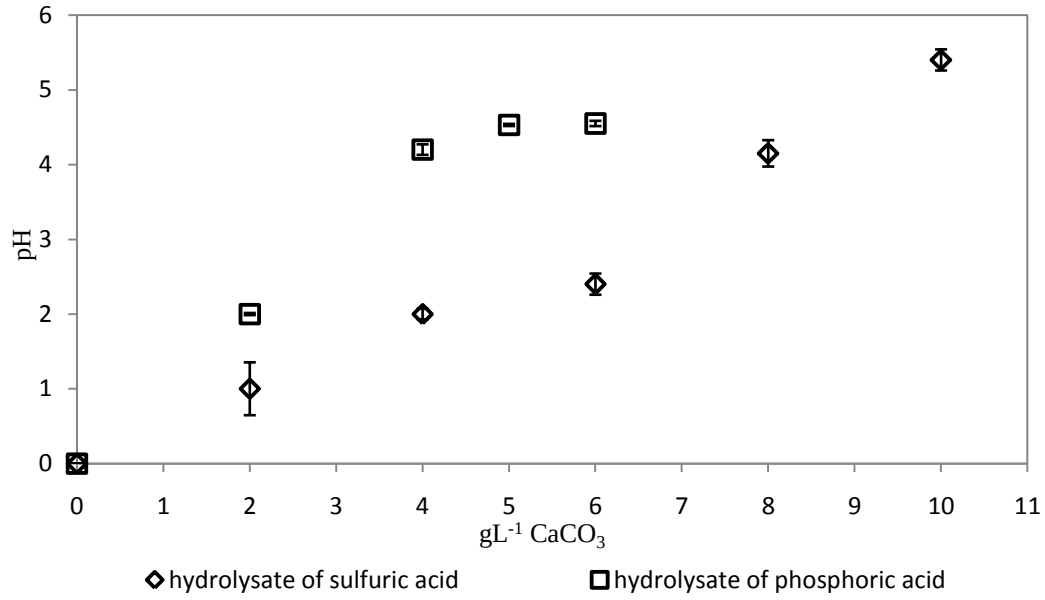


Figure 4.11.Amounts of calcium carbonate for neutralization of hydrolysates

The required amount of $CaCO_3$ for the two defined hydrolysates was shown in Figure 4.11. Due to the high acidity of hydrolysates, 4 gL^{-1} $CaCO_3$ for sulfuric acid hydrolysate and 1 gL^{-1} $CaCO_3$ for phosphoric acid hydrolysate were used to increase pH to approximately pH 2. The amount of $CaCO_3$ that was used for sulfuric acid hydrolysate was much more than used for phosphoric acid hydrolysate. pH of the phosphoric acid hydrolysate could be adjusted with low amount (5 gL^{-1}) of $CaCO_3$ and pH level of phosphoric acid hydrolysate was not changed by increasing amounts of $CaCO_3$ however pH of sulfuric acid hydrolysate continued to increase. This situation may have originated from strong acidity of sulfuric acid.

Before and after neutralization, samples were collected and Table 4.18 shows similar reducing sugar concentrations measured at each step. However the total amounts of sugars recovered were different because the different volumes of hydrolysates were measured before and after (Table 4.19) neutralization. 50 % and 54 % of initial hydrolysis volumes were lost with hydrolysis at 120 ^{o}C, 20 min. and 0.4 % phosphoric acid concentration and 116 ^{o}C, 13 min. and 0.5 % sulfuric acid concentration, respectively. Volumetric losses were at neutralization step and these different volumes were changed according to the type of acid. For example, sulfuric acid was strong acid however phosphoric acid was weaker so volumetric loss for strong acid such as sulfuric acid would be higher.

Table 4.18 shows also reducing sugar concentrations of both sulfuric and phosphoric acid hydrolysates after sterilization. Measured reducing sugar concentrations were increased for both of the hydrolysates. The reason might be hydrolysis of polymeric sugars which could have remained in extracts or evaporation might have an effect by reducing the water ratio.

Table 4.18.Different hydrolysis conditions and released reducing sugar concentrations

Hydrolysis Conditions	Reducing Sugar Concentration (gL^{-1})		
	Before Neutralization	After Neutralization	After Sterilization
120 ^{o}C, 20 min 0.4 % H_3PO_4	34.4	34.1	50
116 ^{o}C, 13 min 0.5 % H_2SO_4	47.6	47.8	66

Table 4.19.Hydrolysate volumes at different hydrolysis conditions

Hydrolysis Conditions	Residual volumes of hydrolysates (%)	
	After hydrolysis [a]	After Neutralization [b]
120 ^{o}C, 20 min 0.4 % H_3PO_4	71	50
116 ^{o}C, 13 min 0.5 % H_2SO_4	73	46

a= (hydrolysate volume after cheesecloth/ initial hydrolysis volume)*100

b= (residula volume after neutralization/ initial hydrolysis voume)*100

4.7. Enzyme Production in Water Extract and Acid Hydrolysates

4.7.1. Exo-PG Production by *A. sojae ATCC 20235*

Hydrolysis at 0.4 % phosphoric acid concentration and 120 ^{o}C in 20 min. and at 0.5 % sulfuric acid concentration and 116 ^{o}C in 13 min. were conducted and then direct and diluted hydrolysates were prepared as fermentation medium by adding nutrient solutions (Table 3.9). pH values of the two hydrolysates were increased up to 2 with $CaCO_3$ then they were adjusted to pH 4.5 with 5N NaOH.

Hydrolysates were diluted with distilled water just after neutralization. Sulfuric acid and phosphoric acid hyrolysates were diluted three and two folds, respectively. Reducing sugar concentrations, pH and exo-PG productions were followed during five-days of fermentation.

4.7.1.1. Exo-PG Production in Phosphoric Acid Hydrolysates

Concentrations of reducing sugar were decreased during fermentation with *A. sojae ATCC 20235* in both twofold diluted and undiluted hydrolysates of phosphoric acid as shown in Figure 4.12. Although sugars were almost depleted after five days of fermentation, significant exo-PG production could not be detected because measured activities for hydrolysate of phosphoric acid was below 1 UmL^{-1}. According to Figure 4.13, low exo-PG production was also validated by pH curve because pH values for both hydrolysates of diluted and undiluted sulfuric acid were nearly constant at level of pH 4 during the first-four days of fermentation. A little decrease was observed but still pH of the fermentation mediums were higher than 3.5.

Pellet formations in both diluted and undiluted hydrolysates of phosphoric acid are shown in Figure 4.14 (a-b). Pellet number was very high in fermentation flasks containing undiluted hydrolysate of phosphoric acid and the pellets were smoother and well-shaped then pellets obtained in hydrolysate of sulfuric acid (Figure 4.14 c-d) although the hydrolysate was used directly.

Hydrolysates of phosphoric acid were used as undiluted in next experiments due to mean diameters of pellets in diluted hydrolysates of phosphoric acid were bigger than the direct use.

4.7.1.2. Exo-PG Production in Sulfuric Acid Hydrolysates

Total sugar concentrations in both diluted and undiluted hydrolysates of sulfuric acid were decreased as expected (Figure 4.12). Although the decreasing rate of undiluted hydrolysate of sulfuric acid was higher than diluted hydrolysate's, their rate of exo-PG production was less than 1 UmL^{-1}.

According to Figure 4.13, low exo-PG production was also validated by pH curve because pH values for both hydrolysates of diluted and undiluted sulfuric acid were nearly constant at level of pH 4 during the first-four days of fermentation. A little decrease was observed but still pH of the fermentation mediums were higher than 3.5.

The fungus, which could not form pellets in undiluted hydrolysate of sulfuric acid (Figure 4.14-c) were able to form smooth pellets when the hydrolysate was diluted (Figure 4.14-d). However, the number of pellets in diluted hydrolysate of sulfuric acid was not similar with the hydrolysate of phosphoric acid.

Exo-PG could not be produced in diluted and undiluted hydrolysates of both phosphoric and sulfuric acid although total reducing sugar concentrations were decreased. As it can be seen in Figure 4.14 which was taken at fourth day of fermentation, *A. sojae ATCC 20235* consumed almost all sugars in the medium for fungal growth in pellets instead of enzyme production.

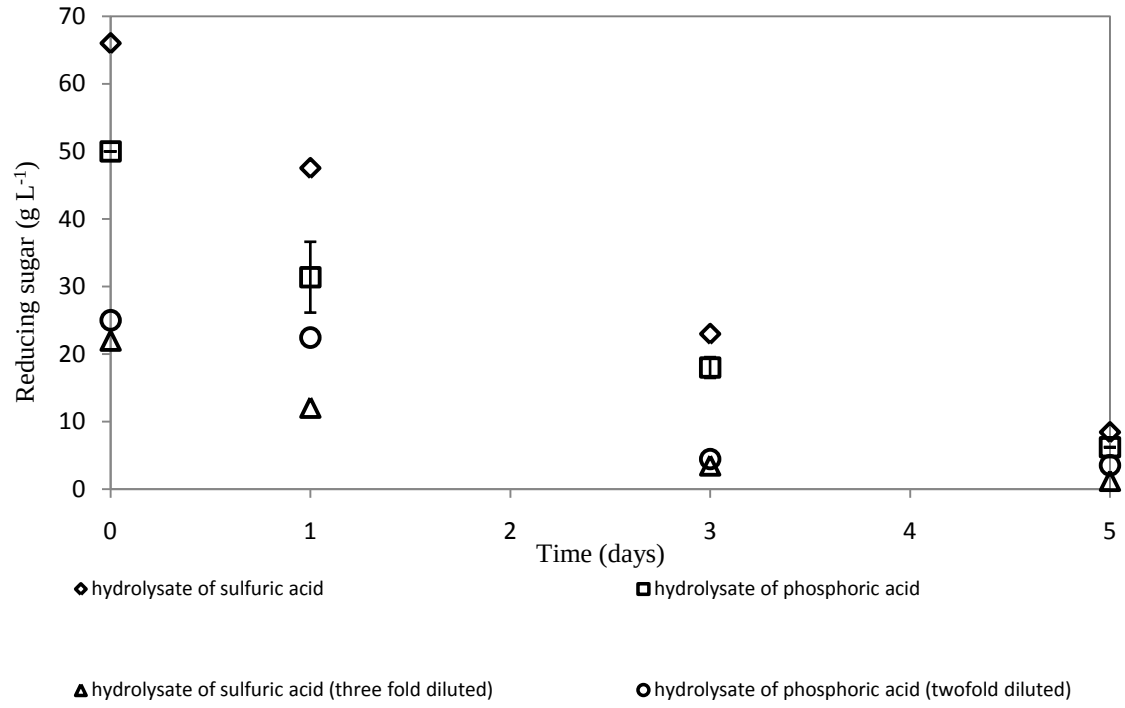


Figure 4.12.Sugar consumption of *A. sojae ATCC 20235* (0.day indicates sugar concentrations after sterilization)

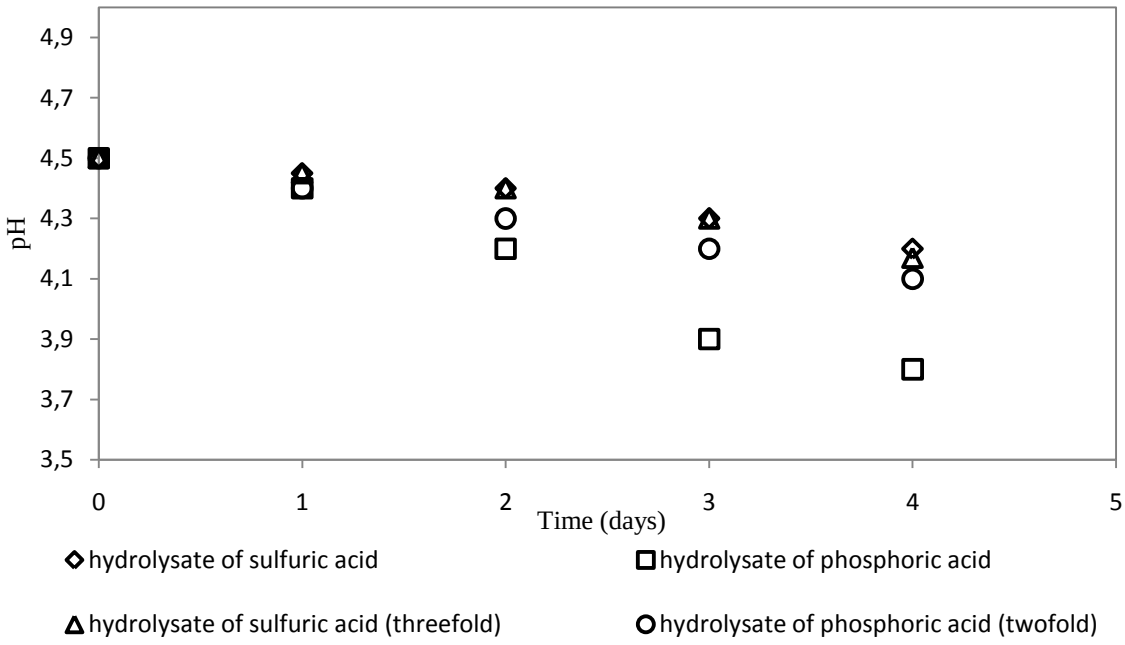
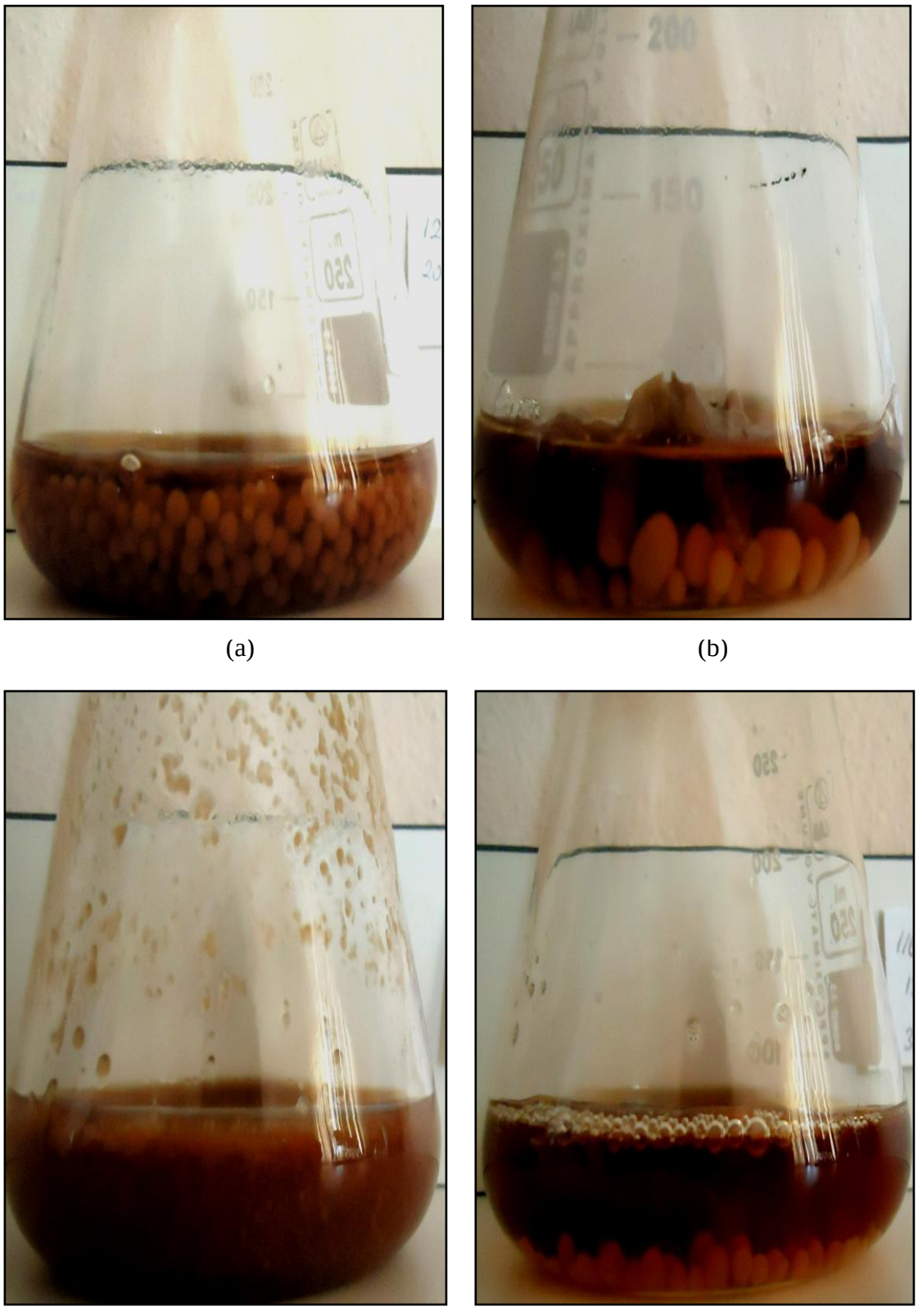


Figure 4.13.pH changes in the course of exo-PG production with *A. sojae ATCC 20235*



(a) (b)

(c) (d)

Figure 4.14.Pellet images from exo-PG production with *A. sojae ATCC 20235*
(a)Hydrolysate of phosphoric acid, (b)Hydrolysate of phosphoric acid (twofold diluted), (c)Hydrolysate of sulfuric acid, (d)Hydrolysate of sulfuric acid (threefold diluted)

4.7.2. Exo-PG Production with mutant *A. sojae*

Mutant *A. sojae* was utilized as well for exo-PG production in hydrolysates of both sulfuric acid and phosphoric acid. Hydrolysates were neutralized with $CaCO_3$ for the exo-PG production with mutant *A.sojae* and pH was adjusted to approximately 4.5. As described in previous experiment; hydrolysates were used undiluted.

4.7.2.1. Exo-PG Production with mutant *A. sojae* in Phosphoric and Sulfuric Acid Hydrolysates

Sugar concentrations in the course of fermentation are shown in Figure 4.15 and a large part of total sugars were consumed up to fourth-day of fermentation by mutant *A. sojae.* Duration of fermentation was limited to four days however there was still significant amount of sugar in the medium because pellets were decomposed at fifth-day of fermentation.

Exo-PG production in hydrolysate of phosphoric acid was much higher than in hydrolysate of sulfuric acid by mutant *A. sojae* as shown in Figure 4.16 while pH changes for hydrolysate of phosphoric and sulfuric acid in the course of the fermentation were similar with *A. sojae ATCC 20235* (Figure 4.17). Exo-PG activity in hydrolysate of phosphoric acid and sulfuric acid were 14.6 UmL^{-1} and 8.4 UmL^{-1}, respectively. Initial sugar concentration in sulfuric acid was more than in phosphoric acid at the beginning of the fermentation. This might have affected the production of exo-PG. And also calcium phosphate was formed at neutralization of phosphoric acid hydrolysate and calcium sulfate was formed at neutralization of sulfuric acid hydrolysate after hydrolyses were completed. Although centrifugation (9500 rpm for 10 min) was done just after neutralization these salts could remain in fermentation media by effecting the exo-PG productions because phosphates could be used as nutrient by organisms (Gámez et al. 2006, Cardona et al. 2010).

Pellet formations in sulfuric and phosphoric acid hydrolysates are shown in Figure 4.18. In sulfuric acid hydrolysate pellets were relatively big (Figure 4.18-a) and in phosphoric acid hydrolysate they were big and not regular (Figure 4.18-b). Although exo-PG values were higher than obtained by *A. sojae 20235*, these pellet formations and this type of fungal growth might cause problems such as limitations of mass transfer in bioreactor studies in large scale.

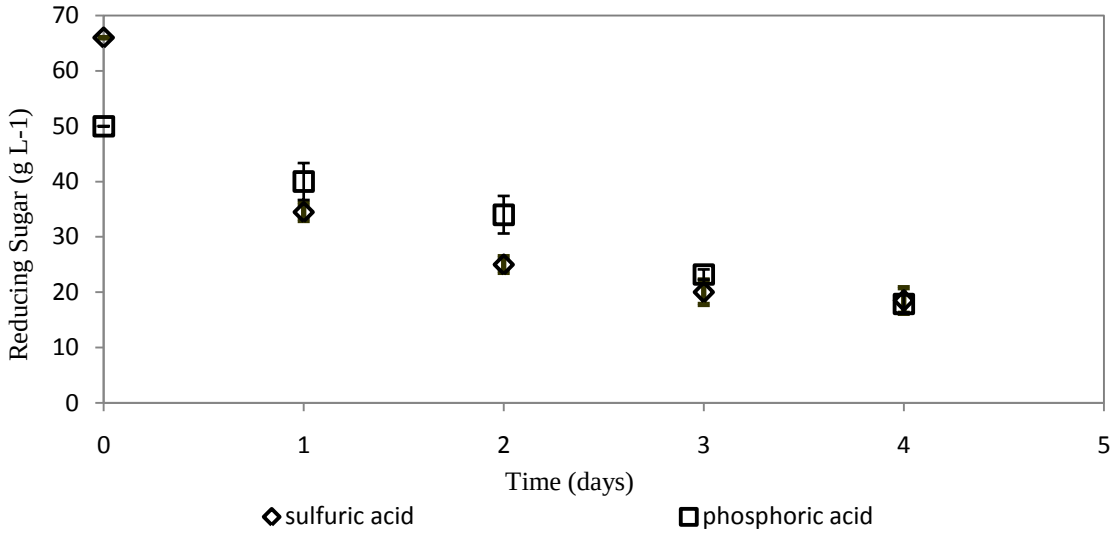


Figure 4.15.Sugar consumption of mutant *A. sojae* (0.day indicates sugar concentrations after sterilization)

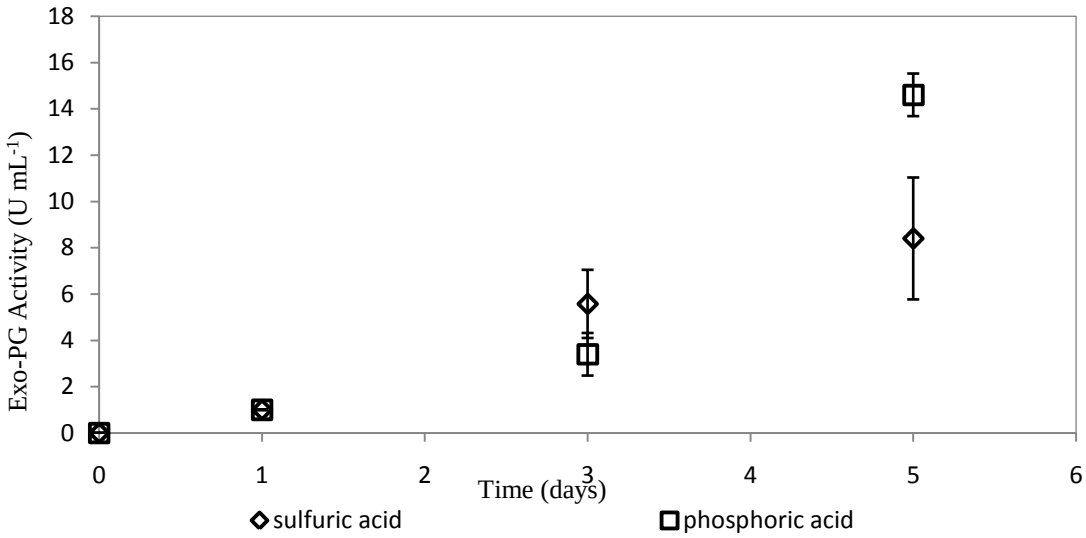


Figure 4.16.Exo-PG production by mutant *A. sojae*

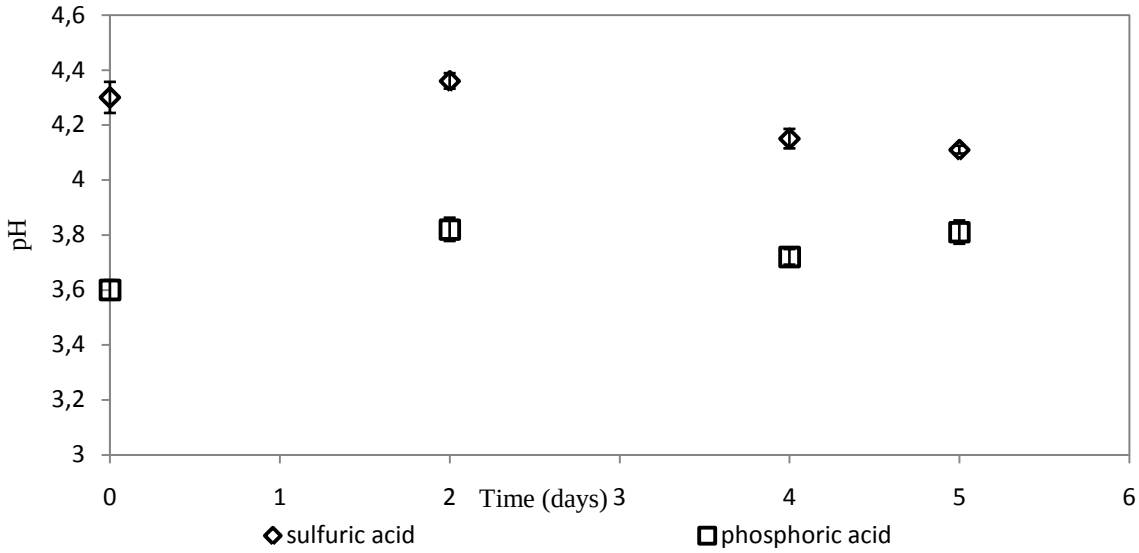
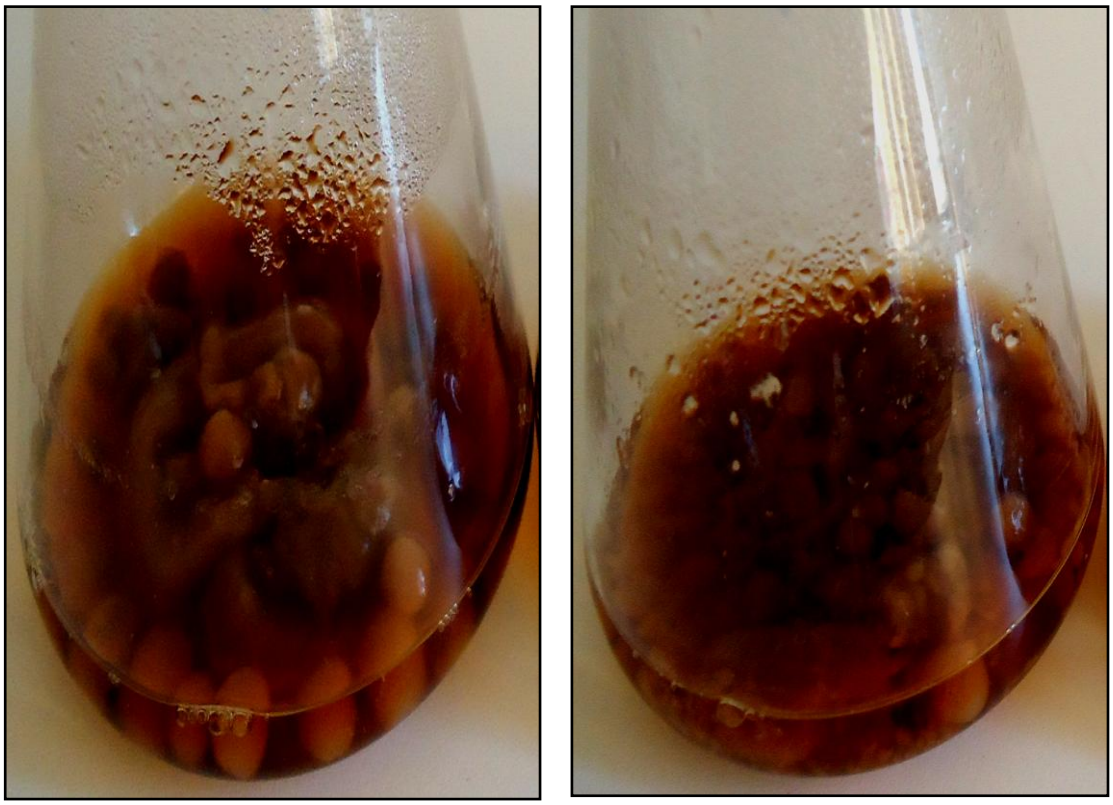


Figure 4.17.pH changes in the course of exo-PG production by mutant *A. sojae*



(a) (b)

Figure 4.18.Pellet images from exo-PG production by mutant *A. sojae* (a) hydrolysate of sulfuric acid, (b) hydrolysate of phosphoric acid

4.7.2.2. Effect of Trace Elements on Exo-PG Production

Mutant *A. sojae* was a better PG producer than *A.sojae 20235*. Additional nutrients were used in fermentation medium in the above studies, but this time trace element solution (Table 3.10) was tested to control whether presence of trace elements might support enzyme production or not. Due to conclusion of Vishniac and Santer (1957), trace elements might support fermentation medium thus Trace element solution was used to test either it could affect enzyme production, fungal biomass or not.

Total sugar concentrations and exo-PG productions in two hydrolysates are shown in Figure 4.19 and Figure 4.20, respectively. Exo-PG production was enhanced in the presence of trace element solution in both cases. Exo-PG production without trace element solution by mutant A.sojae was 8.4 and 14.6 UmL^{-1} in sulfuric and phosphoric acid hydrolysates, respectively. When trace element solutions were added as nutrients, enzyme activity of phosphoric acid hydrolysate was increased up to 18.4 UmL^{-1} at fifth day of fermentation but hydrolysate of sulfuric acid did not give the same response because the enzyme activity value was not increased, even decreased to 5.3 UmL^{-1}. Thus addition of trace element solution increased enzyme production level and the increasing was indicator of relatively low amounts of nutrients in orange peel (Giese et al. 2008).

Pellet formations in the presence of trace element solution are shown in Figure 4.21. There were no pellets in hydrolysate of sulfuric acid (Figure 4.21-a) in the presence of trace element solution on the other hand sizes of pellets were very big in hydrolysate of sulfuric acid without trace element solutions (Figure 4.18-a). In the hydrolysate of phosphoric acid, pellets were more compact and smaller sized (Figure 4.21-b) than pellet formation of fermentation without trace element solution (Figure 4.18-b).

As a summary, hydrolysate of phosphoric acid was a better medium for enzyme production with mutant *A. sojae* in the presence of trace element solution so it could be concluded that fermentations of mutant *A. sojae* might be provided by trace element solutions (Giese et al. 2008). On the other hand, experiments and the results indicate that hydrolysate of sulfuric acid was not suitable for fermentation medium for exo-PG production. On the other hand, hydrolysate of phosphoric acid might promote higher exo-PG enzyme activities using mutant *A. sojae* if the fermentation medium was

supported by additional nutrient solutions .This exo-PG activity values can be optimized with further studies. Pellet size, number and/or density can be also enhanced.

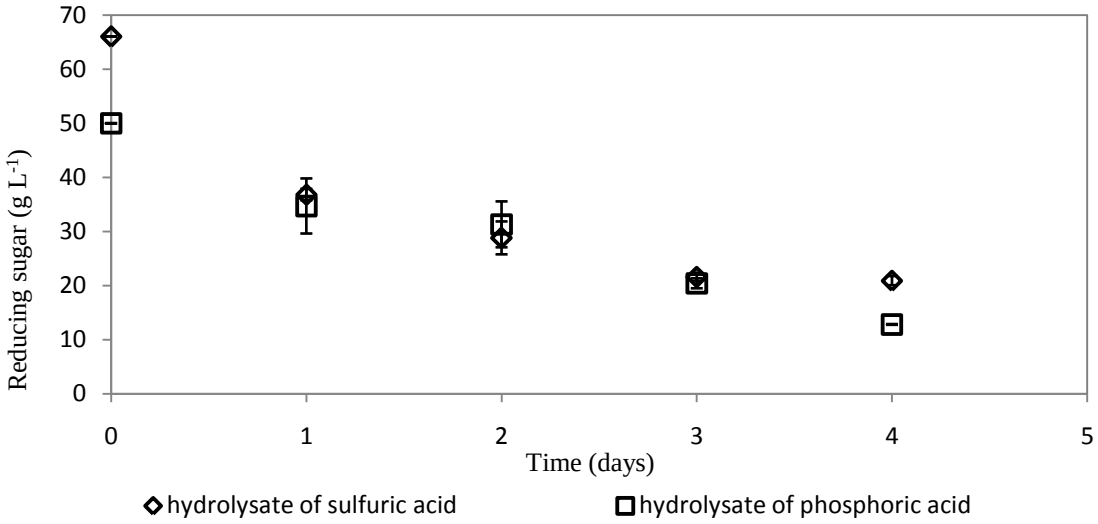


Figure 4.19.Sugar consumption of mutant *A.sojae* in the presence of trace element solution (0.day indicates sugar concentrations after sterilization)

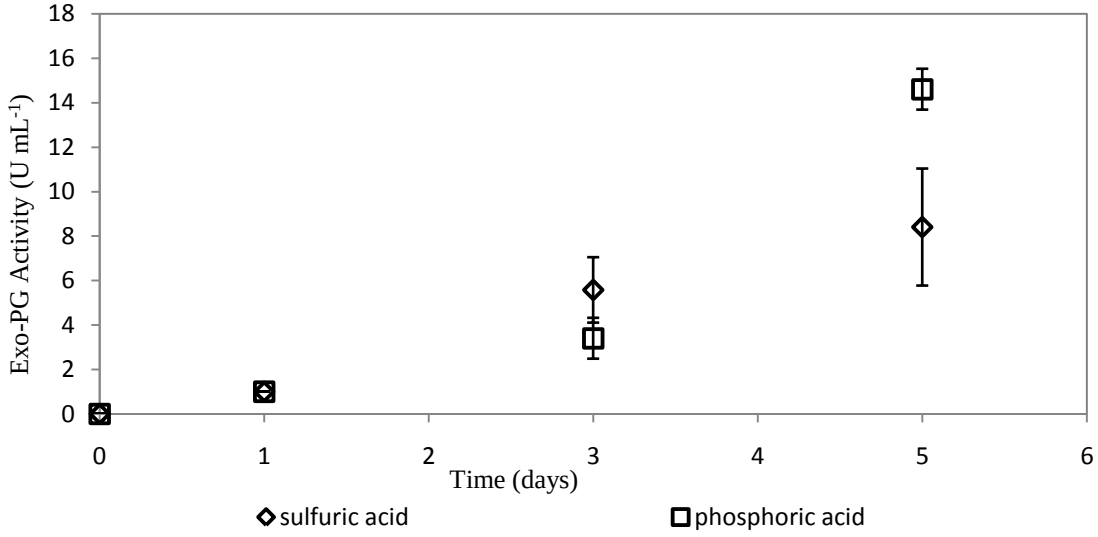
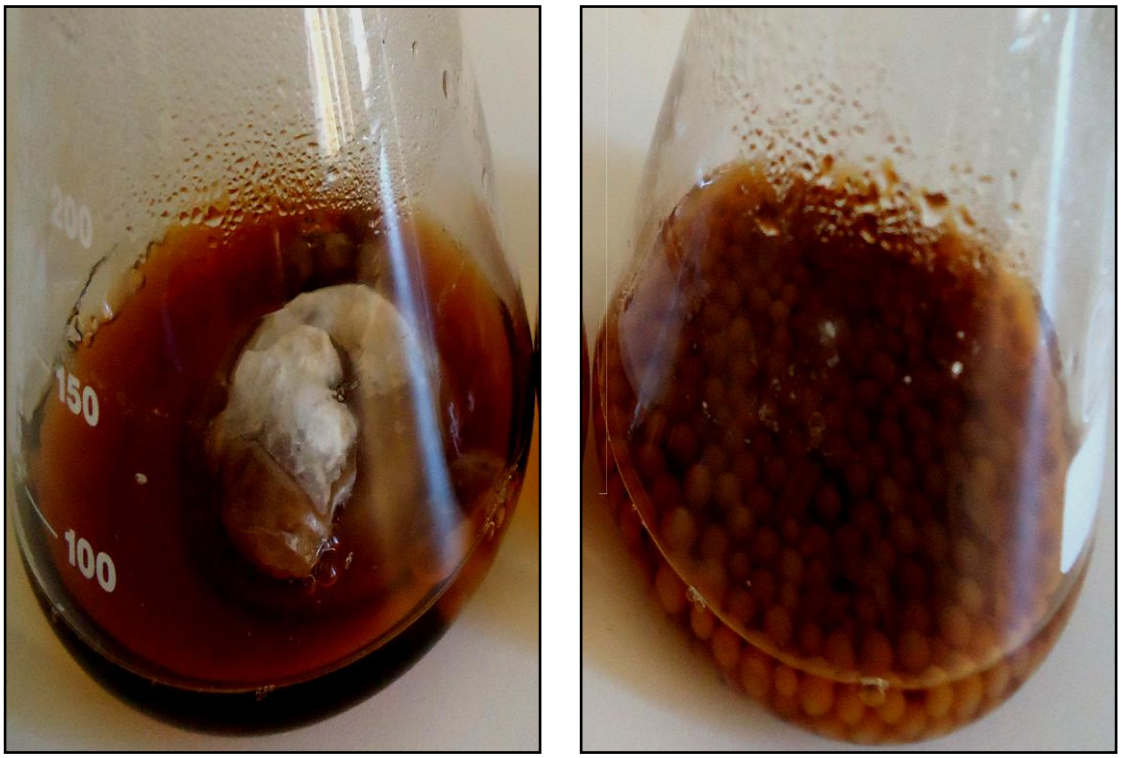


Figure 4.20.Exo-PG production by mutant *A.sojae* in the presence of trace element solution



(a) (b)

Figure 4.21.Pellet images from exo-PG production by mutant *A.sojae* in the presence of trace element solution, (a)hydrolysate of sulfuric acid, (b)hydrolysate of phosphoric acid

4.7.3. Effect of pH on Exo-PG Production in Phosphoric Acid Hydrolysate

Exo-PG activity was highest with mutant *A.sojae* in hydrolysate of phosphoric acid which contained Trace element solution whose pH was adjusted to 4.5. In this part of the study initial pH of hydrolysate before fermentation was adjusted to 6 with different neutralization procedure. The effects of neutralizing agents and initial pH value on the enzyme production were investigated. pH value of hydrolysate of phosphoric acid was increased to 4.2 by adding 4 gL^{-1} $CaCO_3$ and then adjusted to approximately 6 by adding 5N KOH.

The amount of reducing sugar decreased during the course of fermentation (Figure 4.22) and was depleted after eight days. Exo-PG activity was 9.7 UmL^{-1} (Figure 4.23), which was lower than the one obtained in fermentation which was started with an

initial pH of 4.5. During fermentation, pH of the medium varied between 5.7- 4.4 and the pH of the medium was 4.8 when the maximum exo-PG activity was observed (Figure 4.24). pellet formations and densities of both fermentations which were adjusted to initial pH of 4.5 and 6 were not very different from each other (Figure 4.25).

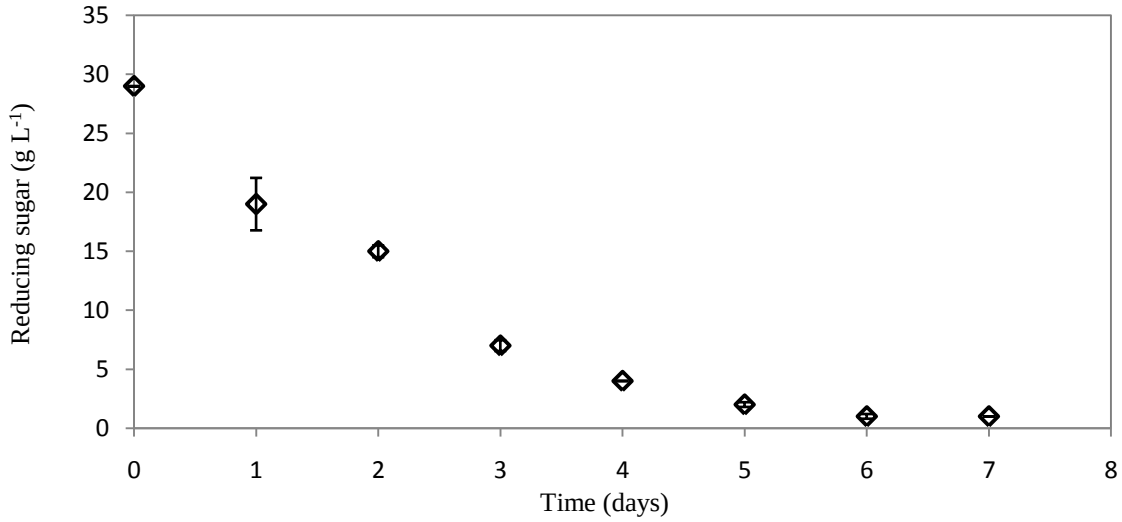


Figure 4.22.Sugar consumption of mutant *A.sojae* in phosphoric acid hydrolysate with different pH adjustment

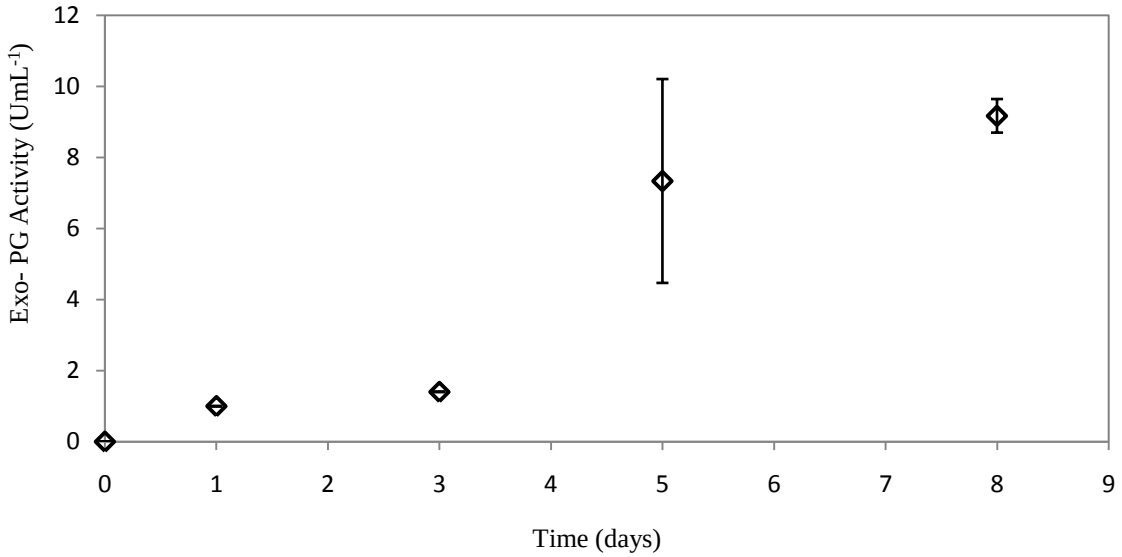


Figure 4.23.Exo-PG production of mutant *A.sojae* in phosphoric acid hydrolysate with different pH adjustment

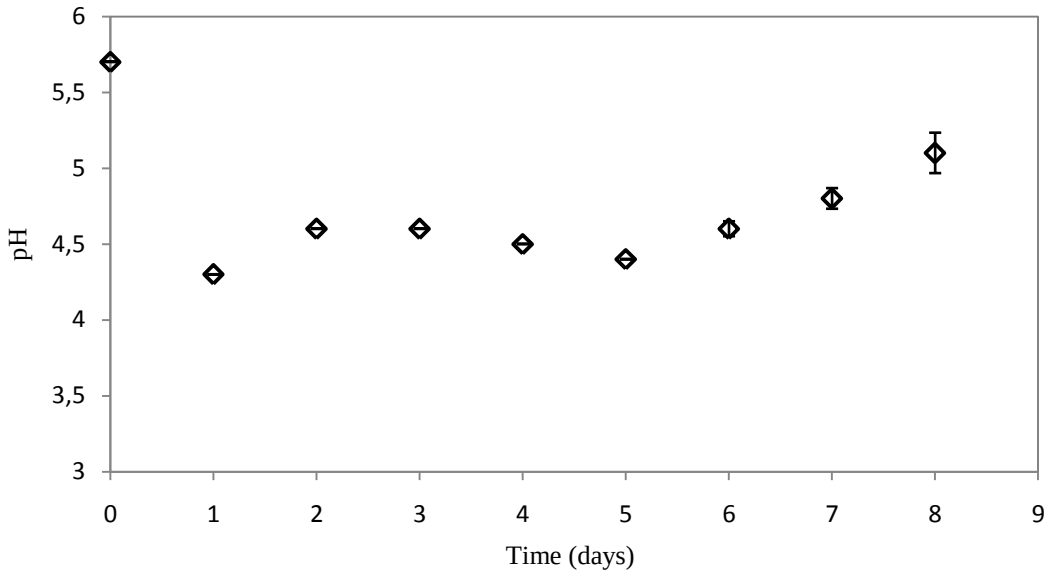


Figure 4.24.pH changes in the course of exo-PG production of mutant *A.sojae* in phosphoric acid hydrolysate with different pH adjustment

Figure 4.25.Pellet image from exo-PG production with mutant *A.sojae* in phosphoric acid hydrolysate with different pH adjustment

4.7.4. Effect of Spore Concentration on Exo-PG Production in Phosphoric Acid Hydrolysate

Spore inoculation which resulted with highest exo-PG activity was $1x10^4$ spore mL^{-1} for experiments up to now. Different spore concentrations for the fermentation of phosphoric acid hydrolysates were tested either to get higher enzyme activities besides acceptable pellet form, size and numbers. The selected concentration levels were $1x10^3$ spore mL^{-1} and $1x10^5$ spore mL^{-1} (Tari et al. 2007). All experiments were done in triplicates.

pH was followed besides the pellet characteristics during fermentations and the pH profile for spore concentration experiment is shown in Figure 4.26. A distinct change of pH was not observed in any flasks and there was also no exo-PG activity in samples (data not shown) however significant amounts of exo-PG activities were obtained with concentration level of $1x10^4$ spore mL^{-1}.

Figure 4.27 shows the image of phosphoric acid flasks that were taken in third-day of the fermentations at a spore concentration of $1x10^5$ spore mL^{-1}. pellet formation in spore concentration of $1x10^3$ spore mL^{-1} were not as sufficient as spore concentration of $1x10^4$ spore mL^{-1}. Pellet production with $1x10^5$ spore mL^{-1} was acceptable than with $1x10^3$ spore mL^{-1} but the forms of pellets and numbers were not sufficient. Pellet formations were not observed in hydrolysates of phosphoric acid at concentration of $1x10^3$ spore mL^{-1}.

These results showed that spore concentration level of $1x10^3$ spore mL^{-1} was inadequate to obtain pellets however the level of $1x10^5$ spore mL^{-1} caused aggregations which made mass diffusions difficult so that it was decided that $1x10^4$ spore mL^{-1} was an acceptable spore concentration for higher exo-PG activities besides pellet formation.

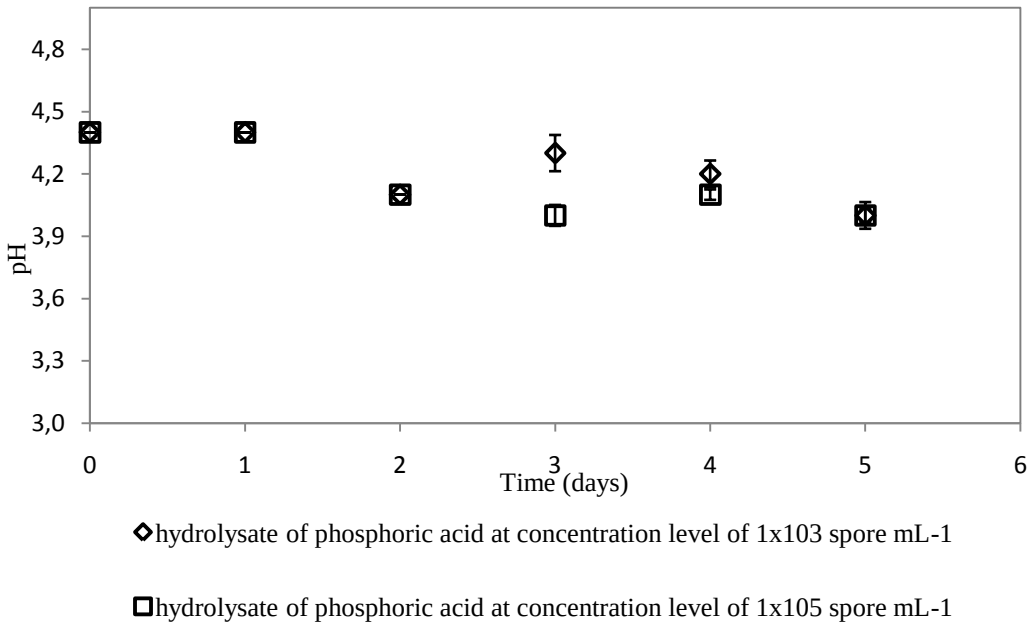


Figure 4.26.pH changes in the course of exo-PG production of mutant *A.sojae* in phosphoric acid hydrolysate at different spore concentrations

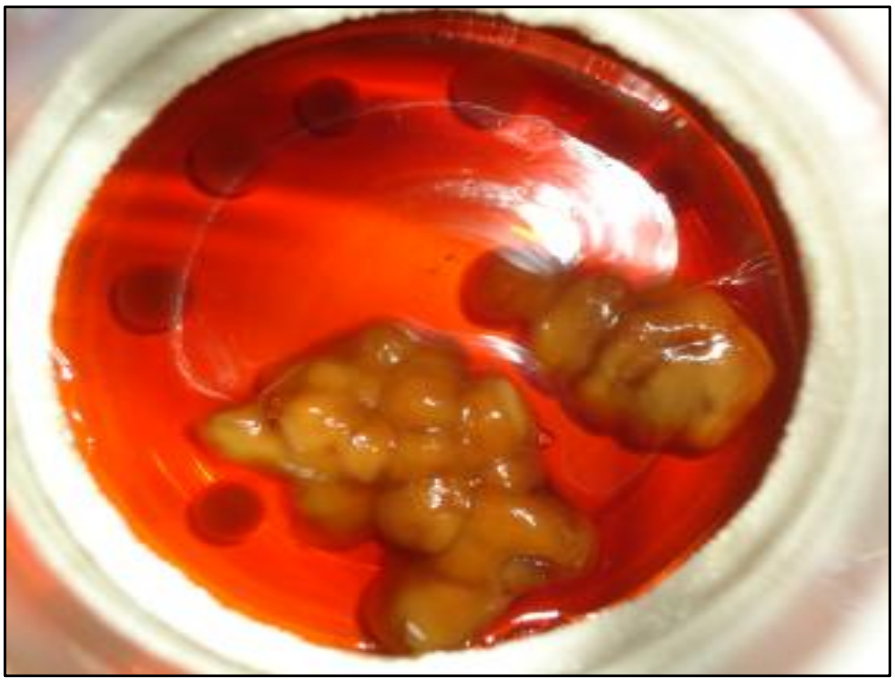


Figure 4.27.Pellet image from exo-PG production with mutant *A.sojae* in phosphoric acid hydrolysate at $1x10^5$ spore mL^{-1} concentration

4.7.5. Exo-PG Production in Water Extract of Orange Peel by Mutant *A.sojae*

4.7.5.1. Neutralization

pH value of extract which was obtained after extraction at 80 ^{o}C in 60 min was measured as 3.9. Thompson et al. 2010 reported the pH of orange peel extract as 3.64 and neutralization of pH may be required thus pH was increased to approximately 4.6. The aim was to eliminate differences between hydrolysates and the extract and provide a suitable medium for fungal growth. However, neutralization was carried out differently. pH was increased using 5N sodium hydroxide instead of calcium carbonate. Trace element solution was also added in the fermentation medium. Changes of pH at each step until fermentation are shown in Figure 4.28.

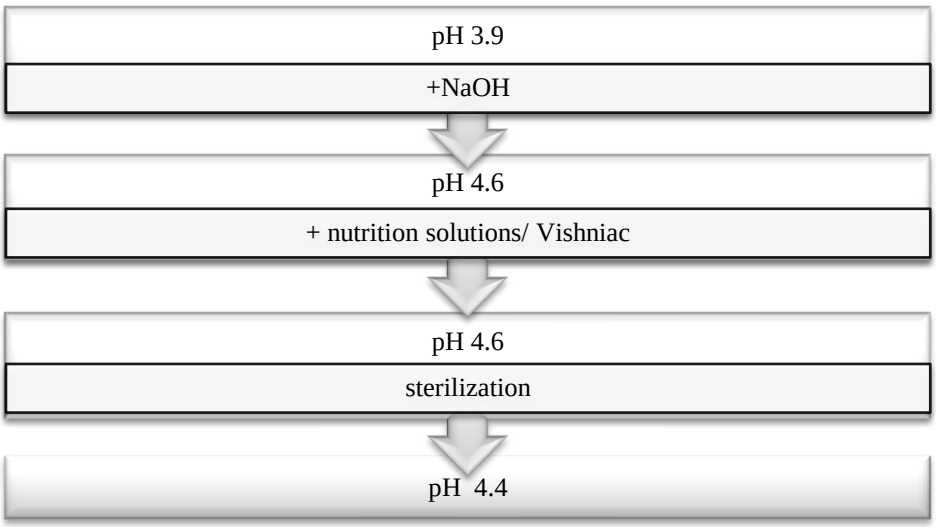


Figure 4.28.pH alterations of orange peel extract before fermentation

4.7.4.2. Fermentation

Mutant *A.sojae* was used as the producer organism for exo-PG production in water extract of orange peel. Amount of reducing sugar (Figure 4.29) and individual sugar content (Figure 4.30) during the fermentation of extract are shown. According to HPLC analysis, mutant *A.sojae* consumed glucose as carbon source with first order kinetics and followed by fructose with a slower rate during the course of the fermentation. Although a majority of sugar was consumed after four days of fermentation, exo-PG production continued to increase up to seventh-day as shown in Figure 4.29. Exo-PG activity (Figure 4.31) was 18.4 UmL^{-1} in phosphoric acid hydrolysate with trace element solution. However it was 19.7 UmL^{-1} and the pH of the fermentation medium while maximum exo-PG production was 4.9 in water extract of orange peel (Figure 4.32). According to individual sugar content (Figure 4.30), the concentration of glucose was 13 gL^{-1} and on the other hand Fontana et al. (2009) obtained the highest exo-PG activity with 22 gL^{-1} initial glucose concentration in wheat bran containing medium.

So, orange peel extract was considered to be a better medium for production of exo-PG because enzymes activities of hydrolysate of phosphoric acid and orange peel extract were not so different (slightly more activity was obtained in the water extract). Besides, using water extract could be economically more attractive since no need for acid and it is safer to handle.

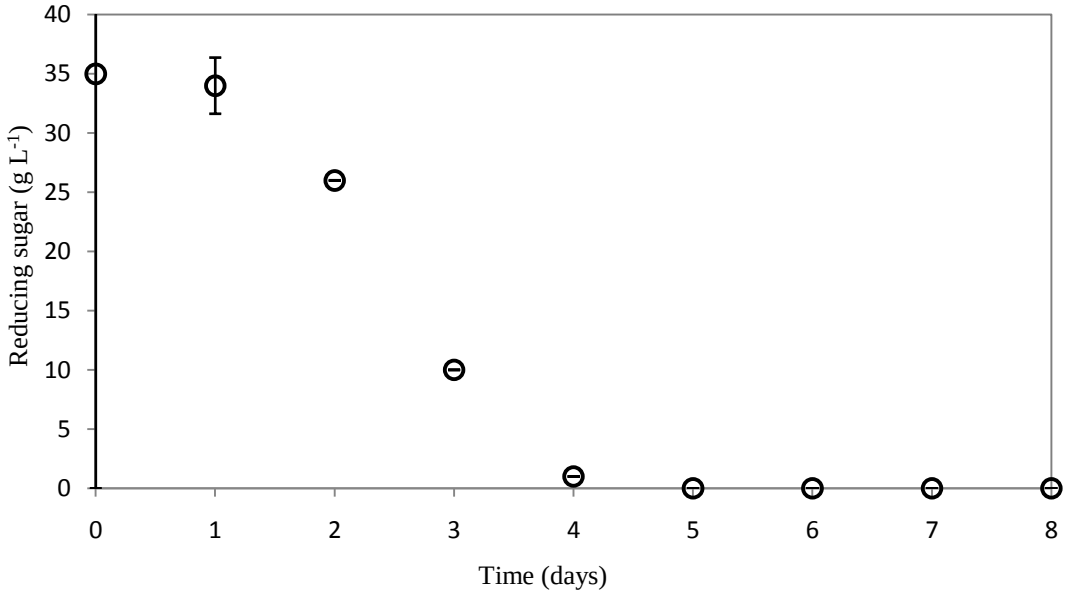


Figure 4.29.Sugar consumption by mutant *A.sojae* in orange peel extract (0. day' indicates sugar concentrations after sterilization)

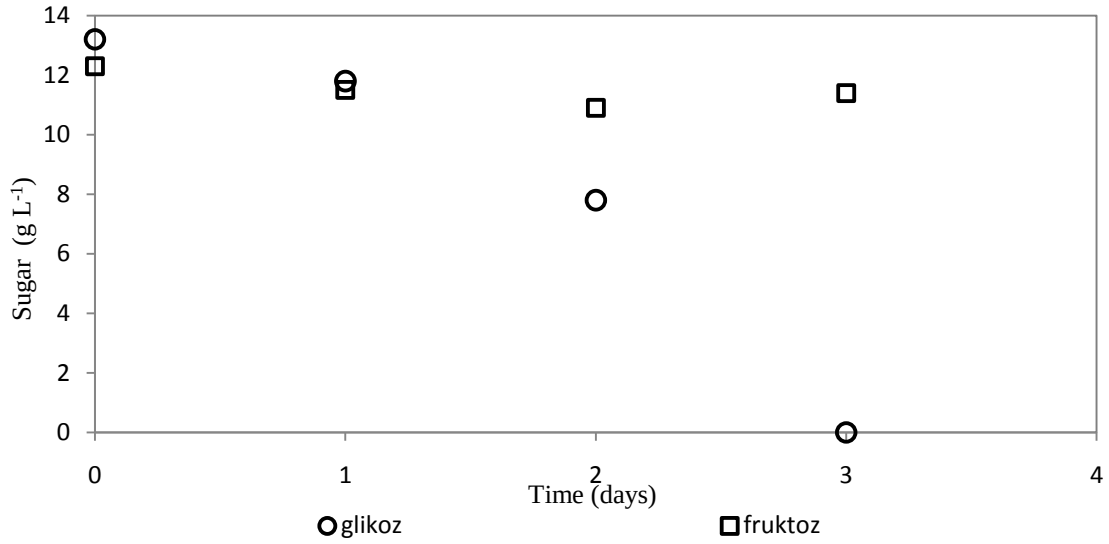


Figure 4.30.Consumption of glucose and fructose by mutant *A.sojae* in the orange peel extract

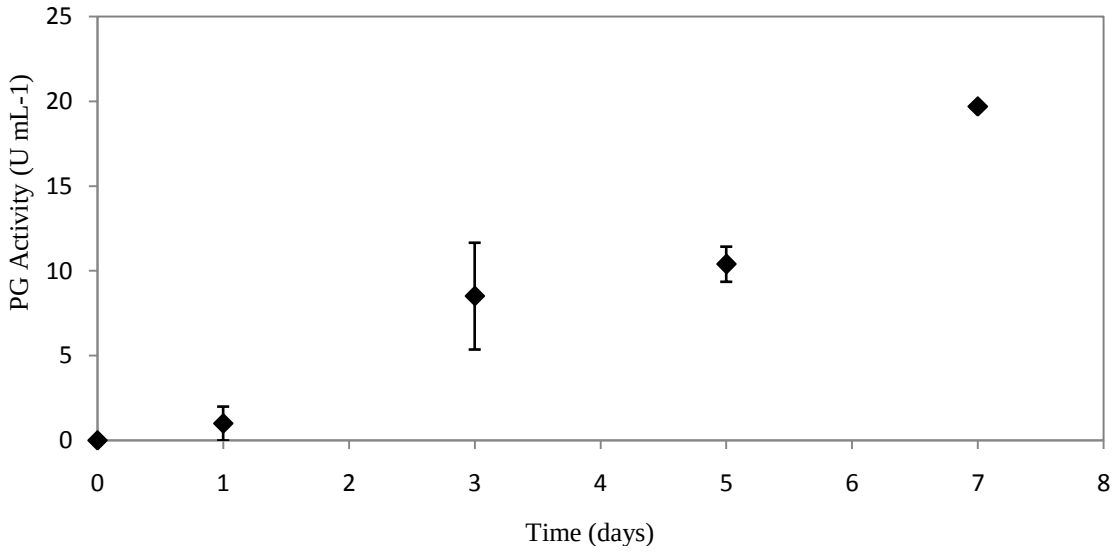


Figure 4.31.Exo-PG production by mutant *A.sojae* in orange peel extract

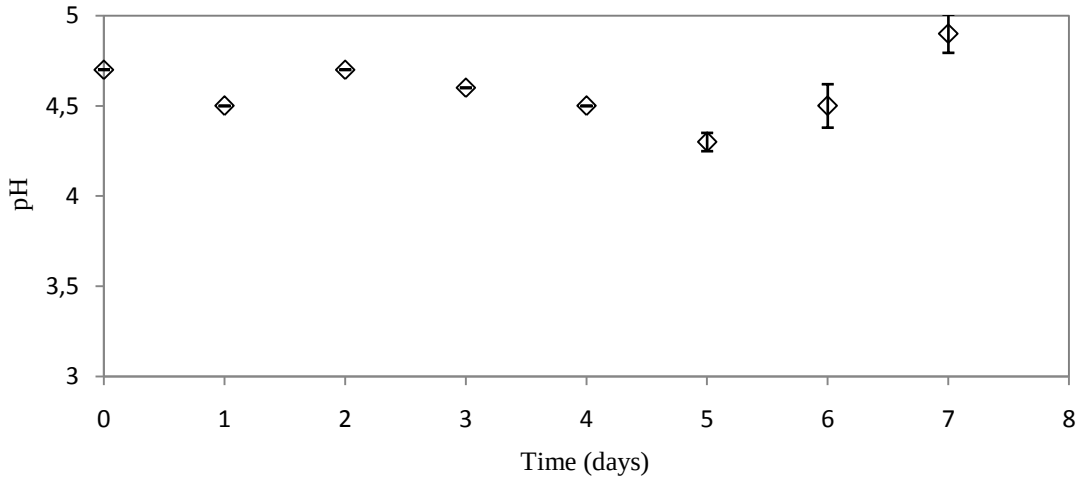


Figure 4.32.pH changes in the course of exo-PG production by mutant *A.sojae* in the orange peel extract

As a summary; *A. sojae ATCC 20235* and mutant *A. sojae* were used as exo-PG producers in different media which were pretreated at different dilute acid conditions. Sulfuric and phosphoric acids were used as catalysts in dilute acid hydrolysis. Hydrolysis conditions for phosphoric acid were optimized by using Design Expert Software and the optimum condition (120 ^{o}C, 35 min. and 4.3 % phosphoric acid concentration) was determined and validated. Another optimum point in the view of reducing sugar (120 ^{o}C, 20 min. and 0.4 % phosphoric acid concentration) was selected as fermentation medium due to the lower acid concentration than the optimum point.

Enzyme productions were carried out with these two acid hydrolysate and exo-PG activities were measured during fermentations. According to the enzyme productions, mutant *A. sojae* produced 14.6 UmL^{-1} exo-PG in phosphoric acid hydrolysate and 8.4 UmL^{-1} in sulfuric acid hydrolysate. Enzyme production of mutant *A. sojae* in phosphoric acid hydrolysate was increased (18.4 UmL^{-1}) with addition of trace element solution however hydrolysate of sulfuric acid did not give favorable response.

In these studies, pH of the fermentation mediums were adjusted to 4.5 after hydrolyses. Initial pH values of the hydrolysates were increased to 6 to control the effect of pH on enzyme production. But the exo-PG activity was decreased at higher pH of fermentation medium. This result provided the suggestion of Jayani et al. (2005), because they were agreed on PG production required acidic conditions.

On the other hand, *A.sojae ATCC 20235* did not produce significant amount of exo- PG however sugar consumption rate of mutant *A.sojae* was almost same. It consumed total sugar in the medium for fungal growth- pellet formation- thus pellet diameters in the both hydrolysate were relatively big.

Exo-PG production in water extract of orange peel (19.7 UmL^{-1}) resulted in almost same activity with phosphoric acid hydrolysate's (18.4 UmL^{-1}). Results after fermentation with orange peel extract highlighted that orange peel, an agro-industrial residue, could be used without any pretreatment. Because of an extra procedure brings with higher operating costs and with high operating costs it will be difficult to scale up.

CHAPTER 5

CONCLUSION

The aim of the study was using carbon sources for exo-PG production. Orange peel as an agro-industrial waste supported fugal growth and induced production of exo-PG due to the rich carbohydrate content.

In previous studies, direct usage of orange peel was mostly preferred instead of conventional liquid system. Exo-PG productions were conducted in acid hydrolysates and water extracts of orange peel in the study. Exo-PG activities were 18.4 UmL^{-1} in phosphoric acid hydrolysate and 19.7 UmL^{-1} in water extract of orange peel while reducing sugar concentrations of phosphoric acid hydrolysate and water extract of orange peel were 34.4 gL^{-1} and 18 gL^{-1}. Thus acid hydrolysate or water extract of orange peel could be used as substrate for exo-PG production however exo-PG values were significantly lower compared to obtain by direct use of orange peel. Also using extracts or acid hydrolysates which contained no solid particles was advantageous in terms of growth form. Pellet production which might be enhanced by absence of solid particles decreased viscosity of media thus rheological problems were eliminated.

Among the indirect usage of orange peel, water extraction and acid hydrolysis were alternative to each other. Exo-PG could be produced in water extract however higher sugar concentrations was obtained by acid hydrolysis and also closer exo-PG activities were obtained in acid hydrolysate and water extract of orange peel. Thus using water extract was more attractive due to economical aspect and no acid consumption

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APPENDIX A

DESIGN EXPERT GRAPHICS

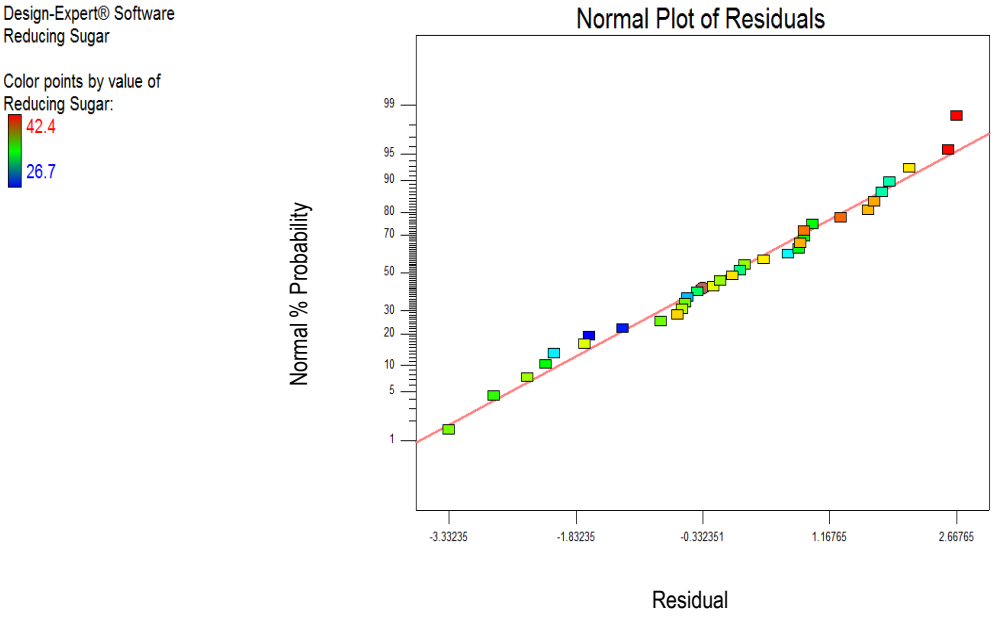

Figure A33.The graphic of normal plot of residuals

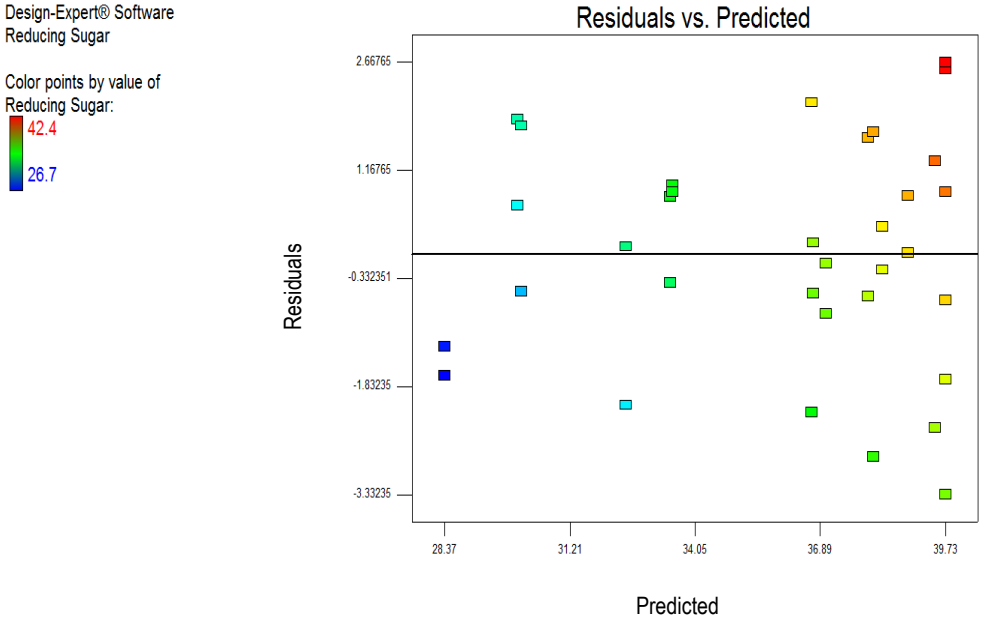

Figure A34.The graphic of residuals vs. predicted values

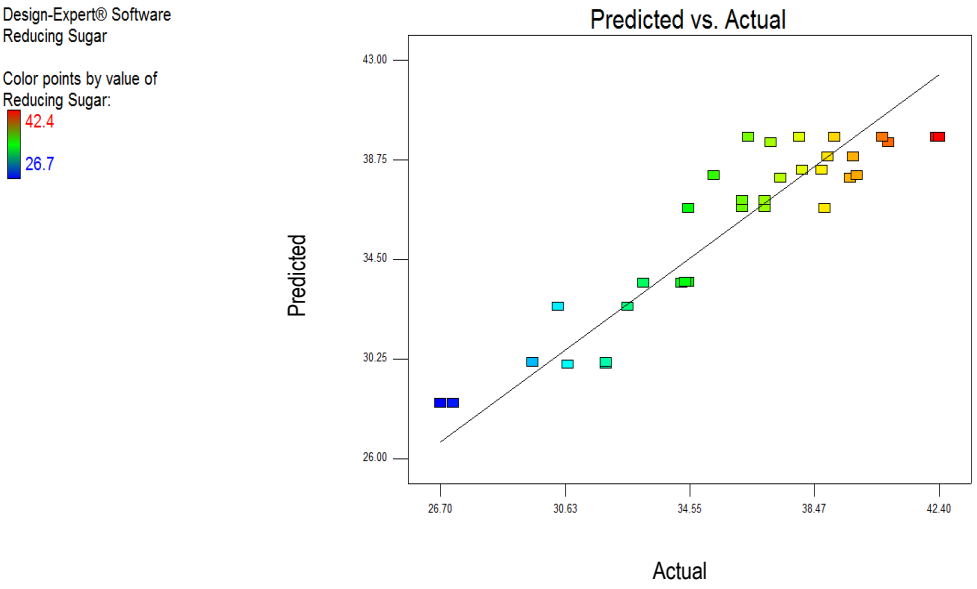

Figure A35.The graphic of predicted vs. actual values

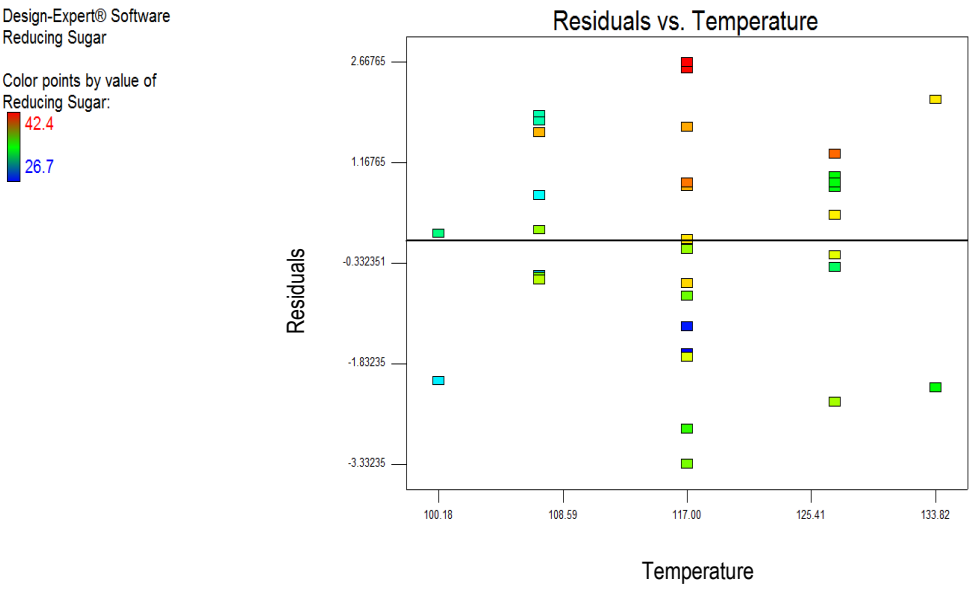

Figure A36.The graphic of residuals vs. temperature

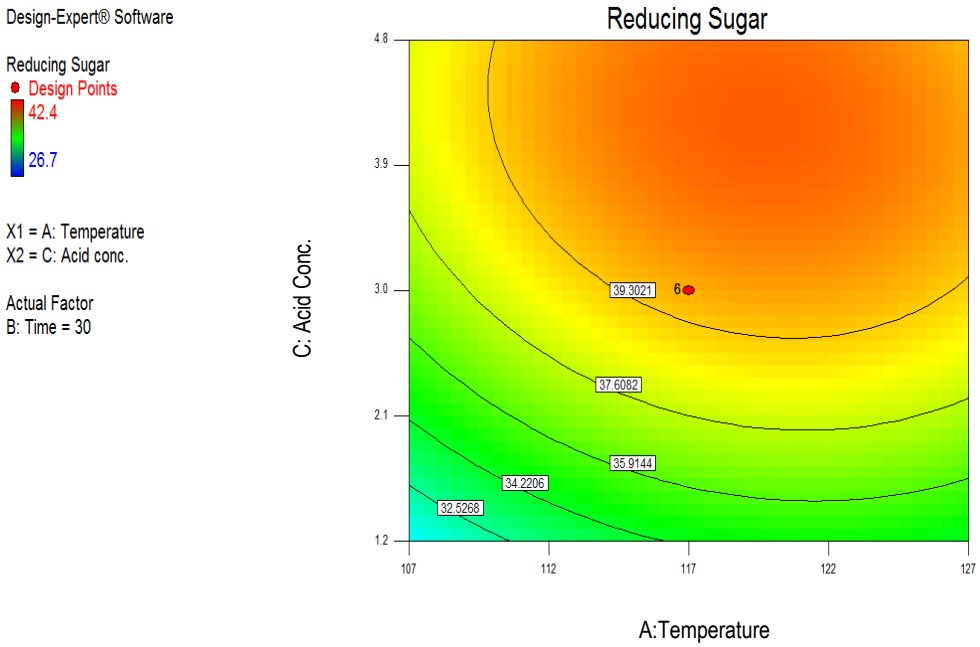

Figure A37.The graphic for reducing sugar distribution with temperature and acid concentration

APPENDIX B

CALIBRATION GRAPHICS

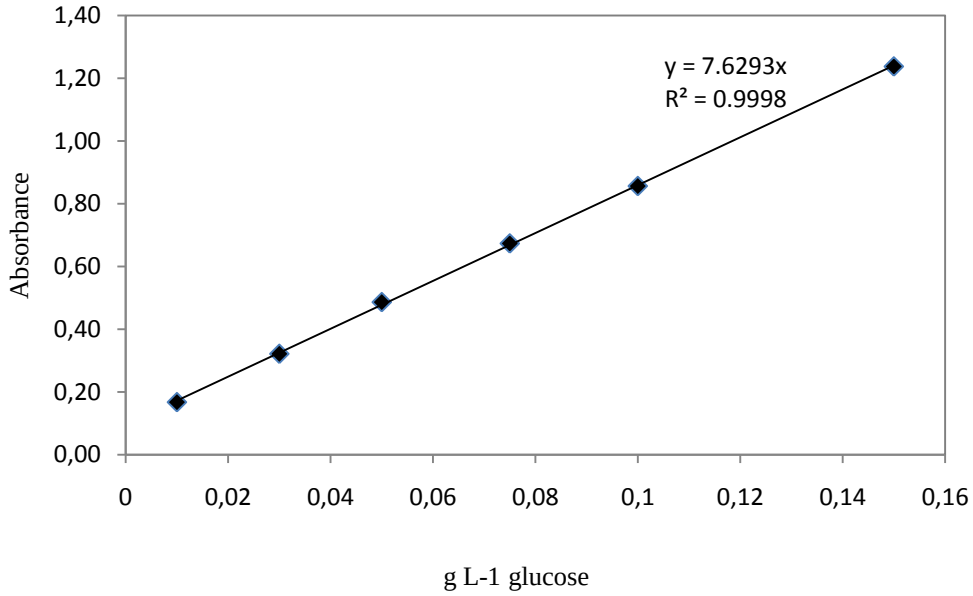

Figure B38.Glucose curve for reducing sugar assays

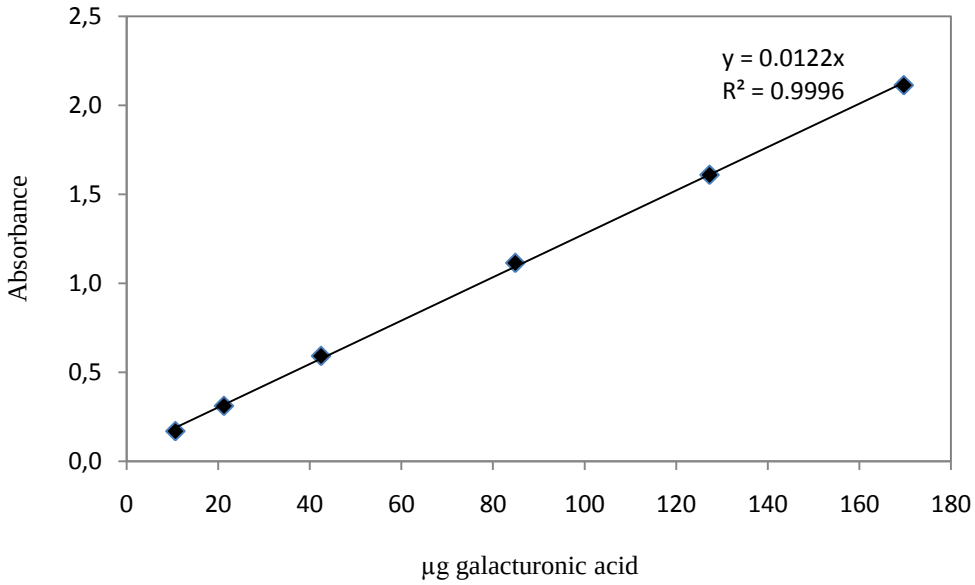

Figure B39.Galacturonic acid curve for PG assays