INVESTIGATIONS ON THE EFFECTS OF CARBON SOURCES AND pH ON EXO-POLYGALACTURONASE PRODUCTION BY *Aspergillus sojae*

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by
Gülten KURU

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We approve this thesis of **Gülten KURU**

Examining committee members:

Assist. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Department of Food Engineering, Izmir Institute of Technology

Prof. Dr. Şebnem HARSA
Department of Food Engineering, Izmir Institute of Technology

Assist. Prof. Dr. Ferda SOYER
Department of Molecular Biology and Genetics, Izmir Institute of Technology

19 December 2013

Assist. Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Supervisor,
Department of Food Engineering
Izmir Institute of Technology

Prof. Dr. Volga Esma BULMUŞ ZAREIE
Head of the Department of
Biotechnology and Bioengineering

Prof. Dr. Tuğrul SENGER
Dean of the Graduate School of
Engineering and Science

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ABSTRACT

INVESTIGATIONS ON THE EFFECTS OF CARBON SOURCES AND pH ON EXO-POLYGALACTURONASE PRODUCTION BY *Aspergillus sojae*

Pectinases are a group of enzymes that are responsible for degredation of pectic substances. Pectinases are produced by many organisms including plants, bacteria and fungi.

The aim of this study was to investigate the effect of different carbon sources and pH on pectinase production by a group of flamentous fungi. For this purpose, *Aspergillus niger*, *Rhizopus oryzae* ATCC 4858, *Aspergillus sojae* ATCC 2035 (*A. sojae* WT) and its mutant type *Aspegillus sojae* MT (*A. sojae* MT) grown in orange peel containing media were evaluated in terms of exo-polygalacturonase (exo-PG), pectin lyase (PL), pectin methyl esterase (PME), and also xylanase and cellulase activities. *A. sojae* WT and *A. sojae* MT were found to be as exo-PG producers. Maximum exo-PG activities were 47.84 U/ml and 108.02 U/ml, respectively.

Exo-PG production by *A. sojae* WT and *A. sojae* MT was evaluated using the medium containing glucose, pectin, pectin/glucose, pectin/fructose and pectin/sucrose as substrate. Additional supply of glucose to the pectin media was found to be more effective than fructose or sucrose. The fermentations in which pH was not maintained, the use of glucose, pectin and pectin/glucose resulted in superior enzyme activities compared to ones obtained with other media compositions.

ÖZET

KARBON KAYNAKLARININ VE pH'NIN *Aspergillus sojae* İLE EKZO-POLİGALAKTURONAZ ÜRETİMİNE ETKİLERİ ÜZERİNE ARAŞTIRMALAR

Pektinazlar bitkiler, bakteriler ve mantarları da içeren birçok organizma tarafından üretilebilen, pektik bileşiklerin degredasyonundan sorumlu olan enzim grubudur.

Bu çalışmanın amacı karbon kaynaklarının ve pH'nın pektinaz üretimine etkilerinin belirlenmesidir. Bu amaçla *Aspergillus niger*, *Rhizopus oryzae* ATCC 4858, *Aspergillus sojae* ATCC 2035 (*A. sojae* WT) ve *Aspegillus sojae* mutantı (*A. sojae* MT) portakal kabuğu içeren sıvı ortamda büyütülerek ekzo-poligalakturonaz (ekzo-PG), pektin liyaz (PL), pektin metil esteraz (PME), ksilanaz ve selülaz üretimleri incelenmiştir, sadece *Aspergillus* suşlarının Ekzo-PG üreticisi olduğu bulunmuştur, *A. sojae* WT ve *A. sojae* MT için sırasıyla 47.84 U/ml ve 108.02 U/ml olarak belirlenmiştir.

Farklı karbon kaynaklarının ekzo-PG üretimi üzerine etkisi, substrat olarak glukoz, pektin ve pektin/glukoz, pektin/fruktoz ya da pektin/sukroz içeren ortamlar kullanılarak incelenmiştir. Ekzo-PG üretimi sırasında substrat tüketim hızı, pH değişimi ve küf morfolojisi incelenmiştir. En yüksek aktivite değerleri pectin/glukoz içeren ortamda elde edilmiştir. Düşük pH değerlerinde exo-PG üretiminin daha fazla olduğu belirlenmiştir. Başlangıç pH'sinin enzim üretimi üzerinde büyük etkiye sahip olduğu, ancak fermentasyon sırasında ortam pH'sine müdahale edilmesi durumunda aktivitenin düştüğü gözlemlenmiştir.

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CHAPTER 1

INTRODUCTION

Pectin is a complex polysaccharide found mainly in the middle lamella and the primary call walls of higher plants (Kashyap et al., 2001) and it constitutes the 35% of primary walls in plants (Caffall et al., 2009).

Pectolytic enzymes, or pectinases are a heterogeneous group of related enzymes that degrades the pectic substances by means of de-polymerization, trans-elimination, or de-esterification. This pectolytic enzymes group mainly consists of exo-polygalacturonase and endo-polygalacturonase, pectin lyase and pectin methylesterase enzymes (Fontana et al., 2012).

Pectinases are commercially important enzymes, owing to have share of almost 5% of enzyme sales around the world (Alimardani-Theuil et al., 2011). This information indicates that pectinases are widely used in industrial applications (Jayani, Saxena et al. 2005), mainly in food industry by the share of 25% in the global sales of the food enzymes, and then textile industry and wastewater treatment due to their ability to degrade pectic substances (Kashyap et al., 2001; Jayani et al. 2005; Fontana et al., 2012).

Pectinases are naturally produced by many organisms including plants, bacteria and fungi (Malvessi et al., 2004). Among these natural producers, fungi have preference over any other organism, because of the feasibility for industrial productions. Fungi have long been used in commercial productions of many valuable products, this may attribute to their easy compatibility to large scale operation modes, safety for human health (Reading et al., 2003; Posch et al., 2013).

In industrial applications, filamentous fungi belonging to the genus Aspergillus are widely used (Heerd et al., 2012), especially *Aspergillus niger* is most preferred specie in many productions (Rodríguez-Fernández et al., 2011) by not only submerged processes but also solid-state processes (Fontana et al., 2012). Recently another specie of *Aspergillus* genus was found to be as a good producer of pectinases, *Aspergillus sojae* (Tari et al., 2008).

It is known that the regulation of pectinolytic enzymes depends on many fermentation conditions like pH, carbon source, growth stage, etc. (Peñalva et al., 2008), but this regulation has not been deeply investigated and characterized yet. Previous published studies suggest that in fungi, pectinase production is regulated depending on mostly pH and carbon source (Malvessi et al., 2004; Fontana et al., 2012).

The aim of this study was to investigate some fungal strains with respect to regulation of pectinase biosynthesis by means of the influence of carbon source and pH. For this purpose, pectinase producing strains were firstly determined, and examined under different culture conditions. The effects of different types and concentrations of carbon sources were evaluated. Additionally, the effect of pH on enzyme production was evaluated using variation of the culture media pH. Enzyme activity and fungal morphology were observed as responses though the fermentation.

CHAPTER 2

LITERATURE VIEW

2.1. The Significance of Fungi in Industry

In the last decades, as a result of great progress in genetic engineering, biotechnology had a great importance almost in every process which generates valuable products as antibiotics, drugs, agricultural regulators, vitamins and enzymes (Reading et al., 2003).

Fungi have long been used in many industrial processes, not only as a food but also processing the food. Moreover, fungi have a great significance in biotechnology as 'biologic agents'. Fungi, furthermore, can be used in textile, waste water treatment industries, and biotransformation of some economically valuable compounds (Sharma et al., 2013).

Aspergillus niger has been used for industrial productions of many enzymes for a long time, not only in submerged fermentations but also solid state fermentations. This fungus has an advantage that is the possession of GRAS (Generally Regarded A Safe) status, which means that the metabolites of this fungus can be used in the food industry (Naidu et al., 1998).

2.1.1. Fungal Enzymes

Among the usage of fungi in biotechnological processes, the most important use is the production of enzymes due to their economic significance (Jayani et al., 2005).

Enzymes are extremely efficient and highly specific biocatalysts, which were discovered in the second half of nineteenth century (Hoondal et al., 2002). Hence they have considerable importance in industry. As a matter of convenience, they can replace many other harmful processes by providing benefits in processes owing to their operation conditions, unlike chemical reaction processes, enzymatic reactions do not

require, for example high pressure operation conditions or do not cause any side reactions (Reading et al., 2003).

Both extracellular and intracellular enzymes can be produced by fungi. However, extracellular enzymes are more commercially important than intracellular enzymes. Fungal enzymes are better than other origins due to the absence of some contaminating enzymes and have easier downstream processing (Reading et al., 2003).

2.2. Pectin

Cellulose, hemicellulose, lignin and pectin are the main components of plant cell wall (Ramos et al., 2010). Pectin is a complex polysaccharide found mainly in the middle lamella and the primary call walls of higher plants (Kashyap et al., 2001). It constitutes the 35% of primary walls in plants (Caffall et al., 2009).

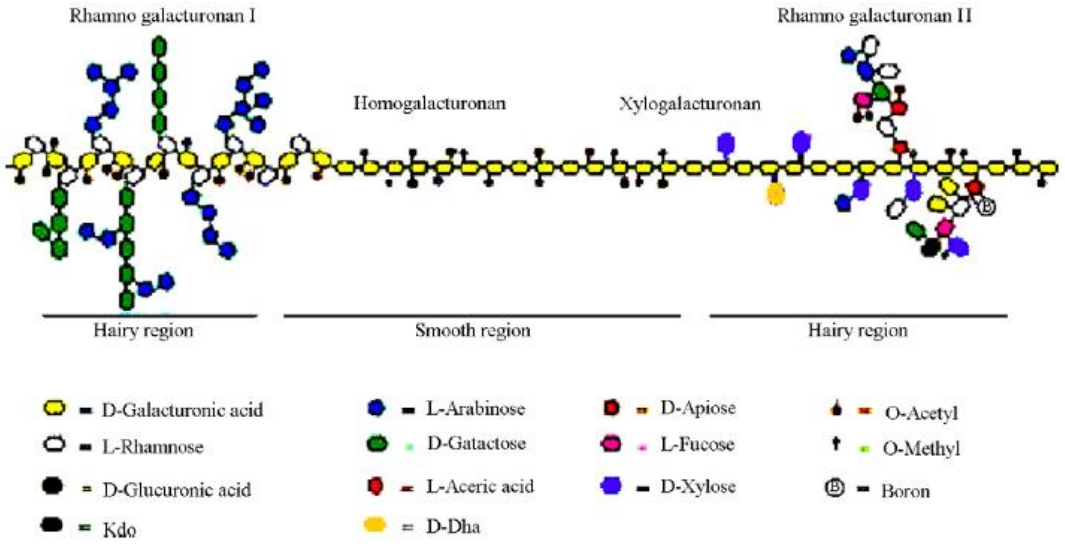


Figure 2.1. The structure of pectin
(Source: Yadav et al., 2009)

The basic unit in pectic substances is galacturonan (α-D-galacturonate). Pectic substances are divided into two types; homogalacturonan ,n which the main polymer chain consist of α-D-galacturonate units linked by (1→4) glycosidic bonds and heterogalacturonan (rhamnogalacturonan) in which the primary chain consist of (1→4)

linked α-D-galacturonates and L-rhamnose units that are β(1→2) and β(1→4) linked to D-galacturonate units (Caffall et al., 2009).

2.2.1. Pectinases and Their Applications

Efficient biodegradation of pectin requires a wide range of enzymes, including enzymes that act on the main chain as well as enzymes acting on the side chains. Classification (Table 2.3), biochemical and physical properties (Table 2.4) of some fungal pectinases are given below.

Pectinases steal market share in many industry according to their action of mechanism, by products or physicochemical properties (Kashyap et al., 2001; Jayani et al., 2005). However, pectinases are classified into two types according to industrial apptication area, as acidic and alkaline pectinases. For example, alkaline pectinases are not compatible to be used in fruit juice industry known as the main application area of pectinases, because of acidic pH of fruit juices (Yadav et al., 2009). But, they have a great demand in textile industry (Kashyap et al., 2001). The paper making, coffee and tea fermentations are some of the application areas of those enzymes (Hoondal et al., 2002). Industrial applications of pectinases can be summarized as given below (Table 2.1).

Pectinases are commercially available at the market, and some of producers are given below (Table 2.2). With an exception of highly purified forms for analytical analysis, commercial pectinase preperations are generally sold as crude forms, including contaminating agents from fermentation broth and other enzymes like cellulose, xylanase etc. However, pectinase enzyme preparations are generally consist of polygalacturonase, pectin methyl esterase and pectin lyase (May et al., 1990; Gummadi et al., 2003).

Polygalacturonases (PG); are divided into two groups as endo- and exo-polygalacturonases based on the nature of reaction. Endo-PG cleaves the substrate randomly while Exo-PG cleaves in end-wise fashion (Gummadi et al., 2003). PGs are known to play a major role in pectin breakdown by cleaving main chain of pectin. PG is a depolymerising enzyme that hydrolysis homogalacturonan or polygalacturonic acid into galacturonic acid monomers by breaking the α-1,4-linkages (Dogan et al., 2008).

Due to its wide range of use, PG enzymes have a great demand in industrial applications.

Table 2.1. Industrial applications of pectinases
(Source: May et al., 1990; Hoondal et al., 2002)

Application	Purpose	Reference
Claud stabilization	To precipitate hydrocolloid matter present in fruit juices	Rebeck, 1990; Grassin and Fauquembergue, 1996
Fruit juice clarification	Degredation of cloud forming pectic substances. Hence, the juice can be easily filtered and processed	Rombouts and Pilnik, 1986; Alkorta *et al*., 1998
Extraction of juice and oil	To overcome the difficulty in pressing fruit pulp to yield juice and oil	Kilara, 1982; Pilnik and Voragen, 1993
Maceration	To break down the vegetable and fruit tissues to yield pulpy products used as base material for juices, nectar as in the case of baby foods, pudding and yogurt	Fogarty and Kelly, 1983
Liquefaction	To break down fermentable plant carbohydrates to simple sugars	Beldman *et al*.,1984
Gelation	To use in gelling low-sugar fruit products	Spiers *et al.*, 1985
Wood preservation	To prevent the wood from infection by increasing the permeability of wood preservative	Fogarty and Ward, 1973
Retting of fiber crops	To release fiber from the crops by fermenting with microorganisms, which degrade pectin	Henriksson et al., 1999
Degumming of fiber crops	To remove the remie gum of remie fiber	Grucharanam and Deshpande, 1986; Zheng et al., 2001
Waste water treatment	To degrade pectic substances in waste water from citsus processing industries	Peterson, 2001; Tanabe et al., 1987
Coffee and tea fermentation	To remove the mucilage coat in coffee bean. To enhance the the tea fermentation and foam forming property of tea	Carr, 1985; Godfrey, 1985

Pectin methylesterase (PME); acts on pectin by removing methoxyl group from pectin or partially esterified homogalacturonan (Niture et al., 2008). In detail PMEs catalayze pectin deesterification by hydrolysis of the ester bond of methylated α-(1→4)-linked D-galacturonosyl units, producing negatively charged polymer and methanol.

Pectin lyases (PL); are one of the pectin degrading enzymes which are able to degrade highly esterified pectin into small compounds via β-elimination mechanism without producing a toxic component like methanol, in contrast with the combination of

PG and PE, which are normally found in commercial enzyme preparations (Whitaker et al., 1990). Pectin lyase is one of the commercially important pectinase, especially fruit juice industry due to esterified pectins found mainly in fruits (Gummadi et al., 2003). Pectin lyases are classified into two according to action mechanism, as Endo-PL that degrades pectic substances in a random fashion yielding 4:5 unsaturated oligomethylgalacturonates and Exo-PL that has not been certainly indentified in terms of action mechanism yet (Yadav et al., 2008; Yadav et al., 2009).

Table 2.2. Commercial Pectinases
(Source: Kashyap et al., 2001)

Supplier	**Location**	**Brand Name**
C. H. Boehringer Sohn	Ingelheim, West Germany	Panzym
Ciba-Geigy, A. G.	Basel, Switzerland	Ultrazyme
Grinsteelvaeket	Aarthus, Denmark	Pectolase
Kikkoman Shoyu, Co.	Tokyo, Japan	Sclase
Schweizerische Ferment, A. G.	Basel, Switzerland	Pectinex
Societe Rapidase, S. A.	Seclin, France	Rapidase, Clarizyme
Wallerstein, Co.	Des Plaines, USA	Klerzyme
Rohm, GmbH	Darmstadt, West Germany	Pectinol, Rohament

Table 2.3. Classification of pectolytic enzymes
(Source: Jayani et al., 2005; Yadav et al., 2009)

Enzyme	E. C. no.	Modified EC Systematic Name	Action Mechanism	Action Pattern	Primary Substrate	Product(s)
Esterase						
1. Pectin methyl esterase	3.1.1.11		Hydrolysis	Random	Pectin	Pectic acid + methanol
2. Pectin acetyl esterase	3.1.1.6		Hydrolysis		Pectin	Pectic acid + methanol
Depolymerizing enzymes						
a. Hydrolases						
1. Protopectinases			Hydrolysis	Random	Protopectin	Pectin
2. Endopolygalacturonase	3.2.1.15	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydrolysis	Random	Pectic acid	Oligogalacturonates
3. Exopolygalacturonase	3.2.1.67	Poly-(1-4)-α-D-galactosiduronate glycanohydrolase	Hydrolysis	Terminal	Pectic acid	Monogalacturonates
4. Exopolygalacturonan-digalacturono hydrolase	3.2.1.82	Poly-(1-4)-α-D-galactosiduronate digalacturonohydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase			Hydrolysis	Terminal	Trigalacturonate	Monogalacturonate
6. Δ4:5 Unsaturated oligogalacturonate hydrolysis			Hydrolysis	Terminal	Δ4:5 $(Galacturonate)_n$	Unsaturated monogalacturonates & saturated (n-1)
7. Endopolymethyl-galacturonases			Hydrolysis	Random	Highly esterified pectin	Oligomethylgalacturonates
8. Endopolymethyl-galacturonases			Hydrolysis	Terminal	Highly esterified pectin	Oligogalacturonates
b. Lyases						
1. Endopolygalacturonase lyase	4.2.2.2	Poly-(1-4)-α-D-galactosiduronate lyase	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	4.2.2.9	Poly-(1-4)-α-D-galactosiduronate exolyase	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galacturonate lyase	4.2.2.6	Oligo-D-galacturonate lyase	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate lyase	4.2.2.10	Poly(methyl galactosiduronate) lyase	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalactorunates)	Unsaturated methyloligogalacturonates
5. Exopolymethyl-D-galactosiduronate lyase			Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalactorunates)	Unsaturated methylmonogalacturonates

Table 2.4. Biochemical and physicochemical properties of some pectolytic enzymes from *Aspergillus* sp.

Source of enzyme	**Nature**	**Molecular weight (kDa)**	**pI**	**Specific activity (U mg^{-1})**	**K_m**	**Optimum temperature (℃)**	**Optimum pH**	**Temperature stability**	**pH stability**	**Reference**
Aspergillus japonicus	Endo-PG	38 (PG I)	5.6	-	-	30	4.0-5.5	-	-	(Hasunuma et al.,. 2003)
	Endo-PG	65 (PG II)	3.3	-	-	30	4.0-5.5	-	-	
	PMGL	-	7.7	-	0.16	55	6.0	-	-	
	PE	46 (PE I)	3.8	-	-	-	4.0-5.5	50	-	
		47 (PE II)	3.8	-	-	-	4.0-5.5	50	-	
Aspergillus niger	Endo-PG	61 (PG I)	-	982	0.12	43	3.8-4.3	50	-	(Singh et al., 2002, Gummadi et al., 2003; Fahmy et al., 2008)
	Exo-PG	32 (PG I)	7.6	186	0.8	35	5.0	50	-	
	PE	-	-	-	1.01	45	5.0	-	-	
Aspergillus awamori	EndoPG	41	6.1	487	-	40	5.0	50	4.0-6.0	(Nagai et al., 2000)
Aspergillus sojae	PL	32	-	-	-	-	5.5	-	-	(Yadav et al., 2009)
Aspergillus oryzae	PL	34	-	-	1.36	40	8.5	-	-	(Yadav et al., 2009)
Aspergillus flavus	PL	38	-	-	0.59	50	8.0	-	-	(Yadav et al., 2008)
Aspergillus ficuum	PL	31.6	-	-	0.60	50	5.0	-	-	(Yadav et al., 2008)

(I) endopolygalacturonate lyase (EndoPGL, E.C. 4.2.2.2); (II) exopolygalacturonate lyase (ExoPGL, E.C. 4.2.2.9).

2.3. Regulation of Pectinase Production in Fungi

Pectinase synthesis is regulated in different organisms depending on species, then fermentation and operation conditions, also carbon source and pH have a significant effect on the enzyme production (Fontana et al., 2012).

It has been reported in the literature that there are a group of genes identified (*pel*A, *ply*A, *pga*A, *pme*A, *pga*I, *pga*II, and *pga*C) encoding pectinases. The expression of these genes depends on various fermentations conditions, and also different expression levels were observed for various genes in the same conditions (Denison et al., 2000; de Vries et al., 2002).

Aspergillus species are widely used to produce enzymes. Hence this specie, especially *Aspergillus niger,* in which the pectinolytic system has been studied in most detail. Many of *A. niger* enzymes that are involved in pectin degradation have been identified. The expression levels of 26 pectolytic enzymes were studied under different growth conditions (de Vries et al., 2002). The study demonstrated that the gene clusters which are responsible for the synthesis of different pectolytic enzymes, response differently for various growth conditions (Akimitsu et al., 2004; Martínez-Trujillo et al., 2009).

As against genetic factors, fermentation conditions also have significant effect on the production of enzymes. Studies on the determination of optimum conditions for pectinase production were made by several workers. With respect to the selection of strains which are the best producers of pectinases, the decision of the fermentation system submerged fermentation (SmF) or solid state fermentation (SSF), media composition, and environmental and operational conditions like pH, temperature, aeration rate, etc. all these parameters regulates the production of pectinases, in terms of both biochemical and physiological aspects of the synthesis of the metabolite.

The regulatory phenomena, among many parameters involved in fermentation conditions, mainly based on pH and carbon source. The productivity of pectinases could be expounded as a function of initial pH and the carbon source in the culture media (Martínez-Trujillo et al., 2008). That can be attributed to their induction-repression or activation-inhibition significant effects on enzyme productions.

2.3.1. pH-Dependent Regulation of Pectinase

Previous studies on the influence of pH on the biosynthesis of pectinases from various microorganisms suggest that pectinase production is associated with the pH of culture medium (Denison et al., 2000; Akimitsu et al., 2004; Fontana et al., 2012).

The studies on the expressions of genes encoding pectinases indicated that there are possibilities of coordinated expression among these genes, and also a coordination with *Pac*C gene which is responsible for pH-dependent expression (Panda et al.,2004).

The initial pH influences the growth of microorganism as well as the production of primary or secondary metabolites (Martínez-Trujillo et al., 2009). It has been demonstrated in the previous studies on the production of PG by *A. oryzae*, pH values close to 4 stimulate the fungal growth since pH values close to 3 stimulate endo- and exo-PG production, however at pH values under 3 resulted in less fungal growth (Malvessi et al., 2004).

The study made by Martinez-Trujillo et al. demonstrated that groth and enzyme production were strongly affected by the initial pH. But, the effect of the initial pH also differs depending on the substrate; glycerol, xylose and polygalacturonizc acid medias resulted in approximately same μ_{max} values in any case of the initial pH, since the μ_{max} values at glucose rhamnose and pectin medias were strongly affected by the initial pH (Martínez-Trujillo et al., 2008).

2.3.2. Carbon Source-dependent Regulation Pectinase

In order to investigate the regulation of pectolytic enzymes, there are several papers, mainly concerns with various carbon sources and nitrogen sources as well as agricultural wastes as inducers for pectolytic enzymes by *Aspergillus* species and the other fungal and bacterial species. Through the experimental studies with microorganisms, all assays demonstrated that the enzyme synthesis is correlated with the quality and concentration of the carbon sources.

Studies on the production of polygalacturonase by *Candida* sp. and *Aspergillus* sp. were showed that exopolygalacturonase production is induced by the addition of galactopyronic acid to pectin (Stratilová, Breierová et al., 1996), also pectic acid have an inducer effect on the production of pectic anzymes since it is used as a carbon source

in the production media (Naidu et al., 1998). It can be concluded that pectin and some of its derivatives induces pectinase production (Nair et al.,. 1995).

The concentrations of carbon sources have a significant effect on the activities of pectolytic enzymes as well as the quality. When high concentrations of pectic acid, glucose or saccharose exist in culture media, exhibited a repression effect on the pectolytic enzymes (Teixeira et al., 2000). The same study demonstrates that Aspergillus japonicus shows the lowest endopolygalacturonase activity when pectin is used as the only carbon source. However, in case pectin supplemented with glucose or saccharose, polygalacturonase activities were higher than pectin, glucose or saccharose alone. The presence of glucose with high concentrations of pectin stimulates the production of exopolygalacturonase, but in the cultures without pectin the exopolygalacturonase activity significantly decreased during the growth (Teixeira et al., 2000).

The studies on the influence of carbon sources on pectinolytic enzyme synthesis, show that it is repressed by glucose and saccharose. When these monosaccharides are used over a certain concentration, they have a repression effect on the production of pectolytic enzymes named catabolic repression (Teixeira et al., 2000; Akimitsu et al., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals

The chemicals used in the study were all analytical grade and listed in Appendix A.

3.2. Microorganisms

Aspergillus niger, *Rhizopus oryzae* ATCC 4858 and two strains *of Aspergillus sojae* were kind gifts of Proffesor Canan Tarı from IZTECH Department of Food Engineering . The second strain, *A. sojae* MT (mutant type) is one of mutant types of this strain, randomly mutated using ultraviolet light exposure at Jacobs University gGmbH, Bremen. It was a kind gift from Professor M. Fernandez Lahore.

3.3. Spore Propagation and Inoculum Preparation

The spore propagation was done in petri dishes of molasses agar containing glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), $FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l) and agar (20 g/l) (Dogan and Tari 2008), using stock cultures which were prepared with 20% (w/v) glycerol-water and stored at -80℃.

The spores used as inoculum were collected from the molasses agar slants incubated at 30℃ for one week. Spores were harvested with approximately 5 ml Tween 80 solution, which contained 0.01% (w/v) Tween 80 and distilled water. The spore suspensions were filtered through a glass funnel loosely packed with cotton wool in order to get rid of agar and mycelia residues. Spores were counted in a counter chamber, Thoma bright line heamocytometer (Marienfield, Germany). The spore suspension was collected in a sterile tube and stored at 4℃ until inoculation, for maximum two weeks.

3.4. Production Media

The culture media containing different carbon sources were tested for the production of pectinases. In addition to the carbon sources some mineral salts which were needed for fungal growth were included in the media, the composition of the media are listed in Table 3.1.

It was reported by Vishniac and Santer (1957) that the Vishniac trace element solution had a positive effect on fungal growth. Therefore the trace element solution was added into the medium, after it has been proved by our previous experiments that it is also had a positive effect on Exo-PG activity. Each flask was inoculated to a final inoculums concentration of $8x10^4$ spore/ml which was optimized in our previous studies.

Cultivations were carried out 5 to 7 days at 30℃ with 200 rpm stirring rate. Samples were collected daily for enzyme activity assays, and every 12 hours for sugar analysis and pellet morphology determination. Indeed change in pH values were measured daily.

Table 3.1. Medium compositions used in the study.

Ingredients	**Concentration (g/l)**
Glucose	
Glucose	20
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	5
Vishniac trace element solution	1 (ml)
Pectin	
Pectin	20
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	5
Vishniac trace element solution	1 (ml)

(cont. on next page)

Table 3.1. (cont.)

Pectin + Glucose	
Pectin	10
Glucose	10
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	5
Vishniac trace element solution	1 (ml)
Pectin + Fructose	
Pectin	10
Fructose	10
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	5
Vishniac trace element solution	1 (ml)
Pectin + Saccharose	
Pectin	10
Saccharose	10
KH_2PO_4	1.5
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	5
Vishniac trace element solution	1 (ml)

3.5. Batch Fermentations

3.5.1. Shake Flask Cultures

The shake flask cultures were conducted in the 250 ml and 500 ml Erlenmeyer flasks containing 75 ml and 150 ml of medium, respectively. Production was carried out in a rotary shaker at 200 rpm, 30℃ for up to 7 days. Samples were collected daily for enzyme activity and sugar analysis.

50% (w/v) glucose solution was used for stepwise addition to the culture madia when glucose was totally consumed. Glucose consumption was followed every 12 hours by HPLC analysis.

3.5.2. Bioreactor Cultures

In order to investigate the effect of pH on Exo-PG of *A. sojae* MT, glucose medium was tested in 1 liter-bioreactor with 0.6 liter of working volume at 30℃, 300 rpm, and 0.5 vvm aeration rate. The bioreactors were equipped with acid and base pumps to automatically maintain pH during the fermentation. To prevent foaming an anti-foaming agent (Sigma Antifoam A, St. Luis, MO, USA) was used.

The experiments were done with duplicates for each condition. The initial pH for all bioreactors was adjusted to 5.0 with 2M KOH before inoculation. The bioreactors were inoculated with fungal pellets. The pellets were obtained by germination from spores suspended in 250 ml shake flasks containing 50 ml glucose medium with $8x10^4$spores/ml, within a rotary shaker at 250 rpm, 30℃ for 48 h.

The first two bioreactors were used without pH control, and the other bioreactors the pH was allowed to drop to the set value. By the time the pH dropped to the set value pH control was initiated and pH was maintained at 3 and 4 by automatic addition of 2M KOH or 2M H_2SO_4.

3.6. Biomass Determination

The biomass was determined by gravimetric method as dry cell weight (g/L). A certain volume of the fermentation broth was filtered through Whatman No.1 filter paper, and then dried to constant weight at 45℃ for approximately 24 hours.

3.7. Analytical Methods

3.7.1. Determination of Reducing Sugar

The reducing sugar assay was performed according to the Nelson-Somogyi method (Somogyi, 1952). The Nelson-Somogyi method depends on the oxidation of reducing sugar (i.e. glucose) by the reduction of the Cu(II) ion to Cu(I), thereafter Cu(I) ions are oxidized back to Cu(II) through the reaction with a colourless hetero-polymolybdate complex. Arsenomolybdate is used for this purpose which is the output of the reaction among ammonium molybdate, sodium arsenate and concentrated sulfuric acid. Arsenomolybdate is responsible for the characteristic color of the mixture, and the absorption changes according to the amount of the reducing sugar present in the reaction mixture. In this study, Varian Cary Bio 100 UV-Visible spectrophotometer was used to measure the absorbance at necessary wave length against water, and the known concentration of reducing sugar was used to draw calibration curve to calculate the exact amount of reducing sugar.

3.7.2. Enzyme Assays

3.7.2.1. Exo-polygalacturonase Assay

Exo-Polygalacturonase (Exo-PG) activity was assayed according to modified procedure based on Nelson-Somogyi reducing sugar assay, adapted by Panda et al. (1999) using 0.4 mL of 2.4 g/L of polygalacturonic acid in sodium acetate buffer pH 4.8 as substrate and 0.1 mL of crude enzyme. The reaction was performed at 40℃ for 30 minutes. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. Galacturonic acid was used as standard for the calibration curve of PG activity. Calibration curve was prepared using different concentrations of galacturonic acid.

One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 micromole of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions.

3.7.2.2. Pectin Methylesterase Assay

Pectin methylesterase (PME) activity was assayed according to Hagerman and Austin (1986) procedure, modified by Yemenicioğlu *et al*. (1999) using 2.3 mL of 0.3 % (w/v) pectin solution prepared in 0.1 M NaCl (pH 7.5), 0.5 mL of 0.01 % (w/v) 0.003 M sodium phosphate buffer pH 7.5 and 0.1 mL crude enzyme. The decrease in absorbance at 620 nm was monitored by using Varian Cary Bio 100 UV-Visible spectrophotometer equipped with a constant temperature cell holder working at 30℃.

The enzyme activity was determined from the slope of the initial linear portion of absorbance versus time curve and one unit of enzyme activity was defined as the amount of enzyme that caused 0.001 changes in absorbance in 1 minute.

3.7.2.3. Pectin Lyase Assay

Pectin lyase (PL) activity was assayed according to modified Albersheim (1966) procedure, using 0.9 mL of 0.25% (w/v) citrus pectin in 0.1 M Tris-HCl buffer pH 8.0 as substrate and 0.1 mL of crude enzyme. Mixture incubated at 40℃. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 235 nm at the beginning and after 1 hour.

The increase in absorbance is a measure of PL activity. One unit of enzyme activity was defined as that amount of enzyme which increases the optical density at 235 nm by 1 in 1 hour under assay conditions.

3.7.2.4. Xylanase Assay

Xylanase activity was assayed according to modified procedure based on Nelson-Somogyi reducing sugar assay and the procedure of Lever *et al*. (1972), using 0.5 mL of 2.5 % (w/v) xylan in 0.1 M sodium acetate buffer as substrate, 1.5 mL of sodium acetate buffer pH 4.5 and 0.5 Ml of crude enzyme. The reaction was performed at 37℃ for 60 minutes. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. The standard curve prepared by plotting the absorbance at 500 nm versus milligrams of glucose.

One unit of enzyme activity was defined as 1.0 mg of reducing sugar from xylan (measured as glucose) per minute at pH 4.5 at 37℃.

$$\text{Activity} = \frac{\text{mg of glucose}}{60 \text{ x } 0.5} \text{ x df U/mL} \quad (3.1)$$

Where, 60 is the reaction time (min), 0.5 is the amount of enzyme in the reaction mixture (ml) and df is the dilution factor of crude enzyme.

3.7.2.5. Total Cellulase Assay

Total cellulose activity was assayed according to the modified procedure of Adney and Baker (1996), using 50 mg of Whatman No:1 filter paper (1.0 x 6.0 cm) as substrate with 1 mL of 100 mM sodium acetate buffer pH 4.8 and 0.5 mL of crude enzyme. The reaction was performed at 45℃ for 60 minutes. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. The standard curve prepared by plotting the absorbance at 500 nm versus milligrams of glucose.

$$\text{FPU} = \frac{0.37}{[\text{enzyme}] \text{ releasing } 2.0 \text{ mg glucose}} \text{ units/mL} \quad (3.2)$$

$$\frac{2.0 \text{ mg glucose}/0.18016 \text{ mg glucose}/\mu\text{mol}}{0.5 \text{ ml enzyme x } 60 \text{ min}} = 0.37 \, \mu\frac{\text{mol}}{\text{min}}.\text{ml} \quad (3.3)$$

The enzyme unit is called as filter paper unit (FPU) and one unit of enzyme activity was defined as the enzyme that causes the release of 2 mg of glucose.

3.7.3. HPLC Analysis

Sugar consumptions of wild and mutant type of *A. sojae* grown in different medium compositions were followed daily by the sugar analysis with High Performance Liquid Chromatography (HPLC) by using The Perkin Elmer Series 200 HPLC system with auto-injector (20 µl), column oven, refractive index detector (RID) and Aminex HPX-87H (1,300 x 7.8 mm, 9µm) column. The standard sugar analysis method which was isocratic at 0.6 ml/min flow rate with 5 mM H_2SO_4 as mobile phase at 65℃ column temperature was used. The serial concentrations of monosaccharides (0.125 to 2 g/l) were used to draw calibration curves.

In consequence of polysaccharides could not be analyzed by HPLC directly, in order to quantify pectin amount a method reported by Rumpunen et. al. (2002) was used after minor modifications. The enzymatic degradation method was validated for citrus pectin degredation into galacturonic acid using 400 µl of commercial pectinase 2% (v/v) in water and 100 µl of fermentation broth containing pectin. A direct proportion was observed between pectin and galacturonic acid, thus serial concentrations of pectin (0.125 to 2 g/l) were degraded by pectinase and used for standard curve. The pectin amount was measured via the standard curve drawn for pectin versus galacturonic acid.

CHAPTER 4

PRELIMINARY INVESTIGATIONS

4.1. Selection of Media for Spore Propagation

The first step in this study was the determination of the best medium for spore propagation. It has been proved that spore production medium has a significant effect on productivity of the processes (Gögus et al., 2006; Krullet al., 2013).

In order to investigate the best effective medium for spore propagation, the fungal strains were grown on different media, molasses agar medium, yeast malt extract agar medium (Gögus et al., 2006), and also potato dextrose agar medium in petri dishes. The incubation was performed at 30℃ until well sporulation, approximately 1 week.

The final sporulation of fungi was evaluated visually. The sporulation was better on molasses agar medium for *A. sojae* than other media as indicated in the study of Gogus et al. (2006), also molasses agar medium provided well sporulation for the other fungal strains. Based on this results molasses agar medium was used for both activation and propagation of fungi.

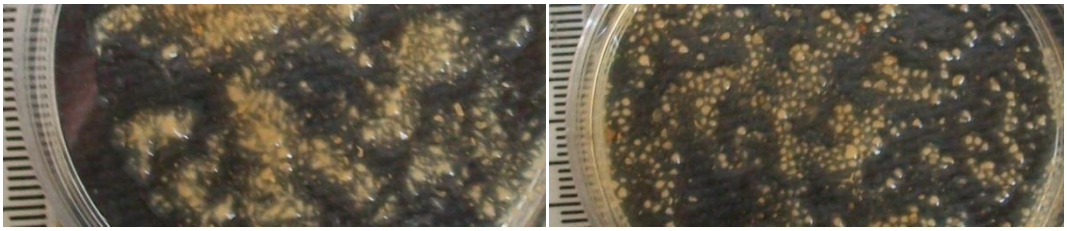
4.2. Medium for Screening Pectinase Producing Fungi

Pectinases are a group of enzymes which includes endo- and exo-polygalacturonase, pectin lyase, pectin methylesterase, etc. Furthermore due to the more stable character of a crude enzyme than a purified enzyme (Tari et al., 2008), most of the commercial pectinases generally includes cellulose, xylanase, protease, etc. (Moyo et al., 2003; Heerd et al., 2012).

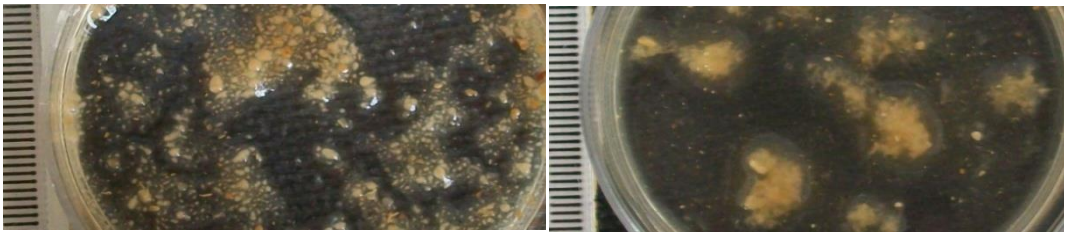
In this study, a number of strains of fungi were examined for Exo-PG, PME, PL, cellulase, and xylanase activities. The shake flask cultures were used in order to determine pectinase producing fungal strains. The media which included 40 g/l of orange peel and 2.75 g/l $(NH_4)_2SO_4$ was used with $2.8x10^3$ spore/ml final spore

concentration. The culture incubated at 30℃ for one week with 200 rpm stirring rate. Samples collected daily for enzyme activity assays.

The results have shown that only the wild and mutant types of *A. sojae* were able to produce only exo-PG under this growth condition. As expected, the mutant type showed more than two-fold activity compared to the wild type. The results were summarized in Table 4.1 and pellet morphologies given by the Figure 4.1.



Aspergillus sojae ATCC 20225 *Aspergillus sojae* MT



Aspergillus niger *Rhizopus oryzae* ATCC 4858

Figure 4.1. Fungal pellet morphologies grown in orange peel medium, images taken at the third day of fermentation.

Table 4.1. Maximum Exo-PG activities of different fungi in OP medium.

	Fungal Strains			
	***A. sojae* MT**	***A. sojae* ATCC 20235**	***A. niger* ATCC**	***R. orayzae* ATCC 4858**
Exo-PG	108.02 ±0.62	47.84 ±0.49	NF*	NF*
PL	NF*	NF*	NF*	NF*
PME	NF*	NF*	NF*	NF*
Xylanase	NF*	NF*	NF*	NF*
Cellulase	NF*	NF*	NF*	NF*

* NF: Not found

4.3. Determination of Optimum Initial Spore Concentration

The pellet formation of filamentous fungi grown in submerged cultures show various growth morphologies as freely suspended mycelia, pellet or clumps depending on fungal strain, the inoculum type, the medium composition and pH, and many other environmental factors (Casas López et al., 2005; Posch et al., 2013). Many publications have reported that the pellet morphology effect the reology of fermentation medium and hence the productivity (Rodríguez Porcel et al., 2005; Posch et al., 2013).

In order to determine the pellet diameter concurrently with the maximum exo-PG activity, glucose medium was used to produce pectinase by *A. sojae* MT with different final spore concentrations ranging from $5x10^3$ to $1.8x10^5$ sp/ml.

The pellet photographs were taken using a digital photo camera (Eastman Kodak M320) and the diameters were measured manually. The average pellet diameters for various final spore concentrations are shown in Figure 4.3. The minimum pellet diameter was observed at $8x10^4$ sp/ml final spore concentration.

According to the results of enzyme activity assays, it has been shown that the initial spore concentration of $8x10^4$ sp/ml provided not only the minimum pellet diameter but also the maximum Exo-PG activity. It has been reported before that there is a correlation between the filament ratio and productivity, because of the effect of filaments on rheological properties (Casas López et al., 2005). This means that minimum ratio of peripheral filamentous region over central compact region also provides higher enzyme activity in many cases.

Furthermore, as mentioned before pH has a major importance on pectinase production as well as on many other processes (Malvessi et al., 2004). In this study, the pH changes were followed during the fermentation for all spore concentrations. However, a significant difference was not observed among the fermentations. At all final spore concentrations it was observed that pH dropped to 2.0 in four days.

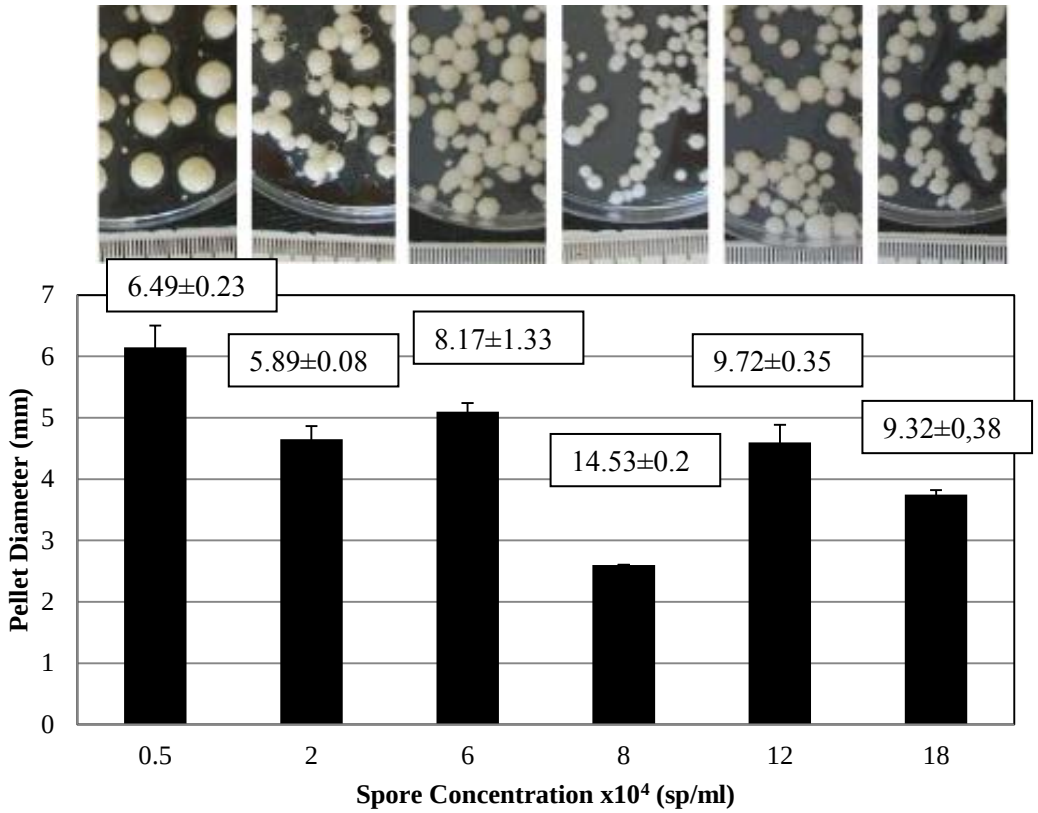


Figure 4.2. The pellet diameters according to the final spore concentrations, exo-PG activities are given in the text boxes.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1. Effect of Carbon Source on Exo-PG Production by *A. sojae*

The aim of this study was to investigate the influence of carbon source and pH on the production of pectinase from some fungal strains. Four strains of fungi were investigated in terms of pectinase production. According to our preliminary works (Chapter 4), only *A. sojae* WT and MT were found as exo-PG producers among the other fungal strains. Exo-PG activities from *A. sojae* WT and *A. sojae* MT grown in orange peel media were 47.84 U/ml and 108.02 U/ml, respectively. Based on these results, exo-PG activity is higher with *A. sojae* MT than *A. sojae* WT when orange peel was used as carbon source.

A. sojae WT and MT could produced exo-PG in glucose, pectin, pectin/glucose, pectin/fructose and pectin saccharose media, varied in a wide range from 1 to 25 U/ml. Results indicated that orange peel was a better inducer than citrus pectin or other purified carbohydrates. The complex structure of orange peel may have provided extra nutrients necessary for enzyme synthesis.

5.1.1. Exo-PG Production by *A. sojae* WT

5.1.1.1. Effect of Different Carbon Sources on pH

Change in pH values during the exo-PG production by *A. sojae* WT grown in various carbon sources are shown in Figure 5.1. The initial pH of the fermentation broths were around 5.0 and decreased down to pH 2.0, in 48 to 96 h depending on carbon source with exception of the presence of pectin alone.

In glucose media pH decreased to 2.5 in 48 h, and decreased to 2.0 in 72 h where it stayed at this value until the end of the fermentation. In pectin/glucose media pH decreased to 2.4 in 72 h, where it stayed at this pH value. In pectin/fructose and

pectin/saccharose media pH decreased to 2.1 in 96 h, and then started to increase after 120 h. The results demonstrated that during the fermentation pH profiles were showed similar trends, except in the pectin medium. In pectin media pH decreased to 4.0 in 48 h, however after 48 h the pH increased up to 8.0. Such a pH profile has been reported for *A. oryzae* in pectin and glucose media by Fontana et al. (2009).

The increasing pH values were also observed by other research groups. Previous studies demonstrated that increasing pectin concentrations in the culture media resulted in extended increase in pH (Malvessi et al., 2004; Fontana et al., 2012). Fungal metabolism may have been associated with this pH trend, production or consumption of organic acids, absorption of nitrogen source and release of H^+ ions. According to the study made by Torrado et al. the intake rates of oxidized and reduced forms of nitrogen acted as a regulator of pH in the range of 4 to 7 (Torrado et al., 1998).

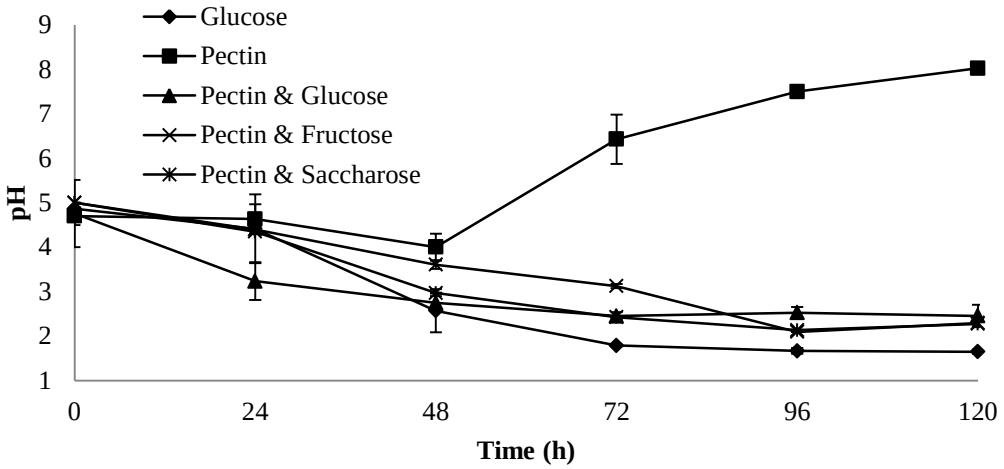


Figure 5.1. Change in pH values during the exo-PG production by *A. sojae* WT grown in different media conditions.

5.1.1.2. Effect of Different Carbon Sources on Exo-PG Activity

The effect of different carbon sources on exo-PG production are shown in Figure 5.2. The pectin/glucose media provided the highest exo-PG activity (21.54 U/ml) compared to the other carbon sources.

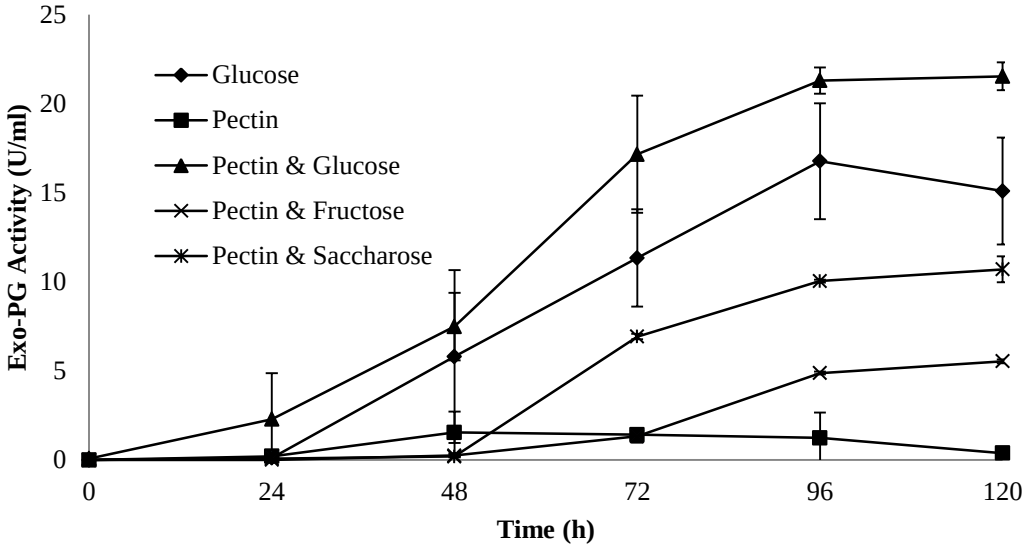


Figure 5.2. Exo-PG activity by *A. sojae* WT grown in submerged culture on various carbon sources.

Pectin/glucose, pectin/fructose and pectin/saccharose yielded 21.54 U/ml, 5.54 U/ml and 10.70 U/ml exo-PG activities, respectively. Due to the fact that pectinase enzymes are known as inducible enzymes (Malvessi et al., 2004; Martínez-Trujillo et al., 2008; Fontana et al., 2012), it was expected that pectin containing media provided more activity. In addition to this, simple sugars may have contributed to the utilization of the complex sources more efficiently. The highest exo-PG activity was observed in pectin/glucose media, which may have been due to the better support of glucose on fungal growth than other sugars.

On the other hand, 1.55 U/ml exo-PG acitivty was observed in the presence of pectin alone. Due to low pH values were necessary to induce exo-PG production, the increase in pH value of pectin media may have caused to low pectinase activity compared to the other media compositions.

When glucose media was not supported with pectin, it yielded 16.77 U/ml exo-PG activity. It may have been as a result of positive effect of low pH values on the exo-PG production (Figure 5.1). The decrease in exo-PG activity after 96 h could be attributed to negative effect of low pH values on the stability of exo-PG. This also proved that *A. sojae* WT is a exo-PG producer even if it grew in a simple sugar which was not the substrate of exo-PG.

5.1.1.3. Sugar Consumption

A. sojae WT was able to metabolize all carbon sources in the culture medium and it metabolized pectin rapidly as well as glucose. This may be due to the other enzymatic activities such as endo-PG. Sugar consumption trends of *A. sojae* WT in glucose, pectin and pectin/glucose are shown in Figures 5.3, 5.4 and 5.5, respectively. As mentioned before the highest exo-PG activity (21.54 U/ml) was obtained in pectin/glucose media.

In glucose medium, the concentration of glucose was 20 g/l at the beginning of the fermentation, after 56 h of incubation almost all glucose was consumed corresponding to utilization rate of 0.36 g/l/h (Figure 5.3). pH dropped to 2.0 in 72 h, at which time all glucose was consumed. Low pH may have resulted in an increased exo-PG production. However, after 96 h exo-PG activity began to decrease. The decrease in exo-PG activity could be explained by the depletion of carbon source, besides low pH value may have had a negative effect on the stability of exo-PG (Fahmy et al., 2008; Tari et al., 2008).

In the presence of pectin in sole carbon source, the concentration of pectin was 20 g/l at the beginning of the fermentation. After 48 h of incubation pectin was consumed totally corresponding to utilization rate of 0.42 g/l/h (Figure 5.4). The utilization rate of pectin was higher than glucose. It may have been attributed to function of other pectinases such as endo-PG.

The highest level of exo-PG activity (1.55 U/ml) was detected after 48 h when pectin was used as carbon source alone. The of growth *A. sojae* in pectin media, resulted in an increase in pH starting after 48 h, while simultaneous decrease in exo-PG production was observed. The pH decreased to 4.0 in 48 h, where pectin was consumed totally. However after 48 h the pH increased up to 8.0., and exo-PG activity had begun to decrease after 48 h of fermentation. The increase in pH could be explained by autolysis of the cells in the absence of carbon source. Lower exo-PG levels could be also attributed to absence of carbon source and high pH values.

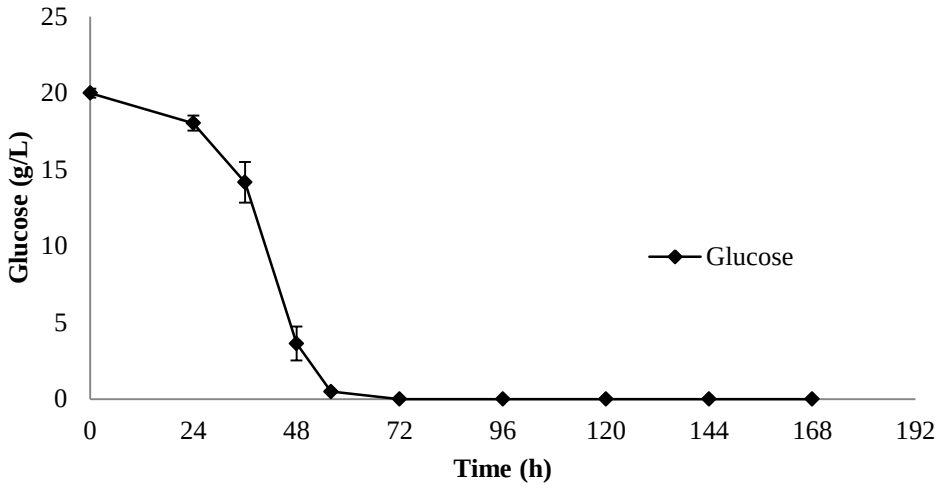


Figure 5.3. Glucose consumption by *A. sojae* WT in glucose media.

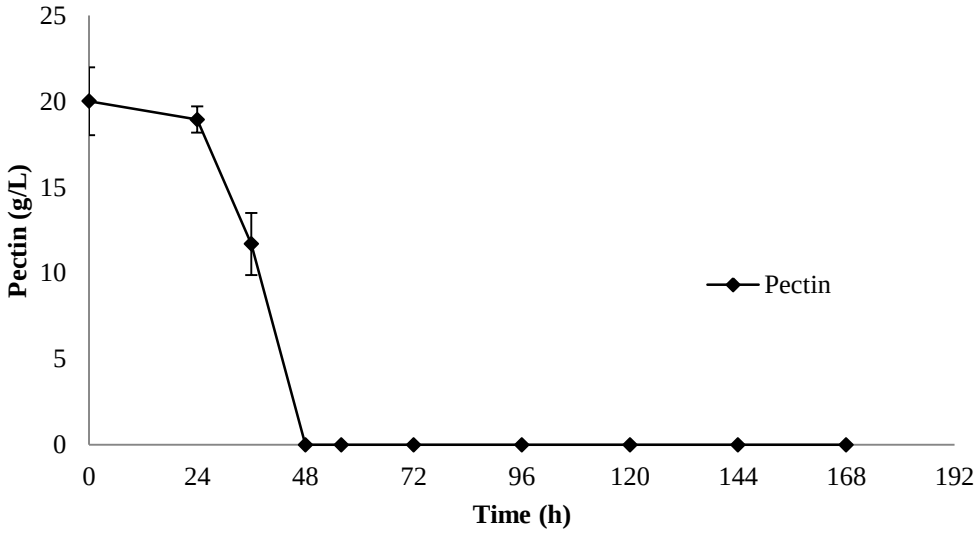


Figure 5.4. Pectin consumption by *A. sojae* WT in pectin media.

In the pectin/glucose medium the initial concentrations of both pectin and glucose were 10 g/l. Glucose was consumed in 36 h of incubation corresponding to utilization rate of 0.28 g/l/h, and pectin was not consumed totally even after 168 h. The presence of glucose prevented the first utilization of pectin. After seven days of incubation 1.74 g/l pectin remained corresponding to a substrate utilization rate of 0.05 g/l/h (Figure 5.5).

The highest level of exo-PG activity was detected (21.54 U/ml) when pectin/glucose was used as carbon source. During growth in that medium, *A. sojae* acidified the culture media, pH dropped to 2.4 in 72 h where it stayed at this pH value. Although pH was low, *A. sojae* continued to utilize remaining pectin albeit at a low rate.

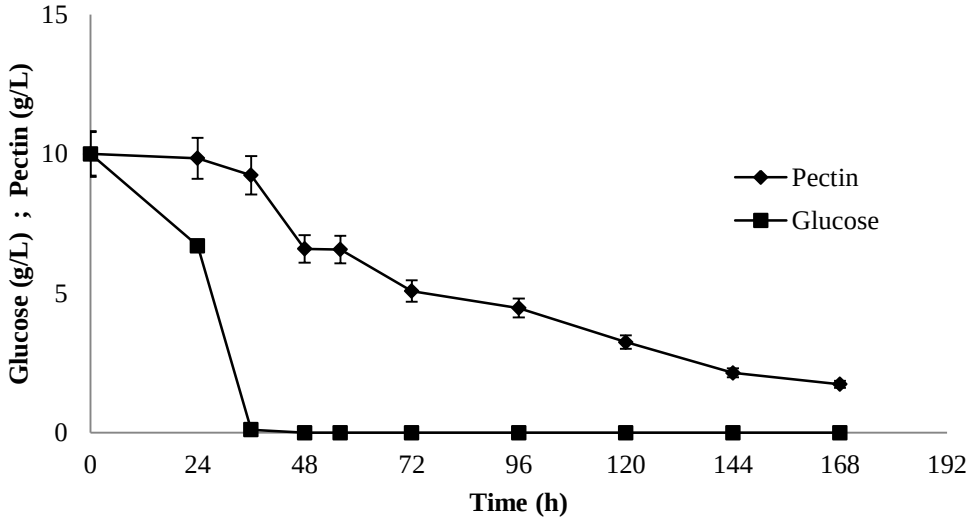


Figure 5.5. Pectin and glucose consumptions by *A. sojae* WT grown in pectin/glucose media.

5.1.2. Exo-PG Production by *A. sojae* MT

5.1.2.1. Effect of Different Carbon Sources on pH

The trend of pH values during the exo-PG production by *A. sojae* MT grown in various carbon sources are shown in Figure 5.6. The initial pH of the fermentation broths were around 5.0 and decreased to at least pH 2.0, varied in 48 to 96 h depending on carbon source with exception of the presence of pectin alone.

In glucose media pH decreased to 2.0 in 48 h, where it stayed at this value until the end of the fermentation. In pectin/glucose media pH decreased to 2.6 in 48 h, and it stayed at this pH value thereafter. In pectin/fructose and pectin/saccharose media pH decreased to 2.1 in 96 h, and then increased to 2.3 at 120 h. The results demonstrated that during the fermentation pH profiles showed similar trends, except the pectin

medium. In pectin media pH decreased to 3.8 in 24 h, however after 24 h the pH increased up to 8.1.

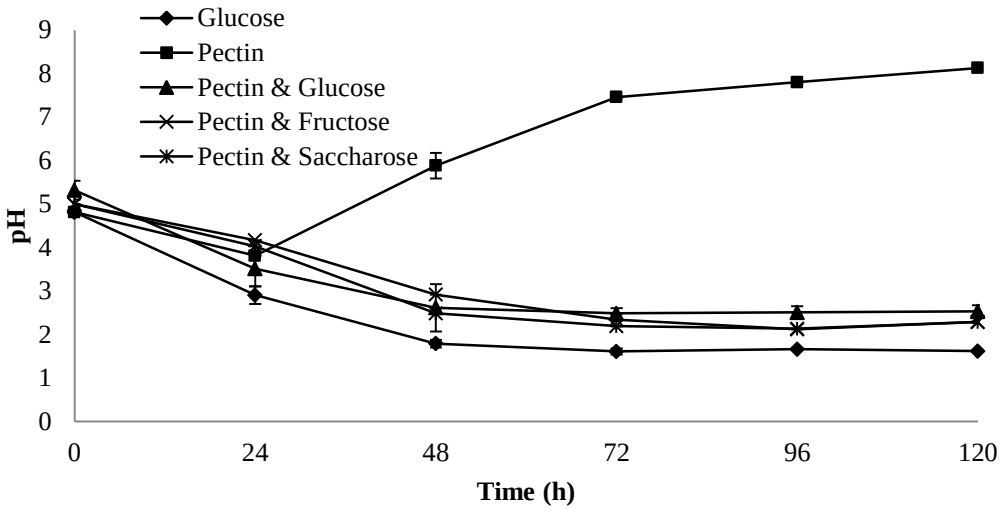


Figure 5.6. Change in pH values during the exo-PG production by *A. sojae* MT grown in different media conditions.

5.1.2.2. Effect of Different Carbon Sources on Exo-PG Activity

Pectin/glucose, pectin/fructose and pectin/saccharose yielded 18.35 U/ml, 5.55 U/ml and 10.01 U/ml exo-PG activities, respectively (Figure 5.7). The highest exo-PG activity was observed in pectin/glucose media, it may have been due to the better support of glucose on fungal growth than other sugars.

In the presence of pectin alone, 1.86 U/ml exo-PG acitivty was obtained. Since low pH values were necessary to induce exo-PG production, the increase in pH value of pectin media may have caused lower exo-PG activity compared to ones obtained with other media compositions.

When glucose media was not supported with pectin, it yielded 12.6 U/ml exo-PG activity. It may have been as a result of positive effect of low pH values on the exo-PG production. The decrease in exo-PG activity after 96 h may have attributed to negative effect of low pH values on the stability of exo-PG.

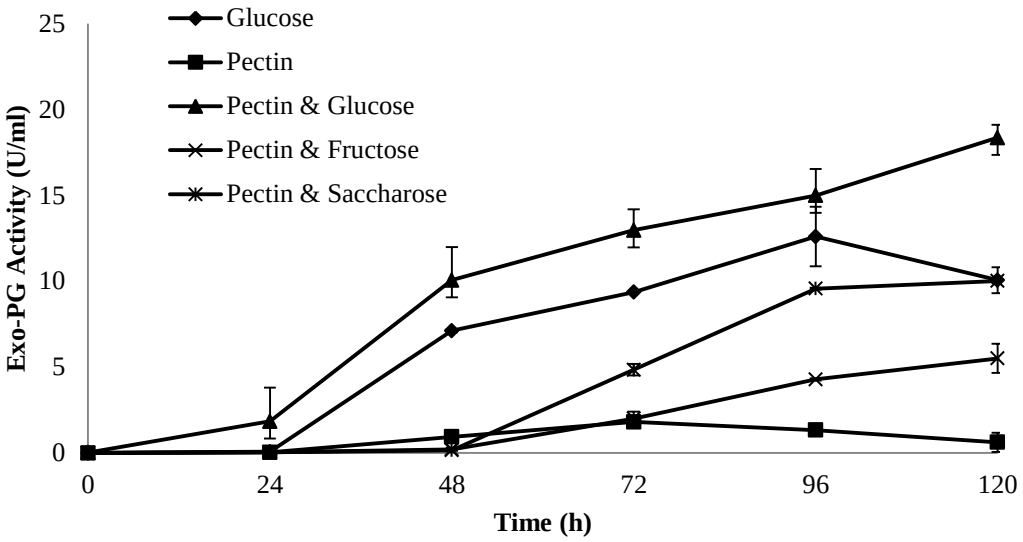


Figure 5.7. Exo-PG activity by *A. sojae* MT grown in submerged culture on various carbon sources.

5.1.2.3. Sugar Consumption

A. sojae MT was able to metabolize all carbon sources in the culture medium similar to the case of *A. sojae* WT.

A. sojae MT was able to metabolize all carbon sources in the culture medium and it metabolized pectin rapidly as well as glucose. This may be due to the other enzymatic activities such as endo-PG. Sugar consumption trends of *A. sojae* MT are shown in Figure 5.8, 5.9 and 5.10, glucose, pectin and pectin/glucose, respectively. As mentioned before the highest exo-PG activity (18.35 U/ml) was obtained in pectin/glucose media.

The concentration of glucose was 20 g/l at the beginning of the fermentation, after 56 h of incubation glucose was consumed totally corresponding to utilization rate of 0.36 g/l/h (Figure 5.8). During growth in glucose media, pH dropped to 2.0 in 48 h, where 0.07 g/l glucose was present in the fermentation broth. Due to *A. sojae* acidified the media, it resulted in an increased exo-PG production. However, after 96 h exo-PG activity began to decrease. In fact, the decrease in exo-PG activity could be explained by the absence of carbon source and high pH value had a negative effect on the stability of exo-PG.

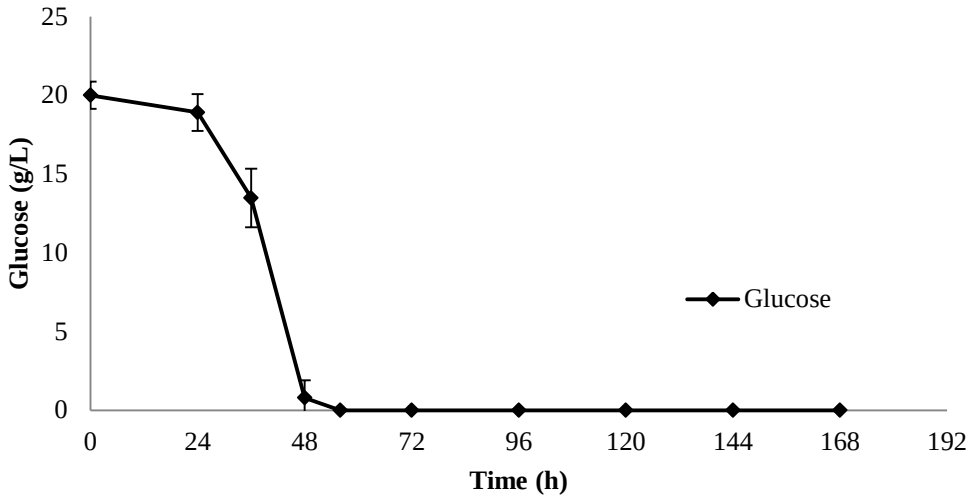


Figure 5.8. Glucose consumption by *A. sojae* MT in glucose media.

The concentration of pectin was 20 g/l at the beginning of the fermentation, after 48 h of incubation pectin was consumed totally corresponding to utilization rate of 0.42 g/l/h (Figure 5.9). The utilization rate of pectin was higher than glucose which was a simple sugar. It may have been attributed to function of other pectinases such as endo-PG.

The highest level of exo-PG activity (1.8 U/ml) was detected after 72 h when pectin was used as carbon source alone. During growth in pectin media, *A. sojae* caused an increase in pH after 24 h. Exo-PG activity decreased after 72 h. The pH decreased to 3.8 in 24 h. However after 24 h the pH increased up to 8.0., and exo-PG activity had begun to decrease after 72 h of fermentation where pH reached to over 7.5. This showed that due to enzyme stability problems, pH values over 7.0 caused to decrease exo-PG activities. The increase in pH could be explained by autolysis of the cells in the absence of carbon source.

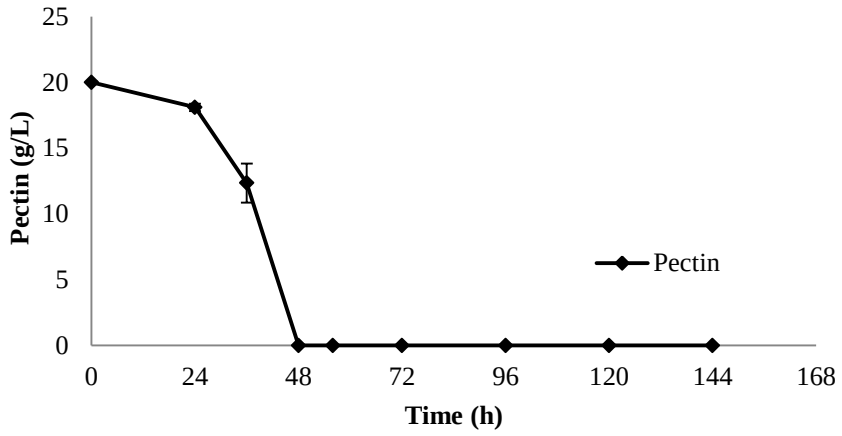


Figure 5.9. Pectin consumption by *A. sojae* MT in pectin media.

In the pectin/glucose media the initial concentrations of both pectin and glucose were 10 g/l. Glucose was consumed in 36 h of incubation corresponding to utilization rate of 0.28 g/l/h, and pectin was not consumed totally in 168 h. After seven days of incubation 3.04 g/l pectin remained corresponding to a substrate utilization rate of 0.04 g/l/h (Figure 5.5).

The highest level of exo-PG activity was detected (18.35 U/ml) when pectin/glucose was used as carbon source. During growth in that medium, *A. sojae* acidified the culture media, pH dropped to 2.5 in 72 h where it stayed at this pH value. Although pH was low, *A. sojae* continued to utilize remaining pectin albeit at a low rate,

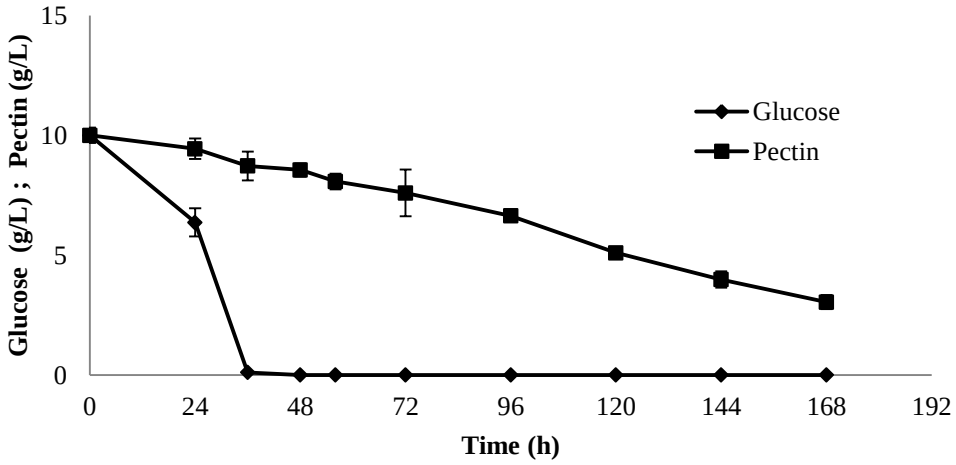


Figure 5.10. Pectin and glucose consumptions by *A. sojae* MT in pectin/glucose media.

5.2. Comparison Beetwen *A. sojae* WT and *A. sojae* MT with Respect to Exo-PG Production and Growth

A comparison was made between the two fungal strains, *A sojae* WT and *A. sojae* MT, according to their growth dynamics in glucose, pectin and pectin/glucose media. Maximum exo-PG activities under different medium conditions are presented in Table 5.2.

Pectin/glucose medium provided the highest activity for both WT and MT types of *A. sojae*, while *A. sojae* WT showed higher activity than *A. sojae* WT almost in all defined media conditions.

In spite of the fact that *A. sojae* MT showed higher exo-PG activity in the medium with orange peel as complex carbon source, it showed lower activity in defined medium than *A. sojae* WT. That may be due to different inducing effect of pectin on exo-PG on two strains. On the other hand, the orange peel is a part of the plant biomass and contains lots of nutrients such as, vitamins, minerals, proteins, etc. in it (Thakur, Singh et al. 1997). Moreover, there are studies showing that even the source of pectin also effected the pectinase production (Pashova, Slokoska et al. 1999). In fact, due to the use of commercial citrus pectin as carbon source in defined media, it has been reported that pectinase activity is highly effected by the type of pectin whether citrus, apple or another source.

It was demonstrated that both *A. sojae* strains were able to metabolize all carbon sources under discussion within approximately 48 h, and generally both showed similar responses under different media conditions in terms of pH profile, sugar consumption rate and growth. The activity of Exo-PG was highest on pectin/glucose, whereas the lowest activity was observed on pectin media.

The growth-pH, growth-exo-PG production and growth-sugar consumption interactions for *A. sojae* WT and MT grown in glucose media were shown in Figure 5.11. *A. sojae* WT biomass concentration was 9 g/l after 72 h, while *A. sojae* MT biomass concentration was 7g/l at the same time. This demonstrated that *A. sojae* WT had higher biomass yield ($Y_{X/S}$= 0.45 g dried biomass/ g substrate) than *A. sojae* MT ($Y_{X/S}$= 0.35 g dried biomass/ g substrate) in glucose media.

The growth-pH, growth-exo-PG production and growth-sugar consumption interactions for *A. sojae* WT and MT grown in pectin media were shown in Figure 5.12.

A. sojae WT biomass concentration was 5.5 g/l after 48 h, while *A. sojae* MT biomass concentration was 6.5 g/l at the same time. This demonstrated that *A. sojae* MT had higher biomass yield ($Y_{X/S}$= 0.275 g dried biomass/ g substrate) than *A. sojae* MT ($Y_{X/S}$= 0.325 g dried biomass/ g substrate) in pectin media.

Table 5.2. Comparison of wild and mutant types of *A. sojae* grown on various carbon sources according to the maximum exo-PG activities.

	Maximum Exo-PG Activity (U/ml)	
	Fungi	
Carbon Source	***A. sojae* 20235**	***A. sojae* MT**
Glucose	16.77 ±3.25	12.60 ±1.73
Pectin	1.55 ±1.16	1.86 ±0.33
Pectin and Glucose	**21.54 ±0.78**	**18.35 ±0.74**
Pectin and Fructose	5.54 ±0.11	5.55 ±0.84
Pectin and Saccharose	10.70 ±0.72	10.01 ±0.12

The growth-pH, growth-exo-PG production and growth-sugar consumption interactions for *A. sojae* WT and MT grown in pectin/glucose media were shown in Figure 5.13. It was observed that *A. sojae* WT biomass concentration was 6 g/l after 72 h, while *A. sojae* MT biomass concentration was 5.7 g/l at the same time. Two growth peaks indicated diauxic growth which occurred due to grown in media containing two types of carbon source (Naidu et al., 1998). First *A. sojae* metabolized glucose, and grew fast, when all glucose had been consumed the fungus started to express the genes to utilize the pectin. Catabolic repression by glucose could be the reason of pectin was not utilize in the first 36 h. It has been known that glucose when added to the media in high concentrations have a repression effect on the exo-PG activity (Teixeira et al., 2000).

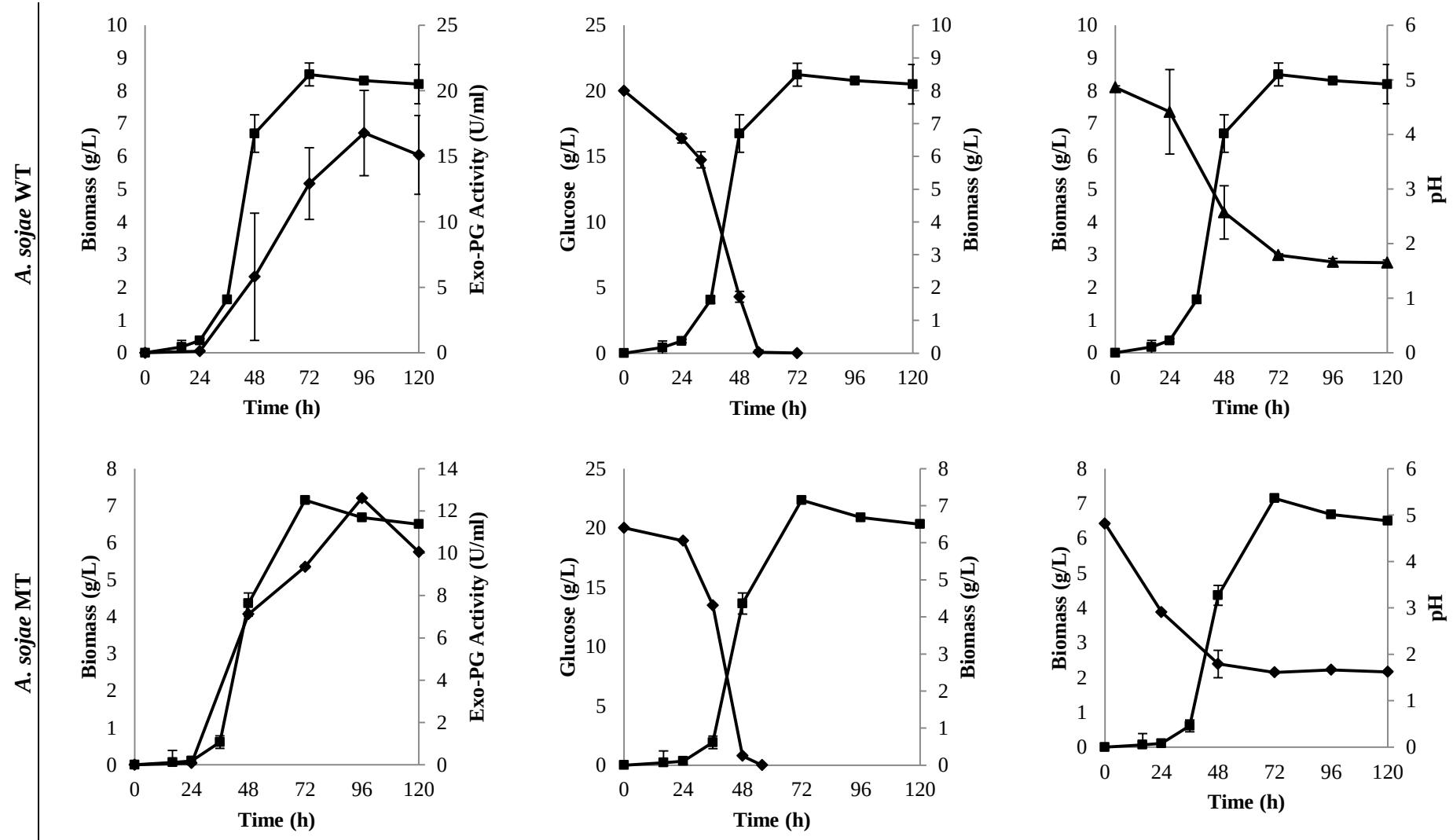


Figure 5.11. Fungal growth (-■-) in glucose media with enzyme activity (-♦-), sugar consumption (-♦-) and pH (-♦-).

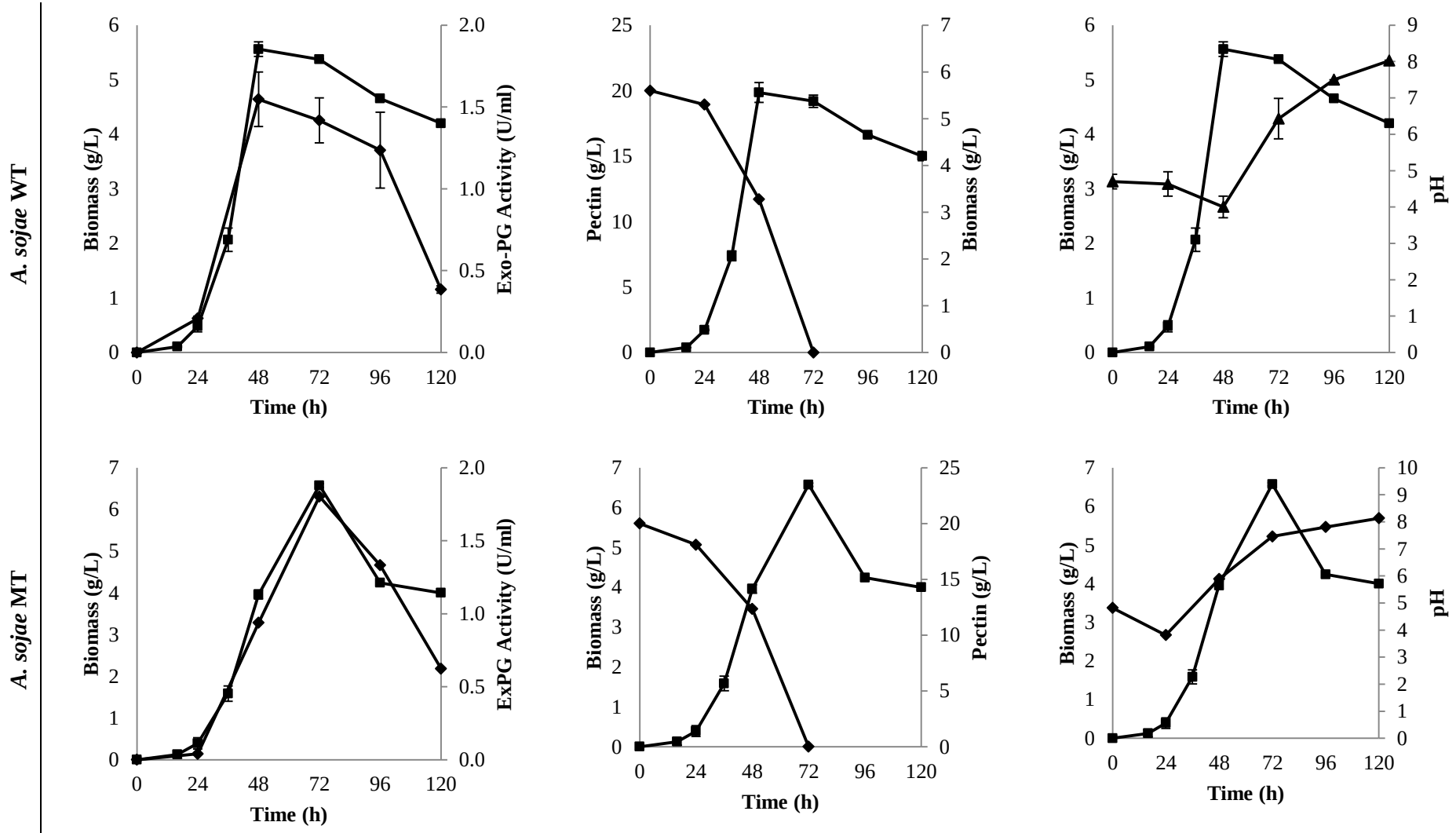


Figure 5.12. Fungal growth (-■-) in pectin media with enzyme activity (-♦-), sugar consumption (-♦-) and pH (-♦-).

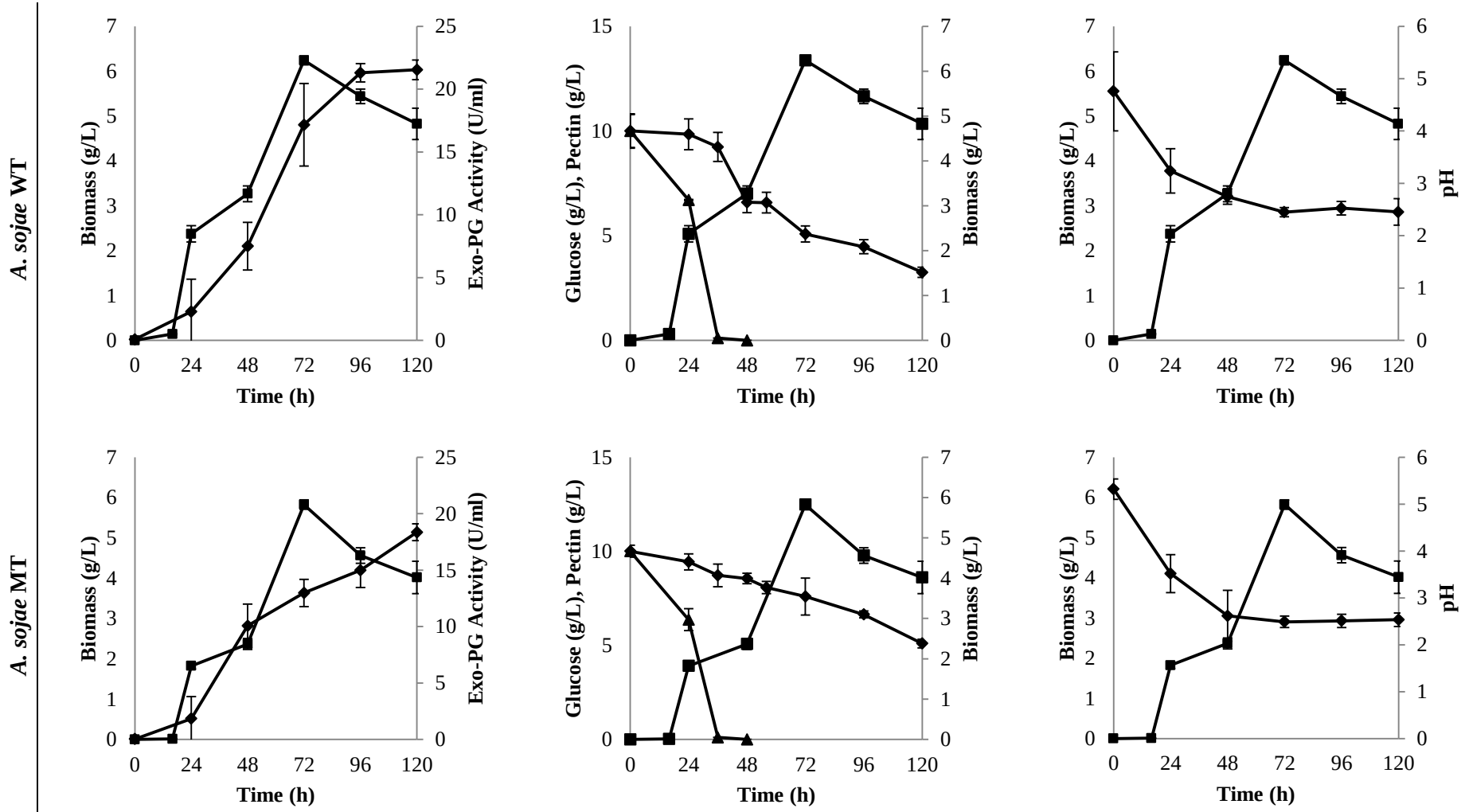


Figure 5.13. Fungal growth (-■-) in pectin/glucose media with enzyme activity (-♦-), sugar consumption (-♦-) and pH (-♦-).

5.3. Effect of Culture pH on the Exo-PG Production

A. sojae MT was grown in glucose media in bioreactors under controlled or uncontrolled pH values by automatic maintenance on pH during growth. Exo-PG activity values at pH 3.0 and pH 4.0, and uncontrolled pH experiments were given in Figure 5.14. The highest exo-PG activity was observed in uncontrolled pH conditions (20 U/ ml).

During the exo-PG production by *A. sojae*, pH rapidly decreases to pH 2.0 approximately in 48 h. Low pH values can inhibit growth of the microorganism, hence also may inhibited the enzyme production or caused to decrease of exo-PG activity due to negative effect of low pH on enzyme stability. Due to the continuous decrease of pH, continuous addition of KOH solution was required during fermentation. This may had a negative effect on enzyme production as well as enzyme stability.

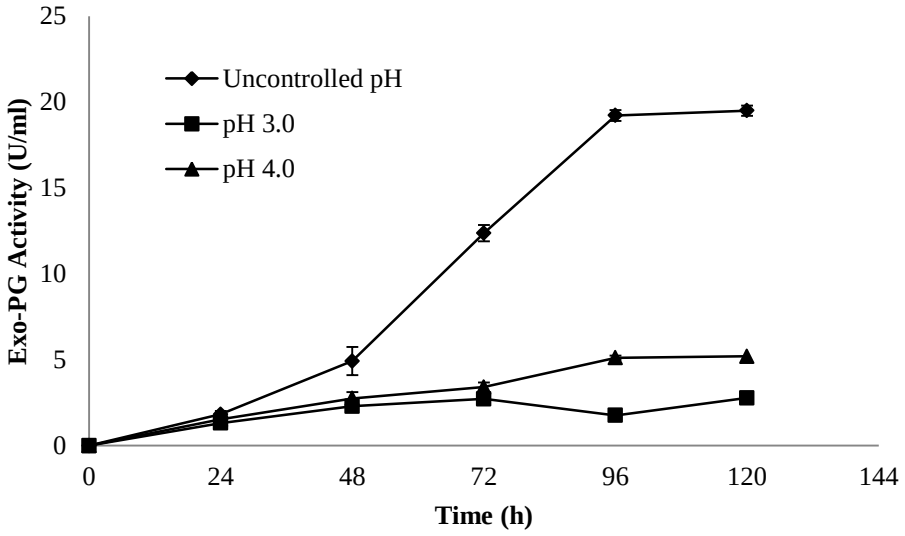


Figure 5.14. Effect of culture pH on the Exo-PG production by *A. sojae* MT.

Fungal morphology was also followed during the incubation in bioreactors. Different pellet morphologies were observed depending on the pH values, this indicated that pH has a drastic effect on pellet forming of fungus. Unlike the case of uncontrolled pH, controlled pH experiments resulted in more filamentous growth or pellets with hairy outer part and with small compact cores (Figure 5.15). Previous studies demonstrated that the initial pH had a significant effect on the pellet formation of

filamentous fungus, and there is a correlation with pellet morphology and enzyme activity (Casas López et al., 2005). Based on these results depending on bioreactor experiments, it can be concluded that *A. sojae* MT provided higher exo-PG activity with the pellet morphology that has more compact core and less hairy region.

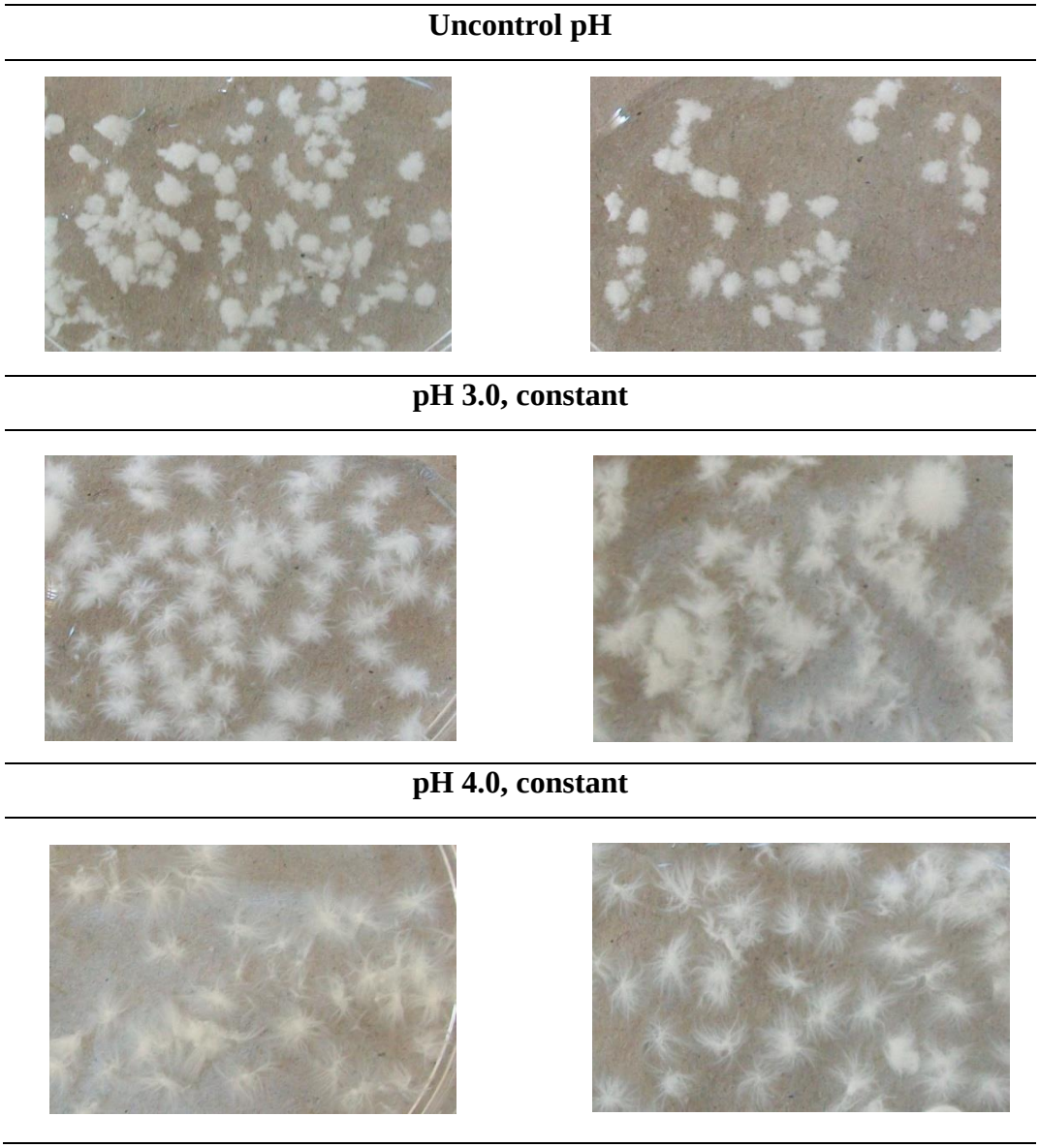


Figure 5.15. Pellet morphologies of *A. sojae* MT grown in bioreactors with different pH values. The right and left columns are replicates of bioreactors.

5.4. Effect of Glucose on the Production of Exo-PG

In order to investigate the effect of the glucose addition on Exo-PG from *A. sojae* WT and *A. sojae* MT, shake flask experiments were done. The effect of glucose was studied by adding a definite amount of sterilized 50% (w/v) glucose solution into the culture broth at a stage of when the glucose in the broth was consumed and exo-PG production was in progress.

Glucose concentration was 20 g/l at the beginning of fermentation, and totally consumed in 56 h corresponding to a utilization rate of 0.36 g/l/h (Figure 5.16). Glucose was added at a concentration of 20 g/l to the media was consumed at a slow rate and, 8.76 g/l glucose remained after 192 h. It was corresponded to a utilization rate of 0.09 g/l/h.

Despite the fact that glucose has repression effect on synthesis of several enzymes (Teixeira et al., 2000), in this study it was observed that glucose provided higher enzyme activity than pectin which had been considered as an inducer of pectinase synthesis. However, addition of glucose to the broth at 72 h caused to stop exo-PG production by *A. sojae* WT and MT for a short time. Thereafter, exo-PG activities increased to maximum values 26.8 U/ml and 24.3 U/ml after 96 h by *A. sojae* WT and MT, respectively. However, after that time point, it had begun to decrease rapidly (Figure 5.16-17). These observations confirmed the repression effect of glucose on exo-PG production. Similarly, there are other reports suggested that addition of glucose caused repression of some pectolytic enzymes by *Aspergillus* sp. (Nair et al., 1995; Panda et al., 2004).

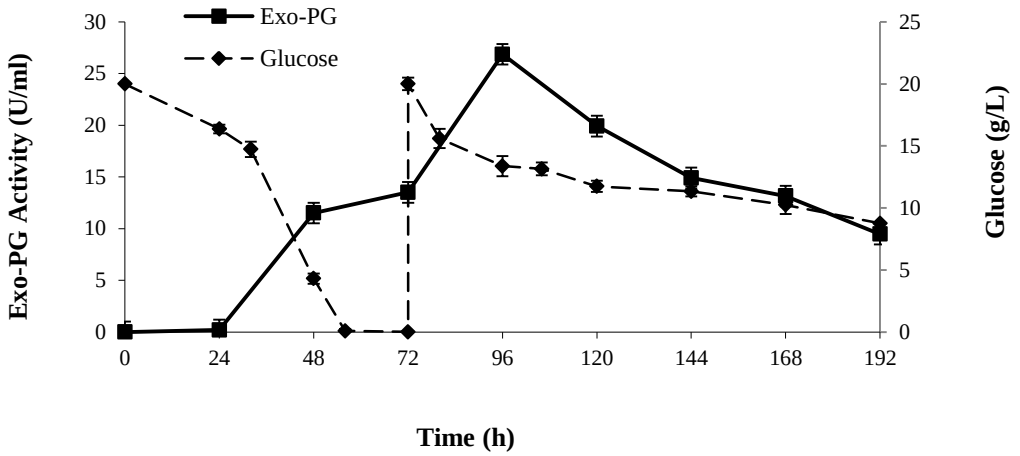


Figure 5.16. Exo-PG production and glucose consumption by *A. sojae* WT with addition of glucose to the broth.

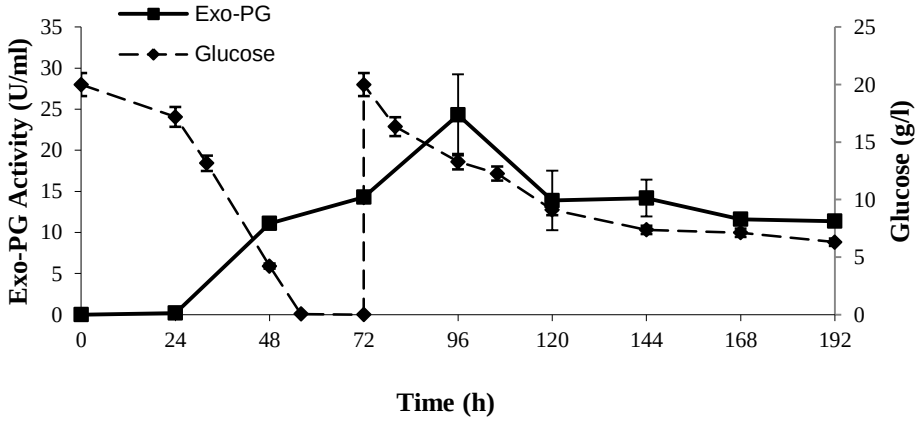


Figure 5.17. Exo-PG production and glucose consumption by *A. sojae* MT with addition of glucose to the broth.

5.5. Cooperative Effect of Glucose and pH on Exo-PG Activity

Shake flask experiments were designed in order to investigate the interaction of glucose consumption and pH on exo-PG production by *A. sojae* WT and *A. sojae* MT. The effects of glucose and pH together were studied by stepwise addition of a defined

amount of sterilized 50% (w/v) glucose solution before glucose was depleted and exo-PG production was in progress. At the same time, in order to eliminate the negative effect of low pH on growth and exo-PG production, pH was manually adjusted to 4.0 by the time pH drops to 2.0 (Figure 5.18-19).

Fungal strains were able to metabolize all glucose in 132 h, and low exo-PG activities were observed (Figure 5.20-21) compared to single addition of glucose without pH control. One potential reason for this was negative effect of increased pH. As it demonstrated in previous sections of the study, it was observed that pH control had negative effect in all experiments.

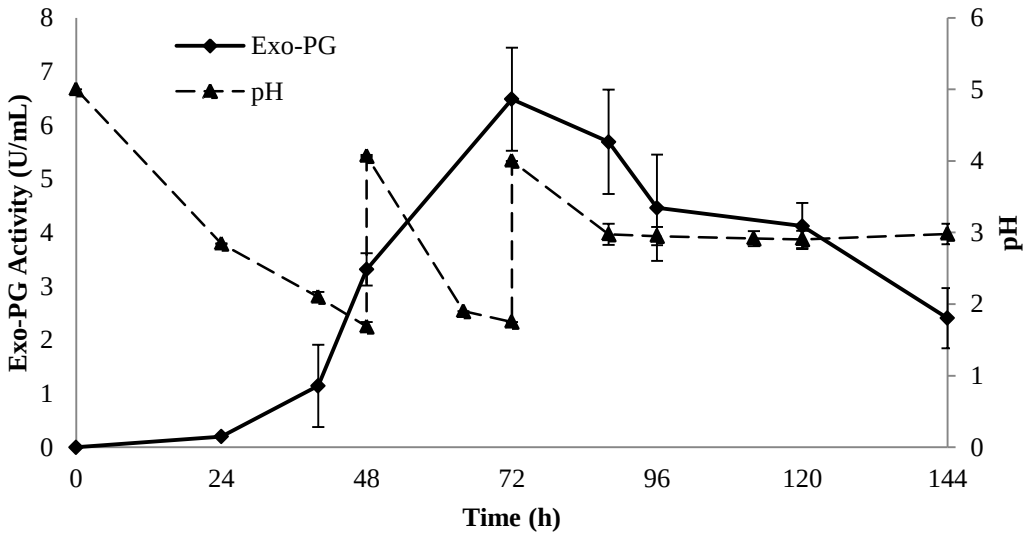


Figure 5.18. Exo-PG production and pH trend of *A. sojae* WT with the stepwise addition of glucose into the broth.

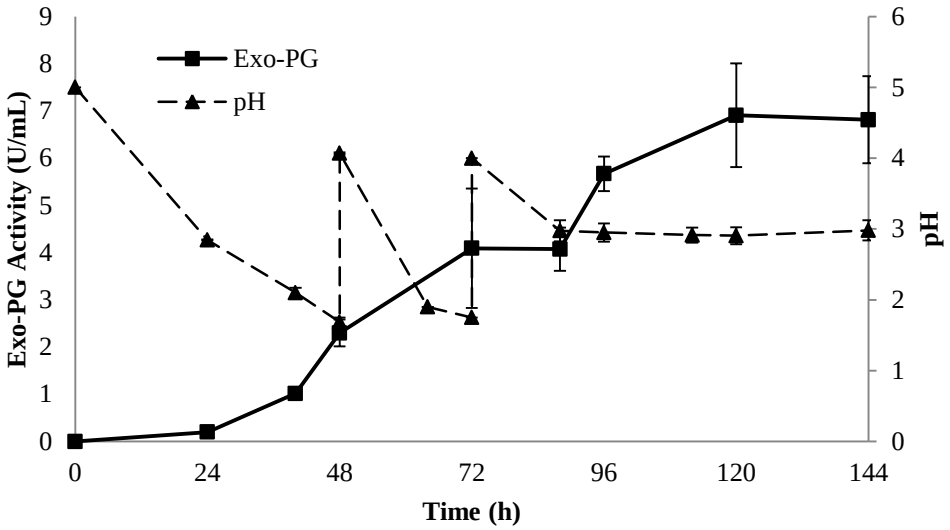


Figure 5.19. Exo-PG production and pH trend of *A. sojae* MT with the stepwise addition of glucose into the broth.

Glucose utilization rates in for each addition step were 0.31, 0.69 0.64 and 0.55 g/l/h for *A. sojae* WT; and 0.27, 0.65, 0.61 and 0.67 g/l/h for *A. sojae* MT, respectively. In early hours of fermentation glucose was consumed and used for mainly biomass, when first glucose added to the broth utilization rate increased and glucose was used for fungus metabolism itself. After second and third addition of glucose, it was observed that utilization rate had decreased due to slow fungal metabolism at stationary phase of growth.

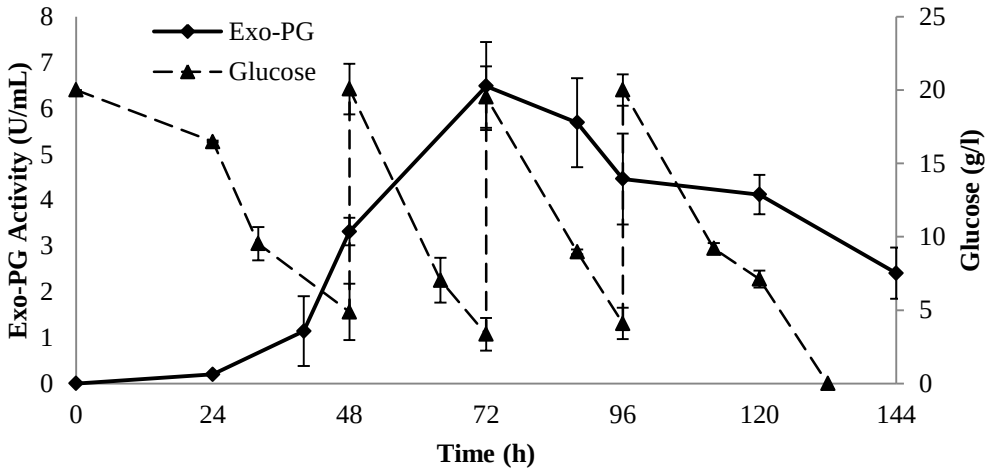


Figure 5.20. Exo-PG production and glucose consumption by *A. sojae* WT with the stepwise addition of glucose into the broth.

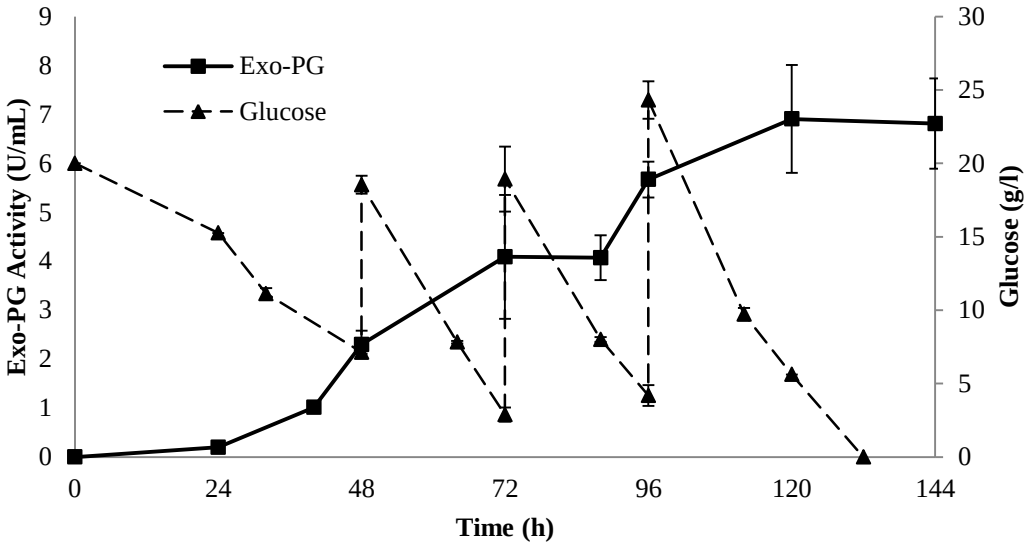


Figure 5.21. Exo-PG production and glucose consumption by *A. sojae* MT, the stepwise addition of glucose into the broth.

5.6. Effect of Glucose on the Production of Exo-PG Activity in Pectin/Glucose Medium

In order to investigate the effect of glucose on exo-PG from *A. sojae* WT and *A. sojae* MT grown in pectin/glucose media which provided highest activities by both of *A. sojae* WT and MT, shake flasks experiments were done.

The effect of glucose was studied by adding a definite amount of sterilized 50% (w/v) glucose solution into the culture broth at a stage of when the glucose level in the broth was consumed.

The interaction of exo-PG activity and pH trends were given for *A. sojae* WT and MT in Figure 5.22-23, respectively. It was observed that pH dropped from 5.0 to 2.0 in 72 h after which time it stayed at this value. Exo-PG activity by *A. sojae* MT increased after 72 h when pH was constant at 2.0, however activity by *A. sojae* WT began to decrease at the same time. This showed that low pH values may have had adverse effects on exo-PG production by *A. sojae* WT.

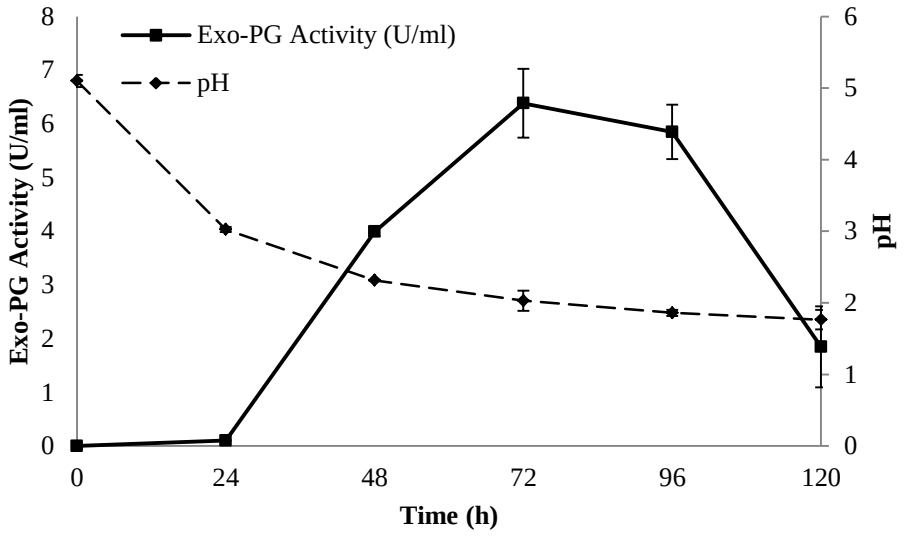


Figure 5.22. Exo-PG production and pH trend of *A. sojae* WT the addition of glucose to the broth.

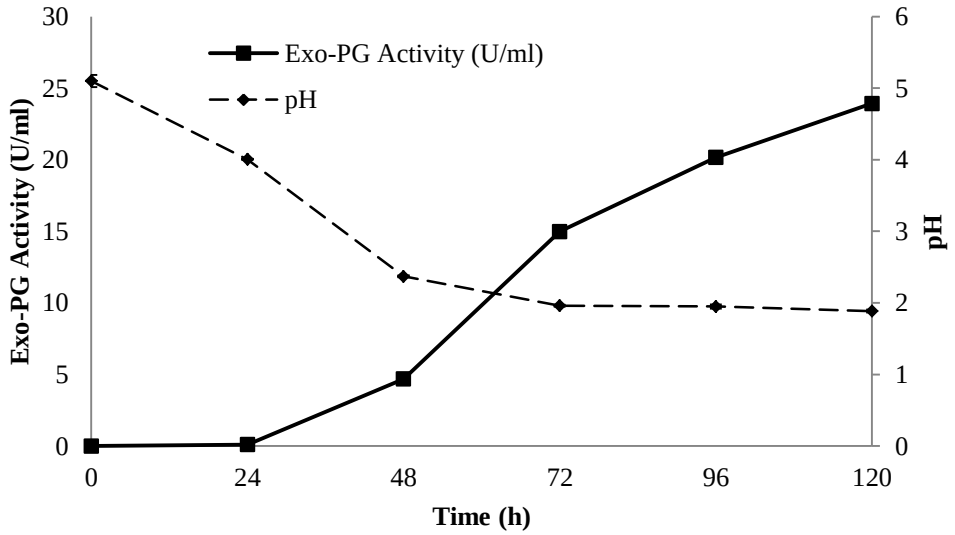


Figure 5.23. Exo-PG production and pH trend of *A. sojae* MT the addition of glucose to the broth.

In the pectin/glucose media the initial concentrations of both pectin and glucose were 10 g/l. Glucose present in broth at the beginning was consumed by both of the strains in 48 h and glucose added to the broth at 48 h was also consumed after 48 h corresponding to a utilization rate of 0.2 g/l/h. Pectin was consumed totally in 96 h

corresponding to a utilization rate of 0.1 g/l/h. Glucose which presented in broth may have had a supporting role on the utilization of pectin the complex carbon source.

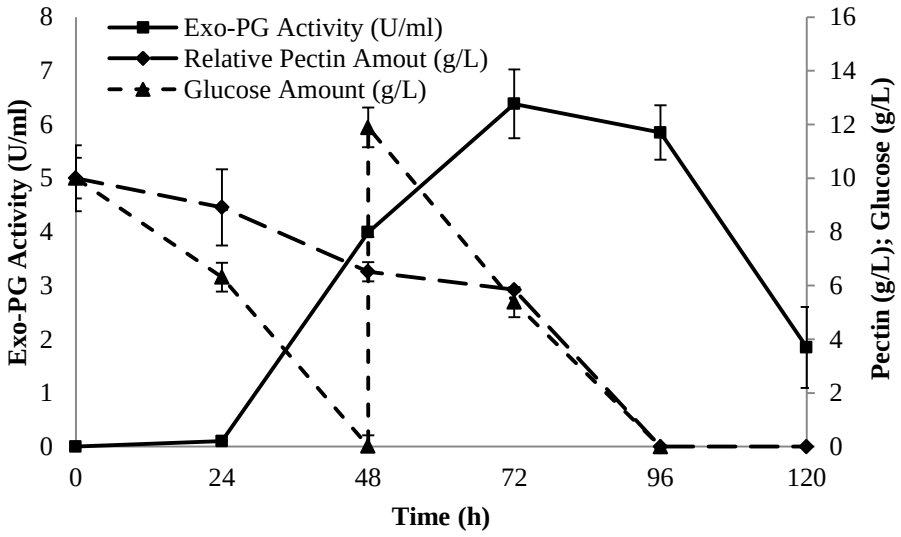


Figure 5.24. Exo-PG production and sugar consumption by *A. sojae* WT the addition of glucose to the broth.

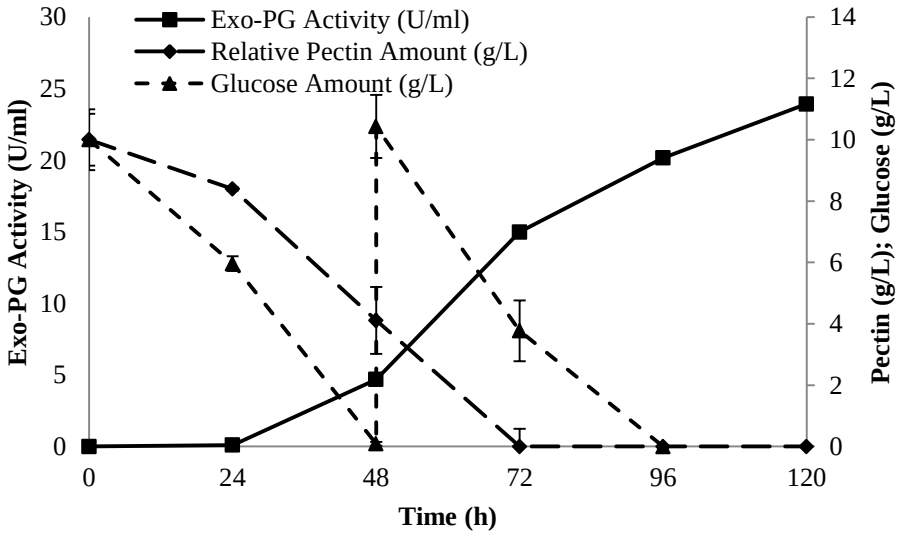


Figure 5.25. Exo-PG production and sugar consumption by *A. sojae* MT the addition of glucose to the broth.

As a result of glucose addition into the pectin/glucose media at 48 h, it was observed that *A. sojae* WT produced 6.3 U/ml at 72 h, and *A. sojae* MT produced 23.9

U/ml at 120 h exo-PG activities. It has been observed that addition of glucose positively effects exo-PG production by only *A. sojae* MT without pH control. This fact showed that glucose had a drastic effect on exo-PG production by *A. sojae*, but the effect was different for *A. sojae* WT and MT (Figure 5.24-25).

Also both fungal strains were able to consume all pectin in broth. It was possible that glucose could be utilized in aiding cell growth as it was more easily utilizable carbon source, while pectin could act as an inducer in the synthesis of exo-PG when both are present in the media.

5.7. Cooperative Effect of Glucose and pH on the Production of Exo-PG Activity in Pectin/Glucose Medium

The effects of glucose and pH on exo-PG production by *A. sojae* WT and *A. sojae* MT were spontaneously investigated similar to previous section. Also pH was adjusted to 4.0 by manually when pH dropped to 2.0 (at 48 h).

In order to eliminate the adverse effect of low pH values, pH was increased to 4.0. After the maintenance of pH, pH dropped to 2.4 at 72 h after which time it stayed at this value (Figure 5.27-28). The decrease in pH after maintenance demonstrated that utilization of carbon source was in progress as well as exo-PG production. Maximum exo-PG activities of *A. sojae* WT and *A. sojae* MT were 15.8 and 16.4 U/ml in 96 h, respectively.

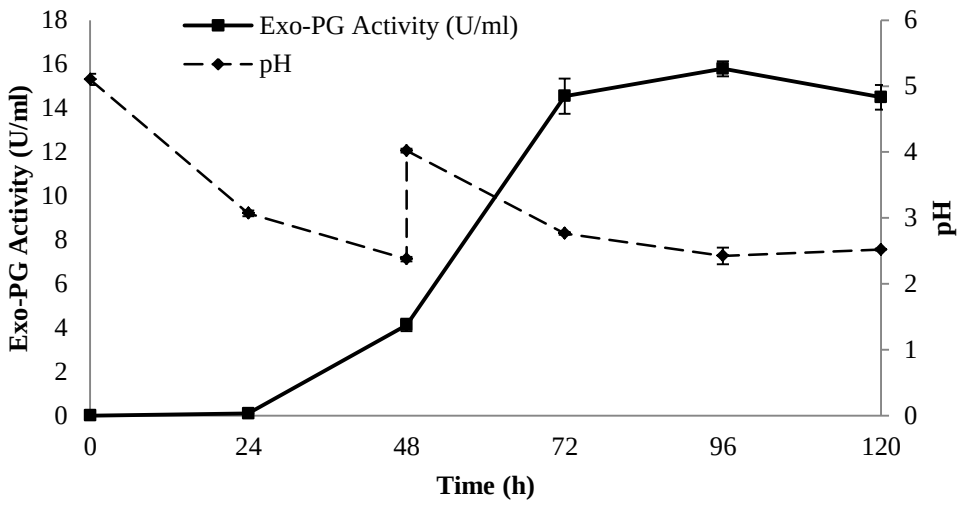


Figure 5.27. Exo-PG production and pH trend of *A. sojae* WT the addition of glucose into the broth.

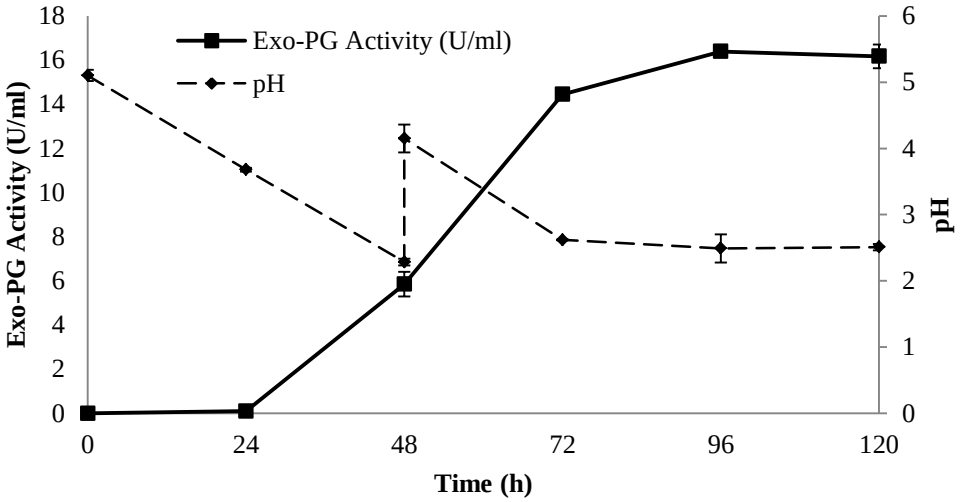


Figure 5.28. Exo-PG production and pH trend of *A. sojae* MT the addition of glucose into the broth.

Sugar consumptions were shown in Figures 5.29 and 5.30. Based on these observations, glucose addition and pH maintenance provided to utilize all carbon sources. Substrate utilization rates (0.2 g/l/h for glucose and 0.1 g/l/h for pectin) were identical to previous experiment which was done without pH control. This demonstrated that substrat utilization rates were not affected by pH maintenance. Exo-PG production by *A. sojae* WT increased from 6.3 to 15.8 U/ml as a result of pH maintenance, while exo-PG production by *A. sojae* MT decreased from 23.9 to 16.4 U/ml. Thus, it can be concluded that two strains of *A. sojae* differently responded to not only glucose addition but also pH maintenance at the same time glucose addition. This could be attributed to differences in their enzyme synthesis metabolisms, although they gave very similar results in terms of sugar consumption and pH trend.

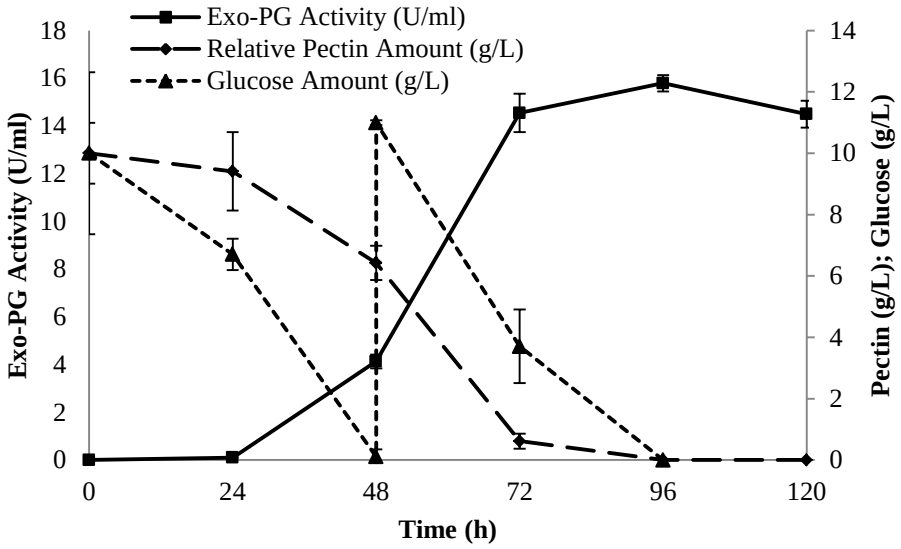


Figure 5.29. Exo-PG production and sugar consumption by *A. sojae* WT the addition of glucose into the broth.

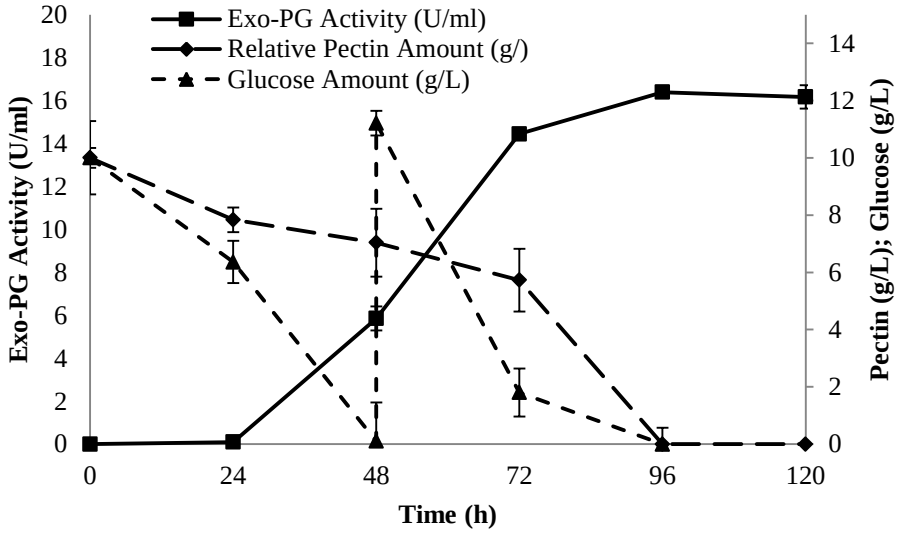


Figure 5.30. Exo-PG production and sugar consumption by *A. sojae* MT the addition of glucose into the broth.

5.8. Effect of Pectin on the Production of Exo-PG Activity from Pectin Medium

The effect of pectin was studied by addition of a defined amount of sterilized pectin solution into the broth at which time all pectin was consumed. The time was determined by previous studies as approximately in 48h.

Addition of pectin to the broth caused a decrease in pH at 48 h due to the acidic character of citrus pectin. The pH trends of *A. sojae* WT and MT were shown in Figures 5.31 and 5.32, respectively. After 48 h pH began to increase up to 8.0. High pH values may have adverse effect on production and stability of exo-PG, hence this could inhibit to reach higher activities during the fermentation.

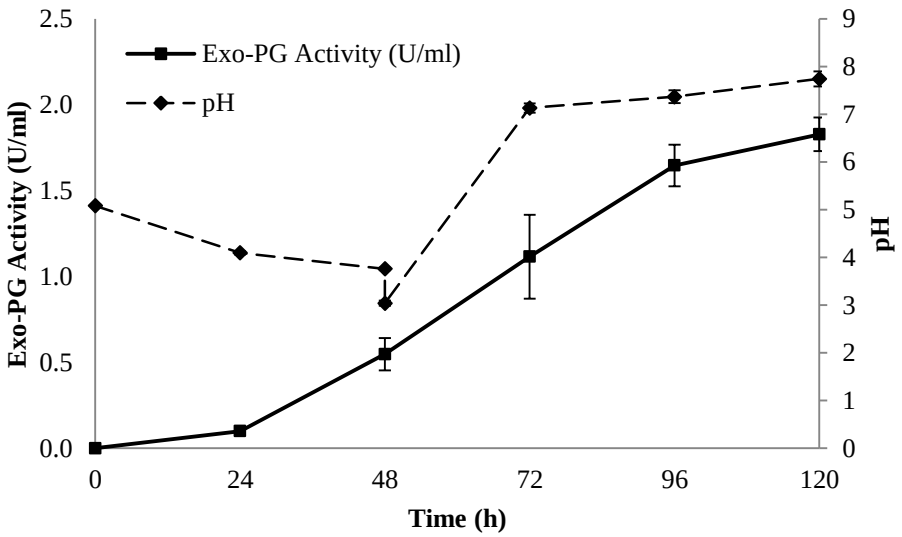


Figure 5.31. Exo-PG production and pH trend of *A. sojae* WT the addition of pectin into the broth.

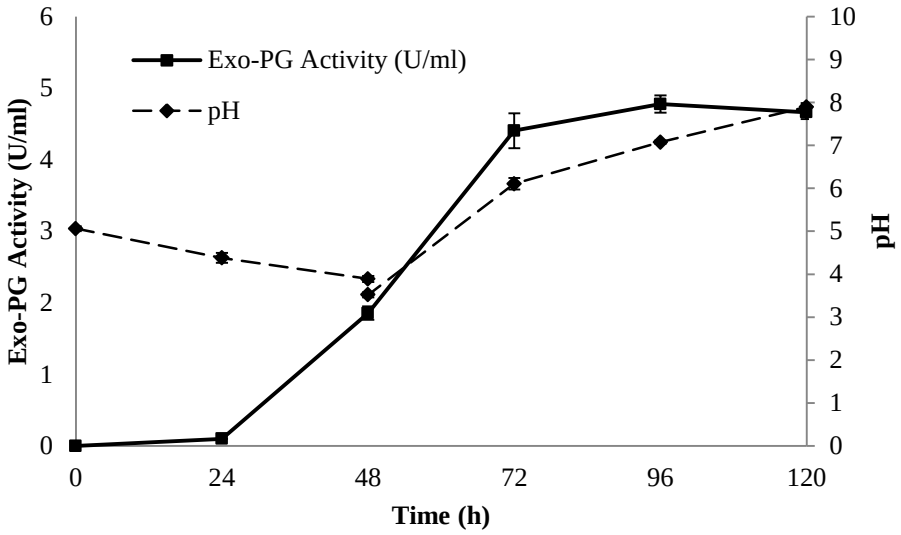


Figure 5.32. Exo-PG production and pH trend of *A. sojae* MT the addition of pectin into the medium.

In pectin medium the initial concentration of pectin was 20 g/l at the beginning of the fermentation, and pectin was consumed in 48 h corresponding to a utilization rate of 0.41 g/l/h. When all pectin was consumed, a defined amount of pectin solution was added to culture broth. Pectin added to the broth was consumed after 72 h corresponding to a utilization rate of 0.07 g/l/h. In fact, the slow utilization of pectin after pectin addition could be explained by adverse effects of high pH values or inhibition exo-PG by addition of pectin.

The addition of pectin into the broth resulted in increased exo-PG activities (Figure 5.33-34). Exo-PG activity by *A. sojae* WT increased from 1.55 to 1.8 U/ml, whereas exo-PG activity by *A. sojae* MT increased from 1.86 to 4.7 U/ml compared to previous exo-PG production in pectin media without pectin addition. The inducing effect of pectin clearly observed for *A. sojae* MT based on 2.5 fold increase of exo-PG activity.

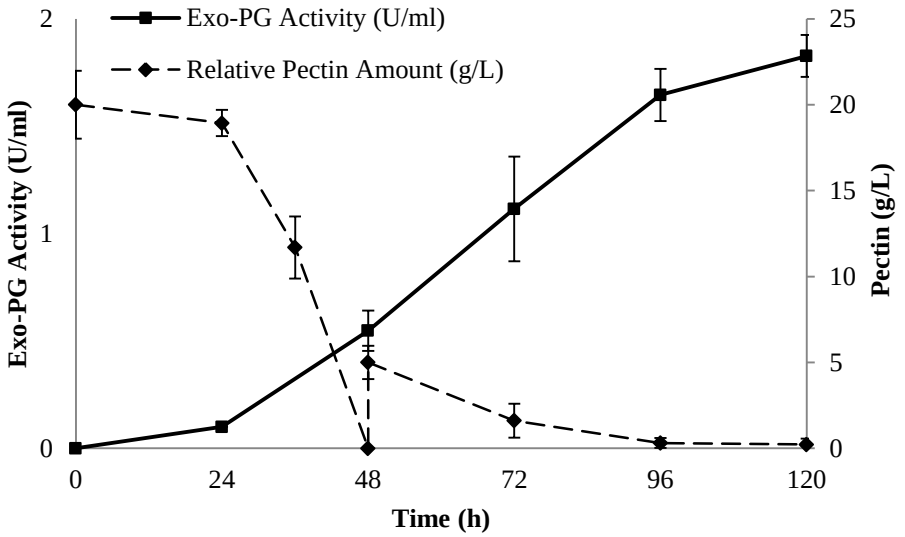


Figure 5.33. Exo-PG production and pectin consumption by *A. sojae* WT the addition of pectin to the broth.

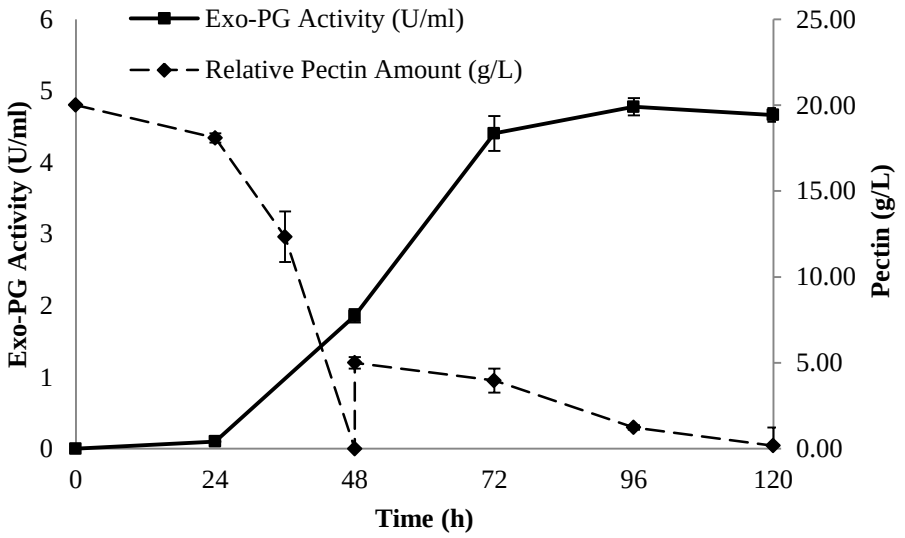


Figure 5.34. Exo-PG production and pectin consumption by *A. sojae* MT the addition of pectin to the broth.

5.9. Cooperative Effect of Pectin and pH on the Production of Exo-PG Activity from Pectin Medium

The interaction between pectin consumption and pH on exo-PG from *A. sojae* WT and *A. sojae* MT was investigated. The effects of glucose and pH were studied by adding a defined amount of sterilized pectin stock solution into the broth at which time all pectin was consumed, also pH was adjusted to 4.0 when pH dropped to 2.0 (Figure 5.35-36).

In pectin medium the initial concentration of pectin was 20 g/l at the beginning of fermentation, all pectin was consumed after 48 h and at which time a defined amount of pectin solution was added to broth. Due to the utilization rates of substrates were equal to the previous experiment pectin addition without pH control, it could be concluded that pH maintenance had no effect on pectin metabolism.

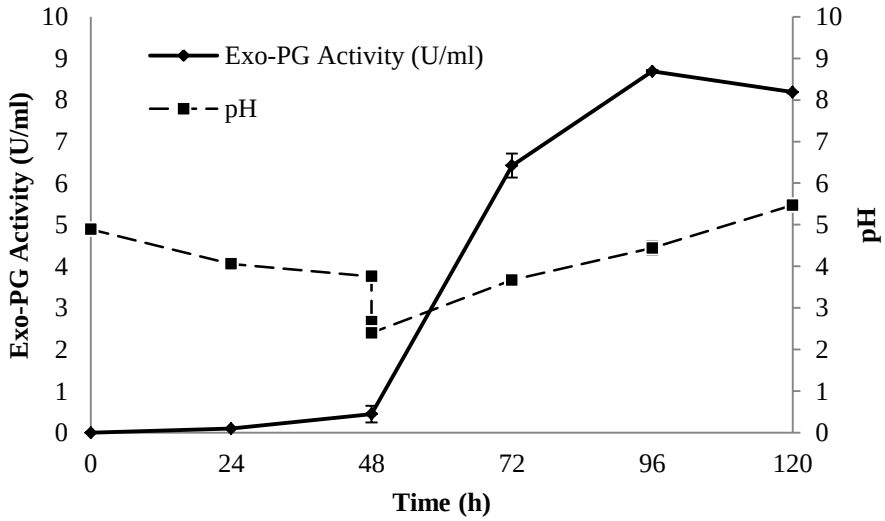


Figure 5.35. Exo-PG production and pH trend of *A. sojae* WT the addition of glucose into the medium.

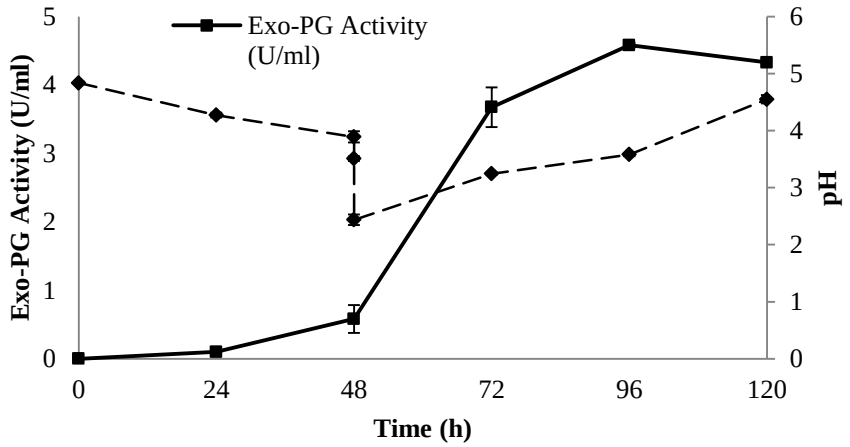


Figure 5.36. Exo-PG production and pH trend of *A. sojae* MT the addition of glucose into the medium.

Results demonstrated that pectin addition caused an increase on the exo-PG activity not only with pH control but also without pH control for of *A. sojae* (Figure 5.37-38). It could be concluded that pectin had an inducer effect on exo-PG production by *A. sojae*. On the other hand, *A. sojae* WT and *A. sojae* MT responded differently to pH control also in pectin media. Exo-PG activity by *A. sojae* WT increased from 1.8 to 8.6 U/ml, whereas exo-PG activity by *A. sojae* MT decreased from 4.7 to 4.5 U/ml after 96 h, compared to previous experiment pectin addition without pH control. While the control of pH had a positive effect on exo-PG production by *A. sojae* WT, *A. sojae* MT showed lower activity under pH control.

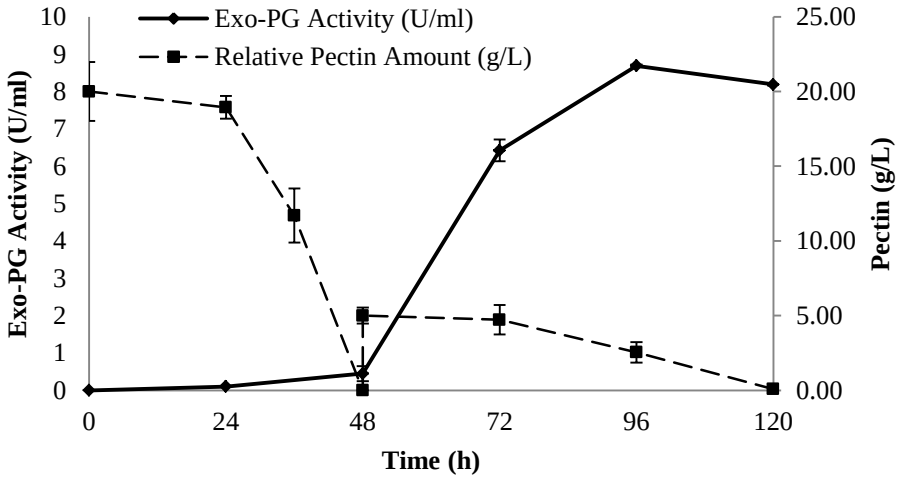


Figure 5.37. Exo-PG production and pectin consumption by *A. sojae* WT the addition of pectin into the medium with pH control.

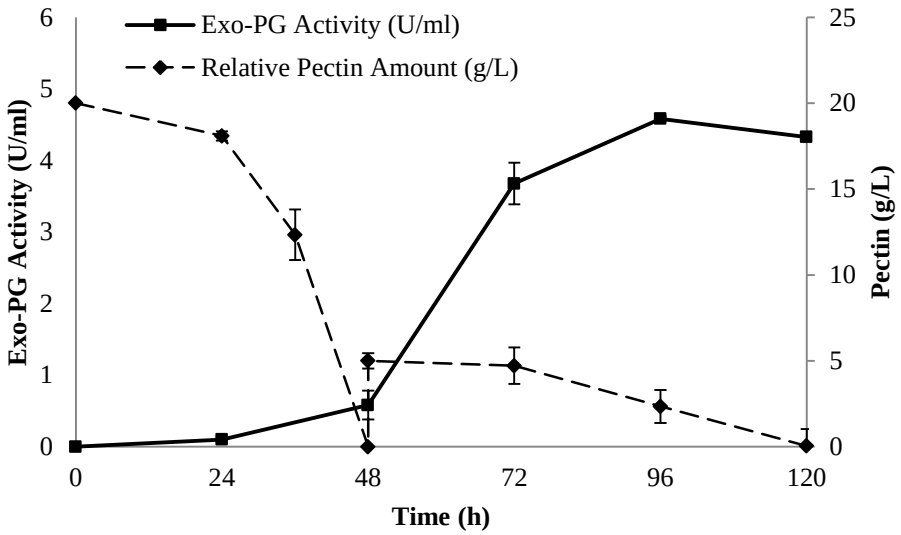


Figure 5.38. Exo-PG production and pectin consumption by A. sojae MT the addition of pectin into the medium with pH control.

CHAPTER 6

CONCLUSIONS

Due to little is known about the regulation of pectinase production by *Aspergillus* sp. and it has not been clearly identified especially for *A. sojae*, this study is an important study demonstrating the influence of carbon source and pH on the production of exo-PG by *A. sojae*.

As previously maintained for different filamentous fungal species including *A. sojae* species, the results presented in this study supported that carbon source and the variation of pH during the fermentation are important factors influencing the synthesis of exo-PG production.

It can be also concluded that pH maintenance during the fermentation have negative effect on the process in terms of exo-PG production. In large scale applications, it will not require pH control reducing the demand for acid and base additions. Hence, this prevents the contamination problems, and also the operational and raw material cost will be significantly reduced.

The lowest exo-PG activity and growth were observed in citrus pectin media. This can be attributed to pH trend in pectin media, and the reduction of flow dynamics as a consequence of the hardening of the medium caused by the excess of pectin.

The results indicate that, additional supply of glucose was found to be more effective than fructose or saccharose to the pectin media. It has been known that high pectin concentrations also induce the pectinase production by *Aspergillus* sp., however high pectin concentration causes reological prolems in culture due to gelatinization property of pectin causing high viscosity values of pectin solutions. Fed-batch operation systems can be used for the production of pectinases, with continuously addition of glucose to get rid of the catabolic repression of glucose or continuously addition of pectin to avoid viscosity problems caused by higher pectin concentrations. The optimization of the large scale production of exo-PG by *A. sojae* are needed to be elucidated by further studies.

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APPENDIX A

CHEMICALS USED IN THE STUDY

Table A.1. List of the chemicals used in the study.

NO	CHEMICAL	CODE
1	Ammonium molybdate tetrahydrate	Sigma 31402
2	Ammonium sulfate	Sigma 31119
3	Bacteriologycal Agar	BD 214010
4	Calcium carbonate	Sigma 12010
5	Carboxymethyl cellulose (CMC)	Aldrich 41928
6	Cobalt(II)chloride hexahydrate	Riedel-De Haën 12914
7	Copper(II)chloride dihydrate	Riedel-De Haën 12914
8	Copper(II)sulfate pentahydrate	Sigma 12849
9	D-(+)-Glucose monohydrate	Sigma 16301
10	D-(-)-Salicin	Sigma S0625
11	D-(+)-Galacturonic acid	Fluka 48280
12	Ethanol 96%	Merck 1.00971
13	Glycerol	Sigma G5516
14	Iron(II)sulfate heptahydrate	Riedel-De Haën 12354
15	Magnesium sulfate heptahydrate	Sigma 63140
16	Malt extract	BD 218630
17	Maltrin	Cargill Starch & Sweeteners
18	Manganese(II)sulfate monohydrate	Riedel-De Haën 13255
19	Molasses	Pakmaya Kemalpaşa Üretim Tesisi
20	Pectin, from citrus peel	Sigma P9135
21	Peptone	BD 211677

(cont. on next page)

Table A.1. (cont.)

22	Polygalacturonic acid	Sigma P3850
23	Potassium hydroxide	AppliChem A3871
24	Potassium chloride	Riedel-De Haën 31248
25	Potassium phosphate monobasic	Sigma 04243
26	Potato dextrose agar	Merck 1.10130
27	Potato dextrose broth	BD 254920
28	Potassium sodium tartrate tetrahydrate	Merck 1.08087
29	Sodium acetate trihydrate	Sigma 25022
30	Sodium arsenate dibasic heptahydrate	Sigma A6756
31	Sodium bicarbonate	Sigma 31437
32	Sodium carbonate	Sigma 13418
33	Sodium carboxymethyl cellulose (CMC)	Aldrich 419311
34	Sodium chloride	Riedel-De Haën 13423
35	Sodium hydroxide	Panreac 141687
36	Sodium dihydrogen phosphate monohydrate	Fluka 71507
37	Sodium phosphate dibasic dihydrate	Riedel-De Haën 04272
38	Sodium sulfate	Sigma 13464
39	Sulfuric acid 98%	
40	Xylan, from beechwood	Sigma X4252
41	Yeast extract	Merck 1.03753

APPENDIX B

REAGENTS FOR ENZYME ACTIVITY ASSAY

Table B.1. The chemical ingredients of copper reagent.

SLOLUTION A*	
Chemical	**Concentration (g/l)**
Na_2CO_3	25
$NaHCO_3$	20
$C_4H_4KNaO_6 \cdot 4H_2O$	33.56
Na_2SO_4	200
SOLUTION B*	
Chemical	**Concentration (g/l)**
$CuSO_4.5H_20$	150
H_2SO_4	1ml

*Mix solutions A and B (25:1)

Table B.2. The chemical ingredients of arsenomolybdate reagent.

Chemical	**Amount**
$\mathbf{(NH_4)_6Mo_7O_{24} \cdot 7H_2O}$	25g in 350ml water
$\mathbf{H_2SO_4}$	21ml
$\mathbf{AsHNa_2O_4 \cdot 7H_2O}$	3g in 125ml water

*Mix solutions and incubate at 37℃ for 24h.

APPENDIX C

CALIBRATION CURVES FOR ENZYME ACTIVITY

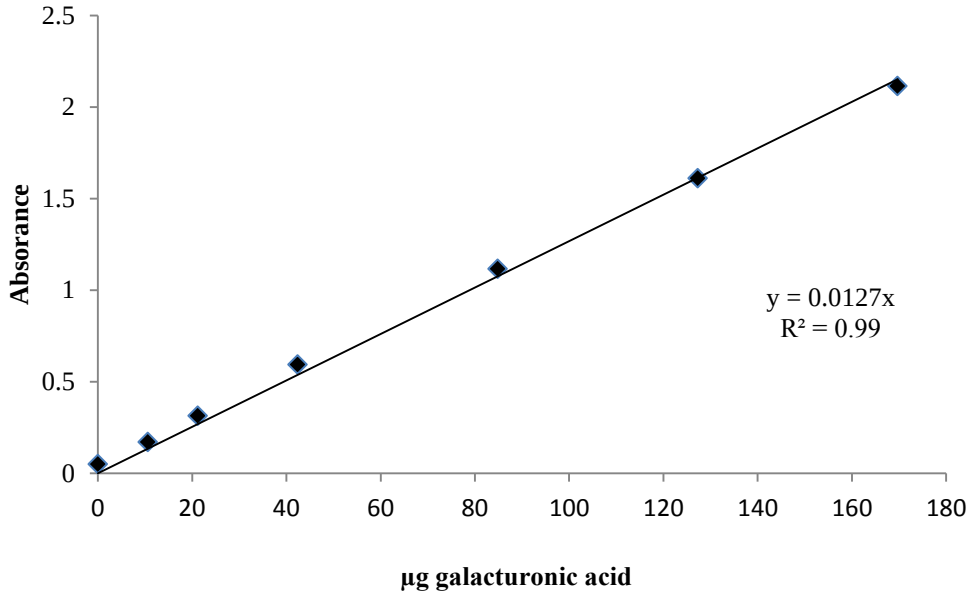


Figure C.1. Calibration curve of galacturonic acid for pectinase activity.

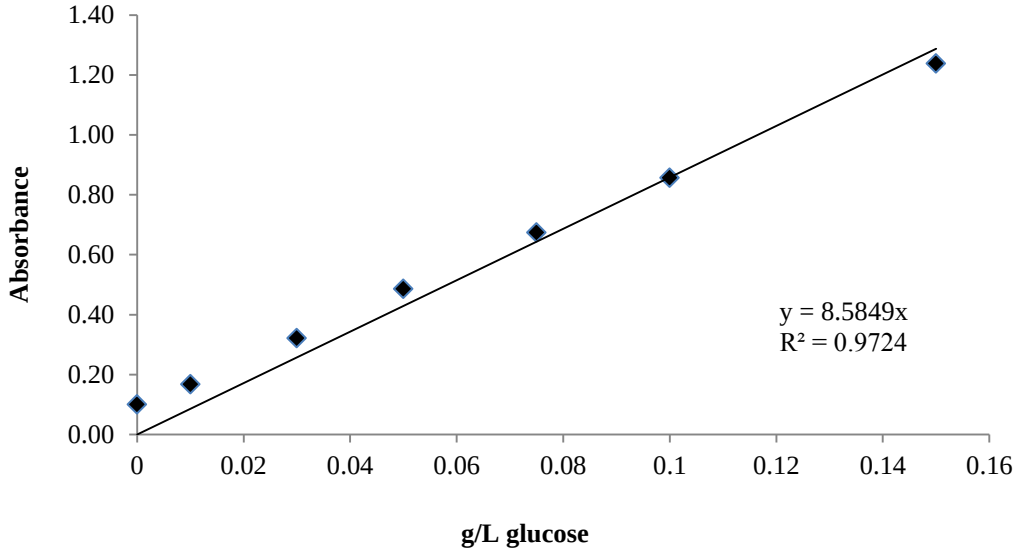


Figure C.2. Calibration curve of glucose for reducing sugar assay.

APPENDIX D

CALIBRATION CURVE FOR PECTIN AMOUNT

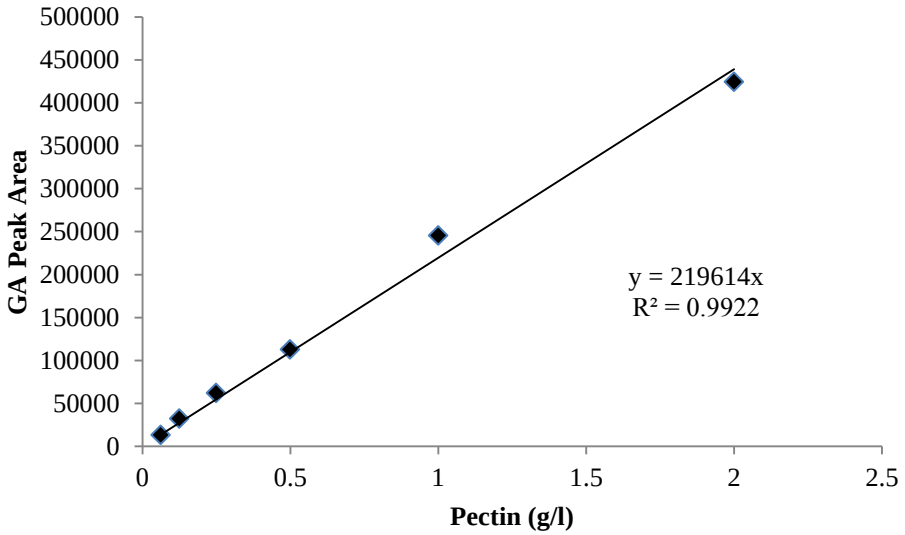
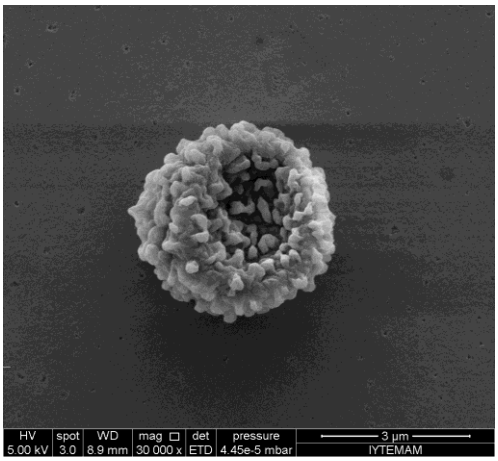


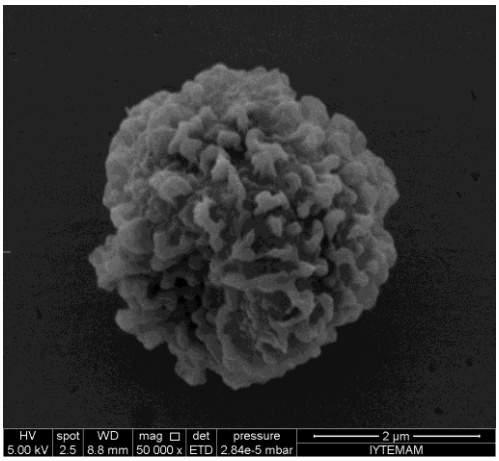
Figure D.1. Calibration curve for pectin amount.

APPENDIX E

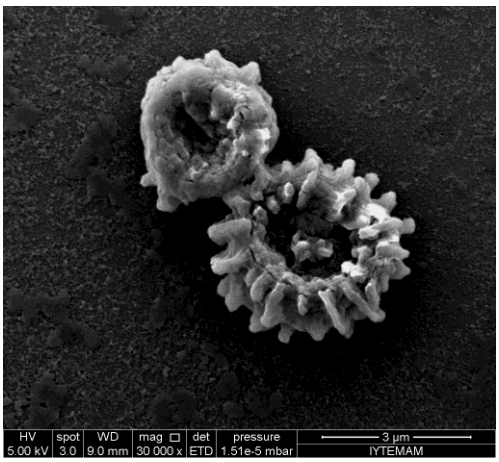
IMAGES TAKEN BY SCANNING ELECTRON MICROSCOPE



Aspergillus sojae ATCC 20225



Aspergillus sojae 20225 MT



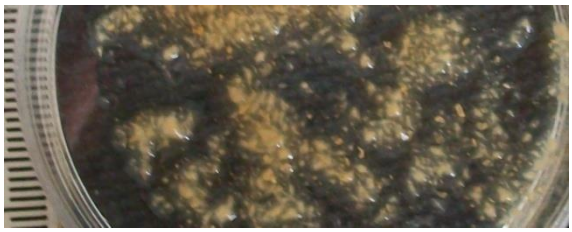
Aspergillus niger

Rhizopus oryzae ATCC 4858

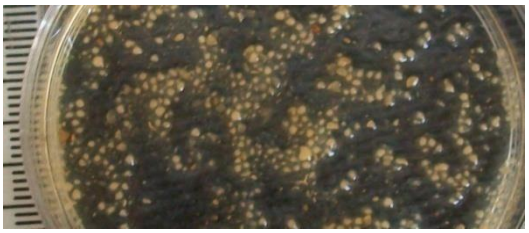
Figure E.1. Fungal spore images taken by scanning electron microscope (Philips XL 30S FEG) of *Aspergillus sojae* ATCC 20225, *Aspergillus sojae* MT, *Aspergillus niger*, and *Rhizopus oryzae* ATCC 4858.

APPENDIX F

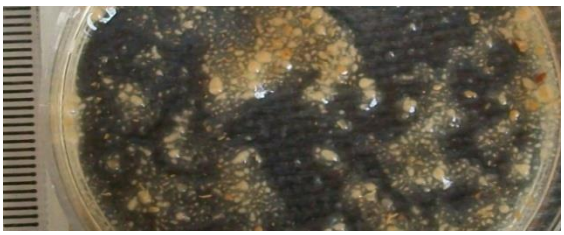
PELLET MORPHOLOGY OF DIFFERENT FUNGAL STRAINS



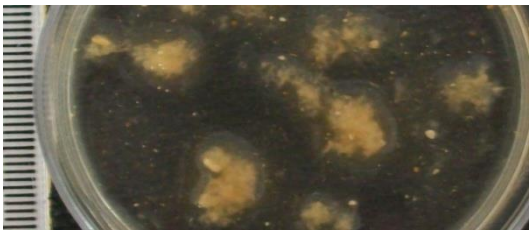
Aspergillus sojae ATCC 20225



Aspergillus sojae MT



Aspergillus niger



Rhizopus oryzae ATCC 4858

Figure F.1. Fungal pellet morphologies grown in orange peel medium, images taken at the third day of fermentation by digital photo camera (Eastman Kodak M320) of *Aspergillus sojae* ATCC 20225, *Aspergillus sojae* MT, *Aspergillus niger*, and *Rhizopus oryzae* ATCC 4858.

APPENDIX G

PELLET MORPHOLOGY OF *A. sojae* MT GROWN IN DEFINED MEDIUM WITH DIFFERENT SPORE CONCENTRATIONS

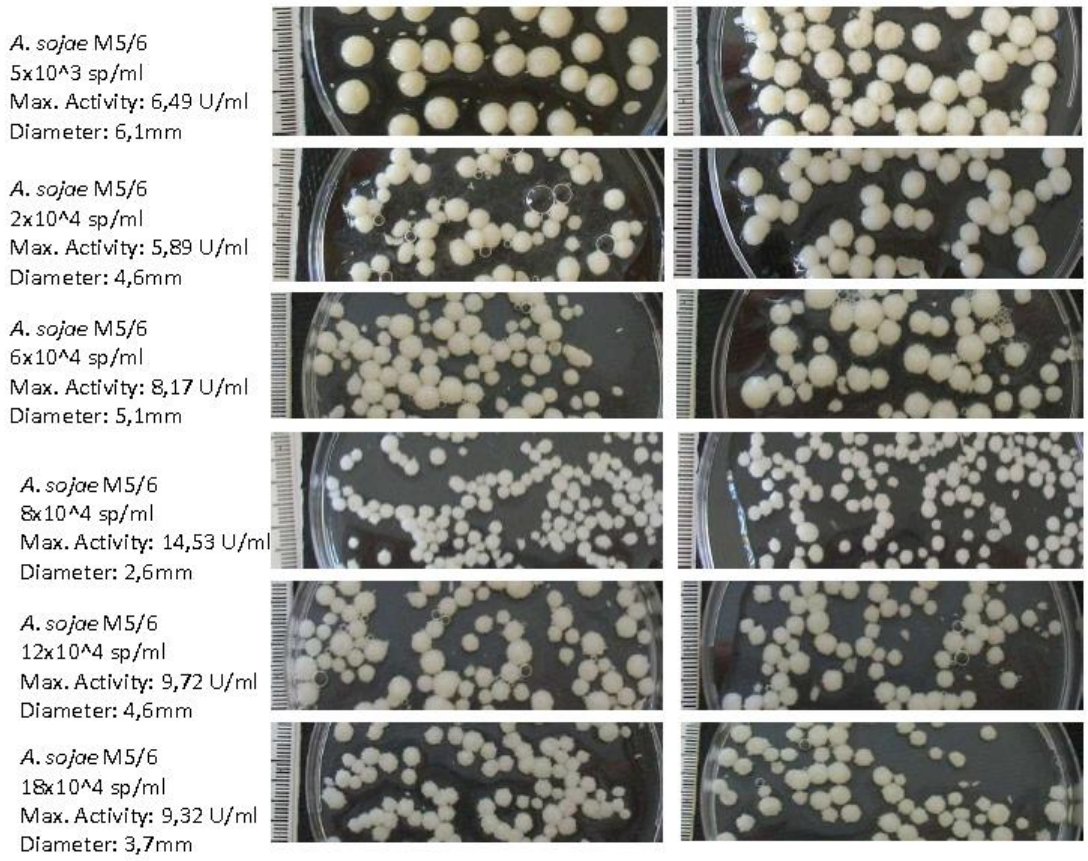


Figure G.1. Fungal pellet morphologies of *Aspergillus sojae* MT grown in glucose medium with different spore concentrations (5x103 to 18x104 spore/mL), images taken by digital photo camera (Eastman Kodak M320). The left and right columns show replicates.

APPENDIX H

PELLET MORPHOLOGIES OF *Aspergillus sojae* ATCC 20225 and *Aspergillus sojae* MT GROWN IN DIFFERENT MEDIUM COMPOSITIONS

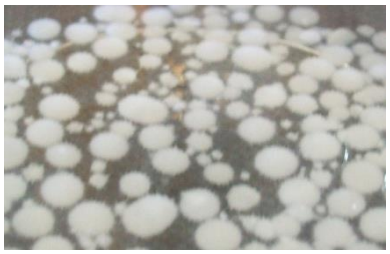
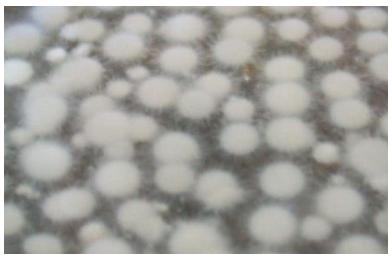
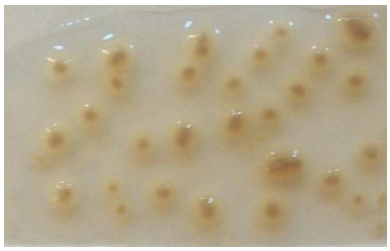
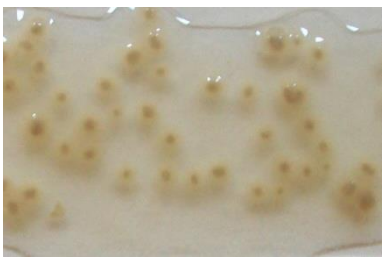
A sojae ATCC 20225	A sojae MT
Glucose Medium	
	
Pectin Medium	
	
Pectin and Glucose Medium	
	

Figure H.1. Pellet morphologies of *A. sojae* grown in different medium compositions, initial pH 5.0.

(cont. on next page)

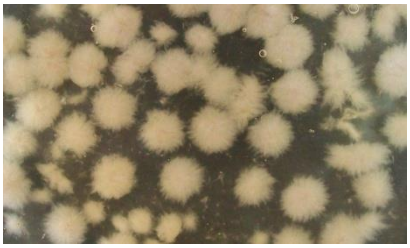
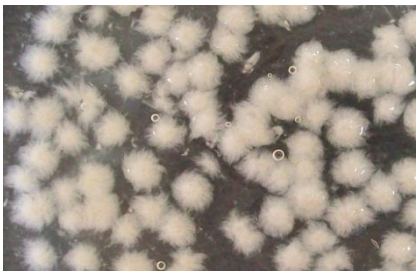
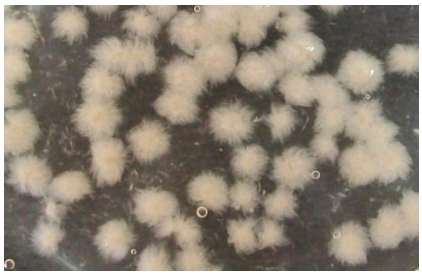
Pectin and Fructose Medium	
	
Pectin and Saccharose Medium	
	

Figure H.1. (cont.)

APPENDIX I

PELLET MORPHOLOGIES OF *A. sojae* MT GROWN IN BIOREACTORS AT DIFFERENT pH VALUES

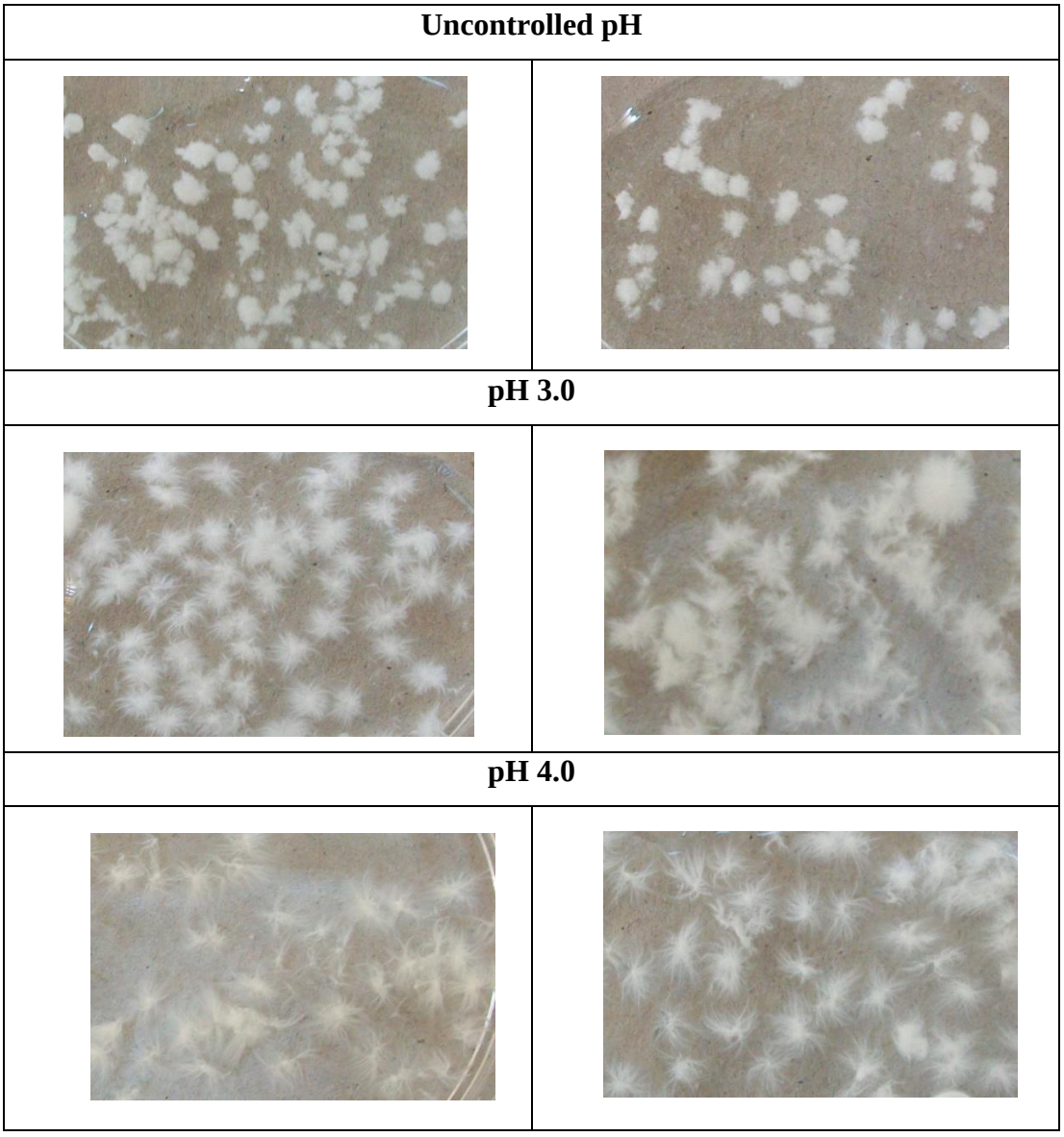


Figure I.1. Pellet morphologies of *A. sojae* grown in bioreactors with different pH values.