PRODUCTION OF PECTINASE FROM *Aspergillus sojae* BY SOLID-STATE FERMENTATION

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ABSTRACT

PRODUCTION OF PECTINASE FROM *Aspergillus sojae* BY SOLID-STATE FERMENTATION

Pectinases are one of the most important enzyme groups that have wide applications in the food industry, especially the fruit juice industry. The production of polygalacturonase from agro-industrial residues by solid-state fermentation leads to not only the acquisition of a value-added product but also the solution of pollution problem caused by the disposal of these residues. In the scope of this study, the production of polygalacturonase from novel mutant *Aspergillus sojae* strains by using solid-state fermentation techniques was investigated. The effects of orange peel concentration, incubation temperature and time, inoculation size and HCl concentration on polygalacturonase activity and spore production from *Aspergillus sojae* M3 were investigated with experimental design and statistical analysis. Various agro-industrial residues and *A. sojae* strains were screened for the production of polygalacturonase and, wheat bran and *Aspergillus sojae* M5/6 pair was selected as the substrate – strain pair. The optimum fermentation conditions for the flask scale production were found as 10^7 spore/g substrate inoculum size, 4 days, 37 °C, 62% initial moisture content, neutral pH, 150-250 μm particle size, 3 times/day agitation frequency and spore inoculum type. The maximum polygalacturonase activity (535.4 U/g substrate) obtained in this study was very competitive with the ones reported in the literature. In contrast to other studies in the literature, high polygalacturonase activity was achieved using wheat bran without the addition of any supplementary nutrients that could increase the cost of the production process. The production of PG from *Aspergillus sojae* M5/6 in the tray and bioreactor scales we also performed.

ÖZET

KATI-KÜLTÜR FERMENTASYONU İLE *Aspergillus sojae*'DEN PEKTİNAZ ÜRETİMİ

Pektinazlar, başta meyve suyu sanayii olmak üzere gıda sanayiinin birçok alanında geniş kullanımı olan en önemli enzim gruplarından biridir. Poligalakturonazların katı-kültür fermentasyonu yöntemiyle tarımsal-sınai atıklardan üretilmesi ile katma değeri olan bir ürünün elde edilmesinin yanı sıra, bu atıkların meydana getirdiği kirlilik problemine de bir çözüm bulunmuş olacaktır. Bu çalışmada, *Aspergillus sojae* mutant suşları kullanılarak katı-kültür fermentasyonu teknikleri ile poligalakturonaz enzimi üretimi üzerinde incelemeler yapılması amaçlanmıştır. Portakal kabuğu rendesi yüzdesi, inkübasyon sıcaklığı ve süresi, inokulasyon oranı ve HCl konsantrasyonu faktörlerinin *Aspergillus sojae* M3'den poligalakturonaz enzimi ve spor üretimi üzerine olan etkisi deneysel tasarım ve istatistiki analiz yardımı ile belirlenmiştir. Pek çok zirai-endüstriyel atık ve *Aspergillus sojae* suşu poligalakturonaz üretimi bakımından taranmış ve buğday kepeği- *Aspergillus sojae* M/6 ikilisi katı substrat-suş ikilisi olarak seçilmiştir. En yüksek poligalakturonaz aktivitesinin elde edilmesi amacıyla erlenlerde optimize edilen fermentasyon koşulları şu şekildedir: inokulasyon oranı 10^7 spor/g substrat, 4 gün inkübasyon süresi, 37 °C inkübasyon sıcaklığı, 62% nem oranı, nötr pH (su ile nemlendirme), 150-250 µm partikül boyutu, günde 3 kez çalkalama ve spor ile inokulasyon. Bu koşullarda elde edilen en yüksek poligalakturonaz aktivitesi (535.4 U/g substrat) literatürde bildirilen benzerleri ile rekabet edebilecek düzeydedir. Literatürde yer alan diğer çalışmalardan farklı olarak bu çalışmada buğday kepeği ile yüksek enzim aktivitesi başka ek besinler kullanılmadan elde edilmiştir. *Aspergillus sojae* M/6'dan poligalakturonaz üretimi tepsi ve biyoreaktör ölçeklerinde de incelenmiştir.

Dedicated to my parents

Meral and Atilla GENÇKAL

for their endless love and support

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CHAPTER 1

INTRODUCTION

Enzymes are key components of textile, ethanol and pharmaceutical industries, and food and beverage manufacturers. Being the largest users of enzymes on a worldwide basis, the total value of the food and beverage enzyme market is estimated to reach $320 million by 2013. As the report published by Freedonia Group, Cleveland, US (2009) enzyme demand is poised to grow at an overall rate of 6.9% each year through 2010. Among the food enzymes pectinases are the complex and diverse group of enzymes that degrade the pectic substances (Gummadi et al., 2007). Pectin and other pectic substances are complex polysaccharides playing an important role in the firmness of plant tissues. Pectinases (or pectinolytic enzymes) holding a share of 25% in the global sales of food enzymes are produced from microbiological sources. These enzymes have variety of applications in food industry such as in clarification of fruit juices, extraction of vegetable oils, curing of coffee and cocoa, refinement of vegetable fibers and in the manufacture of pectin-free starch (Singh et al., 1999; Jayani et al., 2005). Therefore it is of great importance to discover the new pectinase-producing microbial strains and optimize their enzyme production conditions in order to meet this increasing demand in both submerged and solid state fermentations. It is stated that submerged fermentation (SmF) is generally used for the production of industrially demanded enzymes, employing mostly genetically modified strains (Pandey et al., 2000). However, it is necessary to reduce the high production cost of SmF and find alternative methods. At this point, solid-state fermentation (SSF) is a method to be considered as an attractive alternative.

Solid-state fermentation (SSF) is defined as the growth of microbes without free flowing aqueous phase. The utilization of solid substrates is thought to be the oldest method used by human to make microorganisms work for them. As an example, bread making is one of the oldest arts known to man, and archaeological discoveries indicate that Egyptians were making bread using a fermentation process even in 2600 BC. In recent years, SSF has shown much promise in the development of several bioprocesses and products. Since, SSF aims at bringing the cultivated microorganism in tight contact

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with the insoluble substrate to achieve the highest nutrient concentration from the substrate for fermentation, fungi is the most competitive microflora for the bioconversion of solid substrates (Bhargav et al., 2008; Krishna, 2005). The production of industrially important enzymes is of interest since the mid 90's hence nearly all of the enzymes produced by any method can also be produced by solid-state fermentation such as xylanase, cellulase, lipase, pectinase, phytase etc. In Chapter 2, definition, a brief history, advantages and disadvantages of solid-state fermentation technique are discussed.

The industrially important enzymes can be produced by solid-state fermentation (SSF) techniques especially using the fungal metabolisms. Agro-industrial residues are the most popular substrates due to their low-cost and availability. However, there are some important factors that should be optimized in order to maintain microbial growth and enzyme synthesis. These are defined by Pandey et al. (1999) as; pretreatment of the substrate, particle size, water content/water activity, relative humidity, type and size of the inoculum, temperature of the fermenting matter, removal of metabolic heat, period of cultivation and maintenance of the uniformity. The aim of optimization of the process is to increase the productivity and yields by building up a base for a large-scale process. With this perspective, the selection of the type of substrate and formulation of the fermentation medium should be considered to support the growth of the chosen microorganism and synthesis of the target product. The conclusion on the type of microorganism and substrate should be followed by determination of the environmental factors that significantly affect the microbial growth and product formation (Raimbault, 1998). The selection of the optimum parameters should be done considering the measurement and controlling steps at the pilot and large scale processes. The agro-industrial residues used for the production of polygalacturonase were mentioned and some of the most important factors affecting the SSF were thoroughly reviewed in Chapter 2.

Wheat bran is one of the most popular agro-industrial residues preferred by many researchers to produce value-added metabolites by solid-state fermentation from various microorganisms. This popularity is due to its suitability to solid-state conditions with good water holding capacity, having adequate of porosity for gas exchange, being rich in minerals and the high carbon content that is convertible by microorganisms (Taşkın and Eltem, 2008). Wheat bran is not only a nutritive source for the microorganisms but it also serves as an anchorage for the colonization of the cells.

According to the statistics of United States Department of Agriculture (USDA), a total of 18,800,000 tones of wheat were produced and 18,100,000 tones of wheat were consumed in the period of 2010 – 2011 in Turkey (USDA, 2012). Since, about 15-20% (in weight) of wheat bran was reported to be discarded in the wheat flour production process (Dobrev et al., 2007), wheat bran can be accepted as a sustainable by-product for the microbial production of industrially important enzymes by solid-state fermentation. The wheat flour manufacturers often dispose wheat bran because the cost of transportation is more than its worth, and such waste also causes potential environmental concerns (Xie et al., 2008). Moreover, utilization of this by-product to produce a value-added enzyme will also help to solve the pollution problem of wheat flour manufacturers. The research studies focusing on the production of industrially important enzymes by SSF using the wheat bran as the solid substrate were detailed in Chapter 2.

Polygalacturonase (PG) –the target enzyme of this study– is one of the most important pectinolytic enzymes and is classified under depolymerases class of pectinases. The classification and mechanism of pectinolytic enzymes were well described and the definition and biochemical properties of exo-polygalacturonases produced from fungal and bacterial sources were reviewed in Chapter 3.

After brief information on the studies performed with the *Aspergillus sojae* for the production of various metabolites, the studies conducted on production of exo-polygalacturonase enzyme by solid-state fermentation were tabulated in detail in Chapter 3.

The optimized SSF processes were scaled up to produce metabolites or biomass in commercial scales. The types of the bioreactors used at laboratory, pilot or large scale were presented in four classes according to their design and operational properties. The drawbacks and key factors to be considered in the four groups of bioreactors were mentioned in Chapter 4.

In order to determine the potential of *A. sojae* ATCC 20235 (wild type) a study was carried out by Göğüş et al. (2006) using this strain in the production of the PG by submerged fermentation technique. In this previous study, it was proved that this organism has a good potential for PG synthesis with the desired pellet morphology (Göğüş et al., 2006). Additionally, a SSF study again with *A. sojae* ATCC 20235 was performed by Ustok et al. in 2007 using corncob, maize meal and crushed maize as substrates. In this previous study, inoculum size and incubation time factors were

optimized by using response surface methodology techniques. Ustok et al. (2007) have also concluded that *A. sojae* ATCC 20235 can be a good candidate for the production of polygalacturonase. These production studies were followed by the partial purification and biochemical characterization of the polygalacturonase produced by submerged fermentation from *A. sojae* ATCC 20235 (Dogan and Tari, 2008). These previous studies constituted a basis for the idea of screening various mutant strains of *Aspergillus sojae* and agro-industrial residues for the production of polygalacturonase with better enzymatic activity by SSF.

With the abovementioned perspective, a preliminary study was performed to optimize the conditions of SSF process for the production of PG. *Aspergillus sojae* M3 was used as the producer strain and orange peel – wheat bran pair was used as the solid substrate. Factors such as the concentration of orange peel in the wheat bran, concentration of HCl, inoculum size, incubation temperature and time were screened and then optimized with the help of statistical tools. Additionally, the constructed model was numerically optimized and validated by offering the producers various criteria about the PG and spore production. The screening, optimization and validation results were presented in Chapter 6.

In Chapter 7, many *Aspergillus sojae* wild type mutant strains were screened for their PG production potentials. After the selection of the best strain, various agro-industrial residues of the food industry of Turkey were screened for the same purpose. Wheat bran was chosen as the best PG producing substrate. There were many studies in the literature using wheat bran for the production of industrially important enzymes by SSF, however all of them achieved to produce the enzymes with the help of nutritive supplementary liquids added to the residues. One of the originality of this current study is the production of PG having reasonably high activities with the use of only wheat bran and distilled water.

After the selection of *Aspergillus sojae* mutant strain and substrate, the potential of the process was carried out to the next level. The most important factors inoculum size, incubation period, incubation temperature, initial moisture content, moisturizing agent (pH), particle size, agitation and type of inoculum were optimized with the one-at-a-time method. Many parameters such as PG activity, final pH, spore count, specific PG activity, consumption of carbohydrate etc. were monitored to be able to state, present and discuss the metabolic activities of the fermentation process and decide on the

optimum parameters. The results of this detailed parametric study were presented and discussed in Chapter 8.

The biochemical property of an enzyme is very important for the decision of its application area. Therefore pH and temperature stability and optimum activities of the crude enzyme produced under the optimized conditions were expressed in Chapter 9. The behavior of the enzyme with the presence of various compounds and metal ions was also represented. Then, the thermal inactivation kinetics and thermodynamic parameters of the inactivation were revealed. The suitability of the biochemical properties of the produced PG enzyme especially for the fruit juice industry was also discussed in this chapter.

In Chapter 10, the PG production process was scaled up to tray type of SSF. In this part of the study, it was observed that the PG activity showed differences according to the thickness of the substrate bed. As best of our knowledge, there is not any report on the interaction of substrate bed thickness and relative humidity of the environment for the production of metabolites from microbial sources by SSF. Therefore, with the intent of the investigation of this possible interaction, an experimental design was set up and its results were analyzed with the statistical tools. According to these experimental results and statistical analysis, the interaction between the abovementioned factors was determined. Additionally, a kinetic study was performed to be able to monitor the course of the SSF conducted in trays under the partially optimized conditions.

The last part of the study deals with the seven batches which were performed to investigate the PG production by solid-state fermentation in a horizontal drum bioreactor.

Overall objective of this study was to reveal the potential of the SSF process for production of PG from *Aspergillus sojae* mutant strains. For this purpose, the most abundant agro-industrial residues of the food industry of Turkey were screened. In order to improve the PG production by SSF process, the most important factors were optimized. Some of the biochemical properties of the produced enzyme were detremined to present its suitable application areas. A scale-up approach was studied with the aim of transferring the optimized PG production process from the flask scale to the laboratory scale.

CHAPTER 2

SOLID-STATE FERMENTATION

Solid-state fermentation (SSF) is a very ancient technology employed by the eastern countries and has attracted the attention of western countries in recent decades. Due to the engineering and environmental aspects, solid-state fermentation has shown much promise in the development of several products and bioprocesses. The main reasons of interest for the solid-state fermentation are the high productivity, improved product stability and low cost of production. SSF is still in competence with the submerged fermentation (SmF) technique hence, the SSF research activities mostly focused on the bioreactor design, operation of the process and scale-up criteria in the last decade (Mitchell et al., 2000). SSF is also an alternative technique to SmF for the microbial enzyme production. This chapter deals with the definition and brief history of the SSF technique, as well as its advantages and disadvantage. Additionally, a detailed theory and literature survey on some of the important factors affecting the SSF is presented.

2.1. Solid-State Fermentation

Solid-state fermentation (SSF) was defined by many researchers. These definitions all agree on that this process involves solids in the absence or near absence of free flowing aqueous phase (Bhargav et al., 2008; Pandey, 2003; Lonsane et al., 1992). In the solid-state fermentation concept, the solid substrate should possess enough moisture in the absorbed or complex form to support microbial growth and metabolic activities (Raghavarao et al., 2003; Singhania et al., 2009). It was expressed that the use of solid substrates for the growth of microorganisms dates back to the ancient times. The oldest known SSF technique was reported to be bread making by Egyptians in 2600 BC (Krishna, 2005). Other ancient SSF applications can be summarized as cheese making by *Penicillium roqueforti*, Oriental foods and beverages such as tempeh, sorghum, miso, soy sauce, saké and koji process (Krishna, 2005). Koji is an important traditional enzyme preparation produced by the fermentation of steamed rice or other

cereals by *Aspergillus oryzae* which is still used as a starter culture in the soy sauce industry and in fermentation of miso and brewing of sake (Couto and Sanroman, 2006; Ooijkaas et al., 2000). Today, the traditional koji production is accepted as a model process for understanding the principle of the SSF technique.

SSF was classified into two by Krishna (2005) depending on the type of the used microorganism: natural SSF and pure culture SSF. The industrial processes mostly involve pure cultures for the production of targeted product, on the other hand mixed cultures are preferred in the bioconversion processes of agro-industrial residues (composting, ensiling).

In recent years, SSF has shown much promise in the development of several bioprocesses and products. This process has its own advantages over submerged fermentation (SmF) technique as follows (Krishna, 2005; Perez-Guerra et al., 2003):

- The volumetric productivity and concentration of product will be higher compared to SmF.
- Use of raw materials and agro-industrial residues solves the pollution problem of many manufacturers.
- The lower water activity of the fermentation medium reduces the contamination risk especially by bacteria and yeast.
- Capital cost, energy expenditure and cost of downstream process are lower than SmF.
- The media for the microbial growth is usually simple since most of the times it naturally provides the necessary nutrients.
- In some cases the biochemical properties of the SSF product may show difference from the SmF one.
- SSF may need a smaller reactor when compared to SmF to hold the same amount of substrate.
- It is possible that SSF may favor or induce the production of a specific product that may not be produced or poorly produced by SmF.

On the other hand use of SSF may bring some disadvantages such as (Krishna, 2005; Perez-Guerra et al., 2003):

- The agitation of the substrate to obtain homogeneity is difficult.
- Limits the use of organisms that require high moisture levels.

- The substrate may need pretreatment such as size reduction, cooking, hydrolysis that will bring extra cost to the process.

- The determination of biomass during the process is very difficult.

- The in-place monitoring of the process parameters such as pH, oxygen concentration, moisture content, temperature of the bed is difficult.

- Generally, high volumes of inoculum are required compared to SmF.

- Limited knowledge on the scientific and engineering aspects of the process.

- The generated metabolic heat during the process should be removed.

- Aeration will be difficult due to the high concentration of the solid.

- Cultivation times may be longer due to the longer lag times of the inoculated spores.

The main principle of the SSF is to bring the cultivated microorganism in tight contact with the insoluble substrate that simulates the living conditions of many higher filamentous fungi. Typically, the fungi grow on solid substrates such as pieces of wood, seeds, roots and dried parts of the animals in the nature. The hyphal mode of growth enables the fungus to penetrate into the solid substrate that results in colonization of the solid substrate and utilization of the available nutrients. Additionally, good tolerance to low water activity and high osmotic pressure conditions make the fungi the most competitive microflora for the bioconversion of solid substrates (Bhargav et al., 2008; Krishna, 2005; Perez-Guerra et al., 2003; Hölker and Lenz, 2005).

2.2. Substrate

Since, solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials without the presence of free liquid, substrate must have enough moisture to support growth and metabolism of microorganisms. Therefore, selection of substrate is critical for SSF.

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells. The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate.

However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with these. It has also been a practice to pre-treat (chemically or mechanically) some of the substrates before using in SSF processes (e.g. ligno-cellulose), thereby making them more easily accessible for microbial growth (Pandey et al., 1999).

2.2.1. Agro-Industrial Residues

For the SSF processes, agro-industrial residues generally considered as the best substrates for the production of enzymes. For this purpose many agro-industrial residues were used as substrates for polygalacturonase production that are summarized in Table 2.1. Application of these agro-industrial residues in enzyme production processes also solves pollution problems caused by their disposals.

Table 2.1. Some agro-industrial by-products used to produce polygalacturonase by SSF[a]

Microorganism	Substrate	PG activity[b] IU/g
Aspergillus carbonarius	Wheat bran	480.0
Aspergillus carbonarius	Wheat bran	400.0
Aspergillus niger	Wheat bran and glucose	370.0
Aspergillus niger	Pectin	357.5[c]
Aspergillus niger	Wheat and soy bran	152.5[c]
Fusarium moniliforme	Wheat bran and orange pulp	81.0
Penicillium viridicatum	Orange bagasse and wheat bran	71.2
Thermoascus auriantacus	Orange bagasse or wheat bran	43.0
Aspergillus niger	Sugar cane bagasse and pectin	38.0
Aspergillus niger	Wheat bran and dextrose	36.3
Lentinus edodes	Strawberry pomace	29.4
Aspergillus niger	Wheat bran and pectin	27.5
Moniliella sp.	Sugar cane bagasse and wheat bran	26.0
Aspergillus awamori	Grape pomace	25.0
Chaetomium sp.	Palm oil mill fibre	18.9
Penicillium sp.	Sugar cane bagasse and wheat bran	12.0
Aspergillus awamori	Wheat grains	9.6
Aspergillus niger	Sugar cane bagasse and pectin	3.8[c]

[a] Summarized by Favela – Torres et al., 2006.
[b] International units expressed as the amount of enzyme required for liberating one μmol of galacturonic acid per minute
[c] Estimated from the activity value reported (140 U per 5 grams of wheat bran)

2.2.2. Wheat Bran as a SSF Substrate

Wheat bran is the outer layer of the wheat grain that serves to protect the embryo (Figure 2.1). Bran is separated from the wheat kernel during the flour or pasta production processes in order to obtain the endosperm. Bran fraction corresponds to approximately 11% of the total product in dry milling (Dobrev et al., 2007; Xie et al., 2008).
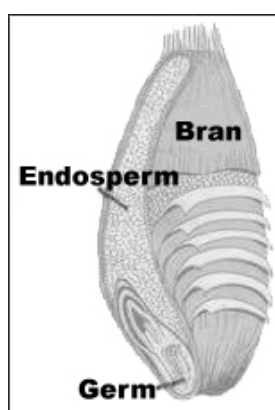
Figure 2.1. Structure of a wheat grain
(Source: AICR, 2012)

The physicochemical composition of wheat bran was stated in many research studies. These data are summarized in Table 2.2.

Table 2.2. Physicochemical properties of wheat bran stated in the literature

Composition	Dobrev et al., 2007	Shankaranad and Lonsane, 1994	Silveria and Badiale-Furlong, 2009
Moisture (%)	12.10	13.2	9.4
Fat (%)	2.59	4.56	5.2
Protein (%)	14.87	11.94	13.8
Total carbohydrate (%)	79.12	62.89	60.1
Starch	24.69	11.8	-
Beta-glucans	13.22	-	-
Arabinoxylans	17.30	-	-
Ash (%)	3.98	5.12	6.3
Fibers (%)	-	-	5.2
Minerals/trace metals	-	-	-
Fe^{2+} (ppm)	-	121.63	-
Cu^{2+} (ppm)	-	16.99	-
Zn^{2+} (ppm)	-	59.74	-
Mn^{2+} (ppm)	-	124.97	-
Mg^{2+} (g/kg)	-	3.24	-
PO_4^{-1} (g/kg)	-	6.98	-

Wheat bran is the most widely used substrate in the SSF experiments. Some of the bioproducts produced using wheat bran with SSF are; xylanase (Antoine et al.,

2010), polygalacturonase (Freitas et al., 2006; Fontana et al., 2005), α-amylase (Balkan and Ertan, 2010), α-galactosidase (Liu et al., 2007), fructosyl transferase (Sangeetha et al., 2004), phytase (Papagianni et al., 2001), nigerloxin (Chakrahadar et al., 2009), L-glutaminase (Kashyap et al., 2002) and gibberellic acid (Corona et al., 2005).

Balkan and Ertan (2007) focused on the optimization of SSF conditions for the production of α-amylase from *Penicillium chrysogenum* using some agricultural by-products. They optimized the initial moisture content, particle size and inoculum concentration parameters for corncob leaf, rye straw, wheat straw and wheat bran. Under optimized conditions wheat bran showed the maximum activity which was 17.5, 30.6 and 28.1 times higher than corncob leaf, rye straw, wheat straw, respectively.

In another study of these researchers, they tested the raw starch digesting α-amylase production potential of wheat bran, rice husks, and sunflower oil meal under solid-state fermentation conditions using *Penicillium brevicompactum*. This time they optimized the conditions of initial moisture content, type of moistening agent (or pH), incubation period, inoculum concentration and incubation temperature for each of the substrate. Similarly, they concluded that wheat bran was the most suitable substrate for the production of α-amylase with high enzymatic activity (Balkan and Ertan, 2010).

Couri et al. (2000) screened the synthesis of a mixture of polygalacturonase, cellulase, xylanase and protease enzymes using an *Aspergillus niger* strain grown on agro-industrial residues. In the preliminary part of their study they tested wheat bran, mango peel, banana peel and cassava bran and they selected the first two substrates as potential bioproducers of abovementioned enzymes. They saw that standard conditions show difference from each other for each of the enzymes. However, the mixture of polygalacturonase, xylanase and protease with highest activities were obtained with wheat bran with a 24 hour-fermentation.

Wheat bran was used as substrate in various solid-state fermentation investigations that aimed to produce polygalacturonase enzyme. For this purpose, Fontana et al. (2005) performed a SSF of an *Aspergillus niger* strain in a media containing wheat bran, salts, and different citric pectin and/or glucose concentrations that focused on the kinetics of fungal growth and polygalacturonase synthesis. They concluded that growth of *Aspergillus niger* and production of polygalacturonase were associated processes. Pectin addition to the media up to 16% enhanced both the growth and PG production. Contrarily, both of them were hindered between the pectin

concentrations of 20 − 30% (w/w). They also observed that addition of glucose over 10% (w/w) decreased the maximum activities of exo- and endo-polygalacturonases drastically.

Taragano and Pilosof (1999) applied the Doehlert design to optimize water activity (0.931 to 0.981), pH (5 to 7) and fermentation time (2 to 8 days) conditions for *Aspergillus niger* 148 pectinolytic activities polygalacturonase (PG; EC 3.2.1.15), pectinesterase (PE; EC 3.1.1.11), and pectin lyase (PL; EC 4.2.2.10) production in solid-state (SSF) and submerged (SmF) fermentation. They employed wheat bran supplemented with 1% citrus pectin in SSF and an aqueous extract of wheat bran (10% w/v) supplemented with 0.5% w/v citrus pectin in SmF and needed amounts of PEG 200, NaOH and HCl to adjust the water activity and pH of the medium. The fermentation type had an important effect on the enzyme activities such that SSF gave 5-fold, 3-fold and 30% higher enzymatic activities of polygalacturonase, pectin lyase and pectinesterase, respectively than the SmF technique. It was found that water activity and incubation time had important effects in the SSF process for the production of polygalacturonase, with the positive effect of time but negative effect of water activity. Additionally, interaction effects were significant between pH and a_w and between pH and time.

2.2.3. Inert Supports

Natural substrates have a major disadvantage, as carbon source constitutes part of their structure. During microbial growth, the solid medium is disintegrated; causing changes in the geometric and physical characteristics of the medium, and consequently heat and mass transfer can be reduced. This can be overcome by use of an inert support with a more or less constant physical structure throughout the process, enabling improved control of heat and mass transfer. Another advantage of using an inert support is its less complicated product recovery. Moreover, it allows the design of adequate production media, and mass balances for more advanced process modeling and process control are more easily established (Krishna, 2005). In any case, the use of inert supports presents economical disadvantages (Perez-Guerra et al., 2003).

2.3. Some Important Factors Affecting the SSF

2.3.1. Effect of Inoculum Size

Krishna (2005) and Pandey (2003) reported the inoculum size being one of the important parameters that should be considered in the SSF processes. It has been shown by many researchers in the literature that the size (or concentration) of inoculum may influence the yield of enzymes of fungal strains. Kumar et al. (2010) optimized the important conditions of SSF for the production of polygalacturonase from mango peels by *Fusarium moniliforme*. In that study they observed that inoculum concentration is an influential factor having a 14.07% contribution among all factors. They also concluded that the increase in inoculum level has resulted in higher polygalacturonase expression up to 12% (w/v).

The inoculum size was optimized by Francis et al. (2002) in which they synthesized alpha-amylase from *Aspergillus oryzae* using spent brewing grains as the solid substrate. They investigated the effect of inoculum size between the ranges of 10^3 to 10^8 spores/g dry substrate (gds). The maximum amylase activity was 6870 U/gds at the 10^7 spores/gds inoculum size level. Below and above of this optimum level, the enzyme activity reduced for 4% and 8% at the sizes of 10^6 and 10^8 spores/gds inoculum, respectively (Figure 2.2).
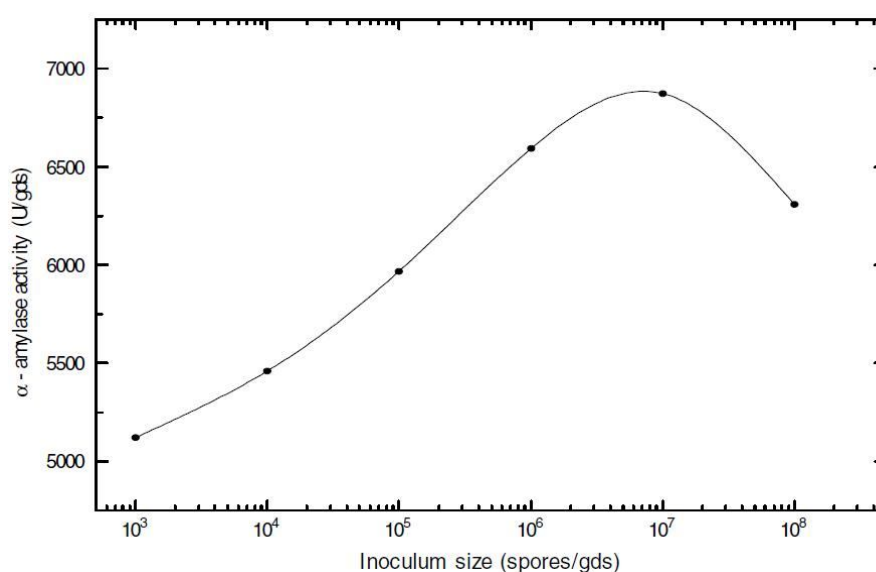
Figure 2.2. Variation in α-amylase production with inoculum size
(Source: Francis et al., 2002)

The important fact in the inoculum size is that there should be a balance between the amount of spores and amount of available oxygen and nutrients i.e. solid substrate and moistening agent to maintain their metabolic activities resulting in enzyme production. The optimum inoculum size in order to produce enzymes may be different for SmF and SSF using the same substrate (Acuna-Arguellez et al., 1995; Patil and Dayanand, 2006a). The inoculum size factor is generally expressed as spores/ml (Kammoun et al., 2008) in SmF, whereas it is expressed as spore/grams of substrate or spore/grams of dry substrate (Diaz et al., 2011) in SSF processes. Additionally, Chen et al. (2011) concluded that inoculum size (content %, v/w) is an effective factor that enhances the biomass formation in SSF process inoculated with *Trichoderma harzianum*.

2.3.2. Effect of Incubation Time

A kinetic study on the SSF process should be performed in order to monitor the targets of the operation. In other words, homogenous and representative samples should be taken periodically and the quantification of the product metabolite or biomass or spores should be made during the process. The constructed time course plots will reveal the development of various parameters such as fungal growth, enzyme production, nutrient consumption, oxygen consumption etc. The incubation should be terminated as soon as the aim of the process is fulfilled. It is important to optimize the incubation period especially in the enzyme production processes, since less time may lead to synthesis of the enzyme with low enzymatic activity and contrary to this; excessive incubation time increases the risk of contamination. Besides, the produced enzyme will be denatured with the generation of metabolic heat or inhibited due to the accumulation of inhibitory secondary metabolites or proteases secreted or due to the shift of pH of the medium at the later stages of the fungal growth (Blandino et al., 2002; Ramachandran et al., 2004; Pal and Khanum, 2010). Though, longer incubation time brings extra cost to the producer and reduces the productivity of the process.

Martin et al. (2004) observed that maximum polygalacturonase activity was achieved with *Moniliella* sp SB9 at 8 days of incubation, whereas incubation for 4 days was sufficient for *Penicillium* sp EGC5, both using a mixture of orange bagasse, sugar cane bagasse and wheat bran (1:1:1) as the solid substrate (Figure 2.3).
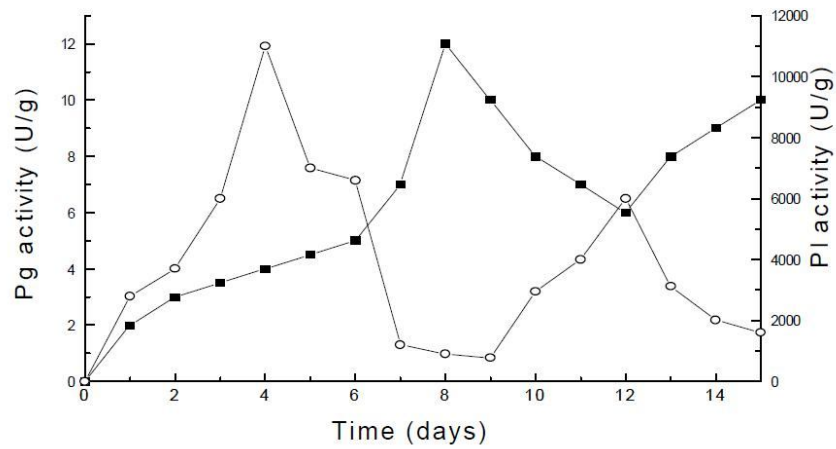
(a)
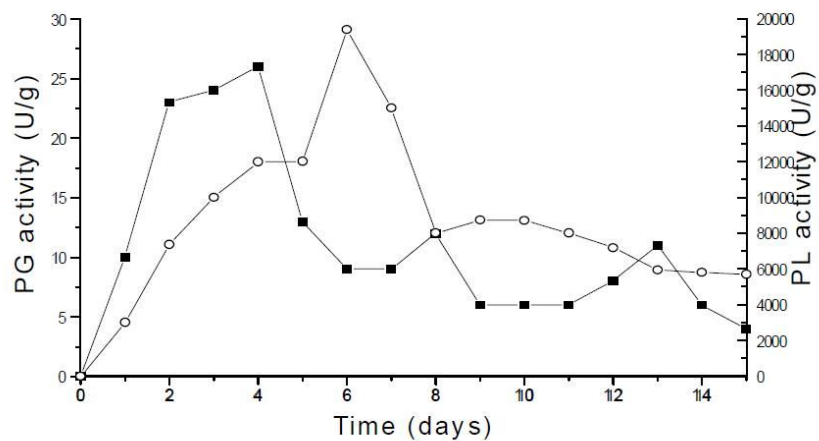
(b)

Figure 2.3. Pectinase production by (a) *Moniliella* sp SB9 and (b) *Penicillium* sp EGC5 on orange bagasse, sugar cane bagasse and wheat bran mixture (1:1:1). [full symbol]= polygalacturonase; [open symbol]= pectin lyase (Source: Martin et al., 2004)

On the other hand, the incubation time required to synthesize the same type of enzyme with SmF or SSF will be different from each other. Alazard and Raimbault (1981) compared the amylolytic enzyme production properties of an *Aspergillus niger* strain by SmF or SSF. They displayed that the maximum amylase activity was obtained at the 60th hour of the cultivation in SSF; however it was obtained at the 40th hour with very similar fermentation media in SmF.

The maximum incubation time may also show difference with the type of the solid substrate. In a study of Silva et al. (2002), they found out that the maximal production of polygalacturonase was achieved after 4, 6, 8 days when the *Penicillium viridicatum* strain was grown on wheat bran, orange bagasse and the mixture of sugar cane bagasse and mango peel, respectively.

2.3.3. Effect of Incubation Temperature

One of the most important environmental factors affecting the development of biological processes is temperature which may lead to protein denaturation, enzymatic inhibition, promotion, or inhibition on the production of a particular metabolite, cells death etc. (Rodriguez-Leon et al., 2008). Today, it is well known that both fungal growth and metabolite production is sensitive to this factor in the solid-state fermentation processes (Smits et al., 1998). Therefore, in order to support the growth of fungi and yield the targeted enzyme from the fermentation medium, the temperature of incubation should be optimized. However, it should be kept in mind that the optimum temperature required for the growth and the product formation could be different from each other. Nevertheless, the optimum temperature of these two should be compatible, e.g. the product formation temperature should be in the range of 20 to 55 °C, where a mesophile fungus is involved in the bioprocess (Bhargav et al., 2008).

The critical issue in the solid-state fermentation is the control of temperature and removal of excess heat generated during the process. Heat is generated due to the metabolic activities of the microorganisms and accumulated in the medium as a result of low thermal conductivity of the substrate or poor aeration/cooling conditions throughout the fermentation (Raimbault, 1998). In case of poor heat removal and occurrence of thermal gradients, the substrate may shrink by losing the moisture in its structure which will result in reduced porosity, poorer heat transfer, decrease in the rate of oxygen transfer through the inner substrate layers and in the solubility of the nutrients. Therefore incubation temperature is a critical parameter that should be optimized and considered well in the scale-up strategies (Raghavarao et al., 2003).

The conventional way of temperature control in SSF systems is the forced aeration that has multiple functions in the process such as supplying oxygen for the growth of the fungus and dissipating the generated heat. However, aeration rate should

be adjusted to a level where microorganism growth is also favored. Otherwise, high aeration rates may reduce the available water of the substrate and adversely affect the fungal growth. Evaporative cooling technique combined with water-saturated air can overcome this problem. The suitable application of mixing to the fermentation medium may also help the dissipation of heat accumulation (Perez-Guerra et al., 2003).

Santos et al. (2004) have studied on the potential of the thermal denaturation of the enzymes with a well-mixed packed-bed solid-state bioreactor by using the protease of *Penicillium fellutanum* as a model system. The mathematical model developed by these researchers to explore the difficulties that would be faced at large scale had shown that even at the high rates of aeration, up to 85% of the enzymes produced by the microorganism can be denatured at the end of the fermentation. This result points out the importance of temperature control during the SSF processes especially in the production of thermolabile bioproducts such as enzymes.

In another study, Lu et al. (2003) examined the effects of water activity and temperature on the xylanase biosynthesis from *Aspergillus sulphureus*. They found out that xylanase synthesis had an optimum temperature between 30 – 33 °C and dropped drastically out of this range in the shake flask part of their study (Figure 2.4a). In the pilot scale part of their study, they observed that lot of heat was generated during the process so that they turned over the medium and ventilated the culture room to remove the metabolic heat which also resulted in the reduction of the water activity of the substrate (Figure 2.4a, b). They achieved to balance the temperature and water activity by increasing the air humidity. These efforts were able to increase the activity of the enzyme by 1.5 fold when compared to natural SSF process.
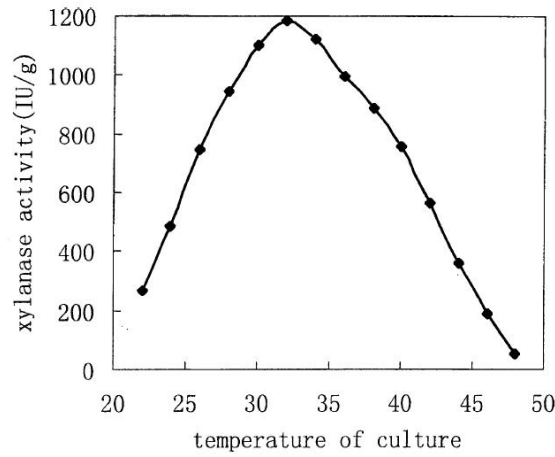
(a)
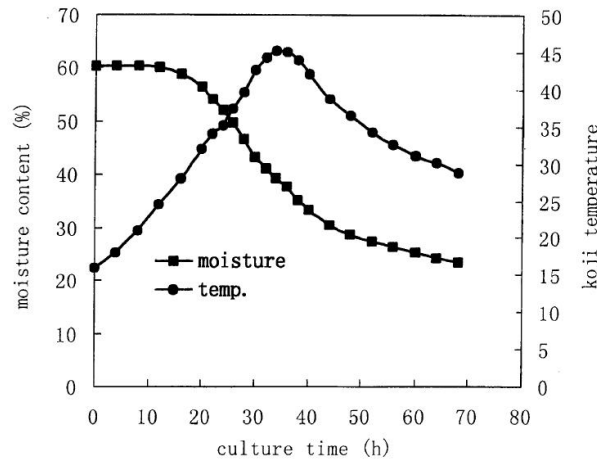
(b)
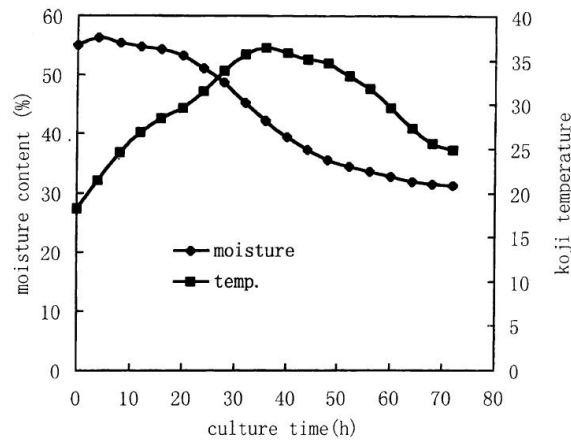
(c)

Figure 2.4. (a) The effect of temperature on the xylanase production carried out in 500-ml flasks, (b) Changes of moisture content and temperature of the medium with fermentation time carried out on pilot-scale SSF naturally, (c) Changes of moisture content and temperature of the medium with fermentation time (carried out on pilot-scale SSF adjusting koji temperature and air humidity in culture room) (Source: Lu et al., 2003)

2.3.4. Effect of Moisture Content and Water Activity

Water exists in the solid-state fermentation systems (i) in a complexed form within the solid matrix, (ii) a thin layer either absorbed to the surface of the particles or (iii) less tightly bound within the capillary regions of the solid (Raimbault, 1998). The other form of the water -free water- is mentioned when the water saturation capacity of the solid substrate is exceeded.

Water has a very vital role in the SSF systems, since it is involved in the critical metabolic activities such as (i) biomass development and metabolic reactions, (ii) enzymatic activities, and (iii) nutrient, extra-cellular metabolite and gas transport for the growth of microorganism or metabolite production (Bellon-Maurel et al., 2003).

The optimum moisture content required for the fungal growth or enzyme production may show difference depending on the type of the substrate. Moisture content below the optimum level may lead to low nutrient diffusion, microbial growth, enzyme stability, substrate swelling and sporulation, whereas above this level the problems will be particle agglomeration, gas transfer limitation and competition with bacteria. In other words, optimum moisture content should be set, where the essentials of the SSF such as fungal growth, metabolite production and physicochemical properties of the substrate meet (Krishna, 2005).

The moisture content of the solid substrate may show variation throughout the SSF process due to the evaporation or consumption of water for the metabolic activities. Therefore, one needs to add water, humidifiers or water-saturated air flow as the fermentation proceeds (Perez-Guerra et al., 2003).

The moisture content required for the fungal growth or secretion of metabolites generally varies between 40 – 80%, yet it heavily depends on the nature of the substrate. For example for the production of pectinases, various strains of *Aspergillus niger* need an optimum moisture content of 65% with the deseeded sunflower head (Patil and Dayanand, 2006a), 70% with wheat bran (Freitas et al., 2006) and 50% with cashew apple dry bagasse (Alcantara et al., 2010). This situation introduces the relationship between the water holding capacity and optimum moisture content (%) of the substrate. Additionally, optimum moisture content determined for the microbial growth and target metabolite secretion can be different from each other.

It is generally accepted that the water requirement of the microorganism is better defined in terms of water activity rather than the water (or moisture) content of the solid substrate (Perez-Guerra et al., 2003; Raimbault, 1998; Bhargav et al., 2008). Water activity (a_w) is a thermodynamic parameter defined as a measure of the availability of water for biological functions and relates to water present in a substrate in free form (Ray, 2004). This parameter is defined by the ratio of the water vapor pressure of substrate to the vapor pressure of pure water at the same temperature (Jay, 2000).

$$a_w = p/p_o \tag{2.1}$$

where p is the vapor pressure of the solution (water in the substrate) and p_o is the vapor pressure of the solvent (pure water)

Raimbault (1998) states that a_w is related to the condensed phase of absorbed water and this concept is related to relative humidity (RH) in the following way:

$$RH = 100 \text{ x } a_w \tag{2.2}$$

Each microbial group has characteristic range of water activity including an optimum value. Ray (2004) defines the minimum a_w values for the growth of these microorganisms as: most molds, 0.8, with xerophilic molds as low as 0.6; most yeasts, 0.85, with osmophilic yeasts, 0.6 to 0.7; most Gram-positive bacteria, 0.90; and Gram-negative bacteria, 0.93. Thus, bacteria require higher water activity values than fungi, which create a valuable advantage for fungi to compete with bacteria in the SSF processes. Water activity is also highly dependent upon the water binding properties and water holding capacity of the substrate. The water absorption capacity is defined as the amount of water (ml) that can be added to certain amount of dry substrate without the appearance of free liquid (Camilios-Neto et al., 2011).

It was observed by many researchers that water activity significantly affects the synthesis of enzymes and growth of microorganisms. Grajek and Gervais (1987) studied the influence of water activity on the biosynthesis of polygalacturonase, D-xylanase and β-glucosidase in the solid culture system of a *Trichoderma viride* strain. They found that water activity and the type of a_w depressor strongly affected the production of these enzymes using sugar beet pulp as the solid substrate. The polygalacturonase and D-xylanase production were maximized at the water activity level of 0.995, whereas it was

between 0.96 - 0.98 for β-glucosidase formation. Acuna-Arguellez et al. (1994) found that *Aspergillus niger* CH4 produced maximum exo-pectinase at 0.98 a_w, however when ethylene glycol was used as the water activity depressor exo-pectinase activity reduced below the a_w values of 0.97 (Figure 2.5). Similarly, Battaglino et al. (1991) obtained the maximum protease activity between the a_w values of 0.982 - 0.986 with the mixture of rice hulls and rice bran by the cultivation of *Aspergillus oryzae*. Additionally, Pandey et al. (1994) clearly observed that both glucoamylase and biomass production of *Aspergillus niger* was correlated with the initial water activity of the substrate.
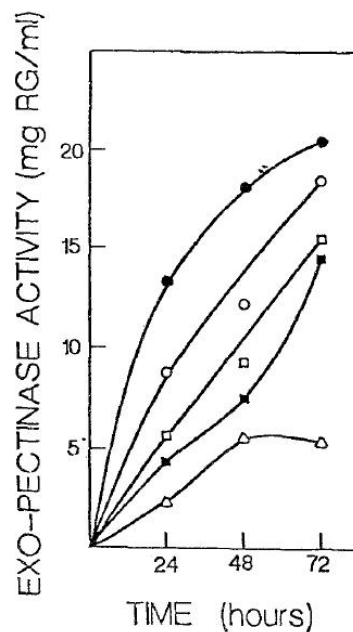
Figure 2.5. Exo-pectinase production by *Aspergillus niger* CH4 in SSF at different values of water activity adjusted with ethylene glycol. (●) 0.98, (○) 0.97; (□) 0.94; (■) 0.92 and (Δ) 0.90 (Source: Acuna-Arguellez et al., 1994)

Since, water activity and moisture content are both very critical factors, these parameters should be monitored. The on-line water activity measurement techniques in the pilot or large scale processes mostly depend on control of the relative humidity of the incubation cell (Bellon-Maurel et al., 2003).

2.3.5. Effect of the Initial pH of the Medium on SSF

The initial pH of the SSF medium may be adjusted via the addition of weak acids or bases, buffers or moistening agents containing mineral salts. However, pH may

shift throughout the process with respect to the metabolic activities of the microorganisms. This alteration may be due to the consumption of the substrate such as ammonium salt consumption may decrease pH, on the contrary hydrolysis of urea in the medium may result in alkali pHs (Bellon-Maurel et al., 2003). Other obvious reason of pH reduction will be the release of organic acids such as citric, acetic or lactic secreted by the microorganisms and vice versa the assimilation of the organic acids present in the prepared media will increase the pH. The trend in the pH variation is mostly dependent on the type of microorganism. Raimbault (1998) reported that with *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. the pH will drop quickly below 3.0 and the strains of *Trichoderma* sp., *Sporotricum* sp. and *Pleurotus* sp. will keep the pH stable between 4 and 5.

It is well known that each microorganism can grow and sustain its metabolic activities between certain pH values, since the pH of the medium can affect the microbial growth through the functioning of cell enzymes and the transport of nutrients into the cell (Jay, 2000). It has been reported by Ray (2004) that the pH range of growth for molds is 1.5 to 9.0; for yeasts, 2.0 to 8.5; for Gram-positive bacteria, 4.0 to 8.5; and for Gram-negative bacteria, 4.5 to 9.0. The wide range of pH that is typical for fungi can be used as an advantage in SSF. The choice of a lower pH -where possible- may prevent or minimize bacterial contamination (Krishna, 2005).

The type of solid substrate plays an important role in the pH trend of the process. Some solid substrates have the ability of resisting the pH changes, which are said to be buffered (Jay, 2000). Therefore it is critical to formulate a medium with a good buffering capacity that should also be compatible with the growth and desired metabolic activities of the fungi (Krishna, 2005). However it should be kept in mind that the substrate possessing a good buffering capacity will result in an increased lag phase of growth for the microorganism due to the tendency of the organism to bring the external environment within its optimum pH growth range (Jay, 2000).

Additionally, the variation in the pH during the SSF process will be caused by the selected nitrogen source in the media formulation (Krishna, 2005).

The main handicap in the control of pH in the SSF processes is that no electrode exists to record the pH in the solid medium due to the lack of free water. The common way of pH measurement is to use of a potentiometric electrode or a pH electrode after suspending the sample in the distilled water. In case of a stable pH requirement researchers prefer to buffer the medium with an adequate mixture of nitrogen

compounds (urea, ammoniac salts), Ca^{2+} salts, or alkaline solution. As the fermentation proceeds, the suitable amount of acid or bases will be added to the medium via the cooling water (Bellon-Maurel et al., 2003).

Patil and Dayanand (2006a) investigated the effect of initial pH (between 0 to 6.5) on the SSF production of endo and exo-polygalacturonase by an *Aspergillus niger* DMF45 strain from deseeded sunflower head. In that study they observed that maximum exo-polygalacturonase activity was obtained around pH 5.0 and it declines sharply below 4.0 and above 5.5 (Figure 2.6).
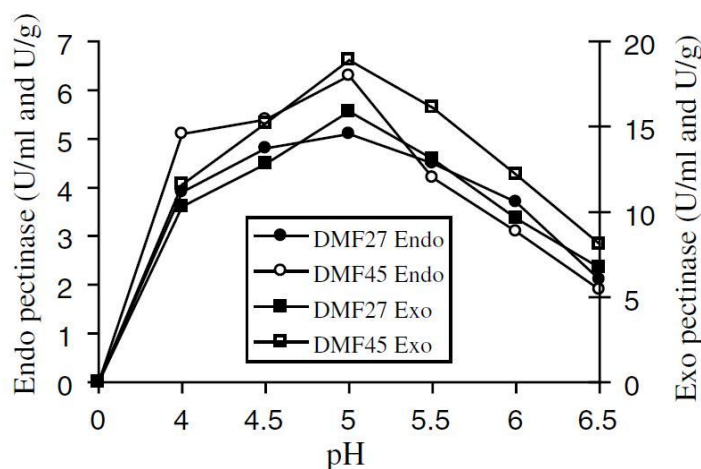
Figure 2.6. Influence of pH on the production of pectinase from sunflower head in submerged (strain DMF 27) and solid-state (strain DMF45) conditions (Source: Patil and Dayanand, 2006a)

Hours et al. (1994) preferred to add three different concentrations of HCl (0.2, 0.3 and 0.4 N, equal to pH 4.5, 3.4, 2.75, respectively) which they called weak, medium and strong acidic conditions to 10 grams of wheat bran. At the end of fermentation (48 h) with an *Aspergillus awamori* strain maximum activity was obtained with the weak initial acidic condition, however when compared to the maximum activity medium and strong acidic conditions gave nearly 50% and 60% lower PG activities respectively.

2.3.6. Effect of Particle Size of the Substrate

Particle size of the substrate is an important factor due to its effects on the microbial growth and heat and mass transfer during the SSF processes (Krishna, 2005). The type of the substrate or matrix is responsible for two critical facts that are: surface

area to volume ratio and void space. The surface of the substrate is the place where SSF occurs and size of the particles is the main factor that determines the total surface area available for the microbial attack. It is clear that for a constant geometry, as the size of the particle decreases, the surface area to volume ratio increases. However, the void space which is occupied with air plays a critical role in the oxygen transfer through the inner parts of the substrate.

At this point it should be stated that particle size of the substrate should be experimentally optimized in order to conduct an efficient SSF process. The use of smaller particle size may support larger surface area exposed to the microbial action and therefore resulting in the increase of product yield. However, the smaller particle size than the optimum range may lead to agglomeration of the substrate which will definitely reduce the volume of void space and cause poor growth due to the insufficient oxygen transfer. On the opposite side, the larger particle size than the optimum range may provide more inter-particle space with suitable respiration and aeration characteristics. Thus, larger particle size denotes limited surface area for microbial attack (Pandey et al., 2000).

The agro-industrial residues which are generally not homogenous in particle size may need pretreatment of size reduction or milling. Since it may bring extra cost to the SSF process, the financial benefit of this pretreatment process should be well defined. In addition, the particle size of the substrate with the rate of air flow is a factor affecting the conductive cooling especially in the fluidized bed SSF bioreactors. It should be kept in mind that the size of substrate particles may show alteration during the SSF process as a result of microbial and enzymatic action, therefore synthetic solid supports may be preferred in the kinetic studies.

Patil and Dayanand (2006a) have studied on the production of pectinases of a potent *A. niger* strain on the dried and ground sunflower heads by SSF. In this study, they screened a wide particle size range of 100 to 600 μm and observed that the particle size of 500 μm is very specific especially for the production of exo-pectinase. Below this value, the exo-pectinase activity decreased gradually as the particle size decreased. It was also reduced roughly about 12% at the 600 μm level (Figure 2.7). A similar result was obtained by Roses and Guerra (2009) with the sugarcane bagasse of very wide particle size (<1, 1–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–14,14–16 and 16–20 mm) obtained by milling. The total amylase activity reduced gradually below and above 6-8

mm level even though total sugar consumption profiles were similar for each particle size range. But unfortunately these researchers did not give a comparison of the enzyme activities at the mentioned particle sizes and the mixed ones may give idea about the necessity of the optimization of the substrate particle size.
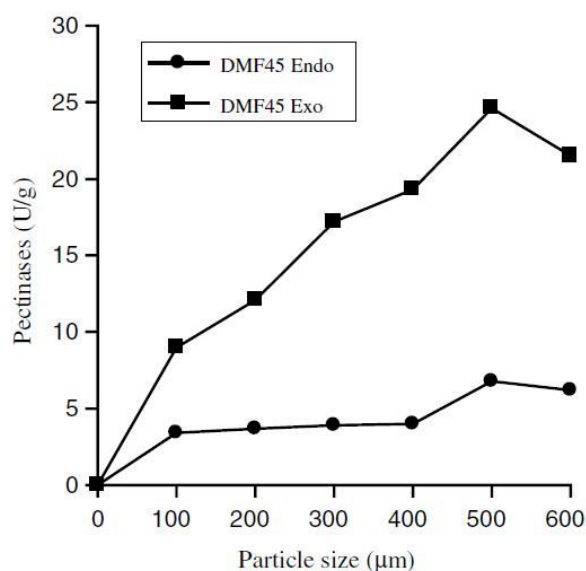
Figure 2.7. Effect of particle size on the production of pectinase from sunflower head in solid-state condition (Source: Patil and Dayanand, 2006a)

On the other hand, Balkan and Ertan (2007) figured out that the optimum particle size for the maximum enzymatic activity may show difference with regard to the type of agro-industrial residue. These researchers screened four kinds of residues at the particle sizes of >1000, 1000, 850 and 600 mm for the production of α-amylase from *Penicillium chrysogenum*. In their study they found that maximum enzyme activity was obtained at >1000 μm for corncob leaf, rye straw, wheat straw, whereas it was 1000 μm for the wheat bran.

2.3.7. Effect of Agitation

The major role of agitation in the SSF medium is maintenance of the homogeneity of the solid substrate. The homogeneity of the substrate implies well distribution of the temperature at every region of the fermentation medium, which will prevent thermal gradients that reduce the efficiency. Moreover, effective mixing may also dissipate the heat generated by the metabolic activities. Therefore, it will be

possible by agitation to inhibit the shrinkage of the substrate particles, formation of the undesired channels and failure of the forced aeration in the solid substrate bed (Krishna, 2005; Schutyser et al., 2003).

Another role of the agitation is to support homogenous water addition to the substrate. Water loss due to evaporation will be blocked by this optional process. This option is also valid in the processes where the nutrient addition during the course of the fermentation is required. Agitation also provides gas-liquid interfacial area for gas to liquid and liquid to gas transfers (Lonsane et al., 1992).

The agitation requirement of the fermentation process should be considered in the substrate selection. The selected solid substrate should not form aggregates or clusters with respect to agitation, otherwise aeration and mixing of the fermentation media will become ineffective. The other important point in the selection of the substrate is that it should resist the shear force caused by the agitation and also should not be physically affected (Krishna, 2005).

On the other hand, agitation is known to have possible negative effects on the substrate and fungal morphology. It may cause substrate porosity due to the compacting of the substrate particles. It was stated by Nigam and Singh (1994) that filamentous fungi can grow on a solid substrate in the absence of free water, hence surface adhesion plays a very important role in fungal growth and mycelial spread. Therefore, main handicap of agitation will be the disruption of the attachment between the fungus and the solid substrate. Additionally, improper agitation may disrupt the fungal mycelia due to the shear forces (Lonsane et al., 1992). In this case, high shear stress should be avoided and an intermittent mixing strategy will be applied in order to supply a balance between the heat removal and mycelial disruption effects of the agitation (Bhargav et al., 2008).

Consequently, it is important to set up a well decided agitation regime with respect to the substrate selection, bioreactor layout, fungal morphology, respiration rate and heat removal requirement in the framework of scale-up strategies. The type of bioreactor is distinctive where tray-type bioreactors have static conditions that do not require agitation, whereas perforated-drum bioreactors or horizontal paddle mixers are designed to maintain continuous or intermittent agitation.

A limited number of research studies were published about the effect of agitation or mixing of the solid substrate on the fermentation process. One of them was published by Lee et al. (2011) who worked on the production of cellulase enzyme using

Aspergillus niger USM AI 1 grown on a mixture of sugarcane bagasse and palm kernel cake in a novel solid-state bioreactor "FERMOSTAT". The effect of mixing intensity on the production of cellulase was investigated with 6, 12 and 24h intervals of mixing intensities. This study revealed that the production of cellulase increased as the mixing intensity used decreased in the fermentation process. They stated the reason of this fact as the disruption of fungal attachment to the solid substrates and damage to fungal mycelial due to shear forces (Figure 2.8).
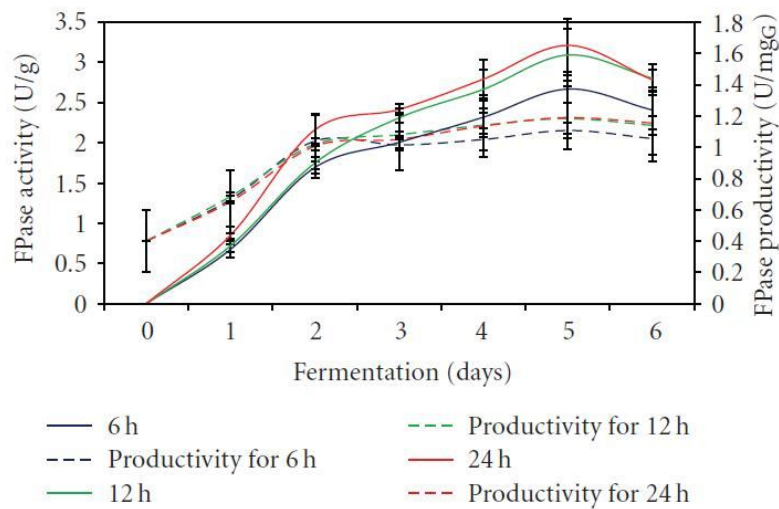
Figure 2.8. Effect of different mixing intensities on FPase production
(Source: Lee et al., 2011)

On the contrary, Gasiorek (2008) found that biomass concentration obtained by continuous mixing was higher than that of intermittent mixing which refuted the general opinion of the damaging effect of agitation on the mycelium. In this mentioned study, the author worked on the effect of agitation on fungal biomass and product concentration during citric acid biosynthesis by solid-state fermentation and for this purpose, *Aspergillus niger* S was cultivated on sugar beet pulp in a horizontal rotating drum bioreactor. The conducted experiments showed that continuous type of agitation caused an increase in the biomass concentration despite of the accumulation of the substrate particles. On the other hand, this state of the fermentation resulted in a slight decrease in the citric acid production with the continuously agitated bioreactor. In the same study, the production of citric acid (g/kg) showed variations between the 2 minute every half an hour, 1 minute every hour and 1 minute every 24 hour intermittently

agitated batches, indicating the importance of the agitation frequency on the product yield by SSF.

2.3.8. Effect of Inoculum Type

One of the most important factors in the operation of a solid-state fermentation process is the selection of the inoculum type which is heavily dependent on the type of the microorganism and scale of the process. Filamentous fungi are the most widely preferred microorganisms in the solid-state fermentation processes which commonly offer two types of inoculum options: spore or vegetative (mycelial) inoculum. It was stated that spore inocula may be prepared form solid-agar cultures by washing off spores with water or in slurry systems or in submerged cultivations using basal salts media. On the other hand, mycelial inoculum may be prepared in defined solid media or in submerged culture as a liquid seed culture (Nigam and Singh, 1994). In both cases the size of the inoculum and viability should be sufficient for the fermentation (Mitchell et al., 2006b).

The spore inoculation has some advantages such as serving as a biocatalyst in bioconversion reactions, convenience, and greater flexibility in the coordination of inoculum preparation, prolonged storability for subsequent use, higher resistance towards toxic effects of bioconversion substrates and products and higher resistance to mishandling during transfers. However, spore inoculation may increase the duration of the fermentation due to its long lag time or the optimum environmental conditions for spore germination and vegetative growth may be different (Krishna, 2005; Ramachandran et al., 2008). But spore inoculation is generally preferred in most of the fungal solid-state fermentation processes designed to produce enzymes (Chutmanop et al., 2008; Gassara et al., 2010; Kumar et al., 2010; Roses and Guerra, 2009). On the contrary, there may be situations where spore production is not effective for the selected strain; hence a liquid seed culture should be prepared as a vegetative inoculum for SSF (Papagianni et al., 2001 and 1999). The fungal morphology (pellets or free mycelia), composition and viscosity of the seed culture medium, agitation and aeration characteristics, age of the seed culture are some of the factors that should be considered in the preparation of the liquid seed culture for the SSF.

At large scale operations, the production of inoculum is usually achieved by using a series of inoculum fermenters of increasing capacities. In that case, the laboratory scale production of spores on the surface of the agar media conducted in test tubes, Petri dishes or culture bottles may be unpractical and appropriate liquid seed cultures fermented in bioreactors under controlled conditions may be preferred. It should be kept in mind that the factors such as environmental conditions, metabolic state of the inoculum cells, viability of the cells and age of the inoculum may show difference at the large scale production of inoculum and they should be optimized again (Lonsane et al., 1992).

An alternative to this will be the production of spores on a low-cost solid substrate. This was tested by Costa et al. (2007) in the optimization process of amyloglucosidase and exo-polygalacturonase production from defatted rice bran using two strains of *Aspergillus niger*. One of the investigated factors in their study was the type of inoculum in which they compared the spore inoculum and fermented bran. The type of the inoculum was found to be a significant factor for the production of both enzymes. However, the maximum amyloglucosidase activity was obtained with the spore suspension inoculum, whereas the maximum exo-PG activity was obtained with inoculation of the fermented bran.

In another study, Sekar and Balaraman (1998) produced an antibiotic - Cyclosporin A- from the fungus *Tolypocladium* sp. by inoculating the strain in the vegetative (produced in a bioreactor) and spore forms to the wheat bran fermented in trays. They observed that the yield of Cyc A obtained by SSF with vegetative inoculum was significantly higher than that inoculated with spores. Their other observation was that the trays inoculated with vegetative inoculum showed better growth than the spore inoculated trays.

CHAPTER 3

PRODUCTION OF EXO-POLYGALACTURONASE FROM *Aspergillus sojae* BY SOLID-STATE FERMENTATION

This chapter deals with the classification and mechanism of pectinolytic enzymes and provides the definition and biochemical properties of exo-polygalacturonases (PGs) produced from fungal and bacterial sources. The industrial applications of pectinolytic enzymes are stated, followed by the discussions on the strain of our interest –*Aspergillus sojae*– and by the studies reported in the literature about its metabolite production. Finally, the published studies on the production of exo-polygalacturonase by solid-state fermentation (SSF) technique are reviewed within the scope of this thesis.

3.1. Pectinolytic Enzymes and Their Classification

Pectic substances (pectins) are the substrates of pectinolytic enzymes (also known as pectinolytic enzymes or pectinases); therefore their roles should be well known in order to understand the mechanism of pectinolytic enzymes. Pectic substances are found as structural polysaccharides in the primary cell wall and middle lamella of higher plants contributing firmness and structure to the plant tissues (Lang and Dörnenburg, 2000; Alimardani-Theuil et al., 2011; Gummadi and Panda, 2003). In pectic substances, α-D-galacturonic acid (galacturonan) units are linked together by α-1,4-glycosidic linkages to form homogalacturonan backbone that alternates with branched (hairy) regions containing rhamnose, arabinose, galactose and xylose as side chains (Gummadi and Panda, 2003; Kashyap et al., 2001). Moreover, the galacturonate units found in plant tissues are esterified with methanol with the rate of about 60-70% (Gummadi et al., 2007). Alkorta et al. (1998) states that The American Chemical Society classified pectic substances into four main types based on their degree of esterification: protopectins, pectinic acids, pectins and pectic acids.

According to this classification, *protopectin* is called to be a parent pectic substance in which carboxyl groups are esterified with methanol with more than 90% degree of esterification. Protopectins are water insoluble pectic substances found in the intact tissue and their carboxyl groups are highly cross-linked with calcium ions or with other polysaccharides. As a result of their restricted hydrolysis soluble pectic substrates such as pectin or pectinic acids are formed. *Pectic acids* are water soluble galacturonan polymers. They contain negligible amount of methoxyl groups and have the 0% degree of esterification. Pectic acids or their acidic salts can be called as pectates. *Pectinic acids* are the galacturonans that have carboxyl groups slightly esterified with methanol. Their degree of esterification may vary between 0 and 75%. Pectinic acids are soluble in water and they individually have the property of forming a gel with sugar and acid or calcium salts where methyl content is suitably low. Pectinic acids or their acidic salts are called pectinates. *Pectins* (polymethyl galacturonate) are water soluble galacturonan polymers having degree of esterification at least 75%. Their carboxyl groups are esterified with methanol. Pectin can be found in cell wall of plants providing rigidity by binding to the structural polysaccharides such as cellulose (Kashyap et al., 2001; Gummadi et al., 2007; Jayani et al., 2005).

Pectic enzymes can be classified into three groups (Jayani et al., 2005):

(I)   Protopectinases: as a result of the action of this enzyme on protopectin, highly polymerized soluble pectins are formed.

(II)   Esterases: catalyze the deesterification of pectin by the removal of methoxy esters forming pectic acid.

(III) Depolymerases: catalyze the hydrolytic cleavage of the α-1,4-glycosidic bonds in the D-galacturonic acid moieties of the pectic substances.

3.2. Exo-Polygalacturonases and Their Biochemical Properties

Polygalacturonases belong to the group of pectic enzymes that involve in the degradation of pectic substances (Niture, 2008). They are classified under depolymerases. Polygalacturonases catalyze the hydrolysis of α-1,4-glycosidic linkages in pectic acid with the introduction of water across the oxygen bridge (Jayani et al., 2005). Polygalacturonases have subgroups depending on the pattern of action (random or terminal).

Endo-PGs (EC 3.2.1.15) are known as poly (1,4-α-D-galacturonide) glycanohydrolase, catalyzes random hydrolysis of α-1,4-glycosidic linkages in pectic acid. This reaction produces oligogalacturonates. They are widely distributed among fungi, bacteria, many types of yeast, higher plants and some plant parasitic nematodes (Kashyap et al., 2001; Gummadi et al., 2007; Jayani et al., 2005).

Exo-PGs (EC 3.2.1.67) are known as poly (1,4-α-D-galacturonide) galacturonohydrolase, catalyzes hydrolysis in a sequential fashion of α-1,4-glycosidic linkages on pectic acid. In contrast, exo-PGs occur less frequently. Exo-PGs can be distinguished into two types: fungal exo-PGs that liberate monogalacturonic acid as the main end product; and the bacterial exo-PGs, that liberate digalacturonic acid as the main end product. Occurrence of PGs in plants has also been reported (Jayani et al., 2005; Kashyap et al., 2001).

The biochemical properties of the microbial polygalacturonases may show variation upon the type of the microbial source, fermentation method and production conditions. Moreover, biochemical properties of PGs should be identified for the decision of its biotechnological application area. Some biochemical and physicochemical properties of exo-PGs produced by various microorganisms are shown in Table 3.1.

Table 3.1 shows that the optimum temperatures of the enzymatic activity of PGs are mostly between 40 and 60 °C. Additionally, most of the PGs show optimum activity at the acidic to neutral pH values at these temperatures. The stability of the produced PGs changes between the temperatures of 40 to 70 °C for approximately 30 min to 1 hour. However, interestingly Kapoor et al. (2000) achieved to produce a thermo-alkali stable polygalacturonase that has an extreme optimum pH of 10.0. This PG also showed a remarkable thermostability by saving 100% of its enzymatic activity at 50 °C for more than 12 h. This was a partially purified polygalacturonase produced from a *Bacillus* sp. isolated from the outer covering of seeds of *Celastrus paniculatus* (Kapoor et al., 2000). Table 3.1 indicates that most of the produced PGs were stable at a wide range of pH values. The PG produced by SSF from *Moniliella* sp. on the mixture of orange bagasse, sugar cane bagasse and wheat bran has the broadest pH stability, since it preserved 70 – 100% of its initial enzymatic activity between 3.0 to 10.0 (Martin et al., 2004). Segel (1976) stated that K_m value can be utilized to compare the enzymes from different organisms to see whether they are identical or not. Acuna-Arguelles et al. (1995) compared the properties of exo-pectinases produced by SSF and SmF technique from

Aspergillus niger CH4. The authors observed that K_m values of the PGs obtained from the same fungal strain were different depending on the type of the fermentation type. Most of the authors determined the molecular weight of the produced exo-PGs with the SDS-PAGE method of Laemmli (1970). Table 3.1 also states that the majority of the PGs have the molecular weight in the range of 30 to 70 kDa.

Table 3.1. Biochemical and physicochemical properties of some exo-polygalacturonases

Source	Fermentation type	Molecular weight (kDa)	pI	Specific activity (U/mg)	K_m (mg/ml)	Optimum temperature (°C)	Optimum pH	Temperature stability	pH stability	Ref
Bacillus sp KSM-P410	plate	45	5.8	54	1.3	60	7.0	50 °C – 15 min	7.0 – 12.0	Kobayashi et al., 2001
Aspergillus niger MIUG 16	SSF	36.3	5.13	1542.2	0.94	40	4.6	-	-	Dinu et al., 2007
Aspergillus sp.	SSF	-	-	-	-	50	5.5	50 °C – 1h	4.0	Freitas et al., 2006
Aspergillus sojae ATCC 20235	SmF	36 - 68	-	-	0.424	55	5.0	65 °C – 1h	3.0 – 7.0	Tari et al., 2008
Thermoascus aurantiacus	SSF	-	-	-	-	65	5.0	60 °C – 1h	3.0 – 9.0	Martins et al., 2002
Moniliella sp.	SSF	-	-	-	-	55	4.5	60 °C – 1h	3.0 – 10.0	Martin et al., 2004
Penicillium sp.	SSF	-	-	-	-	40	4.5 – 5.0	70 °C – 1h	3.0 – 8.0	Martin et al., 2004
Bacillus sp.	SSF	-	-	-	-	60	10.0	50 °C – 12h	8.5 – 12.0	Kapoor et al., 2000
Aspergillus niger CH4	SSF	68, 46.5, 45, 36	-	-	2.05	50	3.0 – 5.0	50 °C – 30 min	3.0 – 6.0	Acuna et al., 1995
Aspergillus niger CH4	SmF	68, 45, 38	-	-	5.42	45	3.0 – 5.0	50 °C – 30 min	4.0 – 5.0	Acuna et al., 1995
Aspergillus sp.	SmF	-	-	-	-	-	-	40 °C – 1h	3.0 – 5.0	Galiotou-Panayotou et al., 1997
Aspergillus giganteus	SmF	69.7	-	419.4	1.16	60	6.0 – 6.5	40 °C – 1h	6.5 – 10.0	Pedrolli and Carmona, 2010

3.3. Applications of Pectinolytic Enzymes

The industrial market for pectinases approached 70 million dollars around the world that corresponded approximately to 5% of global enzyme sales in the year 2011 (Alimardani-Theuil et al., 2011). Pectinases are mainly produced from *Aspergillus niger* (polygalacturonase, pectinesterase, pectinlyase producing strains) for industrial uses (Alimardani-Theuil et al., 2011). However, the produced crude enzyme may have some proteolytic or hydrolytic side activities that can cause undesirable reactions during the industrial applications. Therefore, the produced enzyme may require some purification processes which should be performed in a cost-effective and productive way.

The industrial pectinases can be classified into two types; acidic and alkaline pectinases (Gummadi et al., 2007). Acidic pectinases play an important role in fruit juice industry. The commercial pectinases are commonly used in juice extraction and clarification processes of the sparkling clear juices (apple, pear and grape juices), cloudy juices (citrus juices, prune juices, tomato juice and nectars), and unicellular products where the intent is to preserve the integrity of the plant cells by selectively hydrolyzing the polysaccharides of the middle lamella (Kashyap et al., 2001). On the other hand, alkaline pectinases mainly produced from *Bacillus* spp. are mostly used in the degumming and retting of fiber crops and pretreatment of pectic wastewater from fruit juice industries (Kashyap et al., 2001). Gummadi et al. (2007) have reviewed the industrial applications of pectinases given in Table 3.2. The application areas of pectinases were also reviewed by various authors (Kashyap et al., 2001; Alkorta et al., 1998; Hoondal et al., 2002; Jayani et al., 2005; Alimardani-Theuil et al., 2011).

Table 3.2. Industrial applications of pectinases

Application	Purpose
Cloud stabilization	To precipitate hydrocolloid matter present in fruit juices
Fruit juice clarification	To degrade of cloud forming pectic substances. Hence, the juice can be easily filtered and processed
Extraction of juice and oil	To overcome the difficulty in pressing fruit pulp to yield juice and oil
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
Liquefaction	To break down fermentable plant carbohydrates to simple sugars using enzymes
Gelation	To use in gelling low-sugar fruit products
Wood preservation	To prevent the wood from infection by increasing the permeability of wood preservative
Retting of fiber crops	To release fiber from the crops by fermenting with microorganisms, which degrade pectin
Degumming of fiber crops	To remove the ramie gum of ramie fiber
Waste water treatment	To degrade pectic substances in waste water from citrus processing industries
Coffee and tea fermentation	To remove the mucilage coat in coffee bean. To enhance the tea fermentation and foam forming property of tea

3.4. *Aspergillus sojae*

Filamentous fungi are stated as typical saprophytic microorganisms that can secrete a wide variety of enzymes involved in the decomposition and recycling of complex biopolymers from both plant and animal tissues. These secreted enzymes are mostly hydrolytic and play an important role in fungal nutrition, releasing carbon and nitrogen locked in insoluble macromolecules obtained from the metabolic activities of other organisms. The ability of enzyme production and secretion of the fungi attracted the attention of many researchers to study the production of different kinds of enzymes using filamentous fungi in large scale industrial processes (El-Enshasy, 2007). Among the filamentous fungi, the genus Aspergillus is an important industrial microorganism for large-scale production of both homologous and heterologous enzymes which have a

large potential market (Öztürk et al., 2010). Meyer et al. (2011) mention about 250 named species in the genus Aspergillus, including important industrially exploited species (*A. niger*, *A. oryzae*, *A. awamori*, *A. sojae*, *A. terreus*) and harmful species being pathogenic to animals and/or plants (e.g. *A. fumigatus*, *A. parasiticus*, *A. flavus*). *Aspergillus sojae* is taxonomically classified in section Flavi along with *Aspergillus flavus*, *Aspergillus oryzae*, and *Aspergillus parasiticus*. While *A. flavus* and *A. parasiticus* are fungal contaminants of food and feed, and produce the potent carcinogen, aflatoxin, in contrast, *A. sojae* and *A. oryzae* are used not only for industrial enzyme production but also for fermented food production such as sake (rice wine), miso (bean paste), and shoyu (soy sauce) in eastern Asia. It is generally accepted that *A. sojae* and *A. oryzae* never produce aflatoxin under any culture conditions (Matsushima et al., 2001).

Aspergillus sojae strains were reported to produce many different metabolites such as endo-1,4-β-D-galactanase (EC 3.2.1.89) (Kimura et al., 1998), extracellular leucine aminopeptidase (Chien et al., 2002), β-glucosidase (EC 3.2.1.21) (Kimura et al., 1999), D-amino acid oxidase (D-glutamate, D-aspartate, D-alanine) (Wakayama et al., 1996), endo-1,4-β-xylanase (EC 3.2.1.8) and arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55) (Kimura et al., 1995), alkaline proteinase (EC 3.4.21.14) (Ichishima et al., 1983), chlorogenate hydrolase (EC 3.1.1.42) (Adachi et al., 2008) and exo-polygalacturonase (Göğüş et al., 2006).

Öztürk et al. (2010) used an *Aspergillus sojae* strain (ATCC11906) as the host to introduce the mature protein coding region of the endo-β-1,4-mannanase gene isolated from *Aspergillus fumigatus* IMI 385708. As a result, they have managed to produce industrially important enzyme β-mannanase (EC 3.2.1.78) from a safe recombinant (*A. sojae*) Aspergillus strain instead of a pathogenic (*A. fumigatus*) one (Öztürk et al., 2010). In another study, this research group was able to cultivate an *Aspergillus sojae* expressing the α-galactosidase gene of *Aspergillus fumigatus* IMI 385708 (Gurkok et al., 2011). Matsushima et al. (2001) isolated ten strains from industrial soy sauce producing koji mold which were identified as *Aspergillus sojae*. These strains were distinguished from *Aspergillus parasiticus* morphologically and physiologically and they did not detect any aflatoxin in any culture extracts of *A. sojae* strains.

3.5. Studies on the Production of Exo-Polygalacturonase by Solid-State Fermentation

As can be seen from Table 3.3, wheat bran was the most commonly employed agro-industrial residue for the production of PG by solid-state fermentation (SSF) using many fungi species but especially *Aspergillus niger*. Phutela et al. (2005) has studied the production of pectinase and polygalacturonase from a thermophilic *Aspergillus fumigatus* by SSF. For this purpose they have replaced the pectin content of the basal medium with natural substrates such as; malt sprouts, wheat bran, rice bran and pectin from pomegranate, lemon, banana, orange and *Citrus sinensis* (Var. Mussami). They have observed that wheat bran (625 U/g) was the best natural substrate which was close to pure pectin (642 U/g) with respect to PG activity. Freitas et al. (2006) have conducted a similar study by screening the effects of orange bagasse, wheat bran and sugarcane bagasse and their different mixtures on the PG activity. The maximum exo-PG activity was produced with *Aspergillus* sp. N12 by the wheat bran and orange bagasse mixture (1:1). The literature survey showed that the type of agro-industrial residues screened specifically for PG production by SSF is not broad enough. However, some of the agro-industrial residues screened in order to produce various enzymes can be listed as; aloe-vera skin, pineapple peel, banana peel, rice bran, coba husk, soybean powder, corncob leaf, rye straw and wheat straw (Pal et al., 2010; Fang et al., 2010; Balkan and Ertan, 2007). Utilization of these agro-industrial residues in enzyme production processes not only helps in the production of a value-added product from an economical and sustainable by-product but also solves pollution problems caused by their disposals.

As can be seen from Table 3.3, many of the researchers prefer to add some nutritive or supplementary solutions to the solid-state fermentation media in order to enhance the enzyme activity. However, addition of these solutions may increase the cost of the large scale fermentation process which is contrary to the utilization of economical agro-industrial residues as the low-cost materials. Therefore, the current study will give priority to the screening of the agro-industrial residues without the addition of supplementary materials.

Table 3.3. Studies conducted on production of exo-polygalacturonase enzyme by solid-state fermentation

Reference	Solid substrate	Supplementary medium	Microorganism	Initial moisture content (%)	Water activity	Incubation temperature (°C)	Incubation time (hours)	PG activity
Acuna et al., 1994	Sugar cane bagasse	Basal medium containing (% w/w wet mass): pectin, 1.5; sucrose, 3.14; urea, 0.3; $(NH_4)_2SO_4$, 1.26; KH_2PO_4, 0.65; $MgSO_4.7H_2O$, 0.02; $FeSO_4$, 0.029	*Aspergillus niger* CH4	70	0.98	35	72	20 (mg RG/ml)
Alcantara et al., 2010	Cashew apple dry bagasse	ammonium sulfate	*Aspergillus niger* CCT0916	50 wb	0.99	30	79	11 (U/g)
Blandino et al., 2002	Wheat grains	-	*Aspergillus awamori*	60	-	30	144	9.6 (U/g)
Botella et al., 2005	Grape pomace	-	*Aspergillus awamori*	60	-	30	168	25 (IU/gds)
Castilho et al., 2000	Wheat and soy brans	1.35% w/w $(NH_4)_2SO_4$ (dry basis) and 0.55% w/w HCl (dry basis)	*Aspergillus niger*	40	-	30	22	25 (U/ml)
Costa et al., 2007	Defatted rice-bran	mineral salts solution (g/l): 2 KH_2PO_4; 1.8 Urea; 1 $MgSO_4$	*Aspergillus niger*	50	-	30	96	50.2 (U/g dm)
Couri et al., 2000	Wheat bran and mango peel	0.09% (w:v) ammonium sulphate solution in 0.1 M HCl	*Aspergillus niger* 3T5B8	60	0.9250	32	78	30.75 (U/ml)
Debing et al., 2006	Wheat bran	rice dextrose, ammonium sulfate, tween 80	*Aspergillus niger*	60	-	30–32 27	30 42	36.3 (IU/g dm)

(cont. on next page)

Table 3.3. (Continued)

Reference	Solid substrate	Supplementary medium	Microorganism	Initial moisture content (%)	Water activity	Incubation temperature (°C)	Incubation time (hours)	PG activity
Dinu et al., 2007	Wheat bran, beet marc	mineral solution (w/v): 2.5% ammonium sulphate, 0.33% monopotasium phosphate, and 0.17% magnesium sulphate	*Aspergillus niger* MIUG 16	-	-	28	120	13359 (U total activity)
Fontana et al., 2005	Wheat bran, citric pectin	salt solution (g/l): $(NH_4)_2SO_4$, 4.0; KH_2PO_4, 2.0; $MgSO_4.7H_2O$, 1.0; $FeSO_4.H_2O$, $6.3*10^{-5}$; $ZnSO_4$, $6.2*10^{-5}$; $MnSO_4$, $1.0*10^{-6}$.	*Aspergillus niger* T0005007-2	63	-	30	72	370 (U/g dm)
Freitas et al., 2006	Wheat bran, sugar cane and orange bagasse	0.1% $(NH_4)_2SO_4$ and 0.1% $MgSO_4.7H_2O$	*Aspergillus sp.*	70	-	45	96	10 (U/ml)
Kapoor et al., 2000	Wheat bran and decorticated ramie Fibres	mineral salt solution (pH 7.5) (g:l): KH_2PO_4, 1; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.1; and soil extract 1 ml (v:v)	*Bacillus* sp. MG-cp-2	90	-	30	72	188 (U/ml)
Linde et al., 2007	Wheat or rice brans with commercial orange pectin	0.018 g of KH_2PO_4 and 0.09 g of $MgSO_4$ per gram dry substrate	*Aspergillus niger* and *Aspergillus oryzae*	50	-	30	96	368 (U/g)

Table 3.3. (Continued)

Reference	Solid substrate	Supplementary medium	Microorganism	Initial moisture content (%)	Water activity	Incubation temperature (°C)	Incubation time (hours)	PG activity
Maldonado and Saad, 1998	Sugar cane bagasse	g per 100 g: urea, 0.3; K_2HPO_4, 0.65; $(NH_4)_2SO_4$, 1.26; $MgSO_4$, 0.02; $FeSO_4$, 0.029; pectin, 1.5	*Aspergillus niger*	-	-	30	72	500 (U/l)
Patil and Dayanand, 2006a	Dried deseeded sunflower head	(w/w for solid-state): $(NH_4)_2SO_4$ 0.1; $MgSO_4 \cdot 7H_2O$ 0.5; KH_2PO_4 0.5 and $FeSO_4 \cdot 7H_2O$ 0.0005	*Aspergillus niger* DMF 45	60	-	30	96	35 (U/g)
Rangarajan et al., 2010	Dried orange peel, soybean meal		*Aspergillus niger*	70	-	30	120	6800 (IU/g)
Taragano and Pilosof, 1999	Wheat bran		*Aspergillus niger* 148	55 wb	0.94	28	48	27.5 (U/ 5 g WB)
Taskın and Eltem, 2008	Wheat bran, sugar beet pulp	(% w/v) $(NH_4)_2SO_4$, 1; K_2HPO_4, 0.1; $Mg(SO_4)_2.7H_2O$, 0.1; $FeSO_4. 7H_2O$, 0.001	*Aspergillus foetidus* Ege-K-635	75	-	30	96	385 (U/g)
Ustok et al., 2007	Crushed maize, maize meal or corncob	(g/l): glycerol (45), peptone (18), molasses (45), NaCl (5), (mg/l): $FeSO_4 \cdot 7H_2O$ (15), KH_2PO_4 (60), $MgSO_4$ (50), $CuSO_4 \cdot 5H_2O$ (12), $MnSO_4 \cdot H_2O$ (15)	*Aspergillus sojae* ATCC20235	48.99, 51.04, 50.69	-	30	120-144	29.1 U/g solid
Zheng and Shetty, 1998	Apple pomace	0.5 g of $CaCO_3$, 20 mL of water, and 0.5 g of NH_4NO_3 or 2 mL of fish protein hydrolysate	*Trichoderma*, *Rhizopus*, and *Penicillium* species.	68	0.96	25	96	-

CHAPTER 4

SCALE-UP

The aim of this chapter is to present the key points that should be considered in the scale-up and bioreactor design processes. The information on the solid-state fermentation (SSF) type bioreactors were presented in detail according to the classification of Mitchell et al. (2006b) on the basis of their mixing and aeration strategies.

4.1. Scale-up in SSF

Nigam and Singh (1994) defined the scale-up stages of the solid-state fermentation process as follows:

Flask level: 50-1000 g working capacity for the selection of the organism, optimization of the process and experimental variables in a short time and at low cost. The vessels used are conical flasks and beakers, Roux bottles, jars, and glass tubes.

Laboratory fermenter level: 5-20 kg working capacity for the procedures selection for inoculum development, medium sterilization, aeration, agitation and downstream processing standardization of various parameters, selection of control strategies and instruments, evaluation of economics of the process and its commercial feasibility. The fermenters used are glass incubators, column fermenters; polypropylene bags.

Pilot fermenter level: 50-5000 kg for confirmation of laboratory data and selection of optimized procedures. It facilitates market trials of the product, physiochemical characterization and determination of viability of the process. Most large scale SSFs employ tray type fermenters as in the oldest soy sauce koji process, rotating drum type, horizontal paddle fermenters and mixed layer pilot plant fermenters.

Production fermenter level: 25-1000 tonnes for streamlining the developed process which ultimately leads to a financial return on the investments made so far on process development.

SSF is an ancient method that has attracted the interest of researches in the last few years. Large-scale SSF production studies accelerated as the researchers discovered the use of low-cost by-products produced in excess amounts from agro-industrial processes with the aim of producing value-added metabolites such as enzymes, chemical and biologically active compounds. This approach has necessitated the development of efficient bioreactor systems (Wang and Yang, 2007). Bioreactors are the most critical part of the SSF fermentation process in which the raw substrate is converted into the desired product under controlled conditions (Raghavarao et al., 2003). Although submerged fermentation systems are well developed with respect to the scale-up process, the same methods can not be applied directly to the scaling-up of the SSF due to its unique nature (Mitchell et al., 2000). The major fact in scale-up of the SSF process is to keep the local environmental conditions at or very near optimal values as scale is increased (Mitchell et al., 2006e). The main points that should be considered in the scale-up studies and bioreactor design are summarized as follows:

- The SSF process in large scale brings the need of relatively large amount of inoculum (Lonsane et al., 1992).

- The sterilization of the solid medium needs extra care about the temperature profiles, physicochemical alterations in the medium, thermal degradation of essential nutrients, formation of toxic compounds and nutrient damage (Lonsane et al., 1992).

- The degree of agitation should be decided considering the possible damage of the agitation regime to the fungal hyphae or the final product. Agitation can also damage the substrate or cause agglomeration, but it should be kept in mind that it may enhance oxygen transfer and homogeneity of the substrate and removal of the generated heat as well (Mitchell et al., 2006b).

- The aeration requirement of the system is critical for the aerobic growth. The bioreactor should be designed after the decision of using forced aeration or not. Air not only supplies oxygen but also removes the excess heat (Mitchell et al., 2006e).

- Biological safety of the workers should be maintained during the SSF process by designing a closed bioreactor with filtered outlets (Mitchell et al., 2006b). Such a system may also prevent the entry of contaminants (Raghavarao et al., 2003).

- The loading and unloading of the bioreactor should be well designed (Mitchell et al., 2006b).

- The volume of the bioreactor should be compatible with the volume of the substrate desired to be used at one batch (Mitchell et al., 2006b).

- The maintenance of the water activity is of critical consideration in the design of the bioreactor. Addition of liquid may be required during the process, therefore suitable ports should be added to the bioreactor (Mitchell et al., 2006b).

- On-line measurement of the important variables such as; temperature and water content of the substrate bed, temperature, flow rate, humidity of the inlet air, O_2 and CO_2 concentrations in the outlet gas should be considered in the bioreactor design in order to optimize the growth and product formation (Raghavarao et al., 2003).

- The bioreactor should be designed with respect to the downstream requirements of the process (Lonsane et al., 1992).

- Heat removal is the most critical parameter in SSF bioprocesses because a large quantity of metabolic heat is generated proportional to the level of metabolic activity in the system. Lonsane et al. (1992) stated that the heat removal by conduction and convection is poorer in most SSF systems due to the absence of agitation, a low rate of agitation in agitated systems and the poor thermal conductivity of the solid substrates. Evaporative cooling can be applied by paying attention to the possibility of drying of the substrate.

- A strategy should be developed for the waste management of the large amount of spent solids obtained at the end of the process (Lonsane et al., 1992).

A detailed discussion on the effect of the above-mentioned factors on the SSF process was given in Chapter 3.

4.2. Types of SSF Bioreactors

Mitchell et al. (2006b) have successfully classified the SSF bioreactors on the basis of their mixing and aeration strategies.

- Group I: The bioreactors belonging to this group has static bed, or mixed only very infrequently (i.e., once or twice per day) and air is circulated around the

bed, but not blown forcefully through it. The typical example to this kind of bioreactors is "tray bioreactors".

- Group II: The bioreactors of this group also have a static bed which is mixed only very infrequently (i.e., once per day). But in this type of bioreactors air is blown forcefully though the bed. These are typically referred to as "packed-bed bioreactors".

- Group III: Bioreactors in which the bed is continuously mixed or mixed intermittently with a frequency of minutes to hours, and air is circulated around the bed, but not blown forcefully through it. Two bioreactors that have this mode of operation, using different mechanisms to achieve the agitation, are "stirred drum bioreactors" and "rotating drum bioreactors".

- Group IV: Bioreactors in which the bed is agitated and air is blown forcefully through the bed. This type of bioreactor can typically be operated in either of two modes, so it is useful to identify two subgroups. Group IVa bioreactors are mixed continuously while Group IVb bioreactors are mixed intermittently with intervals of minutes to hours between mixing events. Various designs fulfill these criteria, such as "gas-solid fluidized beds", the "rocking drum", and various "stirred-aerated bioreactors".

4.2.1. Static SSF Bioreactors without Forced Aeration

This category of SSF bioreactors are generally referred as the oldest and simplest ones since they have been used by various civilizations for many centuries for the production of traditional fermented foods such as tempe and soy sauce koji (Durand, 2003; Mitchell et al., 2006a). The typical bioreactor is the tray type used in the Koji process.

Tray-type of bioreactor is composed of wood, plastic or metal trays placed in a climatic controlled chamber. These trays can be perforated or not. The climatic chamber is selected according to the stage of the scale. The scale-up is relatively easier in this type which can be done by increasing the number of trays. The position of the trays inside the bioreactor should also be considered by optimizing the gap between the trays. However, this system has handicaps such as difficulty in sterility maintenance, requirement of large space and labor, separate sterilization of the substrate bed (Mitchell

et al., 2006a; Bhargav et al., 2008; Krishna, 2005). Some operating variables can be controlled such as: the temperature, humidity and flow rate of the air entering the chamber and velocity of the circulation past the tray surface (Mitchell et al., 2006a). These bioreactors offer the ability of studying different process parameters at the same time. The schematic of a koji type-bioreactor (Figure 4.1) was drawn by Durand et al. (2003).
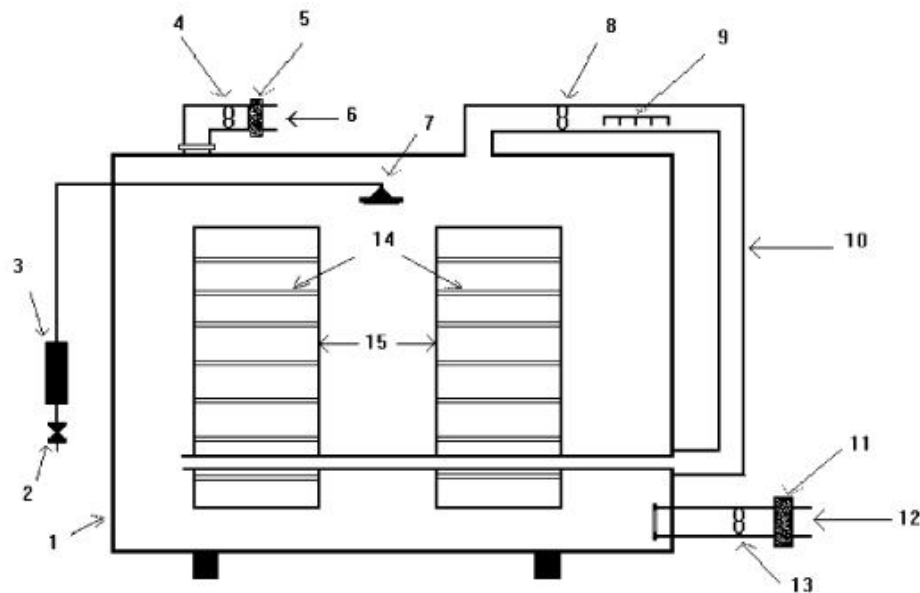
Figure 4.1. Koji-type SSF bioreactor: (1) Koji room, (2) water valve, (3) UV tube, (4, 8, 13) air blowers, (5, 11) air filters, (6) air outlet, (7) humidifier, (9) heater, (10) air recirculation, (12) air-inlet, (14) trays, (15) tray holders (Source: Durand et al., 2003)

The thickness of the solid substrate in the fermentation bed deals especially with the heat and mass transfer characteristics of the tray type of bioreactors. Two of the most important factors affecting the SSF; the oxygen transfer and metabolic heat removal are closely related to the thickness of the substrate. A general comment may be done that large O_2 and temperature gradients will arise in the substrate layer during the fermentation within an individual tray. These limitations should be well described and related parameters should be optimized in order to maintain a healthy growth and effective product formation. Mitchell et al. (2000) indicated briefly that the increasing height of the substrate at the fermentation bed may lead to some limitations on the growth. Especially in the trays without a perforated bottom, the solid substrate at the bottom of the tray bioreactor will be exposed to a limited oxygen transfer and

accumulation of the generated metabolic heat that adversely affects the growth and the product in this region. For these reasons, the scale-up of the tray type of bioreactors should be performed by increasing the area of the tray rather than increasing the bed height. Therefore the thickness of the substrate is a critical factor that should be optimized precisely.

Recently, Bhavsar et al. (2011) performed a scale-up study in enamel-coated metallic trays having dimensions of 28*24*4 cm, 45*30*4 cm, and 80*40*4 cm for the production of phytase from *Aspergillus niger* NCIM 563. They have scaled-up the process from 10 g substrate to 1000 g in a Koji type analogous bioreactor. Some reproducible enzyme activity results were reported that was found to be encouraging for the pilot-scale production. Bhanja et al. (2007) have clearly observed that as the bed height increased (as a result of increasing substrate amount) in a typical tray type-bioreactor, the yield of α-amylase decreased slightly.

4.2.2. Static SSF Bioreactors with Forced Aeration

The bioreactors discussed under this topic are operated under forced aeration conditions in which the substrate bed is not mixed. The packed-bed bioreactors are the typical ones belonging to this group. The basic principle of these bioreactors is the introduction of conditioned air through a perforated plate (sieve) that supports the substrate (Mitchell et al., 2000; Durand, 2003). In packed-bed bioreactors it is possible to control the temperature, oxygen supply and moisture via the adjustable forced air flow (Lu et al., 1997). When packed-bed bioreactors are compared with the tray-type bioreactors, they are thought to allow a better control of fermentation parameters (Ashley et al., 1999). Ashley et al. (1999) state that the temperature rise and lack of oxygen problem in the center of the large tray bioreactors were partially overcame in the packed-bed bioreactors with the use of forced aeration. But the overheating problem could still be faced at the regions close to the outlet air.

Mitchell et al. (2006f) expresses that axial and radial temperature gradients occurring in the bed is one of the most critical facts that should be considered in the design and operation of the packed-bed bioreactors. They also point out that it is impossible to prevent the formation of these temperature gradients, so it should be aimed to minimize the size of these gradients. The other important point is evaporation

of the water from the bed that will lead to the drying out of the bed. Moreover, the height of the bed should be designed by considering the possibility of pressure drop. The growth of the microorganism also affects the pressure drop with respect to filling the inter-particle spaces. In case of pressure drop, aeration through the bed and operational costs may be affected (Mitchell et al., 2006f).

Lu et al. (1997) investigated the production of citric acid using *Aspergillus niger* in a packed-bed SSF bioreactor (700 mm height x 40 mm diameter) shown in Figure 4.2. They observed that the bed loading is the most important operational factor. They also concluded that air flow rate and substrate particle size were also important hence high productivities were achieved by using high air flow rates at the initial period of the fermentation. However, high air flow rate were found to adversely affect the fungal growth due to shear stress (Lu et al., 1997). Therefore, it is desirable to increase heat removal by other techniques (Mitchell et al., 2000).
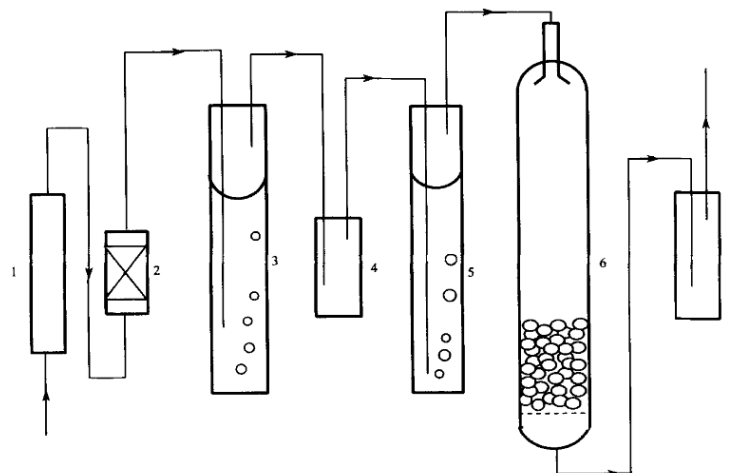
Figure 4.2. Schematic diagram of the packed-bed reactor and ancillary equipment. Rotameter, 1; filter, 2; NaOH solution, 3; water trap, 4; humidifier, 5; packed-bed reactor, 6; and water trap, 7 (Source: Lu et al. 1997)

Astolfi et al. (2011) studied the inulinase production by SSF in a fixed-bed reactor (34 cm diameter x 50 cm height) with working capacity of 2-kg of dry substrate. They have investigated different strategies for feeding the inlet air in the bioreactor (saturated and unsaturated air) as alternative to remove the metabolic heat generated during the microbial growth by evaporative cooling. In that study the authors observed that in the batch mode using unsaturated air, evaporative cooling decreased the mean

temperature of the solid-bed, although the enzyme production was lower than that obtained using saturated air.

4.2.3. Agitated SSF Bioreactors without Forced Aeration

The typical bioreactors of this group are rotating-drum bioreactors and stirred-drum bioreactors in which the aim is to maximize the exposure of each substrate particle to the thermostated air circulating in the headspace (Durand, 2003). The rotating regime of the former bioreactor will be intermittent or continuous. In the intermittently agitated drum bioreactors act as tray bioreactor during the static period and as a rotating drum during the rotation period. The most important difference of the intermittently agitated ones from the trays is that intermittent agitation prevents mycelium from knitting the bed together (Mitchell et al., 2000). Intermittent agitation is thought to prevent the agglomeration of the mycelium and be less damaging to fungal mycelium than the continuous rotation (Sermanni and Tiso, 2008). The important parameters to be controlled are flow rate, temperature and humidity of the air and the frequency, intensity and duration of the agitation action (Mitchell et al., 2000). On the other hand, the design variables such as length and diameter of the bioreactor, inclination of the bioreactor, number, size and location of the baffles (in baffled rotating-drum), location of the inlet and outlet of the aeration system, presence or absence of an external water jacket, requirement of water addition during the process and substrate inlet and outlet design (in continuous operations) should be considered before the construction of a rotating-drum or stirred-drum bioreactor (Mitchell et al., 2006g).

Kalogeris et al. (2003) have determined the suitability of their intermittently agitated rotating-drum bioreactor for the effective control of important operating variables affecting growth of microbes in solid-state cultivation. In their 10-liter capacity system, they managed to control moisture content of the substrate, temperature of the bed (by controlling the temperature of the circulating water at the jacket of the bioreactor) and air flow through the bed for efficient enzyme production.

Stuart et al. (2000) worked on the growth of *Aspergillus oryzae* ACM 4996 on an artificial gel-based substrate and on steamed wheat bran during solid-state fermentations in 18.7 liter rotating-drum bioreactor. They have observed that for gel

fermentations fungal growth decreased as rotational speed increased, presumably due to increased shear. But, for wheat bran fermentations fungal growth improved under agitated compared to static culture conditions, due to superior heat and mass transfer. In this study they concluded that the effects of operational variables on the performance of SSF bioreactors are mediated by their effects on transport phenomena such as mixing, shear, heat transfer, and mass transfer within the substrate bed. In addition, the substrate characteristics affected the need for and the rates of these transport processes.

4.2.4. Agitated SSF Bioreactors with Forced Aeration

This group addresses the continuously agitated bioreactors. The most typical bioreactors of this category are: gas-solid fluidized bed, continuously-stirred aerated bed and rocking drum bioreactor (Sermanni and Tiso, 2008). These types of designs allow addition of water by spraying a fine mist onto the substrate. The water can be dripped through the perforations in the inner drum in the rocking drum bioreactors. By these ways, evaporative cooling will be applied for the removal of the generated heat that makes continuously agitated bioreactors superior to the static bioreactors that allow addition of water but not allow a well distribution of it inside the bed (Mitchell et al., 2000). Nagel et al. (2001) summarized the advantages of using a mixed SSF system as: obtaining a more homogenous culture medium in the bed, uniform distribution of the added materials during the process and enhancement of gas exchange, evaporation, and heat transfer. However, the sensitivity of the microorganism to the shear effects occurred during mixing and mechanical strength of the substrate particles should be considered for the choice of agitation regime (continuous or intermittent).

Continuously agitated and forcefully aerated bioreactors are not widely used. However, Nagel et al. (2001) have used an aseptic horizontal paddle mixer as a continuously mixed SSF bioreactor for the growth of *Aspergillus oryzae* on the whole wheat kernels. In this work they concluded that continuously mixed systems are promising for the simultaneous control of temperature and moisture. Their experimental work showed that the continuous mixing improved temperature control and prevented inhomogeneities in the bed. The continuous mixing regime was not damaging to the mycelial growth of the fungus on the wheat kernels, since the respiration rates were comparable to those in small, isothermal, unmixed beds. Mitchell et al. (2006d)

criticized this fact as the fungus grew underneath the seed coat was protected in this way from the shear forces. The other advantageous result obtained in this study (Nagel et al., 2001) was that the continuous mixing improved the heat transport to the bioreactor wall that reduced the requirement of evaporative cooling. On the other hand the wall cooling was not enough at the 2 m^3 scale, therefore water addition was required. In that case, mixing was again found to have positive effect on the process due to the maintenance of homogenous water addition.

Mitchell et al. (2000) stated that the rocking drum bioreactor consists of substrate held between two perforated drums lying horizontally, encased in an imperforated outer bioreactor shell. The outer two drums are rotated backwards and forth relatively slowly in relation to the inner drum.

In the gas-solid fluidized bed reactors, solid substrate is fluidized by upward airflow supplied by sufficient air velocity. The height of the column should be enough to support this flow. A mixer placed at the bottom of the bed may prevent agglomeration of the substrate. The uniformity of the substrate in the bed can support the increase in the surface area and gas flow which enhance the heat and mass transfer between the substrate particle and the gas phase are considered as the advantages of this bioreactor type (Bhargav et al., 2008; Mitchell et al., 2000).

CHAPTER 5

MATERIALS AND METHODS

The information about the agro-industrial residues, fungal strains and fermentation procedures was represented in detail in this chapter.

5.1. Microorganism and Propagation

Aspergillus sojae ATCC 20235 (wild type) was purchased in the lyophilized form from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. This wild type culture was randomly mutated using ultraviolet light exposure by Jacobs University gGmbH, Bremen and coded as *Aspergillus sojae* M3, M5/6 and M7. According to a modified procedure of Nicolás-Santiago et al. (2006), the mutagenesis was performed at an exposure time at which 90% of spores were inactivated (LD_{90}). The culture was propagated on YME agar plate (Figure 5.1) medium containing malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l), and incubated at 30 °C until well sporulation (1 week). Stock cultures of these strains were prepared with 20% glycerol-water and stored at −80 °C. Isolation of *A. sojae* pyrG deleted mutants by UV irradiation was described by Hartingsveldt et al. (1987) with slight modifications and coded as M7/2. *A. sojae* M7/2 was subjected to transformation with Glucanex Enzyme and Electroporation method which were coded as "vgb-tra" and "electro-vgb", respectively. But, the presence of vgb gene could not be completely confirmed. Therefore, they were called as mutant strains in the current study. The strains *A. sojae* M7/2, vgb-tra and electro-vgb were processed as a part of a M.S. thesis study by Betül Bardakçı (Bardakçı, 2010).

Figure 5.1. *Aspergillus sojae* M5/6 strain grown on YME plate

5.2. Preparation of Inoculum for SSF and SmF Experiments

The spore suspensions used as inoculum were obtained on molasses agar slants formulated by Göğüş et al. (2006): glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), $FeSO_4 \cdot 7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4 \cdot 5H_2O$ (12 mg/l), $MnSO_4 \cdot H_2O$ (15 mg/l) and agar (20 g/l), after the propagation step performed on YME agar using the stock cultures. Slant cultures were incubated at 30 °C for 1 week on the slanted test tubes (Figure 5.2a) and 2 weeks on the slanted 250 ml Erlenmeyer flasks (Figure 5.2b). Spores were harvested from the slants using sterile Tween 80 (0.02%) solution (5 ml for the test tubes, 50 ml for the Erlenmeyer flasks). The spore suspension was collected in a sterile falcon tube and stored at 4 °C until the inoculation step. The spore counts and viability counts were recorded prior to inoculation. Spores were counted manually, using a Thoma bright line hemacytometer (Marienfield, Germany). Besides, spore/g substrate was estimated by dividing the total number of obtained spores to the amount of substrate used in the extraction step.

<div style="text-align:center">(a)   (b)</div>
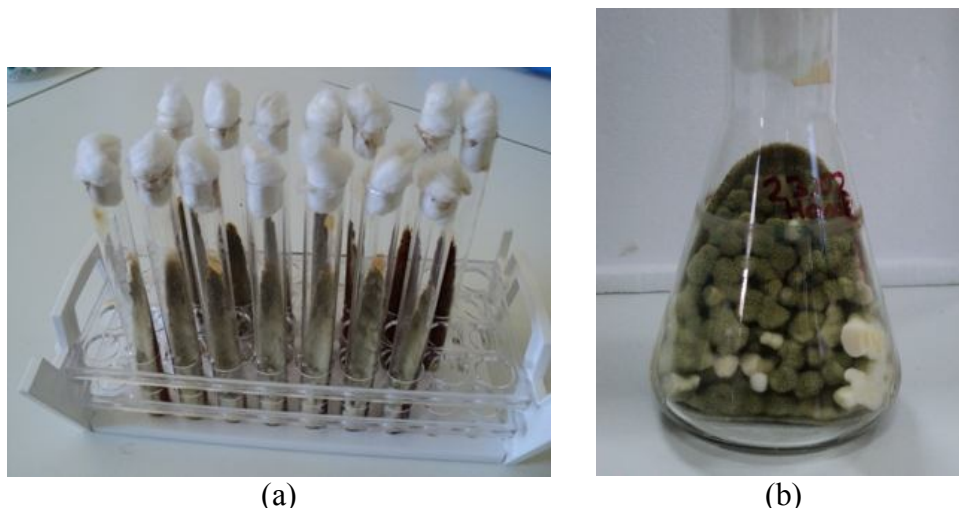
Figure 5.2. Spore production on (a) slanted test tubes, (b) slanted 250 ml Erlenmeyer flasks

The submerged (liquid) seed culture (referred as SmF) was prepared in the 250 ml Erlenmeyer flasks with the 50 ml medium consisting of maltrin (120 g/l), glucose (25 g/l), peptone (2.5 g/l), disodium phosphate (3.2 g/l) and monosodium phosphate (3.3 g/l). Each flask was inoculated with $1.7*10^7$ total spore/50 ml inoculum concentration which was optimized by Tari et al. (2007) and incubated by shaking at 150 rpm, 30 °C for 0, 24, 48 and 72 hours.

5.3. Enzyme Activity, Total Sugar and Protein Assays of Supernatant

Polygalacturonase (PG) activity was assayed according to the modified procedure of Panda et al. (1999) using 2.4 g/l of polygalacturonic acid as substrate at pH 4.8 and 40 °C. The amount of substrate and enzymes used were 0.400 and 0.086 ml, respectively. 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. Galacturonic acid (Sigma, St. Louis, MO) was used as standard for the calibration curve of PG activity. Total sugar content of the crude enzyme was determined with the phenol – sulphuric acid method of DuBois et al. (1955). Protein content of the crude enzyme was analyzed with the Bradford protein content determination method (Bradford, 1976).

5.4. Enzyme Extraction

At the end of the fermentation, appropriate amount of fermented sample was placed in a 250 ml Erlenmeyer flask. To these flasks Tween 80 (0.02%) solution was added with the 1:10 ratio (sample amount: Tween 80 solution). The content was dispersed with a glass baguette until a homogenous mixture was obtained and shaken at 150 rpm and 25 °C for 30 min. Afterwards, the pH of the flask content was measured with a pH meter and the content filtered through the cheese cloth (Figure 5.3) and centrifuged at 4 °C, 5000 rcf for 15 min. The supernatant was separated and used for the enzyme, protein and total sugar assays immediately.

Figure 5.3. Filtration of the flask content through the cheese cloth

5.5. Experimental Design and Statistical Analysis

The experimental design, statistical analysis of data and generation of model graphs were performed by Design Expert (7.0.0 trial version) software.

5.6. Solid-State Fermentation Conducted in 250 ml Erlenmeyer Flasks

Appropriate amount of solid substrate was placed in the 250 ml Erlenmeyer flask. Half of the appropriate amount of liquid calculated to maintain the desired initial moisture content was added to the medium before sterilization and dispersed by a glass

baguette until a homogenous mixture was maintained (Figure 5.4). Other half of the liquid was sterilized and used for the inoculation of the medium including the sufficient inoculum concentration. The content of the flask was mixed well with a sterile glass baguette until the homogenous spread of the inoculum on the solid substrate was obtained. The prepared flask was placed in the static incubator at the desired temperature for sufficient period of time.

Figure 5.4. Various solid substrates prepared for the SSF in the 250 ml Erlenmeyer flasks

5.7. Measurement of pH, Water Activity and Moisture Content of the Solid Substrates

For pH measurement, the mixture of 2.5 g sample and 25 ml distilled water were homogenized with Heidolph, SilentCrusher M (Heidolph Instruments GmbH & Co. KG, Germany) at 10.000 rpm for 1.5 minutes. pH of the homogenized samples were measured with Mettler Toledo Seveneasy pHmeter (Mettler-Toledeo AG, Switzerland) while being stirred on the magnetic stirrer (Figure 5.5a). Water activity of the samples was determined using a Rotronic HygroLab Benchtop Humidity Temperature Indicator (Rotronic AG, Bassersdrof, Germany) (Figure 5.5b). The moisture content of approximately 5 g of the samples were determined with a Precisa XM-60 Moisture Content Analyzer (Precisa Instruments, Diekinton, Germany) by drying the samples at 105 °C until a stable weight was reached (Figure 5.5c). Data were reported on a wet basis and were averages of two determinations.
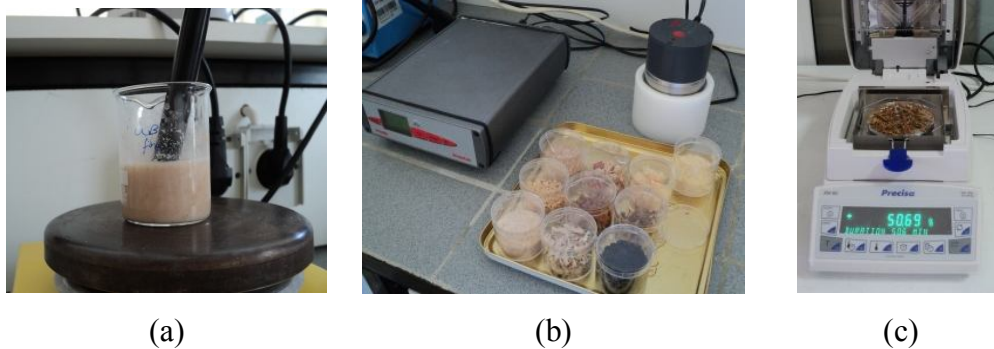
Figure 5.5. Measurement of (a) pH, (b) water activity and (c) moisture content of the agro-industrial residues

5.8. Agro-Industrial Residues

Agro-industrial residues (Figure 5.6) were supplied from Rinatura, Germany (wheat bran, Bremen), Beşler Wheat Flour Company, Gaziantep (wheat bran, Beşler), Beymail, Osmaniye (untreated peanut husk), Sunar Mısır Inc., Adana (corn cob), Konya Şeker Industry and Co., Konya (sugar beet bagasse), Dimes Food Industry and Trade Joint Stock Company, İzmir (grape stalk, apple pomace), Starbucks Coffee Company, Turkey (coffee husk), Konfrut Fruit Juice Concentrates and Purees, Denizli (apricot and peach pomaces). Rest of the residues were supplied from local markets or bakeries or prepared at home. All residues were stored at -18 °C until used. A home-type coffee grinder was used to grind orange peel, soy meat and corn cob. Coffee husk was already ground by the manufacturer. The pH, water activity, moisture content and pretreatment properties of the agro-industrial residues were summarized in Table 5.1.

Table 5.1. Screened agro-industrial residues and their physical properties in original form

Agro-industrial residue	pH	Water activity (a_w)	Moisture content (%) wet basis	Pretreatment for fermentation
Orange peel	4.58	0.525	6.54	-
Orange peel	4.61	0.455	6.91	ground
Soy meat	6.66	0.431	5.78	ground
Wheat bran, Bremen	6.36	0.513	8.86	-
Wheat bran, İzmir	6.23	0.395	8.37	-
Wheat bran, Beşler	6.50	0.697	13.03	-
Peanut husk	5.70	0.487	5.33	ground
Corn cob	6.14	0.643	10.23	ground
Grape stalk	4.25	0.948	59.91	chopped
Sugar beet bagasse	5.80	0.972	73.78	-
Coffee husk	6.50	0.964	62.94	-
Pomegranate pomace	3.63	0.841	61.25	Peels chopped
Orange pomace	4.21	0.830	80.27	Peels chopped
Carrot pomace	5.60	0.855	84.70	Peels chopped
Apple pomace	4.30	0.847	76.50	Peels chopped
Apricot pomace	3.63	0.897	80.50	Peels chopped
Peach pomace	3.96	0.872	82.30	Peels chopped

Figure 5.6. Pictures of agro-industrial residue samples

5.9. FT-IR Analysis of Agro-Industrial Residues

All infrared spectra (4000-650 cm^{-1}) were acquired with a Perkin Elmer Spectrum 100 FT-IR spectrometer (Perkin Elmer Inc., Wellesley, MA). This instrument was equipped with a horizontal ATR sampling accessory (ZnSe crystal) and a deuterated tri-glycine sulfate (DTGS) detector. Horizontal ATR accessory was used to collect the spectral data of agro-industrial residues. The homogenized agro-industrial residues were placed on the ATR accessory for the collection of spectra. The resolution was set at 2 cm^{-1} and the number of scans collected for each spectrum was 32. Air spectra were taken before each sample measurement. ZnSe crystal was cleaned with distilled water in between sample runs. Measurements were taken twice.

5.10. Characterization of Wheat Bran

Two wheat bran samples collected from two different bakeries in İzmir (coded as wheat bran, İzmir and Güzelbahçe) and Hazal Flour and Feed Manufacturing Company, Turgutlu, Manisa (coded as wheat bran, Hazal) were characterized for some of their physical and chemical properties.

5.10.1. Reducing Sugar Content

100 ml of distilled water was added into 5 grams of wheat bran and autoclaved at 105 °C for 5 minutes. After this process, the reducing sugar content at the soluble fraction of wheat bran was analyzed according to modified Nelson-Somogyi method.

5.10.2. Ash Content

Ash content of wheat bran was determined with the help of an ash oven (Protherm, Turkey). The empty crucibles were dried at 650 °C for 2 hours and their tares were recorded. Then 5 grams of wheat bran was added to the crucible and its moisture was removed in a drying-oven at 105 °C for 6 hours. The crucibles with the

dried samples were burned in the ash oven at 650 °C for 12 hours in order to get fully white ash. A desiccator was used to cool the crucibles. Test was done in two parallels.

5.10.3. Dietary Fiber Content

Dietary fiber content of the wheat bran was determined using a Total Dietary Fiber Assay Kit (Sigma-Aldrich). Samples of dried, fat-free foods were gelatinized with heat stable α-amylase and then enzymatically digested with protease and amyloglucosidase to remove the protein and starch present in the sample. Ethanol was added to precipitate the soluble dietary fiber. The residue was then filtered and washed with ethanol and acetone. After drying, the residue was weighed. Half of the samples were analyzed for protein and the others were ashed. Total dietary fiber content was estimated by subtracting the weight of the residue from the total weight of the protein and ash. Test was done in two parallels.

5.10.4. Protein Content

Protein content of the wheat bran was determined with the Kjeldahl method using full-automated nitrogen-protein digestion and distillation system (Gerhardt, Germany). 1 gram of wheat bran was placed in the burning tube. 15 ml of H_2SO_4 was added to the tube and heated to 430 °C until becomes clear. 80 ml of distilled water, 80 ml of boric acid and 70 ml of NaOH was used in the distillation part of the analysis. The coefficient of 6.25 was taken as the protein conversion factor. Test was done in two parallels.

5.10.5. Water Holding Capacity

5 grams of wheat bran was dried in moisture content analyzer at 105 °C until the constant weight was reached. The dried samples were transferred to 50 ml falcon tubes immediately and caps were closed. Then, 1, 3, 5, 7, 9, 11 and 13 ml of distilled water was added to the falcon tubes and mixed with a glass rod in order to obtain homogeneity. Water added falcon tubes were incubated at room temperature for 1 hour

and later centrifuged at 6000 rpm for 20 minutes. After this process, falcon tubes were turned upside down to remove the excess water for 30 minutes. Then the falcon tubes were weighed. Test was done in two parallels.

5.10.6. Particle Size Distribution

Retsch AS 200 Basic (Germany) sieve shaker and 850, 500, 250, 150 and 75 µm mesh size stainless steel sieves (20 cm diameter) and a receiver were employed for the determination of particle size distribution of wheat bran, İzmir and Güzelbahçe. Jeotest (Ankara, Turkey) sieve shaker and a receiver were employed for the determination of particle size distribution of wheat bran, Hazal. Sieving was performed for 15 minutes for each batch having approximately 100 g of wheat bran.

5.11. Effect of Environmental Conditions

The experiments performed to investigate the effect of inoculum size, incubation time and temperature, initial moisture content, pH, particle size and agitation frequency were conducted by using wheat bran, İzmir as the solid substrate. However, wheat bran, Hazal was used for the inoculum type experiments. *Aspergillus sojae* M5/6 was utilized as the fungal strain in all of the experiments under this section. Samples as whole flasks in triplicate were withdrawn at the defined time periods or end of the fermentation process. Final pH, extract volume, PG activity, total sugar and protein and spore count (where sporulation observed) measurements were done for each flask. Consumption of the total carbohydrate values were calculated via the difference between the related run and the initial carbohydrate value of the fermentation medium measured before the inoculation.

5.11.1. Effect of Inoculum Size

10 g of wheat bran was weighed into a 250 ml Erlenmayer flask and inoculated with 10^6, $2*10^6$ and 10^7 spore/g substrate of inoculum concentrations. 12 ml of distilled water (including the inoculum volume) was added to the media and incubation was

done at 22 °C for 4 days. Viability of the inoculated spore solutions at the inoculation day was recorded. Total sugar, protein, spore count and biomass measurements could not be performed.

5.11.2. Effect of Incubation Period

The effect of incubation period was investigated with the help of a time course study. For this purpose, 10 g of wheat bran was moistened with 12 ml of distilled water including the 10^7 spore/g substrate inoculum and incubated at 22 °C. SSF was conducted for 8 days. 3 parallel flasks were removed and analyzed at every 24 hours.

5.11.3. Effect of Incubation Temperature

To investigate the effect of temperature on the PG production and fungal growth fermentation was conducted at the temperatures of 18, 22, 26, 30, 34, 37 and 40 °C. 10 g wheat bran was moistened with distilled water including the inoculum size of 10^7 spore/g substrate and incubated for 4 days.

5.11.4. Determination of Moisture Content and Water Activity Relationship of Wheat Bran

Five grams of wheat bran was dried in moisture content analyzer at 105 °C until the constant weight was reached. The dried samples were transferred to the capped cups of hygrometer and appropriate volume of distilled water was added to reach the desired moisture content (8 to 62 %) immediately. The water added wheat bran was mixed well with a glass rod to obtain homogeneity. The water activity values of the prepared samples were measured with a Rotronic HygroLab Benchtop Humidity Temperature Indicator (Rotronic AG, Bassersdrof, Germany) at 21 °C. Test was done in two parallels.

5.11.5. Effect of Moisture Content or Water Activity

10 g of wheat bran was moistened with the appropriate amount of distilled water (including the 10^7 spore/g substrate inoculum) in order to obtain the moisture content values of 20, 25, 30, 35, 40, 55 and 62%. The inoculated flasks were incubated at 37 °C for 4 days.

5.11.6. Effect of Initial pH

10 g of wheat bran was moistened with the appropriate volume of 0.1 M acetate buffer (for pH 4, 5 and 5.5) or 0.1 M phosphate buffer (for pH 6, 7 and 8) including the spore solution of 10^7 spore/g substrate to obtain 62% moisture content. The inoculated flasks were incubated at 37 °C for 4 days. Distilled water was used instead of buffers as a control flask.

5.11.7. Effect of Particle Size of the Substrate

10 g of 75>, 75-150, 150-250, 250-500, 500-850 and 850< μm particle sized wheat bran fractions were weighed into 3 replicates of 250 ml Erlenmeyer flasks. 10 g of wheat bran (not sized or sieved) in the original form was weighed in 3 replicates of 250 ml Erlenmeyer flasks and used as the control. For both particle sized and original wheat bran containing flasks, half of the appropriate amount of distilled water calculated to maintain 62% initial moisture content was added to the medium before sterilization, whereas other half of the water was sterilized and used for the inoculation of the medium including the 10^7 spore/g substrate of inoculum concentration. The incubation was done at 37 °C for 4 days and a wide beaker full of distilled water was placed in the incubator in order to prevent drying of the solid substrate. Viability of the inoculated spore solutions was recorded at the inoculation day.

5.11.8. Effect of Agitation Frequency

10 g of 150-250 μm particle sized wheat bran was weighed into 3 replicates of 250 ml Erlenmeyer flasks. Half of the appropriate amount of distilled water calculated to maintain 62% initial moisture content was added to the medium before sterilization, whereas other half of the water was sterilized and used for the inoculation of the medium including the 10^7 spore/g substrate of inoculum concentration. The incubation was done at 37 °C for 4 days and a wide beaker full of distilled water was placed in the incubator in order to prevent drying of the solid substrate. The replicate Erlenmeyer flasks were agitated thoroughly by beating the flask on the palm of the hand for about a minute for one, two, three, four and five times a day until the termination of the fermentation. Three replicates of the flasks were not agitated throughout the process and referred as the control of the experiment. Viability of the inoculated spore solutions was recorded at the inoculation day.

5.11.9. Effect of Inoculum Type

0, 24, 48 and 72-hour incubated SmF culture were prepared as indicated in Section 5.2 and inoculated to 10 g of 100-250 μm particle sized wheat bran, Hazal weighed in 2 replicates of 250 ml Erlenmeyer flasks. The initial moisture content of the SSF media of each flask was adjusted to 62%; half of the appropriate amount of liquid calculated to maintain this moisture content came from SmF seed culture (sterile distilled water including 10^7 spore/g substrate spore solution only for the control flasks) and the other half was added as distilled water before the sterilization process. Two replicates of SSF flasks were removed and extracted on the 3rd, 4th and 5th days of the incubation conducted at 37 °C. None of the Erlenmeyer flasks were agitated during the SSF processes due to the unknown effect of the agitation on the viability of the inoculated pellets. A wide beaker full of distilled water was placed in the incubator in order to prevent drying of the solid substrate. The morphology of the pellets obtained in the SmF seed cultures were characterized by using image analysis (Cox and Thomas, 1992). Pellet particles were analyzed for determination of the number of pellet per given volume and pellet size. Image analysis was performed with the software package Image-Pro Plus 4.5.1 (Media Cybernetics Inc., Silver Spring, MD, USA). The size of

the pellet was quantified using the diameter corresponding to a circular area equivalent to the pellet projected area (Lopez et al., 2005).

5.12. Solid-State Fermentation Conducted in Trays

The effect of solid substrate thickness experiments were conducted in the 1500 cc Borcam ® (borosilicate glass) casseroles with inner diameter of approximately 14 cm. These casseroles are; inert, tolerable to the temperatures up to 300 °C, resistant to be autoclaved at 121 °C many times, easy to prepare and clean for the next fermentation and can conduct the heat of the incubating environment well to the solid substrate with a thermal conductivity of (20 °C) 1.14W/m°C. Appropriate amount of wheat bran (Hazal) was placed in each casserole. The thickness of the wheat bran in the casserole was determined by measuring the depth of the flattened wheat bran layer from ten different points (5 points from the inner region and 5 points from the side region) by Torq 150 mm Digital Caliper before the addition of water (Figure 5.7a). The average of these 10 measurements was calculated as the thickness of the solid substrate. Half of the appropriate amount of distilled water calculated to maintain 62% initial moisture content was added to the medium before sterilization and dispersed by a glass baguette until a homogenous mixture was maintained, whereas other half of the water was sterilized and used for the inoculation of the medium including the 10^7 spore/g substrate of inoculum concentration. The content of the container was mixed gently with a sterile glass baguette to maintain homogenous distribution of the inoculated fungal spores. The casserole was covered with 3 piles of sterile bones during the fermentation and also with aluminum foil at the autoclaving and handling steps. The fermentation was performed in the Memmert HCP-108 Humidity Chamber at the desired relative humidity (%) and 37 °C for 4 days (Figure 5.7b). The humidity chamber was cleaned with a diluted disinfectant just before the beginning of the incubation process in order to reduce the risk of bacterial contamination from the surrounding air. Also, sterile distilled water was used at the humidity supplier external tank.

<div align="center">(a)   (b)</div>

Figure 5.7. (a) Measurement of solid substrate thickness in the bed (b) SSF conducted in glass trays

5.13. Biochemical Characterization of the Crude PG Enzyme

The polygalacturonase enzyme that was biochemically characterized was produced by *Aspergillus sojae* M5/6 with wheat bran (Hazal) in the 250 ml Erlenmeyer flask under the optimized conditions (10^7 spore/g substrate inoculum, 4 days of fermentation, 37 °C, 62% initial moisture content, water as the moistening agent, 150 – 250 μm particle size, 3 times/day agitation and spore solution as the inoculum type). All of the experiments (except molecular weight determination) were conducted in 3 parallels.

5.13.1. Effect of pH on the Activity and Stability of PG

The pH values investigated were 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. In these analyses, pH was adjusted using the following buffer systems: acetate (pH 3.0, 4.0, 5.0), phosphate (pH 6.0, 7.0, 8.0), Tris-HCl (pH 9.0, 10.0) and Na_2HPO_4-NaOH (pH 11.0, 12.0). The concentration of each buffer was 0.1M. The optimum pH of PG was determined by standard PG activity assay (Section 5.3) with polygalacturonic acid (2.4 g/l) as substrate dissolved in the buffer systems mentioned. To study the stability as a function of pH, crude enzyme was mixed (1:1) with the buffer solutions

mentioned above and aliquots of the mixture were taken to measure the residual PG activity (%) under standard assay conditions after incubation at 30 °C for 2 hours.

5.13.2. Effect of Temperature on the Activity and Stability of PG

The optimum temperature of the PG was determined by incubation of the reaction mixture (pH 4.8) for 20 minutes, at different temperatures ranging from 25 to 80 °C (25, 30, 40, 50, 60, 70 and 80 °C) and measuring the activity with standard PG activity assay. Before the addition of enzymes, the substrate (2.4 g/l polygalacturonic acid) was pre-incubated at the respective temperature for 10 minutes. The thermostability of the crude PG was investigated by measuring the residual activity after incubating the enzyme at various temperatures ranging from 25 to 80 °C (25, 30, 40, 50, 60, 70 and 80 °C) for 30 and 60 minutes. The incubation medium used was 0.1 M (pH 4.8) sodium acetate buffer system.

5.13.3. Effect of Metal Ions and Various Compounds on PG Stability

The effect of various metal ions and chemical compounds were investigated by incubating the PG enzyme in the presence of reagents given in Table 5.2 with the volumetric ratio of 1:1 at 30 °C for 1 hour. At the end of this incubation period appropriate amount of aliquots were taken and standard PG activity assay was conducted immediately to measure their residual PG acitvities. The control of this set of experiments was the untreated polygalacturonase enzyme and its PG activity was taken as 100%. The concentrations of the acetic acid, citric acid, sucrose, glucose, fructose, gallic acid, malic acid, ascorbic acid and ethanol were chosen according to the chemical characterization of the orange juice and wine obtained from the cv. Kozan of Turkey by high pressure liquid chromatography (HPLC) analyses (Kelebek et al., 2009). The concentration of tartaric acid was decided according to the determination of organic acids in white grape juice by liquid chromatography-mass spectrometry (LC-MS/MS) analysis (Ehling and Cole, 2011). The concentration of the ammonium sulfate was determined according to the maximum limit of the ammonium sulfate that is allowed in the grape cider stated in the wine communication under Turkish Food Codex Regulations (notification no. 2008/67) (Official Gazette, 2009).

Table 5.2. Concentrations of various metal ions and chemical compounds tested for their effects on the stability of the polygalacturonase

Metal Ion/Chemical Compound	Concentration
Acetic acid	0.3 g/l
Citric acid	9.1 g/l
Sucrose	59 g/l
Glucose	32 g/l
Fructose	28 g/l
Gallic acid	3.33 mg/l
Malic acid	1.06 g/l
Tartaric acid	0.9 g/l
Ascorbic acid	0.49 g/l
Ammonium sulphate	1 g/l
$CaCl_2$	0.5 mM
$MgCl_2$	0.5 mM
$CuSO_4$	0.5 mM
KCl	0.5 mM
$FeCl_3$	0.5 mM
$MnCl_2$	0.5 mM
NaCl	0.5 mM
EDTA	0.5 mM
Ethanol	12.6%

5.13.4. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

The enzyme samples were incubated at temperatures of 30, 40, 50 and 60 °C for 30 and 60 minutes for the thermal inactivation kinetic studies. Aliquots were withdrawn and cooled in an ice bath prior to the standard PG activity assay. The residual activity was expressed as % of the initial activity. The inactivation rate constants (k_d) were calculated from the slopes of the curves in the semilogarithmic plot of residual activity versus time and apparent half-lives were estimated using the Equation 5.1. The half-life is known as the time where the residual activity reaches 50%.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{5.1}$$

The Arrhenius plot was used to analyze the variation of k_d according to the temperature (Shuler and Kargi, 2002). The inactivation energy (E_d) was calculated from the Arrhenius Equation as:

$$k_d = k_0 \exp\left(-\frac{E}{RT}\right)$$

(5.2)

or

$$\ln(k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T}$$

(5.3)

The values of E_d and k_0 were estimated respectively from the slope and intercept of the plot of $\ln(k_d)$ versus $1/T$ obtained from Equation 5.3 (R= 8.3145 J mol^{-1} K^{-1}).

5.13.5. Estimation of Thermodynamic Parameters during Inactivation of Polygalacturonase

The enthalpy of inactivation (ΔH^*) for each temperature was calculated according to Equation 5.4.

$$\Delta H^* = E_d - RT$$

(5.4)

The values for the Gibb's free energy (ΔG^*) of inactivation at different temperatures were calculated from the first-order constant of inactivation process by using Equation 5.5.

$$\Delta G^* = -RT \ln\left(\frac{k_d h}{\kappa T}\right)$$

(5.5)

where h (=6.6262x10^{-34} Js) is the Plank constant, and κ (= 1.3806x10^{-23} JK^{-1}) is the Boltzmann constant.

From Equations 5.4 and 5.5 the entropy of inactivation (ΔS^*) of PG was calculated from Equation 5.6 (Bhatti et al., 2006).

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T}$$

(5.6)

5.13.6. Molecular Weight Investigation of PG

The molecular weight of the crude PG enzyme was analyzed by sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). 12% acrylamide containing separating gel (pH 8.5) and 4% acrylamide containing stacking gel (pH 6.5) were employed.

The total protein contents of the samples were measured by Bradford method (Bradford, 1976) and obtained as 0.28, 0.37, 0.42, 0.79 and 8.5 mg/ml for the submerged, flask, drum bioreactor, tray bioreactor PG and commercial pectinase (Pectinase from *Aspergillus niger*, Sigma P2736-50mL) enzymes, respectively. The submerged fermentation-type PG enzyme was supplied by Nihan Göğüş and produced within the concept of her PhD thesis studies under optimized conditions using orange peel as the substrate in the serial type of 1l-scale bioreactor from *Aspergillus sojae* M5/6. The drum bioreactor sample was taken from the 55th hour of the process. The enzyme sample obtained from tray-type fermentation was performed at 70% relative humidity as explained in Section 5.12. The commercial pectinase was diluted to 1/100 with the sodium acetate buffer (pH 4.8), however other samples were not diluted since they have low protein concentrations close to each other. Sample buffer was added to the each sample with the volumetric ratio of 1:1. Then, all of the samples were denatured by boiling in the water bath at 95 °C for 4 minutes and cooled to the room temperature before loading to the gel. A prestained protein molecular weight marker (Thermo Scientific, USA) with the range of 10 – 250 kDa was used as size standards in protein electrophoresis. After these pretreatments, samples (20 µl) and protein marker (3 µl) were loaded on the gel and run on the gel placed in the 20% trichloroacetic acid (TCA) solution (x1) under 100 Volt for 120 minutes. Staining of the gel was applied by silver staining technique. The gel was visualized by BioRad VersaDoc Imaging system (BioRad, USA) and processed by Quantity One Software (BioRad, USA).

5.14. Solid-State Fermentation Conducted in the Drum-type SSF Bioreactor

5.14.1. Set-up of the Horizontal Drum Bioreactor

The schematic diagram of the set-up of the solid-state fermentation (SSF) process conducted in the horizontal drum bioreactor that was designed and constructed by Dr. Sayit Sargin (Bioprocess Group, Dept. of Bioengineering, Ege University) was presented in Figure 5.8. This system was mainly composed of 3 parts namely: steam generator, air humidifier, horizontal drum bioreactor and measurement system. The steam generator was used for the sterilization of the bioreactor and air humidifier. The relative humidity of the air was adjusted to the desired level with the help of the own valves of the air humidifier (Figure 5.9a) and the 2-way PTFE valve added to the system.
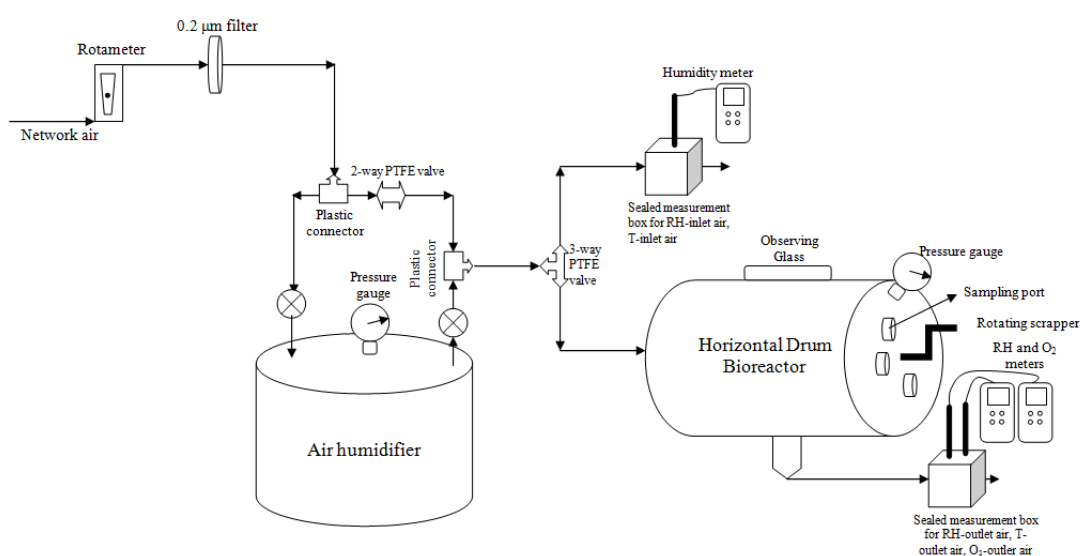
Figure 5.8. Schematic diagram of the horizontal drum SSF bioreactor set-up

Figure 5.9b shows the photo of horizontal drum bioreactor. The main components of the horizontal drum bioreactor were the rotating scrapper inside the main body, water jacket at the bottom half of the main body, observation glass on the top of the main body which was also used as a cap for the loading of the inoculated substrate and the sampling ports on the right side of the bioreactor. There were little holes on the center of the scrapper part for the introduction of air all throughout the main body. This

scrapper was modified by replacing plastic pieces (Figure 5.9c) on the tabs in order to adapt it to the particle size of the substrate (100-250 μm wheat bran). The measurement system was integrated to the bioreactor for monitoring some operation variables. Figures 5.9d and e presents the measurement systems. The humidity and temperature of the inlet and outlet air was measured with a humidity meter (Lutron YK-90HT) that can also measure the temperature. The accuracy of this humidity meter was: $\geq 70\%$; $\pm$ (3% reading + 1% RH) and < 70%; $\pm$ 3% RH. The oxygen concentration of the outlet air was measured with an oxygen meter (Lutron DO-5510 Oxygen meter). The accuracy of this oxygen meter with the polarographic type oxygen probe was $\pm 0.7\%$ O_2 at 23 ± 5 °C.

(a)

(b)

(c)

(d)

(e)

Figure 5.9. Pictures from the SSF process conducted in the horizontal drum bioreactor system

5.14.2. Experimental Procedure of the SSF with Horizontal Drum Bioreactor

The flow of the actions performed during the whole SSF process was diagrammed in the Figure 5.10. In the current section details of each action will be stated.

At the beginning of the process previously cleaned and rinsed (with distilled water) components of the horizontal drum bioreactor were mounted on the main body. Silicon seals were placed between the caps and the main body on the two sides of the bioreactor. The bioreactor was sterilized by passing the steam at 136 °C and 2.2 bars. The sterilization process started when the thermometer and pressure gauge of the bioreactor showed 121 °C and 1.5 psi, respectively and continued by holding the bioreactor at these values for 20 minutes. The hot and pressured steam was supplied by a steam generator (Akkaya Blowtherm, Konya, Turkey). The bioreactor was connected to the compressed air dryer (Drytec VT20, Angleur, Belgium) and dry air was filtered through 0.2 μm Midisart filter and passed through the main body of the bioreactor for the purpose of removing the moisture and cooling for overnight. Simultaneously, air humidifier was filled with 10 liters of distilled water and sterilized with the same method mentioned above, and then left cooling to the ambient temperature.

The fittings of the bioreactor and air humidifier were sterilized in the autoclave (121 °C, 15 min) separately and connected to the bioreactor and air humidifier under aseptic conditions. At the same time, the water circulator (Polyscience temperature controller, Model 912, USA) was connected to the water jacket of the main body and water at 37 °C was circulated for minimum 3 hours before the inoculation until the jacket balanced at 37 °C. This was checked by measuring the temperature of the outlet water with the help of K-type thermocouple and temperature recorder (Fluke IR Thermometer 568).

As the drying and water cycling processes continued, the relative humidity of the inlet air was adjusted to the desired level as described in the modifications part.

PTFE rings (250g rings/1500g wheat bran) of 8*10 mm (Ø*L) were added to the wheat bran with the purpose of maintaining air channels. 1500 g of wheat bran holding the half volume of the required water (for the 62% initial moisture content) and PTFE rings (Figure 5.11a) was autoclaved (121 °C, 30 min) in the plastic autoclave bags and cooled to the room temperature. After cooling, the spore solution (obtained from slanted

flask media, preparation described in Section 5.2) diluted with sterile water was added to the wheat bran (inoculum size: 10^7 spore/g substrate) under aseptic conditions. With this process, the initial moisture content of the fermentation medium became around 62%. The bag containing inoculated wheat bran was shaken rigorously in order to obtain homogenous distribution of the spores all around the substrate.

The inoculated wheat bran was immediately loaded to the bioreactor via the upper cap under aseptic conditions with the help of a gas burner. The upper cap closed immediately and the medium inside was mixed by the rotating scrapper with the aim of establishing the medium and supplying interparticle air between the substrate particles. Following these actions, the inlet air was connected and the air with the desired rate and humidity was supplied to the fermentation medium. The temperature of the inlet air could not be adjusted but could be measured as mentioned before. The 0-hour of the fermentation process was accepted as the time of the loading of the inoculated substrate to the bioreactor.
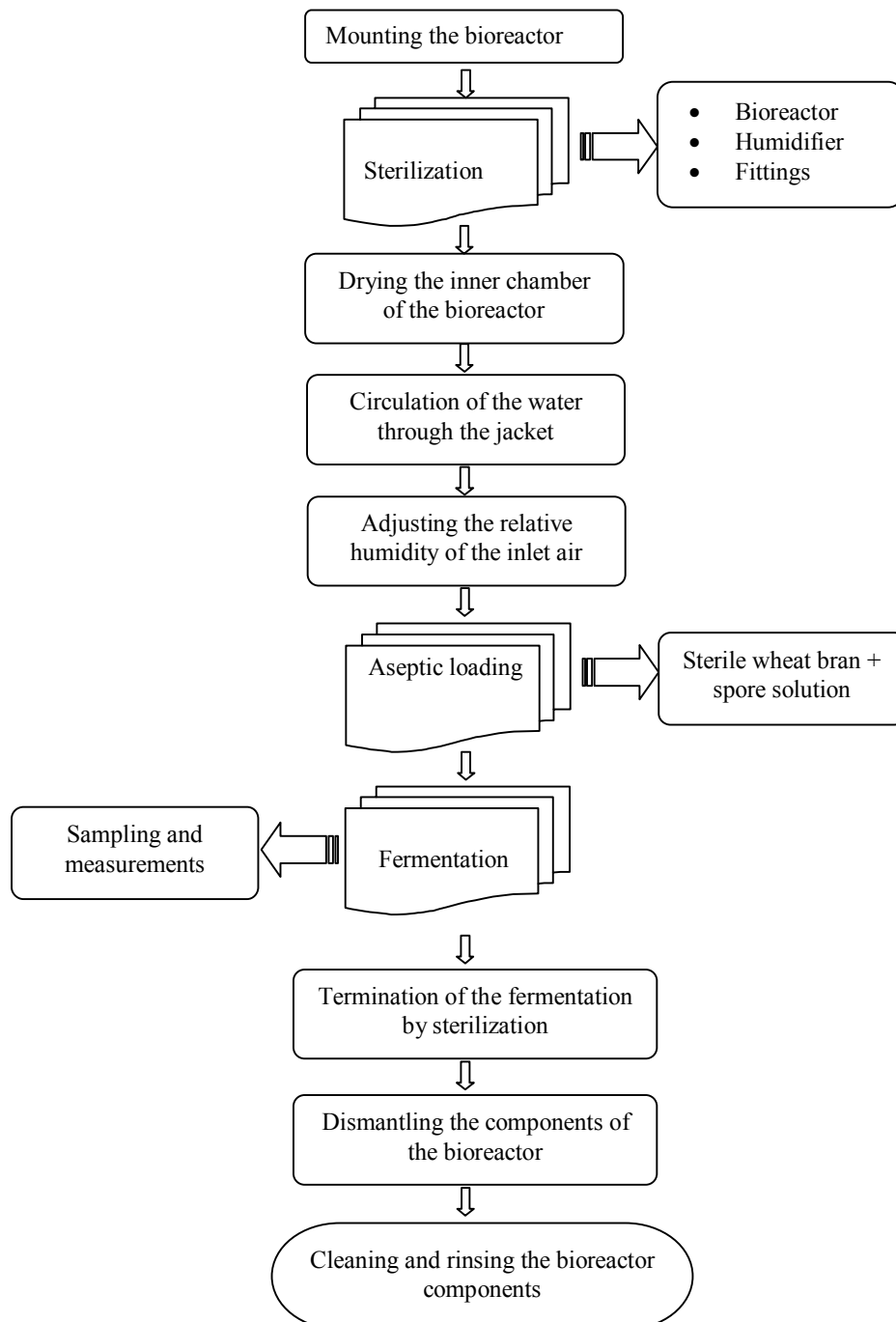
Figure 5.10. Flow diagram of the solid-state fermentation process conducted in the horizontal drum bioreactor

The measurements of the operating variables were performed just before the sampling process. The measured variables and related measurement procedures were as follows;

-    Relative humidity of the inlet air (%): The inlet air valve was turned to the way that direct the inlet air to the sealed box with the relative humidity probe (Figure 5.9d and e). The relative humidity of the air inside the sealed box was observed

for app. 10 minutes until the humidity meter reaches the steady state. Then, the relative humidity of the air was recorded by the humidity meter for 5 minutes. After this 5-minute period, the maximum and minimum recorded relative humidity values were recalled and recorded on the chart. This was operated for two times and finally the average of this four measured relative humidity values were calculated.

- Temperature of the inlet air (°C): The relative humidity probe mentioned in the previous item has the property of temperature measurement. The measurement of the inlet temperature air has the same procedure with the measurement of relative humidity of the inlet air.

- Temperatures of the inlet and outlet water (°C): The K-type probe, replaced in the silicon pipe entering the water jacket of the bioreactor was connected to the IR thermometer. The temperature value on the screen after a 10 second holding period was recorded on the chart.

- Temperature of the jacket wall (°C): The temperature of the bioreactor wall was measured from 4 points: 2 points on the sides and 2 points at the bottom by exposing the infrared rays on these points for 10 seconds. The temperature value on the screen after a 10 second holding period was recorded on the chart.

- Temperature of the water bath (°C): The K-type probe was replaced in the water bath and connected to the IR thermometer. The temperature value on the screen after a 10 second holding period was recorded on the chart.

- Temperature of the headspace of the fermentation medium (°C): This value was read and recorded from the analogue thermometer placed on the side cap of the bioreactor.

- Relative humidity of the outlet air (%): After the measurement of relative humidity and temperature of the inlet air, the 3-way valve was turned to the way that direct the air inside the bioreactor. A silicon pipe (covered with foam) was connected to the outlet at bottom of the bioreactor and to the second sealed box on the other end. The relative humidity of the outlet air was observed for app. 10 minutes until the humidity meter reaches the steady state. Then, the relative humidity of the air was recorded by the humidity meter for 5 minutes. After this 5-minute period, the maximum and minimum recorded relative humidity values were recalled and recorded on the chart. This was operated for two times and finally the average of this four measured relative humidity values were

calculated. The connected pipe was removed after the measurement and dried by passing through dry air in order to prepare the pipe for the next measurement.

- Temperature of the outlet air (°C): The relative humidity probe mentioned in the previous item has the property of temperature measurement. The measurement of the outlet temperature air has the same procedure with the measurement of relative humidity of the outlet air.

- Oxygen concentration of the outlet air (%): The oxygen probe was also inserted in the second sealed box connected to the outlet of the bioreactor. However, just before each oxygen measurement the cap of the box was removed and the oxygen probe was calibrated with zero and 100% oxygen in ventilated environment. Then the cap was closed tightly. The measurement of the oxygen concentration of the outlet air has the same procedure with the measurement of relative humidity of the outlet air. The difference was that the rate of air was adjusted to 1 l/min 5 minutes before the measurement of oxygen concentration of the outlet air. After this measurement, the air rate was adjusted back to the original level.

In order to monitor the fermentation process, at least three samples were taken each day. Approximately 12 grams of sample was taken at each sampling process under aseptic conditions. Just before the sampling, the rotating scrapper inside the bioreactor was turned two times (1 round clockwise, 1 round counterclockwise) in order to obtain a representative sample. Then alcohol was spread on the sampling rod and burned with the gas burner. Samples were taken by digging the sterilized sampling rod to the bioreactor via the sampling port placed on the side cap. The sample was immediately transferred to the sterile 50-ml plastic falcon. Sampling can be seen in Figure 5.11b.

(a)   (b)

Figure 5.11. Pictures of (a) PTFE rings within the biomass and (b) aseptic sampling process

Approximately 3.5 grams of the sample was (PTFE rings separated) used for the moisture content measurement. The 3.5 grams of the sample was dried in the moisture content analyzer (Mettler Toledo HR83 Halogen) at 105 °C for 40 minutes at the standard drying mode. The measured moisture content of the sample was recorded. The decision on the rate and humidity of the inlet air was made depending on the measured moisture content of the sample and the relative humidity of the outlet air. Enzyme extraction procedure was applied to 5 grams of the sample immediately. This procedure was described before in Section 5.4. The supernatant obtained from the extraction process was stored at -20 °C in 15 ml plastic falcon tubes.

At the end of the fermentation period 4 samples were taken and the fermentation terminated by sterilizing the bioreactor via hot and pressurized steam as the same procedure applied at the beginning of the process.

After the sterilization process, the components of the bioreactor were dismantled. The ingredient of the bioreactor was stored for a few days to separate the PTFE rings. The main body and dismantled components of bioreactor was washed with dishwashing liquid and rinsed with distilled water. After the drying of these components the overall cycle was repeated.

CHAPTER 6

PRELIMINARY SSF STUDIES WITH ORANGE PEEL AND *Aspergillus sojae* M3

In this part of the study, a set of experiments were performed in order to investigate the polygalacturonase (PG) and spore production properties of the first mutant *Aspergillus sojae* strain. The important factors affecting these responses were optimized with the help of experimental design and statistical tools. This part of the study constructed the basis of the following parts with respect to set up of solid-state fermentation (SSF) procedures.

6.1. Screening of the Factors Affecting PG Activity and Spore Count

A 2^4 factorial experimental design with double replicate was employed in order to investigate the effects of orange peel concentration (within the wheat bran), HCl concentration, incubation time and inoculum size and their interactions on the PG activity and spore count. The investigated levels of these factors can be found in Table 6.1.

Table 6.1. The investigated factors and their levels in the experimental design of the screening process

Factors	Sign	Actual Factor Levels	
		-1	+1
Orange peel concentration (%)	X_1	5	30
HCl concentration (mM)	X_2	20	200
Incubation time (day)	X_3	4	8
Inoculum size (spore/g substrate)	X_4	10^3	2×10^6

The ANOVA results in Table 6.2 indicated that the constructed model is significant with a p-value of 0.0008 and the most important (p-value<0.0500) factors affecting the PG activity were orange peel concentration (X_1), HCl concentration (X_2), incubation time (X_3) main factors and orange peel concentration-incubation time (X_1X_3)

interaction. According to these results it was found useful to study these factors in order to optimize the conditions that maximize the PG activity even further.

The results showed that neither inoculum size nor its interactions have significant effect on the PG activity. Based on this fact, in the optimization part of this study the inoculum size was fixed at 10^3 spore/g substrate, where the maximum PG activity was obtained.

Table 6.2. ANOVA table for the PG enzyme activity at the screening step (reduced model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Block	41.2	1	41.2		
Model	1936.4	9	215.2	5.3	0.0008
X_1	183.8	1	183.8	4.5	0.0452
X_2	195.5	1	195.5	4.8	0.0395
X_3	936.4	1	936.4	23.1	< 0.0001
X_4	24.7	1	24.7	0.6	0.4440
X_1X_2	85.5	1	85.5	2.1	0.1613
X_1X_3	180.0	1	180.0	4.4	0.0473
X_2X_3	50.8	1	50.8	1.3	0.2758
X_2X_4	139.9	1	139.9	3.5	0.0773
X_3X_4	139.9	1	139.9	3.5	0.0773
Residual	851.3	21	40.5		
Total	2828.9	31			
Std. Dev.	6.4		R-Squared	0.6946	
Mean	15.0		Adj R-Squared	0.5637	
C.V. %	42.5		Pred R-Squared	0.2909	
PRESS	1976.8		Adeq Precision	9.2168	

The ANOVA results in Table 6.3 for the spore count response indicated that orange peel concentration (X_1), orange peel concentration-incubation time (X_1X_3), HCl concentration-incubation time (X_2X_3) have significant effects on the spore count. The maximum spore count was obtained as 1.19×10^9 spore/g substrate at 30% orange peel concentration, 200 mM HCl concentration, 8 days of incubation and using 10^3 spore/g substrate inoculum levels.

Table 6.3. ANOVA table for the spore count at the screening step (reduced model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Block	7.16E+14	1	7.16E+14		
Model	1.94E+18	11	1.77E+17	3.03	0.0164
X_1	3.59E+17	1	3.59E+17	6.16	0.0226
X_2	2.08E+16	1	2.08E+16	0.36	0.5573
X_3	1.81E+16	1	1.81E+16	0.31	0.5834
X_4	2.49E+17	1	2.49E+17	4.27	0.0526
X_1X_2	1.2E+16	1	1.2E+16	0.21	0.6546
X_1X_3	3.52E+17	1	3.52E+17	6.04	0.0237
X_1X_4	1.08E+17	1	1.08E+17	1.85	0.1894
X_2X_3	5.24E+17	1	5.24E+17	9.01	0.0073
X_3X_4	2.1E+14	1	2.1E+14	0.00	0.9527
$X_1X_2X_3$	1.9E+17	1	1.9E+17	3.26	0.0869
$X_1X_3X_4$	1.11E+17	1	1.11E+17	1.90	0.1841
Residual	1.11E+18	19	5.82E+16		
Total	3.05E+18	31			

Std. Dev.	2.41E+08	R-Squared	0.6373	
Mean	4.51E+08	Adj R-Squared	0.4272	
C.V. %	53.49	Pred R-Squared	0.0290	
PRESS	3.14E+18	Adeq Precision	5.6837	

6.2. Optimization of the Factors Affecting the PG Activity and Spore Count

The results of the screening step of this study have shown that orange peel concentration, HCl concentration and incubation time were the most important factors affecting the PG activity. Besides these variables another factor variable (incubation temperature) which was excluded in the screening step due to its known significant effect was included in the optimization step in order to determine the optimum temperature range and its possible interactive effect on other variables. In fact, Krishna (2005) states that; the most important of all the physical variables affecting SSF performance is temperature, because growth and production of enzymes or metabolites are usually sensitive to temperature. Furthermore, many researchers have observed that incubation temperature is a major parameter affecting the production of pectinases in SSF (Krishna, 2005; Patil and Dayanand, 2006a; Shivakumar and Nand, 1995). Patil and Dayanand (2006a) explain this fact, as temperature is known to influence the metabolic rate of the organism involved in the process, which in turn determines the

amount of the end product. It is well known that fungi can grow over a range of 20 to 55 °C, nevertheless the optimum temperature for the growth could be different from that of product formation (Bhargav et al., 2008). Hence, all these factor variables were taken into consideration in the optimization step and their effects were investigated on PG activity and spore count using a Face-Centered Composite Design (FCCD).

The levels of the chosen factors for the optimization process were re-determined according to the results obtained at the screening step and given in Table 6.4. It was observed in the screening step that orange peel concentration and incubation time at their low levels and HCl concentration at its high level, have caused higher PG activities. Based on this, in the optimization step orange peel concentration, incubation time and HCl concentration were decided to be investigated at 2-15%, 3-6 days and 50-250 mM ranges, respectively. Additionally, the range of the incubation temperature was searched at 22-37 °C. These levels were also in accordance with the optimization of the factors affecting the spore count response.

Table 6.4. The investigated factors and their levels in the experimental design of the optimization process

Factors	Sign	Actual Factor Levels		
		-1	0	+1
Orange peel concentration (%)	X_1	2	8.5	15
HCl concentration (mM)	X_2	50	150	250
Incubation time (day)	X_3	3	4.5	6
Incubation temperature (°C)	X_4	22	29.5	37
Inoculum size (spore/g substrate)	-		10^3	

The most important factors affecting the PG activity were selected (p-value<0.1000) according to the ANOVA results of the responses and a model was developed (Table 6.5). This analysis indicated that, incubation time-incubation temperature (X_3X_4), orange peel concentration-incubation temperature (X_1X_4), orange peel concentration-incubation time (X_1X_3) interactions and quadratic forms of incubation time (X_3^2) and temperature (X_4^2) were the most important factors. The orange peel concentration (X_1), incubation time (X_3) and incubation temperature (X_4) main factors were found to be insignificant terms, but they were included in the model because of their strong interactions with the incubation time and temperature i.e. X_3X_4, X_1X_3, X_1X_4. HCl concentration (X_2), on the other hand, was not included in the model due to its insignificant effect on the PG activity.

The ANOVA (Table 6.5) gave the p-value of the model as 0.0383. This indicated that the constructed model was significant and the terms included in this model had important effect on the PG activity. Moreover, the lack-of-fit value of the model (0.9224) proved the validity of the factors included in the model.

Table 6.5. ANOVA table for the PG enzyme activity at the optimization step (reduced model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Block	35.6	1	35.6		
Model	284.4	8	35.5	3	0.0383
X_1	0.6	1	0.6	0	0.8299
X_3	25.7	1	25.7	2	0.1838
X_4	9.8	1	9.8	1	0.4045
X_1X_3	41.0	1	41.0	3	0.0975
X_1X_4	37.2	1	37.2	3	0.1131
X_3X_4	71.4	1	71.4	5	0.0327
X_3^2	71.3	1	71.3	5	0.0328
X_4^2	84.2	1	84.2	6	0.0216
Residual	271.0	20	13.6		
Lack of fit	164.9	16	10.3	0	0.9224
Pure error	106.1	4	26.5		
Cor Total	591.0	29			

Std. Dev.	3.68	R-Squared	0.5120	
Mean	33.66	Adj R-Squared	0.3168	
C.V. %	10.94	Pred R-Squared	0.1101	
PRESS	616.52	Adeq Precision	5.5677	

The model equation that expressed the PG activity in terms of coded factors was as follows:

$$PG\ activity\ (U/g\ substrate) = 33.42 - 0.19X_1 - 1.19X_3 - 0.74X_4 + 1.52X_1X_3 + 1.60X_1X_4$$
$$+ 2.12X_3X_4 - 4.74X_3^2 + 5.16X_4^2 \quad\quad (6.1)$$

The response surface plots obtained according to the above model can be seen in Figure 6.1. The Figure 6.1a shows that high PG activity was obtained at mid level of time and low levels of orange peel concentration. Nevertheless, the low level of orange peel concentration and both high and low levels of temperature resulted in high PG activity (Figure 6.1b). According to Figure 6.1c, at the mid level of time and both high and low level of temperature, PG activity was also high.
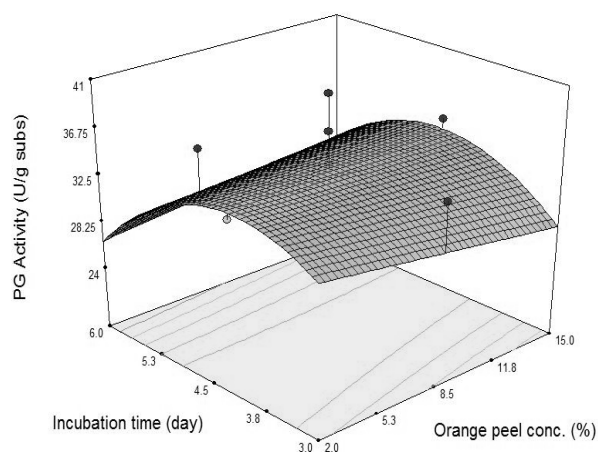
(a)
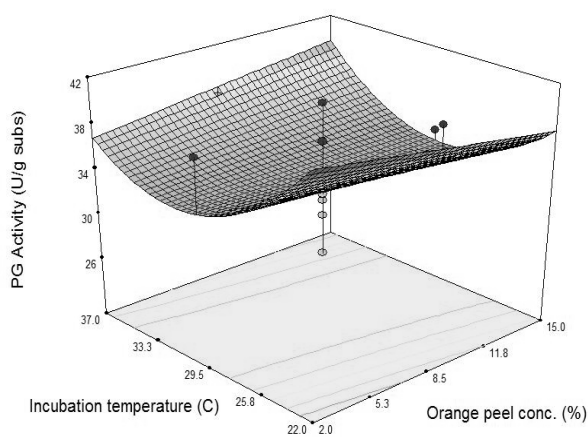
(b)
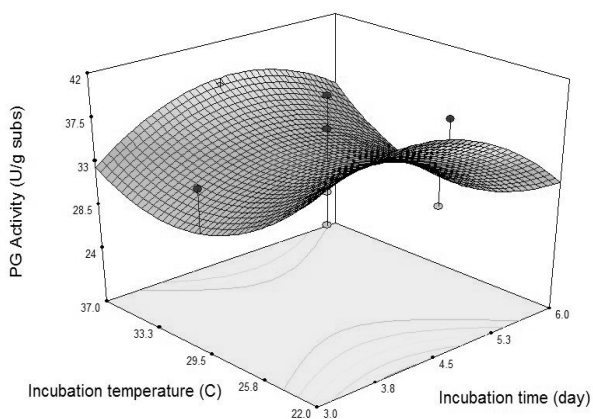
(c)

Figure 6.1. (a) Response surface plot showing the interaction between the orange peel concentration and incubation time (constant values; HCl conc.: 150 mM, incubation temp.: 29.5 °C) (b) Response surface plot showing the interaction between the orange peel concentration and incubation temperature (constant values; HCl conc.: 150 mM, incubation time: 4.5 days) (c) Response surface plot showing the interaction between the incubation time and incubation temperature (constant values; orange peel conc.: 8.5%, HCl conc.: 150 mM)

It was concluded from the ANOVA results of the other response –spore count– of the FCCD that incubation temperature (X_3) and time (X_4) main factors, their interaction with each other (X_3X_4) and quadratic forms of incubation time (X_3^2) and temperature (X_4^2) were the most important factors affecting this response (Table 6.6). Therefore, the mathematical model developed for the spore count in terms of coded factors is as follows:

$$\text{Spore count (spore/g substrate)} = 8.89*10^9 - 1.48*10^9 X_3 - 2.31*10^9 X_4 - 1.46*10^9 X_3 X_4$$
$$- 2.43*10^9 X_3^2 - 4.32*10^9 X_4^2 \tag{6.2}$$

Table 6.6. ANOVA table for the spore count at the optimization step (reduced model)

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-value	p-value
Block	4.04E+19	1	4.04E+19		
Model	3.46E+20	5	6.92E+19	15.41	< 0.0001
X_3	3.93E+19	1	3.93E+19	8.75	0.0070
X_4	9.57E+19	1	9.57E+19	21.33	0.0001
X_3X_4	3.43E+19	1	3.43E+19	7.65	0.0110
X_3^2	1.87E+19	1	1.87E+19	4.16	0.0530
X_4^2	5.91E+19	1	5.91E+19	13.16	0.0014
Residual	1.03E+20	23	4.49E+18		
Lack of fit	4.97E+19	19	2.62E+18	0.20	0.9943
Pure error	5.35E+19	4	1.34E+19		
Cor Total	4.9E+20	29			

Std. Dev.	2.12E+09	R-Squared	0.7702
Mean	5.11E+09	Adj R-Squared	0.7202
C.V. %	41.49	Pred R-Squared	0.6819
PRESS	1.43E+20	Adeq Precision	8.8583

Compared to the SSF study of Ustok et al. (2007) using wild type *Aspergillus sojae*, where the maximum PG activity was determined as 31.7 U/g solid, the investigated factors and their levels in this study have improved the PG activity by at least 25% that gives a rough estimate about the PG producing potential of *Aspergillus sojae* M3. Similarly, this optimization process achieved to increase the spore count production approximately by 56 times relative to Ustok et al. (2007)'s SSF study. These improvements may be attributed to i) the result of mutation and screening process, ii) the optimization routine, and iii) the change in the cultivation system.

6.3. Numerical Optimization and Validation of the Constructed Model for PG Activity

In order to provide the end-user with certain alternatives depending on their point of interest, following criteria summarized in Table 6.7 were used for the numerical optimization of the two responses. The aim of the Criterion-I was to reach maximum PG activity, for the PG production processes with no limitation on the spore production. On the other hand, Criterion-II gave the optimum conditions for the processes in which spore production is the main purpose. However, Criterion-III serves for the processes where both high PG activity and spore production should be obtained. The optimal solution of the obtained models on the basis of the Criterion-I gave the optimum PG activity and spore production conditions as: 2% orange peel concentration, 50 mM HCl concentration, 22 ºC incubation temperature and 3.8 days of incubation time. This result is in accordance with the conditions obtained in the response surface plots of the PG activity response (Figure 6.1a, b, c). The optimization results for the Criterion-II given in Table 6.7 states that, when compared with the optimum conditions of maximum PG activity (Criterion-I), nearly 1.5 more incubation day and slightly higher temperature was required in order to obtain maximum spore count. Furthermore, the optimum conditions will be kept very close to the ones obtained with Criterion-I when both maximum PG activity and spore count is the target.

The utilization of the optimum conditions for a spore production process (Criterion-II) will lead to yield 159% more spores than the process of a maximum PG activity (Criterion-I). However, it should be kept in mind by the end user that maximum spore production conditions will cause a reduction of about 24% in the PG activity. The Criterion-III supplies the conditions of both maximum PG activity and spore count, which may result in an insignificant decrease in the PG activity (1.4%) besides a significant increase (81%) in the spore count relative to the Criterion-I. This criterion (III) may also lead to obtain 23% more PG activity, but a 30% less yield in the spore count when compared with the Criterion-II.

The graphical optimization of the responses built up the overlay plot where the contours of interest for the various response surfaces were superimposed. The overlay plot shown in Figure 6.2 was constructed with the contours of PG activity more than 35.0 U/g substrate and the spore count contours between $9.0*10^9$ and $2.0*10^{10}$ spore/g substrate. This overlay plot, given in Figure 6.2, demonstrated an optimum region

(shaded in gray) in which each point would represent a combination of fermentation conditions that would give the maximum PG activity and spore count. As can be seen in Figure 6.3, optimum region for the maximum PG activity and spore count can be outlined as the incubation period of 4.4 to 5.3 days and 24.0 to 28.0 ºC incubation temperatures. This overlay plot was prepared for the visual presentation of the optimum conditions where the conditions meet the proposed criteria and can be used practically for the decision of the conditions at the preparation step of the fermentation process.

In order to validate the models constructed for the PG activity and spore count in the optimization step, four experiments were conducted with the optimum conditions suggested by the software (Design Expert 7.0.0 trial version) with the highest desirability values (0.891-0.995). The conditions of validation experiments and the actual and predicted values and their error percentages were summarized in Table 6.7.

The error percentages of the validation experiments were in the range of 1.5 to 24.1%, which indicated the good compatibility of the model with the experimental results. Moreover, the percentages of the errors of the predicted values given in Table 6.7 demonstrated the accuracy and reliability of the constructed models for PG activity and spore count in the optimization step of this study.

Table 6.7. Numerical optimization and results of validation experiments

Criterion	X_1 (%)	X_2 (mM)	X_3 (°C)	X_4 (day)	PG activity (U/g substrate)			Spore count (spore/g subs)		
					Predicted	Actual	Error rate (%)	Predicted	Actual	Error rate (%)
I	In Range 2.0	Minimize 50	In Range 22.0	In Range 3.7	Maximize 42.3	40.5	4.4	In Range 3.7×10^9	3.2×10^9	15.3
II	In Range 2.0	Minimize 50	In Range 26.6	In Range 5.3	In Range 32.1	28.1	14.2	Maximize 9.6×10^9	10.0×10^9	3.9
II	In Range 9.7	Minimize 50	In Range 26.6	In Range 5.3	In Range 32.1	36.5	12.1	Maximize 9.6×10^9	11.1×10^9	13.7
III	In Range 2.0	Minimize 50	In Range 22.0	In Range 4.3	Maximize 41.7	41.1	1.5	Maximize 6.7×10^9	5.4×10^9	24.1

Additionally, one treatment chosen randomly forming the shaded area of the overlay plot given in Figure 6.2 was validated experimentally. The conditions of this treatment was; 2% orange peel and 50 mM HCl concentrations, 4.6 incubation days and 26 °C incubation temperature. The predicted PG activity and spore count values were

validated with the errors of 0.77% and 8.05%, respectively.
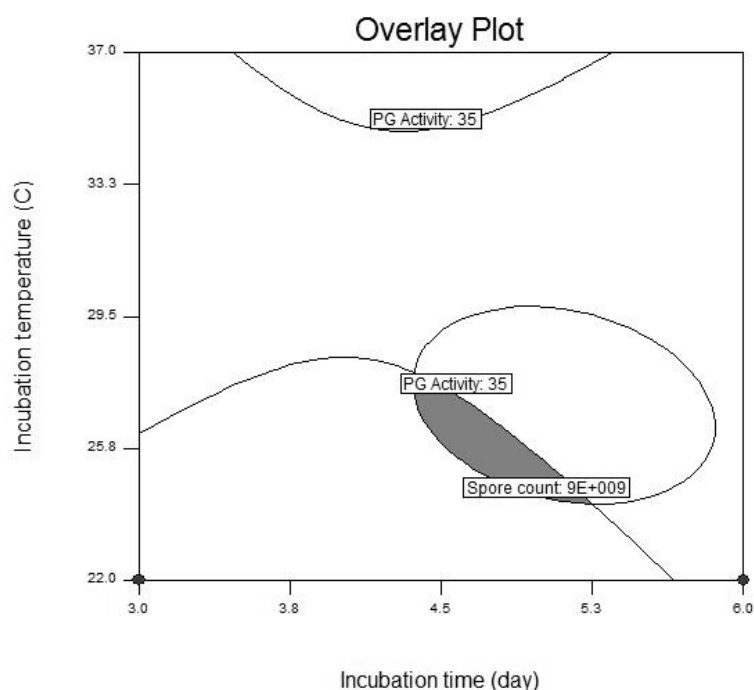
Figure 6.2. Overlay plot of PG activity (U/g substrate) and spore count (spore/g substrate) in response to incubation temperature and incubation time (orange peel conc.= 2 g/l, HCl conc.= 50 mM). Optimum area is shaded in grey.

As a conclusion, this part of the study showed that optimum conditions favoring both PG and spore production from *Aspergillus sojae* M3 were determined as; 2% orange peel and 50 mM HCl concentrations, 22 °C and 4.3 days of incubation. The overall results obtained in this chapter indicated the potential of *Aspergillus sojae* M3 for the production of industrially important enzyme PG and its adaptability to the solid-state fermentation conditions that utilize agro-industrial residues such as orange peel and wheat bran. It was also observed that the enzymatic activity or the spore production properties of the strain can be enhanced upon the optimization of the important factors with the help of experimental design and statistical analysis tools.

CHAPTER 7

SCREENING OF AGRO-INDUSTRIAL RESIDUES AND *Aspergillus sojae* STRAINS

In the previous chapter, the potential of a mutant *Aspergillus sojae* strain for the production of polygalacturonase (PG) by solid-state fermentation (SSF) technique that utilizes the agro-industrial residues was observed. This potential was optimized with the help of experimental design and statistical analysis tools. This chapter includes the investigation of PG production by SSF using the new mutant strains grown on various kinds of agro-industrial residues that are some of the major residues of the food industry in Turkey.

7.1. Screening of Agro-industrial Residues with *Aspergillus sojae* M5/6

At the beginning of this part of the study a mutant strain coded as *Aspergillus sojae* M5/6 was supplied which gave high polygalacturonase (PG) activity values in the submerged fermentation studies. Therefore, it was decided to continue with this new strain in order to be able to compare the PG activity results of SSF with the submerged part.

The optimum PG activity was obtained at 22 °C with *Aspergillus sojae* M3 in the previous part (Section 6.3), however in this part it was decided to set the incubation temperature at 30 °C in order to be compatible with the properties of the bioreactor that is planned to be used in further studies. Since this bioreactor does not have a cooling property, the incubation temperature was chosen as 30 °C. As can be seen from Table 5.1, apple pomace has the maximum moisture content (76.5%) among the screened substrates; therefore it was decided to fix the moisture content of each substrate at 80% by the addition of appropriate amount of distilled water. The inoculation size was hold at $2*10^6$ spore/g substrate ($2*10^7$ total spore) that was optimized by Ustok et al. (2007) for the PG production by SSF from *A. sojae* wild type. Since the PG production of each

residue was unknown at this point, the incubation time was extended to 6 days. The PG activity results of the obtained solid substrates can be seen in Table 7.1.

Table 7.1. Screened substrates with *Aspergillus sojae* M5/6

Substrate and amount	PG activity (U/g substrate)	Spore count (spore/g substrate)	Final pH
Orange peel, unground (10g)	0	0	4.68
Orange peel, fine (10g)	0	0	4.89
Soy meat (10g)	24.2	$8.53*10^7$	7.70
Wheat bran, Bremen (10g)	0	$5.57*10^8$	7.77
Wheat bran, İzmir (10g)	6.1	$5.23*10^8$	7.78
Corn cob (10g)	0	$1.03*10^8$	5.91
Apple pomace (10g)	0	0	3.57
Grape stalk (10g)	0	0	4.37
Sugar beet bagasse (10g)	0	$3.98*10^8$	5.25
Coffee husk (10g)	0	0	5.18
Sugar beet bagasse (5g) + Orange peel fine (5g)	0	$3.44*10^8$	4.55

At this stated fermentation conditions, PG activity was obtained only with soy meat and fine wheat bran as 24.2 and 6.1 U/g substrate, respectively, which were relatively low values compared with the previous results in Section 6.3 and spore count was low as well. This activity loss might have been the results of inappropriate incubation temperature or moisture content selection.

7.2. Comparison of *Aspergillus* strains

At this point, various *Aspergillus* strains were tried to be developed by mutation in our laboratory. The names of the mutant strains that were developed or obtained from other project partners were: *Aspergillus sojae* wild type, M5/6, M7, M7/2, electro-vgb and vgb-tra. In order to search for their PG production potential in SSF, a factorial design with 24 runs was conducted. Other factors were selected as incubation temperature and HCl concentration since their effects on the PG activity of these strains were not investigated yet. Wheat bran (İzmir) of 10 g was used as the solid media that was thought to be suitable for the fungal growth of each strain. 12 ml distilled water

(with 0 or 50 mM HCl) was added to each flask. The inoculation size was hold at $2*10^6$ spore/g substrate. Table 7.2 gives the runs and responses of the employed design.

Table 7.2. Factorial design and response values for the comparison of the strains

Std	Run	Aspergillus strain type	Incubation temperature (°C)	HCl concentration (mM)	PG activity (U/g substrate)
11	1	M5/6	30	0	5.4
10	2	vgb tra	30	0	7.1
9	3	M7/2	30	0	4.6
20	4	M7	30	50	0
16	5	vgb tra	22	50	10.6
6	6	electro vgb	22	0	6.8
21	7	M7/2	30	50	3.1
13	8	wild type	22	50	5
4	9	vgb tra	22	0	22.5
3	10	M7/2	22	0	62.7
17	11	M5/6	22	50	7.6
23	12	M5/6	30	50	0
22	13	vgb tra	30	50	5.2
2	14	M7	22	0	25.3
15	15	M7/2	22	50	55.5
8	16	M7	30	0	3.5
5	17	M5/6	22	0	3.2
7	18	wild type	30	0	8.7
14	19	M7	22	50	49.7
24	20	electro vgb	30	50	0
19	21	wild type	30	50	5.6
18	22	electro vgb	22	50	11.4
12	23	electro vgb	30	0	0
1	24	wild type	22	0	3.9

Figure 7.1a and b indicates that PG activities obtained at 22 °C of incubation temperature were higher than that of 30 °C for all of the strains (except wild type in the absence of HCl). At the 22 °C level, M7/2 gave the maximum PG activity among the investigated strains both in the absence and presence of HCl with 62.7 and 55.5 U/g substrate, respectively. The ANOVA given in Table 7.3 states that HCl concentration was not a significant ($p < 0.05$) factor with the p-value of 1.000, and it's interactions with the other factors were also insignificant. Therefore in the next step of screening, HCl was not added to the medium in order to reduce the fermentation cost.

Table 7.3. ANOVA table for the factorial design (reduced model)

Source	Sum of Squares	df	Mean Square	F-value	p-value (Prob > F)
Model	7122.2	18	395.7	8.92	0.0119
A-*A. sojae* strain	2364.3	5	472.9	10.65	0.0107
B-Incubation temperature	2035.0	1	2035.0	45.85	0.0011
C-HCl conc.	0.0	1	0.0	0.00	1.0000
AB	2501.1	5	500.2	11.27	0.0094
AC	182.3	5	36.5	0.82	0.5828
BC	39.5	1	39.5	0.89	0.3886
Residual	221.9	5	44.4		
Cor Total	7344.1	23			
Std. Dev.	6.7		R-Squared	0.9698	
Mean	12.8		Adj R-Squared	0.8610	
C.V. %	52.0		Pred R-Squared	0.3038	
PRESS	5112.8		Adeq Precision	10.5	
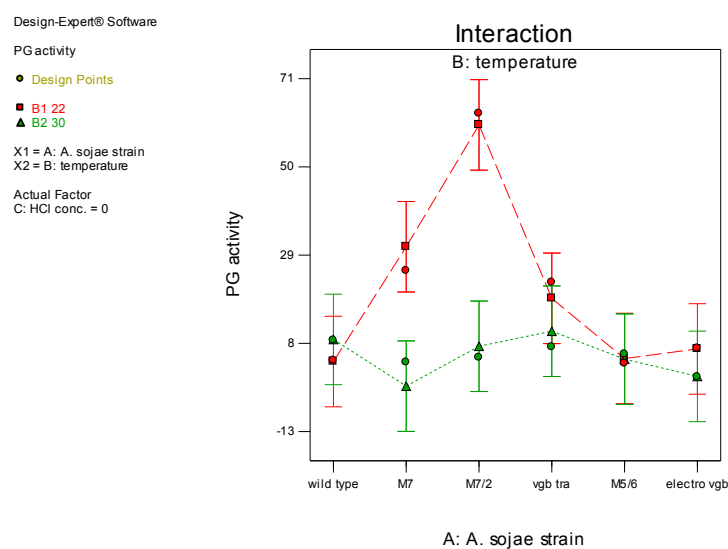
(a)

Figure 7.1. (a) Interaction between *A. sojae* strain and incubation temperature factors (HCl conc.: 0 mM), (b) Interaction between *A. sojae* strain and incubation temperature factors (HCl conc.: 50 mM)

(cont. on next page)

Design-Expert® Software

PG activity

● Design Points

■ B1 22
▲ B2 30

X1 = A: A. sojae strain
X2 = B: temperature

Actual Factor
C: HCl conc. = 50

Interaction

B: temperature

PG activity

A: A. sojae strain
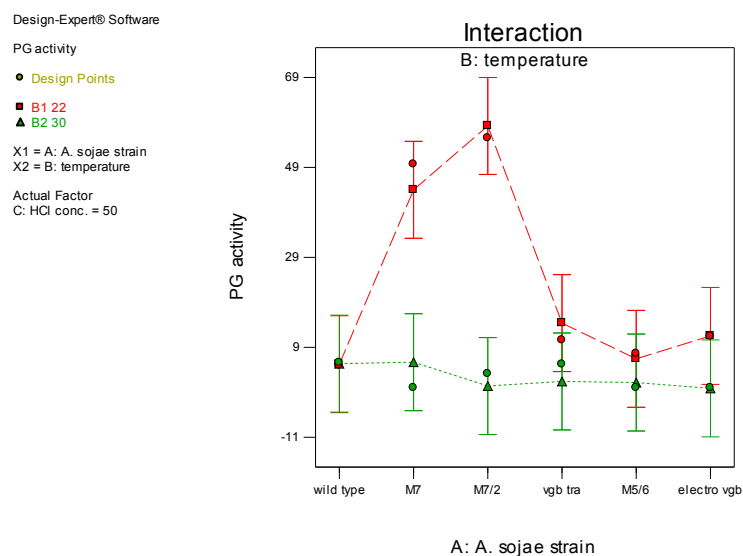
(b)

Figure 7.1. (cont.)

7.3. Screening of Agro-industrial Residues with *Aspergillus sojae* M7/2

In the previous step (Section 7.2), the maximum PG activity was obtained with *Aspergillus sojae* M7/2, at 22 °C and in the absence of HCl. Therefore in this step, various agro-industrial residues and some of their mixtures were screened for their PG activities with *Aspergillus sojae* M7/2 at 22 °C and in the absence of HCl. For this purpose, solid substrates were inoculated with $2*10^6$ spore/g substrate and incubated at 22 °C for 6 days. The agro-industrial residues, their water activity values (with 12 ml of distilled water addition) and final pH values were given in Table 7.4. The initial pH values of the unmixed samples can be found in Table 5.1.

Table 7.4. Mixture ratio, water activity (in SSF) and final pH values of the agro-industrial residues

Residue	Ratio	Water activity	Final pH
Orange peel, fine	-	0.971	4.78
Soy meat, fine	-	0.965	7.85
Wheat bran, Beşler	-	0.976	7.23
Wheat bran, İzmir	-	0.985	6.91
Corn cob	-	0.981	5.78
Peanut husk	-	0.962	7.05
Grape stalk	-	0.970	4.16
Sugar beet bagasse	-	0.966	5.30
Coffee husk	-	0.972	5.55
Pomegranate pomace	-	0.927	3.30
Orange pomace	-	0.925	4.19
Carrot pomace	-	0.991	6.51
Apple pomace	-	0.865	4.13
Apricot pomace	-	0.902	4.37
Peach pomace	-	0.916	4.95
Sugar beet bagasse + orange peel, fine	1:1	0.965	5.40
Wheat bran, İzmir + orange peel	4:1	0.974	6.44
Wheat bran, Beşler + molasses	1:1	0.842	5.00
Wheat bran, Beşler + pomegranate pomace	1:1	0.933	6.63
Wheat bran, Beşler + orange pomace	1:1	0.957	7.11
Wheat bran, Beşler + carrot pomace	1:1	0.959	7.26
Wheat bran, Beşler + apple pomace	1:1	0.974	6.72
Wheat bran, Beşler + apricot pomace	1:1	0.970	7.17
Wheat bran, Beşler + peach pomace	1:1	0.974	7.07

No PG activity was found in the SSF conducted with the agro-industrial residues given in Table 7.4, even though five of the samples were randomly selected and analyzed once again. Since, all of the conditions of 10th run on Table 7.2 were same as the wheat bran, İzmir in Table 7.4; a PG activity around 60 U/g substrate was expected. On the other hand, fungal growth was observed on the solid substrates on the last day of the fermentation. As a result of this part of the study, it was decided to continue with a relatively well-known (from the simultaneous submerged fermentation studies) mutant strain -*Aspergillus sojae* M5/6-, since *Aspergillus sojae* M7/2 did not have any repeatability to produce PG enzyme. But before the next SSF experiments with the abovementioned (Table 5.1) agro-industrial residues, their sugar compositions were investigated using the FT-IR analyses.

7.4. FT-IR Analysis of Agro-Industrial Residues

The aim of the FT-IR analysis was to determine the sugar compositions of the agro-industrial residues qualitatively. For this purpose, the sugar compositions of the agro-industrial residues were compared with the standards of glucose, fructose, sucrose and pectin using FT-IR analysis. The FT-IR spectra were given in Figure 7.2 and sugar compositions of the agro-industrial residues were summarized in Table 7.5. As a general comment on these analyses, pectin was overlapped with the fructose which gave more dominant peaks; therefore pectin could not be determined in the presence of fructose.
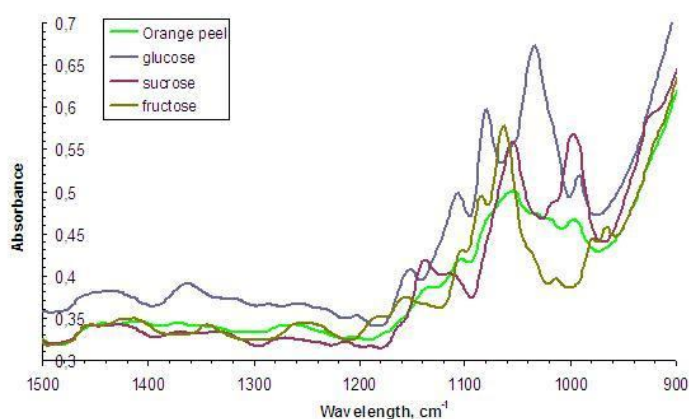
(a)
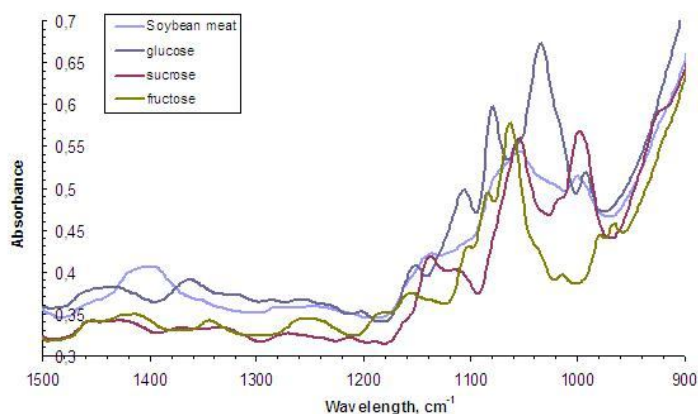
(b)

Figure 7.2. FT-IR analyses of the agro-industrial residues: (a) orange peel, (b) soybean meat, (c) wheat bran, İzmir, (d) peanut husk, (e) corncob, (f) sugar beet bagasse, (g) coffee husk, (h) orange pomace, (i) carrot pomace, (j) apple pomace

(c)

(d)

(e)

Figure 7.2. (cont.)

(f)

(g)

(h)
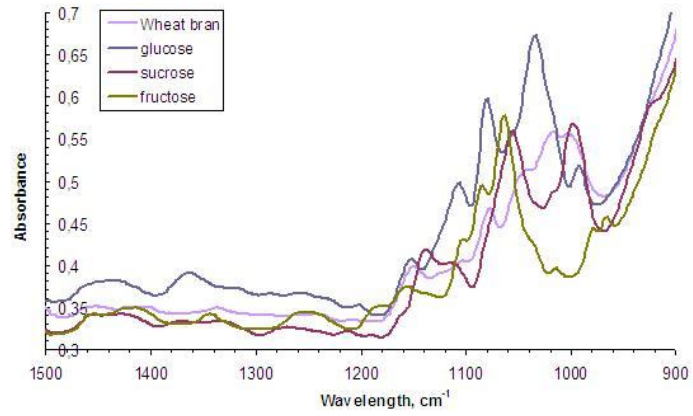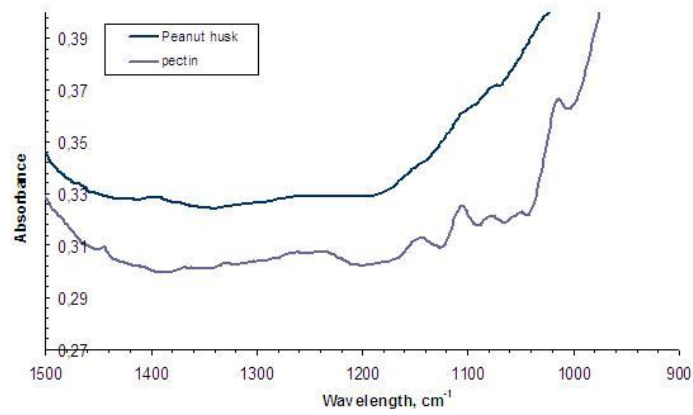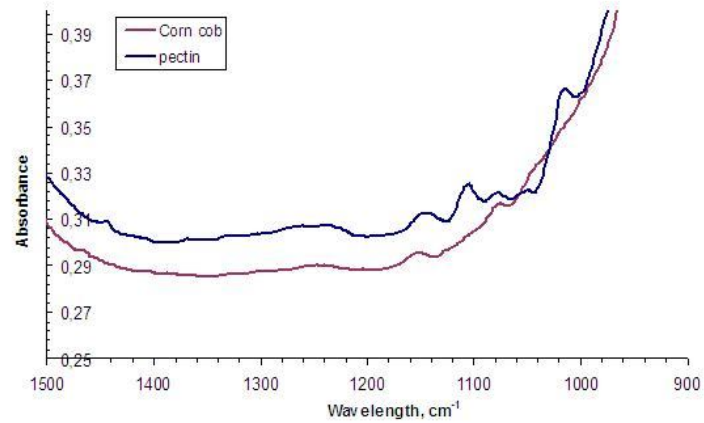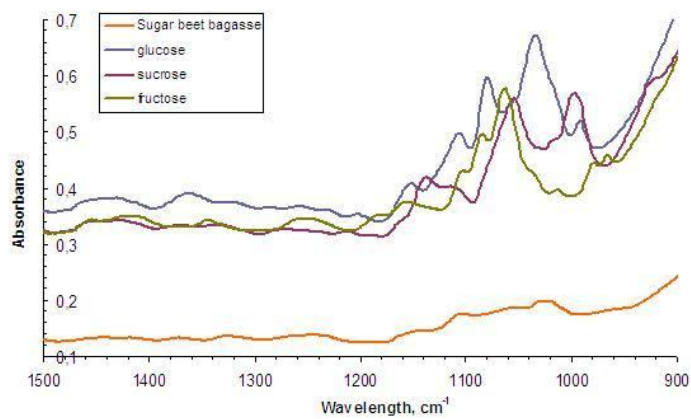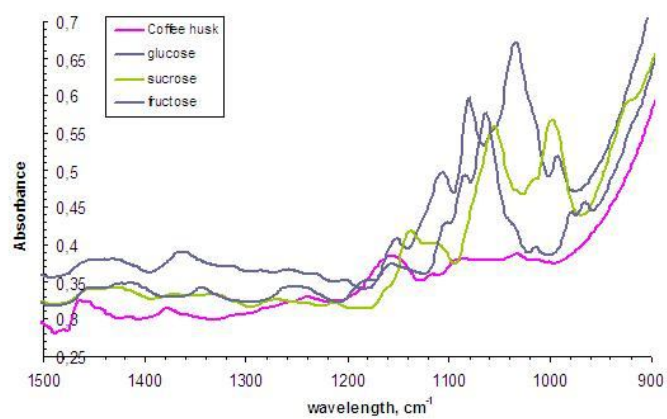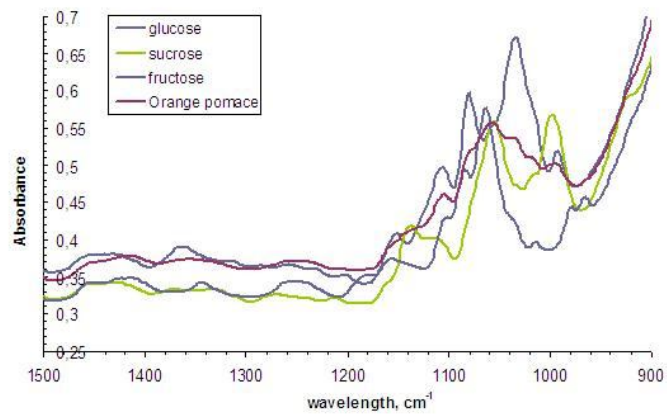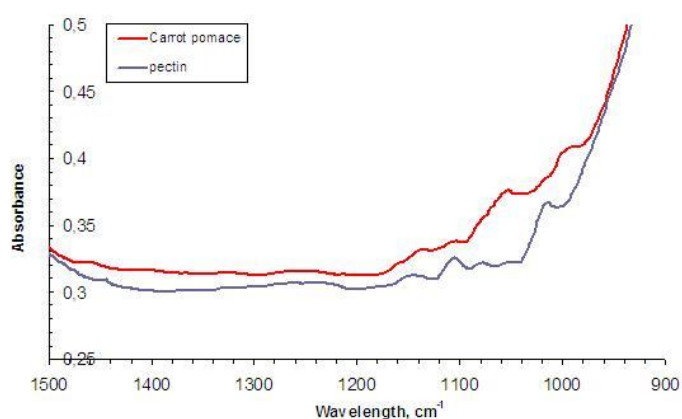
Figure 7.2. (cont.)

(i)
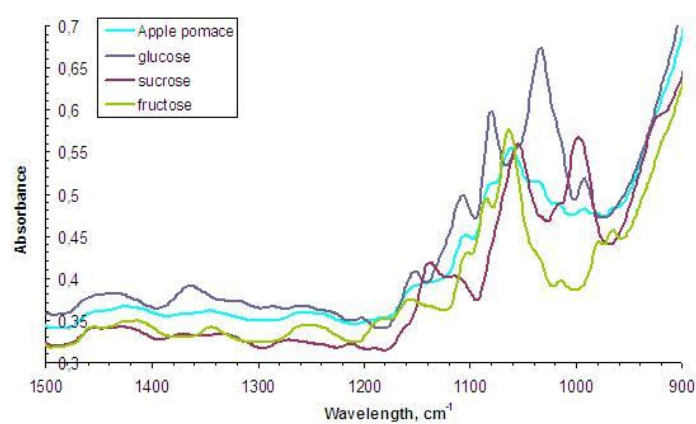
(j)

Figure 7.2. (cont.)

Table 7.5. Glucose, fructose, sucrose or pectin compositions of agro-industrial residues
determined by FT-IR analysis

Agro-industrial residue	Sugar composition
Orange peel	Fructose
Soybean meat	None
Wheat bran, İzmir	Glucose
Peanut husk	None
Corn cob	Pectin
Sugar beet bagasse	None
Coffee husk	None
Orange pomace	Fructose
Carrot pomace	Pectin
Apple pomace	Fructose

7.5. Production of PG on Various Agro-industrial Residues by *Aspergillus sojae* M5/6

In this part of the study, PG production potential of various substrates were screened using 10^7 spore/g substrate inoculation size, 52% moisture content, 22 °C incubation temperature, 5 days of incubation and *Aspergillus sojae* M5/6 as the fungal strain. Substrates were moistened with distilled water or a supplementing liquid consisting of 8 g/l ammonium sulfate, 3.3 g/l monosodium sulfate and 3.2 g/l disodium sulfate. The formulation of this solution was optimized in a submerged fermentation study. The PG activity, final pH and spore count results are given in Figure 7.3.

Figure 7.3a illustrated that when distilled water was used as the moistening agent; PG could be produced with ground soy meat and wheat bran (İzmir). It was determined that (Figure 7.2c) wheat bran includes glucose that is generally essential to the microorganisms to grow and sustain its metabolism. It is thought that glucose content of the wheat bran may have facilitated the growth of fungus especially as the penetrative hyphae penetrate into the solid via the liquid filled pores of the substrate followed by the synthesis of the hydrolytic enzymes (Hölker and Lenz, 2005). The other PG producing residue (moistened with water) was ground soybean meat which does not contain any glucose, fructose, sucrose or pectin with regard to its FT-IR analysis (Figure 7.2b), but it probably contains a sufficient amount of nitrogen that helped the strain to produce PG. The third residue that helped the production of PG was corn cob residue (moistened with water) containing pectin (Figure 7.2e) that might have induced PG production and possibly supported a large surface area for the anchorage of fungal cells. However, when supplementing liquid was used, PG was produced in ground soy meat, wheat bran and corn cob. It can be clearly observed from Figure 7.3a, that PG activity obtained with wheat bran was 4 times higher when the supplementing liquid was added. On the other hand, the three pomaces were insufficient for the PG production even though they contain fructose or pectin (Figure 7.2h, i, j). This is possibly due to their low pHs or limited surface area available for the fungal growth.

The spore count results (Figure 7.3b) indicated that wheat bran, corn cob and coffee husk were able to produce spores in the SSF process when moistened with water. In the presence of supplementing liquid spores were produced in wheat bran and coffee husk. The final pH values given in Figure 7.3c shows that PG and spore production was occurred in the agro-industrial residues that have final pH values at the neutral region.

Hence, little or no PG activity or spore production was available especially with the pomaces that were in the acidic region at the end of the fermentation.
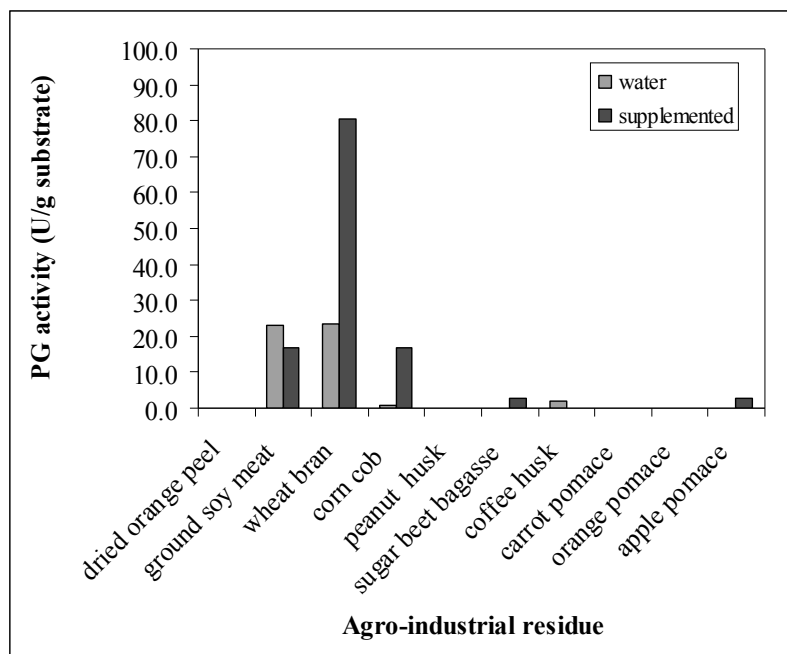
(a)
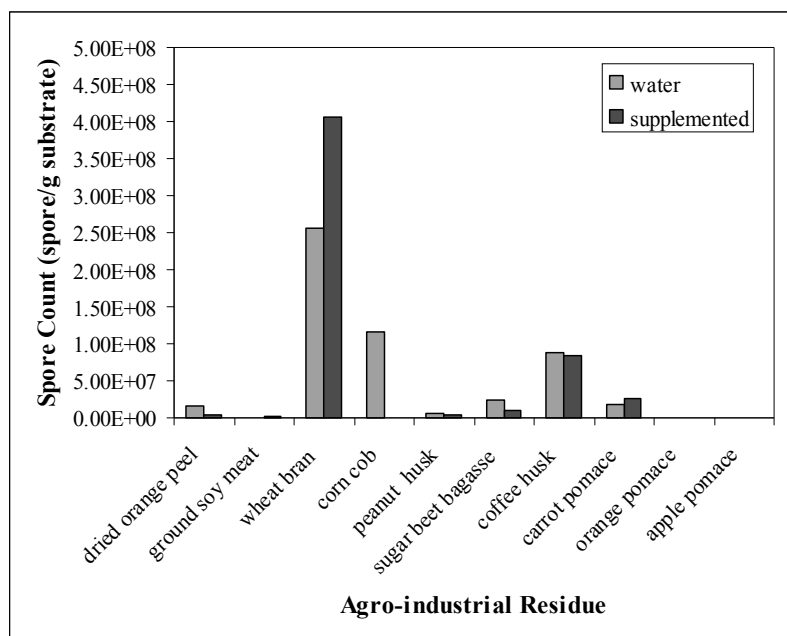
(b)

Figure 7.3. (a) PG activities, (b) spore counts and (c) final pH values of various agro-industrial residues moistened with water or supplementing liquid
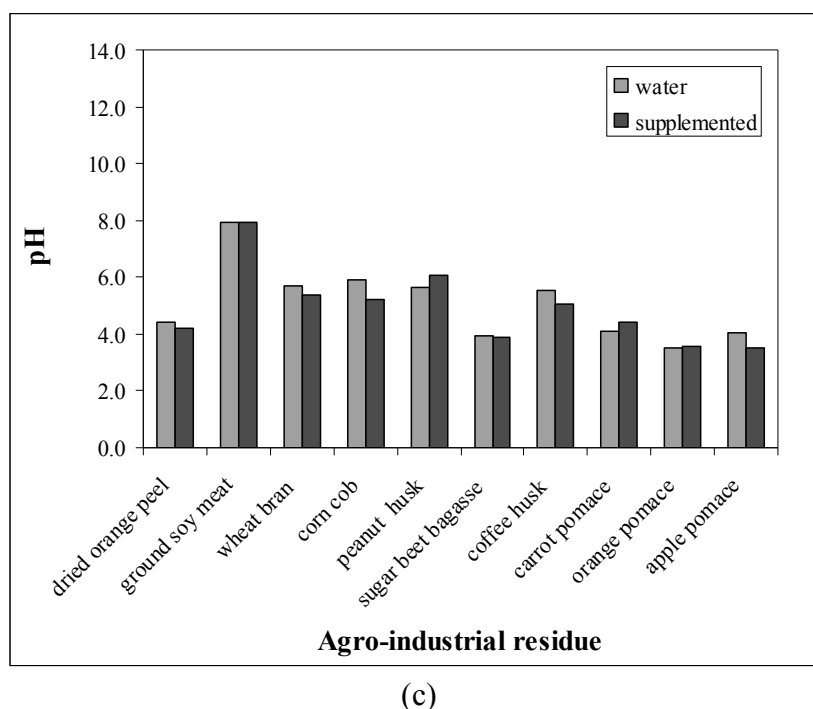
(c)

Figure 7.3. (cont.)

This set of experiments showed that unlike the previous parts (Section 7.1 and 7.3), relatively high PG activity was achieved with some of the residues. This result confirmed the PG production potential of *Aspergillus sojae* M5/6 by SSF. However, some important factors such as pH, temperature, inoculum size, pretreatment type or particle size might not be suitable for PG production from all of the residues. At this point it was decided to choose wheat bran and *Aspergillus sojae* M5/6 as the model residue and strain pair in order to understand the parametric requirements favoring the PG production by SSF considering the most important factors.

CHAPTER 8

EFFECT OF IMPORTANT FACTORS ON THE PRODUCTION OF POLYGALACTURONASE

In this chapter, *Aspergillus sojae* M5/6 and wheat bran was selected as the mutant strain – solid substrate pair in order to build up a model for the polygalacturonase (PG) production by solid-state fermentation (SSF). For this purpose, most important factors affecting the fungal growth and enzyme synthesis were investigated with one-at-a-time method. The process indicators such as PG activity, specific PG activity, final pH, spore count or consumption of total sugar were monitored and plotted in order to present the mechanism of the performed SSF batch.

8.1. Effect of Inoculum Size

Inoculum size is known to be an important factor that affects the enzyme production (Balkan and Ertan, 2010; Battaglino et al., 1991; Padmanabhan, 1993) and should be optimized to obtain the maximum PG activity. Inoculum size was generally defined with the unit of "spore/gram substrate" instead of "total spore" in the SSF studies (Aguilar et al., 2008; Linde et al., 2007; Chakradhar et al., 2009).

The effect of inoculum size was plotted in Figure 8.1 and this plot indicated that the maximum PG activity was obtained with 10^7 spore/g substrate. The PG activity increased by 58% with the inoculation of 10^7 spore/g substrate with respect to $2*10^6$ spore/g substrate. Therefore 10^7 spore/g substrate inoculum size was used in the remaining part of this study.
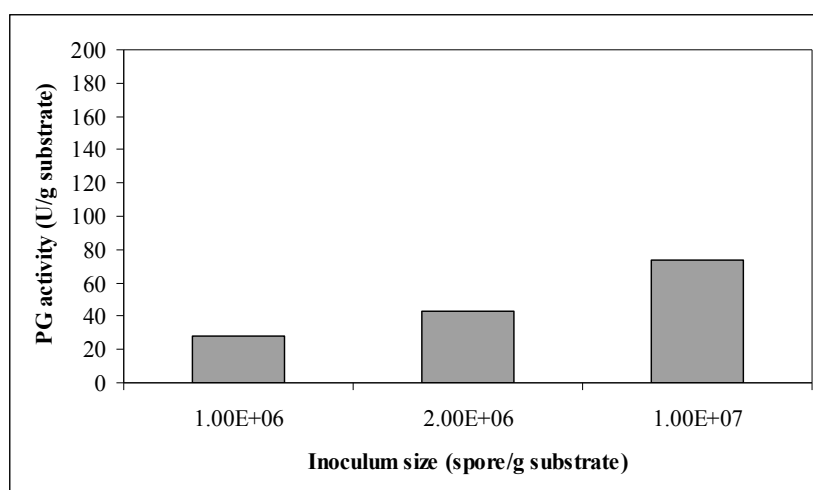
Figure 8.1. Effect of inoculum size on the PG activity (SSF conditions: 22 °C, 4 days, 55% moisture content)

8.2. Effect of Incubation Period

The effect of incubation period was investigated with a time course study. The PG activity, spore count, final pH, specific activity and total carbohydrate profiles were monitored during the 8 days of fermentation and presented in Figure 8.2.

The PG activity production of *Aspergillus sojae* M5/6 on wheat bran was monitored for 8 days. According to the PG profile in Figure 8.2a, no PG was produced by the microorganism on the first and second day of the fermentation, suggesting that PG content of the wheat bran was negligible and presence of the enzyme was only due to the fungal synthesis. It is possible that the metabolic activities of the fungal spores were mostly focused on germination on the first and second day of the SSF. For this purpose, it is thought that the microorganism preferred to use the free sugar in the environment without the need of synthesizing the pectinolytic enzymes. As can be seen in Figure 8.2d, the total carbohydrate showed an increasing trend on the first and second days, which indicated the breakdown of complex sugars into simple ones for the growth of microorganisms. The pH of the medium reduced slightly to 4.62 due to the metabolic activities of the fungi until the second day (Figure 8.2b). The spore count was nearly zero until the 3rd day which was probably because of the germination activities. After the second day, the PG profile increased sharply, and a maximum PG activity (136.9 U/g substrate) was achieved on the 4th day of the incubation. The total carbohydrate suddenly decreased to the level of 5.45 g/l on the 3rd day, so that the pectinolytic

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enzymes such as polygalacturonase began to be synthesized between the 3rd and 4th days with the aim of utilizing the other carbon sources in the media. Then the total sugar concentration in the media reduced to 2.92 g/l on the 4th day and nearly stayed at the same level until the end of the fermentation. This was interpreted as the metabolic activities related to the breakdown of sugars with the help of hydrolytic enzymes terminated at the end of the 4th day. Hence, the PG activity sharply reduced to 13.2 U/g substrate on the 5th day of the process and it increased very slightly to 19.8 U/g substrate during the last 3 days of the process. The specific activity profile given in Figure 8.2c followed a similar trend with the PG activity profile. After the PG production and total sugar consumption ceased on the 4th day, sporulation started to increase showing a slight drop on the last day of the fermentation. At the same time, pH of the media began to pass into the basic side and reached up to pH 8. For these reasons, the incubation period was hold at 4 days for the remaining part of this study. Martin et al. (2004) had also obtained the maximum PG activity on the 4th day of the incubation in their time course study for the production of polygalacturonase from a *Penicillium* sp. strain using a mixture of orange bagasse, wheat bran and sugarcane bagasse. However, Botella et al. (2005) found the maximum exo-PG activity on the 25th hour of the fermentation with an *Aspergillus awamori* strain cultivated on grape pomace.
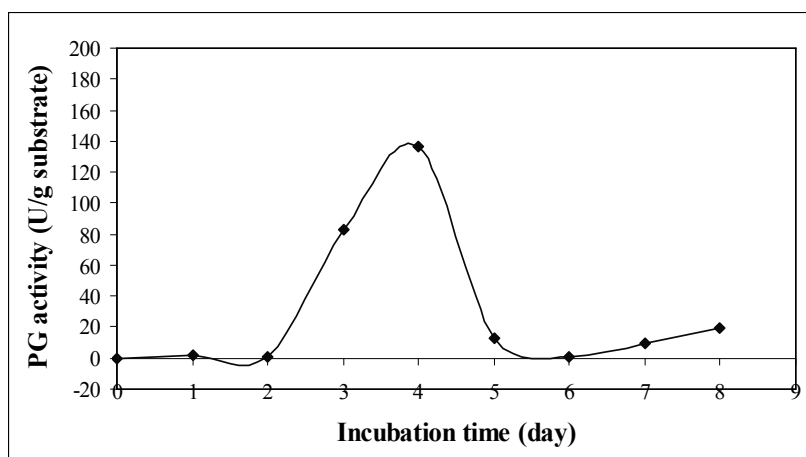
(a)
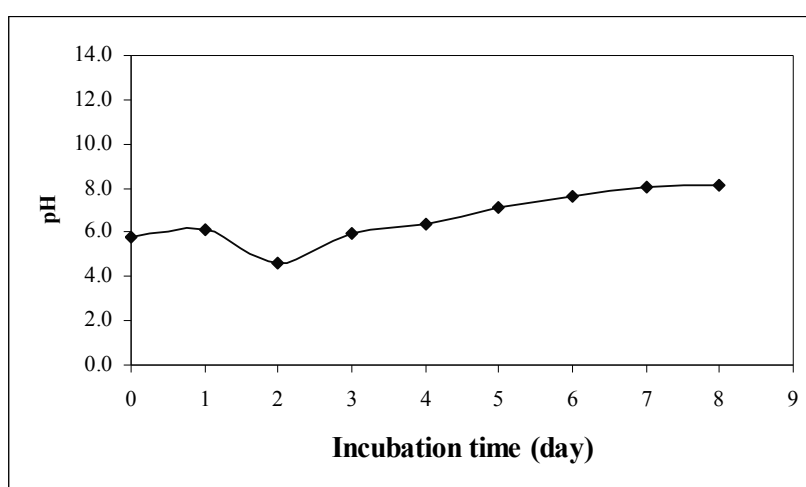
(b)
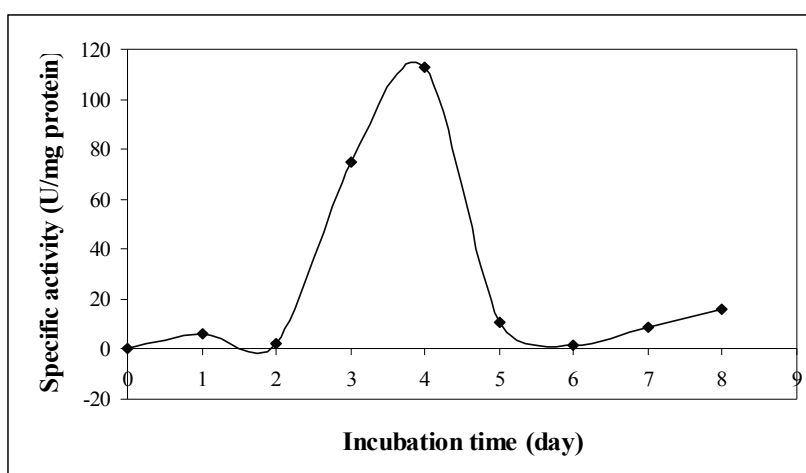
(c)

Figure 8.2. (a) PG activity, (b) pH, (c) specific activity, (d) total carbohydrate and (e) spore count profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 22 °C, 55% moisture content with water)

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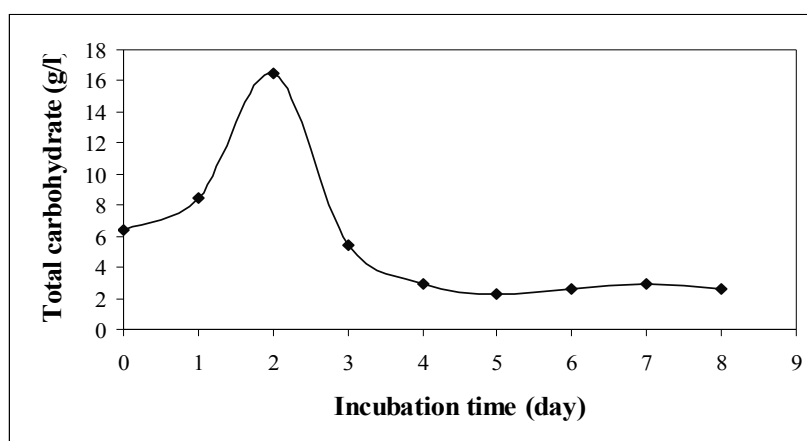
(d)
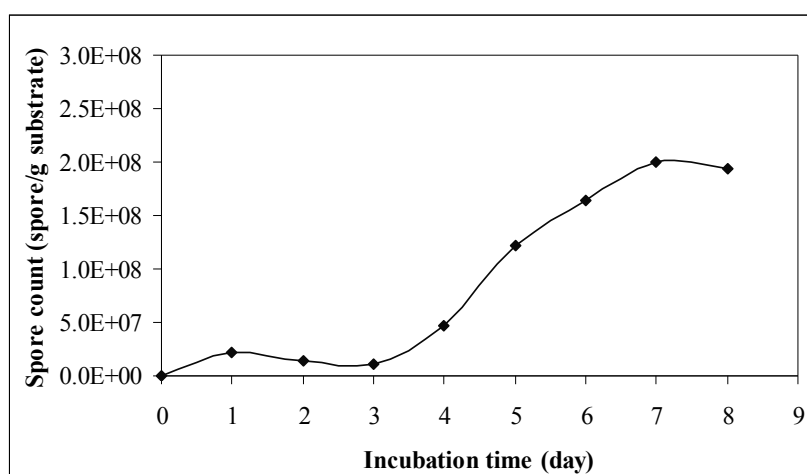
(e)

Figure 8.2. (cont.)

According to the time course part of the study, the maximum PG activity was obtained on the 4th day of the fermentation. Therefore, it was decided to conduct SSF process for 4 days on the remaining part of the optimization study (unless otherwise indicated). Moreover, productivity of this process was calculated as: 1.43 U g substrate^{-1}h^{-1} on the 4th day of the incubation.

8.3. Effect of Incubation Temperature

The temperature of the incubation is a critical parameter in the enzyme production by SSF since it has known to affect the structure of the enzyme and growth of the microorganism (Kumar et al., 2010; Roses and Guerra, 2009). In the current study, the effect of incubation temperature was investigated at a wide range from 18 to

40 °C. The PG activity, spore count, final pH, specific activity and consumption of total carbohydrate values obtained at the end of a 4 day-incubation period was illustrated in Figure 8.3.

The PG activity results given in Figure 8.3a presented that no PG activity was available at 18 °C. This was probably due to the lack of growth of the inoculated fungal spores, as can be seen on Figure 8.3e no spore count was obtained at this temperature (18 °C). The maximum PG activities obtained were 159.3 and 86.1 U/g substrate at 37 and 22 °C, respectively. This result was in agreement with our previous findings given in Section 6.2. At 40 °C of incubation, PG activity reduced for nearly 66% compared to 37 °C, which was probably due to the generation of excess heat that denatured the enzyme or evaporated the moisture of the medium. The PG activity was approximately 14% higher when compared with the maximum PG activity of the time course study (Figure 8.2a). It can be seen from Figure 8.3a, 22 and 37 °C are very characteristic temperatures for the production of PG, since very low PG activities could be achieved at the other temperatures namely 18, 26, 30, 34 and 40 °C. The final pH belonging to the 22, 37 and 40 °C incubation temperatures, where high PG activity was obtained, were between pH 5.3 and 6.1 (Figure 8.3b). The specific activity values calculated for 37 and 40 °C were higher than 22 °C, pointing out the presence of other kinds of proteins in the supernatant apart from PG enzyme (Figure 8.3c). The consumption of total carbohydrate plot (Figure 8.3d) indicates that no carbohydrate was consumed at 18 °C which supports the other findings for this temperature. On the other hand, total carbohydrate was mostly consumed at 26 °C, however, at this temperature the fungus entered to the dormancy and formed spores and as a result did not need to synthesize PG enzyme in excess (Figure 8.3e). The consumption of total carbohydrate, at the 37 and 40 °C temperatures were lower, probably due to the formation and consumption of more simple carbohydrates in the fermentation medium with the help of secreted hydrolytic enzymes such as PG. This result is in accordance with the time course of the consumption of total carbohydrate given in Figure 8.2d. Interestingly, no spore production was attained at 37 and 40 °C temperatures although high PG activities were achieved. These results supported the idea that the sporulation of *Aspergillus sojae* M5/6 is not associated with its PG production potential. The literature indicates a wide range of incubation temperature recorded for the maximum pectinase activities. Patil and Dayanand (2006a) recorded the maximum exo-pectinase activity at 34 °C employing *Aspergillus niger* DMF45 and deseeded sunflower head in SSF. Hours et al.

(1988) determined the maximum pectinase activity at 30 °C with *Aspergillus foetidus* and apple pomace. Freitas et al. (2006) obtained the maximum PG activity at 50 °C with an *Aspergillus* sp. strain and wheat bran and orange bagasse mixture. Conclusively, it was decided to continue to the next experiments holding the incubation temperature at 37 °C. The productivity of the process at the 37 °C incubation period was 1.66 U g substrate^{-1} h^{-1} which was slightly higher (16.1%) than that of the 4th day of the time course study.
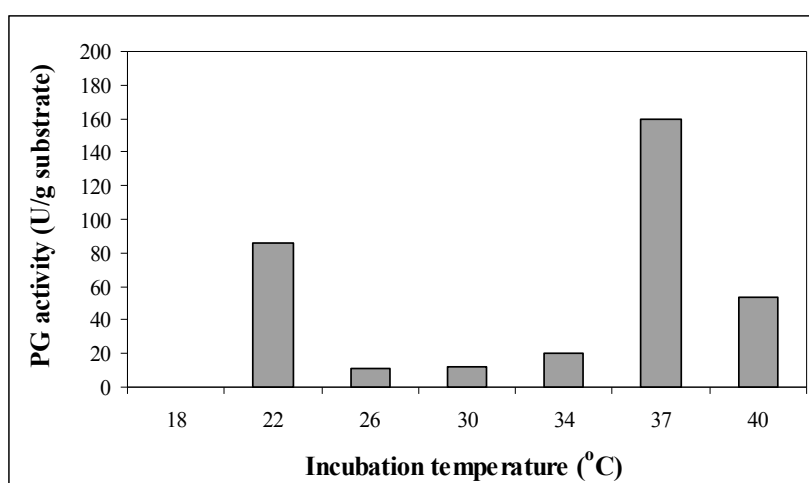

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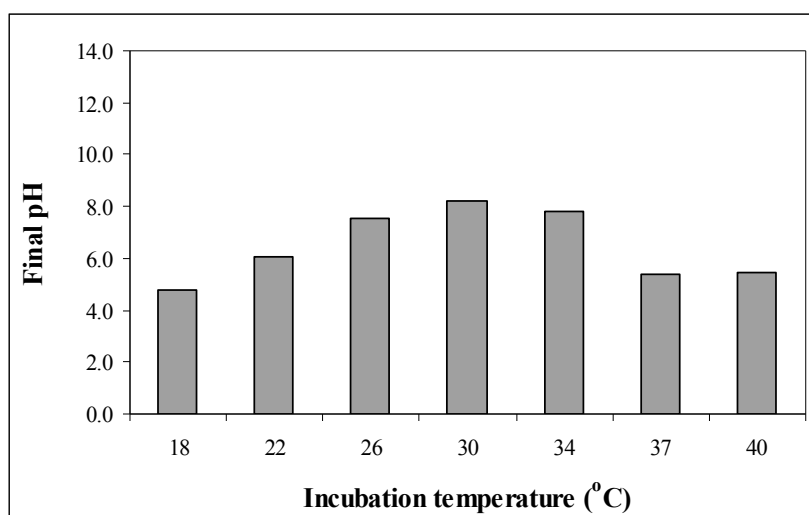
(b)

Figure 8.3. (a) PG activity, (b) final pH, (c) specific activity, (d) consumption of total carbohydrate and (e) spore count profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 4 days, 55% moisture content with water)

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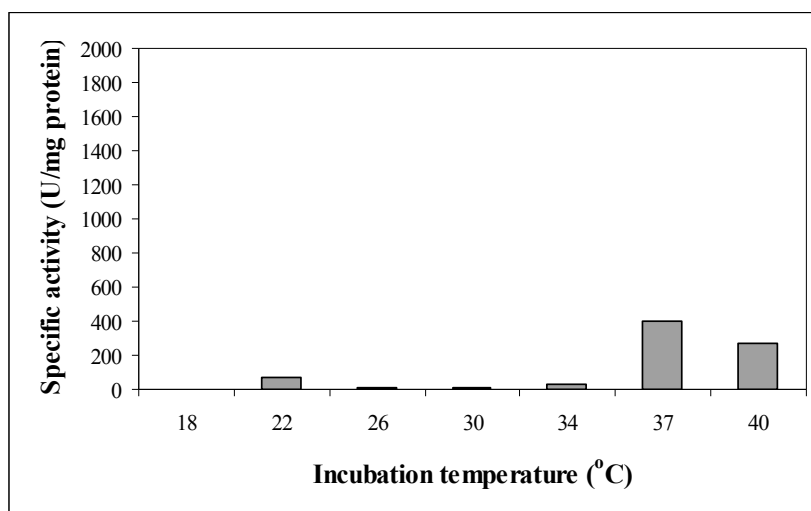
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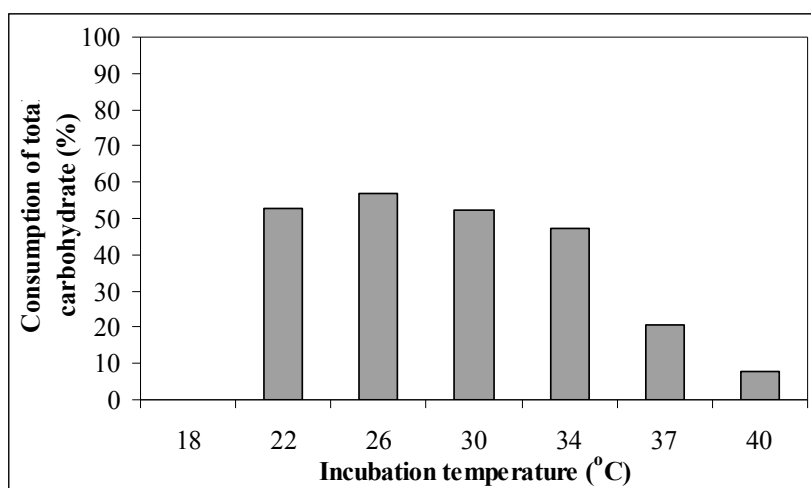
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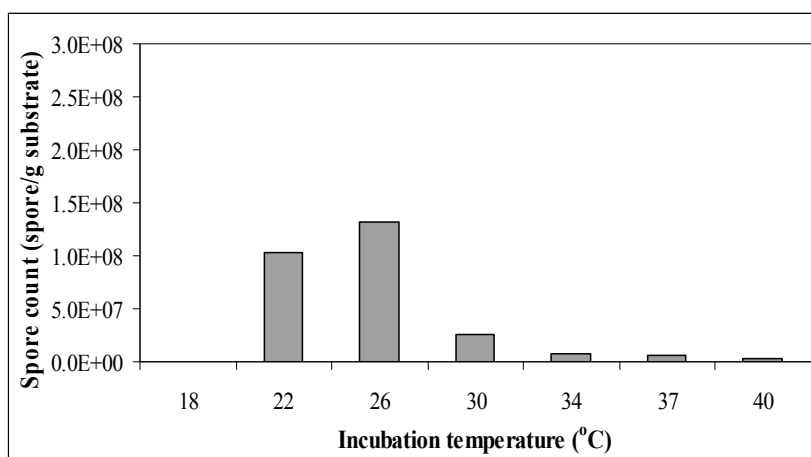
(e)

Figure 8.3. (cont.)

8.4. Effect of Moisture Content or Water Activity

For most of the living systems, the need of water is critical, even in SSF systems which occur in the lack of free water. Therefore, water requirements of microorganisms should be well established and provided by the fermentation medium for the maintenance of the metabolic activities. The amount of water available for the microorganism varies depending on the characteristics of the substrate such as porosity, water holding capacity, matrix forming ability etc. This phenomenon can be better defined with water activity term instead of moisture content (Battaglino et al., 1991). With this perspective the relationship between the moisture content and water activity of the wheat bran was illustrated in Figure 8.4.

The Figure 8.4 explained that water activity of the wheat bran varied significantly between the moisture content levels of 8 – 30%. However, between 30% to 62% moisture contents, the water activity increased very slightly between 0.946 and 0.998. This plot (Figure 8.4) clearly demonstrated that the water activity of the wheat bran is closely related with the moisture content. The other outcome of this plot was to show that water activity of the wheat bran can be adjusted with the help of distilled water. This is important because most of the researchers preferred to adjust the water activity of the solid medium by adding some amount of polyols. They observed that polyols were successful for the regulation of the medium to the desired level of water activity, but they caused reduction in the enzymatic activity (Acuna-Argüelles et al., 1994; Grajek and Gervais, 1987; Diaz-Godinez et al., 2001). Once the water activity of the wheat bran adjusted with the appropriate moisture content, it may be regulated by the relative humidity of the air throughout the pilot or large scale fermentation processes.

The current study was conducted at the upper limit of 62% moisture content where water activity was at its maximum (0.998) and no further moisture content level was tried since rate of free water in the environment would increase above this level which would be contrary to the principle of SSF (Raghavarao et al., 2003). The SSF was performed at 37 °C since maximum PG activity was obtained at this temperature in Section 8.3. The PG activity, final pH, specific PG activity and consumption of total carbohydrate responses were analyzed and given in Figure 8.5.

The effect of moisture content can be clearly seen from Figure 8.5a, that the fungus was not able to synthesize any PG between the range of 20 – 35% moisture content (equal to 0.656 to 0.957 a_w). The moisture content (or water activity) at this range was more likely to be insufficient for the growth of fungus. The maximum PG activity was achieved with 62% moisture content. This PG activity was 48% higher than that of 55% moisture content level which was also employed in the previous steps of the optimization (Section 8.1, 8.2 and 8.3) indicating the progressive effect of optimization on the SSF processes. The wheat bran particles swelled as they received more distilled water which corresponded to the increased surface area. Also, as the medium homogenized well before the fermentation, the gaps between the swollen particles made oxygen available for the inoculated spores for vegetative growth. Additionally, the high content of moisture possibly increased the solubility of the nutrients found in the structure of the wheat bran. Moreover, this maximum PG activity (250.8 U/g substrate) was enhanced for 57.4% when compared to the maximum activity obtained in the effect of temperature part of the study (Figure 8.3a). This situation indicated the importance of the determination of the optimum conditions that favor the production of the PG activity.

The optimum moisture content determined in this study (62% equal to 0.998 a_w) was compatible with the ones reported in the literature. The reported initial moisture content values varied from 40 to 70% (Blandino et al., 2002; Freitas et al., 2006; Castilho et al., 2000) for the production of pectinase from various solid substrates using different fungal strains. Patil and Dayanand (2006a) found similarly that exo-pectinase activity was achieved at 65% moisture content of the substrate and activity declined above this moisture content level using deseeded sunflower head and *Aspergillus niger* DMF45. Kapoor et al. (2000) determined the optimum moisture content as 90% for the PG production from *Bacillus* sp. MG-cp-2 cultivated on the decorticated ramie. On the other hand, Kashyap et al. (2003) reported the maximum pectinase activity was achieved at 75% moisture content using wheat bran and *Bacillus* sp. DT7. These results indicated the strong possibility of bacterial contamination when moisture content more than 65% was employed in the present system. Therefore extra care should be taken at the pilot and large scale productions with *A. sojae* M5/6 for the control of moisture content levels to reduce the risk of bacterial contamination.

The final pH values were very close between the moisture content levels of 20 – 35% where no PG was produced (Figure 8.5b). This result enhanced the hypothesis of

the reduction in the pH of the medium due to metabolic activities of the fungus. Similar with the results obtained in the effect of temperature study (Section 8.3), pH of the medium reduced close to 5.3 when PG was produced by the fungus. The specific enzyme activity (Figure 8.5c) at 62% was superior to the 40 and 55% moisture contents and also the maximum specific activities obtained in Sections 8.2 and 8.3. This indicated as the fermentation conditions were optimized, *A. sojae* M5/6 produced a purer polygalacturonase. The consumption of total carbohydrate varied between the moisture content levels of 20 − 35% even though there was no PG activity (Figure 8.5d). This will be explained not with the metabolic activities but the difference in solubility of the sugars at each level of moisture of the substrate. Among the PG producing moisture content levels; carbohydrates were consumed more at 55%. Spore count was zero for each moisture content level which is in accordance with 37 °C investigation in the Section 8.3.

This part of the study expressed that initial moisture content of the substrate is a critical factor that should be optimized for improved enzyme activities. The productivity of the process was developed for 57.2% with respect to the previous step (Section 8.3) and obtained as 2.61 U g substrate^{-1} h^{-1} at the 62% moisture content level.
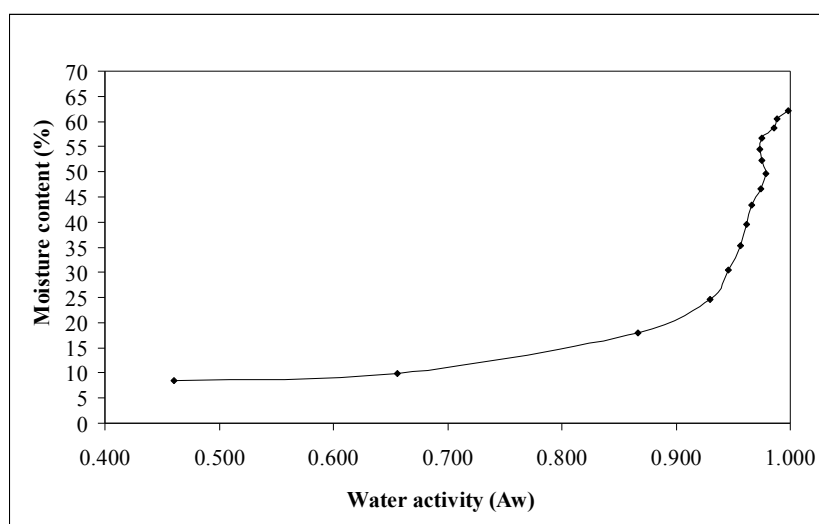
Figure 8.4. Relationship between the water activity and moisture content of wheat bran
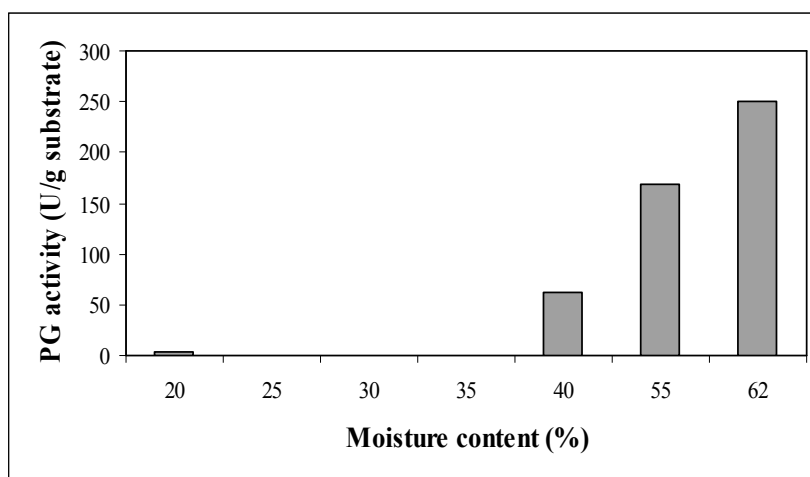
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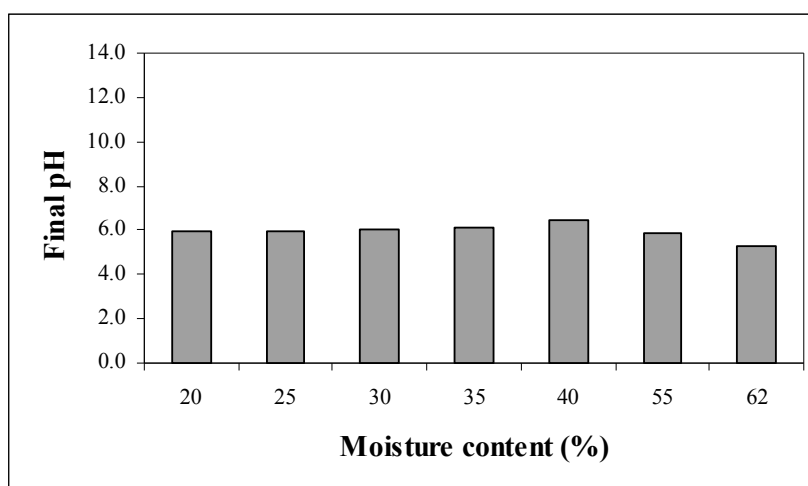
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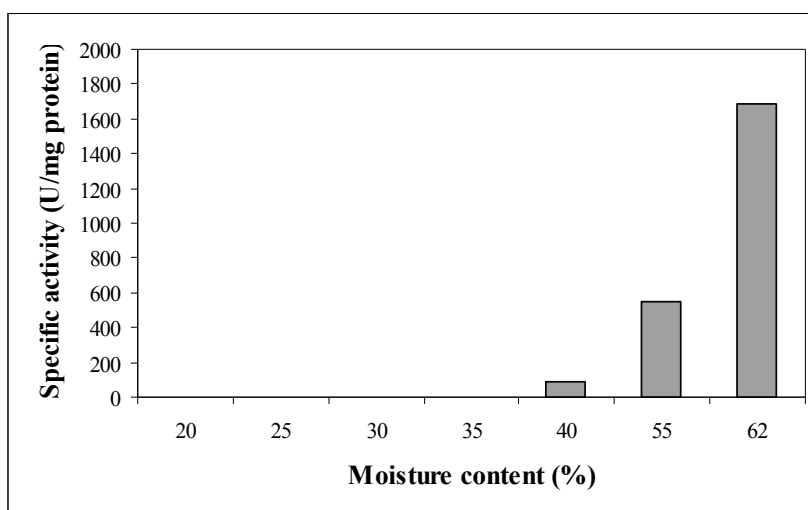
(c)

Figure 8.5. (a) PG activity, (b) final pH, (c) specific activity and (d) consumption of total carbohydrate profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, moistened water)

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(d)
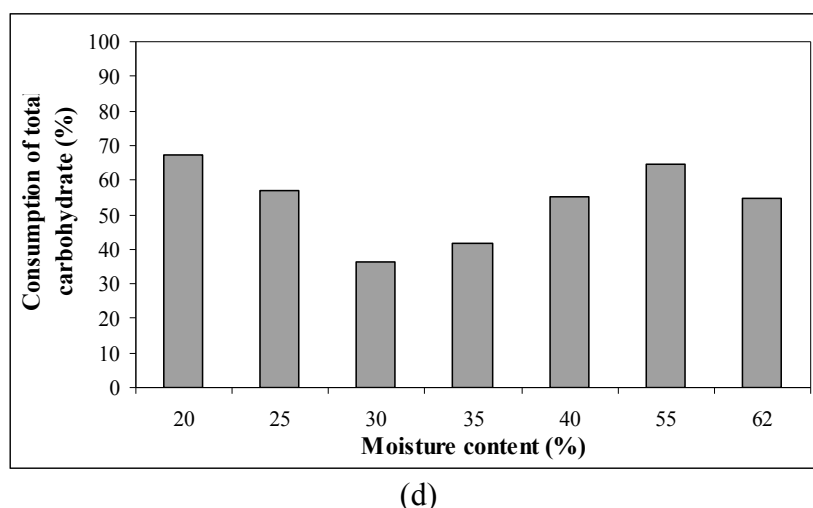
Figure 8.5. (cont.)

8.5. Effect of Initial pH of the Medium

The initial pH of the medium is known to effect physiological phenomena of the cell such as fungal growth and sporulation, production of enzymes and stability of the produced enzyme (Taragano and Pilosof, 1999). This critical factor was investigated by adding various buffers (pH 4.0 – 8.0) or distilled water to the fermentation media as the moisturizing agent. For this purpose, PG activity, final pH, specific PG activity and consumption of total carbohydrate responses were analyzed and given in Figure 8.6.

The Figure 8.6a illustrated that no PG activity was obtained at the pH 4.0 and 5.0. The maximum activities were obtained with pH 6.0 and distilled water which had close pH values with each other. However, *A. sojae* M5/6 was able to synthesize 47% more PG activity with the distilled water moistened medium than the pH 6.0 buffer moistened one. This result indicated the presence of own buffering capacity of the wheat bran that did not need any addition of buffer. This fact is in accordance with the low-cost economical view of the current SSF. In case of any nitrogen source addition to the wheat bran in the future studies, the effect of pH should be reviewed. The maximum PG activity obtained in this part of the study was very close to the maximum PG activity obtained at the 62% moisture content of the previous part of this study (Figure 8.5a). The specific activity on the other hand was nearly half of the value obtained at 62% moisture content (Figure 8.6c), which might be due to the variations in the soluble protein content of the wheat bran.

The initial pH of the medium moistened with distilled water was approximately 6.01. At the end of the fermentation, it reduced to pH 5.17 which was in accordance with the final pH values presented in Figure 8.3b for 37 °C and in Figure 8.5b for 62% moisture content. The initial and final pH values for the distilled water moistened run were also compatible with the pH stability of the polygalacturonases produced from fungal sources reported in the literature (Freitas et al., 2006; Martins et al., 2002). This means that the produced PG may not lose its activity and keep it well until the termination of the process. The growth of the strain was positively affected by the neutral pH of the medium, since some *Aspergillus* sp. were reported to produce other hydrolytic enzymes at the neutral pH ranges such as amylases, xylanases and cellulases that might support their metabolic activities (Spier et al., 2006; Roses and Guerra, 2009; Mamma et al., 2008; Jecu, 2000).

There was not any visual indication of growth in pH 4.0 and 5.0, where Figure 8.6d supported this fact with the unconsumed carbohydrates found in the medium at the end of the fermentation. Among the remaining initial pH values, consumption of total carbohydrate was slightly higher in distilled water moistened SSF, supporting the suitable growth of the fungus at the optimum conditions favoring the PG production.

Water was found to be the best moisturizing agent to obtain maximum PG activity in this part of the study. This fact is an advantage to reduce the raw material cost and may increase the overall productivity with the lack of pH adjustment stage. Additionally, the neutral nature of the fermentation medium throughout the process is advantageous for the operational safety and waste management.

The productivity of the process was 2.20 U g substrate^{-1} h^{-1} for the water moistened run. Approximately, 15.7% reduction in the productivity with respect to the previous part (Section 8.4) was because of the reduction in the maximum PG activity (as explained above). The measurement of pH during the process is very difficult due to the lack of free water in the medium, so in the case of pH monitoring requirement, aseptic sampling possibilities should be considered during the process.

(a)
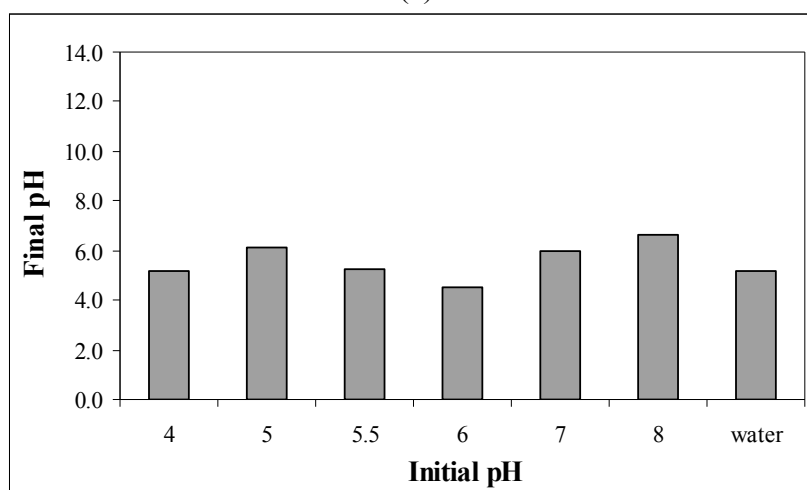
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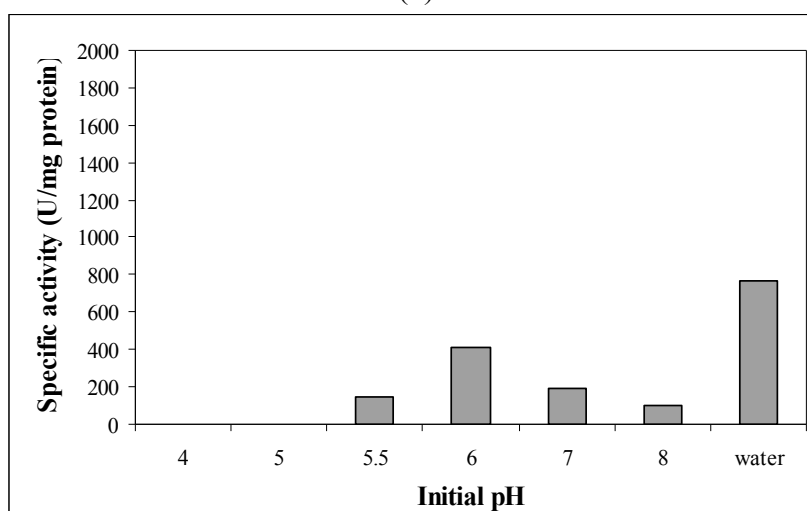
(c)

Figure 8.6. (a) PG activity, (b) final pH, (c) specific activity, (d) consumption of total carbohydrate profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content)

(d)
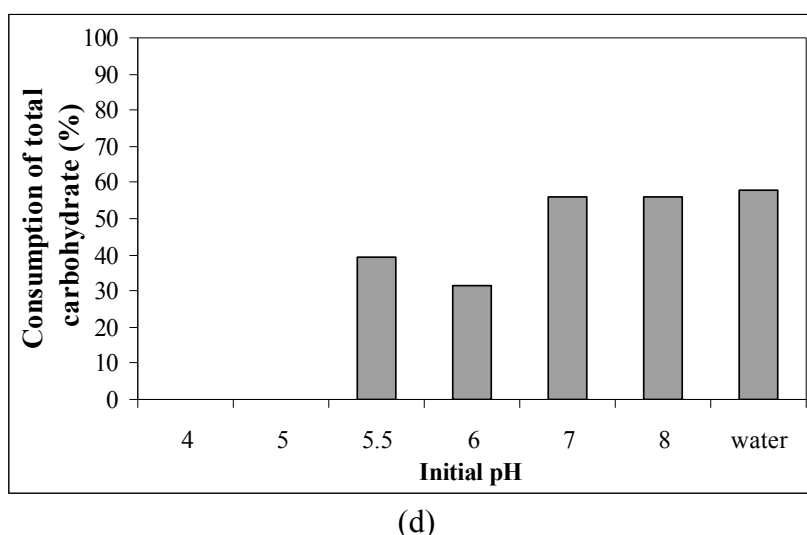
Figure 8.6. (cont.)

8.6. Effect of Particle Size of the Substrate

Particle size of the utilized solid substrate is an important factor that should be investigated, since it may directly affect the heat and mass transfer and indirectly affect the growth and metabolite synthesis during the SSF process. Particle size of the substrate establishes the surface area that is available for the microbial attack; however, it may lead to agglomeration resulting in poor oxygen transfer and fungal growth. Therefore, it is crucial to investigate and decide the requirement for employment of a specific particle size in the fermentation. For this purpose the influence of various wheat bran sizes (particle size distribution given in Figure 7.2) on PG production from *A. sojae* M5/6 was investigated and their PG activity, final pH, specific PG activity and con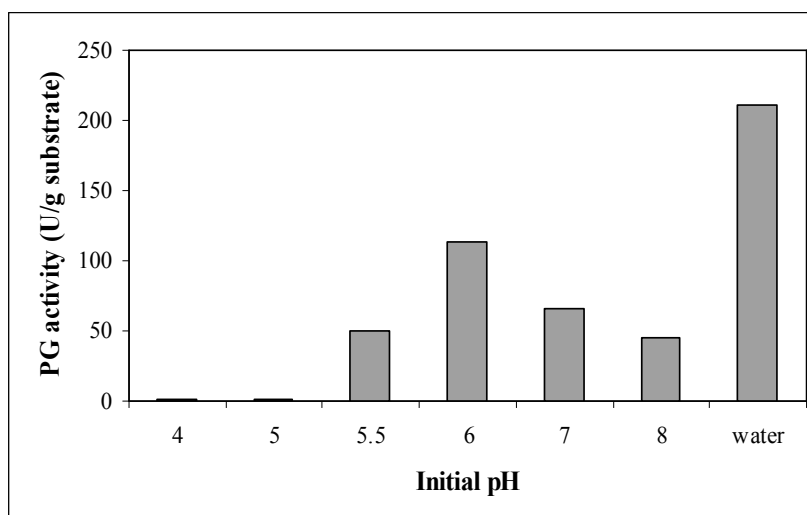sumption of total carbohydrate responses were analyzed and compared with the control which is the original form of the wheat bran without any size distribution (Figure 8.7).

As can be seen in Figure 8.7a, the PG activity obtained with the control (not sized) is in accordance with the previous PG activities obtained under similar conditions. However, the results given in Figure 8.7a clearly pointed out that the particle size range of 150 to 250 μm was very specific for the PG production, where 92% more PG activity was obtained at this particle range when compared to the control (not sized). On the other hand, no or very low PG activity was obtained with the remaining particle size ranges. This result indicated that a particle size separation of the

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wheat bran to obtain the range of 150 to 250 μm is inevitable since it significantly increased the PG activity. At the ranges of 500-850 μm and 250-500 μm the substrate probably could not serve sufficient surface area to the fungus for attachment or the size and structure of these particles did not support the fungal hyphae to grow alongside the solid particle. On the other hand, in the 150 to 250 μm sized particles the fungus was possibly able to use the surface liquid film as moisture and nutrient source and to penetrate into the cracks and pores. For this purpose the growing tips of the fungal hyphae produced polygalacturonase to convert pectic acid into metabolizable sugars. Another possibility is the collection of some nutritive fractions of the substrate produced during the wheat bran separation process such as germ and endosperm particles at the 150 to 250 μm range. At 75-150 and 75> μm particle size ranges, agglomeration of the small particles may have reduced the interparticle space and prevented the oxygen transfer to the aerial hyphae at the inner parts of the fermentation medium. The final pH results in Figure 8.7b confirmed the PG activity results, such that at the 500-850, 250-500 and 75-150 μm particle size ranges (no PG activity found) final pH values were on the basic side (>pH 7.0), whereas in the control, 150 to 250 and 75> μm particle size ranges the fermentation media resulted into the acidic region (<pH 7.0). The specific activity obtained at 150 to 250 μm was 550% higher than that of control which showed that this fraction of the wheat bran contained less soluble impurities coming from the substrate that may affect the specific activity results. The consumption of total carbohydrate results (Figure 8.7d) supported the maximum production of PG at the 150 to 250 μm range. The net concentration of consumed total sugar (at the end of a 4-day fermentation) was zero, probably because the fungus produced hydrolytic enzymes such as PG to breakdown the complex sugars into simple ones, then utilized these simpler sugars and reduced it to the initial sugar content level that the substrate supplies. A relatively high consumption level was observed for 75> μm particle size range, however the composition of the total sugar of the substrate at this fraction was probably not metabolizable for the fungus resulting in poor growth (visual observation) and low PG activity production. The consumption of total sugar concentration for the control was compatible with the one obtained in the initial pH part having the similar conditions (moistened with water). Additionally for the ranges of 500-850 and 250-500 μm; the consumption of total sugar concentrations were low, meaning that initial total sugar concentrations of these ranges were also insufficient for the fungal growth or the

total sugar content of these media were effectively consumed by the fungus without the need of PG enzyme secretion to the environment to breakdown the complex pectic compounds.

This part of the optimization resulted in the maximum activity obtained with a specific particle size of the wheat bran – 150 to 250 μm –. The use of this specific wheat bran fraction may certainly bring extra cost to the process as a pretreatment stage. It is predicted that sieving only needs capital cost and low operational cost but probably may decrease the overall productivity. However, it is thought that the lack of supplementary liquid addition and approximately 90% increment in the PG activity obtained with the 150 to 250 μm fraction of the wheat bran may compensate the cost of this pretreatment.

When the productivity of the fermentation process was calculated it was seen that a significant increase of 40% was obtained with the use of 150 to 250 μm fraction compared to the previous step (Section 8.5). With this perspective, it was decided to conduct the next optimization experiments with the 150 to 250 μm fraction of the wheat bran.

(a)
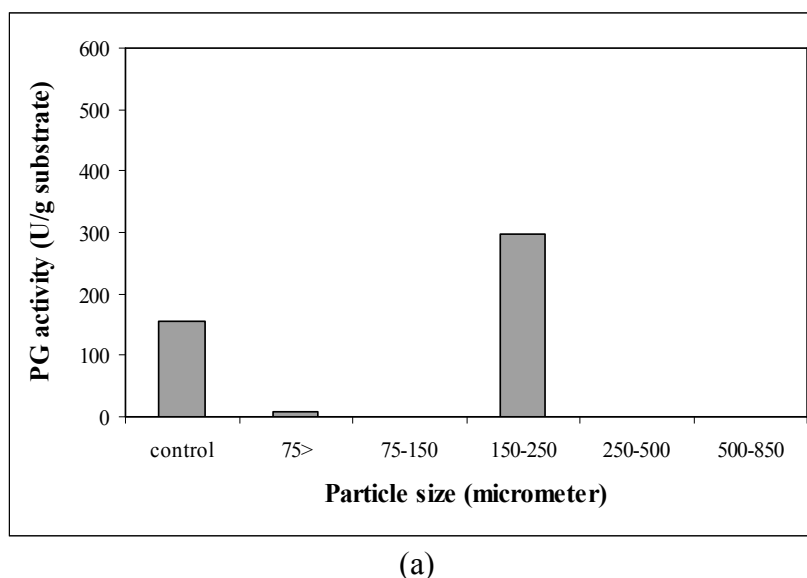
Figure 8.7. (a) PG activity, (b) final pH, (c) specific activity, (d) consumption of total carbohydrate profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content with water)
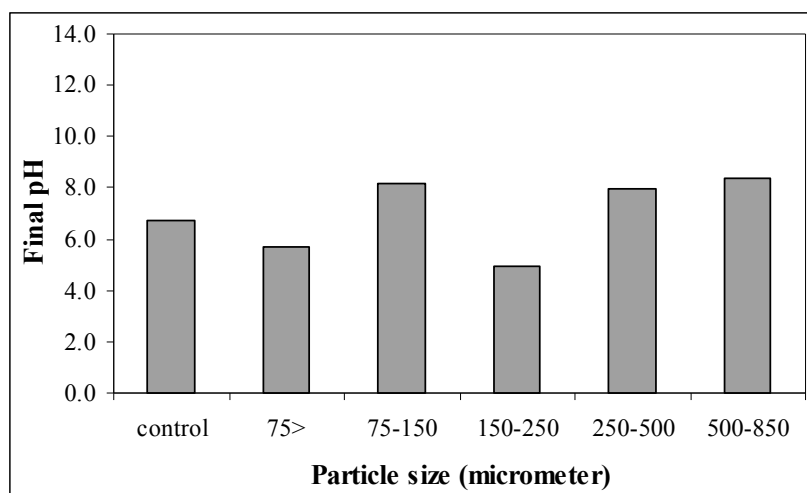
(b)
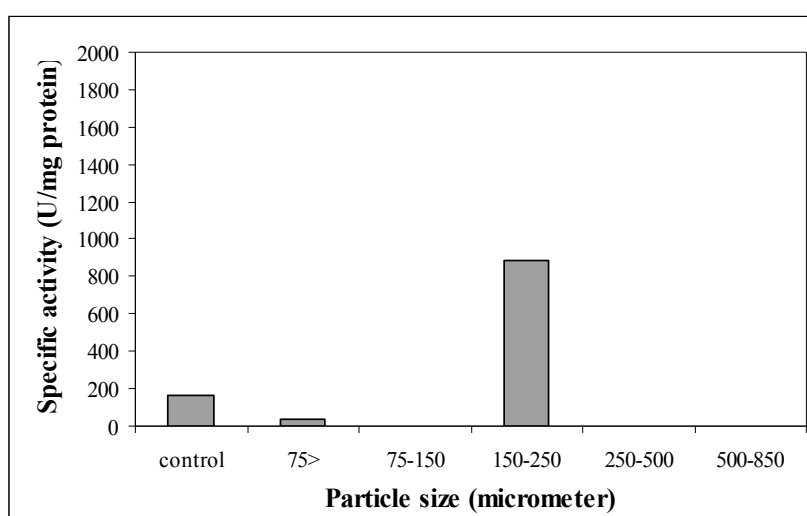
(c)
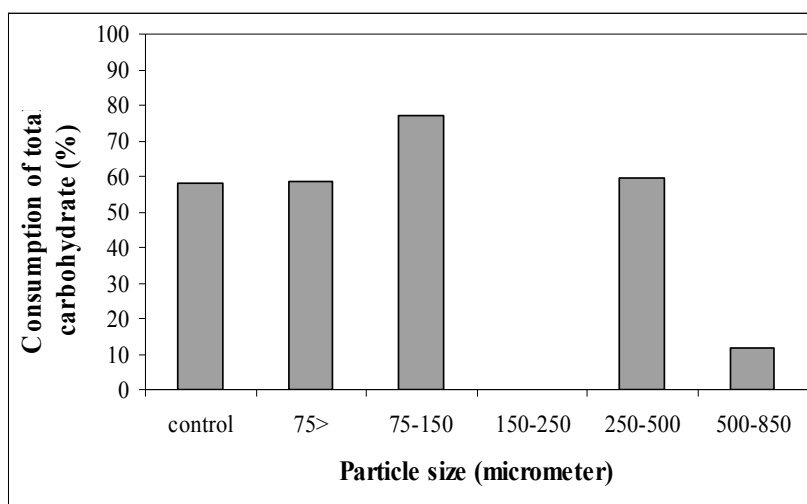
(d)

Figure 8.7. (cont.)

8.7. Effect of Agitation Frequency

One of the key factors in the SSF process is agitation of the fermentation media during the process since it plays crucial roles such as homogenization of the substrate and added water, prevention of the thermal gradients, removal of the excess heat and supply of the oxygen demand of the fungal growth. However, an unsuitable agitation regime may adversely affect the fungal SSF process due to the disruption of the mycelial growth and product formation. Therefore, a lab-scale study should be conducted to determine the agitation requirement of the process with respect to its own target. A SSF process may be agitated or non-agitated which is generally called as static. In case of the operation of the agitation, these facts should also be cleared by the lab-scale experiments; selection of the continuous or intermittent type of agitation, decision of the frequency and intensity of the agitation, observation of the physical affects of agitation on the substrate, cost/benefit balance of the agitation within the whole process (Mitchell et al., 2006a). Hence, in this part of the study agitation frequency of the SSF process with respect to PG production from *A. sojae* M5/6 was investigated. For this purpose, PG activity, final pH, specific PG activity and consumption of total carbohydrate responses were analyzed and compared with the control which was static during the whole process (Figure 8.8).

This set of experiments was held with 150-250 μm particle sized wheat bran. The PG activity of the control was in accordance with that one performed in the Section 8.6 under the same conditions. The Figure 8.8a showed that the PG enzyme was achieved to be produced at all agitation frequency levels resulting in the final pH values of less than 5.0 (Figure 8.8b), but the maximum enzymatic activity was obtained with the fermentation media agitated for 3 times per day. The agitation of the SSF medium for 3 times per day was able to enhance the PG activity by 17.2% compared to the static medium, however, obtained PG activities were lower than the control at all other agitation levels. It is possible that for 3 times agitated per day runs the generated metabolic heat was able to be removed or oxygen could be transferred to the fermentation medium very homogenously as a result of agitation. These facts probably led to a proper fungal growth and gave the fungus the chance of reaching the nutrients not only at its limited surrounding area as in the static fermentation but also to the fresh ones at different regions of the medium as a result of the movement of the fungus due to

the agitation action. However, Figure 8.8a showed that 1 or 2 times per day agitation was not sufficient to achieve a PG activity more than the static runs. The Figure 8.8d supported that the fungi of the 1 and 2 times per day agitated runs could not be able to reach and consume as much carbohydrate as the 3 times per day runs. On the other hand, agitation of the fermentation medium more than 3 times per day adversely affected the PG activity. The low pH values of the fermentation media denoted in the Figure 8.8b supported the existence of the metabolic activity intended to produce PG enzyme for the 4 and 5 times per day agitated runs, however there was a strong possibility that the mycelial growth of the fungus was partly damaged. Hence the attachment of the fungal branches to the solid substrate was negatively affected by the excess frequency of the agitation which resulted in the lack of penetration of these branches into the cracks and pore of the substrate in order to utilize the nutrients in the fermentation medium. The low consumption of total carbohydrate values (compared to the control) at the end of the fermentation for the 4 and 5 times per day agitation levels (Figure 8.8d) confirmed this statement. Additionally, more agglomeration of the substrate was observed visually at the 4 and 5 times per day agitation levels. Moreover, Figure 8.8c indicated that with the agitation of the SSF medium for 3 times per day, the specific activity was increased significantly by 73.8% compared to the static runs. This increment supported the theory that as the fermentation conditions are optimized purer PG enzymes could be produced without the need of any purification process. A limited number of parametric SSF studies were conducted on the effect of agitation on the enzyme production. One of them was performed by Diaz et al. (2009) who investigated the production of exo-polygalacturonase, CMC-ase and xylanase by *Aspergillus awamori* grown on grape pomace in a laboratory scale rotating bioreactor. In this study the researchers focused on the effect of agitation types at different aeration flow rates on the enzyme production by conducting experiments with the agitation types of: static, intermittent (1 min and 2*10min) and continuous, and the aeration rates of 0, 9, 120 and 200 ml/min. They found that for the exo-PG production the type of agitation did not show a significant difference with low aeration flow rates (0 and 9 ml/min), but low enzymatic activities were recorded. However, as the aeration flow rate increased to 120 ml/min the maximum exo-PG activities were measured with the static and 1 min/day intermittent agitation types, meaning that the aeration provided sufficient oxygen for the enzyme producing metabolic activities of the fungus hence the presence of an intense agitation was not essential. It was also stated that at the 120 ml/min aeration level, the

agitation frequency values more than 1 min/day reduced the exo-PG activity due to the possible disruption of the fungal mycelia. The results obtained in that study revealed the presence of a close relationship between the aeration and agitation during the SSF process. It was also indicated that the requirement of agitation show difference depending on the type of microorganism, nature of the substrate, environmental conditions and the type of target product.

The productivity of the process obtained with the 150 to 250 μm fraction (Section 8.6) was improved for 54.5% with respect to the 3 times/day run. This part of the study may provide an insight to the agitation regime consideration of a possible bioreactor design. The results indicated that not a significant difference was achieved with the 3 times/day agitation compared to the static control. However, in the upper scale, agitation may be selected as a tool for the removal of possible generated metabolic heat and moisture or the homogenous sampling. In that case, this set of experiments has shown the importance of the assignment of an optimum agitation regime on the PG activity.

(a)
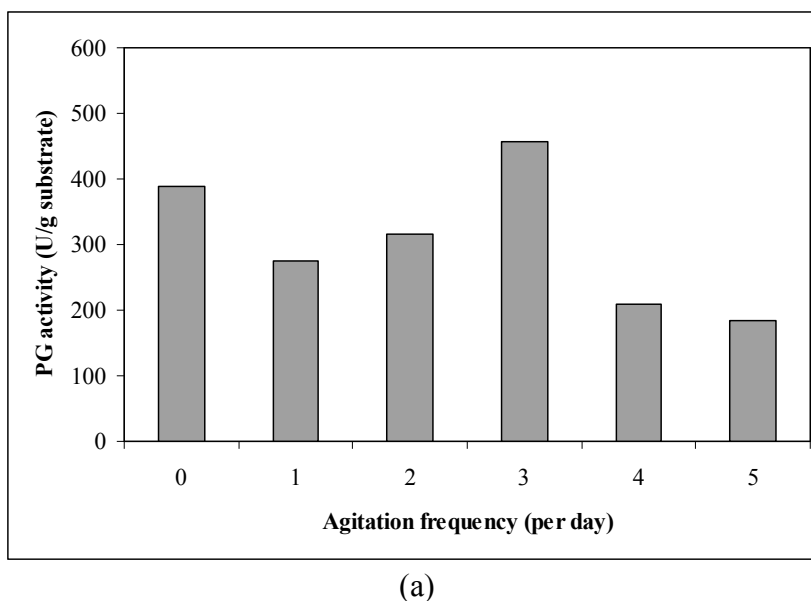
Figure 8.8. (a) PG activity, (b) final pH, (c) specific activity, (d) consumption of total carbohydrate profiles of PG production of *Aspergillus sojae* M5/6 (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content with water, substrate particle size: 150-250 μm)

(cont. on next page)
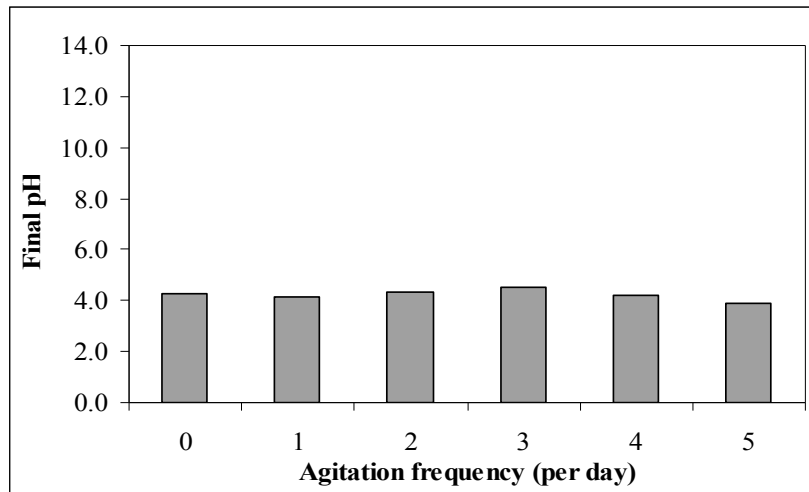
(b)
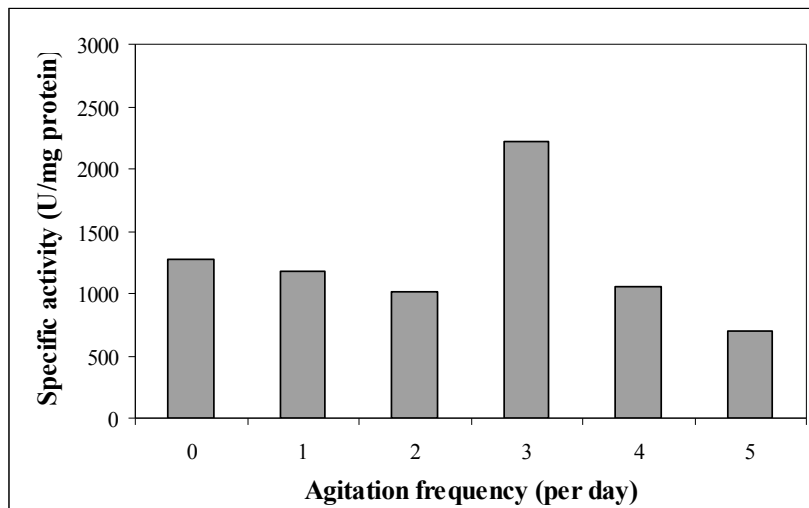
(c)
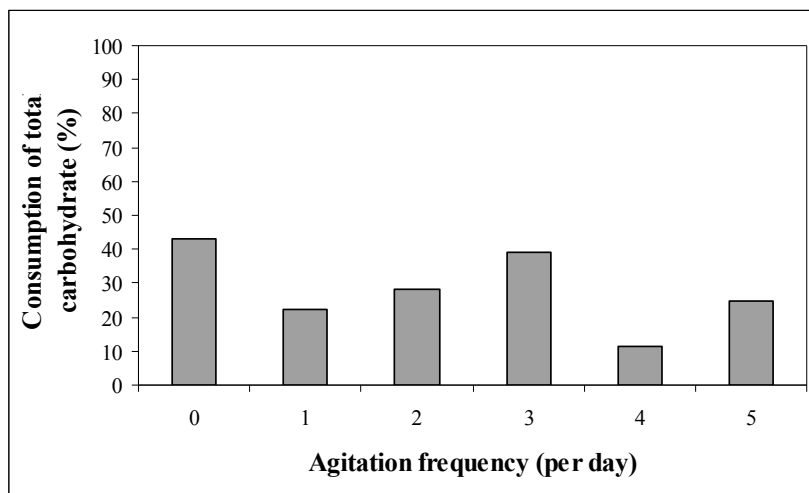
(d)

Figure 8.8. (cont.)

8.8. Transition to a New Wheat Bran as the Substrate and Characterization of Wheat Bran

Until this point of the study some of the fermentation conditions such as inoculum size (10^7 spore/g substrate), incubation time (4 days) and temperature (37 °C), initial moisture content (62%), pH (neutral), particle size (150-250 μm) and agitation frequency (3 times/day) were optimized step-by-step in the 250 ml shake flasks in order to maximize the PG enzyme activity. The maximum PG activity was obtained as 456.6 U/g substrate at the end of that investigation period. However, at the end of this part of the study a problem was encountered. The wheat bran, İzmir supported from a bakery in the Güzelbahçe was used successfully during the optimization part (until the end of the Effect of Agitation Frequency). But unfortunately this wheat bran was depleted. Then many samples (22) were taken from various bakeries (8), flour manufacturers (8), supermarkets (3), feed wholesaler (1) and research institutes (2) and screened for their PG enzyme production capacities under the conditions of 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content adjusted with water. At the end of a long screening period, finally the PG activity close to that of wheat bran, İzmir could be obtained with a sample and it was supplied with a large amount from Hazal Flour and Feed Manufacturing Company, Turgutlu, Manisa. 5 samples were taken from the newly supplied batch of the wheat bran (not sized) and the PG activities were obtained as 228, 118, 136, 102 and 128 U/g substrate after the SSF process again held at 10^7 spore/g substrate, 4 days, 37 °C and 62% moisture content adjusted with water. Then it was decided to continue with this batch of wheat bran, since the conditions optimized up to that point was about the growth and PG producing metabolism of the fungus but not the characteristics of the wheat bran. Additionally, the 100 – 250 μm size fraction of this new wheat bran gave a relatively high PG activity of 535.4 U/g substrate which made our decisions clear about conducting the further experiments with this wheat bran coded as Hazal. Also, after the addition of distilled water and/or inoculum, the rheology of this new wheat bran was also very suitable to be mixed properly without any formation of dough-like structure. But this is a general problem that should always be considered in the upper scales of solid-state fermentation. In the large scale SSF production processes it should be kept in mind that there will be variability between the batches of the substrate (Raimbault, 1998). The producer should supply the substrate from a very trustable and good practicing manufacturer because the variability between the batches

of the wheat bran may increase due to the origin, variety and quality of the wheat, variation of the operation parameters such as conditioning temperature in the processing of the wheat, technology of the manufacturing equipment and storage conditions of the wheat (reviewed due to the verbal information given by the manufacturing companies).

Some of the physical and chemical characteristics of the wheat bran, İzmir, Güzelbahçe (one of the screened wheat bran samples with no PG activity) and Hazal were determined and illustrated in Table 8.1. When the determined composition values of the wheat bran types were compared with the literature (Table 2.2) it could be seen that protein, ash, moisture contents were compatible with the reported results. Additionally there is not any significant difference between these wheat bran types with respect to the investigated properties illustrated in Table 8.1 and Figure 8.9.

Table 8.1. Physical and chemical characteristics of the wheat bran

Component	Wheat bran, İzmir	Wheat bran, Güzelbahçe	Wheat bran, Hazal
Moisture (%)	9.36	5.23	9.39
Total protein (%) *	15.73	15.53	12.71
Reducing sugar (%) **	2.34	1.42	3.03
Ash (%) **	3.24	3.46	2.61
Dietary fibers (%) *	66.12	53.26	58.69
pH	6.23	5.57	5.77
Water activity (a_w)	0.395	0.574	0.482
Water holding capacity (%) **	128.64	172.88	121.99

*on wet basis
**on dry basis
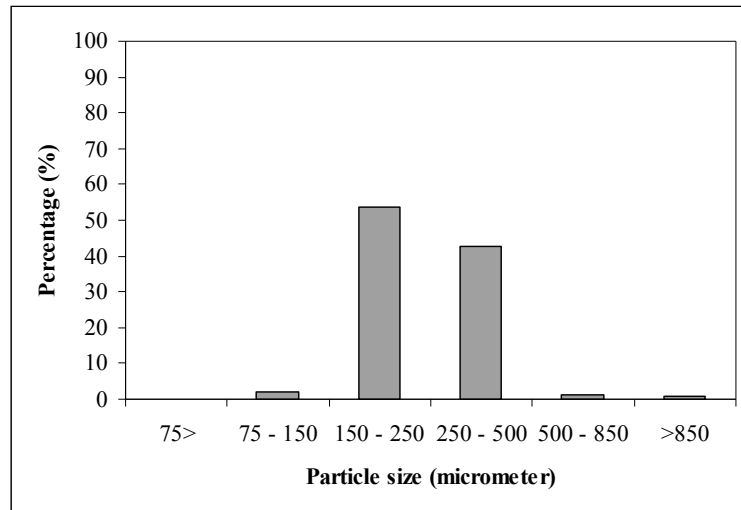
(a)
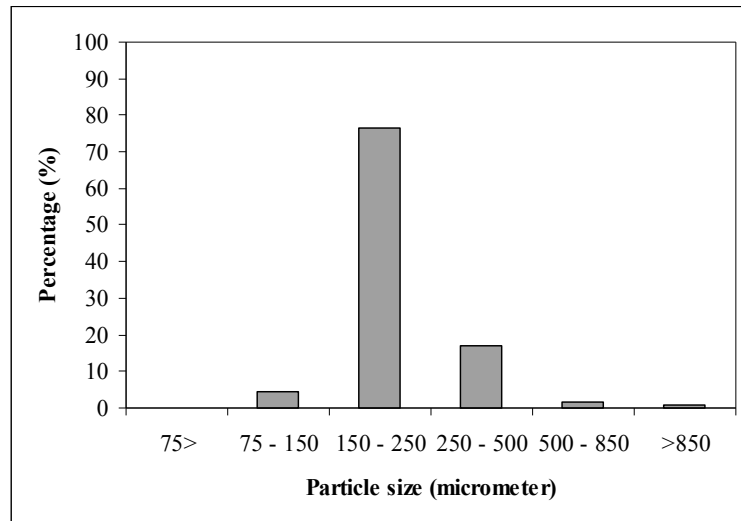
(b)
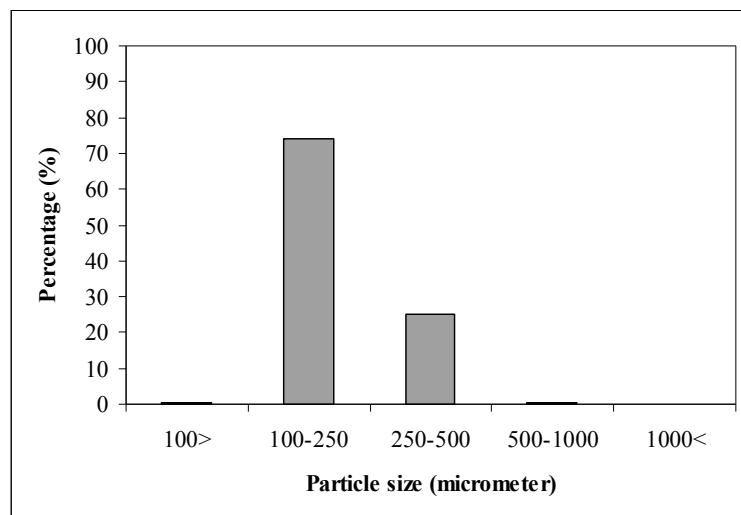
(c)

Figure 8.9. Particle size distributions of (a) wheat bran, İzmir, (b) wheat bran, Güzelbahçe, (c) wheat bran, Hazal

8.9. Effect of Inoculum Type

The objective of this part of the study was to investigate the effect of inoculation of liquid seed culture to the SSF process. The motivation of this investigation was to see whether a vegetative inoculum could be used for the inoculation of the SSF medium, since the production of inoculum by SmF in the bioreactors (larger volumes and controlled conditions) will be more practical than the production of spore solutions (on the slants) at the pilot or industrial scale SSF processes. For this purpose, *Aspergillus sojae* M5/6 was grown as a preculture in the submerged medium (formulated in Section 5.2) formulated and run under the optimized conditions reported by Tari et al. (2007). In this mentioned study, medium formulation, agitation speed and inoculation ratio factors were optimized with the aim of producing PG enzyme and obtaining the desired pellet morphology of the wild type *Aspergillus sojae* ATTC 20235 (Tari et al., 2007). Our preliminary studies indicated that same growth characteristic (pellet form) was also observed for the submerged fermentation of mutant strain *Aspergillus sojae* M5/6 using the medium given in the Section 5.2. Therefore 0, 24, 48 and 72h grown SmF seed cultures were inoculated to the SSF media and the PG activity, final pH, specific activity and consumption of total carbohydrate values of these SSF runs were monitored for 3, 4 and 5 days. The reason of this experimental setup was to determine whether there would be a reduction or not in the duration of the fermentation that would also reduce the cost of the operation.

The SmF media were inoculated with the concentration of $1.7*10^7$ total spore/50 ml inoculum and agitated at 150 rpm which were the conditions reported to produce smaller sized pellets of *Aspergillus sojae* ATTC 20235 (Tari et al., 2007). The pellet count, average pellet size and observations about the pellets obtained in the 0, 24, 48 and 72h grown SmF seed cultures were given in the Table 8.2. The distribution of pellet number and pellet size were given in Figure 8.10 for the 24, 48 and 72h grown SmF seed cultures.

Table 8.2. Morphologic information of the pellets obtained in the precultures

Sample	Pellet number per ml	Average pellet size (mm)	Observation
0 h SmF	0	0	No pellets
24 h SmF	170	0.4218 (±0.2044)	Pellets with hairy region, regular shape
48 h SmF	151	0.4659 (±0.3680)	Pellets with hairy region, irregular shape
72 h SmF	116	0.6752 (±0.4314)	Pellets with hairy region, irregular shape

As can be seen in Figure 8.10 large numbers of small pellets (<0.5 mm) were obtained in the 24 and 48h-incubated SmF cultures, however number of the relatively larger sized pellets was higher in the 72h incubated SmF cultures. The pictures of the pellets given in Figure 8.11 also presented that smaller, more uniform and more regular pellets and also some free filamentous mycelia were obtained in the 24h incubated SmF culture. This means that as summarized in Table 8.2 smaller and numerous pellets were inoculated to the SSF media via the 24h-incubated SmF culture which probably led to a homogenous and effectively distributed inoculation process. Also the presence of the more free filamentous mycelia might be effective in the adaptation of the fungus to the solid environment after the submerged one with respect to easy attachment to the solid by penetrating into the cracks and pores. Additionally, when we compared the pictures of 24h SmF runs (Figure 8.13g, h, i) with the 48 and 78h SmF runs (Figure 8.13j, k, l, m, n, o) it was clearly observed that the latter group had more aerial hyphae on the surface of the substrate that might alter the metabolic activities of the fungus. These points could be the reason of obtaining higher PG activity with the inoculation of 24h SmF preculture (having the smallest average pellet size) compared to the other seed culture inoculated SSF batches. A similar result was obtained by Papagianni et al. (2001) who noted that inoculation of the mixture of small pellets and filamentous mycelia of *Aspergillus niger* to the solid-state fermentations resulted in increased phytase activity.

On the other hand, when the seed culture inoculated SSF runs were compared with the one inoculated with a spore solution of 10^7 spores/g substrate concentration it could be clearly seen that none of the seed culture inoculum type could affect the PG

activity in a positive manner. On the contrary, Sekar and Balaraman (1998) found that the yield of Cyclosporin A (an antibiotic) was higher with the vegetative inoculation than the spore inoculation to the SSF media. They also determined that the yield of Cyc A showed variation due to the size of the inoculum.
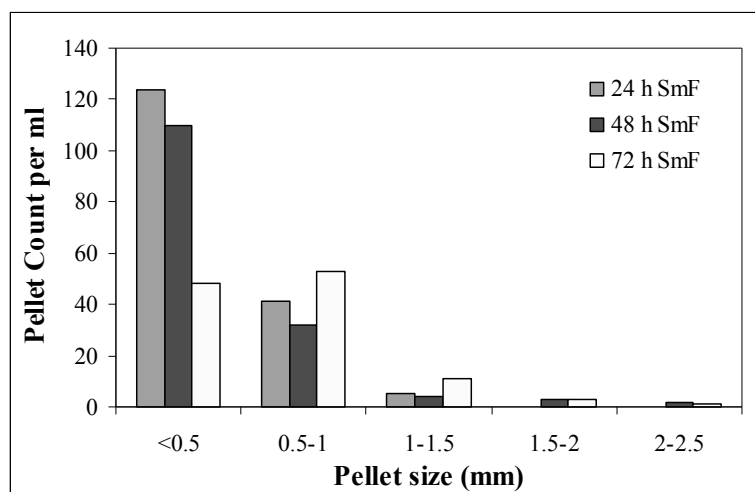
Figure 8.10. Pellet size and pellet count distribution of the SmF seed cultures fermented for 24, 48 and 72 hours
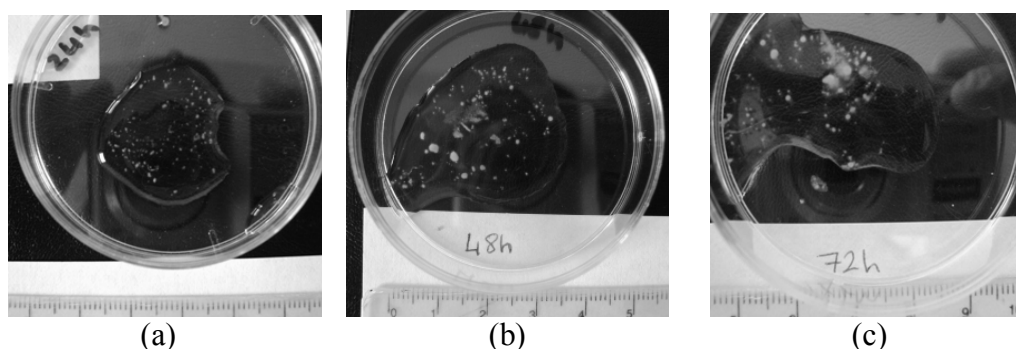
Figure 8.11. Pictures of the pellets obtained from (a) 24h, (b) 48h and (c) 72h SmF precultures

The aim of inoculating the 0h SmF culture to the SSF flasks was to investigate whether SmF medium composition (Section 5.2) has any nutritive or inducing effect that might repress the effect of the seed cultures on the solid substrate to produce the PG enzyme. The morphology of the spore inoculated and 0h SmF inoculated runs were very similar as can be seen in Figure 8.13a, b, c and d, e, f, respectively. But the results

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plotted in Figure 8.12a revealed that 0h SmF inoculations (composed of just the spores and SmF media) reduced the PG activities compared to the control that was inoculated with spores via distilled water. It is thought that the major reason for this fact is the catabolite repression phenomenon. A similar result was reported by Patil and Dayanand (2006b) that as the glucose concentration increased the pectinase activity (produced by *Aspergillus niger* DMF45 growing on the deseeded sunflower head) tended to reduce after a critical concentration. This effect can probably be explained by the catabolite repression of the enzyme due to the abundant presence of a readily metabolizable substrate in the SmF media i.e. glucose.

As can be seen in Figure 8.12d, the consumption of total sugar increased as the incubation day proceeds from 3 to 5 days for 0h SmF runs. But, at the 24h SmF hour runs, the consumption of the total sugar reduced on the 5th day which had probably induced the synthesis of PG enzyme. Therefore, one of the highest PG activities among the SmF inoculated batches was obtained at the 5th day of the 24h pellet inoculated run.

The use of different age of SmF inoculum did not change the final pH of the SSF media (Figure 8.12b). This is an important finding because the content (media formulation and produced metabolites) of the SmF inoculum should not alter the pH of the SSF medium in a negative way.

Another interesting result of this set of experiments was that the specific activity obtained with the inoculation of the 24h seed culture was 14% and 30% higher than that of the control at the 4th and 5th days of the SSF process (Figure 8.12c). The fungus probably did not need to synthesize most of the other hydrolytic enzymes to utilize the nutrients in the solid medium due to the readily metabolizable carbon coming from the high glucose content of the SmF medium (Section 5.2) and therefore the amount of proteins other than PG enzyme might be reduced and specific activity increased. This result showed that inoculation of the SSF with a SmF seed culture is a potential application for the production of PG enzyme in the pilot or large scale bioreactors. However our results indicated obviously that the composition of the SmF media, morphology of the fungus and fermentation conditions of the seed culture production process play the key roles in the development of a seed culture inoculated SSF process.
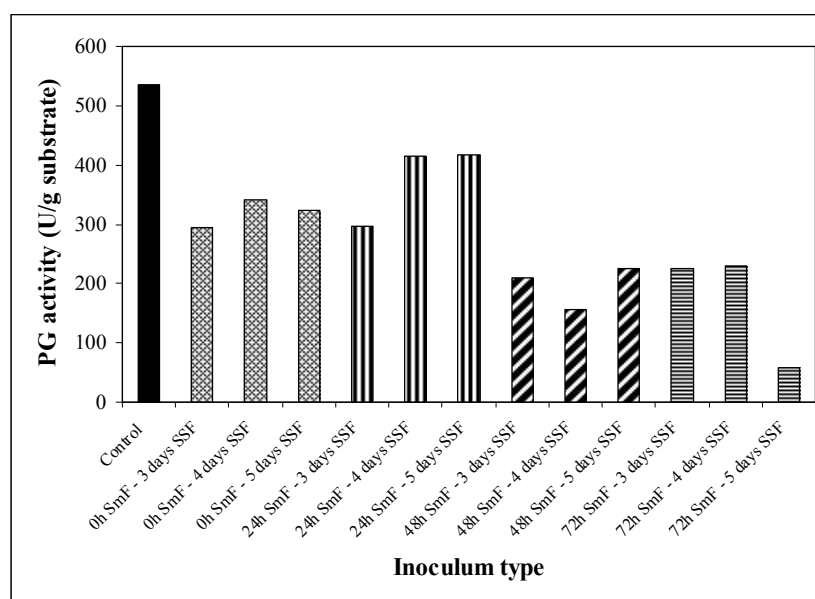
(a)
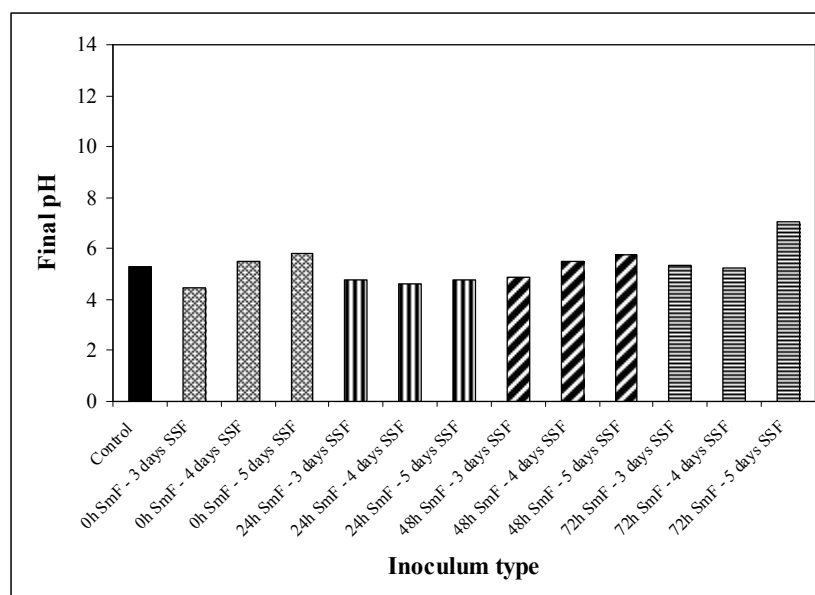
(b)

Figure 8.12. (a) PG activity, (b) final pH, (c) specific activity, (d) consumption of total carbohydrate profiles of PG production of *Aspergillus sojae* M5/6 by SSF inoculated with various inoculum types

(c)

(d)

Figure 8.12. (cont.)

Spore – 3 days (a)	Spore – 4 days (b)	Spore – 5 days (c)
0h SmF – 3 days (d)	0h SmF – 4 days (e)	0h SmF – 5 days (f)
24h SmF – 3 days (g)	24h SmF – 4 days (h)	24h SmF – 5 days (i)
48h SmF – 3 days (j)	48h SmF – 4 days (k)	48h SmF – 5 days (l)
72h SmF – 3 days (m)	72h SmF – 4 days (n)	72h SmF – 5 days (o)
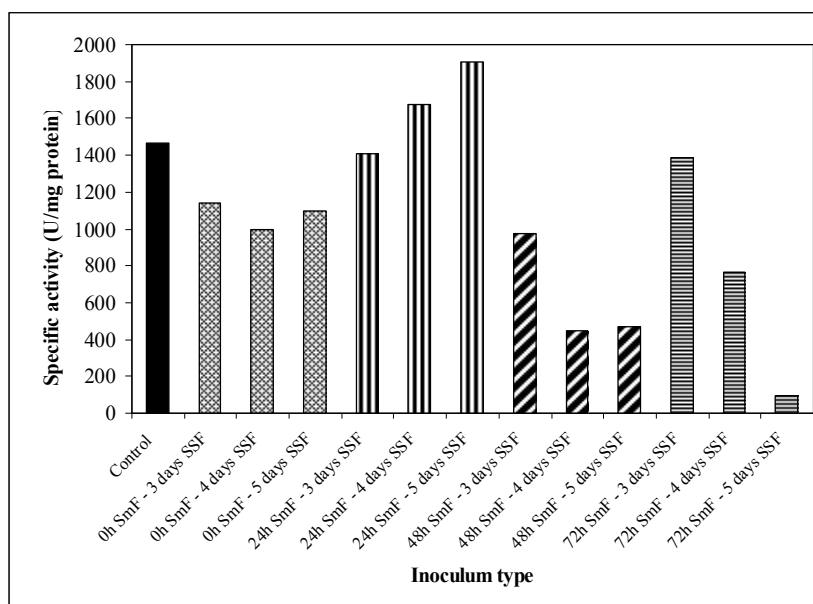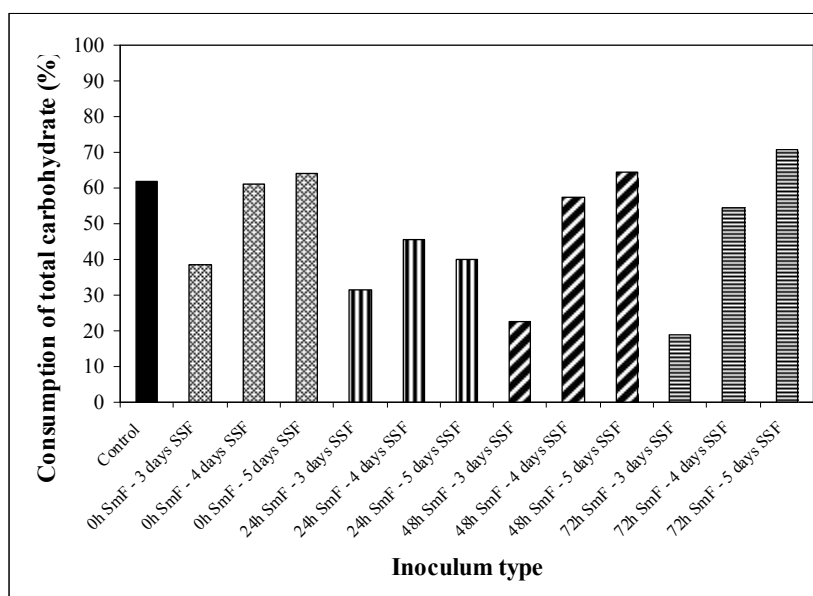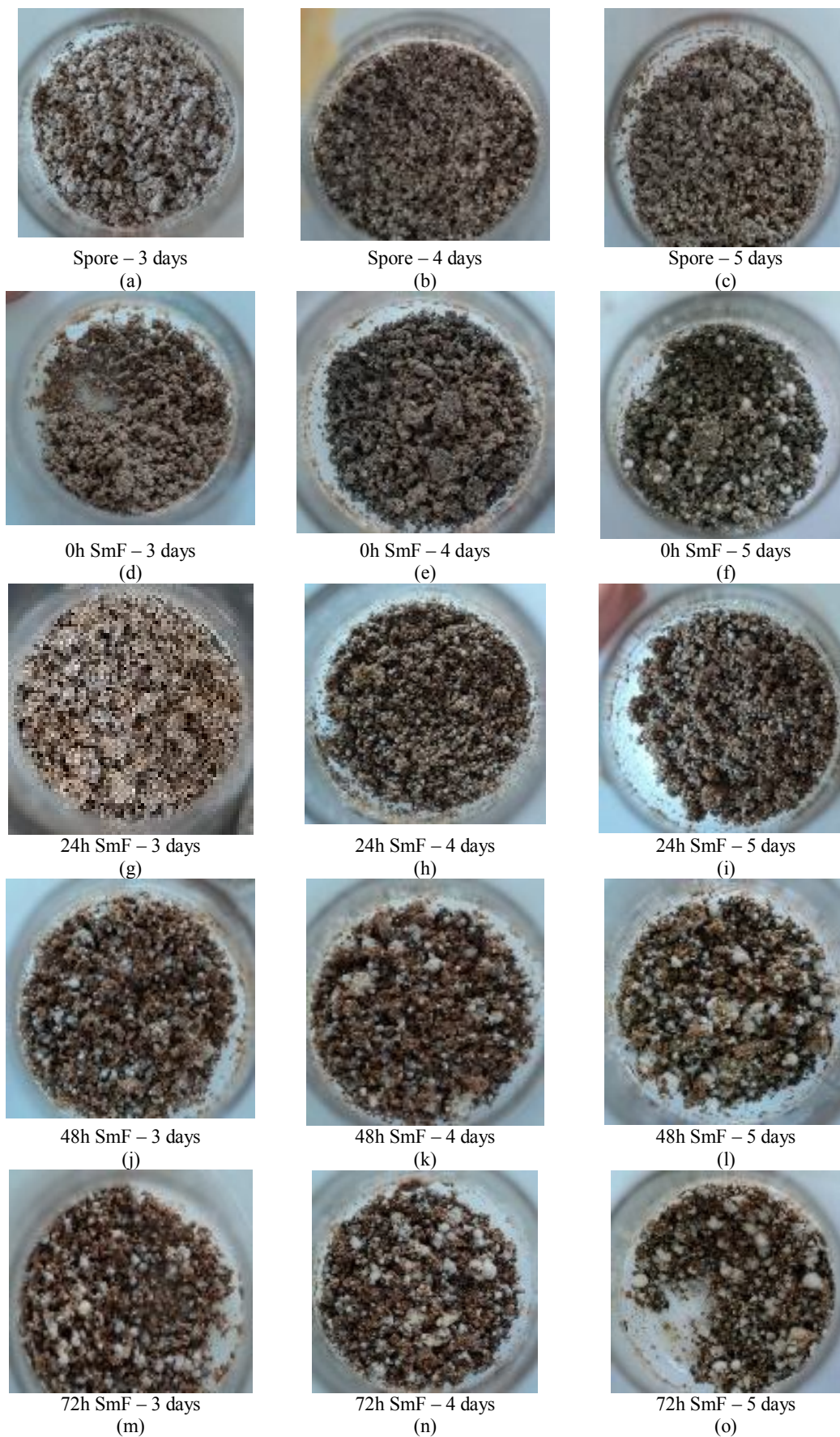
Figure 8.13. Pictures of the spore and pellet inoculated flasks at the end of 3rd, 4th and 5th day of solid-state fermentation

The results of this chapter indicated the importance of optimization of the fermentation conditions in the SSF process for enzyme production. Hence, the PG activity was enhanced from 73.4 U/g substrate (Section 8.1) to 456.6 U/g substrate with wheat bran, İzmir (Section 8.7) and 535.4 U/g substrate with wheat bran, Hazal (Section 8.9) as a result of a series of optimization process. The productivity of the process was also improved from 1.43 (Section 8.2) to 4.75 U/g substrate^{-1}h^{-1} (Section 8.7) with wheat bran, İzmir and 5.58 U/g substrate^{-1}h^{-1} with wheat bran, Hazal at the end of the optimization part of the study.

When we compare these results with the literature of those who have expressed the unit of the enzymatic activity as similar as the current study (in Section 5.3), the maximum PG activities (Section 8.7 and 8.9) obtained in this study is superior to those of Patil and Dayanand (2006a), Alcantara et al. (2010) and Ustok et al. (2007) with a rough range of 10 – 40 U/g detailed in Table 3.3. Besides, the maximum PG activity of the current study is even higher than the higher range of the PG activities of 368, 370 and 385 U/g reported by Linde et al. (2007), Fontana et al. (2005) and Taşkın and Eltem (2008), respectively. The utilized microbial strains and substrates can be found in Table 3.3. At this point, it should be noted that the authors preferred to use nutritive supplementation to the fermentation media (see Table 3.3) in all of these mentioned reports contrary to our process that is composed of only wheat bran and water.

On the other hand, the maximum productivity value of the current study (5.58 U/g substrate^{-1} h^{-1}) obtained with the spore inoculated wheat bran, Hazal (Figure 8.12a) was lower than those calculated as 6.15 and 7.71 U/g substrate^{-1} h^{-1} from the data of Linde et al. (2007) and Fontana et al. (2005), respectively. However, it was in line with the productivity of Taşkın and Eltem (2008) calculated as 5.53 U/g substrate^{-1} h^{-1}.

The results obtained at the incubation temperature and agitation frequency will be evaluated at the design of bioreactors for larger scale production processes. On the other hand, particle size effect and type of inoculum parts gave information on the preparation stage of the upper scale SSF process.

CHAPTER 9

BIOCHEMICAL CHARACTERIZATION OF THE CRUDE POLYGALACTURONASE

The biochemical characterization of polygalacturonase (PG) enzyme produced from *Aspergillus sojae* M5/6 with wheat bran (Hazal) at the flask scale under the optimized conditions (10^7 spore/g substrate inoculum, 4 days of fermentation, 37 °C, 62% initial moisture content, water as the moistening agent, $100 - 250$ μm particle size, 3 times/day agitation and spore solution as the inoculum type) was presented in this chapter. The purpose of the biochemical characterization was to understand the activity or stability behavior of the produced enzyme under different conditions in order to evaluate its potential applications in the area of biotechnology or food engineering.

9.1. Effect of pH on the Activity and Stability of PG

The results of the effect of pH on the PG activity were presented in Figure 9.1. The pH values of the reaction mixture were adjusted by various buffers ranging from 3.0 to 12.0 as detailed in Section 5.13.1. As can be seen in Figure 9.1, the produced PG enzyme showed significant activity only at the acidic region ($4.0 - 5.0$) and its activity was very low (below 20%) at the very acidic side (pH 3.0) and also above pH 5.0. It is thought that with the change in pH, the ionization of essential active site amino acid residues were affected, which are involved in substrate binding and catalysis, i.e., breakdown of substrate into products (Bhatti et al., 2006). The enzyme found to be a typical acidic pectinase displaying its activity on the polygalacturonic acid under the acidic region of pH 4.0 to 5.0. This result confirmed the use of reaction mixture at pH 4.8 in all of the enzymatic activity assays of the PG conducted in this study. Some biochemical properties of the fungal PGs were nicely reviewed by Niture (2008) recently. In that study, it could be seen that most of the fungal PG enzymes stated in the literature have maximal activity at the acidic pH range of $2.0 - 5.5$. Dinu et al. (2007) reported a PG enzyme having an optimum activity at 4.6 against sodium

polygalacturonate. This purified PG (with chromatographic methods) was obtained from an *Aspergillus niger* strain and lost its activity rapidly beyond 5.0 similar to the PG produced in the current study. Another PG produced by *Thermoascus aurantiacus* was also reported to lose most of its activity out of the range of 3.5 to 5.5 (Martins et al., 2004). The PG enzyme produced in the current study was thought to have potential in the enzymatic clarification (depectinization) of the fruit juices which mostly have acidic pH values.
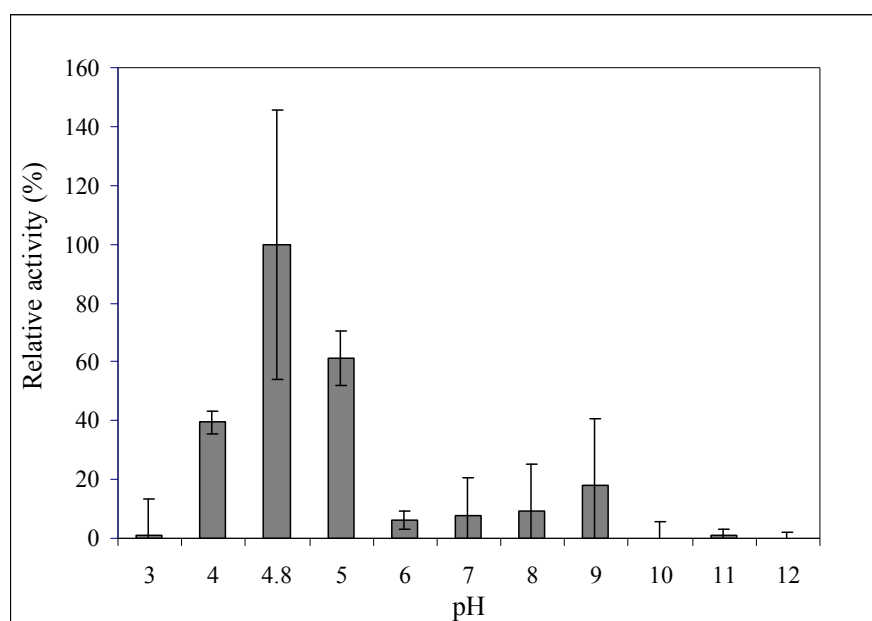
Figure 9.1. Effect of pH on the polygalacturonase activity

The stability of the *Aspergillus sojae* M5/6 PG was investigated in the pH range of 3.0 to 12.0. The residual activities (%) which remained after incubating the enzyme at various buffers (Section 5.13.1) for 2 hours at 30 °C were presented in Figure 9.2.

The study conducted on the stability of the PG showed that enzyme was stable in the pH range of 3.0 – 7.0 by saving at least 65% of its activity. The stability of the enzyme was maximized at pH 3.0 and it sharply decreased above 8.0. Martin et al. (2004) reported two SSF polygalacturonases obtained from *Moniliella* sp. and *Penicillium* sp. preserving 70-100% of their initial activities between pH 3.0 - 10.0 and 3.0 – 8.0, respectively. Most of the PG enzymes stated in the literature lost more than 70% of their stability beyond the pH value of 9.0 (Freitas et al., 2006; Martins et al., 2002; Acuna et al., 1995). The PG enzyme of the current study was superior to the crude PG enzyme produced by submerged fermentation from *Aspergillus sojae* ATCC

20235 which was reported to preserve its stability (more than 65%) only at pH 5.0 and 6.0 (Tari et al., 2008). It is thought that the stability of *Aspergillus sojae* M5/6 PG is compatible with the fruit juice processing requirements. Additionally the stability range of this PG should be considered in the packaging and storage of the possible end-product.
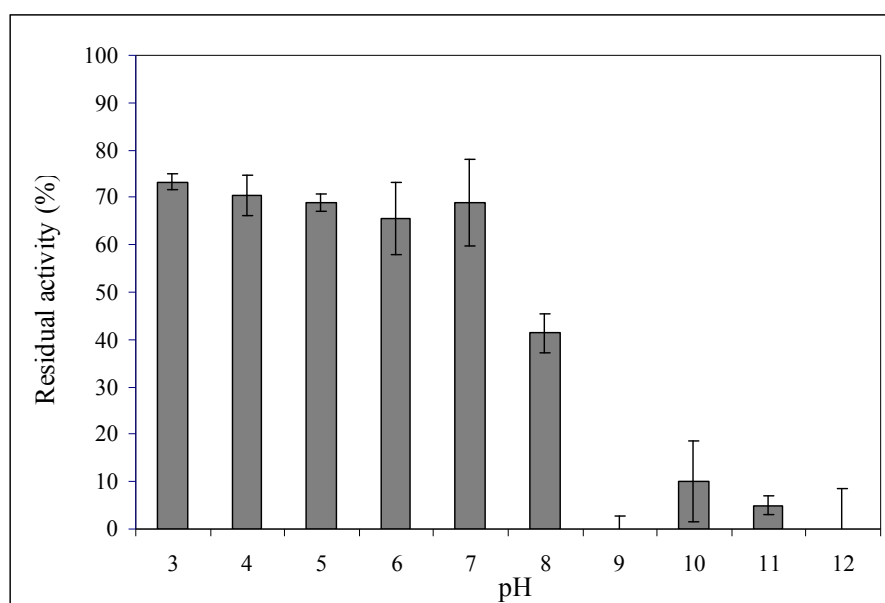
Figure 9.2. Effect of pH on the stability of polygalacturonase

9.2. Effect of Temperature on the Activity and Stability of PG

The effect of temperature on the PG activity was determined by the incubation of the reaction mixture (pH 4.8) for 20 minutes at different temperatures ranging from 25 to 80 °C. The results of the experiments are presented in Figure 9.3.

The PG could show activity over a broad range of temperature (25 – 80 °C). However, the optimum activity for the PG was 40 °C and it retained its 99% activity at 50 °C. Most of the researchers determined the optimum activity of their exo-PG enzymes (fungal or bacterial) between 40 and 60 °C (Dinu et al., 2007; Freitas et al., 2006; Kapoor et al., 2000). Ortega et al. (2004) have found the optimum PG activities of three commercial pectinases Rapidase C80 (Gist–Brocades), Pectinase CCM (Biocon) and Pectinex 3XL (Novozyme) as 55, 50 and 50 °C, respectively. This indicated that PG of *Aspergillus sojae* M5/6 has compatible activity with these three commercial enzymes. The optimum temperature of activity of the *Aspergillus sojae* M5/6 PG (40-50

°C) is very suitable for fruit juice clarification (enzymatic depectinization) applications which are generally held between 30 – 50 °C. But *Aspergillus sojae* M5/6 PG had shown sensitivity at 60 °C and could not be active at this temperature, therefore in case of its use in the fruit juice clarification process, the pasteurized juice should carefully be cooled to the temperatures below 60 °C before the addition of this enzyme.
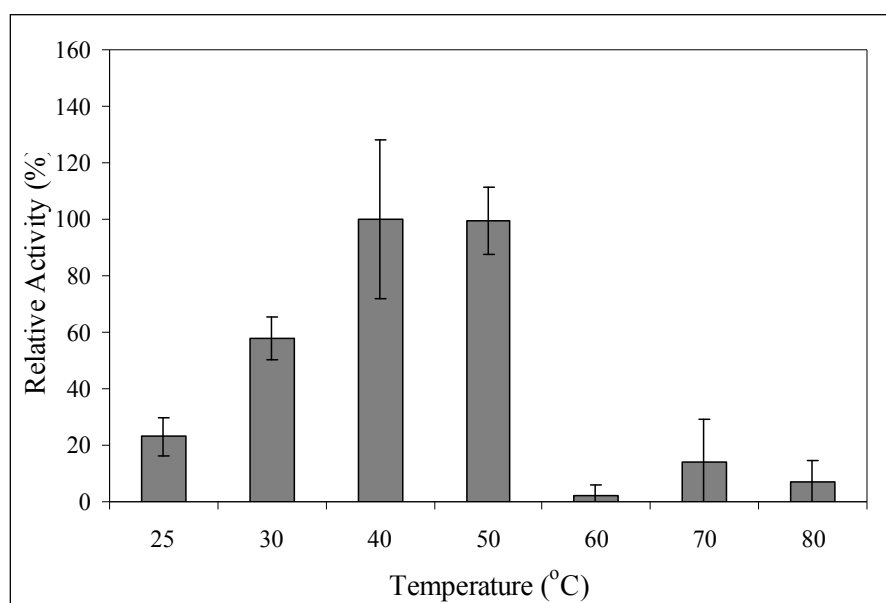
Figure 9.3. Effect of temperature on the polygalacturonase activity

In order to investigate the thermostability of the PG, the enzyme was incubated at various temperatures between 25 – 80 °C for 30 and 60 minutes. The residual activities (%) after this incubation periods were plotted in Figure 9.4.

As can be seen in Figure 9.4, there was not any significant difference between 30 and 60 minutes with respect to the thermostability of the enzyme. The *Aspergillus sojae* M5/6 PG could preserve more than its 50% stability between 25 and 50 °C, both for 30 and 60 minutes. This property indicated that the PG has sufficient thermostability at the enzymatic depectinization of fruit juices held between 30 – 50 °C. Besides, the enzyme was found to have potential to be utilized at the cold depectinization process (about 20 °C) of the fruit juice as well. However, the enzyme could not present any thermostability above 50 °C. The thermostability range of *Aspergillus sojae* M5/6 PG was narrower when compared to the other studies (Martin et al., 2004; Martins et al., 2002; Tari et al., 2008).
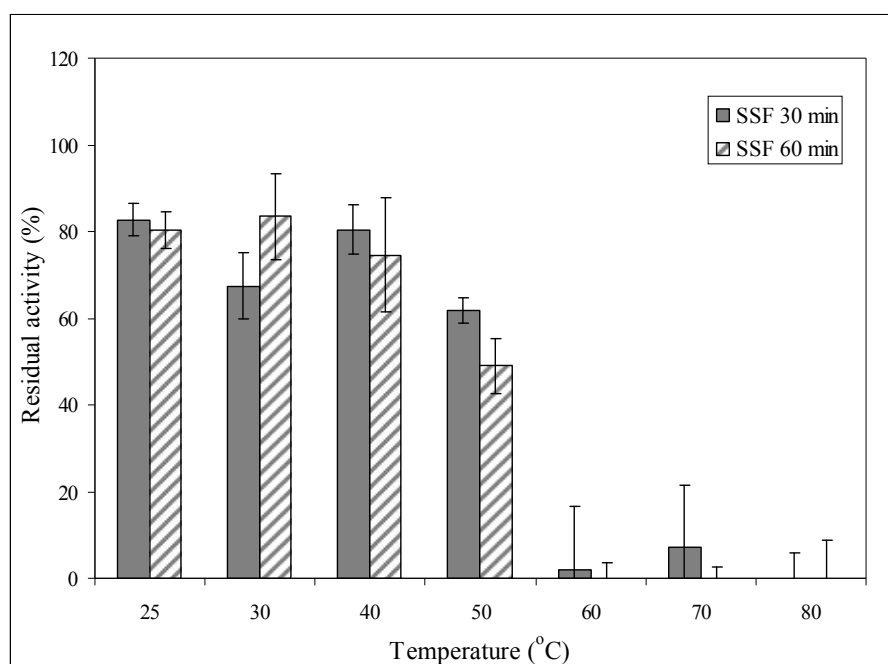
Figure 9.4. Effect of temperature on the polygalacturonase stability

9.3. Effect of Metal Ions and Various Compounds on PG Stability

The effect of 12 compounds and 7 metal ions on the stability of the PG activity was investigated by incubating the PG enzyme in the presence of these reagents with the volumetric ratio of 1:1 at 30 °C for 1 hour. The residual PG activity (%) was determined with the standard PG activity assay (Section 5.3) and tabulated in Table 9.1.

The PG enzyme activity was enhanced by 19 and 17% in the presence of acetic acid and citric acid, respectively that were representing the total acidity of the orange juice (Kelebek et al., 2009). Kapoor et al. (2000) reported a PG produced by SSF from *Bacillus* sp. MG-cp-2 that was also enhanced in the presence of 1 mM citric acid. Moreover, the activity of the *Aspergillus sojae* M5/6 PG increased in the presence of sucrose and glucose and reduced slightly (17.67%) in the presence of fructose which were experimented at their detected levels in the orange juice by Kelebek et al. (2009). The other result was the presence of organic and hydroxybenzoic acids that were gallic, malic, tartaric and ascorbic acids (found commonly in the orange and grape juices) significantly stimulated the activity of the PG with the rates of 35%, 29.5%, 22.5% and 32.8%, respectively. These mentioned results were good indicators for the potential of the produced PG enzyme in the fruit juice (especially orange and grape) and wine industry.

The PG enzyme was very stable in the presence of ammonium sulfate which was allowed in the grape cider (for the prevention of pulp oxidation) stated in the wine communication under Turkish Food Codex Regulations (notification no. 2008/67) (Official Gazette, 2009). It also showed stability in the presence of 12.6% ethanol which was measured by Kelebek et al. (2009) in the orange wine. During the wine production process, pectic enzymes can be added while grapes are being crushed, before or after the fermentation of the must or after the fermentation when the wine is ready for bottling. It was also reported that the addition of pectic enzymes at the last stage (after fermentation) increases filtration rate and clarity. However, it was pointed out that the supplemented enzyme level should be adjusted allowing for the inhibitory effect of the alcohol on the enzyme (Kashyap et al., 2001). As can be seen in Table 9.1, *Aspergillus sojae* M5/6 PG was not inhibited in the presence of 12.6% ethanol, in fact it was stimulated by 13%. This property of the produced enzyme indicates its potential application in the clarification of the wines.

There are a few reports on the effect of metal ions and compounds on the pectinases produced from solid-state fermentation. Therefore, the current PG of this study was also compared to the pectinases produced by SmF in addition to the SSF pectinases reported in the literature. With this perspective, the polygalacturonase of *Aspergillus sojae* M5/6 produced by SSF as described in the current study was quite stable in the presence of the most of the cations tested, unlike the other pectinase extracts produced either by SSF or SmF reported in the literature.

The PG enzyme activity was stimulated with the Mn^{2+}, Cu^{2+}, Mg^{2+} and Na^+ that indicated the possibility of acting as salt or ion bridges that stabilize the enzyme in its active conformation and might protect the enzyme against thermal denaturation or this is a metalloprotein needing a cofactor for higher activities (Correa et al., 2010; Balkan and Ertan, 2010). On the contrary, many PGs were reported to be inhibited by these cations. For example, PG (by SmF) of *Mucor circinelloides* (Thakur et al., 2010) was inhibited with the presence of 1 mM Mg^{2+}, Mn^{2+} and Cu^{2+}, extracellular PG (by SmF) of *Thermoascus aurantiacus* (Martins et al., 2007) was inhibited by 2mM of Mn^{2+} and Mg^{2+} additionally, polygalacturonase (by SmF) of *Trichoderma harzianum* (Mohamed et al., 2006) was inhibited by 1 mM of Mg^{2+}, Mn^{2+} and Cu^{2+}. The effect of metal ions could be important in the use of raw materials with a high salt content (Bhatti et al., 2006).

On the other hand, *A. sojae* M5/6 PG was stable in the presence of 0.5 mM EDTA. This result revealed that the PG of *A. sojae* M5/6 was not dependent on the presence of metals otherwise it could not show activity in the presence of a metal chelator i.e. EDTA.

Among the investigated metal ions only the presence of $CaCl_2$ and $FeCl_3$ inhibited the PG activity by 7 and 24%, respectively. This result is compatible with the PG of *Sporotrichum thermophile* Apinis (Kaur et al., 2004) for 5 mM of Ca^{2+} and extracellular pectinase (by SSF) of *Acrophialophora nainiana* (Celestino et al., 2006) for 12.5 mM of Ca^{+2}, whereas the PG (by SSF) of *Bacillus* sp. MG-cp-2 (Kapoor et al., 2000) was stimulated with the 1mM Ca^{2+} ion. The exopolygalacturonases (by SmF) of *Streptomyces* sp. QK-11-3 and *Bacillus* sp. strain KSM-P443 were similarly reported to be inhibited by Fe^{3+} reported by Beg et al. (2000) and Kobayashi et al. (2001), respectively.

When we compare PG of *A. sojae* M5/6 PG (SSF) with the PG of *Aspergillus sojae* ATCC 20235 (wild type) produced by SmF technique by Dogan and Tari (2008), some differences in the behavior of these two PGs were observed. For example, wild type *A. sojae* PG was completely inhibited by 1mM Mn^{2+} after an incubation period of 30 minutes contrary to the mutant *A. sojae* PG. Additionally, wild type *A. sojae* PG was similarly stimulated by 1 mM KCl and NaCl, but it was inhibited by Mg^{2+} and Cu^{2+} unlike the mutant *A. sojae* PG. These results indicated that microbial source and fermentation type are the two factors significantly affecting the biochemical properties of the exopolygalacturonases.

Table 9.1. Effect of various metal ions and compounds on the stability of PG activity

Metal Ion/Chemical Compound	Concentration	Residual PG Activity (%)
Control	-	100.00
Acetic acid	0.3 g/l	118.99
Citric acid	9.1 g/l	116.86
Sucrose	59 g/l	117.16
Glucose	32 g/l	118.66
Fructose	28 g/l	82.33
Gallic acid	3.33 mg/l	135.03
Malic acid	1.06 g/l	129.48
Tartaric acid	0.9 g/l	122.48
Ascorbic acid	0.49 g/l	132.83
Ammonium sulphate	1 g/l	141.55
$CaCl_2$	0.5 mM	93.08
$MgCl_2$	0.5 mM	117.02
$CuSO_4$	0.5 mM	107.48
KCl	0.5 mM	108.81
$FeCl_3$	0.5 mM	76.28
$MnCl_2$	0.5 mM	126.51
NaCl	0.5 mM	107.82
EDTA	0.5 mM	124.83
Ethanol	12.6%	113.02

9.4. Kinetics of Thermal Inactivation and Estimation of the Inactivation Energy

In general temperature is the most important variable in all biological systems and in enzymatic processes as well. Enzymes are referred to be complex labile proteins and biocatalysis occur under non-natural conditions, where the native properties of the enzymes can be significantly altered. Then it is not surprising that the temperature have a profound impact not only in enzyme activity but also in its stability. As a general rule, as the temperature increases, the reaction rate of the catalyzed chemical reaction also increases, besides the rate of the enzyme inactivation increases too. The thermal inactivation of the enzyme is a result of weakening of the intermolecular forces that are responsible for the 3D structure of the enzyme. The thermal inactivation causes a reduction in catalytic capacity of the enzyme. Therefore, the knowledge on the thermal inactivation of the enzyme is critical to properly evaluate the performance of the enzyme under the process conditions (Illanes et al., 2008).

The inactivation of pectolytic enzymes is assumed to follow first order kinetics (Naidu and Panda, 2003). The first-order deactivation rate constants were calculated from the slope of semi logarithmic plot of residual activity versus time (Figure 9.5) and presented in Table 9.2 for the temperatures of 30, 40, 50 and 60 °C. The k_d (deactivation rate constant) values indicated that the enzyme was stable most at 30 °C, but a rapid loss in activity obtained at 60 °C. Similarly, the calculated k_d values for the exo-PG produced from *Aspergillus niger* by SSF increased significantly after 50 °C (Diaz et al., 2011).

Half-life values of the enzyme were estimated from the calculated k_d values using the Equation 5.1 and tabulated in Table 9.2. The half-life dramatically reduced from 231.05 min at 30 °C to 2.007 min at 60 °C. A similar trend was reported by Ortega et al. (2004) for the commercial pectinases. In that study, half-life values (in phase II) were 209.74 and 409.79 min for Rapidase C80 and Pectinase CCM at 40 °C, whereas they reduced to 7.80 and 61.78 min at 60 °C, respectively.

The inactivation energy (E_d) of *A. sojae* M5/6 polygalacturonase was determined as 125.3 kJ/mole from the slopes of the linear curve plotted by 1/T versus ln (k_d) using Equation 5.3 (Figure 9.6). This value is in accordance with the E_d obtained as 128.91 kJ/mole for the exo-PG of *Aspergillus niger* by Diaz et al. (2011) and 1.64 times higher than the Pectinase CCM, but 1.16 and 1.27 times lower than those of RapidaseC80 and Pectinex 3XL. The partially purified PG of *A. sojae* ATCC 20235 (wild type) was reported to have a superior E_d of 286.2 kJ/mole (Dogan and Tari, 2008), but an approximate E_d of 152 kJ/mole was obtained with the crude PG of the same strain (Tari et al., 2008). These results revealed that the current PG holds potential use in the applications below 60 °C, however a further purification may enhance some of its biochemical properties.

Table 9.2. Kinetic parameters for thermal inactivation of *Aspergillus sojae* M5/6 PG

T (°K)	k_d (min^{-1})	$t_{1/2}$ (min)
303	0.0030	231.049
313	0.0049	141.459
323	0.0119	58.248
333	0.3454	2.007
E_d (kJ/mole)	125.3	
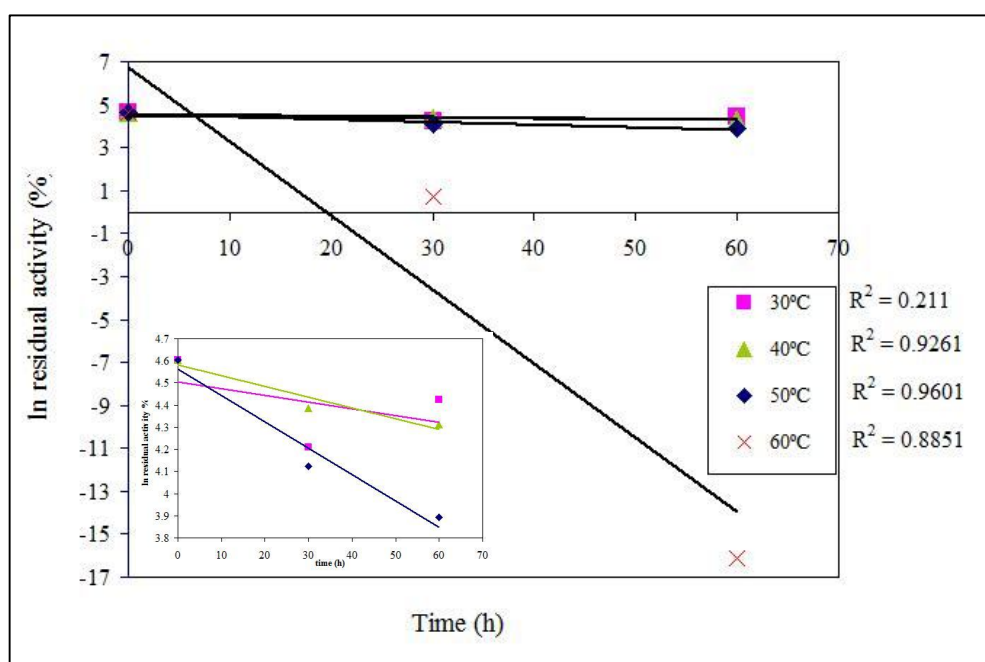
Figure 9.5. First-order plots of the effect of thermal denaturation of *A. sojae* M5/6 polygalacturonase and closer view for trendlines of 30, 40 and 50 °C
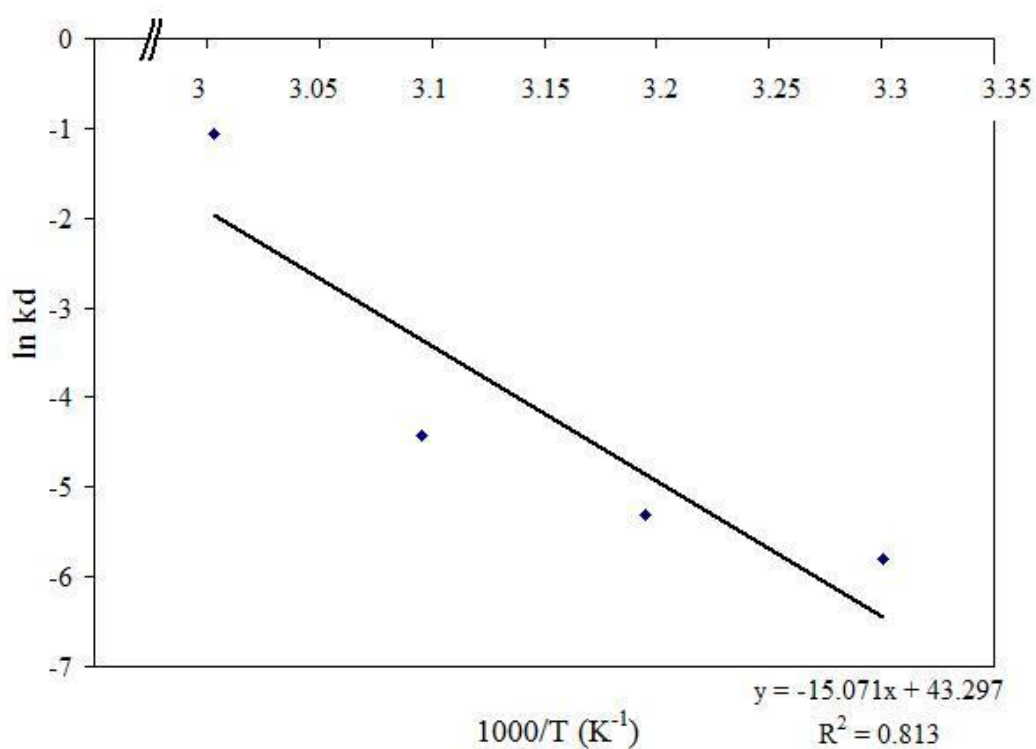
Figure 9.6. Arrhenius plots for determination of energy of thermal inactivation of *A. sojae* M5/6 polygalacturonase

9.5. Estimation of Thermodynamic Parameters during Inactivation of Polygalacturonase

The changes in the enthalpy (ΔH^*) and entropy (ΔS^*) for the thermal inactivation of the polygalacturonase were calculated according to the Equation 5.4 and 5.6 by the transition state theory (Naidu and Panda, 2003). Positive ΔH^* values were obtained with the investigated temperatures indicating the endothermic nature of the inactivation reaction. The free energy of inactivation (ΔG^*) decreases slightly as the temperature increased. This means that less energy is needed for inactivation of the enzyme at 60 °C, which is in accordance with the thermal stability results given in Section 9.2.

Naidu and Panda (2003) predicted that entropy values provide information regarding the degree of solvation and very likely the degree of compactness of protein molecule. The increase in entropy indicates opening up of the enzyme structure (Bhatti et al., 2006). As can be seen in Table 9.3, ΔS^* did not change significantly with the increasing temperature, then thermal deactivation did not imply any relevant variation in the enzyme tertiary structure. Moreover, Ortega et al. (2004) stated that large activation enthalpy is the characteristic of protein denaturation reaction. However, ΔH^* values did not vary with the increasing temperature, supporting the idea of enzyme unfolding may not be the rate-determining step for the irreversible thermal inactivation of PG under the conditions assayed.

Table 9.3. Thermodynamic parameters for thermal inactivation of *A. sojae* M5/6 polygalacturonase

T (°K)	ΔH^* (kJ/mol)	ΔG^* (kJ/mol)	ΔS^* (kJ/mol.K)
303	122.78	88.89	0.11
313	122.70	90.63	0.10
323	122.61	91.23	0.10
333	122.53	84.81	0.11

9.6. Molecular Weight Investigation of Polygalacturonase

The molecular weight investigation of the crude PG enzymes from submerged (SmF), flask, bioreactor and tray scales (SSF) were performed by comparing the protein

bands with the molecular weight marker within the range of 10 – 250 kDa. It should be noted that the produced PGs run on the lane 2 to 5 were crude enzymes therefore a single protein band was not expected. Hence, the exact protein band representing the produced exo-polygalacturonase enzyme could not be determined. However, the aim of this part of the study was to present the protein band profile of the produced crude enzymes and the molecular weight ranges that they belong to.

When the protein bands of SmF (lane 2) compared with the SSF (lanes 3, 4 and 5) protein bands given in Figure 9.7, it can be seen that different kind of proteins were produced by *Aspergillus sojae* M5/6 with the SSF technique. This is probably due to the nature of the SSF in which the fungus is in tight contact with the substrate where it penetrates into the cracks of the substrate. With this respect, fungus is not exposed only to the water-soluble part of the substrate as in SmF technique. In general, complex form of the agro-industrial residues induce the synthesis of various kinds of hydrolytic enzymes to be consumed by the microorganism. Additionally, the protein band profiles of SmF and SSF enzymes were very different indicating the change in the biochemical properties of the polygalacturonases depending on the fermentation type.

The SSF proteins were determined within the range of 18 to 64 kDa which can be accepted as compatible with the molecular weight range of the exo-polygalacturonases (30 to 70 kDa) listed in Table 3.1.

As can be seen in Figure 9.7, the protein band profiles of the three SSF enzymes were similar to each other. The protein bands at the 27 kDa were obtained in all SSF enzymes (lanes 3, 4 and 5), but it was more distinct for the flask scale enzyme (lane 3). The other identical band was obtained at c.a. 37 kDa for the SSF enzymes (lanes 3, 4 and 5), which was also obtained with the commercial pectinase (lane 6). Another comment on the Figure 9.7 will be the broad protein band profile of tray PG (lane 5) compared with the flask scale SSF PG (lane 3). This may indicate the attempt of the fungus to adapt to a different medium (tray) by synthesizing more kinds of hydrolytic enzymes. The last common protein bands were obtained at c.a. 41 and 44 kDa for the SSF enzymes (lanes 3, 4 and 5).
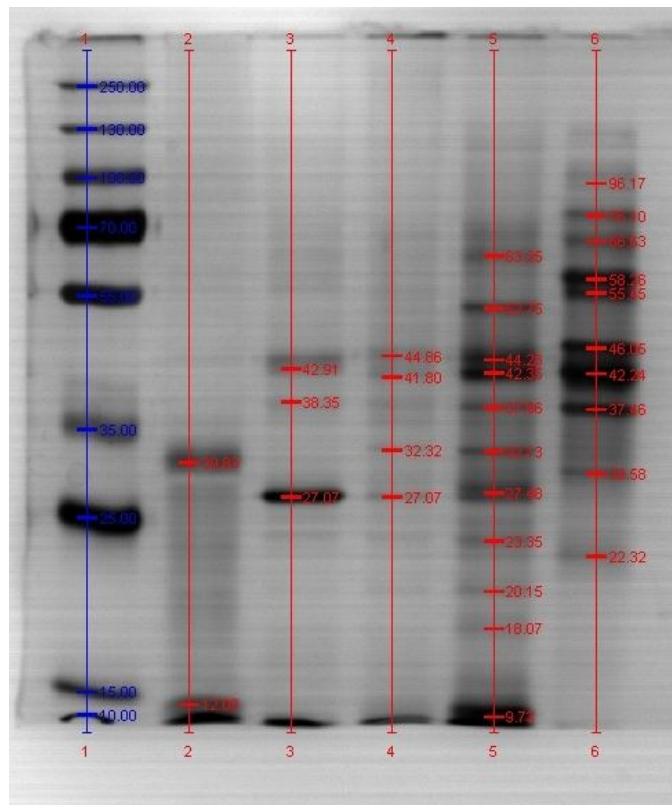
Figure 9.7. SDS-PAGE profiles of lane 1: molecular weight marker, lane 2: PG from SmF, lane 3: PG from flask, lane 4: PG from bioreactor, lane 5: PG from glass tray, lane 6: commercial pectinase

CHAPTER 10

SCALE-UP STUDIES

This chapter is composed of the solid-state fermentation (SSF) studies conducted on the trays and horizontal drum bioreactor for the production of polygalacturonase (PG) from *Aspergillus sojae* M5/6 strain using 100-250 µm wheat bran (Hazal).

10.1. Polygalacturonase Production with Tray Type SSF

The effect of solid substrate thickness and its interaction with the relative humidity of the environment were studied under this section. The production kinetics under the partially optimized conditions was also investigated.

10.1.1. Effect of Solid Substrate Thickness on the PG Activity

The thickness of the substrate placed in the fermentation bed of the tray type of bioreactors has importance with respect to the formation of oxygen and temperature gradients in the bed. As the microorganisms used in the SSF is almost in direct contact with the gaseous oxygen in the air and water content is quite low compared to the SmF, the overall reaction involves the transport of oxygen and water into the microbial biomass, the generation of metabolic heat due to respiration and the transport of heat and carbondioxide from the interior of the substrate into the gas phase. However, due to the heat and mass transfer resistances in the biomass, concentration and temperature gradients will occur which should be minimized in the design of a proper SSF bioreactor (Raghava Rao et al., 1993). At this point, the thickness of the substrate in the bed becomes a critical factor and should be optimized in order to prevent overheating and guarantee the aerobic conditions in the static type of the bioreactors. With this perspective, the substrate thickness in the fermentation bed was investigated in the present study. It was difficult to measure the thickness of the substrate placed in the Erlenmeyer flasks due to its bottleneck and conical shape, therefore the SSF was

conducted in the round borosilicate glass casseroles (see Section 5.12). They were placed in a humidity chamber adjusted to 90% relative humidity (RH) rather than a regular incubator in order to prevent the drying of the substrate due to the wider area exposed to air and circulation of the heated air (37 °C) inside the incubator. This set of experiments also had the characteristic approach to an upper scale i.e. tray type bioreactor. 50, 75 and 100 g of wheat bran (Hazal, 100 – 250 μm) was placed in the casseroles with the measured thicknesses of 7.9, 11.6 and 14.0 mm and the accumulation density (Bhanja et al., 2007) values of 1.137, 1.705, 2.274 g substrate/cm^2 of fermentation bed, respectively. The amount of substrate per unit bed area values were calculated to express the amount of substrate that should occupy the area of 1 cm^2 in any other container with different dimensions. Additionally, no agitation was applied to the fermentation medium during the incubation period with the intent of preventing possible contamination. The PG activity, final pH, specific PG activity and consumption of total carbohydrate results were presented in Figure 10.1.

As can be seen in Figure 10.1a, the PG activities obtained for three of the investigated thicknesses were significantly lower than the PG activity (535.4 U/g substrate, the control in the Section 8.9) obtained in the Erlenmeyer flask scale under the optimized conditions (except the relative humidity of the environment). This reduction in the PG activity addressed that the optimized conditions achieved at the down scale should be reviewed in the framework of the scale-up strategies before their use at the upper scale. The reason of this low PG activity may be the selection of an unsuitable relative humidity level. Sekar and Balaraman (1998) observed that the optimum relative humidity of the environment (95%) led to an increase of 48% (compared to 80% RH) in the yield (g/kg of bran) of the target metabolite Cyclosporin A. The views taken from each tray at the end of the fermentation period (Figure 10.2) supports this idea. As can be seen from these (top and inner) views, the fungus and wheat bran complexes agglomerated and formed relatively large aggregates at all thicknesses probably because of the high relative humidity (90%) of the environment. These aggregates may limit the transfer of oxygen to the interparticle spaces and result in poor growth of the fungus (Figure 10.2).

When a comparison was made among the PG activities obtained for each thickness (Figure 10.1a), the results indicated an inverse proportion between the bed thickness and PG production. This result revealed the limitation of the oxygen and moisture as the depth of the bed increases. Sargin and Öngen (2003) similarly observed

approximately 38% reduction in the xylanase activity of *Trichoderma longibrachiatum* as the substrate thickness increased from 0.5 cm to 1.0 cm at the non-aerated condition. In another study, Bhanja et al. (2007) observed that the yield of α-amylase (from *Aspergillus oryzae* IFO-30103) increased slightly with the increase of bed height from 0.5 to 1.0 cm. However, the yield of α-amylase declined with the further (2, 3 and 4 cm) increase in the bed height in the enamel coated metallic trays (30*25*4 cm). Another concern was the temperature gradient occurring throughout the bed. Chen et al. (2005) have placed probes in the trays every 3 cm bed height (0, 3, 6 and 9 cm) from the bottom to the top of the bed. With this method, they have observed nearly 2.5 °C medium temperature difference between 0 and 6 cm levels of the bed (with the air pulsation method).

The specific activity results for 7.9, 11.3 and 14.0 mm thicknesses plotted in the Figure 10.1c were 76%, 88% and 95% lower than the one obtained previously at the flask-scale under similar conditions (spore inoculated run in Section 8.9). It is thought that the reason of these low specific activities was; high protein contents of the crude enzymes rather than their low PG activity values. This idea was supported with the relatively (compared to flask) higher number of protein bands obtained with the enzyme extract of tray (lane 5 in Figure 9.7). Even the SSF was conducted with the same substrate and same fungus strain, the tray type of fermentation is quite different from the flask type. The most important difference is the increase in the contact area of the fermentation medium with the air. This means the fungus is exposed to more oxygen and may show difference in the metabolite synthesis behavior during the adaptation period. The total carbohydrate consumption given in the Figure 10.1d showed that the fungus was able to consume the carbohydrate to a reasonable level and but could not reduce the pH under 6.0.
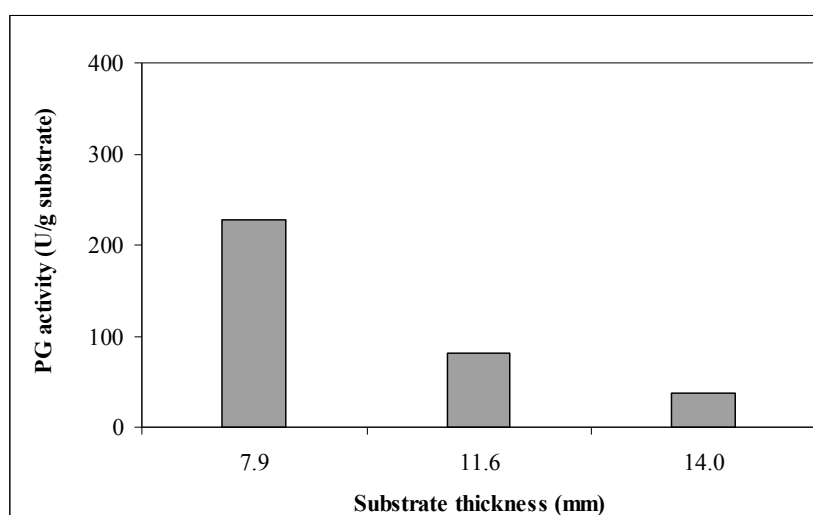
(a)
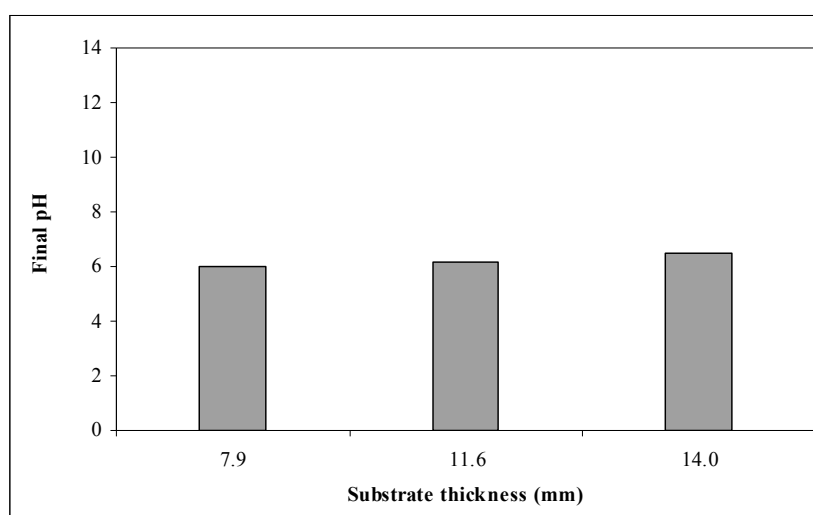
(b)

Figure 10.1. (a) PG activity, (b) final pH, (c) specific activity, (d) total carbohydrate profiles of PG production by *Aspergillus sojae* M5/6 fermented in the glass casseroles (SSF conditions: 10^7 spore/g substrate, 4 days, 37 °C, 62% moisture content with water, substrate particle size: $100 - 250$ μm, relative humidity: 90%)

(c)

(d)

Figure 10.1. (cont.)

| 7.9 mm - top | 11.6 mm - top | 14.0 mm - top |
| (a) | (b) | (c) |

| 7.9 mm – close shot | 11.6 mm – close shot | 14.0 mm – close shot |
| (d) | (e) | (f) |
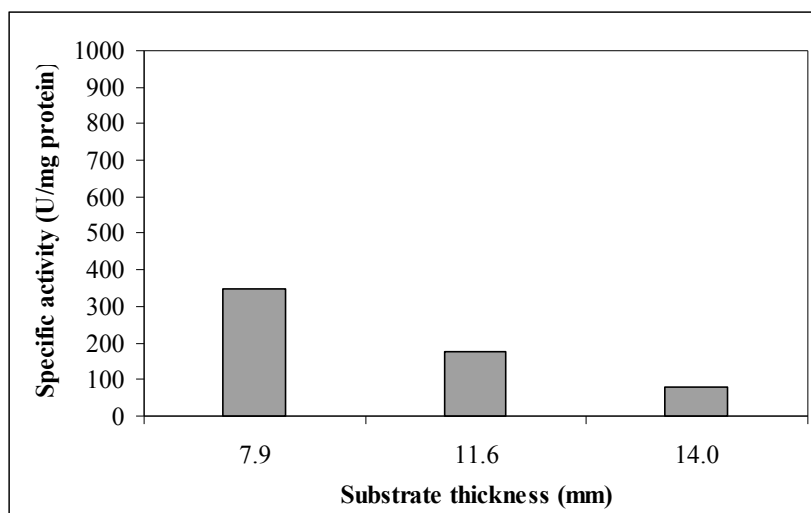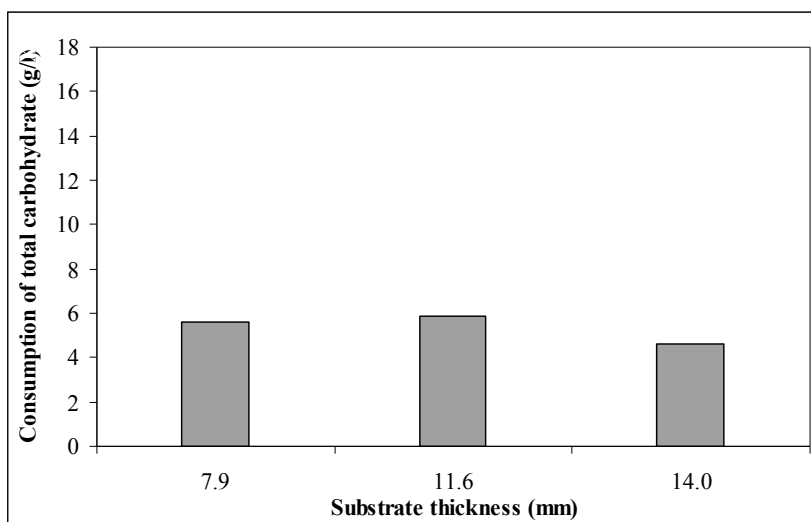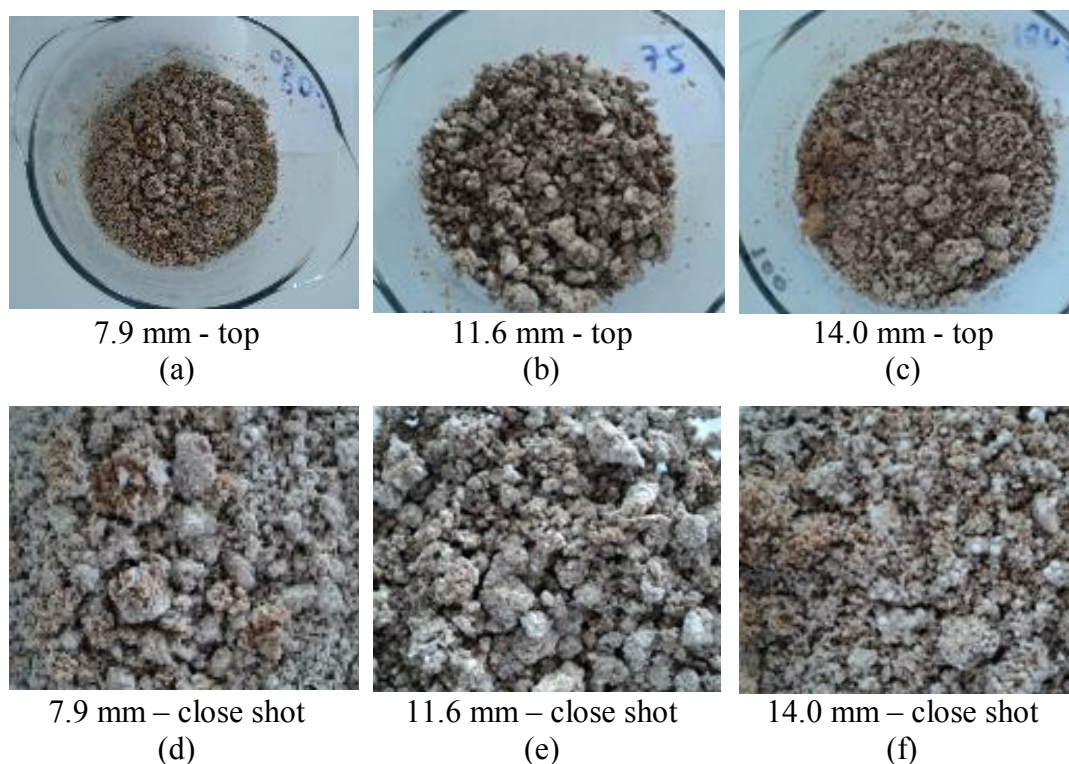
Figure 10.2. Top and close shot pictures of tray batches with 7.9 mm, 11.6 mm, 14.0 mm thicknesses

10.1.2. Effect of Relative Humidity and Solid Substrate Thickness on the PG Activity

In the previous section (10.1.1) it was observed that the thickness of the substrate has a significant effect on the PG production capacity of *Aspergillus sojae* M5/6 at 90% RH. Many studies showed that RH of the environment also plays an important role in the SSF (Madeira Jr et al., 2011; Sekar and Balaraman, 1998; Ito et al., 2011). Lu et al. (2003) clearly stated that the RH of the chamber controls the moisture of the fermentation medium and indirectly the product yield in the koji type of fermentation. In our case, an interaction was expected between the thickness of the bed and RH of the environment. Therefore, experimental design and statistical analysis tools were utilized.

For this purpose, a factorial D-optimal design was used with two factors; thickness of the bed and relative humidity of the environment and 3 levels. Each run was conducted in identical glass trays (Section 5.12). The thickness of bed factor was represented with the amount of substrate placed in the tray. The runs of the experimental design and related PG activity responses were given in Table 10.1.

Table 10.1. Experimental design and actual PG activity values

Std	Run	A: Relative Humidity (%)	B: Substrate Amount (g)	PG Activity (U/g substrate)
4	1	70	75	298
1	2	70	50	114
5	3	80	75	237
3	4	90	50	217
2	5	80	50	264
8	6	80	100	37
10	7	70	50	136
9	8	90	100	68
12	9	90	100	37
11	10	80	75	269
6	11	90	75	116
7	12	70	100	73

The results of the experimental design were analyzed with the DesignExpert (version 7.0) software and the ANOVA table was given in Table 10.2. According to this analysis, a significant (p-value<0.05) model was constructed including the terms A, B and AB. The p-values of these terms indicated that RH itself did not affect the PG activity production between 70 to 90% levels; however it had a strong interaction with the substrate thickness as expected. This interaction was also plotted in Figure 10.3. The 100 g substrate amount runs gave the lowest PG activities (Figure 10.3). On the other hand, as the RH decreased, the PG activity increased at the 75 g substrate amount. And maximum PG activity (298 U/g substrate) was obtained at the 70% RH with 75 g substrate amount that is equivalent to 11.5 mm initial bed thickness (dry substrate). The pictures of the 1st, 10th and 11th runs (Table 10.1) with 75 g batches fermented at 70, 80 and 90% RH conditions were presented in Figure 10.4a, b and c, respectively. When, the top views of the trays fermented at 70, 80 and 90% (Figure 10.4a, b, c) were examined, there can be seen relatively large sized substrate-fungus aggregates in the tray fermented at 90% RH (Figure 10.4c) compared to the 70 and 80% RH batches (Figure 10.4a, b). The fermentation media of 70 and 80% RH batches were more homogenous in shape with smaller aggregates. This may be one of the reasons of achieving higher PG activities with the 70 and 80% RH batches. On the other hand, Figure 10.4 a and b presented some dried parts on the surface of the fermentation media which was far less in the Figure 10.4c. The close shot view showed that (Figure 10.4f) the 90% RH batch had a different morphology with the presence of denser white aerial

hyphae grown on the wheat bran. The presence of more aerial hyphae and production of low PG activity was similarly observed at the flask scale (48h and 72h SmF inoculated SSF runs) in Section 8.9.

Table 10.2. ANOVA table for the PG activity results in the tray SSF production

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	100360.5	8	12545.1	30.5	0.0086
A-R. humidity	5272.9	2	2636.5	6.4	0.0826
B-S. thickness	54835.9	2	27418.0	66.6	0.0033
AB	28504.1	4	7126.0	17.3	0.0206
Pure Error	1234.5	3	411.5		
Cor Total	101595.0	11			
Std. Dev.	20.3		R-Squared	0.9878	
Mean	155.5		Adj R-Squared	0.9554	
C.V. %	13.0		Pred R-Squared	N/A	
PRESS	N/A		Adeq Precision	14.9	
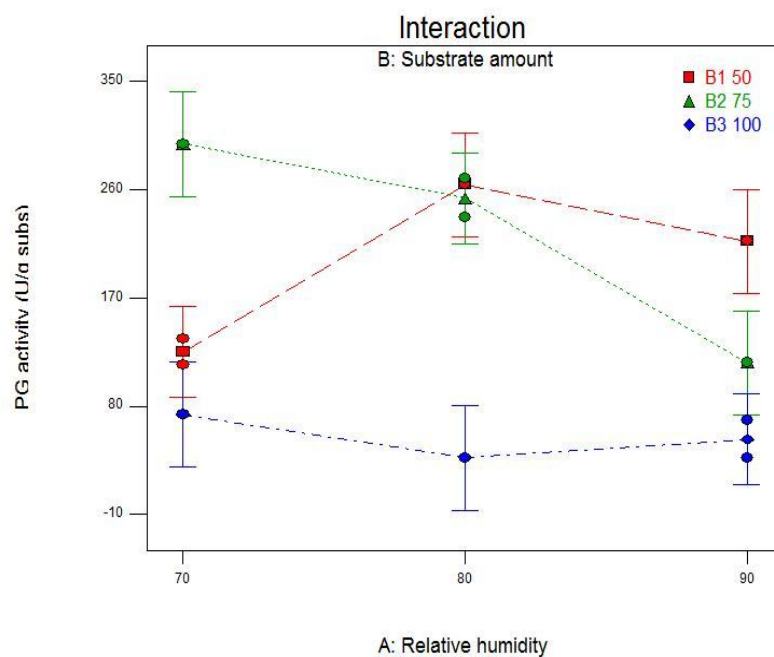
Figure 10.3. The interaction plot of relative humidity and substrate amount (thickness)
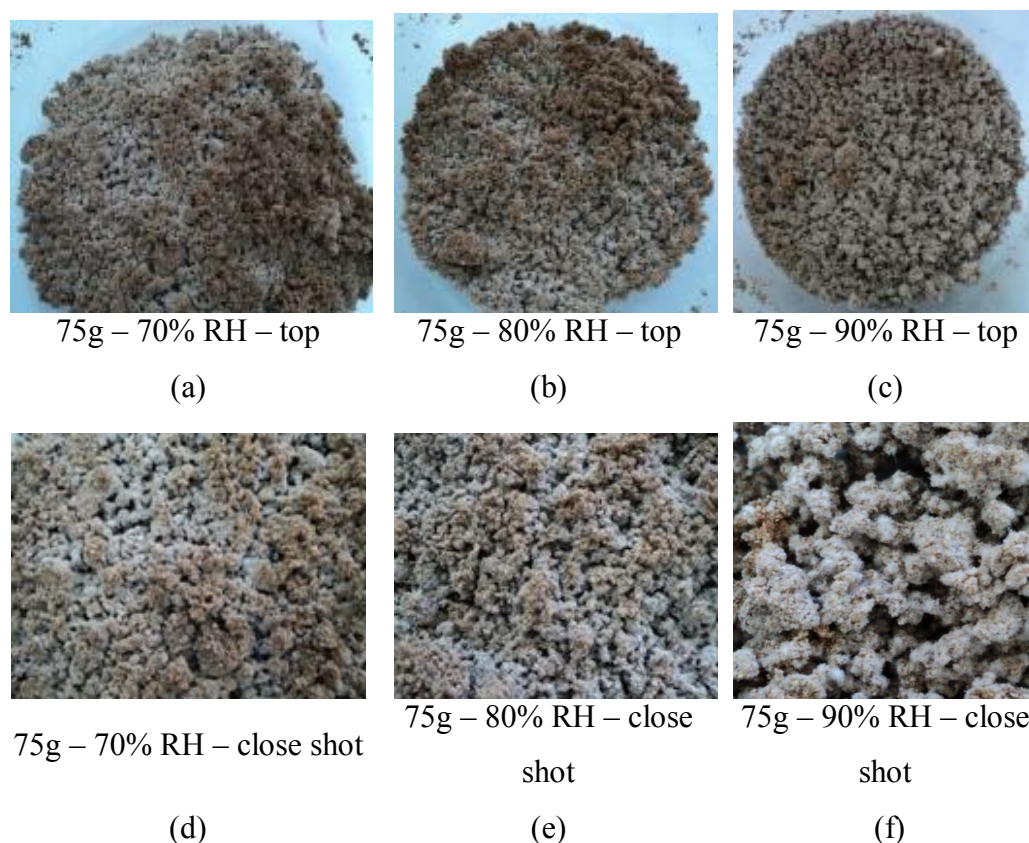
Figure 10.4. Top and close shot pictures of tray batches with 75 g substrate (app. 11.5 mm bed thickness) fermented at various relative humidity levels

As a result of this part of the study the PG activity could be enhanced for 31% compared to the first tray type SSF given in Section 10.1.1. Additionally, 75 g of substrate amount (c.a. 11.5 mm thickness) and 70% relative humidity conditions were selected for the next kinetic tray SSF study. The approach to the tray-type of bioreactor presented in this part of the study produced reproducible and encouraging results for a further optimization under pilot-scale conditions.

10.1.3. Kinetic Study on the PG Production with Tray Type SSF

The production process of PG from *Aspergillus sojae* M5/6 in the trays were monitored for 4 days under the conditions of 10^7 spore/g substrate inoculum size, 37 °C of incubation temperature, 62% initial moisture content, water as moistening agent and 70% RH of the chamber by loading 75 g of 150-250 μm particle sized wheat bran. Two of the trays were withdrawn each day and analyzed immediately for their PG activity, final pH, moisture content, water activity, specific PG activity and consumption of total

carbohydrate measurements during the 4 days of the process. The average of the measurements of two trays was presented in Figure 10.5.

A relatively high PG activity was obtained on the third day of the fermentation (Figure 10.5a) unlike the PG activity profile of the flask scale SSF (Figure 8.2a). But the PG activity decreased on the 4th day contrarily to the flask scale profile given in Figure 8.2a. It is thought that reason of the reduction in PG activity on the 4th day may be the significant decline in the moisture content and water activity on the same day as can be seen in Figure 10.5c and d. The water activity of the fermentation medium could be preserved at 0.998 even the moisture content reduced to 37% until the 3rd day. But on the 4th day, water activity sharply reduced to 0.862 probably due to the large contact area of the tray exposed to the air. The air was circulated in the chamber in order to maintain uniform temperature and humidity distribution automatically by the incubator. But, the incubator does not allow the user to control the rate of the air circulation. This fact was probably the reason of the reduction in the moisture content and water activity on the last day of the SSF. This result indicated the importance of aeration rate in the PG production by SSF. It is thought that the difference in the PG activity of the batches fermented under the same conditions (4th day of the current experiment and 1st run of the experimental design in Section 10.1.2) was due the compositional variations between the wheat bran batches. The fluctuation occurred on the 4th day of the fermentation was also reflected to the final pH, specific PG activity and consumption of carbohydrate profiles given in Figure 10.5b, e and f.

The productivity of the process conducted under optimized conditions was calculated as 2.43 U/g substrate^{-1} h^{-1} for the 4th day of the SSF. For an approximate comparison with the literature; in a similar scale-up study (Bhavsar et al., 2011) performed from flask scale to tray scale, the productivity of the phytase from *Aspergillus niger* fermented on the 28*24*4 cm trays with 100 g of wheat bran moistened with an optimized medium was 1.55 IU/ g substrate^{-1} h^{-1}.
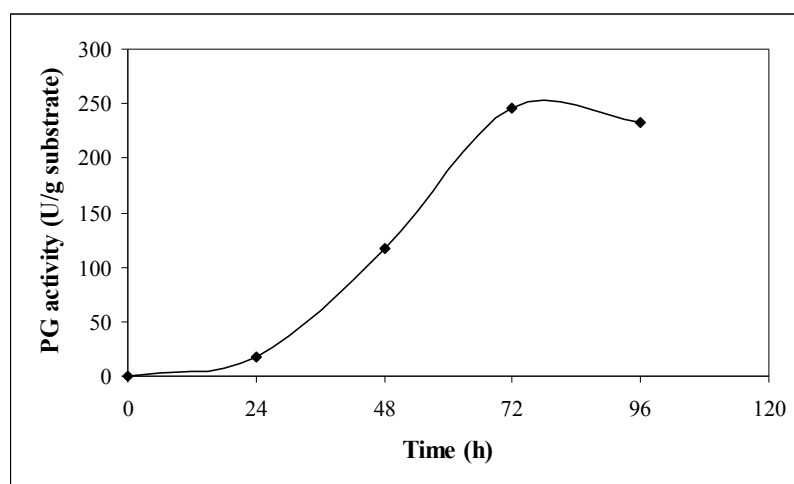
(a)
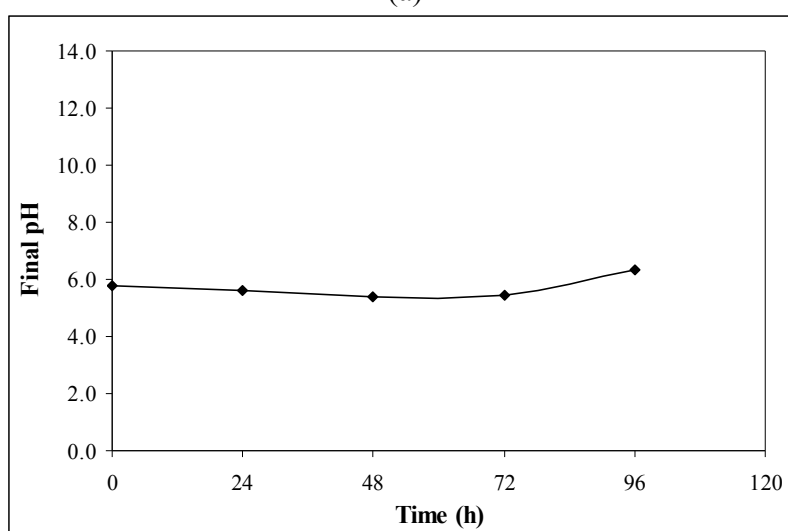
(b)
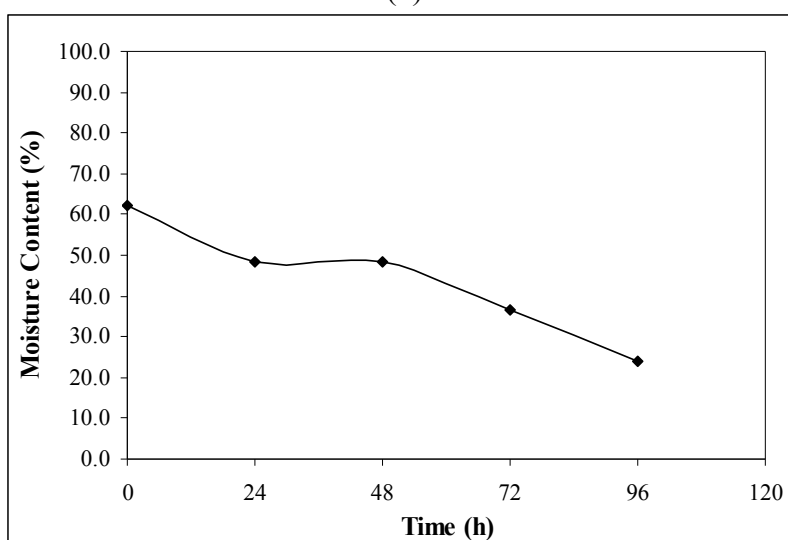
(c)

Figure 10.5. Kinetic profiles of (a) PG activity, (b) final pH, (c) moisture content, (d) water activity, (e) specific activity, (f) consumption of total carbohydrate profiles of *Aspergillus sojae* M5/6 by SSF conducted in trays
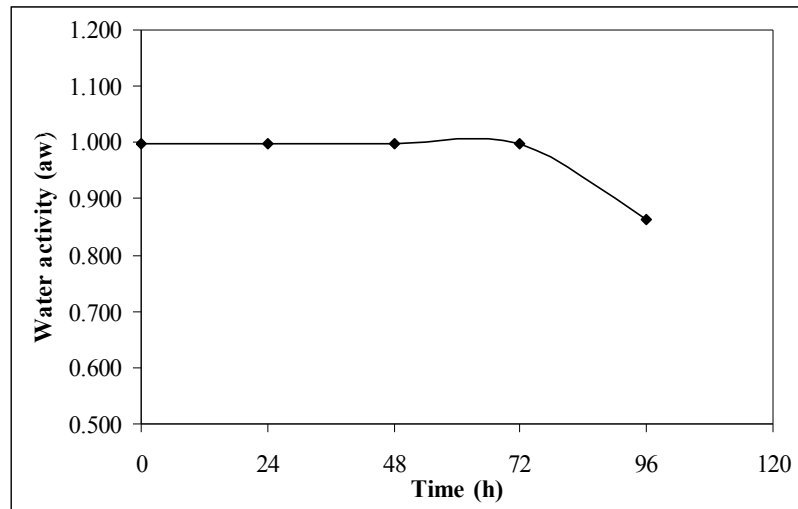
(d)
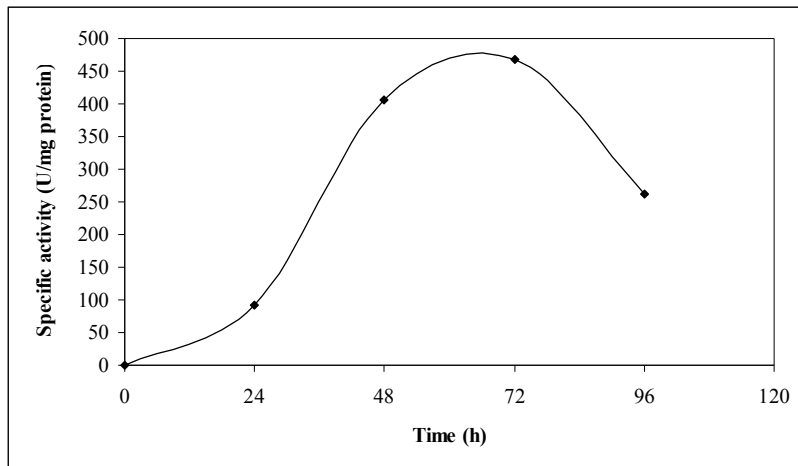
(e)
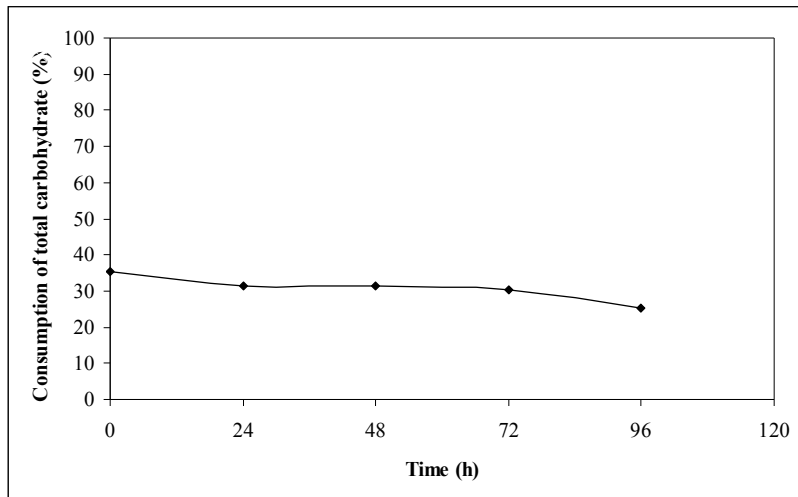
(f)

Figure 10.5. (cont.)

10.1.4. Polygalacturonase Production with Horizontal Drum Bioreactor

The experimental studies on the production of PG from *Aspergillus sojae* M5/6 in the horizontal drum bioreactor were presented in this section. The experimental set up and procedures were detailed in Section 5.14.1 and 2. This set of experiments was performed in the Bioprocess Laboratory of Department of Bioengineering in Ege University.

In order to investigate the PG production in the horizontal drum bioreactor 7 batches were performed in total. In the first batch, the decisions on the measurements, their set up and sampling plan were tested. The air flow rate was set to 1.5 l/min and hold at this value through the process in the second batch. The relative humidity of the inlet air was adjusted between 30 and 60% during the process. The initial growth of the fungus was observed but the moisture content of the biomass rose to 66.8% at the end of 3rd day. Therefore, the RH of the inlet air was relatively reduced, but it was observed that the growth of the fungus became less frequent on the substrate. The final pH of the biomass increased to 6.94. No PG activity could be obtained from this batch.

The air flow rate was set again to 1.5 l/min and hold at this value through the process in the third batch. This time the RH of the inlet air was kept at a relatively low range i.e. 10 − 35%. The decision of the RH was made upon the moisture content of the sample. But the raise of the moisture content of the sample to 68% could not be prevented with this method. Again no PG activity could be obtained at the end of the third batch. The growth was also poor.

The initial air flow rate was set to 0.8 l/min in the fourth batch. The moisture content of the sample increased to 66% on the third day when the RH of the inlet air was around 10%. At this point it was decided that the moisture content of the fermentation medium could not be controlled by adjusting the RH of the inlet air. Then a trial was done on this batch. The air flow rate was increased from 0.8 to 3 l/min and it was observed that the moisture content of the medium reduced to 58% in 24 hours. Some indications of growth were observed. Then, some more trials were done to understand the relationship between the air flow rate and moisture content of the fermentation medium on the remaining part of this batch.

In the fifth and sixth batches, the moisture content of the fermentation medium was tried to be kept at 62% level by cascading both the RH of the inlet air (in the range

of 1 – 13%) and air flow rate (in the range of 1 – 8 l/min). This batch was relatively successful compared to the previous ones with respect to the control of the moisture content of the fermentation medium. The observed growth was good, but it did not have the usual morphology observed in the flask scale. PG activity values obtained in the fifth and sixth batches were 8 and 11 U/g substrate, respectively.

The last batch was performed with the same principle, however much experience was gained from the previous batches on the moisture control. The measured parameters and the actions taken upon these measurements at the seventh batch were represented in Table 10.3.

Table 10.3. Measured parameters of the seventh batch of the horizontal drum bioreactor

Fermen-tation time (h)	Moisture content of the sample (%)	Relative humidity of the inlet air (%)	Temperature of the inlet air (%)	Relative humidity of the outlet air (%)	Temperature of the outlet air (%)	Volume of collected water from the outlet of bioreactor (ml) / Collection period	Action taken after the sampling and measurements
0	59.30	16.50	23.86	-	-	-	humidified air connected, air rate: 1 l/min
4	58.99	19.20	23.92	96.45	29.12	-	air rate: 1 l/min
8	58.83	12.58	24.31	100.00	29.54	-	air rate: 3 l/min
23	60.80	11.72	23.31	100.00	28.92	33 ml / 8 - 23 h	network air connected, air rate:7 l/min
27	62.02	2.73	23.80	100.00	29.60	10.5 ml / 23-27 h	air rate: 8 l/min
30	59.67	1.32	24.91	100.00	31.09	7 ml / 27-30 h	air rate: 8 l/min
34	60.36	1.20	24.94	100.00	31.35	7.5 ml / 30-34 h	air rate: 7 l/min
48	62.85	3.55	24.03	100.00	30.25	29.5 ml / 34-48 h	air rate: 7 l/min
51	64.78	-	-	100.00	-	11 ml / 48-51 h	air rate: 7 l/min
55	57.69	3.74	24.47	100.00	30.41	7.5 ml / 51-55 h	air rate: 6 l/min
72	60.82	3.01	24.70	100.00	30.57	15.5 ml / 55-72 h	air rate: 5 l/min
75	58.59	-	--	100.00	-	3 ml / 72-75 h	air rate: 3 l/min
79	55.17	4.47	26.07	100.00	31.59	3 ml / 75-79 h	air rate: 2 l/min
99	55.42	7.74	25.63	100.00	31.02	11.5 ml / 79-99 h	end

The last batch performed with the details given in Table 10.3 and analyses of the samples collected from this batch were represented in Figure 10.6. It can be seen from Figure 10.6a that the maximum PG activity was obtained at the 55th hour of the process as 14.3 U/g substrate. This was a very low activity compared to the flask and tray scale productions. It can be clearly seen from the Figure 10.6 a, b, c and d, a breakdown was occurred in the 72nd hour of the process. The oxygen consumption of the fungus reduced, the pH began to increase, the protein of the fermentation medium increased dramatically and the consumption of the carbohydrate also decreased. Those were the indicators that the process was no more favoring the growth of the fungus and its metabolic activities of PG production. The increase in the protein indicated that the metabolic activities of the fungus was altered (from the usual kinetic obtained at the flask and tray scales) and it began to synthesize proteins other than PG. The presence of these proteins might have induced the growth and the production of PG by the fungus.

(a)
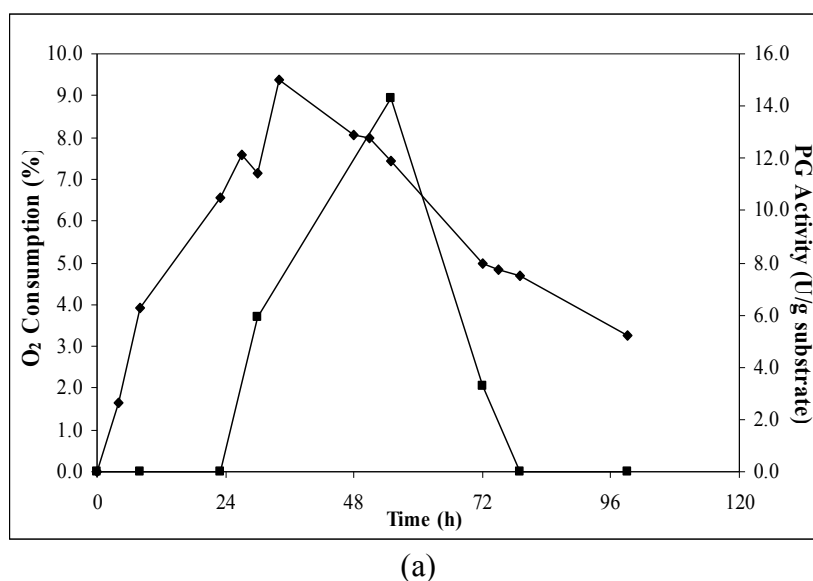
Figure 10.6. Kinetic profiles of (a) PG activity [full square] and O_2 consumption [full diamond], (b) final pH, (c) protein concentration, (d) consumption of total carbohydrate of *Aspergillus sojae* M5/6 by SSF conducted in horizontal drum bioreactor
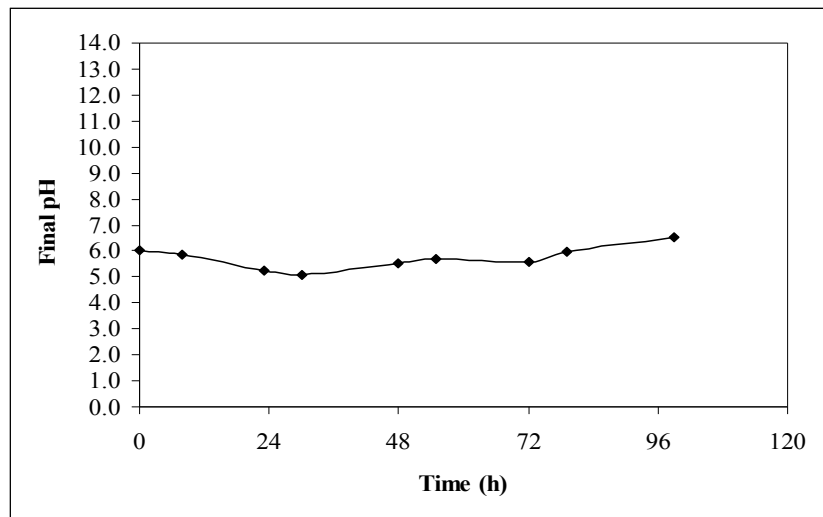
(b)

(c)

(d)

Figure 10.6. (cont.)

166
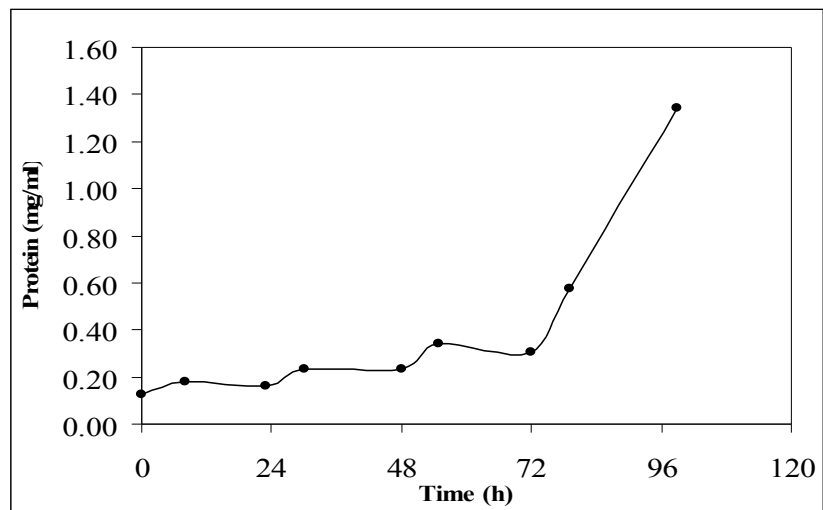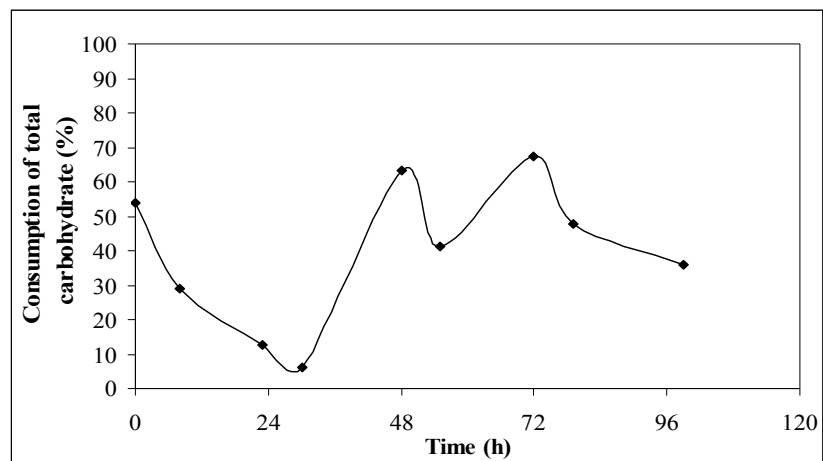
The reasons of obtaining low PG activity in the SSF process conducted in the horizontal drum bioreactor may be as followings.

- The rate of the air flow was cascaded during the fermentation in order to control the moisture content of the fermentation medium. This was a valid method when combined with the cascading the RH of the air. However, in the period especially between 24th and 72nd hours, high air flow rates had to be applied for the moisture content control. This action led to excess drying on the substrate especially on the surface of the fermentation bed. The over drying of the substrate probably resulted in poor growth on the dry regions. In Section 8.4, the negative effect of low moisture content on the PG activity was also determined at the flask scale. In order to prevent the over drying of the surface, more than 3 times/day agitation had to be applied during the process. This agitation frequency also negatively affected the growth. The negative affect of agitation other than the optimum level was indicated in Section 8.7. It can be concluded with the help of this fact and the visual observations, the mycelial growth of the *Aspergillus sojae* M5/6 on the wheat bran was disrupted by the agitation.

- The particle size of the wheat bran (100-250 μm) was not suitable for its use in a horizontal drum bioreactor. Large agglomerates occurred during agitation because of the compacting parts between the scrapper and the bioreactor wall. However, it was stated in Section 8.6 no PG activity could be obtained with larger particle size.

- The optimum process temperature was 37 °C. The difference between this relatively high incubation temperature and the room temperature (especially at night) resulted in condensation of water vapor inside the bioreactor on the glass upper cap and upper walls of the bioreactor (non-jacketed part). The condensed water droplets came together in time and soaked into the fermentation medium through the side walls of the bioreactor. Because of this mechanism, the lower parts of the substrate became over moistened. Then, the control of the moisture content of the fermentation medium became more difficult. This fact might also have a negative affect on the growth of the fungus.

CHAPTER 11

CONCLUSIONS

Solid-state fermentation has gained remarkable attention in recent years with respect to its low cost and potential of value added product formation. This study includes the production of polygalacturonase (PG) from *Aspergillus sojae* mutant strains by solid-state fermentation technique using the agro-industrial residues as the substrate. A detailed literature survey on the solid-state fermentation technique, pectinolytic enzymes and production of polygalacturonase by solid-state fermentation technique was stated. The experimental results performed for the investigation of the production of polygalacturonase from *Aspergillus sojae* mutant strains by solid-state fermentation technique were presented. Based on the obtained experimental results, following remarks can be concluded from the study;

1-   The optimum conditions favoring both polygalacturonase and spore production from *Aspergillus sojae* M3 were determined as; 2% orange peel and 50 mM HCl concentrations, 22 °C and 4.3 days of incubation. An overlay plot was drawn for the demonstration of the optimum conditions favoring both polygalacturonase and spore production. It can be concluded that overlay plots can be used practically for the decision of the conditions at the preparation step of the fermentation process. The optimum conditions favoring only maximum PG activity and only maximum spore production were also determined and presented. The overall results obtained in this part of the study indicated the potential of *Aspergillus sojae* M3 mutant strain for the production of industrially important enzyme polygalacturonase and its adaptability to the solid-state fermentation conditions that utilize agro-industrial residues such as orange peel and wheat bran.

2-   A comparison study was performed between the mutant *Aspergillus sojae* strains. The *Aspergillus sojae* mutant strain M7/2 was found to synthesize the maximum PG activity (55.5 U/g substrate) as a result of this comparison. However, after a set of agro-industrial residue screening experiments *Aspergillus sojae* M7/2 did not show any repeatability to produce

polygalacturonase enzyme. At this point, it can be concluded that the developed strains in the laboratory environment should be tested for the repeatability for a period of time as well as its metabolite producing capability. Therefore, it was decided to continue with a relatively well-known (from the simultaneous submerged fermentation studies) mutant strain *Aspergillus sojae* M5/6.

3-   FT-IR analysis was used successfully to determine the sugar compositions of 10 kinds of agro-industrial residues, qualitatively.

4-   10 kinds of agro-industrial residues were screened for the PG producing potentials with the *Aspergillus sojae* M5/6. As a result of this screening process, the maximum PG activity (among the water moistened residues) was obtained with the wheat bran, İzmir as 23.3 U/g substrate. This result confirmed the PG production potential of *Aspergillus sojae* M5/6 by SSF. However, it has also shown that the important factors affecting the solid-state fermentation should be well optimized in order to obtain a higher PG acitivty.

5-   For this purpose, most important factors affecting the fungal growth and enzyme synthesis were investigated with one-at-a-time method with *Aspergillus sojae* M5/6 and wheat bran selected as the mutant strain – solid substrate pair. As a result of this detailed optimization process, the most suitable factor levels favoring the PG production were found as 10^7 spore/g substrate inoculum size, 4 days of fermentation, 37 °C of incubation temperature, 62% initial moisture content, water as the moistening agent, $100 - 250$ μm particle size of wheat bran, 3 times/day agitation and spore solution as the inoculum type. The PG activity could be enhanced from 73.4 U/g substrate to 456.6 U/g substrate with wheat bran, İzmir and to 535.4 U/g substrate with wheat bran, Hazal as a result of a series of optimization process. The productivity of the process was also improved from 1.43 to 4.75 U/g substrate^{-1}h^{-1} with wheat bran, İzmir and 5.58 U/g substrate^{-1}h^{-1} with wheat bran, Hazal at the end of the optimization part of the study. These achieved PG activity and productivity values were competitive with those obtained from various microbial strains reported in the literature.

6-   As a result of a wide screening action performed to find identical wheat bran it was deduced that in the large scale SSF production processes it should be kept in mind that there will be variability between the batches of the substrate. The producer should supply the substrate from a very trustable and good practicing manufacturer because the variability between the batches of the residue may

increase due to the origin, variety and quality of the raw material, variation of the operation parameters such as conditioning temperature in the processing of the substrate, technology of the manufacturing equipment and storage conditions of the raw material. Additionally, the rheology of the substrate during the moistening and fermentation should also be considered in the selection of the substrate.

7- The produced polygalacturonase enzyme should be compatible with the targeted application. Therefore, some of the biochemical properties of the polygalacturonase, produced under the optimized conditions at the flask scale were determined. The produced PG showed optimum activity at the pH range of 4.0 – 5.0. The investigation on the stability of the PG showed that enzyme was stable in the pH range of 3.0 – 7.0 by retaining at least 65% of its activity. The optimum activity for the polygalacturonase was 40 °C and it retained its 99% activity at 50 °C. The *Aspergillus sojae* M5/6 polygalacturonase could preserve more than its 50% stability between 25 and 50 °C, both for 30 and 60 minutes. The enzyme was found to be stable in the presence of most of the tested compounds and metal ions. It was only inhibited in the presence of fructose, $CaCl_2$ and $FeCl_3$. The results obtained from this part of the study indicated that the *Aspergillus sojae* M5/6 polygalacturonase was suitable for the clarification (depectinization) of the fruit juices and wine, especially orange and grape juices and wines.

8- The investigation on the kinetics of thermal inactivation revealed that the k_d (deactivation rate constant) value was calculated as 0.003 min^{-1} at 30 °C, but increased to 0.3454 min^{-1} at 60 °C. The half-life value at 30 °C was determined as 231.05 min, however it reduced to 2.0 min at 60 °C. The inactivation energy (E_d) of *A. sojae* M5/6 polygalacturonase was determined as 125.3 kJ/mole. The thermodynamic parameters; enthalpy (ΔH^*), free energy of inactivation (ΔG^*) and entropy (ΔS^*) for thermal inactivation of *A. sojae* M5/6 polygalacturonase were found to be stable with the increasing temperature. The molecular weight of the polygalacturonases produced in flask, tray and horizontal drum bioreactor were found to be within the range of 18 to 64 kDa.

9- The first part of the tray type of fermentation showed that the thickness of the substrate has a significant effect on the polygalacturonase production capacity of *Aspergillus sojae* M5/6 at 90% relative humidity. The maximum PG activity

obtained in this part was 227 U/g substrate which addressed that the optimized conditions achieved at the down scale, should be reviewed in the framework of the scale-up strategies before their use at the upper scale.

10- The significance of interaction between the thickness of the bed and relative humidity of the environment was determined with the help of experimental design and statistical analysis tools. As a result of this part, the PG activity could be enhanced for 31% compared to the first tray type SSF. Additionally, 75 g of substrate amount (c.a. 11.5 mm thickness) and 70% relative humidity were selected as the PG production favoring conditions.

11- The kinetic study conducted in the trays revealed the presence of reduction in the water activity on the 4th day of the SSF process under stated conditions. The productivity of the process conducted under optimized conditions was calculated as 2.43 U/g substrate^{-1} h^{-1} for the 4th day of the SSF.

12- The major problem faced in the horizontal drum bioreactor was the condensation of water droplets on the bioreactor walls. These droplets then drained inside the fermentation medium. This led to the over moistening of the substrate. As a result of this fact, the growth of the fungus was not proper and the PG production was negatively affected as well.

Based on the obtained experimental results, further studies should be performed on the following issues:

1- The important factors such as initial moisture content, type of inoculum, particle size and agitation frequency should be optimized for each agro-industrial residue other than the wheat bran for the polygalacturonase production from *Aspergillus sojae* strains. The pretreatment methods should be investigated for each residue in order to increase the process efficiency.

2- The side enzymatic activities of the produced crude enzyme should be screened. This study will also be effective on the selection of a different agro-industrial residue (other than wheat bran) and application area of the produced enzyme.

3- The produced polygalacturonase enzyme should be purified. The purification procedure should be economical as much as possible serving the low-cost principle of the SSF processes.

4- The optimum factors should be reviewed for the tray type of fermentation. However, this set of experiments should be performed under the conditions of controlled aeration rate.

5-   The heat and mass transfer occurring in the fermentation bed is important to design a pilot or large scale bioreactor. A detailed study with experimental and numeric parts, on the heat and mass transfer phenomena of the solid-state fermentation should be performed.

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