SCALE UP STUDIES AND DETERMINATION OF GROWTH AND POLYGALACTURONASE FORMATION KINETICS OF *Aspergillus sojae* MUTANT STRAIN

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ABSTRACT

SCALE UP STUDIES AND DETERMINATION OF GROWTH AND POLYGALACTURONASE FORMATION KINETICS OF *Aspergillus sojae* MUTANT STRAIN

The complex structure of the submerged fungal fermentations makes the kinetic parameter estimation difficult due to the problems like settling of mycelial clumps, less uniform distribution of biomass and because of the use of heterogeneous medium. Therefore in thecurrent study the aim was to kinetically evaluate the growth and production of polygalacturonase by *Aspergillus sojae* mutant strainin 1 l serial bioreactor systemby applying different techniquesto biomass data of whole orange peel (WOP) fermentation. With this perspective, spectrophotometric technique, sample blank subtraction method, adaptation of a new sampling port, application of cube root kinetics and different linearization methods and glucoseamine content determination methods were applied. With the new sampling port, reasonable biomass results were obtained due to the provision of homogeneous sampling.

Overall, maximum PG activity was achieved at 600 rpm agitation speed, 1.5 vvm aeration rate and uncontrolled pH conditions. The highest polygalacturonase activity (249.49 U/ml) obtained was quite higher than our previous findings. The maximum specific growth rate (μ) of *Aspergillus sojae* mutant strain was estimated as 0.21 h^{-1} and 0.40 h^{-1} for WOP and EOP, respectively. Fed-batch fermentation technique was observed to increase the PG production yield 60% and 54% more than the batch fermentation of WOP and EOP, respectively.Moreover, crude PG enzyme showed significant activity at the acidic pH region (4.0 – 5.0) and was stable in a broad pH range (3.0 –11.0). Optimum temperature for maximum PG activity was observed at 60°C, however the enzyme could not present any thermostability above 40°C.

ÖZET

Aspergillus sojae MUTANT SUŞUNUN BÜYÜME VE POLİGALAKTURONAZ ÜRETİM KİNETİKLERİNİN BELİRLENMESİ VE ÖLÇEK BÜYÜTME ÇALIŞMALARI

Sıvı ortam küf fermentasyonlarının kompleks yapısı, misel kümelerinin çökmesi, biyokütlenin tekdüze dağılmaması ve heterojen besi ortamı kullanımı nedeniyle kinetic parametrelerin belirlenmesini zorlaştırmaktadır. Bu nedenle bu çalışmada portakal kabuğu fermentasyonu biyokütle değerleri için farklı teknikler uygulayarak 1 l seri biyoreaktör sisteminde *Aspergillus sojae* mutant suşu büyüme ve poligalakturonaz üretim kinetiklerinin belirlenmesi amaçlanmıştır. Bu bakış açısı ile spektrofotometrik teknik, örnek körü çıkarma yöntemi, yeni bir örnekleme portu adaptasyonu, glukozamin içeriği tayini, küp kök kinetiği ve farklı linearizasyon yöntemleri uygulanmıştır. Yeni örnekleme portu ile homojen örnekleme sağlanması nedeniyle makul biyokütle sonuçları elde edilmiştir.

Genel olarak, en yüksek PG aktivitesi 600 rpm karıştırma hızı, 1,5 vvm havalandırma hızı ve kontrolsüz pH koşullarında elde edilmiştir. Elde edilen en yüksek poligalakturonaz aktivitesi (249,49 U / ml) daha önceki bulgularımıza göre oldukça yüksektir. *Aspergillus sojae* mutant suşunun maksimum spesifik büyüme hızı (μ), sırasıyla portakal kabuğu ve ekstrakte edilmiş portakal kabuğu ile 0,21 s^{-1} ve 0,40s^{-1} olarak belirlenmiştir. Yarı-kesikli fermentasyon tekniği ile kesikli fermentasyona göre PG üretim verimindeportakal kabuğu ve ekstrakte edilmiş portakal kabuğu ile sırasıyla %60 ve %54 artış gözlenmiştir. Ayrıca, ham PG enzimi asidik pH değerlerinde (4.0 - 5.0) önemli oranda aktivite gösterdiği ve geniş bir pH aralığında (3.0-11.0) stabil olduğu belirlenmiştir. Yüksek PG aktivitesi için optimum sıcaklık ise 60°C olarak belirlenmiştir, ancak enzim 40°C' nin üzerindeki sıcaklıklarda termostabilite gösterememiştir.

Dedicated to my parents
Hacer and Aydın GÖĞÜŞ
for their endless love and support

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CHAPTER 1

INTRODUCTION

Nowadays, fungal enzyme production is an important and fast growing sector in the fermentation industry. According to San Jose the global market for industrial enzymes is forecast to reach US$ 3.74 billion by the year 2015 (San Jose, 2011). Among these enzymes pectinases are one of the most important industrial enzymes whose production occupies about 10% of the overall manufacturing of the enzyme preparations (Pedrolli et al., 2009). They have wide applications in textile processing, degumming of plant fibres, pectic wastewater treatment, papermaking, and in food industry (coffee and tea fermentations, fruit juice extraction and clarification, oil extraction, improvement of chromaticity and stability of red wines). Polygalacturonase (PG; EC 3.2.1.15), which is aimed to be produced in this study is a depolymerizing pectinase, which catalyzes the hydrolysis of α-1.4 glycosidic linkages in homopolygalacturonan backbone (Nakkeeran et al., 2012).

It is reported that pectinase producing organisms are plants, filamentous fungi, bacteria and yeasts. However, they are mainly produced from fungal species (Gomes et al., 2011). It is advantageous to produce enzymes by using microorganisms because they are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulations to increase the yield (Dhillon et al., 2004). In industry mostly molds like *Aspergillus niger, Coniotryrium diplodiela, Penicillum* and *Rhizopus* species are used, due to the excretion of up to 90% of the enzyme into the culture medium (Blandino et al., 2001; Souza et al., 2003; Gomes et al., 2011). In this study a mutant strain of *Aspergillus sojae*, an industrially important species which is used in food fermentations such as soy souce and bean paste was used for PG production. *Aspergillus sojae* belongs to *Aspergillus* section *Flavi* like *Aspergillus oryzae* (Chang, 2006), was not considered as a potential PG producer so far. Filamentous enzymes from fungi are good models for industrial applications since they can produce large quantities of extracellular enzymes, their cultivation is feasible and production is cost effective in large bioreactors (Lara-Marquez et al., 2011).

Pectinases can be industrially produced by submerged (SmF) and solid-state fermentations (SSF). It is known that about 90% of all industrial enzymes are produced by SmF using genetically modified microorganisms due to several process advantages over SSF. The drawbacks of SSF can be summarized as the difficulties in scale-up and control of process parameters such as pH, temperature, oxygen transfer and moisture. Additionally, low mixing efficiency and higher product impurities increases the product recovery costs of SSF. However, today SSF gained some attention due to the possibility of using cheap agro-industrial wastes as substrates (Nakkeeran et al., 2012). Therefore we aimed to use one of these cheap agro-industrial wastes (orange peel) in SmF.

Biomass is an essential parameter in kinetic studies and for the characterization of the optimum growth and sporulation. But there are some problems faced in submerged fungal fermentations causing difficult sampling because of the heterogeneous distribution of biomass, settling of mycelial clumps, fouling of fermenter probes and impellers and growth along the feed lines. Also the use of heterogeneous complex media such as wheat bran or orange peel causes difficulties to measure fungal growth during fermentation (Prosser and Though 1991; Koutinas et al., 2003). To solve these problems many scientists reported indirect methods to estimate fungal biomass. These methods can be summarized as, the determination of biological activity (ATP, enzymatic activity, respiration rate, or immunological activity), the nutrient consumption, the analyses of cell constituents such as chitin/glucosamine, ergosterol, nucleic acids and proteins (Scotti et al., 2001; Yingyi et al., 2012). Recently, near-infrared spectroscopy (NIRS) has been used to measure components from the composite sample spectral information (Vaidyanathan et al., 2001). Garcia et al (2010) and Schoustra Punzalan (2012) and measured the mycelial growth rate by fungal colony measurement technique in a petri dish. Koutinas et al (2003) estimated fungal growth with a technique based on on-line measurements of CO_2 evolution during fungal fermentation on whole wheat flour. Aynsley et al.(1989) and Fu et al.(2010) studied the fungal biomass by the determination of the morphological characteristics of the fungus such as the measurement of its hyphal length.

In our preliminary study we demonstrated that this strain has considerable potential to be a candidate for industrial polygalacturonase production with a pellet morphology preferred by the industry (Gogus et al., 2006; Tari et al., 2007; Ustok et al., 2007). Therefore, the aim of this study was to expand this research to larger scale with the same objectives of increasing polygalacturonase production. Likewise, another

objective was to optimize the fermentation conditions and develop a low cost industrial media formulation for the production of polygalacturonase enzyme. In summary the goal of the proposed study was to scale up the preliminary study first to 1 l using serial bioreactor system, followed by 20 l and finally generate essential informations for mutant *Aspergillus sojae* fermentations.

Initially, enzyme production was optimized in shake flask systems (0.25 l scale) utilizing industrial low cost media formulations via statistical experimental design tools. The effect of agitation speed, aeration rate, pH, and carbon / nitrogen sources on biomass levels and on PG biosynthesis was evaluated. In order to run such experiments an overproducing mutant strain waschosen which was obtained after classical mutagenesis and selection procedure performed by Jacobs University Bremen, Germany.

The optimized system was scaled in a stirred tank bioreactor (1-20 l scale) under batch and fed-batch mode of operation. Investigations were performed on the effect of i) aeration rate, ii) agitation speed, iii) feeding type and iv) dissolved oxygen concentrations on i) biomass and ii) PG production. Factors were changed one at a time and a cascading procedure was employed. Different carbon and nitrogen sources were tested as well as their state and concentration. Furthermore, kinetic parameters were predicted in order to quantitatively understand the fermentation. Batch fermentations at different concentrations of the primary carbon source were conducted from where μ_{max} and K_s values were estimated for the chosen mutant *A.sojae* strain.

Moreover biochemical characterization of the crude PG enzyme was performed where the optimum pH and temperature values as well as its stability were determined. Thermodynamic parameters, thermal inactivation kinetics thereby the inactivation energy were also estimated. Finally, molecular weight of PG enzyme produced by WOP and EOP state were compared.

CHAPTER 2

FUNGI

Filamentous fungi are eukaryotic and typically saprophytic microorganisms that affect our lives in many areas such as medicine, agriculture, basic science and they secrete wide array of enzymes responsible from the decomposition and recycling of complex biopolymers from both plant and animal tissues (Wang et al., 2005; El-Enshasy, 2007). Fungi have been proven useful in many medical and biotechnological applications due to their unique metabolic activities, and as a source of degradative enzymes and secondary metabolites. Approximately 60% of the 260 commercial enzymes originate from fungal sources (Erjavec et al., 2012). These microorganisms are characterized as having branched filamentous structures or hyphae that has typical diameters of 2-18μm. The morphological forms of *Aspergillus* species are given in Figure 2.1. While higher fungi include *Aspergillus, Penicillium, Trichoderma* and *Fusarium* species lower fungi include *Rhizopus* and *Mucor* species (Ward, 2012). Industrial fungi which are generally used in large scale production processes are; *Penicillium chrysogenum, Aspergillus niger,Trichoderma reesei* and *Aspergillus oryzae* (Posch et al., 2012). The production of homologous or heterologous fungal proteins by filamentous fungi is usually very efficient and has accessible production levels of grams per liter. Especially *A. niger* and *A. oryzae* are well known strains that have been used in fermentation industry and regarded as safe (GRAS) in accordance with the Food and Drug administration (FDA). Hence it is necessary to develop an expression system for these microorganisms. This makes filamentous fungi attractive hosts for the production of secreted heterologous proteins (El-Enshasy, 2007). Filamentous fungal fermentations are generally used for the production of commercial products such as primary metabolites including organic acids (citric, gluconic, fumaric, kojic, itaconic acid and fatty acids), secondary metabolites especially human therapeutics, like penicillin. Some of fungi produce polysaccharides and biosurfactants. Some of them are food materials like mushrooms, single cell protein (SCP)/biomass or are components in fermented foods. Most of them are producers of commercial enzymes such as amylases, amyloglucosidases, cellulases, pectinases, laccases, phytase, proteases, microbial

rennets, lipases and glucose oxidase (Liao et al., 2007; Ward, 2012). *Aspergillus* species, and particularly *A. niger* and *A. oryzae*, play a dominant role in the production of these enzymes. Filamentous fungi offer very attractive, safe and cheap expression systems for the high level production of heterologous proteins.

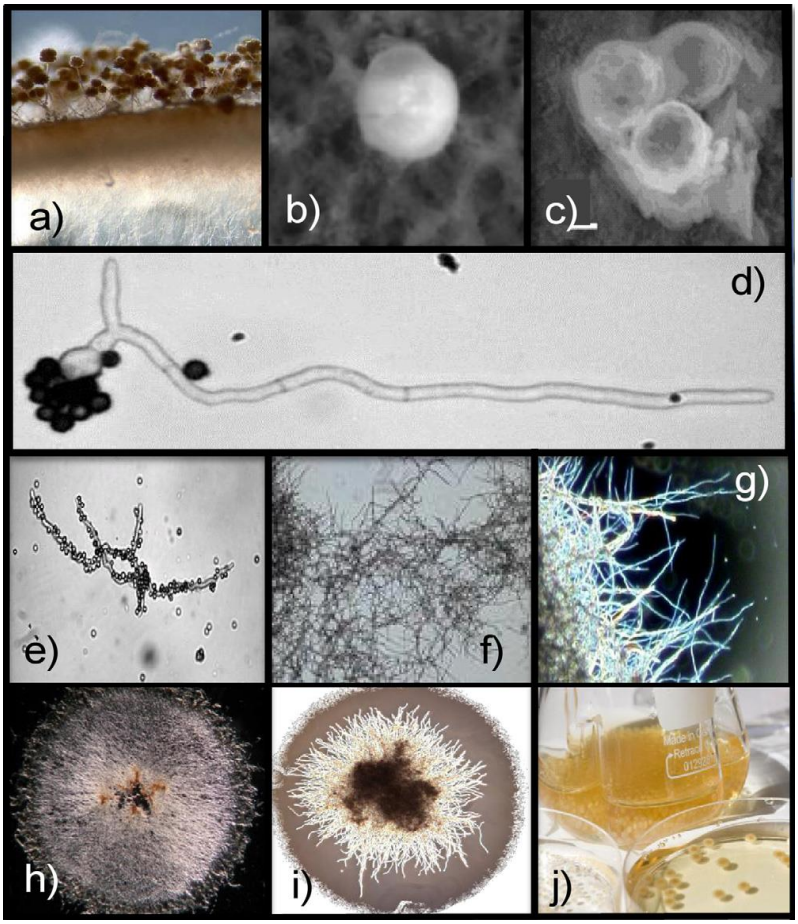


Figure 2.1.Morphological forms of *Aspergillus*sp.: (a) profile view of conidiophores (diameter 200 _m) on solid agar medium, (b) single spore, (c) spore package (spore diameter 5 _m), (d) germinated tube (length approx. 250 _m), (e) coagulated type of mycel, in which single ungerminated spores adhere to germinated hyphal tubes (length approx.100 _m), (f) dispersed mycel, (g) exposed hyphae of a pellet ("pellet hair") (length approx. 100 _m), (h) pellet slice (diameter approx. 1000 _m), (i) hairy biopellet (pellet diameter approx. 1000 _m), and (j) submerged biopellets (Source: Krull et al., 2013).

2.1. *Aspergillus sojae*

The genus *Aspergillus* is a group of filamentous fungi with large number of species. It is reported that the genus *Aspergillus* consists of more than 180 species. Most of them degrade plant polysaccharides and are used for the large scale production of industrial homologous and heterologous enzymes (Fawole and Odunfa, 2003; Ward, 2012). Most of the industrially important enzymes have been extracted from fungi of the genus *Aspergillus*. This genus is an important model organism for fungal enzyme production. Among these, *Aspergillus niger* is the single largest fungal source of enzymes (Subramaniyam and Vimala, 2012).

Aspergillus sojae strains have typical olive conidial color large conidial diameters and are taxonomically classified in section Flavi like *Aspergillus flavus, Aspergillus oryzae* and *Aspergillus parasiticus.* Among these *Aspergillus flavus* and *Aspergillus parasiticus* are known as fungal contaminants of food and feed, which produce the potent carcinogen, aflatoxin. However *Aspergillus sojae* and *Aspergillus oryzae* are used for industrial enzyme production and fermented food productions like sake (rice wine), miso bean paste) and soy sauce which is a condiment of global appeal with a multi-billion dollar market worldwide (Matsushima et al., 2001; Takahashi et al., 2004; Chang et al., 2007). No isolates of *Aspergillus sojae* and *Aspergillus oryzae* areknown to produce aflatoxins under any culture conditions (Matsushima et al., 2001; Takahashi et al., 2002; Chang et al., 2007). Some of the enzymes produced by *Aspergillus* species were summarized in Table 2.1.

Table 2.1. Enzyme production by *Aspergillus* species

Enzymes	**Microorganism**	**Substrate**	**Type of fermentation**	**Reference**
Cellulase	*Trichoderma reesei* and *Aspergillus niger*	Cellulose and lactose	SmF	Ahamed and Vermette, 2008
Pectinase	*Aspergillus niger*	Wastewater	SSF	Bai et al., 2004
Polygalacturonase	*Aspergillus awamori*	Wheat	SSF	Blandino et al., 2002
Cellulase	*Aspergillus niger*	Sugarcane bagasse	SSF and SmF	Cunha et al., 2012
Lovastatin	*Aspergillus terreus*	Lactose	SmF	Casas Lopez et al., 2005
α-amylase	*Aspergillus niger* ATCC 16404	Orange waste powder	SmF	Djekrif-Dakhmouche et al., 2006
Endoxylanase	*Aspergillus awamori* ZH-26	Wheat bran	SmF	Li et al., 2006
Fructosyl transferase	*Aspergillus oryzae* CFR 202	Agrocultural by-products	SSF	Sangeetha et al., 2004
Naringinase	*Aspergillus niger MTCC 1344*	Molasses	SmF	Puri et al., 2005
Mannanase	*Aspergillus niger UAM-GS1*	Copra paste	SSF	De Nicolas-Santiago et al., 2006

Accordingly, Kimura et al. (1995) described the purification and characterization of xylanase and arabinofuranosidase from *A.sojae* by solid state fermentation. In another study of Kimura et al. (1999)a β-glucosidase was purified from a solid culture of *A.sojae.* Moreover extracellular leucine aminopeptidase from *A.sojae* was purified by Chien et al. (2002). Also the production of exo-polygalacturonase from *A.sojae* was first reported by our research group (Göğüş et al., 2006). Furthermore mannanase production from recombinant *A.sojae* was optimized by Öztürk (2008).

2.2. Fungal Growth

Microbial growth is the most essential response of microorganisms to their physiochemical environment. Growth is a result of both replication and change in cell size (Shuler and Kargı, 2002). Many aspects of growth and the underlying growth kinetics are similar to those of yeasts and unicellular bacteria, but the filamentous growth form increases colony and culture heterogeneity (Prosser and Tough, 1991). It is recognized that optimal design and control of fermentation systems requires quantitative estimates of microbial growth, substrate depletion and product formation (Goudar and Strevett, 1998).

There will be a proportionate increase in all aspects of biomass, such as cell number, dry weight and the nucleic acid and protein content of the population. Growth may hence be monitored by measuring any of these or other convenient attributes of the population including optical density. A variety of methods are available to predict cell growth by direct or indirect measurements. Cell dry weight, cell optical density, cell turbidity, cell respiration, metabolic rate and metabolites are quite suitable for analyzing cell growth, substrate utilization and product formation (Najafpour, 2007).

Biomass concentration is one of the most basic parameter that should be followed during the course of fermentation. Most of the fermentation parameters like, yield coefficient, specific growth rate and oxygen uptake rate need biomass data. However when a particulate and insoluble substrate is used, the determination of dry mass becomes difficult or impossible. The use of complex media with particles are often used in fungal fermentations but the information on such fermentations with respect to growth, kinetics and morphology is scarce in the literature. The use of a complex medium may influence morphology and growth kinetics and will make

biomass determination difficult due to the need of the separation of the biomass from the substrate. Cui et al., (1998a) found that fungal cells mainly grew on the wheat bran particles when the initial spore concentration was higher than $1.3x10^5$ ml^{-1}. However when the initial spore concentration was lower than $1.8x10^4$ ml^{-1}, cells grew in pellets without adhering to the wheat bran particles.

Among the biomass determination methods spectrophotometric turbidity measurement is mostly used for single cell cultures,however this is not directly applicable for fungi, since it needs pretreatment like homogenization. This method cannot be applied at all if a particulate and insoluble substrate is used.

Chitin concentration measurement can also be used as a measurement of biomass concentration however chitin content of hyphae may change with age and growth conditions. Therefore the chitin content per biomass will not be constant and may lead to poor accuracy (Cui et al., 1998a).

Measured parameter must be known to be proportional to criterion of growth being considered and must directly correlate to the increase in biomass (protoplasm).

The parameters that could be measured for the index of fungal growth are as follows;

1. Change in linear dimensions (colonies, hyphae)
2. Change in mass or weight
3. Change in cell number
4. Change in volume
5. Change in metabolic activity
6. Change in quantity of cell constituent
7. Change in optical density.

1. *Linear measurements of increase in hyphal length using microscopic techniques* is an excellent nondestructive method but difficult to perform.

Lopez-Isunza et al., (1997) indicates that micrometric measurements during hyphal growth of *Aspergillus niger* with labeled compounds may help in the estimation of growth kinetic parameters. Aynsley et al., (1989) developed a model which is capable of predicting changes in the rates of hyphal extension, branching, and fragmentation during mycelial growth of *Penicillium chrysogenum* in submerged culture and consequently variations in the hyphal growth unit. But the model is limited with the requirement of over forty parameters, many of which are immeasurable.

2. *Dry weight* is probably one of the best measures for mold growth only when

linear measurements are appropriate. It is destructive method and requires culture sampling which must be precise. Because of the nonhomogenity of the mold culture it is nearly impossible to uniformly sample.

3. *Estimates of cell numbers* is not suitable for molds because hyphae of any length would yield only one viable count.

Martinez-Trujillo et al. 2009 measured cell growth as dry weight by filtering the sample through a Millipore membrane, previously dried to constant weight. The retained cell mass was dried at 80^{o}C until constant weight was reached.

4. *Turbidity or optical density* is not useful for molds because hyphae do not always form homogenously.

5. *Metabolic activity*: measured by,

- Product formation
- Substrate utilization
- Enzymatic activity

Because of generally high levels of endogenous reserves, fungi often show high rates of O_2 uptake and CO_2 evolution in the absence of growth. This may cause misleading data.

Biomass formation and substrate depletion were described by a model used to estimate growth kinetic parameters of *Penicillium chrysogenum* in batch culture(Goudar and Strevett 1998).

6. *Compositional changes* is good for certain balanced growth situations, e.g.DNA

The doubling time of DNA production during the “logarithmic phase” gives an indirect estimate of nuclear doubling. However, direct estimates of cell doubling are not feasible in a multicellular fungus such as *Aspergillus* (Dorn and Rivera 1966).

According to Krishna (2005) biomass can be estimated indirectly from physical measurements such as temperature, effluent gas composition, light reflectance, composition changes, including estimation of cell components, as analyzed by infrared spectrophotometry, and by variation of the dielectric properties. Other indirect methods include determination of biological activities such as ATP, enzymatic activities, DNA assay, respiration rate (oxygen consumption and carbon dioxide release), and immunological activity, nutrient consumption, and analyses of cell constituents such as chitin, glucosamine, nucleic acids, ergosterol, and protein. But some of these approaches have drawbacks as enzymatic activity and ergosterol content vary during

fungal development, and nutrient consumption can be applied only in axenic conditions. Microscopic observation using a scanning electron microscope is another way of estimating fungal growth. Image analysis by computer software, Confocal Microscopy, based on specific reaction of fungal biomass with specific fluorochrome probes and reflectance infrared spectrophotometry are the other methods of estimating fungal growth (Krishna 2005). Moreover, for fungal growth measurement Boswell et al., (2003) explainedtessellated agar droplet system where fungal growth was considered in a heterogeneousenvironment. In that technique molten agar (MSM) was pipetted onto the petri dishes forming a hexagonal array comprising 19 circular droplets. Each agardroplet was 10mmin diameter and the droplets were separated at their closest pointof contact by a nutrient-free gap of 2 mm. A 4 mm diameter inoculum of *R. solani* was placed onto the central droplet and the petri dish was then sealed to prevent dehydration and contamination and inspected daily over a period of 7 days.

Fermentations may be carried out as batch, continuous and fed-batch processes. The mode of operation isto a large extent dictated by the type of product being produced (Stanbury and Whitaker, 1984). The illustration of a typical fermentation process is given in Figure 2.2.

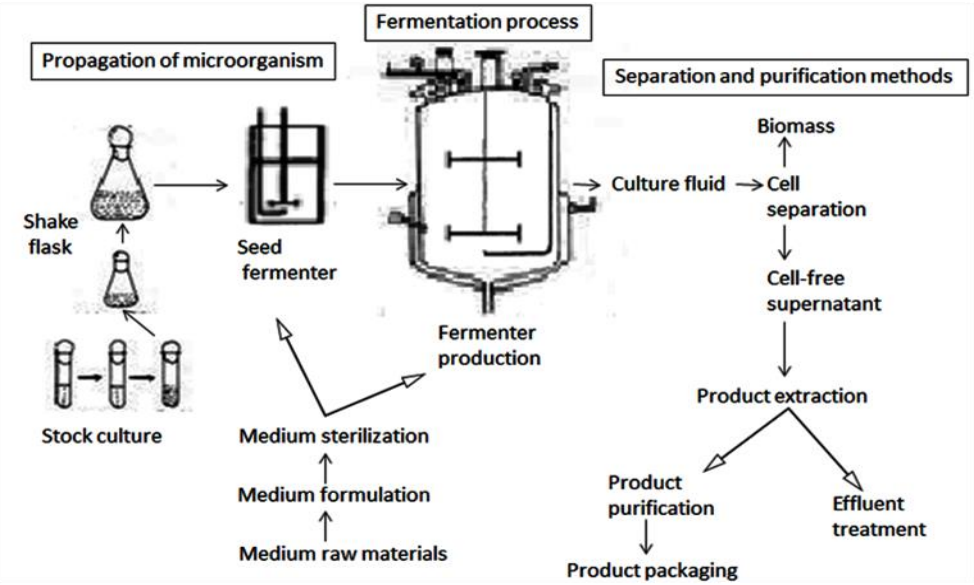


Figure 2.2.Schematic representation of atypical fermentation process (Source: Diaz-Montano, 2013).

2.2.1. Batch Culture

Microorganisms can be cultured in closed systems (batch system) or in open systems (continuous flow systems). Batch culture is a closed system without any inlet or outlet stream, as nutrients are prepared in a fixed volume of liquid media (Figure 2.4).After the inoculation the microorganisms gradually grow and replicate. As the cell propagates the nutrients are depleted and end products are formed(Najafpour, 2007).

Batch growth has a number of phases: lag, exponential growth, stationary and death (Figure 2.3). In the lag phase no growth occurs, microbial population remains constant because fungal cells or spores adapt to the new environment which means the formation of enzymes and intermediates to support the maintenance of growth (Waites et al., 2001; Papagianni, 2004). The duration of this phase mostly depends on the physiological state and morphology of the fungus and on the level of inoculum. While the spore inocula require a germination period, pelleted inocula requires a certain degree of mechanical disruption. Physiological adaptation period includes synthesis of enzyme systems required for substrate utilization, or removal of inhibitory compounds carried over with the inoculum (Papagianni, 2004). In a commercial process the length of the lag phase should be reduced as much as possible by using a suitable inoculum (Stanbury and Whitaker, 1984).

Once there is an appreciable amount of cells, they will grow very rapidly, the cell number increases, exponentially; this phase is known as the exponential growth phase. The rate of cell synthesis sharply increases; the linear increase is shown in the semi-log graph with a constant slope representing a constant rate of cell population. At this stage carbon sources are utilized and products are formed(Najafpour, 2007).

The rate of product formation depends upon the state of the cell population, environmental condition, temperature, pH, media composition and morphology of the microorganism. A similar balance can be formulated for microbial biomass and cell concentration. The exponential phase of the microbial growth in a batch culture is defined by:

$$\frac{dX}{dt} = \mu X \tag{2.1}$$

where x is the concentration of microbial biomass, t is time, in hours and μ is the

specific growth rate, in hours^{-1}.

There is no cell removal from the batch vessel and the cell propagation rate is proportional to specific growth rate, μ (h^{-1}). During any period of true exponential growth, equation (2.1) can be integrated to provide the following equation (Stanbury and Whitaker, 1984; Najafpour, 2007):

$$x_t = x_0 e^{\mu t} \tag{2.2}$$

where x_0 is the biomass concentration at the start of exponential growth, x_t is the biomass concentration after time t.

Taking natural logarithms, equation (2.2) gives;

$$\ln x_t = \ln x_0 + \mu t \tag{2.3}$$

For cells in exponential phase, a plot of the natural logarithm of biomass concentration versus time, a semilog plot, should yield a straight line, whose slope will equal to μ (Stanbury and Whitaker, 1984; Waites et al., 2001).

The hyphal growth rate depends on the strain of the fungus and physicochemical environmental conditions. On solid media, exponential growth results from autocatalysis through exponential production of branches, each of which extends at a linear rate. A reduction in the specific growth rate occurs when the fungus enters an unfavorable growth environment such as the limitation of a required nutrient, the development of an adverse pH value or the accumulation of end products of metabolism that are inhibitory (Papagianni, 2004). The effect of limiting substrate concentration on the specific growth rate (μ) may be expressed by the Monodequationfor microbial growth (Waites et al., 2001).

The effect of substrate concentration on specific growth rate (μ) in a batch culture is defined by the Monod rate equation. The cell density increases linearly in the exponential phase. When substrate (S) is depleted, the specific growth rate (μ) decreases. The Monod equation is described in the following equation:

$$\mu = \mu_m \frac{S}{K_S + S} \tag{2.4}$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate in h^{-1}, K_s is saturation or Monod constant and S is the substrate in $g.l^{-1}$. K_s is equal to the substrate concentration at $\mu = 0.5\mu_{max}$. Although eucaryotes tend to grow more slowly than bacteria, filamentous fungi such as *Aspergillus* and *Penicillium* have maximum specific growth rates in the order 0.1-0.3 h^{-1}, equivalent to doubling times of 2-7 h (Papagianni, 2004).

By performing a series of batch fermentations, each with a different initial limiting substrate concentration, the specific growth rate (μ) for each experiment can be determined. These data can then be used to estimate both μ_{max} and the saturation constant (K_s) by simply taking the reciprocal values in the Monod equation and rearranging equation 2.4 to give the linearized form of the Monod equation:

$$\frac{1}{\mu} = \left(\frac{K_S}{\mu_m}\right)\frac{1}{S} + \frac{1}{\mu_m} \qquad (2.5)$$

The plot of $1/\mu$ against $1/S$ should produce a straight line with the intercept on the y-axis at $1/\mu_{max}$ and a gradient equal to K_s/μ_{max}. Hence, the key parametersof the fermentation, K_s and μ_{max} can be determined and a complete quantitative description can be given of the growth events occurring during a batch culture (Waites et al., 2001).

Another parameter that characterizes fermentation is the doubling time (t_d) which was calculated from the following equation;

$$t_d = \ln 2 / \mu_{max} \qquad (2.6)$$

After the exponential phase, finally, rapid utilization of substrate and accumulation of products may lead to stationary phase where the cell density remains constant. In this phase, cell may start to die as the cell growth rate balances the death rate. It is well known that the biocatalytic activities of the cell may gradually decrease as they age, and finally autolysis may take place. The dead cells and cell metabolites in the fermentation broth may create toxicity, deactivating remaining cells. At this stage, a death phase develops while the cell density drastically drops in the presence of toxic secondary metabolites. The death phase shows an exponential decrease in the number of living cells in the media while nutrients are depleted. In fact the changes can be detected by monitoring the pH of the media (Najafpour, 2007).

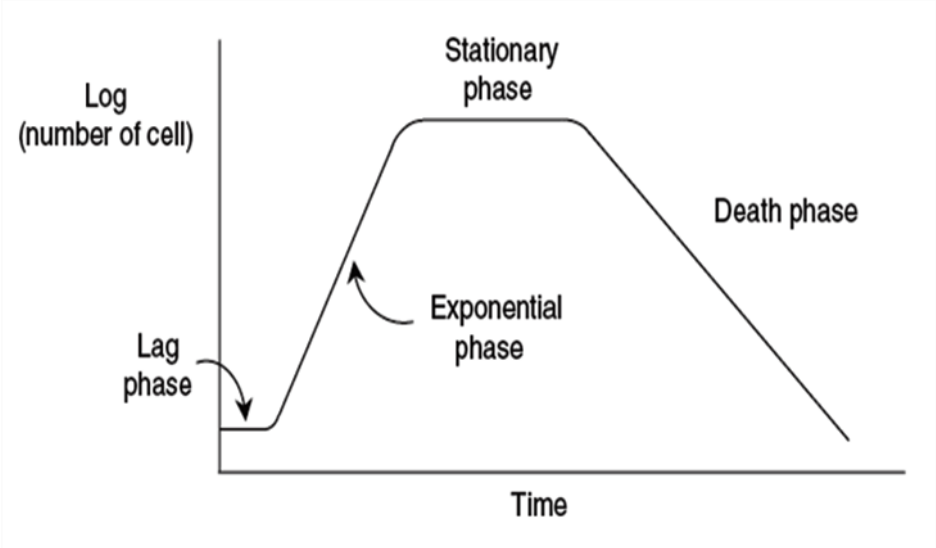


Figure 2.3. Typical batch growth of a microbial culture
(Source: Najafpour, 2007).

The batch culture is a simple, well-controlled vessel in which the concentration of nutrients, cells and products vary with time as the growth of the microorganism proceeds. Material balance in the reactor may assist in following the biochemical reactions occurring in the media. In batch fermentation, living cells propagate and many parameters of the media go through sequential changes with time as the cells grow. The following parameters are monitored while the batch process continues:

- Cells and cell by-product
- Concentration of nutrients
- Desirable and undesirable products
- Inhibition
- pH and temperature

The objective of a good process design is to minimize the lag phase period and maximize the length of exponential growth phase (Najafpour, 2007).

Batch fermentation may be used to produce biomass, primary metabolites and secondary metabolites. For biomass production, cultural conditions supporting the maximum cell production should be used. For primary metabolite production, conditions to extend the exponential phase with product excretion and for secondary metabolite production, conditions giving a short exponential phase and an extended stationary, or production phase, or conditions giving a decreased growth rate in the log

phase resulting in earlier secondary metabolite formation should be used (Stanbury and Whitaker, 1984).

2.2.2. Continuous Culture

Continuous culture of microorganisms means the continuous supply of fresh medium to the culture vessel, with continuous removal of spent medium and culture fluid at the same rate (Figure 2.4.). The reactor is continuously stirred and a constant volume is maintained by incorporating an overflow weir or other leveling device (Prosser and Tough, 1991; Waites et al., 2001). Continuous culture is generally proceeded by growth of the fungus in batch culture to stationary phase. When fresh medium supply is started growth proceeds and material from the vessel is washed out, until the concentration of the medium is reduced to a level at which it limits specific growth rate (Papagianni, 2004). The composition of the feeding medium is usually designed such that the limiting substrate is the component of interest such as carbon or nitrogen source (Prosser and Tough, 1991). If medium is fed continuously to such a culture at a suitable rate, a steady state is achieved at which the formation of new biomass is balanced by the loss of cells from the vessel. The flow of medium into the vessel is correlated to the volume of the vessel by the term dilution rate (D). This equals the number of reactor volumes passing through the reactor per unit time and is expressed in units of reciprocal time, per hour(Waites et al., 2001).

$$D = \frac{\mathrm{F}}{\mathrm{V}} \tag{2.7}$$

where D = dilution rate (per hour), F = flow (L/h) and V = reactor volume (L).

Addition of fresh medium into the reactor can be controlled at a fixed value, therefore the rate of addition of the rate-limiting nutrient is constant. Within certain limits, the growth rate and the rate of loss of cells from the fermenter will be determined by the rate of medium input. The changes in biomass and substrate concentrations during growth in continuous culture can be described by the following equation:

$$\frac{\mathrm{dX}}{\mathrm{dt}} = \mu\mathrm{X} - \mathrm{DX} \tag{2.8}$$

where μ is described by the Monod equation, X is the biomass concentration and D is the dilution rate. If D is less than μmax a steady state is established. By setting this equation equal to zero, the steady state biomass and substrate concentrations as functions of dilution rate can be obtained. Additionally, in steady state, the specific growth rate will be equal to the dilution rate (Papagianni, 2004). At fixed flow rates and dilution rates under constant physical and chemical operating conditions like steady state conditions, the specific growth rate of the microorganism is dependent on the operating dilution rate, up to a maximum value equal to μmax. As the dilution rate increases above μmax, compelete wash-out of the cells occurs due to the insufficient time to double cells before wash out of the reactor. The point at which this is avoided is the critical dilution rate (Dcrit) (Waites et al., 2001).

The continuous system, where the growth rate of the culture is controlled by its chemical environment, which is the availability of one limiting component in the medium is often described as a chemostat. An other type of continuous culture system, where the concentration of cells in the culture is kept constant by controlling the flow of medium such that the turbidity of the culture is kept within certain limits, is termed as turbidostat (Stanbury and Whitaker, 1984; Waites et al., 2001).

The problems of filamentous growth in submerged batch culture worsen in continuous culture. Wall growth increases continuously and maintenance of steady state is difficult. Breakage of mycelia to provide new centers for growth may be a solution. Furthermore, maintenance of the stability of filamentous fungi in prolonged culture is difficult (Papagianni, 2004).

2.2.3. Fed Batch Culture

In fed-batch systems fresh medium or medium components are fed continuously, intermittently or as a single feed supplement; the volume of the batch increases with time as there is no removal of the culture (Waites, 2001).

Most of the large scale industrial fungal fermentations involve fed batch culture where biomass is grown initially in batch culture until a chosen component of the substrate is fully utilized. Then fresh nutrient is added and the culture volume continually increases (Figure 2.4.). The aim is to promote product formation rather than biomass. Because of the high levels of active biomass present in the medium, fresh

substrate is converted immediately. Thus metabolism is directed towards product formation instead of biomass and substrate utilization and catabolite repression are minimized. Different from the continuous culture, in fed batch culture systems specific growth rate is not constant; it rather decreases as the culture volume increases (Papagianni, 2004).

The use of fed-batch in the fermentation industry takes advantage of the fact that residual substrate concentration can be maintained at very low level in such a system. A low residual level of substrate can be advantageous in:

- Removing repressive effects of rapidly utilized carbon sources and maintaining conditions in the culture within the aeration capacity of the fermenter.
- Avoiding the toxic effect of a medium component (Stanbury and Whitaker, 1984).

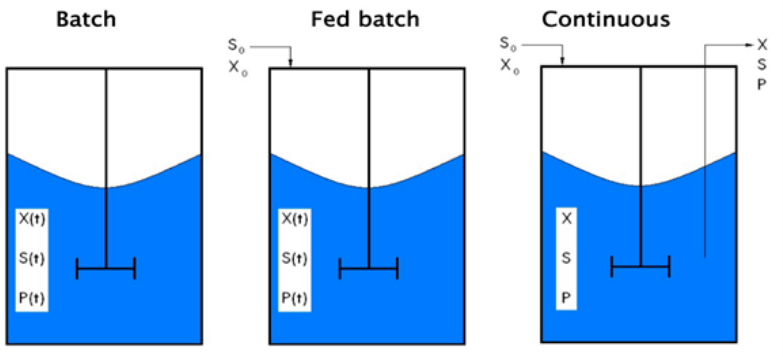


Figure 2.4. Fermentation process modes; X: biomass, S: substrate, P: product, t: time (Source: Diaz-Montano, 2013).

CHAPTER 3

SUBMERGED FERMENTATION

Fermentation is the biological conversion ofcomplex substrates into simple compounds by variousmicroorganisms such as bacteria and fungi. During this metabolic breakdown, microorganisms also release severaladditional compounds different from the usual products offermentation, such as carbon dioxide and alcohol. Fermentation has been widely used for the production of a wide variety of compounds that are highly beneficial to individualsand industry. Over the years, fermentation techniques have gained great importance due to their economic andenvironmental advantages. Ancient techniques have been further modified and refined to maximize productivity. This has alsoinvolved the development of new machinery and processes. Two main fermentation techniques have emerged as a result ofthis rapid development: Submerged Fermentation (SmF) and Solid State Fermentation (SSF) (Subramaniyam and Vimala, 2012).

Submerged fermentation (SmF) utilizes free flowing liquid substrates and broths, where substrates are utilized quite rapidly and thus need to be constantly supplemented. Furthermore, SmF is mainlypreferred for the production of secondary metabolites that need to be used in liquid form since;these compounds are secreted into the fermentation broth making the purification easier (Subramaniyam and Vimala, 2012).

Approximately, 90% of the industrial enzymes are produced by submerged fermentation (Belmessikh et al., 2013). However the production cost of submerged fermentations is high; therefore it is necessary to reduce the production cost for example by using agro-industrial wastes such as orange peel. SmF has several process advantages over SSF which are the ease of scale up and control of process parameters such us pH, temperature, oxygen transfer and moisture, higher mixing efficiency and easier product recovery (Nakkeeran et al., 2012).Additional advantages of SmF was described by Lin and Chen (2007) as a greater mycelia production in a more compact space over a shorter time with a lower chance of contamination, thus it may be a preferred route for the production of some valuable metabolites, including exopolysaccharides and triterpenoids. Furthermore SmF ensures the standardized

quality and year around production (Shih et al., 2008). Subramaniyam and Vimala (2012) explained the reasons why SmF is widely used in industrial fermentations, being that SmF supports the utilization of genetically modified organisms more than SSF and the ease of handling regarding the production of various enzymes using SSF.

Submerged cultures of filamentous fungi are mostly used to produce commercially important metabolites including many antibiotics, enzymes and cholesterol lowering drugs (Casas Lopez et al., 2005). Filamentous organisms show different morphological growth forms than bacterial and yeast cultures in aerobic submerged cultivations which affect the suspension characteristics. The morphological characteristic of the filamentous microorganisms have significant effect on rheological properties of the cultivation medium which are reflected directly in the production of microbial metabolites. In fungal submerged fermentations the growth morphology can vary from discrete compact pellets of hyphae to a homogeneous suspension of dispersed mycelia (El-Enshasy, 2007). The growth morphology is determined by the genetic material of the fungal species, the nature of the inoculum, chemical (medium composition) and physical (temperature, pH, mechanical forces) culturing conditions (Papagianni, 2004; Krull et al., 2013; Nitayavardhana et al., 2013). The mycelial growth causes more viscous fermentation broth due to the easy entanglement of the hyphae. Non-Newtonian rheological behavior is common for fungal fermentations leading to relatively low viscosities in regions with high shear rates (near the impeller) and very high viscosities in region with low shear rates (near the wall). The high viscosity and pseudoplastic behavior of the suspension cause many problems during cultivation such as; decreasing the mass transfer, heat transfer and requiring more power input for mixing (El-Enshasy, 2007).

Many investigators haveattempted to obtain optimal submerged culture conditionsfor exo-biopolymer production from several fungi. Among the fungal SmF studies, Park et al. (2001) optimized submerged fermentation conditions for the mycelial growth and exo-biopolymer production by *Cordyceps militaris*in shake flask culture. Tang and Zhong, (2003) investigated effect of oxygen supply on the submerged fermentation of *Ganoderma lucidum.* A comperative study on solid and submerged fermentation on tomato pomace for neutral protease production by*Aspergillus oryzae* was performed by Belmessikh et al. (2013). Chi Chiang and Huang Chiang (2013) determined the processing characteristics of submerged fermentation of *Antrodia cinnamomea* inairlift bioreactor. Enhanced enzyme production from mixed cultures of

Trichoderma reesei RUT-C30 and *Aspergillus niger* LMA in a stirred tank bioreactor was performed by Ahamed and Vermette (2008). Cui et al. (1998a) used complex media like wheat bran for submerged fermentation of *Aspergillus awamori*and quantified the biomass concentration in complex medium.Also Fontana and Silveira (2012) studied polygalacturonase production by *Aspergillus oryzae* in stirred tank and internal-external loop airlift reactors. Additionally recent studies showed that in solid state fermentation gene expression and protein secretion mechanism might be different from that in submerged fermentation. For instance, although in submerged cultures some enzyme activities are found mainly in the cell wall of the mycelia, in solid state cultures same enzyme activities are found mainly in the medium, but a little in the cell wall (Wang et al., 2005). This proves the fact that culture conditions strongly influences the protein secretion.

3.1. Effects of Cultivation Conditions

It is known that different culture conditions, including those during the inoculum preparation step, results in different fungal morphologies and consequently affect enzyme production (Cunha et al., 2012). The influence of aeration and agitation (Tang and Zhong, 2003; El-Enshasy et al., 2006; Lin et al., 2010), pH and temperature (Bailey, 1990; Park et al., 2001; Fang and Zhong, 2002; Malvessi and Silveira, 2004), medium composition (Park et al., 2001; Malvessi and Silveira, 2004; Belmessikh et al., 2013), strain type (Khairnar et al., 2009), inoculum size and age of spores (Mojsov, 2010), metal ions (Couri et al., 2003) and bioreactor design (Ahamed and Vermette, 2008) have been investigated.

3.1.1. Medium Composition

Filamentous fungi are heterotrophic organisms; they require organic compounds as carbon and energysource. Organic compounds like glucose, fructose and sucrose that support growth are rapidly used. Polysaccharides, aminoacids, lipids, organic acids, proteins, alcohols and hydrocarbons are also consumed (Papagianni, 2004).

For the design of successful laboratory experiments, pilot scale development and manufacturing processes, development of a suitable medium formulation is an essential

stage (Stanbury and Whitaker, 1984). The media used in the submerged industrial fermentations favor both growth and product formation. Fungi require water, molecular oxygen, an organic carbon source, a nitrogen source and several other elements (Papagianni, 2004). Some of the common substrates used in SmF are soluble sugars, molasses, liquid media, fruit and vegetable juices and waste water (Subramaniyam and Vimala, 2012). In submerged fermentation processes, the cost of the carbon source is known to be the main contributor of the overall production cost (Ahamed and Vermette, 2008). Therefore it is essential for industrial fermentations to reduce the cost of the medium by using cheaper sources like agro-industrial wastes. Blandino et al.(2001) studied the utilization of whole wheat flour for the production of extracellular pectinases by some fungal strains and proved that the whole wheat flour acted as a good nutrient source for the cultivation of the microorganisms and exo- andendo-polygalacturonases (PG) produced in submerged culture. But the use of complex media in submerged fermentations of filamentous fungi affects growth kinetics and morphology of the fungi. In a study performed by Cui et al. (1998a) wheat bran as a complex media was used for the fermentation with *Aspergillus awamori* and results were compared with the studies on synthetic medium. They found that wheat bran was not consumed completely as glucose or sucrose and fungal growth and adhesion varied due to the inoculum and the kinetics with complex medium was the same with synthetic medium. Dairy wastes were also used for the yeast polygalacturonase production by Murad and Foda (1992).

The waste of citrus fruits has been used in some researches as a carbon source for enzyme production. Khamseh and Miccio (2012) compared the batch, fed-batch and continuous fermentations of the enzymatic hydrolysis of orange peel wastes. Djekrif-Dakhmouche et al. (2006) optimized the culture medium for α-amylase production by *Aspergillus niger* on orange waste powder. El-Sheekh et al. (2008) produced pectinase by the submerged fermentationof *Aspergillus carneus*utilizing the orange juice industry scraps. Lemon peel was used as a sole carbon source by Larios et al. (1989) for endo-polygalacturonase production by *Aspergillus* sp.Pectinase production from orange peel extract anddried orange peel solid as substrates using*Aspergillus niger*was performed by Rangarajan et al. (2010). Citrus peelis animportant agro-industrial byproduct which can be a rich carbon source for microbial growth and simultaneous polygalacturonase and xylanase production by SSF (Mamma et al., 2008). Citrus peel solid byproduct is approximately 50% of the fresh fruit weight(Rodriguez-Fernandez et al., 2011).Orange production generates large amount of residues, mainly the peel and segment membranes

which are rich in soluble and insoluble carbohydrates (Zhou et al., 2011). The disposal of these residues poses a challenge for many factories, which often pelletize the peel and use it as animal feed or as a pectin precursor (Rodriguez-Fernandez et al., 2011). However orange peel is rich in pectin which is the inducer for the pectinase. Therefore orange peel is a suitable carbon source and inducer for the pectinase production (Zhou et al., 2011).In the thesis dry orange peel was used which is rich in pectin, cellulose and hemicellulose as a fermentation substrate. In some of the studies the composition of orange peels were given in Table 3.1.;

Table 3.1. Composition of dry orange peels
(Source: Zhou et al., 2011; Mamma et al., 2008).

Component	**% (w/w dry basis) (Zhou et al., 2011)**	**% (w/w dry basis) (Mamma et al., 2008)**
Crude fat	3.6 ± 0.1	3.9 ± 0.1
Water soluble materials	43.1 ± 1.1	41.1 ± 0.1
Glucose	15.6 ± 0.3	14.6 ± 0.4
Fructose	16.5 ± 0.6	15.5 ± 0.5
Sucrose	10.9 ± 0.2	10.9 ± 0.3
Pectin	14.7 ± 0.3	14.4 ± 0.3
Protein	7.7 ± 0.1	7.9 ± 0.1
Cellulose	16.7 ± 0.5	16.2 ± 0.5
Hemicellulose	11.8 ± 0.3	13.8 ± 0.3
Ash	1.2 ± 0.1	1.7 ± 0.1
Lignin	1.2 ± 0.02	1.0 ± 0.02

Nitrogen may be supplied as ammonia, nitrate or as organic compounds like aminoacids or proteins. Also industrial raw materials such as yeast extract, corn steep liquor, whey powder, beet and cane molasses, soy flour can be used as nitrogen source (Papagianni, 2004). Lack of nitrogen in the medium strongly affects fungal growth and metabolite production (Papagianni, 2004).

Besides, the optimization of fermentation media represents an important cost and time factor in bioprocess development. In the optimization process suitable reaction conditions (pH, temperature and medium composition) for the respective biological system to maximize or minimize economically or technologically important process variables such as product concentration, yield, selectivity and raw material cost are among the main goals (Weuster-Botz, 2000). Due to the large number of quantitative and qualitative process variables and the metabolic complexity of microorganisms, the classical method of changing a variable one at a time in order to optimize performance is impractical (Weuster-Botz, 2000; Djekrif-Dakhmouche et al., 2006). Therefore the methods of statistical experimental design are used in many studies.

3.1.2. Culture pH

The pH of the medium is one of the most important factors for fermentations. The pH of a solution is defined as a measure of the H^+ ion concentration present in the solution (Papagianni, 2004). Medium pH affects the cell membrane function, cell morphology and structure, salt solubility, ionic state of substrates, nutrient uptake and product biosynthesis. Microbial cells generally grow within a certain pH range, for example, conidial fungi can grow over a wide range of pHs. Most of them can tolerate a pH range 4-9 but their optimum growth and sporulation pH is near neutral pH. Metabolite formation is also affected by medium pH (Fang and Zhong, 2002; Papagianni, 2004). Malvessi and Silveira (2004) investigated that *A. oryzae* growth was favored by pH close to 4, although a drop of pH to around 3 was needed for polygalacturonase production. Fawole and Odunfa (2003) evaluated some factors affecting production of pectic enzymes by *Aspergillus niger* and found thatpH had marked effects on the production of pectic enzymes with the best condition being pH 5. It was found by Martinez-Trujillo et al. (2009) that the production of pectinolytic enzymes is not only related to the initial pH of culturemedium, but also to its evolution during fermentation.

The balanced use of carbon and nitrogen sources will also help to control the medium pH by the buffering capacity of the proteins, peptides and amino acids in the medium. During the fermentation especially in bioreactors pH may be controlled by the

addition of ammonia, sodium hydroxide and sulphuric acid (Stanbury and Whitaker, 1984).

3.1.3. Aeration and Agitation

Most of the fermentationprocessesareaerobic and require oxygen. Oxygen should be in solution in order to be used by the microorganisms for the complete oxidation of glucose. The oxygen demand of an industrial fermentation processes is provided by aerating and agitating the fermentation broth (Stanbury and Whitaker, 1984).

Industrial metabolite production by filamentous fungi can be regulated by the dissolved oxygen tension of the medium (Papagianni, 2004). Oxygen is a limiting parameter in fermentations due to its low solubility and low volumetric mass transfer in bioreactors. In order to maintain the dissolved oxygen (DO) tension at high levels, oxygen is used instead of air. For example, oxygen was found to be the most significant factor that influenced the lovastatin production reported by Casas Lopez et al. (2005). The dissolved oxygen tension may affect the productivity, cell autolysis, fungal morphology, etc. (Cui et al., 1998a,b). In the aerobic fermentation systems oxygen behaves as substrate for energy production. It supports the cell growth and product formation directly or by the morphological change. Adversely, oxygen can be toxic or can cause undesirable morphological change (Wang et al., 2005). Potumarthi et al. (2007) evaluated the effects of aeration and agitation on alkaline protease production by submerged fermentation in a batch STRusing *Bacillus licheniformis* NCIM-2042 and stated that agitation and aeration have a significanteffect on protease production. Effect of volumetric power input through aeration and agitation on pellet morphology and product formation of *Aspergillus niger* was studied by Lin et al. (2010). They defined the effect of high aeration and agitation rates on the filamentous fungi fermentation in STR as; enhanced oxygen supply, better mixing and better mass and heat transfer. However, it requires higher energy input and may cause higher mechanical stress on the fungal pellets which may lead to morphological changes.

In submerged fermentations, agitation is also an important factor for good mixing and heat and mass transfer. In aerobic fermentation processes mixing is essential for sufficient oxygen transfer throughout the vessel. Although there is efficient mass

and heat transfer around the impellers due to the high energy dissipation, there may be inadequate supply of oxygen in the other parts of the vessel because of the poor mixing. Therefore fungal growth and product formation may be strongly affected by the mass transfer gradients across the vessel. Agitation creates a shear force within the vessel which can affect the fermentation by damaging the cell structure, causing morphological changes, influencing the growth rate and product formation (Papagianni, 2004). Cui et al. (1998b) found that *Aspergillus awamori* can grow faster at higher agitation rates. However, in that study they also determined that the fungal growth rate at 400 rpm was lower than 250 rpm and this was explained by two different effect of power input on growth. Primary effect was described as the effect on the gas-liquid mass transfer which in turn influences the dissolved oxygen tension and affects the growth indirectly. Other one was mechanical forces which may affect the fungal morphology, metabolism and can damage fungi (Cui et al., 1998b). El-Enshasy et al. (2006) also evaluated the agitation effect on morphology and protein productivefractions of filamentous and pelleted growth forms ofrecombinant *Aspergillus niger*and revealed that the growth morphology and protein production of *A. niger* was strongly influenced by power input variations. Furthermore in that study withincreasing power input the growth morphology changed frompelleted to filamentous growth and increased the protein production capability. However,increasing power input also exposed the fungus to higher shear stress, resulted in lower biomass yields and increasedrespiration and acid formation (El-Enshasy et al., 2006).

Additionally the increase in the biomass concentration makes the culture broth, highly shear thinning, which affects the mixing and oxygen transfer in the vessel. However, as the fungal growth and enzyme production yields are strongly influenced from the oxygen supply to the medium, the scale up considerations in industrial fermentation processes mainly focus on gas-liquid mass transfer predictions (Gabelle et al., 2012).

3.2. Scale-up

Fungal fermentations are known to be complex processes, in which several problems occur. Scale up and optimization of these processes still needs to be evaluated. The major problems faced in large scale fungal fermentations are viscous and non-

Newtonian media, bad mixing, poor mass transfer and difficulty in the control of morphology (Cui et al., 1998b). During the scale up of fermentations, it is important to apply the optimum K_La found in the small scales, to the larger scales. This can be achieved by changing the operational conditions, like air-flow rate and agitation speed (Stanbury and Whitaker, 1984). Rocha-Valadez et al. (2006) reported that shake flasks generate lower levels of hydromechanical stress than stirred tanks.

Once a fungus has been decided to be used in the fermentation process, research starts under laboratory-scale conditions using 1-10 l fermenters for the examination of media formulation and feeding strategies (batch, fed-batch, continuous, etc.) and the selection of fermentation system (stirred tank, airlift, packed bed, solid state, hollow fibre, etc.). Additionally, reactor configuration, pH control, dissolved oxygen, foam and temperature should be considered. After the optimization of product yield, process scale-up is usually performed; primarily to pilot scale of 10-100 l and finally to industrial scale of 1000- 100 000 l or more. The conditions in the large-scale fermenters are not the same with the smaller scale or laboratory systems. Therefore during scale up, product yields decreases. Yield is affected by the following factors during scale up process (Waites et al., 2001);

1. Choice of medium, cheaper nutrient sources are mostly used for large scale applications for a cost effective production.
2. Inoculum type, quality, quantity and the inoculum propagation procedures.
3. Degradation of heat-labile compounds in the industrial-scale sterilization protocols, which affects the final quality of the medium.
4. Profiles of nutrient, temperature, pH and oxygen gradients are needed in larger scale fermenters although they are not experienced in smaller, well mixed fermenters.
5. Foam generation, shear forces and carbon dioxide removal rate can be altered during scale up.

In the study performed by Pollard et al. (2006) the scale up, from pilot scale to(0.07, 0.8, and 19 m^3) production scale (57 m^3)forthe intracellular production of the pharmaceutically important secondary metabolite, pneumocandin from*Glarea lozoyensis* was described. They claimed that a scale up process requires the establishment of oxygen delivery and mixingefficiency in the bioreactor, coupled with process sensitivity and characterizationstudies that together define the process-limiting andcritical scale-up parameters.

Junker et al. (2004) summarized the key elements to be considered for a successful initial pilot plant scale-up as followings, based on experiences during model cultivations:

1. Minimization of culture pelleting and morphology, particularly in shake-flask and fermentor seed stages.
2. The use of media suitable for the large scale, containing fewer solids, defined components and commercially available bulk nutrients, possessing a reasonable viscosity.
3. Optimizing operating conditions for pilot scale from laboratory scale.
4. Establishment of reproducibility for similar fermentation vessels (within ±20% if possible) to fully evaluate potential benefits of changes in the medium or process.
5. Evaluation of on-line and off-line data to determine the key parameters for improved process performance.

Junker et al., (2009) have developed pilot-scale fermentation for an antifungal compound produced by a filamentous fungus.The process was scaled up to the15,000 l working volume based on constant aeration rate(vvm) and peak impeller tip speed. In that study they concluded that although process scale up resulted with high productivity, high broth viscosity(5,000–6,000 cP at 16.8 s^{-1}) was a problem during fermentation.

CHAPTER 4

PECTINASES

Pectinases include a number of related enzymes involved in the breaking down of pectic substances (Jayani et al., 2005). The complex heteropolysaccharide called pectin is present in the middle lamella and the primary cell wall of higher plants. The American Chemical Society classified pectic substances into four main classes; protopectins, pectinic acids, pectins and pectic acids. Among them only protopectins are water-insoluble, the others are totally or partially soluble in water (Alkorta et al., 1998).Chemically, pectic substances are a group ofcomplex colloidal polymeric materials, composed largely ofa backbone of anhydrogalacturonic acid units. Thecarboxyl groups of galacturonic acid are partially esterifiedby methyl groups and partially or completely neutralized bysodium, potassium or ammonium ions (Jayani et al., 2005). These carbohydrate polymers support the cohesion of other cell wall polysaccharides and proteins. Since pectic substances account for 0.5 – 4.0 % of the fresh weight of plant material, the heterogeneous group of pectinolytic enzymes is of significant importance in industrial processes, e.g. as processing aids for fruit juice extraction and clarification.

Depending on the substrate preference, reaction mechanism, and action pattern pectinases are classified in three main groups:

1. Protopectinases: catalyze the solubilization of protopectin to the highly polymerized soluble pectin.
2. Esterases: catalyze de-esterification of pectin by the removal of methoxy esters.
3. Depolymerases: depolymerizing enzymes are classified as hydrolases and lyases, which either catalyze the hydrolytic cleavage with the introduction of water across the oxygen bridge or break the glycosidic bond by a transelimination reaction.

Depending on the action pattern, i.e. random or terminal, these enzymes are termed as endo or exo enzymes. A brief classification of several pectinolytic enzymes is mentioned in Table 4.1. Pectin degradation requires the combined action of several enzymes(Kashyap et al., 2001; Jayani et al., 2005).

Table 4.1. Classification of pectinases
(Source: Jayani et al., 2005).

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

Since the 1940s, pectinases have been exploited for many industrial applications. The largest industrial application of these enzymes is in juice and wine production for the extraction, clarification, and filtration by enzymatic breaking down of the cell wall, and for the maceration of fruits and vegetables. According to Gummadi et al. (2007) there are two types of industrial pectinases; acidic and alkaline. Acidic pectinases are generally used in the fruit juice industriesand wine making, often come from fungal sources, especiallyfrom *Aspergillus niger*.Alkaline pectinases are mainly used in the degumming and retting of fiber crops and pretreatment ofpectic wastewater from fruit juice industries. These enzymescome mostly from bacterial sources.Furthermore pectinases are also used for oil extraction, removal of citric fruit peels, degumming of plant bast fibres, animal feed production, and waste water treatment, etc. (Kashyap et al., 2001; Gummadi and Panda, 2003). Almost all of the commercial pectinases preparations derive from fungal sources. Commonly, the species *Aspergillus niger* is used for the industrial pectinase production.Some of the commercial pectinase suppliers are; C.H. BoehringerSohn Germany, Ciba-Geigy, A.G. Switzerland,Grinsteelvaeket Denmark, KikkomanShoyu, Co. Japan, Schweizerische Ferment, A.G. Switzerland, Societe Rapidase, S.A. France, Wallerstein, Co. USA and Rohm, GmbH Germany (Kashyap et al., 2001).

In industry the most extensively used pectinases are the polygalacturonases (PG). PGs catalyze the hydrolytic cleavage of the glycosidic linkages of polygalacturonates (pectates) by endo and exo splitting mechanism. PGshows no activity on highly methylated pectin.Endo PGs break the homogalacturonanbackbone into oligogalacturonates whereas exo-PGs break downpolygalacturonates to di- and monogalacturonates. Therefore viscosity reductionmethod is more suitable for endoPG activity determination as the decrease in viscosity is relatively high. ExoPG activity can be determinedby measuring the reducing sugars formed due to hydrolysis.Endo PGs are widely distributedamong fungi, bacteria and yeast whereas exoPGs are widely distributed in*A. niger*, *Erwinia* sp. and in some plants such as carrots, peaches, citrus and apples. EndoPGs often have molecular weights in the range of 30–80 kDa and pI ranging between 3.8 and 7.6 on the other hand the molecular weight of exoPGs vary between 30–50 kDa and their pI ranges between 4.0 and 6.0. Most endoPGs have optimum pH in the acidic range of 2.5–6.0 and an optimum temperature of 30°C–50°C. Moreover, K_m values of endoPGs are in the range of 0.14–2.7 mg/ml for pectate (Jayani

et al., 2005; Gummadi et al., 2007). In table 4.2 biochemical properties of some of the PGs are given.

Table 4.2. Biochemical characterization of some polygalacturonases

Source of PGs	**Nature**	**Molecular weight (kDa)**	**Optimum temperature (°C)**	**Optimum pH**	**Reference**
Aspergillus niger	-	-	40	5	Fawole and Odunfa, 2003
Mucor flavus	Endo	40	45	3.5-5.5	Gadre et al., 2003
Fusarium moniliforme	PG I PG II	38 30.6	45 40	4.8 5.3	Niture and Pant, 2004
Trichoderma harzianum	-	31	40	5	Mohamed et al., 2006
Penicillium viridicatum RFC	Exo	24	60	6	Silva et al., 2007
Streptomyces lydicus	Endo	43	50	6	Jacob et al., 2008
Aspergillus sojae	Exo	36.53-68	55	5	Tarı et al., 2008
Aspergillus niger ATCC 9642	-	-	37	5.5	Gomes et al., 2011
Aspergillus niger (MTCC 3323)		34-69	45	4.8	Kant et al., 2013

CHAPTER 5

MATERIALS AND METHODS

5.1. Microorganism

Aspergillus sojae ATCC 20235 was purchased 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 according to a modified procedure of De Nicolás-Santiago et al. (2006). The propagation of the cultures was done on YME agar plate medium (Figure 5.1) containing malt extract (10 g/l), yeast extract (4 g/l), glucose (4 g/l) and agar (20 g/l) at 30°C until well sporulated, according to the procedure given by Gogus et al. (2006). Stock cultures of these strains were prepared with 20 % glycerol-water and stored at -80°C.

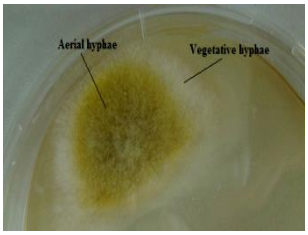


Figure 5.1. *Aspergillus sojae* mutant strain grown on YME slants.

5.2. Preparation of Inoculum

The inoculum for either shake flasks or bioreactor was obtained on molasses agar slants optimized by Gogus et al. (2006) after the pre-activation step performed on YME agar using the stock cultures. Molasses agar (Figure 5.2a) contains; glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), $FeSO_4 \cdot 7H_2O$ (15 g/l), KH_2PO_4 (60 g/l), $MgSO_4$ (50 g/l), $CuSO_4 \cdot 5H_2O$ (12 mg/l), $MnSO_4 \cdot H_2O$ (15 mg/l) and agar (20 g/l). After one week of incubation at 30°C, spores were harvested by the addition of 5 ml

Tween 80 water (0.02% (v/v)) per slant. The spore suspension (Figure 5.2b) was counted using Thoma bright line hemacytometer (Marienfield, Germany) and the suspensions were stored at 4°C until the inoculation. Inoculation rate was $2.8x10^3$ spore/ml for both bioreactor and shake flask experiments.

Figure 5.2.(a) Spores grown on molasses agar slants,(b)spore suspension used for inoculation.

5.3. Enzyme Activity, Total Protein and Total Carbohydrate Assays

Polygalacturonase (PG) activitywas assayed according to the modified procedure given by 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 0.4 and 0.086 ml, respectively. Galacturonic acid (Sigma, St. Louis, MO) was used as standard for the calibration curve (Table B.1). 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.

Thetotalprotein contents of the samples were determined according to the method described by Bradford (Bradford, 1976) with BSA as a standard used to measure the total protein in fermentation broth. Standard curve used for total protein calculations was given in Table B.2.

The phenol-sulfuric acid Method was used to determine the total carbohydrate content in fermentation broth (DuBois et al., 1956). Glucose was used as standard and the constructed standard curve was given in Table B.3.

In order to determine the protein content difference of the two substrate state, Kjeldahl method using full automated nitrogen-protein digestion and distillation system (Gerhardt, Germany) (conversion factor was 6.25).

5.4. Biomass Determination

The biomass expressed as dry cell weight (g/l) was determined by means of gravimetric method. The fermentation broth was filtered through the dried and pre-weight Sartorius whatman filter discs grade: 389, followed by drying to constant weight at 95°C, overnight.

5.5. Yield and Productivity Calculation

Biomass production yield coefficient, $Y_{X/S}$ (mg biomass/mg substrate) and enzyme production yield coefficient, $Y_{P/S}$ (U activity /mg substrate)based on substrate consumption were calculated by;

$$Y_{X/S} = -\frac{\Delta X}{\Delta S} \text{ and } Y_{P/S} = -\frac{\Delta P}{\Delta S} \quad (5.1)$$

Volumetric production rate for biomass (q_x) and enzymatic activities (q_{xE}) were calculated by (Najafpour, 2007; Trujillo et al., 2009);

$$q_x = \frac{\text{Produced biomass (X)}}{\text{time period}} = \frac{X_2 - X_1}{t_2 - t_1} = \frac{\text{mg dry biomass}}{\text{ml.h}} \quad (5.2)$$

and

$$q_{xE} = \frac{\text{Produced enzyme activity (E)}}{\text{time period}} = \frac{E_2 - E_1}{t_2 - t_1} = \frac{U}{\text{ml.h}} \quad (5.3)$$

5.6. Calculation of the Growth Kinetic Parameters Specific Growth Rate (μ) and Saturation or Monod Constant (K_S)

The effect of substrate concentration on specific growth rate (μ) in a batch culture is described with the relation known as the Monod rate equation given below:

$$\mu = \mu_m \frac{S}{K_S + S} \tag{5.4}$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate in h^{-1}, K_S is saturation or Monod constant and S is substrate in $g.l^{-1}$. The linearized form of the Monod equation is:

$$\frac{1}{\mu} = \left(\frac{K_S}{\mu_m}\right)\frac{1}{S} + \frac{1}{\mu_m} \tag{5.5}$$

Specific growth rates (μ) of the culture at different substrate concentrations were determined from the slope of semi-natural logarithmic plot of biomass vs. time and finally from the intercept and the slope of the 1/μ vs. 1/S plot μ_m and K_s values were calculated (Najafpour 2007).

5.7. Media Optimization Studies for the Maximum Polygalacturonase Production

Media optimization study was performed in three steps; screening, pre-optimization and finally the optimization step by using statistical analysis tools.

5.7.1. Production Medium and Fermentation

For the development of low-cost industrial media formulation shake flask experiments were conducted in 250 ml Erlenmeyer flasks each containing 50 ml of sterilized media composed of orange peel or corn meal as complex carbon source and maltrin or glucose as additional carbon source at different concentrations determined by

the experimental design. Orange peel was purchased from a local market in Bremen, Germany. Ammonium sulphate as the nitrogen source and sodium di-hydrogen phosphate and di-sodium hydrogen phosphate as the phosphate sources were added to the medium at the constant amounts of 8 g/l, 3.3 g/l and 3.2 g/l, respectively. The agitation speed ranged between 150-350 rpm and incubation time ranged between 5-7 days (according to the experimental design). Temperature was maintained at 30°C.

5.7.2. Experimental Design and Statistical Analysis

Experiments were started with the screening of the process variables and then continued with the pre-optimization and optimization experiments. Contour plots and data analysis were done by Design Expert 7.0.0 Trial Version (Stat-Ease Inc., USA) in both screening and optimization stages. The following equation was used for coding of the actual values of the factors between the range of (-1) to (+1).

$$x = [\text{actual} - (\text{low level} + \text{high level})/2)] / [(\text{high level} - \text{low level})/2] \quad (5.6)$$

In the screening part of this study, general factorial design with 24 runs was performed in order to determine the effects of additional carbon sources, complex carbon sources and the incubation time on the polygalacturonase (PG) activity.

In the pre-optimization step, according to the results of screening experiments orange peel, maltrin and glucose concentration levels were determined in order to be used in the optimization study. Agitation rate, another important parameter of submerged fermentations, was also included in the pre-optimization process as a factor in order to screen its effect on the PG activity. Hence, 30 experiment combinations determined by Face Centred Central Composite Design were employed using the given factors.

In the optimization step Face Centred Central Composite Design was used with the enlarged factor levels and optimum maltrin and orange peel concentrations were determined for the maximum PG activity. Finally, a second-order polynomial regression equation was fitted to the response data:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j + \boldsymbol{\varepsilon} \quad (5.7)$$

where Y is the predicted response, k the number of factor variables, β_0 the model constant, β_i the linear coefficient, X_i the factor variable in its coded form, β_{ii} the quadratic coefficient, β_{ij} the interaction coefficient and ε is the error factor.

5.8. Determination of the Process Parameters in 1 l Scale Serial Bioreactor System

Performance of the bio-reaction system in submerged fermentation 1 l scale (750 ml working volume) on PG production was evaluated under batch mode, in terms of the effects of aeration, agitation speed, pH, and dissolved oxygen concentrations using cascading procedure. Media formulation optimized for the max. PG production in shake flasks by using statistical tools in the previous section (Section 5.7) was used and given as follows;

- 34 g/l orange peel
- 142 g/l maltrin
- 8 g/l ammonyum sulphate
- 3.3 g/l monosodium phosphate
- 3.2 g/l disodium phosphate

The other fermentation conditions were as 30^oC temperature, $2.8x10^6$ spore/l inoculation rate. As one parameter (aeration, agitation speed or pH) was optimized, it was used in the other experiment, then the other parameter was optimized and it was continued like this. The bioreactor was Sartorius BIOSTAT Qplus-6 MO, Germany serial bioreactor used in the Biotechnology and Bioengineering Center Laboratory (Figure 5.3).At the end of fermentation the collected samples were filtered for biomass determination and their enzyme activity, total protein and total carbohydrate content were determined on supernatant obtained by centrifugation of the broth at 4620 g for 15 min.

Figure 5.3. Sartorius BIOSTAT Qplus-6 MO serial bioreactor system.

5.9. Preliminary Study for the Effect of Substrate Concentration on PG Activity and Growth

The aim of this study was to investigate the effect of different substrate concentrations on PG activity and biomass formation. With this perspective six different orange peel concentrations were specified as followings; 60 g/l, 40 g/l, 20 g/l, 15 g/l, 10 g/l, 5 g/l. In order to have a cost effective media formulation additionally only 2.75 g/l ammonium sulphate was used as a nitrogen source. The other fermentation conditions were the optimized conditions in the previous section (Section 5.8) as uncontrolled pH, 600 rpm agitation speed, 30^{o}C temperature, $2.8x10^{6}$ spore/l inoculation rate and 1 vvm aeration rate. Sartorius BIOSTAT Qplus-6 MO serial bioreactor was used in the Biotechnology and Bioengineering Center Laboratory for this experiment.

5.10. Application of Different Techniques for the Determination of Fungal Biomass

Due to the heterogenous distribution of biomass and presence of orange peel particles in the fermentation, biomass sampling thereby determination of the biomass was difficult in the previous section (Section 5.9). The biomass profile of that preliminary study was not reasonable. Therefore a couple of experiments were

performed in order to determine a biomass determination procedure for fungal submerged fermentations with particulated substrates.

5.10.1. Determination of Optical Density (OD)

The fermentation was performed in 250 ml Erlenmeyer flasks containing 50 ml medium. Separate flasks were prepared for each sampling day and for 40g/l and 60 g/l orange peel concentrations. Besides the given concentrations of orange peel, 2.75 g/l ammonium sulfate was also added to the medium. They were inoculated at $2.8x10^6$ spore/l inoculation rate and incubated at 30^oC, 350 rpm for 7 days in a shaker. Every 24 hours 10 ml sample was taken from both of the orange peel concentrations and filtered through Sartorius filter discs grade: 389. Afterwards the filters were dried at 95^oC overnight for dry weight determination. From the supernatant total carbohydrate and PG activity assays were performed.

The remaining 40 ml sample was used for the spectrophotometric biomass determination. The samples were homogenized in Heidolph Slient Crusher M homogenizer at 13000 rpm for 4 minutes. The optical density of diluted, homogenized broth was measured (450 nm) spectrophotometrically against water.

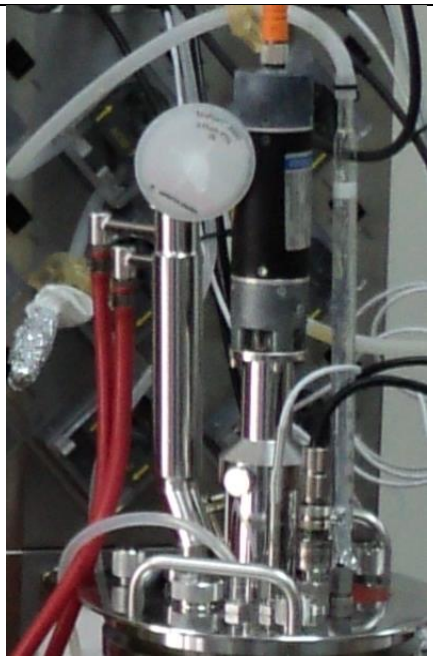
5.10.2. Subtraction of Sample Blank in OD and Biomass Calculations

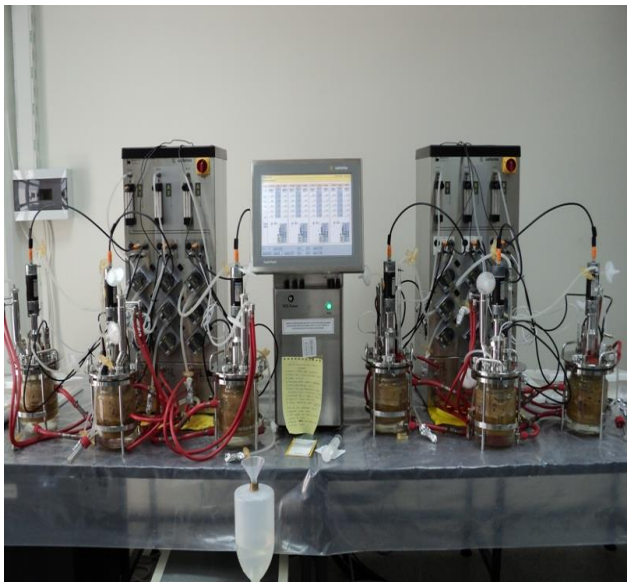
Blanks of the sampling time points were calculated from the total carbohydrate concentration of that time point and subtracted from the sample OD readings.

For the sample blank calculation un-inoculated media at different orange peel concentrations (5, 10, 15, 20, 40, 60 g/l) were prepared, sterilized and 10 ml of the media was filtered as described above. Then the filters were dried at 95^oC overnight for dry weight determination. From the supernatant total carbohydrate concentrations were determined. 40 ml of the media was used for the spectrophotometric biomass determination as described above. From the results, OD vs. total carbohydrate concentration and dry weight vs. total carbohydrate concentration standard curves were drawn. From the total carbohydrate concentration of every sampling time point, estimated OD and dry weight values for every time pointwas determined. These values were subtracted from the sample OD readings and dry weight calculations as blank.

5.10.3. Using a New Sampling Port Adapted to the Serial Bioreactor System

In order to determine the growth characteristics of *A. sojae* mutant strain a set of bioreactor experiments were conducted with six different orange peel concentrations specified as, 20 g/l, 25 g/l, 30 g/l, 40 g/l, 50 g/l, 60 g/l. The concentrations were chosen higher than the previous experiments in order to obtain pellet growth which provides more homogeneous broth rather than clumps obtained at low concentrations (5 g/l, 10 g/l, and 15 g/l). Also by the use of new sampling system, sampling was thought to be more homogeneous. New sampling system was generated by 25 ml glass pipettes whose narrower tips were cut off in order to obtain wider sampling port. They were assembled to the bioreactor vessels by liquid silicon tubing and the flow was provided by the peristaltic pump of the bioreactor. The system is shown in Figure 5.4. Besides the given concentrations of orange peel, 2.75 g/l ammonium sulfate was also added to the medium. The fermentation was carried out in a 1 l Sartorius BIOSTAT Qplus-6 MO serial bioreactor with a working volume of 750 ml at uncontrolled pH, 30°C, 600 rpm agitation speed, 1 vvm aeration rate and 2.8×10^6 spore/l inoculation rate. At the end of fermentation samples were centrifuged at 5000 rpm for 15 minutes and filtered for biomass determination and enzyme activity, total protein and total carbohydrate content were determined on the supernatant.





(a) **(b)**

Figure 5.4. Pictures of (a) one of the constructed sampling system and (b) Sartorius BIOSTAT Qplus-6 MO serial bioreactor system.

5.10.4. Glucosamine Content Determination for the Estimation of Biomass

Among the indirect methods for fungal biomass monitoring, glucosamine is considered as a good biomass indicator. As it is known glucosamine is a component found in fungal cell wall. Therefore a modified procedure of Sardjono et al.(1998)was used to estimate fungal growth indirectly from glucosamine content. They performed this assay in SSF but it was modified for SmF. About 0.25 ml of sample was hydrolized with 2.5 ml of 4 M HCl for 3 h at 100°C in sealed tubes. The hydrolysates were diluted to 25 ml with mobile phase after cooling. Samples were diluted 10 fold and pH was adjusted to 1-3. Then they were filtered from the 0.45 μm filter and transferred to the HPLC vials. Glucosamine concentrations were measured by HPLC, Aminex HPX-87 H (Biorad) column at 60°C, 0.6 ml/min flow rate with refractive index detector. As a standard D-Glucosamine hydrochloride 98% was used.

Shabrukova et al.(2002) defined that, the hydrolysis of chitin in the concentrated acids proceeds with the formation of acetic acid and D-glucosamine salt, and the

hydrolyses of its deacetylated derivative, chitosan, results in the formation of D-glucosamine salt:

conc. HCI

$[C_6H_9O_5(NH\text{-}COCH_3)]_n \rightarrow n\ CH_3COOH + n\ C_6H_9O_5(NH_2 \cdot HCl)$

chitin — aceticacid — D-glucosamine hydrochloride

conc. HCl

$[C_6H_9O_5(NH_2)]_n \rightarrow n\ C_6H_9O_5(NH_2 \cdot HCl)$

chitosan — D-glucosamine hydrochloride

In weak acids chitin is hydrolized with the formation of N-acetyl-D-glucosamine and salts of D-glucosamine (Shabrukova et al 2002):

HCl, H_2O

$[C_6H_9O_5(NH\text{-}COCH_3)]_n \rightarrow xC_6H_9O_5(NHCOCH_3)$ + N-acetyl-D-glucosamine

chitin+ $yC_6H_9O_5(NH_2{*}HCl) + yCH_3COOH$

Glucosamine content was determined through shake flask experiments performed with the same media formulation used in the bioreactor experiment; 2.75 g/l ammonium sulfate and six different orange peel concentrations 20 g/l, 25 g/l, 30 g/l, 40 g/l, 50 g/l, 60 g/l. Fermentation conditions were $2.8x10^6$ spore/l inoculation rate, 30°C and 350 rpm agitation rate. Six Erlenmeyer flasks of 500 ml in size, filled with 100 ml media were used. During 5 days of incubation, 2 samples of 1 ml volume were aseptically drawn everyday and used for glucosamine determination by HPLC.

5.11. Estimation of the Kinetic Parameters by Using Cube-Root Kinetics and Different Linear Equations in order to Linearize the Monod Equation

It is known that the rate of growth will increase with the amount of viable biomass present which was discussed initially in Section 2.2.1. in detail.

However, in some of the studies performed with fungal sources it was stated that, pelleted cultures are assumed to follow cube-root kinetics where this kinetic is described by the Equation 5.8 (Znidarsic et al., 1998; Papagianni, 2004; El-Enshasy, 2007; Feng et al., 2010);

$$X^{1/3} = X_0^{1/3} + kt \tag{5.8}$$

where X represents the biomass concentration (g/l) and k is the biomass growth rate constant.Therefore this kinetic was also applied to the biomass data by plotting $X^{1/3}$ versus t plot giving a slope of k. For all of the substrate concentrations k values were calculated in the phase of linear increase.

In order to express the fungal growth, the exponential growth rate was used and specific growth rate (μ) was described by Monod expression and its linearized form Lineweaver-Burke equation which were explained in detail in Section 5.6:

$$\frac{1}{\mu} = \left(\frac{K_S}{\mu_m}\right)\frac{1}{S} + \frac{1}{\mu_m} \tag{5.9}$$

Finally by using Lineweaver-Burke plot, from the intercept and the slope of the $1/\mu$ vs. $1/S$ plot, μ_m and K_s values were calculated (Najafpour, 2007).

Other linear equations used to linearize the Monod equation are given below.

- *Hanes-Woolf plot*: $S/\mu = (1/\mu_m)\, S + K_s/\mu$ (5.10)

  A plot of S/μ versus S is linear with intercept of K_s/μ_m and slope of $1/\mu_m$.

- *Wolf Augustinsson-Hofstee:* $\mu = (-K_s)\, \mu/S + \mu_m$ (5.11)

  A plot of μ versus μ/S is linear with intercept of μ_m andslope of $-K_s$.

- *Eadie-Scatchard plot*: $\mu/S = (-1/K_s)\, \mu + \mu_m/K_s$ (5.12)

  A plot of μ/S versus μ is linear with intercept of μ_m/K_s andslope of $(-1/K_s)$.

5.12. Effect of the Substrate Concentration and its State on the PolygalacturonaseActivity and Biomass

5.12.1. Shake Flask Experiments

This study was performed in order to determine the effect of substrate state on biomass, PG activity and morphology prior to the main study which was planned to be performed with extracted orange peel (EOP) in serial bioreactor to determine the kinetic parameters. This preliminary experiment in shake flasks will provide knowledge if the extraction process can simplify biomass determination or not without causing a decrease in PG production.

Again by using previously described media formulation and fermentation conditions, fermentations with the extracted orange peel and not extracted dry orange peel was performed at different orange peel concentrations (5, 10, 15, 20, 40, 60 g/l). Extraction was performed according to the modified procedure of Banerjee et al.(1993). For extraction, substrates were prepared at suitable amounts in 100 ml, for each concentrations of orange peel. Then they were autoclaved at 121^{o}C for 15 minutes, allowed to cool and filtered through cheese cloth. Only 50 ml of the extracted volume was used for fermentation in 250 ml Erlenmeyer flasks. Similarly, not extracted whole orange peel (WOP) was prepared for fermentation at the same orange peel concentrations for 50 ml volume and all of the flasks were autoclaved at 121^{o}C for 15 minutes. After coolingthey were inoculated at 2.8x10^{6} spore/l inoculation rate and incubated at 30^{o}C, 350 rpm for 7 days in a shaker. At the end of the fermentation biomass, PG activity and total carbohydrate analysis were performed according to the procedures given above.

5.12.2. Serial Bioreactor Experiments

In order to compare the growth kinetics of *A. sojae* mutant strain in the whole orange peel and extracted orange peel and to investigate the effect of different substrate concentrations at different states on PG activity and biomass formation a set of bioreactor experiments were conducted with six different orange peel concentrations

specified as, 60 g/l, 40 g/l, 20 g/l, 15 g/l, 10 g/l, 5 g/l. Besides the given concentrations of orange peel, 2.75 g/l ammonium sulfate was also added to the medium. The fermentation was carried out in a 1 l Sartorius BIOSTAT Qplus-6 MO serial bioreactor with a working volume of 750 ml at uncontrolled pH, 30°C, 600 rpm agitation speed, 1 vvm aeration rate and $2.8x10^6$ spore/l inoculation rate located at the Biotechnology and Bioengineering Center Laboratory of İzmir Institute of Technology. At the end of fermentation samples were filtered for biomass determination and enzyme activity, total protein and total carbohydrate content were determined on supernatant obtained by centrifugation of the broth at 5000 rpm for 15 min.

Extraction process of 750 ml media in 1 l serial bioreactors was performed according to the modified procedure of Banerjee et al.(1993). For extraction, substrates were prepared at suitable amounts in 1 l, for each concentrations of orange peel. Then they were autoclaved at 121°C for 15 minutes, allowed to cool and filtered through sterile cheese cloth. Only 750 ml of the extracted volume was used for fermentation in 1 l scale serial bioreactors. Also whole dry orange peel was prepared for fermentation at the same orange peel concentrations for 750 ml volume and bioreactors were autoclaved at 121°C for 15 minutes. When cooled, these were inoculated and incubated for six days.

5.13. Effect of Scale up to 20 l on PolygalacturonaseActivity

The aim was to investigate the effect of scale up (20 l) on the PG activity using the formerly mentioned media formulation (Section 5.9) with orange peel concentration (40 g/l) and 2.75 g/l ammonium sulphate. Two parallel experiments were performed in Jacobs University, Bremen with fully automated Techfors, INFORS bioreactor (Figure 5.5). The other fermentation conditions were as 600 rpm agitation speed, uncontrolled pH, 30^oC temperature, $2.8x10^6$ spore/l inoculation rate and 0.5 vvm aeration rate. Although the optimized aeration rate in 1 l scale was 1 vvm, the maximum attainable rate in this system was 0.5 vvm, thereforethis was used in these experiments. In addition, foaming problem during fermentation was experienced which increased the usage of high amount of antifoam.

Figure 5.5. 20 l scale Techfors, INFORS bioreactor

5.14. Fed Batch Fermentation

In order to evaluate theeffectof fed-batch fermentation system on the PG activity and biomass, five different bioreactor experiments were performed. The fermentation was carried out in a 1 l Sartorius BIOSTAT Qplus-6 MO serial bioreactor with a working volume of 750 ml at 30°C, 600 rpm agitation speed, 1 vvm aeration rate and $2.8x10^6$ spore/l inoculation rate. Fermentation was started with the medium formulation of 40 g/l whole orange peel and 2.75 g/l ammonium sulphate for all of the fermentations. As only the orange peel was used in the medium it was considered as the limiting substrate in the experiments and fermentation was started in batch mode. Fed batch operation started after 48 h and 72 h of the fermentation with a feeding rate of 33 ml/min. Feeding media, types and time were summarized in Table 5.1.In the course of fermentation, samples were drawn at certain intervals from the each fermentation system and the samples were analyzed for PG activity, biomass, total protein and total carbohydrate content.

Table 5.1. Summary of the feeding conditions.

Fermentation	Feeding type	Feeding media	Feeding time
1	Intermittently	40 g/l OP	after 48.h
2	Intermittently	40 g/l OP+ammonium sulphate	after 48.h.
3	single supplement	40 g/l OP+ammonium sulphate	after 48.h.
4	Intermittently	40 g/l OP+ammonium sulphate	after 72.h.
5	Intermittently	20 g/l OP+ammonium sulphate	after 48.h.

5.15. Biochemical Characterization of the Crude PG

The crude enzyme used in this study was obtained from the study given in section 5.13. The enzyme was chosen as the one giving the maximum activity at optimum conditions of that experiment (60 g/l WOP and 2.75 g/l ammonium sulphate).

5.15.1. 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 and12.0. In these analyses, pH was adjusted using the following buffer systems: acetate (pH3.0, 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 ofPG was determined by standard PG activity assay (Section 5.3) with polygalacturonicacid (2.4 g/l) as substrate dissolved in the buffer systems mentioned. To study thestability as a function of pH, crude enzyme was mixed (1:1) with the buffer solutionsmentioned above and aliquots of the mixture were taken to measure the residual PGactivity (%) under standard assay conditions after incubation at 30 °C for 2 hours.

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

The optimum temperature of the PG was determined by incubation of thereaction mixture (pH 4.8) for 20 minutes, at different temperatures ranging from 25 to80 °C (25, 30, 40, 50, 60, 70 and 80 °C) and measuring the activity with standard PGactivity assay. Before the addition of enzymes, the substrate (2.4 g/l polygalacturonicacid) was pre-incubated at the respective temperature for 10 minutes. Thethermostability of the crude PG was investigated by measuring the residual activity afterincubating 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 (pH4.8) sodium acetate buffer system.

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

The enzyme samples were incubated at temperatures of 30, 40, 50 and 60 °C for30 and 60 minutes for the thermal inactivation kinetic studies. Aliquots were withdrawnand cooled in an ice bath prior to the standard PG activity assay. The residual activitywas expressed as % of the initial activity. The inactivation rate constants (k_d) werecalculated from the slopes of the curves in the semi logarithmic plot of residual activityversus time and apparent half-lives were estimated using the Equation 5.13. The half-lifeis known as the time where the residual activity reaches 50%.

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{5.13}$$

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

$$k_d = k_0 \exp\left(-\frac{E}{RT}\right) \tag{5.14}$$

or

$$\ln(k_d) = \ln(k_0) - \left(\frac{E}{R}\right)\frac{1}{T} \qquad (5.15)$$

The values of E_d and k_0 were estimated respectively from the slope and interceptof the plot of $\ln(k_d)$ versus 1/T obtained from Equation 5.15 (R= 8.3145 J mol^{-1} K^{-1}).

5.15.4. Estimation of Thermodynamic Parameters during Inactivationof Polygalacturonase

The enthalpy of inactivation (ΔH*) for each temperature was calculatedaccording to Equation 5.16.

$$\Delta H^* = E_d - RT \qquad (5.16)$$

The values for the Gibb's free energy (ΔG*) of inactivation at differenttemperatures were calculated from the first-order constant of inactivation process byusing Equation 5.17.

$$\Delta G^* = -RT\ln(\frac{k_d h}{\kappa T}) \qquad (5.17)$$

where *h* (=6.6262x10^{-34} Js) is the Plank constant, and κ (= 1.3806x10^{-23} JK^{-1}) is theBoltzmann constant. The entropy of inactivation (ΔS*) of PG wascalculated using Equation 5.18 (Bhatti et al., 2006).

$$\Delta S^* = \frac{(\Delta H^* - \Delta G^*)}{T} \qquad (5.18)$$

5.15.5. Molecular Weight Investigation of PG and Comparison of the Molecular Weights

The molecular weight of the crude PG enzyme was analyzed by sodium dodecylsulphate gel electrophoresis (SDS-PAGE) method (Laemmli, 1970). 12% acrylamidecontaining resolving gel (pH 8.5) and 4% acrylamide containing stacking gel (pH 6.5)were employed.Protein concentrations of the samples were determined by nanodrop measurements and Bradford assay. According to these protein concentrations, 20 µl of the crude enzyme, diluted commercial enzyme and the molecular weight marker (Fermentas SM0431) were each treated with 20 µl sample buffer (62.5 Mm Tris-HCl, pH 6.8, 20% Glycerol, 2% SDS, 5% β-Mercaptoethanol, 0.5% (w/v) bromophenol blue) and denaturated at 95ºC for 10 minutes (marker was incubated for 5 minutes).ThePG enzyme was producedwithin the concept of the study described in the section 5.11., under optimized conditions using orangepeel as the substrate in the serial type of 1l-scale bioreactor from mutant *A. sojae*.

10 µl of the prepared samples were loaded into the gel. The electrophoresis was performed at 100 volts for 2 hours. After the electrophoresis, the gel was left in 20% TCAsolution for fixation for 30 minutes and then stained with 0.05% Commassie Brilliant Blue R-250 for 30 minutes. Then the gel was de-stained in de-staining solution 2 times for 15 minutes and overnight. The apparent molecular weight of the enzyme was estimated using the standard marker proteins.

CHAPTER 6

MEDIA OPTIMIZATION STUDIES FOR THE MAXIMUM POLYGALACTURONASE PRODUCTION

The optimization of fermentation media represents a significant cost and time in bioprocess development. The aim of optimization is to determine suitable reaction conditions like pH, temperature and medium composition for any biological system in order to maximize or minimize economically or technologically crucial process variables such as product concentration, yield or raw material cost. However in optimization studies, mostly there are large number of quantitative or qualitative process variables and the use of microorganisms or cells cause metabolic complexities in processes. Therefore to reduce the number of experiments, methods of statistical experimental design are used in many studies (Weuster-Botz, 2000). In this study screening, pre-optimization and optimization steps were performed by using statistical experimental design tools and the investigated factors and their levels were summarized in Table 6.1.

Table 6.1. The investigated factors and their levels in the experimental design of (a) screening, (b) pre-optimization, (c) optimization processes.

Factor	**Actual factor levels**		
	-1	**0**	**+1**
Complex carbon source	Orange peel	-	Corn meal
Additional carbon source	Maltrin	-	Glucose
Incubation time	5	6	7
Design type	General factorial design		

(a)

Factor	**Actual factor levels**		
	-1	**0**	**+1**
Orange peel	3	16.5	30
Maltrin	0	60	120
Glucose	0	15	30
Agitation	150	250	350
Design type	Face Centred Central Composite Design		

(b)

Factor	**Actual factor levels**		
	-1	**0**	**+1**
Orange peel	20	50	80
Maltrin	0	75	150
Design type	Face Centred Central Composite Design		

(c)

6.1. Screening of the Media Composition and Incubation Time

General factorial design is mostly used in screening experiments when there are different numbers of levels of each numeric or categorical factors are present. Therefore in this study general factorial design with 24 runs was employed in order to determine the effects of additional carbon sources, complex carbon sources and the incubation time on the polygalacturonase (PG) activity. Analyzed factors and their levels were decided by considering our previous experiments (unpublished results) performed with *A. sojae* strain for the investigation of submerged fermentation media composition. Consequently, (due to their high PG activity potential) maltrin (100 g/l) and glucose (20 g/l) as additional carbon sources, orange peel (10 g/l) and corn meal (10 g/l) as complex carbon sources and incubation time (5, 6 and 7 days) were screened by using statistical techniques to investigate their interactive effects.

The investigated factors and their levels are given in Table 6.1a. Figure6.1a, b and c showed that the maximum PG activity was achieved when orange peel was used as the complex carbon source and harvested at the end of 6 days of incubation. The PG activity results of the 24 experiments performed with general factorial design showed that the maximum PG activity was obtained as 90.66 U/ml (Figure 6.1a).

The ANOVA analysis showed that the generated model was significant for the determination of the effects of examined factors on the PG activity (p-value<0.1) (Table A.1). Among the investigated factors the most important factor affecting the PG activity was complex carbon sources (p-value<<0.001). However, additional carbon sources and incubation time had no significant effect on the PG activity at the studied levels (p-value>0.1). Besides the lack of fit value was insignificant (p-value>0.1) indicating that the investigated factors were efficient for the model construction.

Since, additional carbon sources (maltrin and glucose) at the studied levels did not result into major difference in PG activity and were close to each other, both of their concentrations were taken as additional factor variables besides orange peel in the pre-optimization step. The incubation time on the other hand was fixed at 6 days where the maximum PG activity was observed.

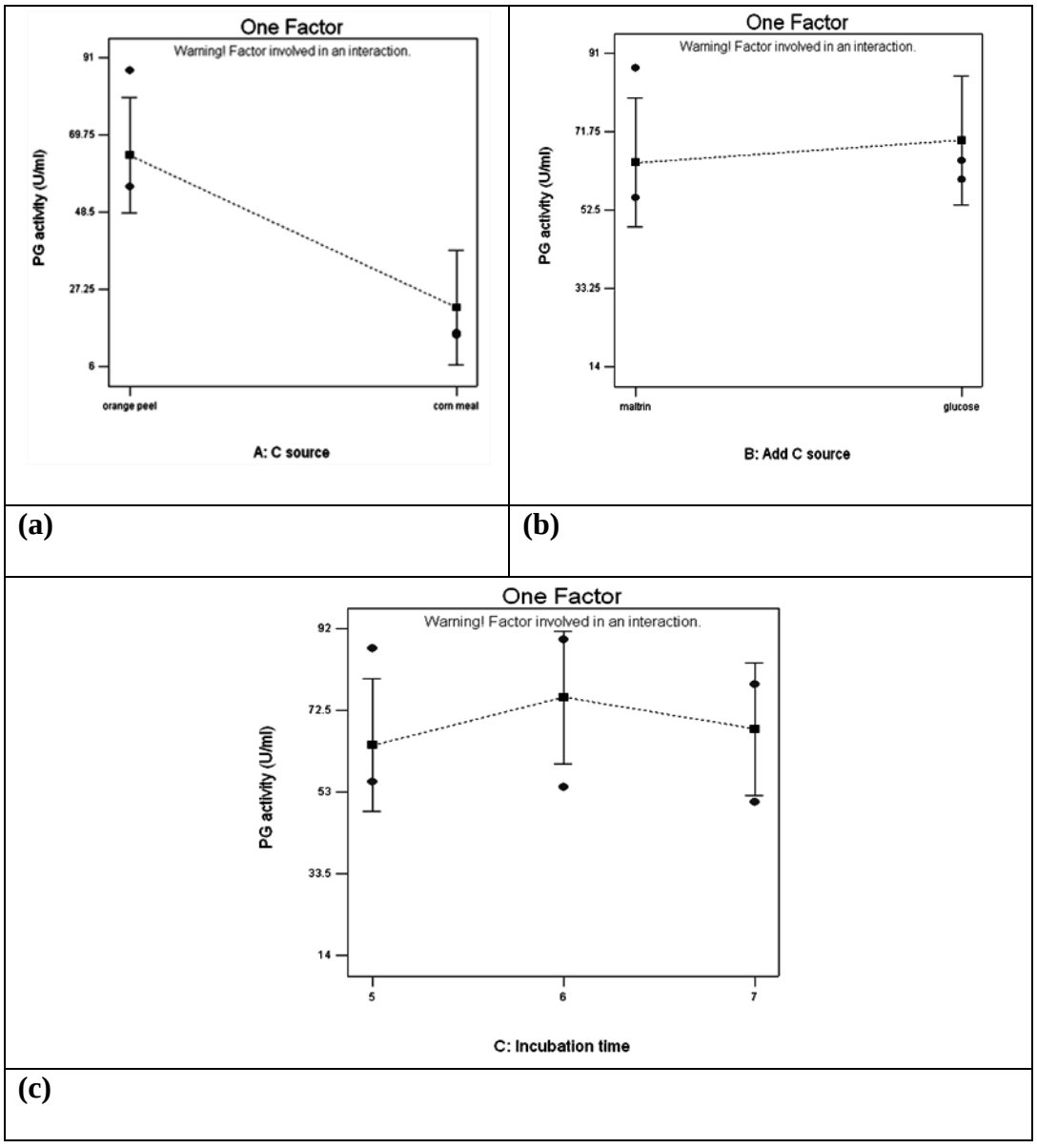


Figure 6.1. (a) Effect of complex carbon sources on the PG activity (hold at mid-levels; additional C source: maltrin. incubation time: 5 days). (b) Effect of additional carbon sources on the PG activity (hold at mid-levels; complex C source: orange peel. incubation time: 5 days). (c) Effect of incubation time on the PG activity (hold at mid-levels; complex C source: orange peel. additional C source: maltrin)

6.2. Pre-optimization Study for the Determination of the Levels of the MediaComponents and Agitation Rate

According to the results of screening experiments a pre-optimization study similar to screening study was performed considering the significant factors orange peel, maltrin and glucose concentrations in order to gain understanding about their factor levels and interactive effects on PG activity. Furthermore, agitation rate, another important parameter in submerged fermentations, was also included in the optimization process as a factor variable. Hence, 30 experiment combinations determined by Face Centered Central Composite Design (FCCD) was performed with factors of orange peel concentration (A), maltrin concentration (B), glucose concentration (C) and agitation rate (D) at the levels of (3-30 g/l), (0-120 g/l), (0-30 g/l) and (150-350 rpm), respectively (Table 6.1b).

The ANOVA (Table A.2) indicated that the agitation rate (D) had no significant effect (p-value=0.91) on the PG activity at the chosen levels. Also the interactions of the agitation rate (D) with orange peel (A) and maltrin (B) were insignificant terms ($p>0.1$). On the other hand orange peel (A) was found to be the most important factor on the PG activity with a p-value of $<<0.001$. However, glucose (C) and maltrin (B) had insignificant effect on PG activity ($p>0.1$) but as the p-value of maltrin (B) was close to the significance level of $p>0.1$ it was included in the optimization study.

The contour plot given in Figure 6.2 showed that the high concentrations of both maltrin (60 - 120 g/l) and orange peel (25 - 30 g/l) gave the maximum PG activity. However it was clear from Figure 6.2 that for both maltrin and orange peel concentrations beyond the selected levels, PG activity still had an increasing trend. Therefore in order to determine the exact optimum levels of the factors and to construct a model with these factors, an optimization process with enlarged factor levels was conducted.

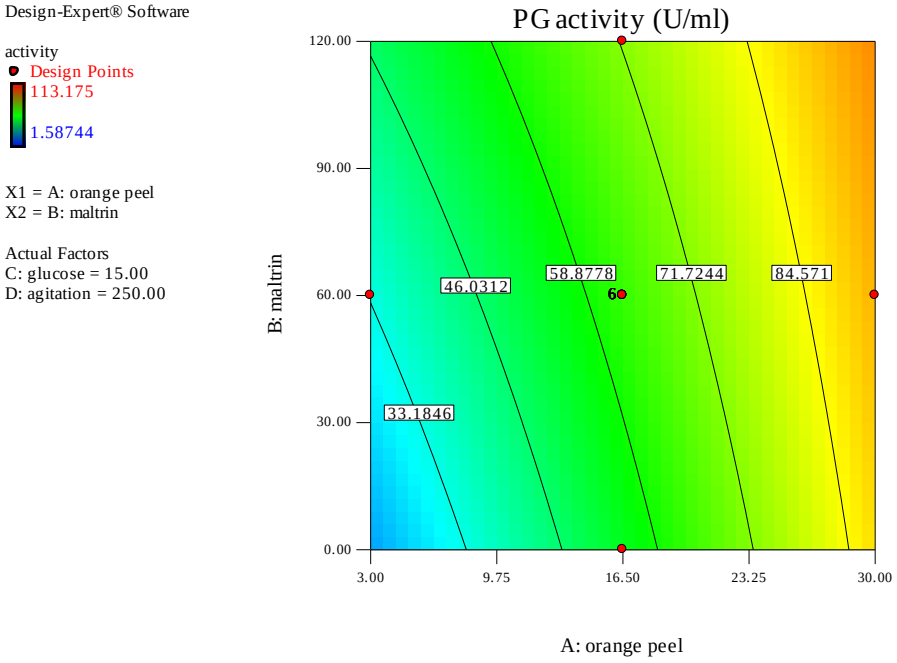


Figure 6.2. Interaction of orange peel concentration and maltrin concentration factors in the pre-optimization study (glucose: 15 g/l. agitation speed: 250 rpm).

6.3. Optimization Study of Media Components

In order to determine the optimum maltrin and orange peel concentrations for the maximum PG activity, Face Centred Central Composite Design (FCCD) was used with the enlarged factor levels (Table 6.1c). Therefore the factors orange peel (A) and maltrin (B) were investigated between 20-80 g/l and 0-150 g/l, respectively. The other media components were the same as the previous screening and pre-optimization experiments.

Variance analysis of the PG activity values (Appendix A3) showed that the quadratic form of maltrin (B^2) had an insignificant effect on the PG activity with a p-value of 0.88 ($p>0.1$) thus it was not included in the model. Moreover, although orange peel seemed to be an insignificant term at the studied level, it was included in the model due to its significant interaction with maltrin and its significant effect seen in the pre-optimization process. According to ANOVA (Table A.3), the constructed model was significant ($p<<0.001$) to describe the PG activity with a R^2 value of 0.90 and expressed in the following equation.

$$PG\ activity = 88.17 - 4.55*A + 10.12*B - 19.48*AB - 30.99*A^2 \quad (6.1)$$

The contour plot given in Figure 6.3 indicated that maximum PG activity was achieved at the high level of maltrin (B) and mid-level of orange peel (A) concentrations. As a result optimum orange peel and maltrin concentrations for the production of maximum PG activity (97.01 U/ml) from *A. sojae* strain were determined as 42.35 g/l and 137.09 g/l, respectively. This result was obtained at 30^{o}C temperature, 250 rpm agitation rate and 6 days of incubation conditions.

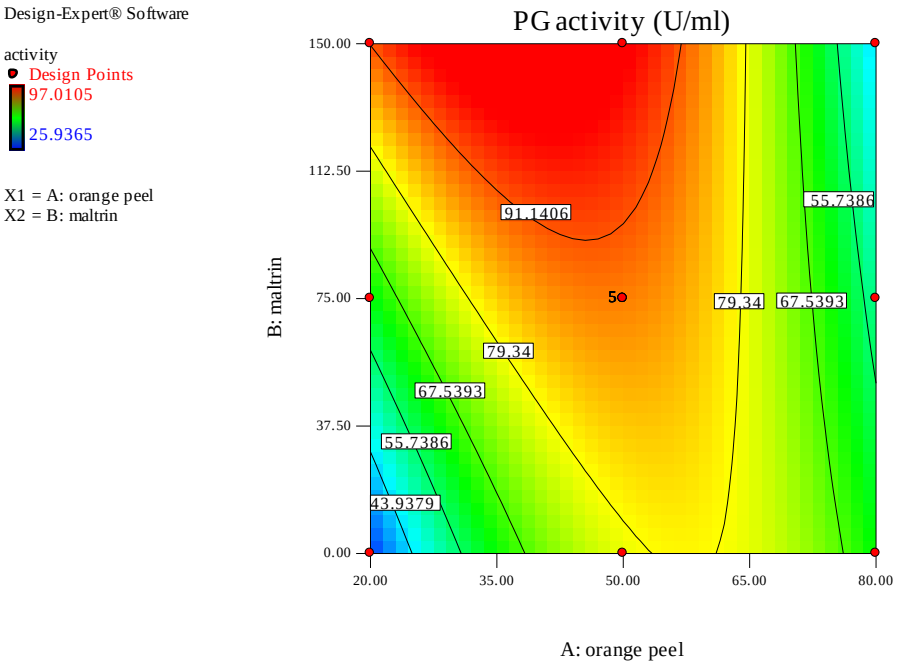


Figure 6.3. Interaction of orange peel concentration and maltrin concentration factors in the optimization study.

6.4. Validation of the Constructed Model for PG Activity

A model was constructed for maximum PG activity in the optimization process. This model was validated by performing three experiments at the optimum conditions which were given by Design Expert 7.0.0 software. These experimental conditions and the actual and predicted PG activity values were summarized in Table 6.2.

According to Table 6.2 there was a 5.31 % difference between the predicted PG activity values given by the software (99.51 ± 1.63 U/ml) and the actual PG activity values obtained by the validation experiments (104.79 ± 7.58 U/ml). Validation experiments also showed that the empirical model constructed in the optimization process was a reliable and accurate model for the prediction of the maximum PG activity produced by *A. sojae* between the studied concentration ranges.

Indeed, this validated optimum media formulation was decided to be used in bioreactor studies for further determination of optimum pH as it is easier to control the pH in bioreactor systems as opposed to shake flasks. For this purpose, the concentrations (orange peel: 33.98 g/l and maltrin: 142.06 g/l) in the second experimental combination (Table 6.2) at which the highest experimental value of PG activity was reached were used in the bioreactor experiments. Nevertheless, the other media components were as followings; ammonium sulphate (8 g/l), sodium dihydrogen phosphate monohydrate (3.3 g/l) and disodium hydrogen phosphate dehydrate (3.2 g/l) like in the shake flask experiments.

In our previous studies performed by Oncu et al. (2007) we found maximum PG activity as 6.73 U/ml, which was way below the current study. Gomes et al. (2011) achieved maximum PG activity of 51.82 U/ml with *A. niger* using pectin as carbon source which was again lower than our findings. To best of our knowledge there are no studies in literature that report exactly the effect of low cost media formulation such as orange peel and corn meal on the production ofPG by *A. sojae* in submerged fermentations. In fact this will be one of the initial studies contributing to the literature with potential high PG activity of possible industrial significance.

Table 6.2. Validation experiment combinations of optimization study.

Run	Orange peel (g/l)	Maltrin (g/l)	Predicted PG activity (U/ml)	Actual PG activity (U/ml)
1	38.01	140.84	100.75	107.13
2	33.98	142.06	100.11	110.93
3	44.19	127.78	97.66	96.31

It is reported that, pectinase production by filamentous fungi varies according to the strain, the composition of the growth medium and the cultivation conditions (pH, temperature, aeration, agitation and incubation time) (Souza et al., 2003). The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of microbial population. The most important ones among them are medium pH, agitation speed and aeration rate. It is known that at low pH values the cell permeability increases which in turn facilitate the transport of nutrients into the cell. Also the surface properties of spores were denoted to be affected by the medium pH level where the coagulation of the spores is thought to be increased due to the electrostatic interaction between spores, themselves (Pazaouki and Panda, 2000). The dissolved oxygen tension may affect the productivity, cell autolysis, fungal morphology, etc. It supports the cell growth and product formation directly or by the morphological change. Agitation is important for efficient mixing, mass and heat transfer; it creates shear forces, causing changes in morphology, growth and product formation, it may also cause damage to the cell structure(Cui et al., 1998; Kim et al., 2003; Malvessi and Silveira, 2004). In the light of these facts, in this study the optimum medium conditions with respect to PG activity were tried to be determined and their interrelationships discussed. Initially, the carbon source optimization in shake flasks was conducted as it could be easily performed in shake flasks. However, it was not feasible to conduct the optimization of otherfermentation conditions (pH,aeration, agitation) in shake flasks. Thus, it was decided to perform the fermentation condition optimization experiments in 1 l small scale (750 ml working volume) serial bioreactors in which controlling the parameters was easy and automatic.

CHAPTER 7

DETERMINATION OF THE PROCESS PARAMETERS IN 1 L SCALE SERIAL BIOREACTOR SYSTEM

7.1. The Effect of pH on PG Activity and Biomass

In order to determine the effect of pH, which is an important fermentation parameter, four different fermentations were performed at uncontrolled pH, pH 6, pH 5 and pH 4. pH was fixed at pH 6, 5 and 4 by automatically adding 4 N NaOH and 4 N H_2SO_4. Fermentation conditions were adjusted as 30^oC temperature, 600 rpm agitation speed, 1 vvm aeration rate and 750 ml working volume.

Changes in the amount of PG activity, specific enzyme activity, biomass and total carbohydrates as a function of time were shown in Figure7.2. Moreover, PG enzyme yield and productivities were calculated for each pH experiment and results were given in Table 7.1. Enzyme assay results showed that PG production remained at low levels at pH 4 (Figure7.2a). Only 18.8 U/ml PG activity was obtained at this pH. On the other hand specific PG activity curve (Figure 7.2b) pointed out a relatively higher value (232.49 U/ mg protein) which indicated that nutrients were used for enzyme production rather than biomass formation. Figure 7.2c denoted low amount of biomass (10.71 mg/ml) formation at this pH. It was also observed that carbohydrate was not completely consumed (Figure 7.2d). Therefore it can be concluded that insufficient transfer of nutrients to the cell interiors may have resulted in lower biomass and enzyme production. On the other hand the second highest enzyme yield and productivity was observed at pH 4 (Table 7.1).

It was observed that PG activity remained at such a low level like 1.6 U/ml when pH was set to 5 (Figure 7.2a). Furthermore specific PG enzyme activity was found to be 10.55 U/mg protein (Figure 7.2b). Figure 7.2c and Figure 7.2d pointed out that efficient carbohydrate utilization contributed to higher amount of biomass production (18.31 mg/ml). It can be deduced that the strain used carbohydrate for biomass production instead of enzyme production due to the fact that high acidity in the fermentation medium favors the production of PG enzyme rather than biomass. The

lowest enzyme yield and productivity value was obtained due to the low amount of enzyme production when pH was set to 5 (Table 7.1).

Activity results pointed out that enzyme production was the lowest (only 1.2 U/ml PG activity) at pH 6 (Figure 7.2a). Moreover the specific PG activity and biomass formation were also found to be the lowest (6.83 U/mg protein and 9.8 mg/ml, respectively) compared to the others (Figure 7.2b and. Figure 7.2c). However, carbohydrates were effectively consumed under this condition (Figure 7.2d). Due to the lowest enzyme production, enzyme yield and productivity values were also the lowest at this pH (Table 7.1).

Considering the enzyme activity results maximum PG activity (93.48 U/ml) was obtained at uncontrolled pH condition and this value was 75.21, 56.90 and 4.97 times higher than those obtained under pH 6, 5 and 4 conditions, respectively (Figure 7.2a). Figure 7.2b showed that specific PG activity exhibited the same profile with PG activity indicating PG enzyme produced under uncontrolled pH condition did not contain impurities. Furthermore the highest biomass production (21.18 mg/ ml) was observed when pH was not controlled during the fermentation (Figure 7.2c). During the course of fermentation studies it was monitored that initial pH value of the fermentation medium was approximately 4.8-5.0 (Figure 7.1). Fang and Zhong (2002) suggested that initial pH level of fermentation medium had a significant effect on cell membrane function, cell morphology and structure, solubility of salts, ionic state of substrate, and transfer of various nutrient compounds into the cell and product synthesis. As stated earlier, because of the initial pH was around 5, nutrient compounds were considered to dissolve better in case of uncontrolled pH and pH 5. However, it was observed that carbohydrates in the medium were not used during the first 24 hours of fermentation under uncontrolled pH condition while it was rapidly and efficiently used at pH 5 (Figure 7.2d). After the first 24 hours-period of fermentation, pH of the medium started to decrease along with initiation of microbial proliferation, where cell metabolism accelerated and carbohydrate consumption started. It was reported that low pH values affected cell permeabilization, and transfer of nutrients inside the cell was much more effective at this condition (Oncu et al., 2007). As a general observation, the amount of total carbohydrates showed an increase after initiation of microbial growth due to the decomposition of maltrin by microbial cells. Because maltrin which is a maltodextrin needs to be broken down smaller components in order to be used by microorganisms. Afterwards, the amount of total carbohydrates in the medium was found to be lower as a

result of the consumption of carbohydrates by the cells. It can be concluded that carbohydrates were not completely metabolized at uncontrolled pH condition considering total sugar analysis results (Figure 7.2d). Obtaining the highest PG activity at uncontrolled pH value indicated that nutrients were mostly used for enzyme production under this condition. Furthermore the highest enzyme yield and productivity were reached under uncontrolled pH (Table 7.1). The best yield and productivities for both endo and exo-PG activities produced by *Aspergillus oryzae*were reported to be achieved at the freely decreased pH condition in the experiments conducted by Malvessi and Silveira (2004). This finding is similar to the results of this current study. However, Malvessi and Silveira (2004) reported lower exo-PG activity (54 U/ml) than the one found in this research.

It was noted that pH value of the medium decreased to 2 at the 118^{th} hour of the fermentation when the highest enzyme activity was obtained under uncontrolled pH condition (Figure 7.1). Right along with obtaining the highest enzyme activity, working under high acidic condition reasonably reduces the risk of microbial contamination which can occur in the fermentation medium. In fact this is an important advantage of this medium and these processing conditions, which can be considered in industrial fermentations that are prone to contamination problems. Also uncontrolled pH condition will significantly reduce the acid and base consumption, which is not required in this case. Therefore uncontrolled pH condition was selected for further fermentation studies.As a conclusion, giving the highest enzyme yield and productivity, at the same time the highest PG activity, uncontrolled pH was the optimum pH for the maximum PG production by *A. sojae* strain.

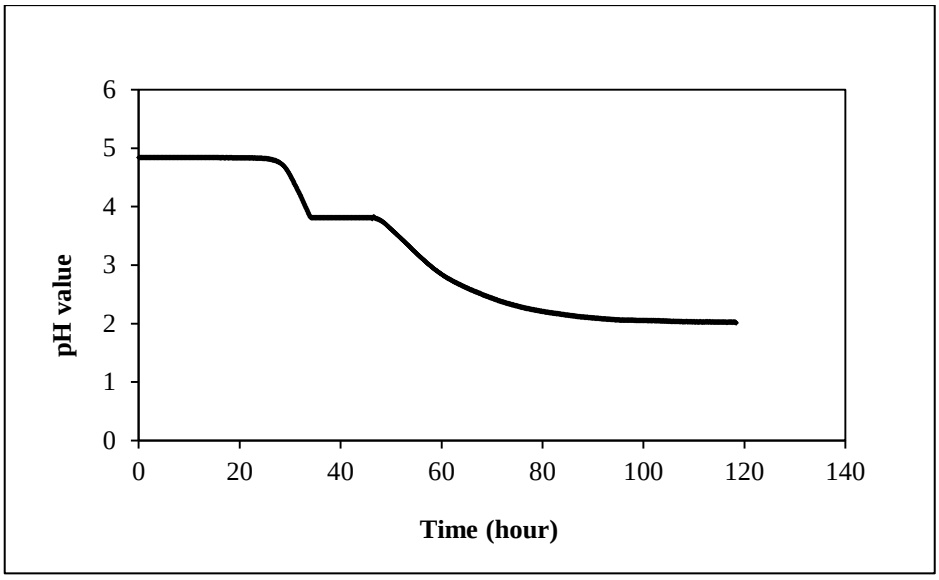


Figure 7.1. pH profile under uncontrolled condition

The profiles of dissolved oxygen (DO) for each pH experiment were given in Figure 7.3. Although total carbohydrate consumption was similar for all of the pH conditions (Figure 7.2d), at uncontrolled pH and pH 4, DO level decreased to 0%. However at pH 5 and pH 6 DO didn't drop below 10% and 40% levels, respectively (Figure 7.3). Additionally maximum PG production was achieved under the uncontrolled pH condition (Figure 7.2a) at which DO level decreased to 0% levels which may be due to the quick consumption of dissolved oxygen for the PG production.

Finally the optimum pH condition was determined as uncontrolled pH in this study, thus from now on in the subsequent experiments this parameter was taken constant as uncontrolled.

Table 7.1. Yield and productivity values for pH experiments.

	Uncontrolled pH	**pH 6**	**pH 5**	**pH 4**
Yield ($Y_{P/S}$) (U /mg substrate)	17.66	0.003	0.007	0.28
Productivity (U /ml.h)	0.78	0.004	0.008	0.19

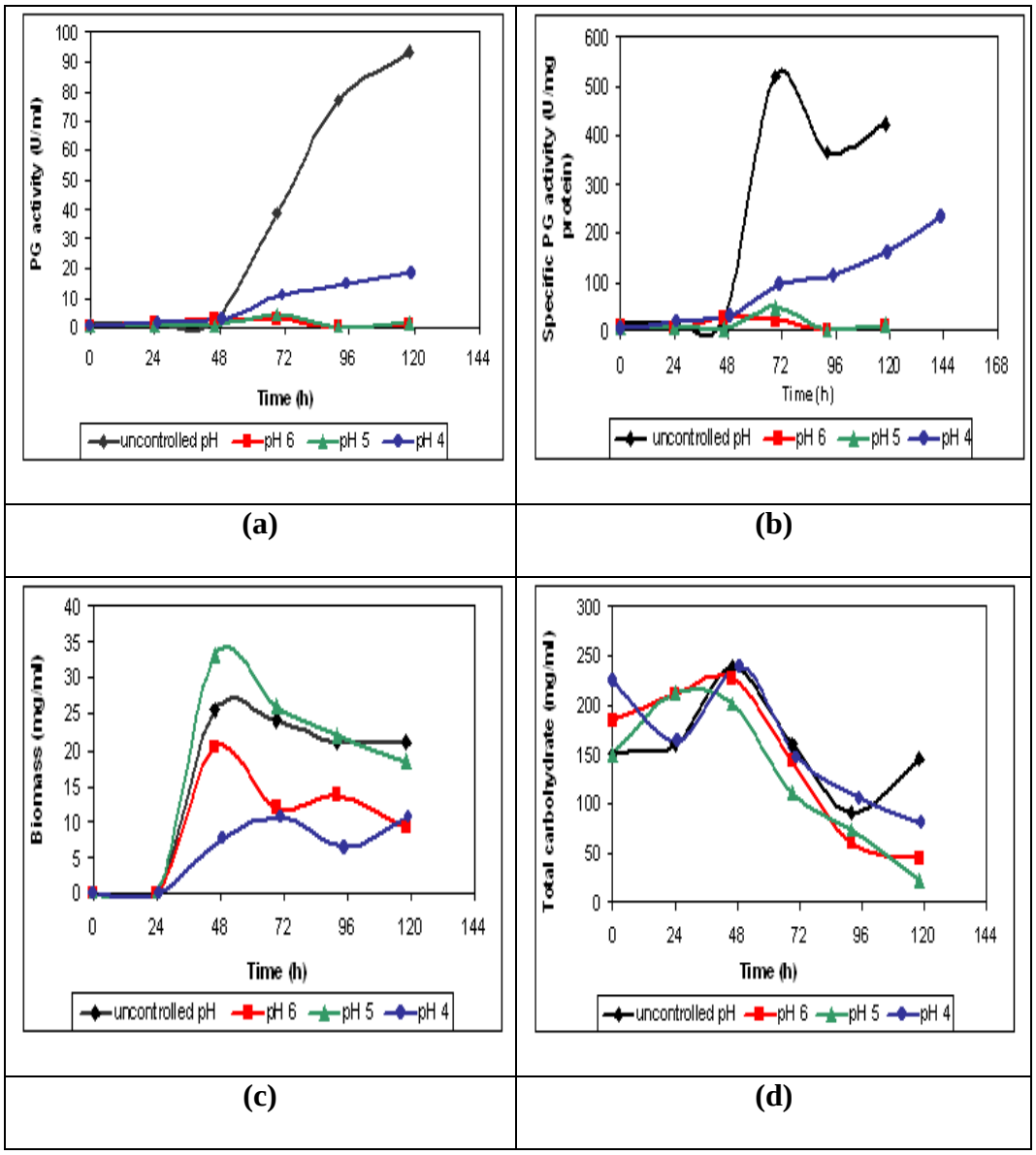


Figure 7.2. Profiles of pH experiment performed at 600 rpm, 30^{o}C temperature and 1 vvm aeration conditions (a) PG activity, (b) specific PG activity, (c) biomass and (d) total carbohydrate.

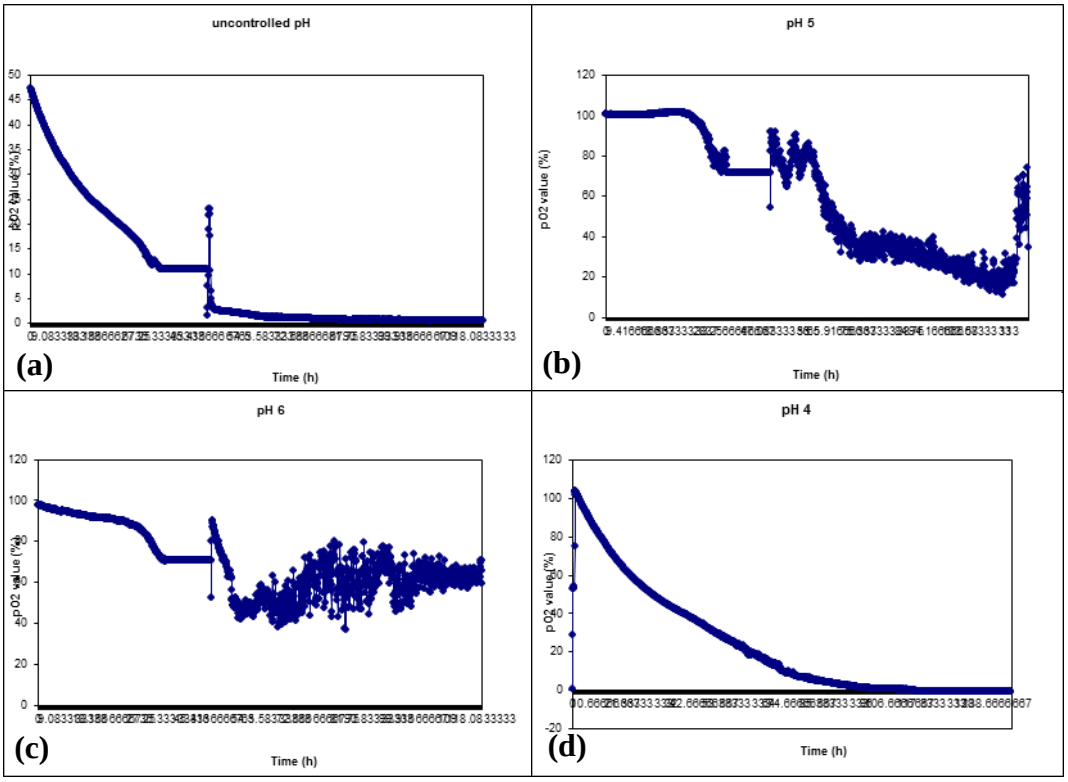


Figure 7.3. Dissolved oxygen profiles of pH experiment performed at 600 rpm, 30°C temperature and 1 vvm aeration rate conditions (a) uncontrolled pH, (b) pH 5, (c) pH 6 and (d) pH 4.

7.2. The Effect of Agitation on PG Activity and Biomass

There are studies in literature indicating that agitation speed creates shear stress which changes the morphology and growth of microorganisms and even causes damage to cell structure (Cui et al., 1998; Papagianni 2001). Therefore fermentations were carried out at three different agitation speeds (800, 600 and 300 rpm), constant aeration rate (1 vvm), uncontrolled pH and 30°C temperature conditions. Fermentation volume was 750 ml in the 1 l scale serial Sartorius bioreactor. As the optimum pH condition was determined in the previous section it was taken constant as uncontrolled pH. The studied agitation speeds were determined according to the literature. There are many researchers that have used 600 rpm in fungal fermentations as Casas Lopez et al. (2005) used 300, 600 and 800 rpm in lovastatin production by *Aspergillus terreus* in a 5 L working volume bioreactor. Rodriguez Porcel et al. (2005) used agitation speed of 300, 600 and 800 rpm in the fermentation of *Aspergillus terreus.* Abd-Aziz et al. (2008) used 120 and 600 rpm for the chitinase production using *Trichoderma virens* UKM1 in 2 L

stirred tank reactor. For the recombinant protein production by *Aspergillus oryzae* Amanullah et al. (2001) used 3 different agitation speeds (525, 675 and 825 rpm). Bioreactor working volume of this study was 750 ml which is relatively small scale and the agitation speeds of 600 rpm and 800 rpm are available for small scales. For the scale up experiments according to the tip speed and impeller speed, agitation rate decreases.

Considering all the agitation speeds studied, growth was faster at 800 rpm (Figure 7.4c). Maximum biomass obtained at 72.h (36.15 mg/ml) was 1.65 and 3.27 times higher than 600 and 300 rpm, respectively. Slow growth at 300 rpm can be explained by inadequate DO level which dropped to 0% level after 48.h and then increased to 10% after 72.h (Figure 7.5b). At the other agitation speeds DO level did not drop under 40% (Figure 7.5a and c). It can be concluded that mutant *A. sojae* strain can grow faster at higher agitation speeds.

According to Figure 7.4a PG activity reached its maximum level (120 U/ml) at the end of 168.h at 600 rpm agitation speed. Maximum PG activity obtained at 600 rpm was 1.45 and 1.31 times higher than the PG activities obtained at 800 and 300 rpm, respectively. Maximum specific PG activity was observed at 300 rpm, this was followed by 600 and 800 rpm, respectively(Figure 7.4b). Therefore, PG enzyme obtained at 300 rpm contained lower amount of impurities than the other agitation speeds. There is an opposite relationship between biomass and specific PG activity indicating that enzyme production wasn't associated with growth. In addition, the reason of higher total carbohydrate consumption at 300 rpm (Figure 7.4d) could be explained by the possible use of substrate for PG production.

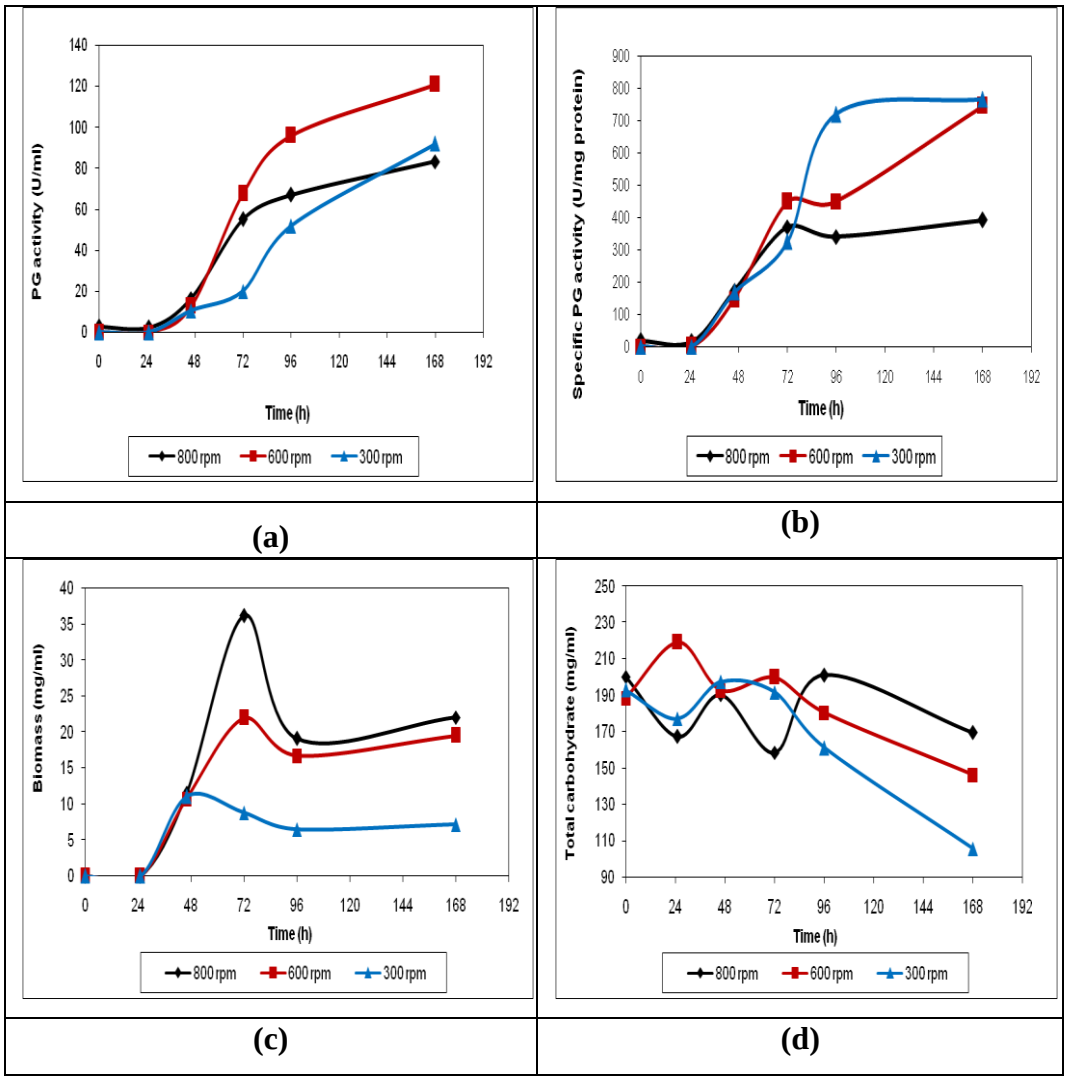


Figure 7.4. Profiles of agitation experiment performed at uncontrolled pH, 30^{o}C temperature and 1 vvm aeration conditions (a) PG activity, (b) specific PG activity, (c) biomass and (d) total carbohydrate.

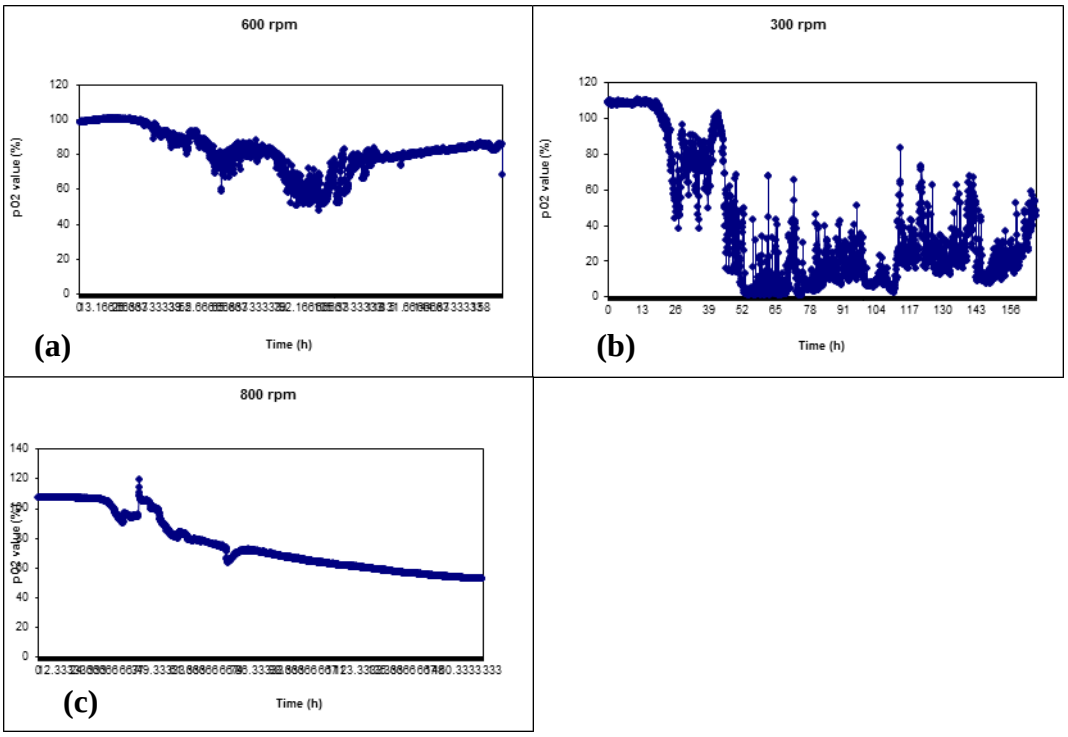


Figure 7.5. Dissolved oxygen profiles of agitation experiment performed at uncontrolled pH, 30°C temperature and 1 vvm aeration rate conditions (a) 600 rpm, (b) 300 rpm, and (c) 800 rpm.

PG production yields and productivities for each agitation speed was calculated and given in Table 7.2.

Table 7.2. Yield and productivity values for agitation speed experiments.

	800 rpm	**600 rpm**	**300rpm**
Yield ($Y_{P/S}$) (U/mg substrate)	2.60	2.87	1.05
Productivity (U/ml.h)	0.48	0.72	0.55

As a result, 600 rpm agitation speed giving the maximum PG activity and the highest yield and productivity was fixed for the next experiments.

7.3. The Effect of Aeration on PG Activity and Biomass

As it is known fungi require molecular oxygen to grow which can be providedthrough aeration at bioreactor conditions. Aeration causes an increase in growth and product formation by improving mass transfer properties of microbial cells (Cui et al., 1998; Sinha et al., 2001;Kim et al., 2003; Papagianni, 2004). Thus in this study, 3 different fermentations in which aeration was fixed at 1.5, 1 and 0.5 vvm were performed in order to investigate the effect of aeration on PG production and growth of mutant *A. sojae* strain. Optimum agitation speed (600 rpm) and optimum pH (uncontrolled pH) found in previous experiments were fixed in this experiment.

As shown in Figure 7.6a and 7.6c although maximum biomass was achieved at highest aeration condition (1.5 vvm) maximum PG activity was achieved at 1 vvm at the end of fermentation (168 h). Biomass reached its maximum value (44.41 mg/ml) at the end of 143 h and at 1.5 vvm aeration rate (Figure 7.6c). When dissolved oxygen profiles (Figure 7.7b) were examined, rapid decrease of DO at 1.5 vvm aeration rate werecorrelated to high biomass production.

Maximum PG production (120.78 U/ml) was obtained at 1vvm at the end of 168 h (Figure 7.6a). However at 1.5 vvm PG activity increased faster and reached 120.40 U/ml at 119 h and then it decreased. Specific PG activity reached its highest value also at 1 vvm aeration rate that can be clearly seen in Figure 7.6b. This revealed the presence of small amount of impurities in PG produced at 1 vvm aeration rate.

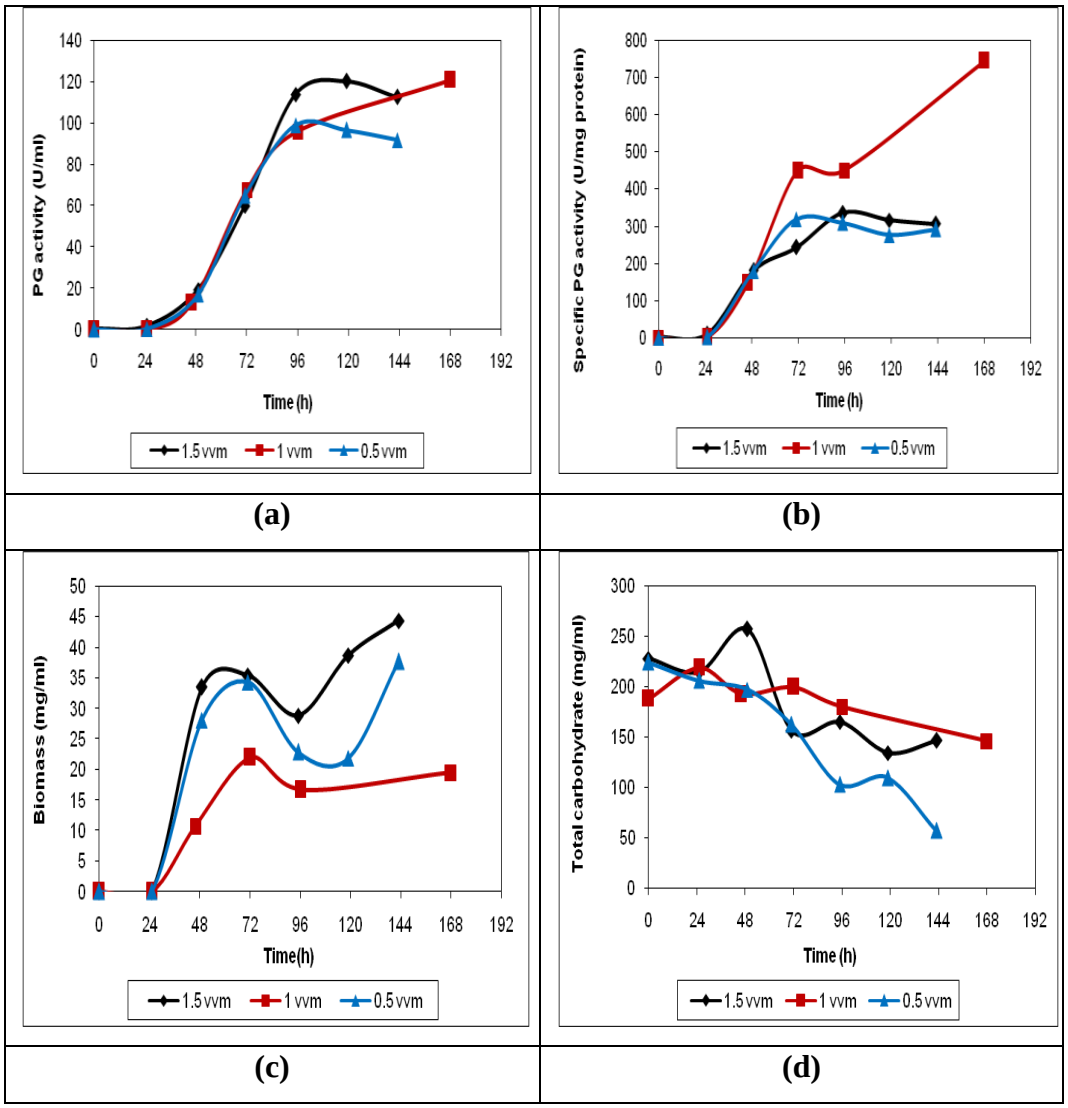


Figure 7.6. Profiles of aeration experiment performed at 600 rpm, 30^{o}C temperature and uncontrolled pH conditions (a) PG activity, (b) specific PG activity, (c) biomass and (d) total carbohydrate.

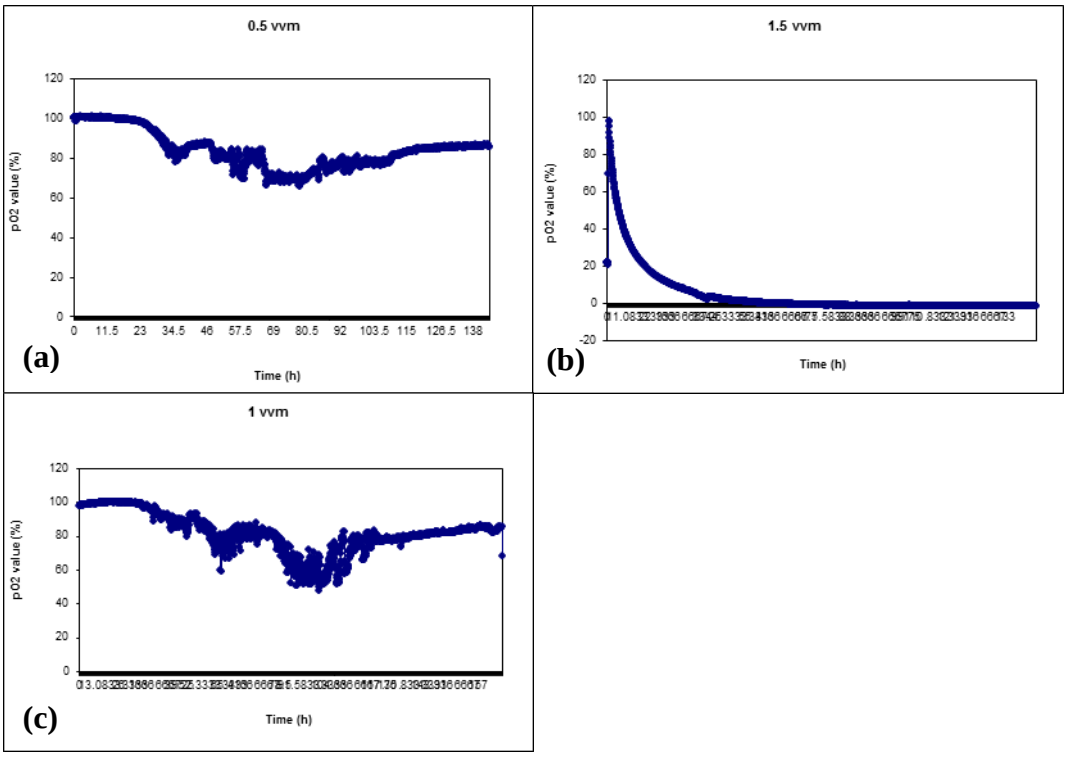


Figure 7.7. Dissolved oxygen profiles of aeration experiment performed at 600 rpm, 30^{o}C temperature and uncontrolled pH conditions (a) 0.5 vvm, (b) 1.5 vvm and (d) 1 vvm.

When yield and productivities given in Table 7.3 were examined, maximum values were obtained at 1 vvm aeration rate. Therefore it was decided to set the aeration rate at 1vvm in the next cascading experiment and other experiments.

Table 7.3 Yield and productivity values for aeration experiments.

	1.5 vvm	**1 vvm**	**0.5 vvm**
Yield ($Y_{P/S}$) (U activity /mg substrate)	1.37	2.87	0.55
Productivity (U /ml.h)	0.64	0.72	0.008

7. 4. The Effect of Dissolved Oxygen Concentration (DO) on PG Activity and Biomass

Besides the oxygen demand of fungi for growth, production of some industrial metabolites by fungi is affected by the amount of dissolved oxygen in the medium (Papagianni 2004). Therefore, to maximize product formation it is important to keep DO level of the medium above the critical level. With this point of view, it was aimed to maintain constant dissolved oxygen above 40% saturation level to prevent oxygen depletion and thus to increase the product formation. Maintanance of constant DO level above 40% was achieved by three different cascading experiments. In the former experiment agitation speed was cascaded between 300-800 rpm and aeration rate was fixed at 1vvm which was the previously optimized value in order to maintain constant DO level at 40%. In the second one aeration rate was cascaded between 2-0.5 vvm and agitation speed was fixed at 600 rpm which was the previously optimized value in order to maintain constant DO level at 40%. In the last biorector, agitation speed and aeration rate were cascaded together between the value ranges specified above in order to maintain constant DO level at 40%.

According to Figure 7.8a the highest value of PG activity (114.49 U / ml), was obtained by agitation + aeration cascading, this value was 1.05 and 1.22 times higher for aeration and agitation cascading, respectively. Also the specific PG activity obtained by aeration cascading was higher than the others which indicated that controlling the DO level with aeration cascadingcould result in PG activity with low impurities (Figure 7.8b).In aeration cascading experiment maximum biomass (41.18 mg/ml) obtained at the end of 71.h. was 1.14 and 1.49 times higher than agitation cascading and agitation+aeration cascading experiments, respectively (Figure 7.8c). The PG activity increased faster with aeration cascading but after 72 h it almost remained constant however agitation cascade andagitation+aeration cascading experiments showed similar PG and specific PG activity profiles and their activity increased slowly but at the end (168 h) reached similar activities.

As it is seen in Figure 7.9b., in aeration cascading experiment, the drop of DO level below 40% later than the other cascading experiments caused aeration to start adjusting DO level later.In the case of agitation cascading, due to better oxygen transfer (by the effect of agitation), DO level in the medium started to drop faster because of

faster consumption. Therefore due to the ensurance of better oxygen transfer with agitation, agitation+aeration cascading experiment resulted in higher PG activity.

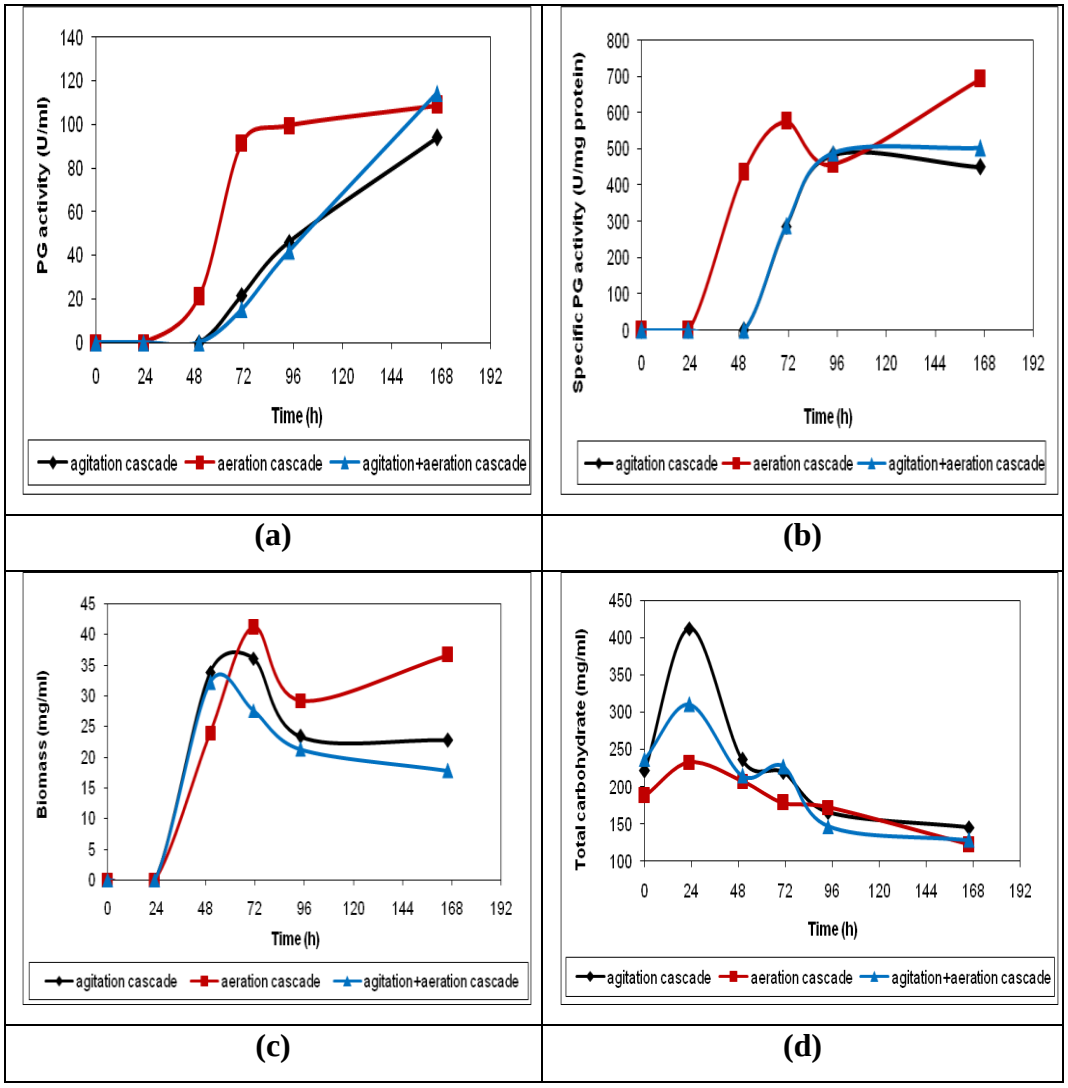


Figure 7.8. Profiles of cascading experiment performed at 600 rpm, 30^{o}C temperature, 1 vvm and uncontrolled pH conditions (a) PG activity, (b) specific PG activity, (c) biomass and (d) total carbohydrate.

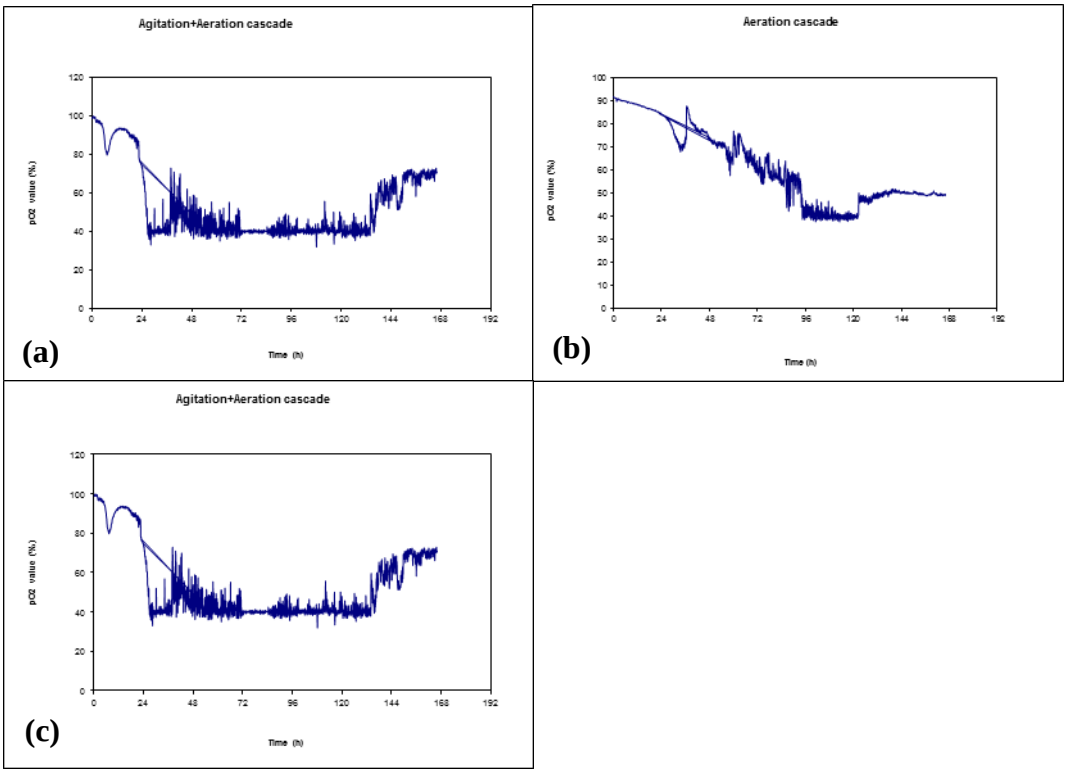


Figure 7.9. Dissolved oxygen profiles of cascading experiment performed at 600 rpm, 30^{o}C temperature, 1vvm and uncontrolled pH conditions (a) agitation cascading, (b) aeration cascading and (d) agitation+aeration cascading.

With respect to the yield and productivity values given in Table 7.4,relatively high yield coefficient was calculated for aeration cascadinghowever productivity values were similar for all the cascading experiments. This result was in accordance with the PG activity profile given in Figure 7.8a.

Table 7.4. Yield and productivity values for cascading experiments.

	Agitation cascade	**Aeration cascade**	**Agitation+aeration cascade**
Yield ($Y_{P/S}$) (U /mg substrate)	1.24	1.65	1.05
Productivity (U /ml.h)	0.57	0.66	0.69

CHAPTER 8

PRELIMINARY STUDY ON THE EFFECT OF SUBSTRATE CONCENTRATION

Highest PG activity (88.55U/ml) was obtained at 95.h. with 40 g/l orange peel concentration (Figure 8.1a). It was clear from Figure 8.1a that PG activity increased with an increase in substrate concentration except for 60 g/l orange peel concentration. This casecould be explained by catabolite repression. Maldonado and Saad (1998) indicated that high sugar concentration stimulated pectinase production in solid state fermentation, whereas in submerged fermentation this production was inhibited, probably by catabolite repression.

As the orange peel concentration increased, DO level decreasebecame faster after 24.h. as it can be observed from Figure 8.1c. At the 60, 40, 20 and 15 g/l orange peel concentrations the DO level dropped below 40% saturation level at the end of 48.h. (Figure 8.1c). At the beginning of fermentation pH started around 4.50 and decreased to 2.93 at the end of 95.h. (Figure 8.1d)

Actually the main purpose of this experiment was to determine the growth pattern of mutant *A. sojae* strain and its growth kinetics,unfortunately it was not possible to estimate growth kinetics from the biomass profilespresentedin Figure 8.1b. Therefore it was decided to determine an alternative way for biomass determination inorder to eliminate the unfavorable effects of whole OP particles and heterogenous fermentation broth on biomass determination.

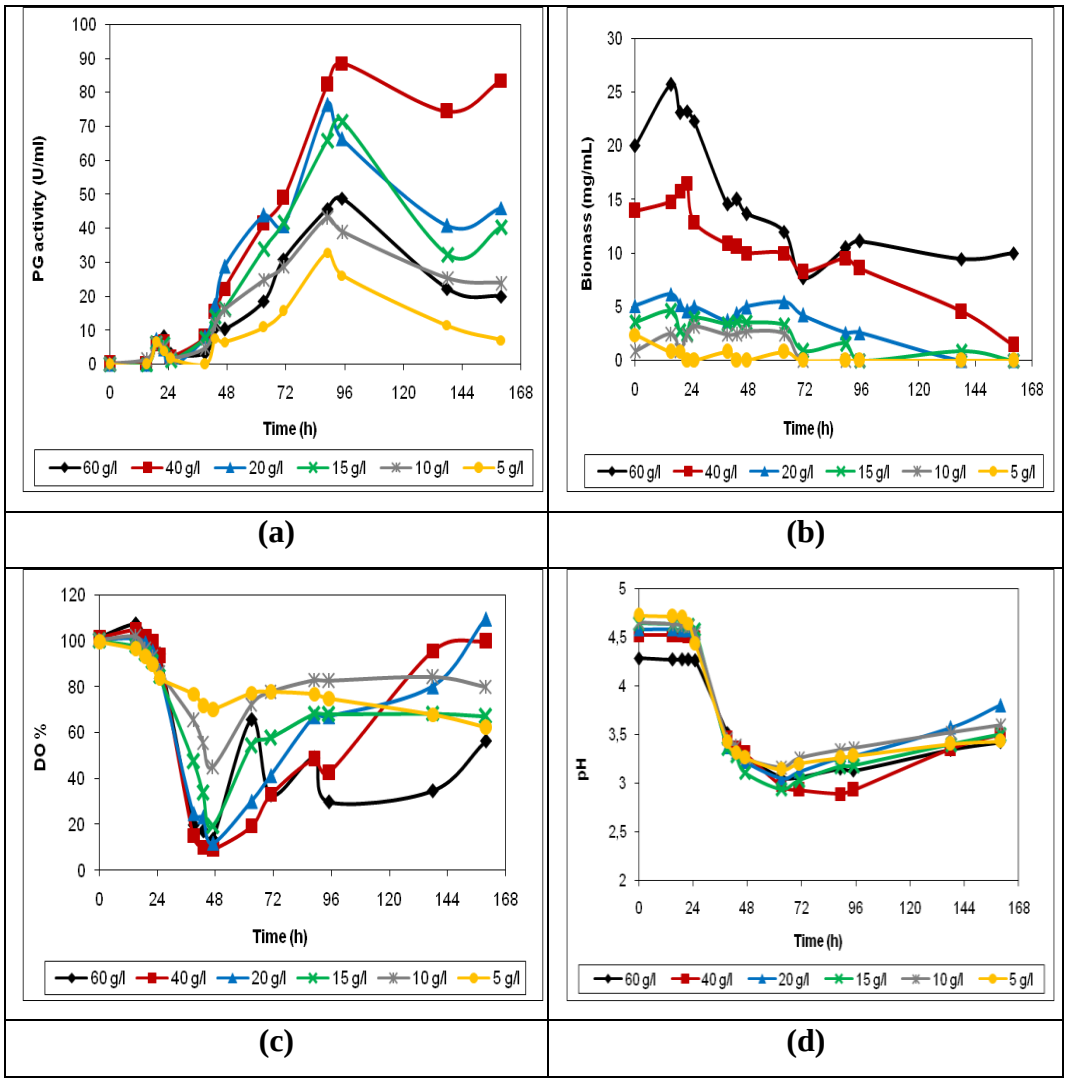


Figure 8.1. Profiles of substrate concentration experiment performed at 600 rpm, 30°C temperature, 1 vvm and uncontrolled pH conditions (a) PG activity, (b) biomass, (c) DO (%) and (d) pH.

CHAPTER 9

APPLICATION OF DIFFERENT TECHNIQUES FOR THE DETERMINATION OF FUNGAL BIOMASS

In the previous study (Chapter 8) it was stated that it was not possible to calculate the growth kinetic parameters due to the problems in biomass determinations encountered in many fungal fermentations. These problems make sampling difficult and may be summarized as, heterogeneous distribution of biomass, settling of mycelial clumps, fouling of fermenter probes and impellers and growth along feed lines (Prosser and Tough 1991). Hence, it was essential primarily to apply different biomass determination techniques and determine the most feasible one.

9.1. Spectrophotometric Determination of Biomass and the Effect of Sample Blank in OD and Biomass Calculations in Shake Flasks

In order to determine the biomass with spectrophotometric technique and dry weight technique, submerged fermentation was performed by using the media formulation and fermentation conditions described in materials and method section (Section 5.10.1) at 40 and 60 g/l orange peel concentrations. Total carbohydrate and PG activity profiles aregiven in Figure 9.1at which sampling was done every 24 hours. PG profile was similar for both of the concentrations with the maximum PG activity of 109 U/ml. Total carbohydrate concentrations at the sampling times were used to estimate the sample blanks for OD and dry weight calculations.

For the sample blank calculations non-inoculated media was prepared at different orange peel concentrationsand autoclaved as described in the materials method section. OD, dry weight and total carbohydrate values were determined accordingly. The carbohydrateconcentrations at the sampling times of fermentation (Figure 9.1) were inserted into the related standard curves (Figure 9.2a and b) for the blank calculations of OD and dry weight measurements.

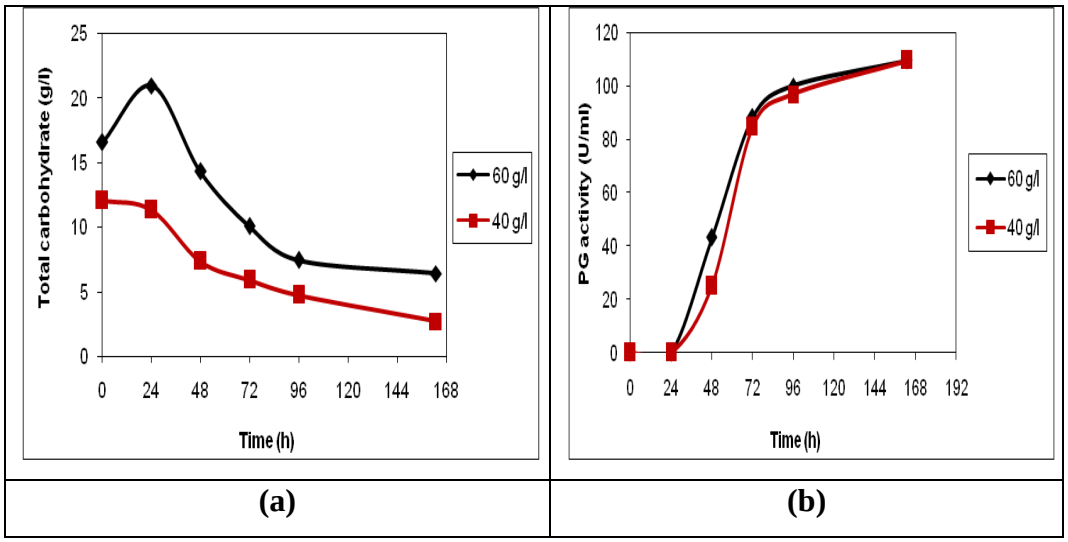


Figure 9.1. Profiles of shake flask fermentation (a) Total carbohydrate, (b) PG activity.

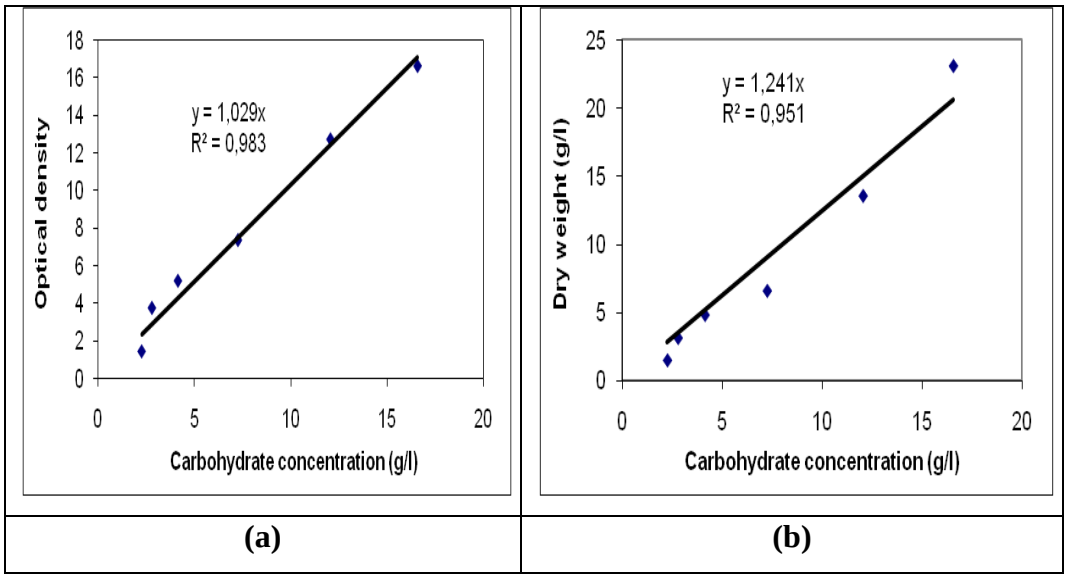


Figure 9.2. Standard curves for the sample blank calculations (a) OD change with total carbohydrate concentration, (b) dry weight change with total carbohydrate concentration.

Figure 9.3a and b showed the OD and dry weight profiles of the homogenized fermentation broth at 40 and 60 g/l orange peel concentrations calculated by the subtraction of the sample blanks for every sampling time which were calculated as described before. The optical density (450 nm) of homogenized, diluted broth was plotted against mycelial dry weight in Figure 9.4a. Samples of broths for both of the concentrations homogenized for 4 minutes and the correlations between optical density

and dry weight were given in Figure 9.4a. Although there was no exact correlation between optical density and dry weight, OD measurements were converted to dry cell mass by using correlation curve given in Figure 9.4a and the resulted profile was shown in Figure 9.4b.

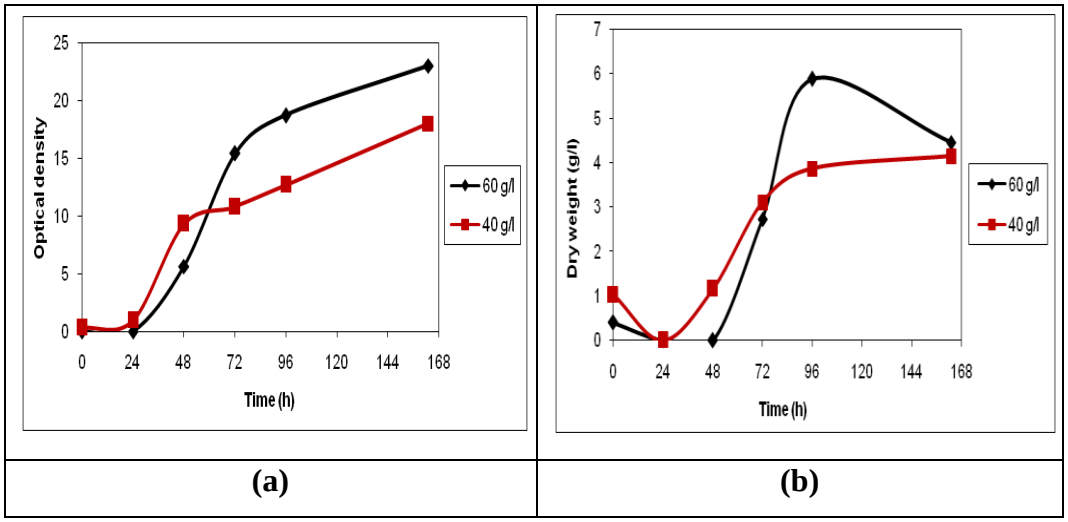


Figure 9.3. Optical density (450 nm) and dry weight profiles calculated by the use of sample blank (a) OD profile, (b) dry weight profile.

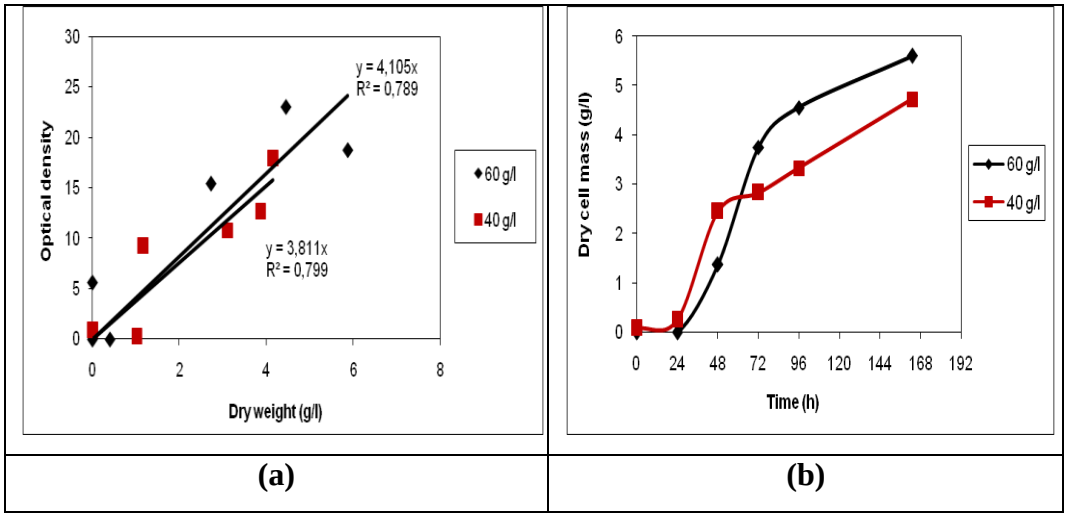


Figure 9.4. Conversion of optical density to dry cell mass (a) optical density vs. dry weight plot for the correlation, (b) dry cell mass profile calculated in homogenized broth by spectrophotometric measurement.

In order to investigate the difference between specific growth rate values (μ) calculated by the spectrophotometric measurements and dry weight measurements, the semi-natural logarithmic plots (Figure 9.5a and b) were drawn from the dry weight (Figure 9.3b) and dry cell mass (Figure 9.4b) profiles. From the slopes of the linear parts of the semi-natural logarithmic plots, specific growth rates (μ) were calculated for both spectrophotometric and dry weight measurements.

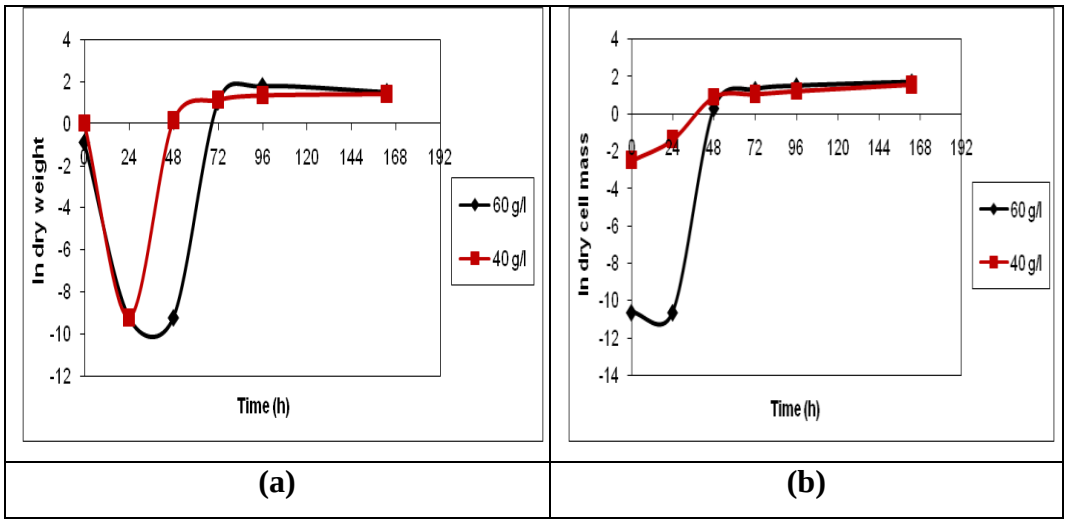


Figure 9.5. Semi-natural logarithmic plots of (a) dry weight, (b) dry cell mass

The specific growth rates for 60 and 40 g/l orange peel concentrations calculated by spectrophotometric and dry weight technique were given in Table 9.1. It is clear from the table that there was a problem at 40 g/l concentration where the specific growth rates of the two techniques were very different from each other.

Table 9.1. Specific growth rates calculated for 40 and 60 g/l orange peel concentrations by using spectrophotometric and dry weight method.

	μ (h^{-1})	
S (g/l)	**Spectrophotometric measurement (dry cell mass)**	**Dry weight measurement**
60	0.25	0.23
40	0.05	0.22

As the sampling in shake flasks was difficult for the described time intervals the experiments of the other substrate concentrations (20, 15, 10, 5 g/l) were not performed and with two concentration point μ_m value could not be calculated. However the aim of this preliminary study was to see if the spectrophotometric technique could be used instead of dry weight measurement. As we can see from Table 9.1 and the correlation curve in Figure 9.4a, spectrophotometric technique in homogenized broth was not very useful for *A.sojae*mutant strain fermentation with orange peel as substrate.

9.2. Effect of the Substrate Concentration on the PG Activity and Biomass at High Substrate Concentrations Using an Adapted New Sampling Port

In order to eliminate the difficulty in sampling, a new sampling system using 25 ml glass pipettes whose narrower tips were cut off in order to obtain wider sampling port was constructed for every vessel and assembled to the bioreactor vessels by liquid silicon tubing (Figure 5.4). Flow was provided by the peristaltic pump of the bioreactor. At first 3 fermentations using 3 of the bioreactor vessels were conducted with 5, 10 and 15 g/l whole orange peel (WOP) concentrations but it was seen that formation of clump morphology rather than pellet morphology hindered the homogeneous sampling. Biomass profiles were given in Figure 9.6. Therefore, it was decided to perform 6 fermentations with higher WOP concentrations (20, 25, 30, 40, 50, 60 g/l) those forming pellet morphology, which provided homogeneous broth thereby easy sampling.

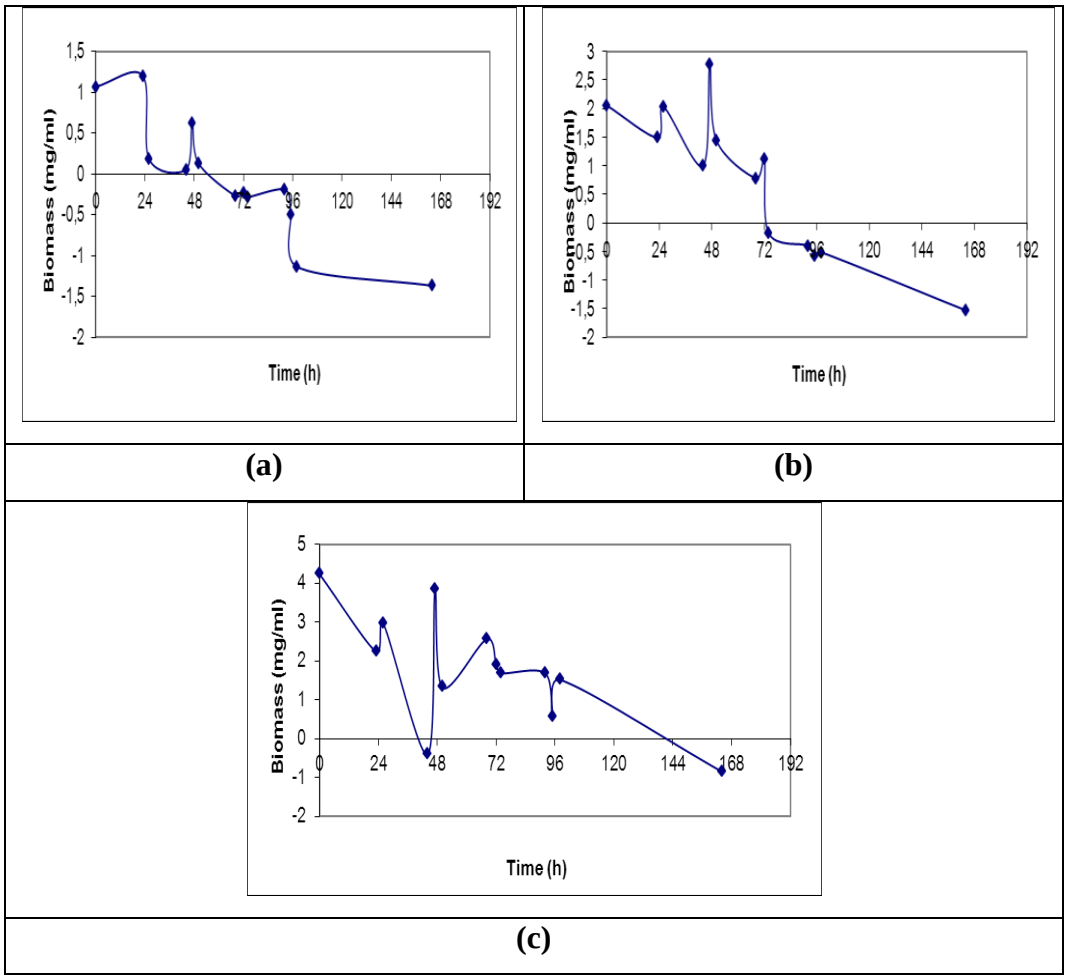


Figure 9.6. Biomass profiles of (a) 5 g/l, (b) 10 g/l, and (c) 15 g/l OP concentrations.

Biomass profiles obtained at 6 different WOP concentrations are presented in Figure 9.7c. It is clearly observed that higher concentrations (20, 25, 30, 40, 50, 60 g/l) gave better biomass profiles than the lower concentrations (5, 10 and 15 g/l). As the substrate concentration increased biomass increased, too and biomass formation started after 24 hours of fermentation for all of the concentrations except for 60 g/l. At 60 g/l, biomass decreased continuously, maybe due to the more viscose structure of the broth. Maximum PG activity (249.49 U/ml) was achieved with 60 g/l WOP concentration at the end of the fermentation (139.h) (Figure 9.7a). Total carbohydrate consumption profiles (Figure 9.7b) showed that for all the concentrations total carbohydrate started to be consumed after 24th hour due to the increase in PG activity and biomass observed after the same hour. This finding was also supported with the decrease observed in DO levels and pH after 24th hour (Figure 9.7d and 9.7e). DO level decreased below 40% level at 48th hour for all substrate concentrations except for 20 g/l.pH decreased to 2.5-3

from 4.6 starting value after 24.h due to the product formation and remained stable at this value until the end of the fermentation during stationary and death phases (Figure 9.7e).

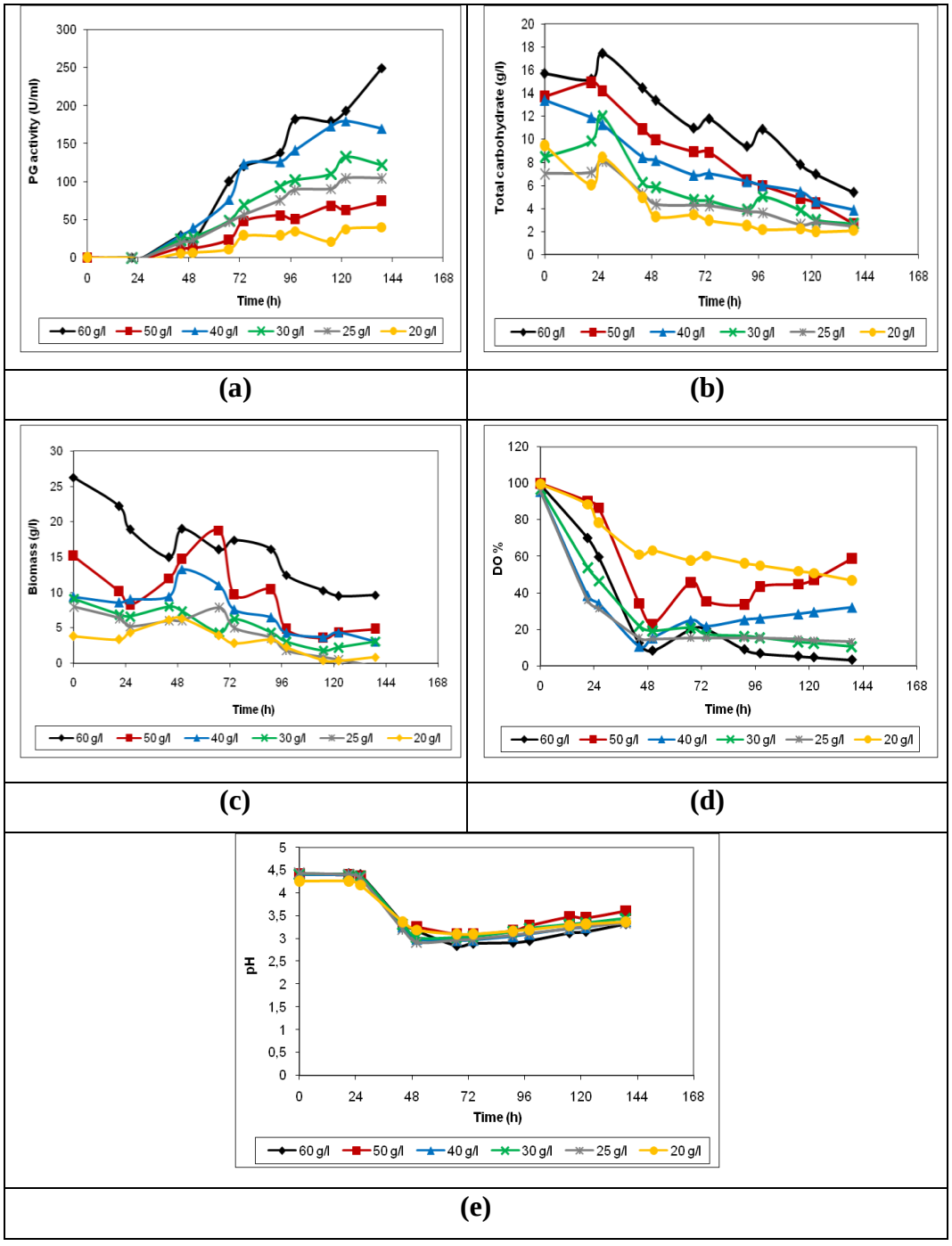


Figure 9.7. Profiles of thedifferent substrate concentrations (a)PG activity, (b) Total carbohydrate, (c) Biomass, (d) DO %, (e) pH.

9.2.1. Estimation of the Kinetic Parameters using Cube-Root Kinetics and Different Linear Equations in order to Linearize the Monod Equation.

A kinetic study or the kinetics of a process is determined by the variation of one or several variables during the fermentation period. From this point of view, any available (measured) variable can be chosen to determine the kinetic characteristics. The selected variable isthen related to other factors in the process. It is essential to take into account how these factors are related and which of these are dependent or independent factor variables. Factor identification and their relationships eventually will permit the establishment of appropriate models describing the process, postulate studies for process optimization, determine control criteria etc. It must be underlined that kinetic means necessarily the determination of at least one variable with time bearing in mind that time; at the same time, could be explicit or implicit in the models, which represent the process kinetics (Rodriguez-Leon et al., 2008). Fungal kinetics may vary as the culture conditions change. Maximum growth rate (μ_{max}) should be determined as an input for process optimization, modeling and scale-up. Fungal growth kinetics has been studied by some researchers that consider a classic kinetics for filamentous fungi like other microorganisms including a lag and an exponential growth phase. On the other hand some other researchers believe that the growth kinetics of filamentous fungi can be fitted to cubic model (Ardestani, 2012).

It is known that growth kinetics of filamentous fungi in submerged culture are quite similar to those of unicellular organisms that reproduce by binary fission. However, because of the practical difficulties that hinder studies of filamentous organisms in submerged culture, growth kinetics of filamentous organisms are based mainly on the studies with unicellular organisms. In bioreactor studies attachment of the biomass to the bioreactor walls, agitators, probes and baffles can lead to heterogeneity. Therefore growing and non-growing biomass areas inside the bioreactor influence the overall growth kinetics (Papagianni, 2004).

In a fermentative process, the variable used to establish the process kinetics has been usually the variation of biomass (synthesis) in the system, variation of substrate content, variation of a product (metabolite), determination of O_2 consumed or CO_2 evolved, heat evolved, etc. Among these variables, biomass synthesis during the process

and substrate consumption determination has to be conducted following the procedures that represent kinetically any fermentation process (Rodriguez-Leon et al., 2008).

In this study specific growth rates for each of the OP concentrations (20, 25, 30, 40, 50, and 60 g/l) were calculated by using the semi-natural logarithmic plots (Figure 9.8) and cubic root plots (Figure 9.9). The results of both of the approaches were given in Table 9.2 and it was seen that R^2 values of the two approaches were not very different from each other (Figure 9.8 and 9.9). Additionally it was clear from the plots that logarithmic phase started after 24^{th} hour for all of the concentrations but its duration changed according to the substrate concentration.

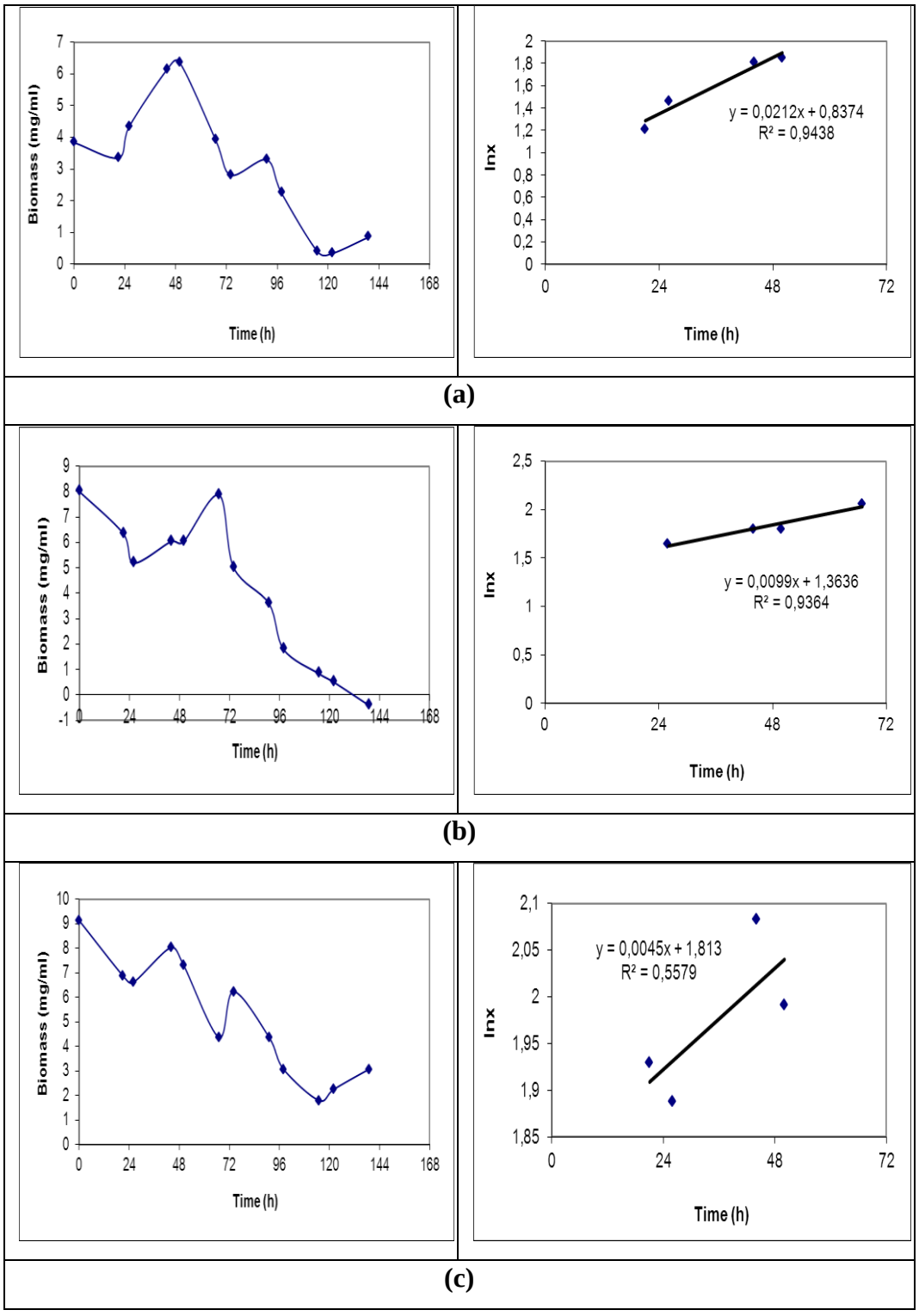


Figure 9.8. Semi-natural logarithmic plots for (a) 20 g/l, (b) 25 g/l, (c) 30 g/l, (d) 40 g/l, (e) 50 g/l and (f) 60 g/l OP concentrations.

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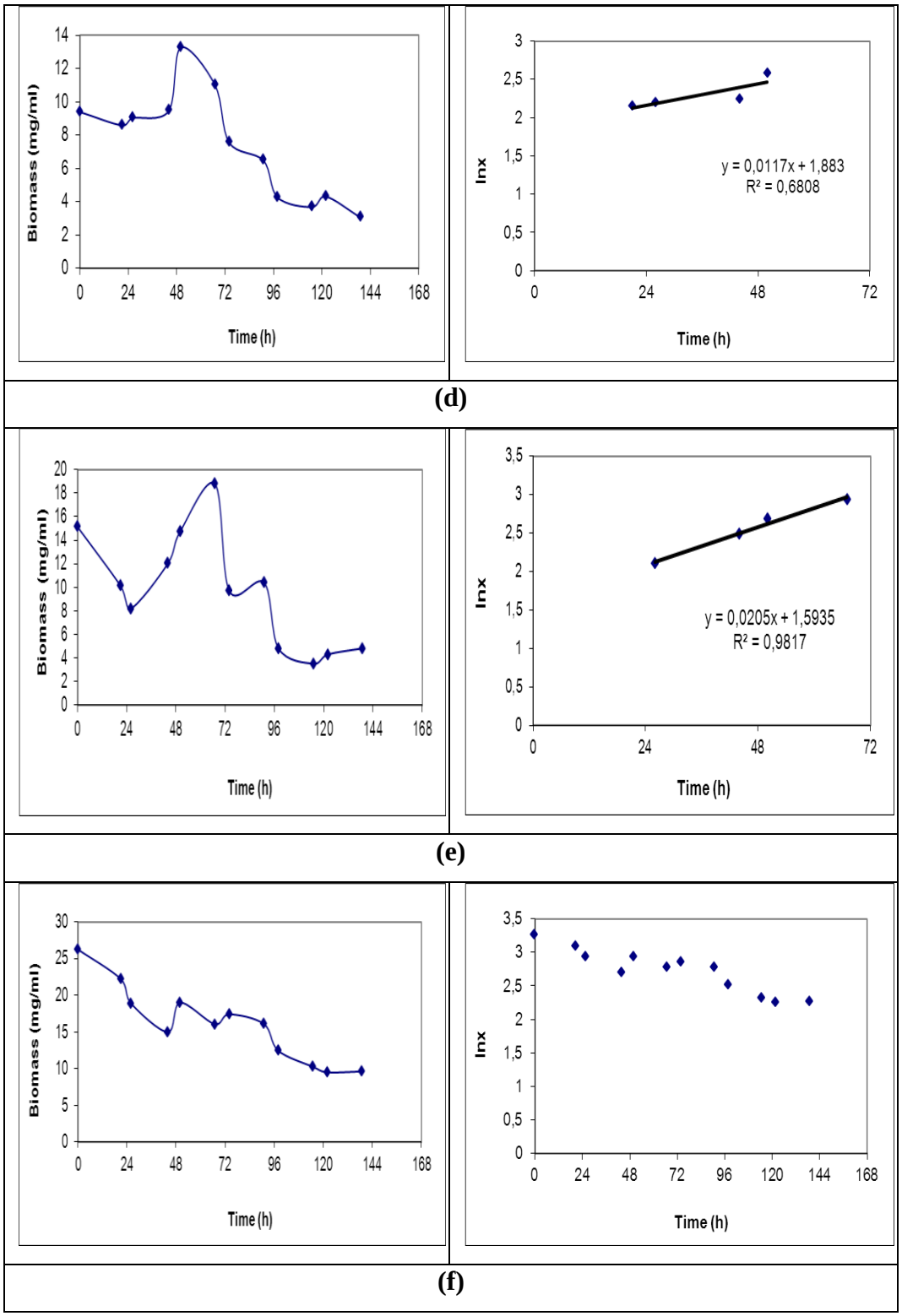

Figure 9.8. (cont.)

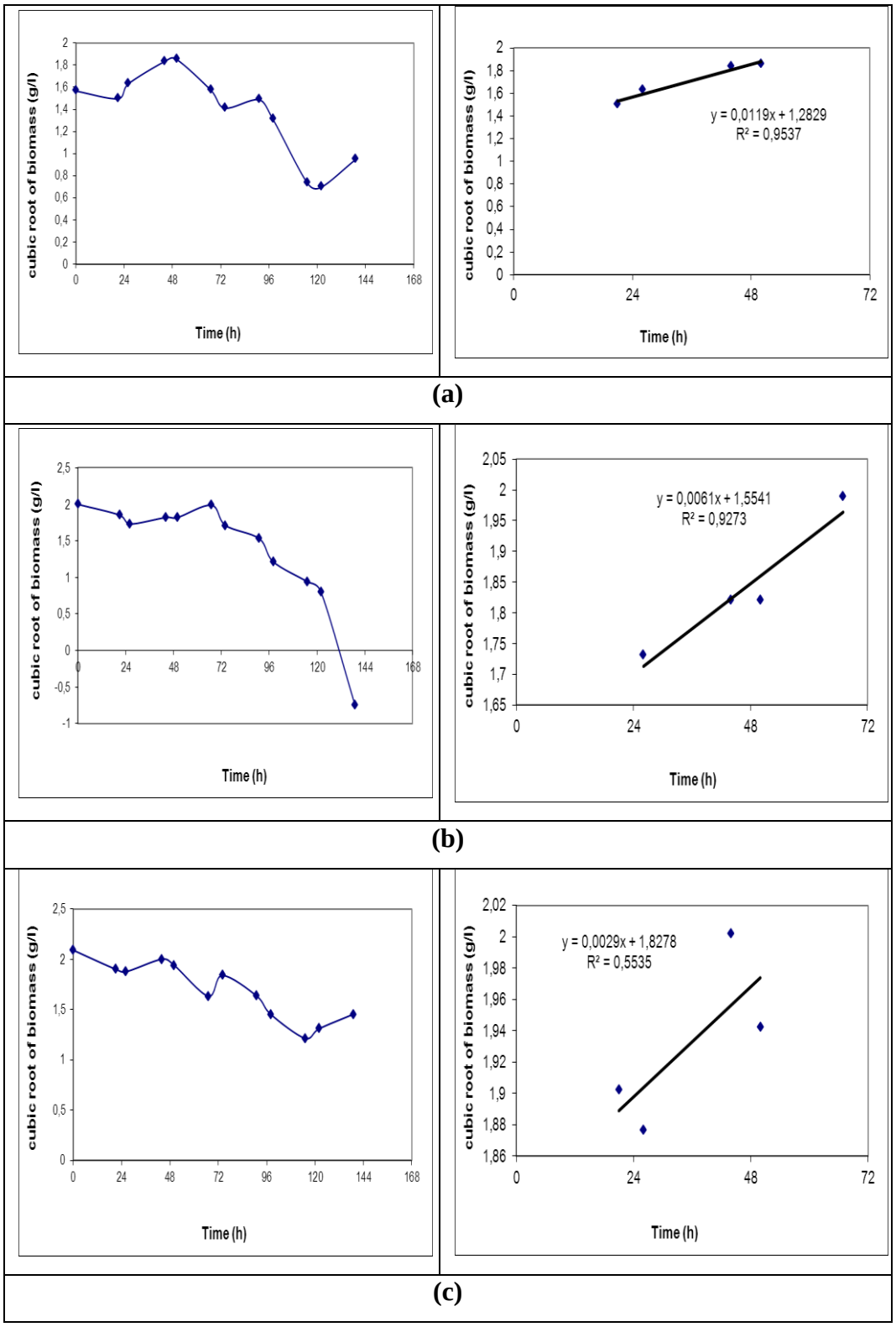

Figure 9.9. Cubic root of biomass versus timeplotsfor (a) 20 g/l, (b) 25 g/l, (c) 30 g/l, (d) 40 g/l, (e) 50 g/l and (f) 60 g/l OP concentrations.

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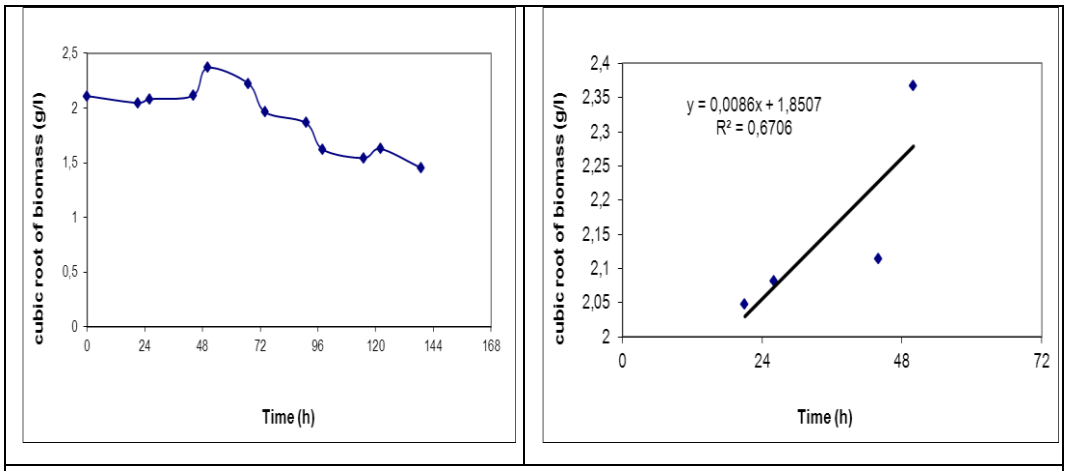
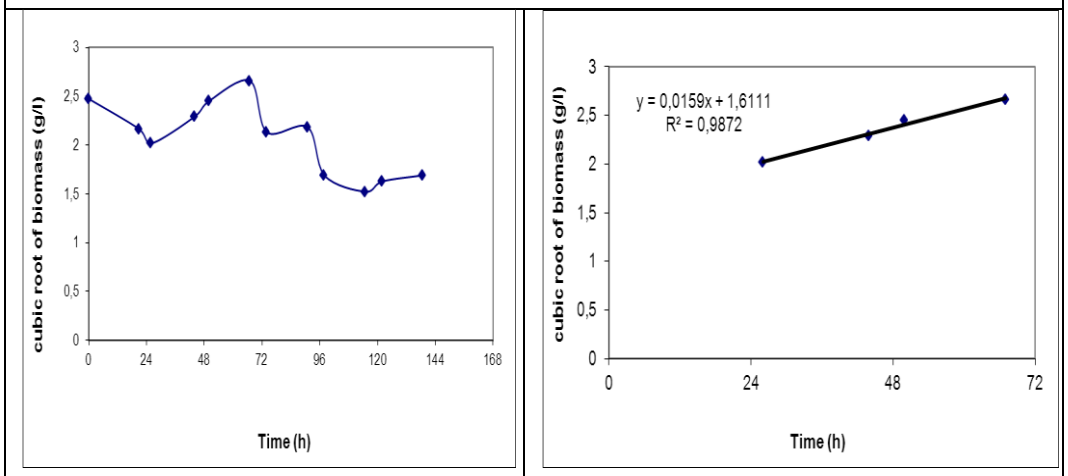
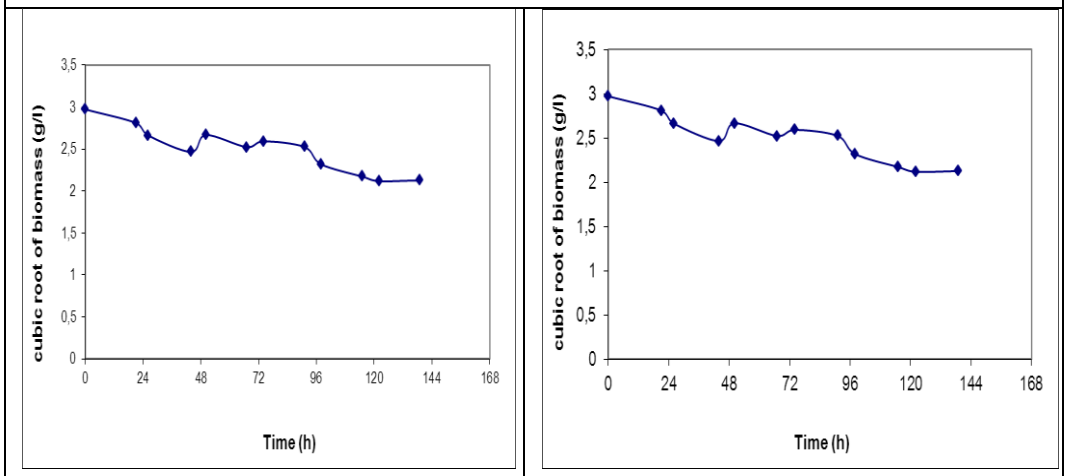


(d)



(e)



(f)

Figure 9.9.(cont.)

Table 9.2. Specific growth rates (μ) calculated by two approaches.

μ (lnx vs t)	**μ ($\sqrt[3]{x}$ vs t)**	**S**
0.0212*	0.0119*	20*
0.0099	0.0061	25
0.0045	0.0029	30
0.0117*	0.0086*	40*
0.0205*	0.0159*	50*

*μ values and concentrations used in Lineweaver Burke plot.

It was obvious from the Table 9.2 that higher specific growth rates were obtained with the semi-naturallogarithmic approach than the cubic-root approach; however the change of the specific growth rate with the change in substrate concentration was same for both of the approaches. It can be also concluded that the growth of *A.sojae* mutant strain grows faster at 20 and 50 g/l whole orange peel concentrations with both of the approaches.

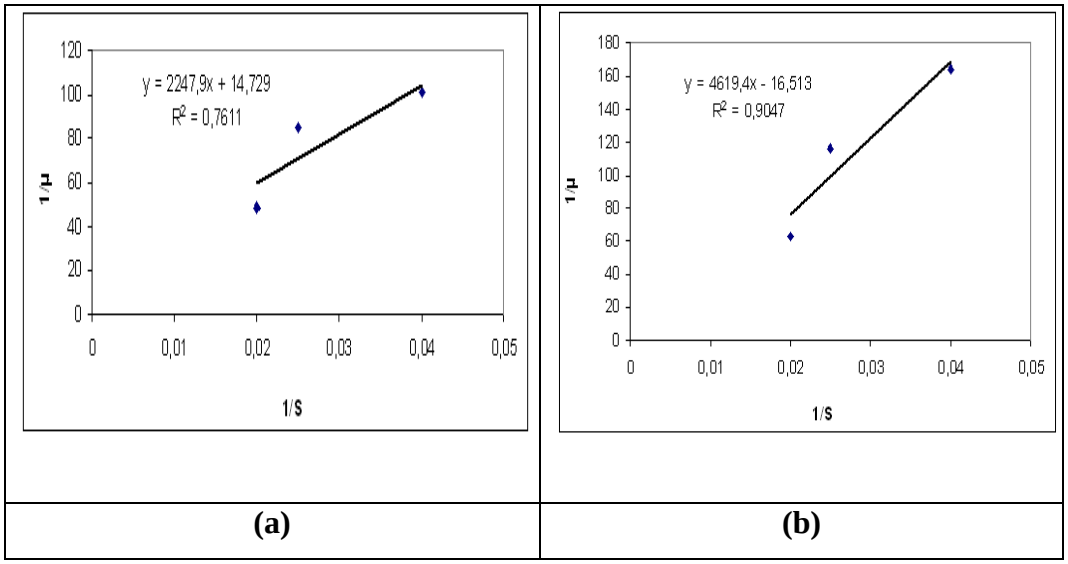
According to Kelly et al. (2006) productivity of filamentous fungi like *A. niger* is related to the morphology based on macroscopic growth. Each product has its own optimum morphology, pelleted or freely dispersed mycelia. Therefore it is hard to determine process parameters for the process. For the ease of process development, in batch culture many researchers defined biomass (X) as a cubic function of time according to the Equation 5.8 described in Section 5.11 (Papagianni, 2004; Kelly et al., 2006;El-Enshasy 2007). This cube root approach was also used for the calculation of specific growth rates.

Additionally, in order to estimate the kinetic parameters, maximum growth rate (μ_{max}) and substrate saturation constant (K_s), three different approaches described in Section 5.11 were applied. These approaches cover the linearization of Monod equation. In this sense the calculated μ values (Table 9.2) were fitted to the Monod equation and the equation was linearized by three different methods in order to calculate μ_{max} and K_s values from the slope and intercept of the plots.

According to the results of the Lineweaver Burke plot (Figure 9.10a) the maximum specific growth rate of *A. sojae* mutant strain was estimatedas 0.068 h^{-1} with naturallogarithmic approach. In literature similar μ_{max} values were found such as

Favela-Torres et al (1997) determined the maximum specific growth rate (μ_{max}) as 0.134 h^{-1}in submerged fermentation for *A. niger*. Bettin et al (2011) found μ_{max}as 0.038 $h^{-1}$for *Pleurotus sajor-caju*. Also in a study performed by *Penicillium chrysogenum* Goudar and Strevett (1998) found μ_{max}value of 0.103 h^{-1}. However Cui et al (1998) determined maximum specific growth rate for *A. awamori*as 0.27 h^{-1}which is relatively higher than the one calculated in the current study, indicating slower growth of *A. sojae* mutant strain than *A. awamori*. Nevertheless, maximum specific growth rate could not be calculated by cubic root approach as the intercept of the plot was negative.

The saturation constant for substrate in the Monod expression (K_s) is an indirect measure of the affinity of the organism for the substrate (Koutinas et al., 2003). In this study the saturation constant of WOP for *Aspergillus sojae* mutant strain was estimated as152.62 g/l which is quite high indicating low affinity of *Aspergillus sojae* mutant strain for WOP from the Lineweaver Burke plot with naturallogarithmic approach.



(a) **(b)**

Figure 9.10. Lineweaver Burke plots. Specific growth rate (µ) calculated by (a) ln X versus time plot and (b) $\sqrt[3]{X}$ versus time plot.

Furthermore from the Hanes Woolf plot (Figure 9.12) the maximum specific growth rate of *Aspergillus sojae* mutant strain was estimated as0.022 $h^{-1}$and 0.027 h^{-1} with naturallogarithmic approach and cubic root approach, respectively. Saturation constant of WOP for *Aspergillus sojae* mutant strain was estimated as 17.38 g/l and

57.29 g/l with naturallogarithmic approach and cubic root approach, respectively which were lower than that of Lineweaver Burke plot indicating higher affinity of *Aspergillus sojae* mutant strain for WOP. However other two approaches (Wolf Augustinsson-Hofsteeand Eadie-Scatchard) did not give reasonable results as their intercepts were negative (Figure 9.11 and 9.13).

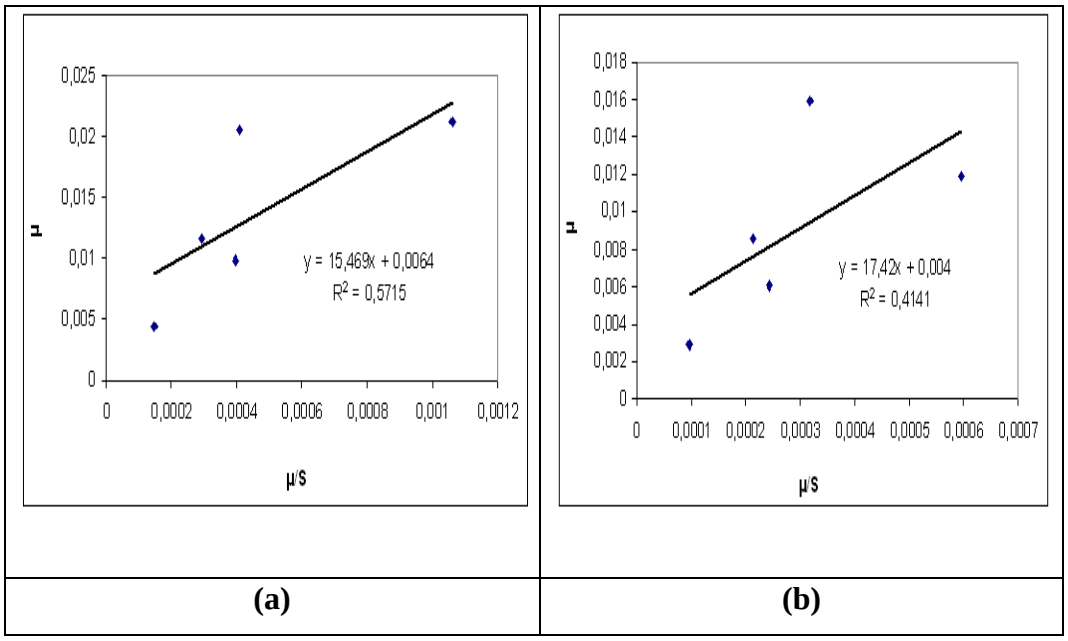
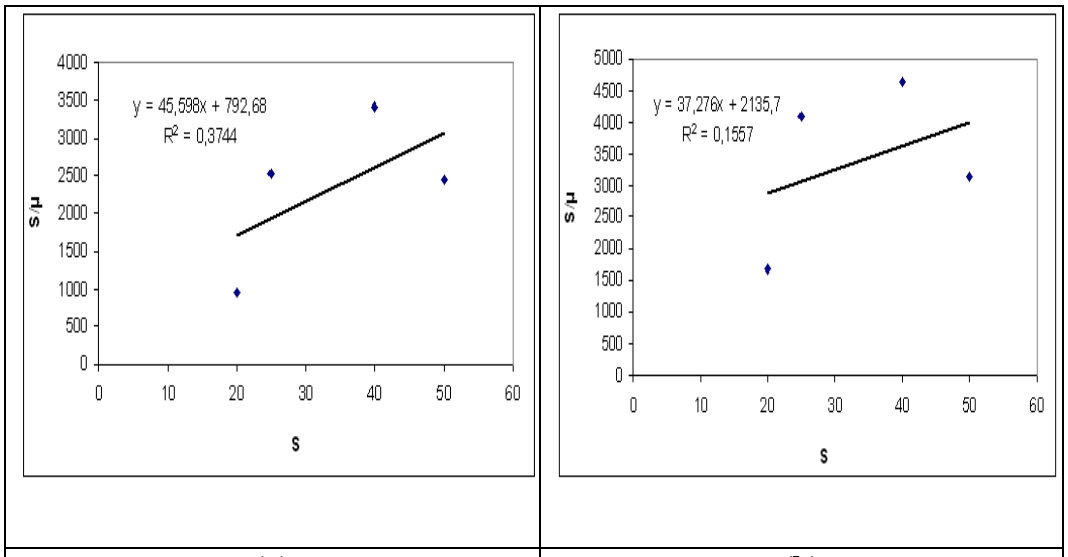
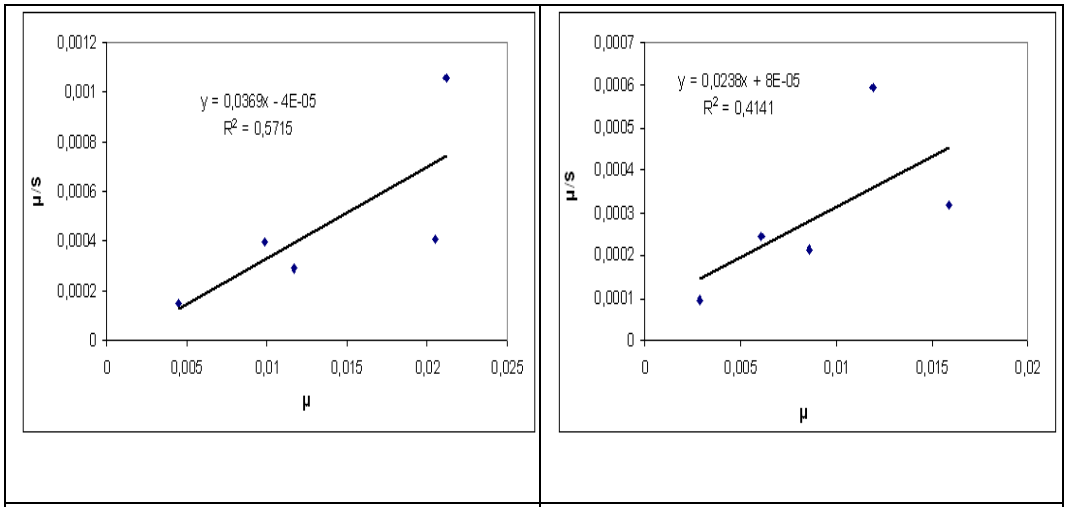


Figure 9.11. Wolf Augustinsson-Hofstee plots. Specific growth rate (μ) calculated by (a) ln X versus time plot and (b) $\sqrt[3]{X}$ versus time plot.



(a) **(b)**

Figure 9.12. Hanes Woolf plots. Specific growth rate (μ) calculated by (a) ln X versus time plot and (b) $\sqrt[3]{X}$ versus time plot.



(a) **(b)**

Figure 9.13. Eadie-Scatchard plots. Specific growth rate (μ) calculated by (a) ln X versus time plot and (b) $\sqrt[3]{X}$ versus time plot.

9.3. Glucosamine Content Determination for the Estimation of Biomass

The measurement of a cell wall constituent glucosamine is an indicator well adapted to the estimation of fungal development. The content of glucosamine and its change during the cultivation of *Aspergillus sojae* mutant strain on WOP was determined and the profiles were given in Figure 9.14 for all of the substrate concentrations. It was observed from the profiles that initial glucosamine concentrations were very high. This could be because of the fact that WOP as a substrate also had glucosamine content preventing us to see the change of glucosamine coming from biomass production. Koutinas et al. (2003) defined that most of the components to be measured such as DNA, RNA, glucosamine and chitin are present in both fungal cells and wheat. This also supported our statement of glucosamine coming from the substrate preventing to indicate the growth using the glucosamine content.

HPLC analysis showed that the samples giving the same peak with standard D-Glucosamine hydrochloride 98% at 6.8^{th} minute of analysis was an indicator of the accuracy of the method.

Although we achieved the same peaks with the samples and standards, from the glucosamine profiles it was not possible to determine the growth due to the glucosamine content present in the WOP.

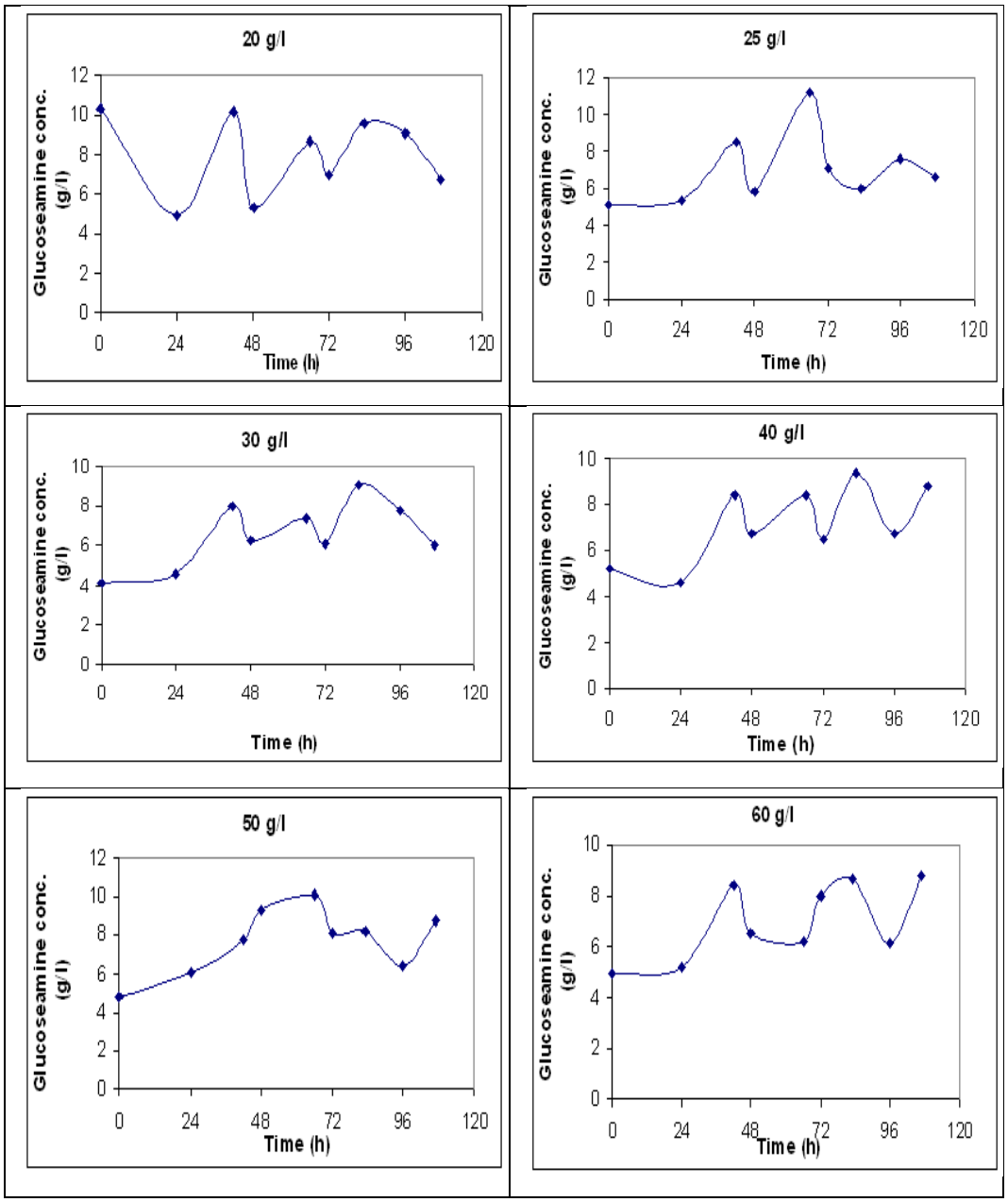


Figure 9.14. Glucosamine profiles for each of the substrate concentrations.

Nevertheless when developing such methods for fungal biomass quantification, the goal is to have a good correlation with fungal biomass measured by dry weight technique, which is impossible to be directly quantified in heterogeneous systems (Marin et al., 2005). Therefore if there is a problem in the determination of dry weight it is very hard to develop a new method.

CHAPTER 10

EFFECT OF THE SUBSTRATE CONCENTRATION AND ITS STATE ON THE PG ACTIVITY AND BIOMASS

10.1. Shake Flask Experiments

This section was performed in shake flasks in order to see if it could be easier to determine the biomass in extracted orange peel (EOP) in the absence of particles which was a problem in whole orange peel (WOP) to determine the biomass. It was thought to provide a basis in shake flasks for further 1 l scale serial bioreactor studies.

According to the results, PG activity increased as the substrate concentration increased and the PG values obtained with whole orange peel (227.47 U/ml) were higher than with extracted orange peel (153.07 U/ml) (Figure 10.1a). Dhillon et al 2004 also found that when submerged and semisolid orange peel fermentations with *Aspergillus niger* were compared for the pectinase activity, more enzyme activity was obtained with the semisolid substrate. Biomass was increasing linearly with the substrate concentration and the difference between the biomass of whole substrate and extracted substrate was also increasing as the substrate concentration increased (Figure 10.1b). Total carbohydrate concentration left at the end of fermentation was same for both of the substrate states at low concentrations of orange peel (5, 10, 15 g/l). For higher concentrations of orange peel (20, 40, 60 g/l) residual carbohydrate concentrations of whole orange peel fermentation was greater than that of extracted orange peel fermentation (Figure 10.1c). This result indicates that carbohydrate utilization was same in both of the substrate states. After 15 g/l orange peel concentration, residual carbohydrate concentration was even higher in whole orange peel indicating that carbohydrate was utilized better in extracted orange peel after this concentration. On the other hand this carbohydrate consumption was not converted to PG activity but might have been converted to biomass because we could not see the real biomass values in whole orange peel due to the orange peel particles in the medium. It is clearly seen from Figure 10.1d that final pH of fermentation broth decreased with an

increase in the substrate concentrations until the 20 g/l orange peel concentration, after this for both of the substrate states pH remained constant.

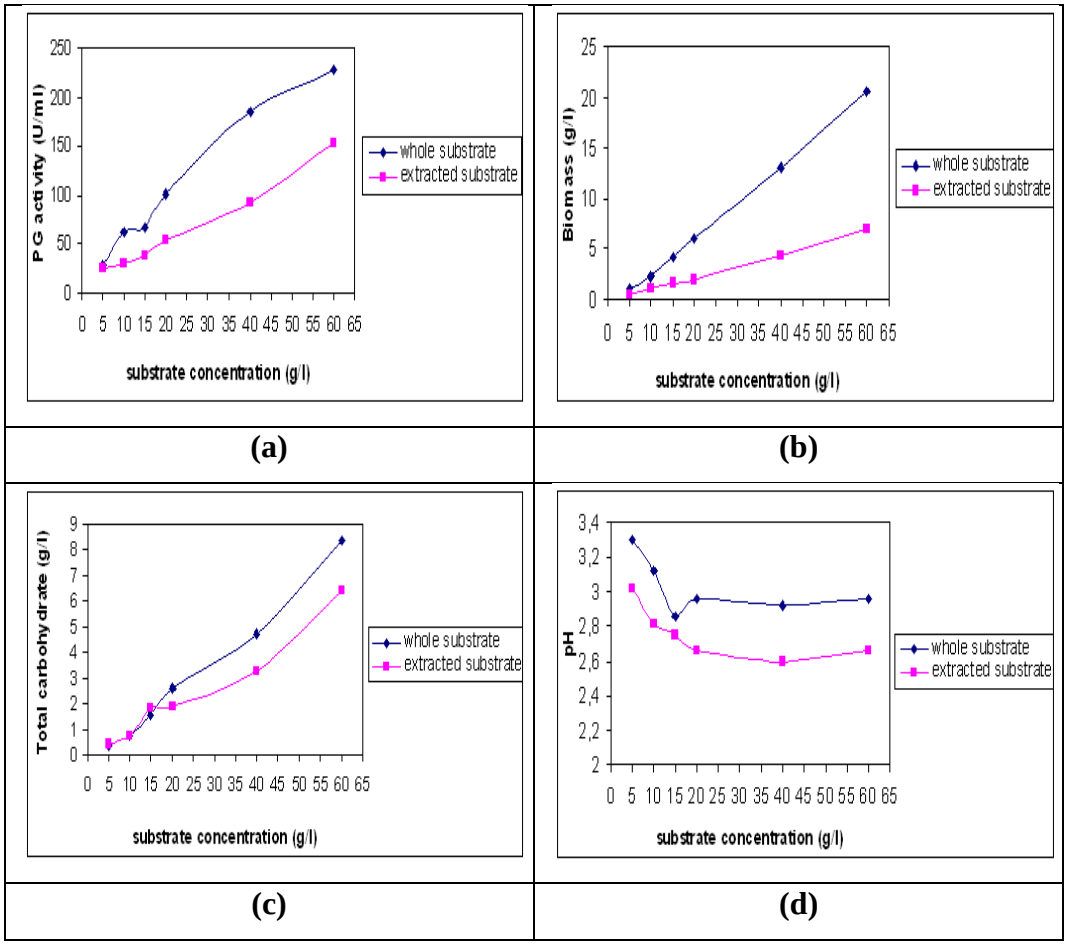


Figure 10.1. Comparison of whole orange peel and extracted orange peel fermentations (a) PG activity, (b) biomass, (c) total carbohydrate and (d) pH profiles.

Furthermore in order to determine the effect of orange peel state on the morphology of the fermentation the photographs of the flasks were taken at the end of the fermentation and given in Figure 10.2. Flask images show that by using extracted orange peel, clearer fermentation broth with smooth and spherical pellets was obtained, however more viscous and turbid fermentation broth with small and irregular pellets were obtained when whole orange peel was used as substrate. It can be concluded that extracted orange peel will be advantageous due to the ease of downstream processing with clear and non-viscous broth.

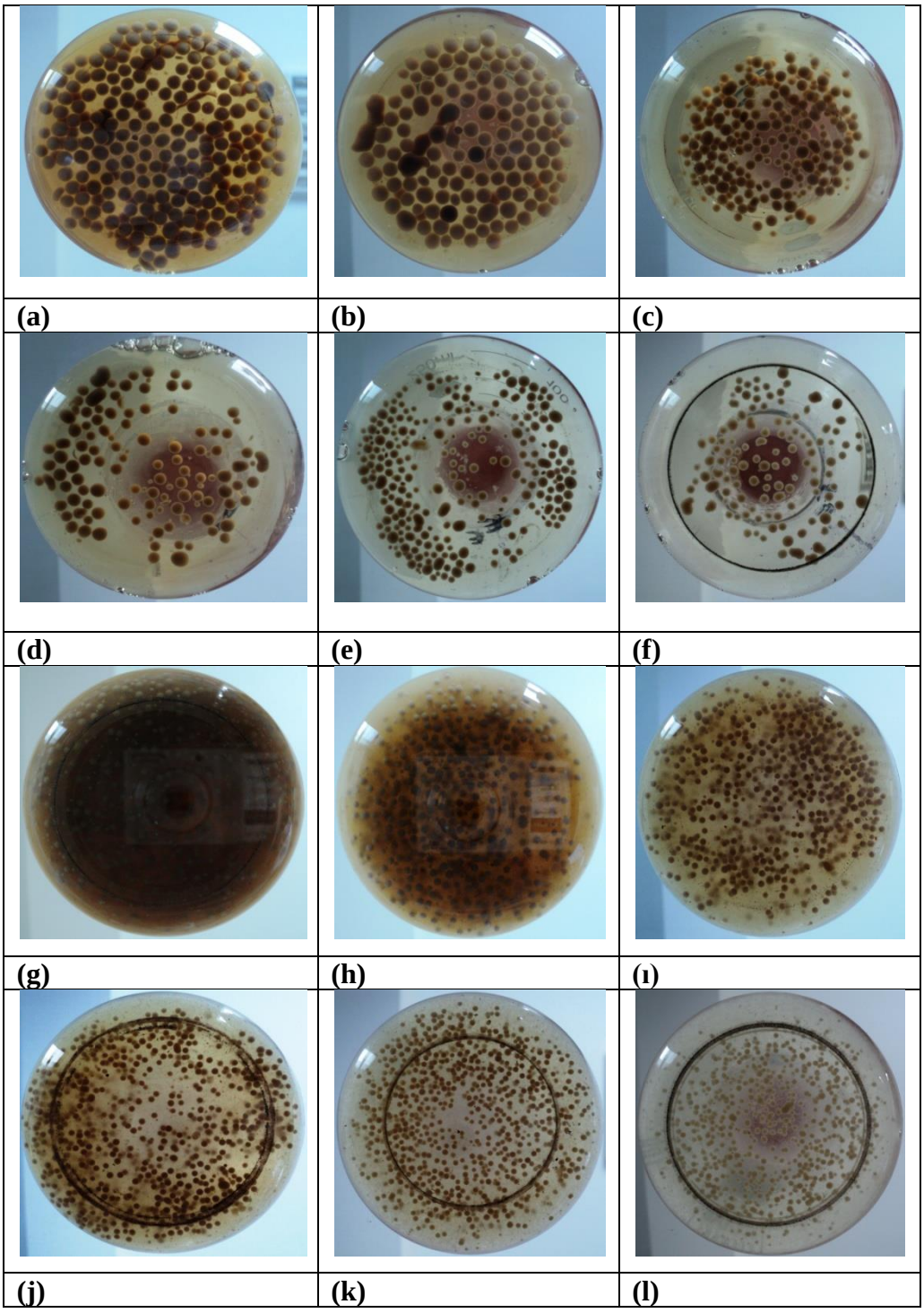


Figure 10.2. Images of fermentation broths at the end of fermentation with extracted orange peel (a) 60 g/l, (b) 40 g/l, (c) 20 g/l, (d) 15 g/l, (e) 10 g/l, (f) 5 g/l and whole orange peel (g) 60 g/l, (h) 40 g/l, (ı) 20 g/l, (j) 15 g/l, (k) 10 g/l, (l) 5 g/l.

In conclusion the PG activity and biomass was found to be effected by the state of orange peel. Rangarajan et al.(2010) found similar pectinase activity in fermentations

performed with orange peel extract and orange peel solid by changing the nitrogen source. This may create a starting point for the future studies.

10.2. Serial Bioreactor Experiments

For both of the whole orange peel and extracted orange peel fermentations it was clear from the Figures 10.3a and 10.4a that PG production started after 24.h of fermentation. According to the results, the highest enzyme activity (108.87 U/ml) was obtained with 40 g/l orange peel concentration at the end of the fermentation (144.h) with WOP. At the end of the fermentation with EOP maximum PG activity (71.20 U/ml) was also achieved at 40 g/l orange peel concentration. But at the 120.h of the fermentation 60 g/l EOP concentration gave the highest PG activity (78.85 U/ml). Different from the WOP fermentation there was a big difference between the low concentrations of orange peel (20, 15, 10, 5 g/l) and high concentrations of orange peel (40, 60 g/l) in EOP fermentation in terms of PG activity (Figure 10.3a). This could be attributed to the utilization of carbohydrate for biomass production at low orange peel concentrations in EOP fermentations. Figure 10.3d indicated that biomass concentrations were very similar for all of the EOP concentrations except for 60 g/l.

Total carbohydrate concentration started to decrease after the 24.h for both EOP and WOP concentrations (Figure 10.3b and 10.4b). This can be the evidence of the use of carbohydrate for the PG production as PG activity increased also after 24.h. It was clear from the Figure 10.3b and 10.4b that there was no significant difference between the total carbohydrate concentrations and profiles of EOP and WOP fermentations.

Higher specific PG activities obtained with the WOP fermentation showed that PG activity obtained with this substrate state had lower side activities than that of EOP fermentation (Figure 10.3c and 10.4c). This may be due to the complex structure of WOP stimulating the production of PG enzyme.

In EOP fermentation DO level did not drop below 40% except for 10 g/l.On the other hand in WOP fermentation DO level dropped below 40% at 60 and 40 g/l concentrations at 48.h which may be the result of higher PG activity and biomass production (Figure 10.3e and 10.4e). pH decreased to 2.5-3 from 4.6 starting value after 24.h due to the product formation and remained stable at this value until the end of the

fermentation during stationary and death phase for both of the substrate states (Figure 10.3f and 10.4f).

While the biomass value was maximum at 40 g/l WOP concentration (12.44 g/l), it was maximum at 60 g/l EOP concentration (3.6 g/l), which was relatively low. As it is seen from Figure 10.4d in WOP fermentation, for 40 and 60 g/l orange peel concentrations logarithmic phase was approximately between 48. and 72. hours of fermentation but for the other concentrations it was observed between 24. and 48. hours of fermentation indicating that as the substrate concentration increased, the culture entered the logarithmic phase later. This statement was not valid for EOP fermentation as it is seen in Figure 10.3d, exponential phase started approximately after 24. h for all of the orange peel concentrations.This could be attributed to the complex structure of WOPwhich might have causeddelay in adaptation of the fungus to the environment and therefore, delayed the production of the necessary metabolites for growth.

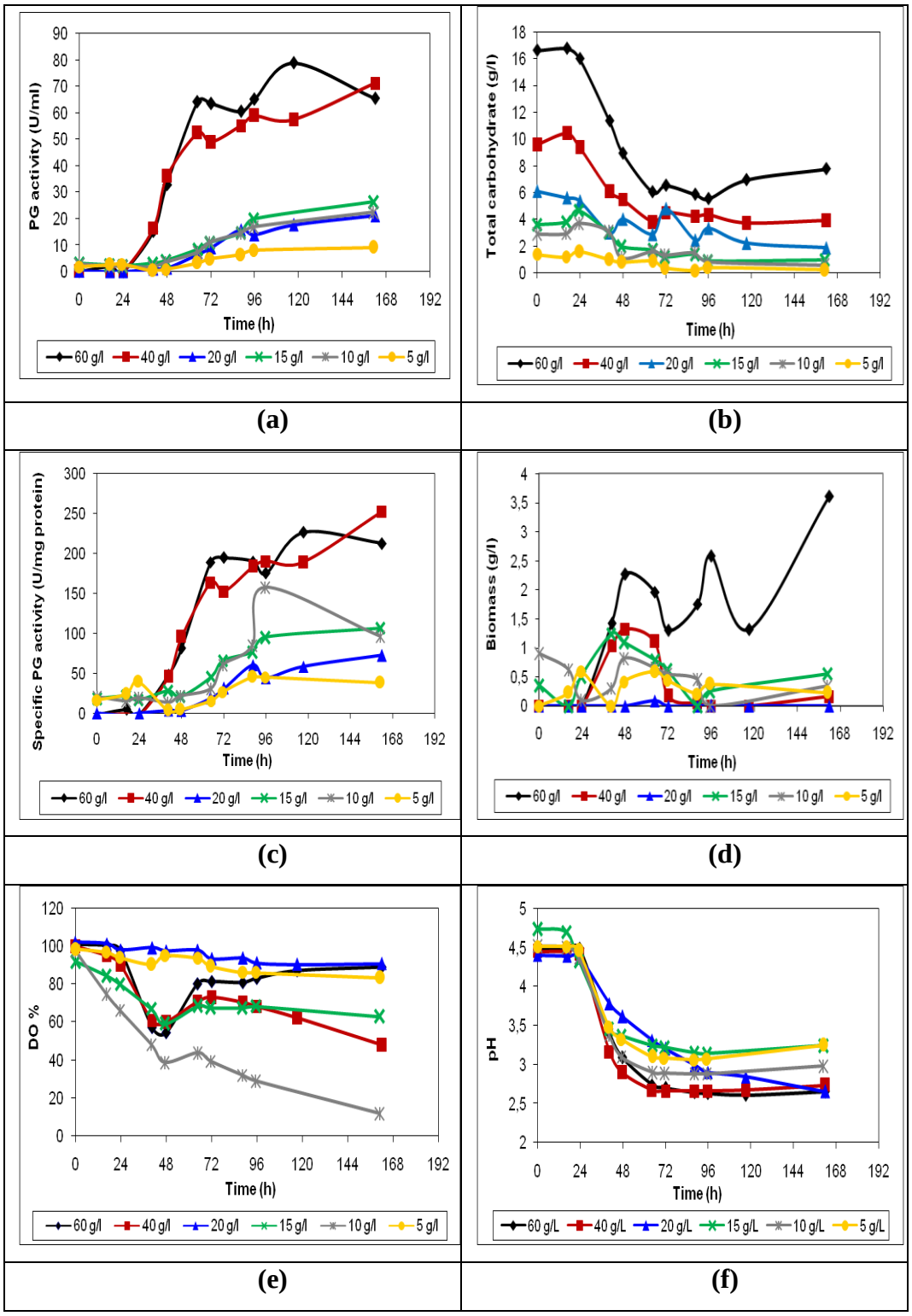


Figure 10.3. Profiles of thedifferent substrate concentrations of EOP (a)PG activity, (b) Total carbohydrate, (c) Specific PG activity, (d) Biomass, (e) DO %, (f) pH.

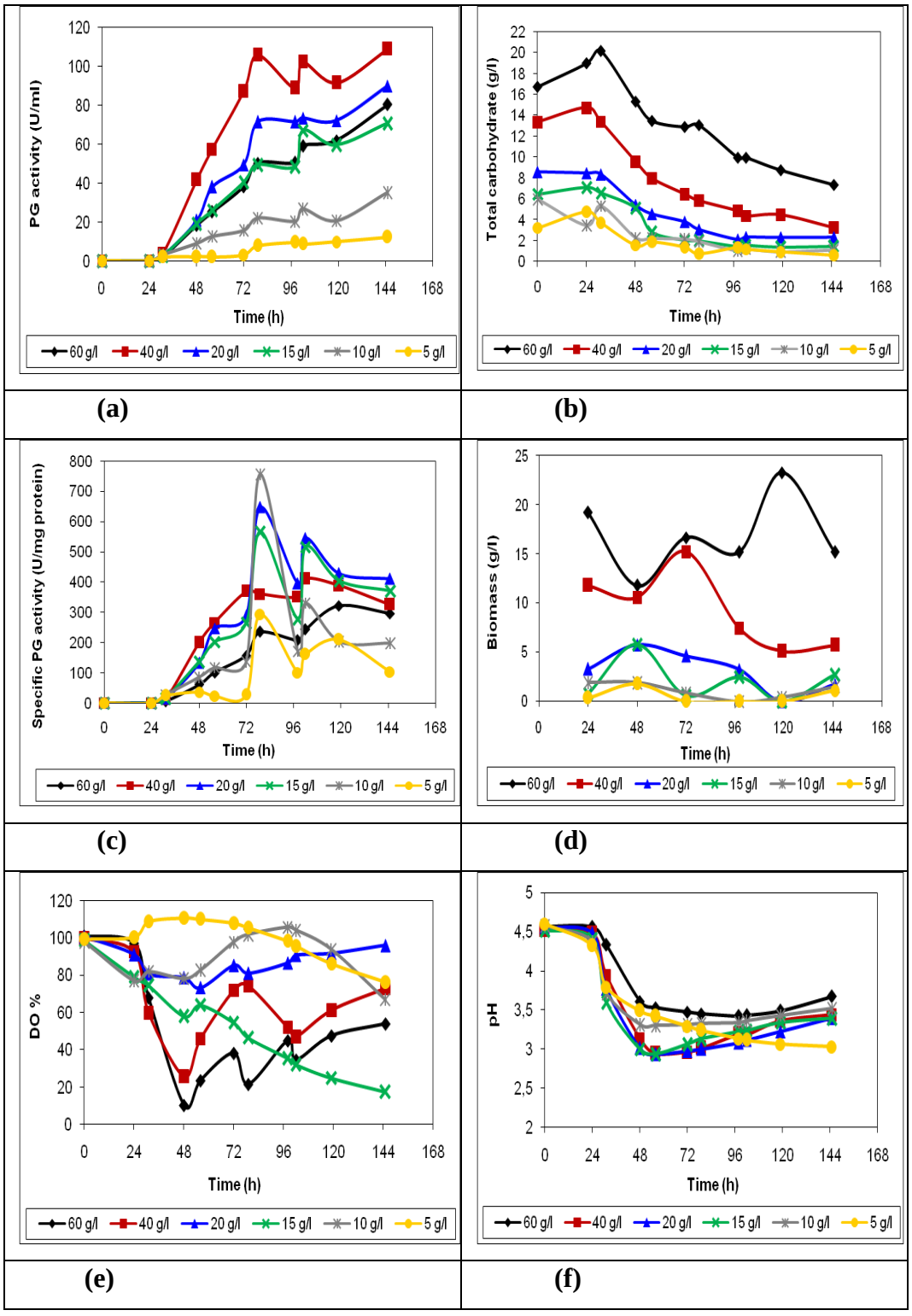


Figure 10.4. Profiles of thedifferent substrate concentrations of WOP (a) PG activity, (b) Total carbohydrate, (c) Specific PG activity, (d) Biomass, (e) DO %, (f) pH.

As a result WOP resulted in 35 % more PG activity and 71 % more biomass compared to EOP. Maximum PG activity and biomass was obtained at the end of the fermentation with 40 g/l and 60 g/l concentrations, respectively for both of the substrate states. The maximum biomass and PG activity versus substrate concentration plots were very similar in WOP and EOP fermentations except for the quantities (Figure 10.5). Both PG activity and biomass production were higher in WOP fermentation probably due to the complex structure of the WOP. It can be seen from the plots that biomass and PG activity increased with increasing substrate concentration but at 60 g/l concentration PG activity decreased again (Figure 10.5). The reason for this decrease can be because of the catabolite repression as described previously.

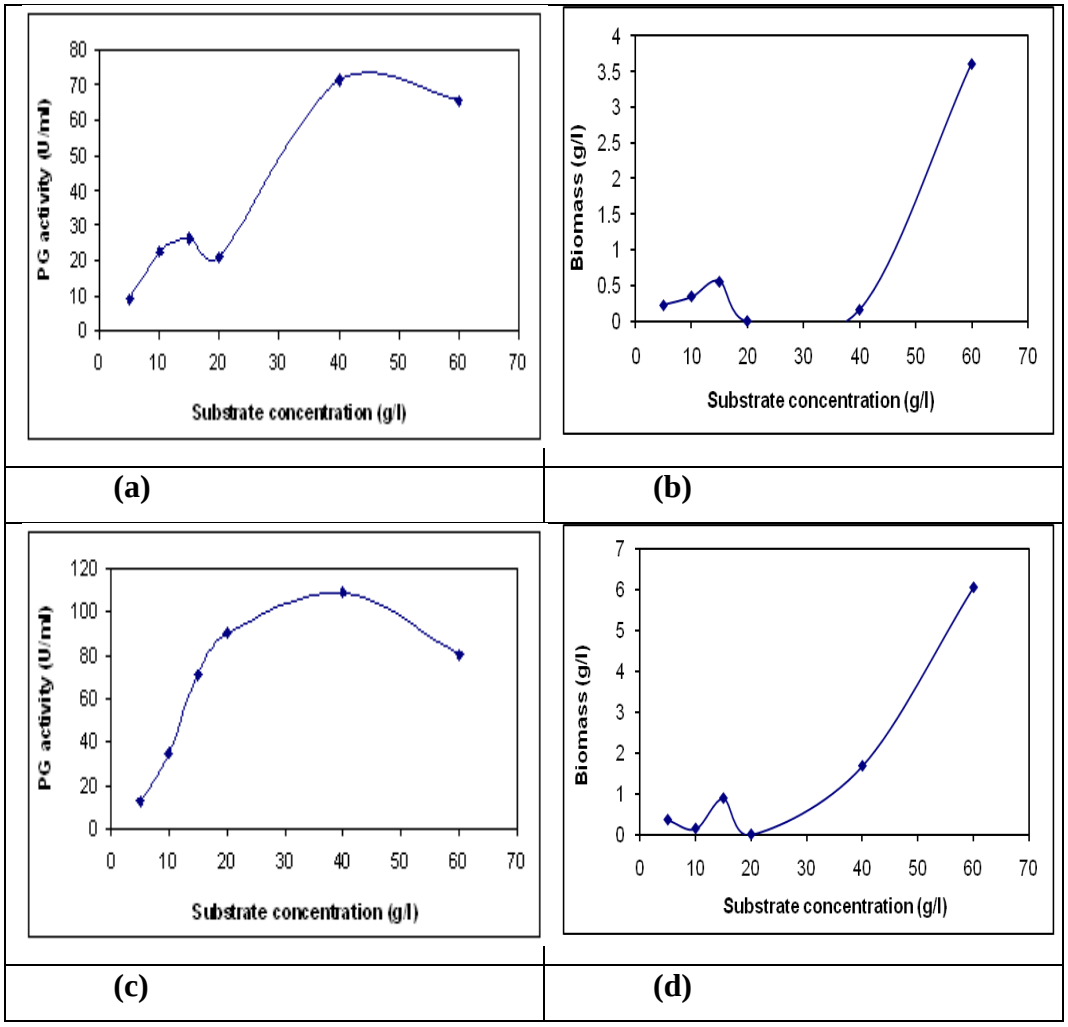


Figure 10.5. Change in PG activity and biomass with the concentration of EOP (a), (b) and WOP (c), (d).

The PG enzyme yield coefficients and productivities calculated for all of the orange peel concentrations of WOP showed that although the highest PG production per substrate was obtained at 20 and 15 g/l concentrations, productivity was maximum at 40 g/l substrate concentration (Table 10.1). For EOP the highest PG production per substrate and productivity were obtained both at 40 g/l. WOP resulted in 41% more PG productivity compared to EOP. Also these results were in accordance with the PG profile given in Figure 10.3a and 10.4a.

Table 10.1. PG activity yield and productivity values of different substrate concentrations at different states.

Substrate state						
	Product (PG activity) yield ($Y_{P/S}$) (U activity/mg substrate)					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
EOP	7.42	12.56	4.95	8.84	8.59	6.42
	Productivity (U/ml.h)					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
	0.41	0.44	0.13	0.14	0.12	0.05
	Product (PG activity) yield ($Y_{P/S}$) (U activity/mg substrate)					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
WOP	8.59	10.77	14.47	14.35	7.21	4.69
	Productivity (U/ml.h)					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
	0.55	0.75	0.62	0.49	0.24	0.09

10.2.1. Estimation of the Kinetic Parameters by Construction of the Standard Curve to Calculate the Sample Blanks of the WOP Biomass Data.

The biomass values of the WOP experiment was calculated using sample blanks of the defined sample times as described in the materials and method section using the

standard curve given in Figure 10.6 and the new biomass profile is given in Figure 10.7. Also the two biomass profiles were compared in Figure 10.8.The specific growth rates of both WOP and EOP fermentations were calculated for 5, 10, 15, 20, 40 and 60 g/l orange peel concentrations by using the semi-natural logarithmic plots (Figure 10.9 and 10.10) given in Table 10.2.

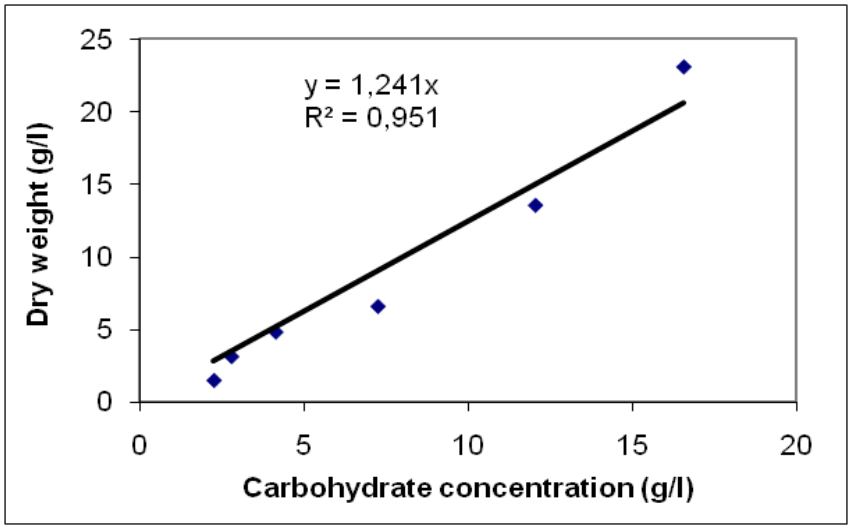


Figure 10.6. Standard curve for the sample blank calculation of WOP.

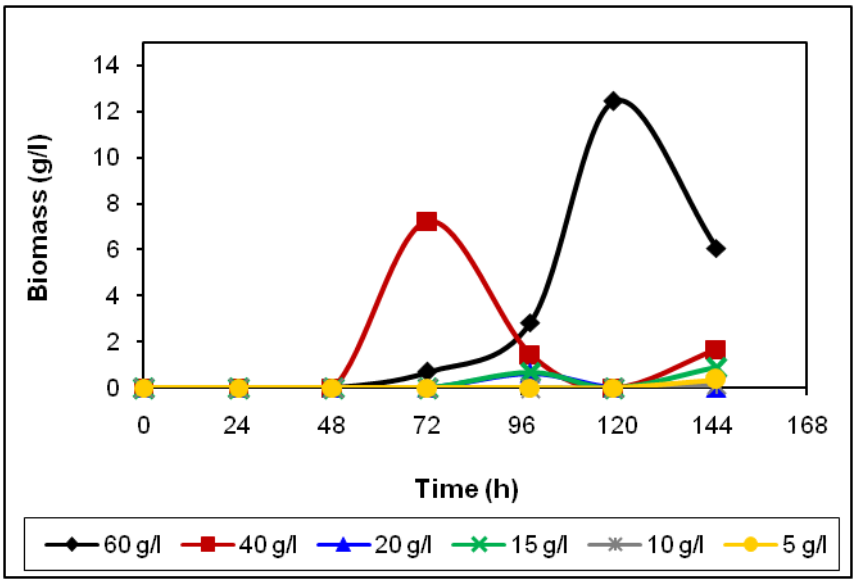


Figure 10.7. Biomass profile of the WOP experiment which was calculated again by the subtraction of sample blanks.

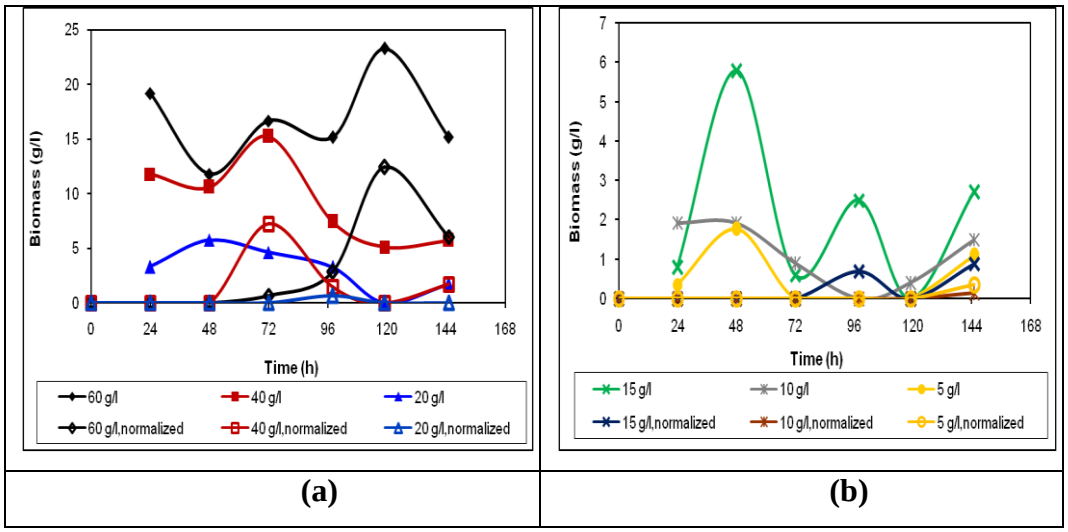


Figure 10.8. Comparison of the biomass profile of WOP with and without subtraction of sample blanks (a) 20, 40 and 60 g/l, (b) 5, 10 and 15 g/l.

In Figure 10.9 and 10.10 semi-natural logarithmic plots of WOP and EOP were given and from these plots the specific growth rates of each substrate concentration were calculated. Afterwards the effect of substrate concentration on the specific growth rate was plotted for WOP and EOP (Figure 10.11).

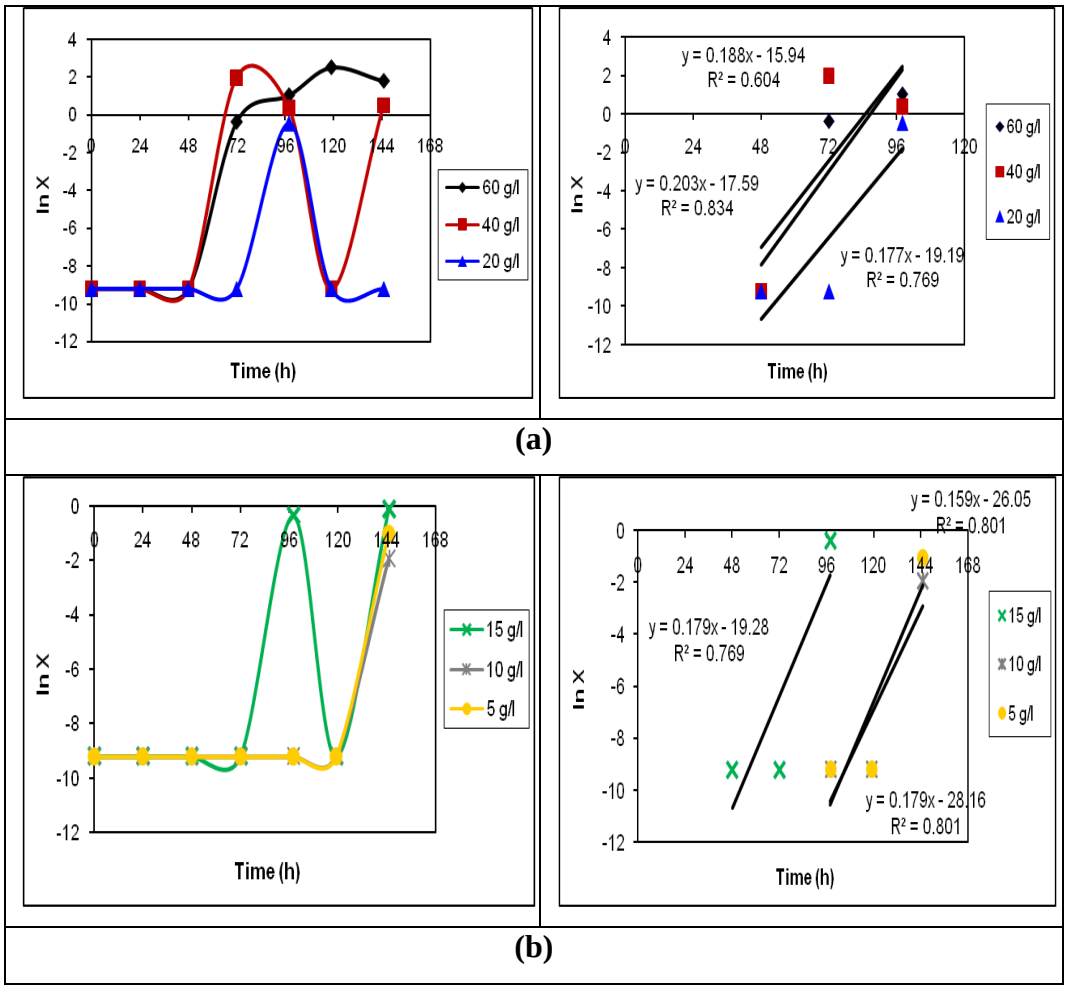


Figure 10.9. Semi-natural logarithmic plots of WOP (a) 20, 40 and 60 g/l, (b) 5, 10 and 15 g/l.

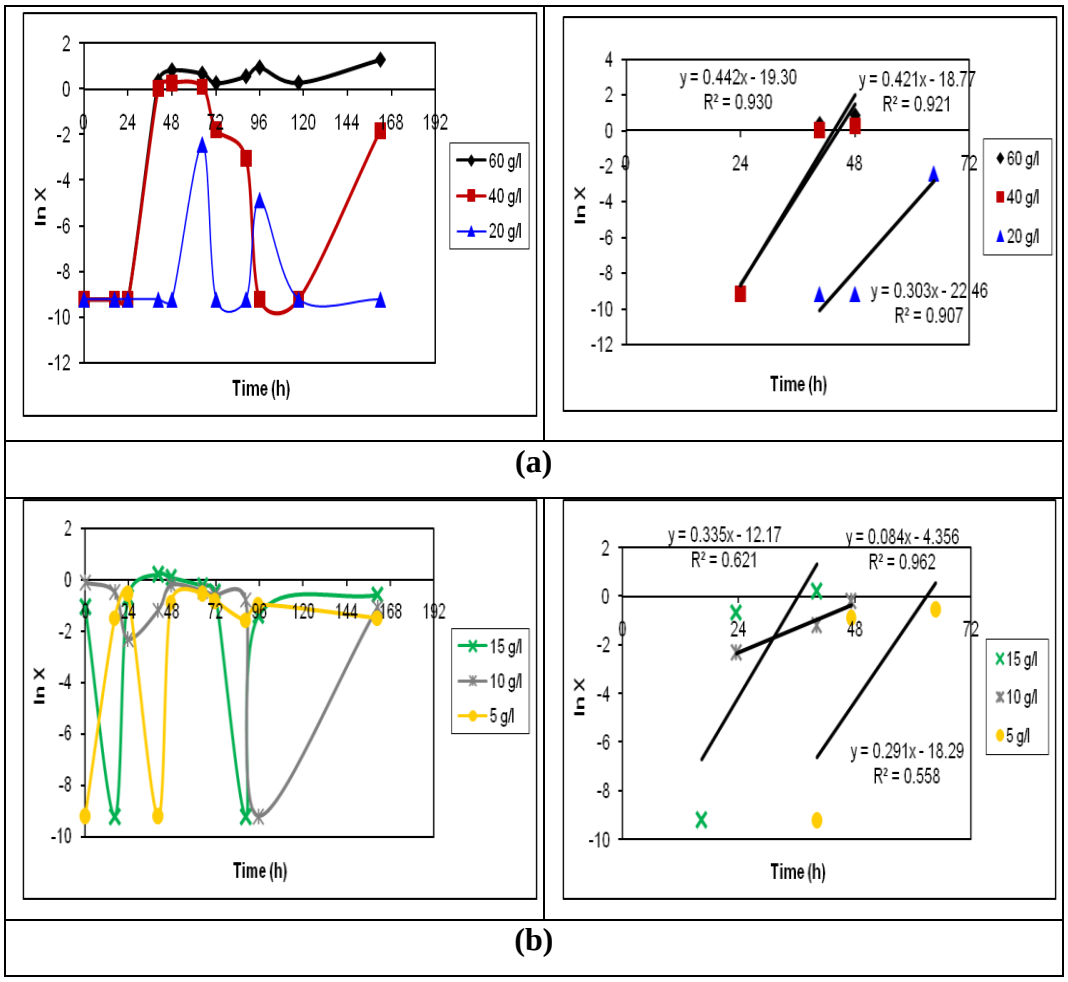


Figure 10.10. Semi-natural logarithmic plots of EOP (a) 20, 40 and 60 g/l, (b) 5, 10 and 15 g/l.

Figure 10.11 shows the effect of substrate concentration on the specific growth rate, indicating that specific growth rate decreased by a decrease in substrate concentration.

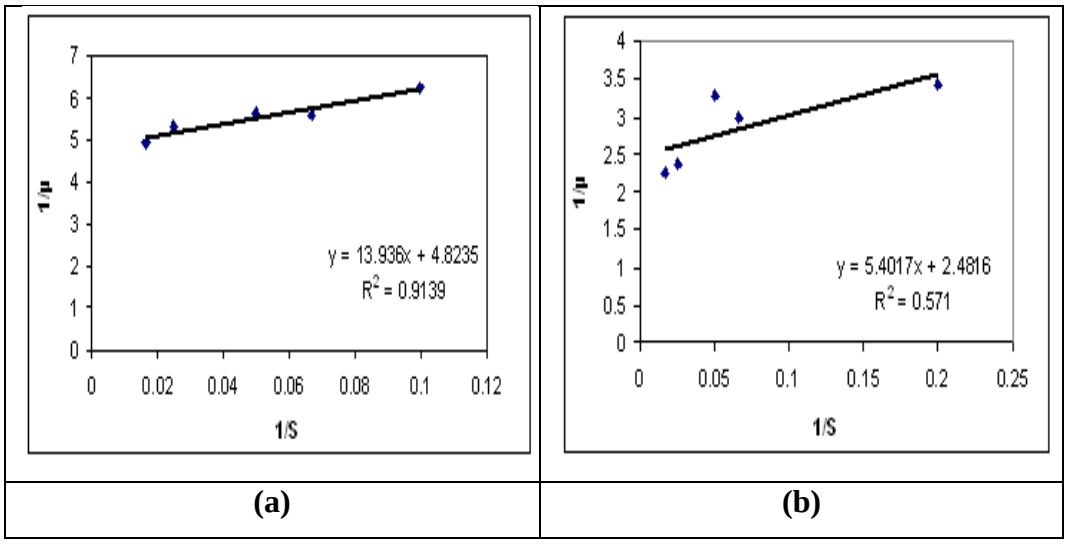


Figure 10.11. The effect of substrate concentration on the specific growth rate for (a) WOP and (b) EOP.

From the slope and intercept of the Figure 10.11, μ_{max} and K_S values were calculated for both WOP and EOP and given in Table 10.2. According to the results the maximum specific growth rate of *Aspergillus sojae* mutant strain was estimated as 0.21 h^{-1} and 0.40 h^{-1} for WOP and EOP, respectively. These values indicate that the growth of *Aspergillus sojae* mutant strain in EOP was faster than that of WOP. Prosser and Tough 1991 defined that maximum specific growth rate values for eukaryotes are generally lower than for prokaryotes and for fungi such as *Aspergillus* and *Penicillium* are typically in the order of 0.1 to 0.3 h^{-1}. As it is obvious from the statement, our findings are compatible with the literature. Also Koutinas et al 2003 estimated fungal growth by using on-line measurements of CO_2 during fermentation and found μ_{max} as 0.28 h^{-1}. In another study performed with *Aspergillus awamori* maximum specific growth rate was estimated as 0.27 h^{-1} (Cui et al., 1998a).

The saturation constant for substrate in the Monod expression (K_s) is measure of the affinity of the organism for the substrate (Koutinas et al., 2003). In this study the saturation constants of WOP and EOP for *Aspergillus sojae* mutant strain was estimated as 2.89 g/l and 2.18 g/l, respectively (Table 10.2). It was clear that there was not so much difference between the affinity of *Aspergillus sojae* mutant strain for the WOP and EOP. Koutinas et al 2003 estimated the saturation constant of glucose and fructose for *Aspergillus awamori* as 0.01 g/l which was very low compared to our findings indicating higher affinity of *Aspergillus awamori* for glucose and fructose.

Doubling time or mean generation time (t_d), is the time required for a given population to double in number. When the doubling times (t_d) of WOP (3.34 h.) and EOP (1.42 h.) were compared it was seen that in WOP *Aspergillus sojae* mutant strain doubles in number later than in EOP (Table 10.2).

Table 10.2. Kinetic parameters estimated for WOP and EOP.

Substrate state	**μmax (h^{-1})**	**Ks (g/l)**	**t_d (h)**
WOP	0.21	2.89	3.34
EOP	0.40	2.18	1.72

The biomass yield coefficients ($Y_{X/S}$) of WOP and EOP fermentations given in Table 10.3 showed that the biomass yields were lower in EOP than WOP. This could be because of the inadequate and simple structure of the EOP compared to the complex structure of WOP.

Table 10.3. Biomass yield and productivity values of different substrate concentrations at different states.

Substrate state	Biomass yield based on substrate consumption (mg biomass/mg substrate consumed) $Y_{x/s}$					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
	0.41	0.03	0	0.08	0	0.20
EOP	**Productivity (mg dry biomass/ml.h)**					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
	0.02	0.001	0	0.001	0	0.001
	Biomass yield based on substrate consumption (mg biomass/mg substrate consumed) $Y_{x/s}$					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
WOP	0.65	0.17	0	0.18	0.03	0.14
	Productivity (mg dry biomass/ml.h)					
	60 g/l	**40 g/l**	**20 g/l**	**15 g/l**	**10 g/l**	**5 g/l**
	0.04	0.012	0	0.006	0.001	0.003

Growth parameters of some of the fungal species and the used substrates were summarized in Table 10.4.

Table 10.4. Growth parameters of some of the fungi.

Fungus	$\mu_{max}(h^{-1})$	K_s (g/l)	Substrate	Reference
Penicillium chrysogenum	0.103	0.145	Glucose	Goudar and Strevett 1998
Aspergillus awamori	0.28	0.01	Wheat flour	Koutinas et al 2003
Aspergillus awamori	0.16-0.25 0.07-0.1	- -	Synthetic medium Wheat bran	Cui et al 1998
Aspergillus nidulans	0.73	-	Glucose	Trinci 1969
Aspergillus sojae mutant strain	0.207 0.403	2.89 2.18	WOP EOP	Current study

Consequently these findings showed that WOP produced higher PG activity and biomass than EOP. The reason for this was assumed to be the complex structure of WOP. In order to determine the difference in their protein contents, Kjeldahl Nitrogen Determination Method was performed and it was found that WOP had higher protein content (% 5.17 ± 0.13) than EOP (% 0.20 ± 0.01). Different from the ammonium sulphate amount added into the fermentation medium, WOP itself had higher protein content than EOP.

CHAPTER 11

EFFECT OF SCALE UP TO 20 L ON PG ACTIVITY

For a fermentation process development scale-up is an important issue to be considered. In order to expand a process from lab-scale to commercial scale, it must be characterized and validated by some controls to evaluate its reliability and reproducibility. Generally it is known that large-scale fermentation processes give lower yield than laboratory scale due to the factors affecting the process yield. Therefore in this study it was aimed to determine the PG production in 20 l scale and compare the PG activity with the small scale studies. The experimental procedure was explained in materials and method part, Section 5.13.

As shown in Figure 11.1a, PG activity showed rapid increase after 72.h. and reached its maximum value at the end of 140.h. (101.38 U / ml).Dissolved oxygen level dropped down to the level 4.4% at the end of 96.h. with an increase in PG activity (Figure 11.1c). This activity value was very similar to the activity values obtained in the previous 1 l scale bioreactor experiments.

Specific PG activity similar to PG activity reached its maximum value at 140.h. with a value of 262.46 U/mg protein as seen in Figure 11.1b. When the change in protein concentration over time was examined, it can be said that protein concentration profile was similar to the profile of PG activity and increase in protein concentration occurred after 72. h. may be due to the increase in PG activity.In addition, the stabilization of protein concentration after 96 hours, could be explained by constant DO level of about 5% after 96 hours (Figure 11.1c).At the beginning of fermentation pH started around 5.23 and decreased to 2.04 at the end of the 146.5 hours of fermentation (Figure 11.1d).

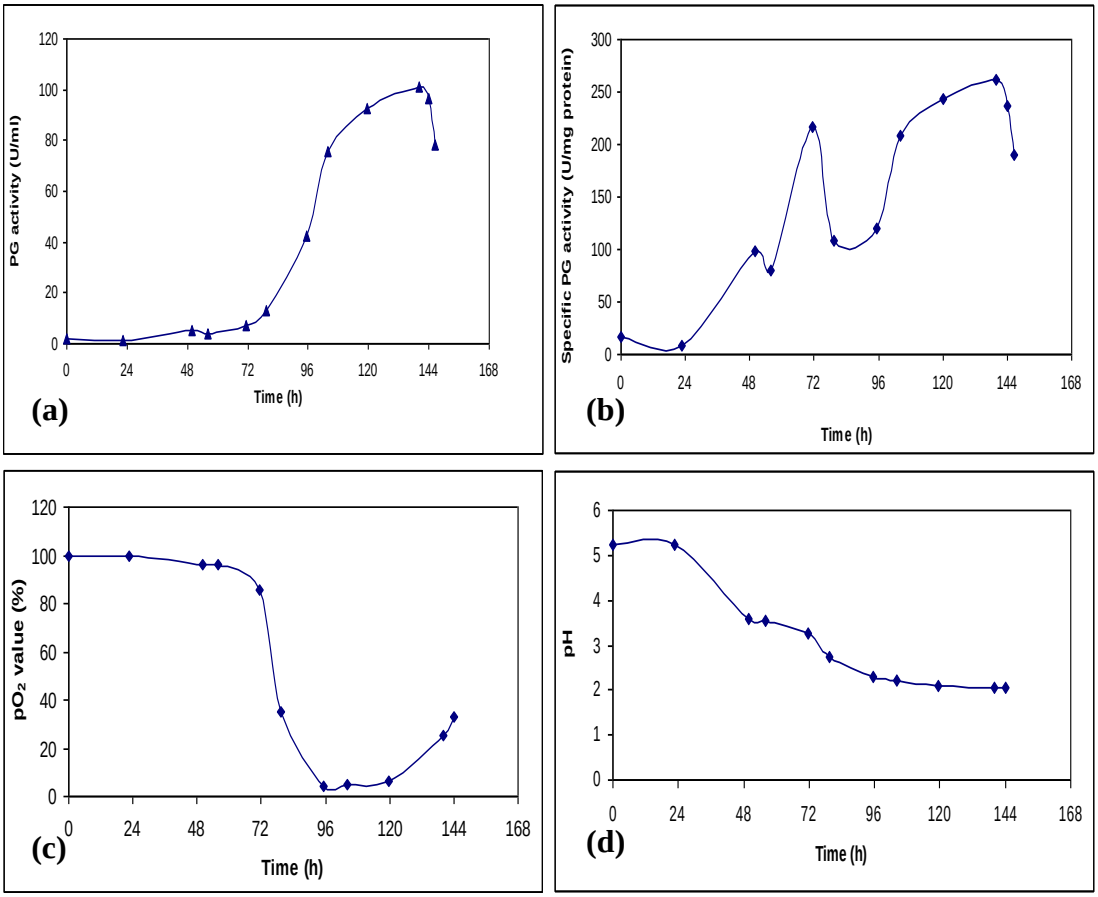


Figure 11.1. Profiles of first 20 l scale bioreactor experiment performed at 600 rpm, 30^{o}C temperature, 0.5 vvm and uncontrolled pH conditions (a) PG activity, (b) specific PG activity, (c) DO (%) and (d) pH.

A second fermentation parallel to the one described above was conducted under the same conditions.According to Figure 11.2a as in the previous experiment there was an increase in PG activity after 60.h. but this increase was not more than 73.53 U/ml at the end of 123 hour. In addition, as shown in Figure 11.2c the rapid increase in the activity profile at this range led dissolved oxygen fall quickly to the level of 27% after 60 hours. This activity value was lower than the previous parallel fermentation and 1 l scale fermentations. Maximum specific PG activity (133.29 U / mg protein) was obtained at 123.h. like the PG activity (Figure 11.2b).

At the beginning of fermentation pH started around 5.18 and decreased to 2.19 and 2.05 at the end of 96.h. and 156.h., respectively (Figure 11.2d). This result was compatible with our previous results.

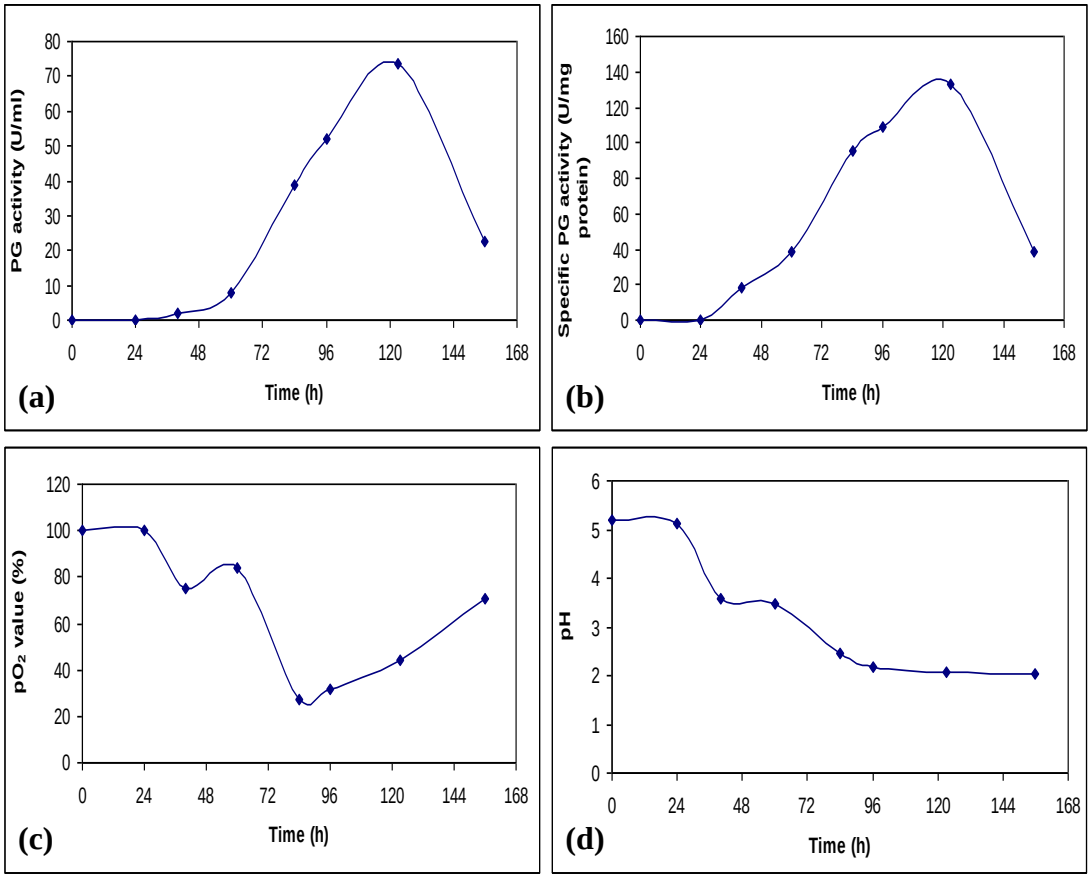


Figure 11.2. Profiles of second 20 l scale bioreactor experiment performed at 600 rpm, 30^{o}C temperature, 0.5 vvm and uncontrolled pH conditions (a) PG activity, (b) specific PG activity, (c) DO (%) and (d) pH.

As a result, average PG activity (87.45 U/ml) obtained in this 20 l scale bioreactor experiments was similar to the PG activity (91.76 U/ml) obtained at 0.5 vvm aeration rate conducted previously at 1 l scale. This result also confirmed the reproducibility of the conditions regardless of scale.

CHAPTER 12

FED BATCH FERMENTATION

Fed-batch cultivation is the most frequentlyused method in the industry (Pedersen et al., 2000). It reduces the substrate inhibition which means the inhibition of growth and product formation due to the high concentration of sugar in fermentation medium (Stanbury and Whitaker, 1984). As it is stated, many enzymes are exposed to carbon-catabolite repression; therefore fed-batchcultivations can be used to grow the fungus primarily at low carbon concentrations in order to increase product formation (Pedersen et al., 2000). With this point of view it was decided to perform five different fed batch fermentations in order to increase the PG production. The substrate feeding was started according to the results of Section 10.2 (Figure 10.4b) when total carbohydrate level of 40 g/l initial WOP concentration decreased to 8 g/l after 48.h. This process enabled the maintenance of low substrate concentration during fermentation, which was necessary for achieving a high product formation rate by avoiding catabolite repression. Also DO and pH level decreased to their minimum levels of 20% and pH 3 at 48.h, respectively in that study which were used for the decision of feeding time. The feeding conditions were summarized in Table 5.1 in Section 5.14. Consequently, it was found that, the highest PG activity (186.78 U/ml) was obtained with the single supplement of 40 g/l orange peel + 2.75 g/l ammonium sulphate medium formulation fed after 48.h (Figure 12.1). This value was approximately 42% and 62% higher than the batch fermentation of WOP and EOP discussed in Section 10.2 (Figure 10.3a and 10.4a). Since the data of that study were used in order to decide the feeding time they served as control to evaluate the effect of fed-batch fermentation on PG activity and biomass. Furthermore current PG production achieved with fed batch fermentation of 40 g/l WOP concentration was 4% higher than the batch fermentation of 40 g/l WOP presented in Section 9.2 (Figure 12.1 and 9.7a).

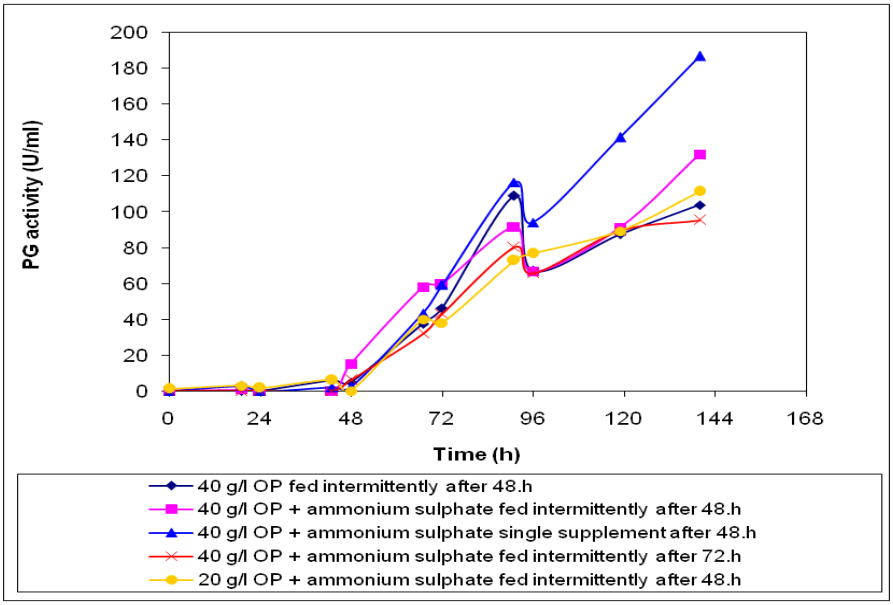


Figure 12.1. PG activity profiles of each fed batch fermentation.

Total carbohydrate consumption was similar for all of the feeding conditions. At the end of each fermentation total carbohydrate concentration fell to approximately the same value (~2 g/l) (Figure 12.2).

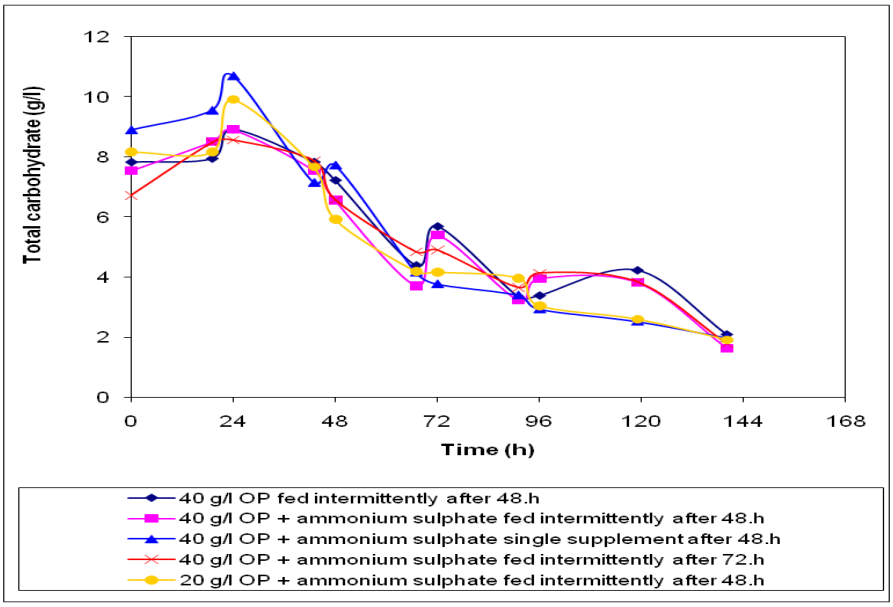


Figure 12.2. Total carbohydrate profiles of each fed batch fermentation.

There were no specific PG activities up to the 96.h, for all of the fed batch fermentations. However at 96.h. specific PG activities reached their maximum values (Figure 12.3). The maximum specific activity was obtained withthe single supplement of 40 g/l orange peel + 2.75 g/l ammonium sulphate medium formulation fed after 48.h indicating that PG activity obtained with this condition possesed less side activities.

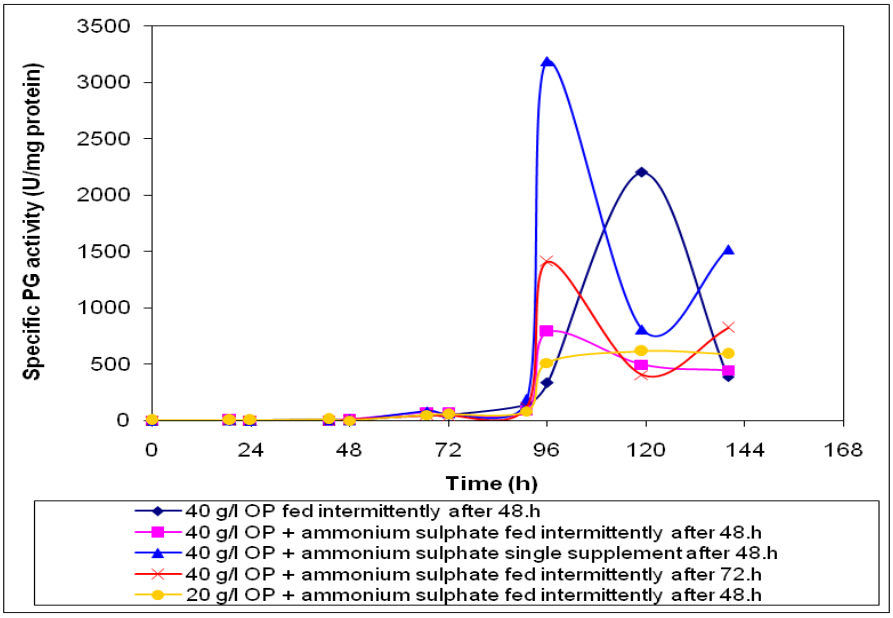


Figure 12.3. Specific PG activity profiles of each fed batch fermentation.

Biomass production was maximum (15 g/l) at 96.h, when 40 g/l OP was fed intermittently after 48.h (Figure 12.4). This result indicated that presence of nitrogen source such as ammonium sulphate in the fermentation medium promoted product formation, but not the biomass production (Figure 12.1 and 12.4). Therefore in the absence of ammonium sulphate, when the feeding was performed intermittently biomass production could be stimulated.

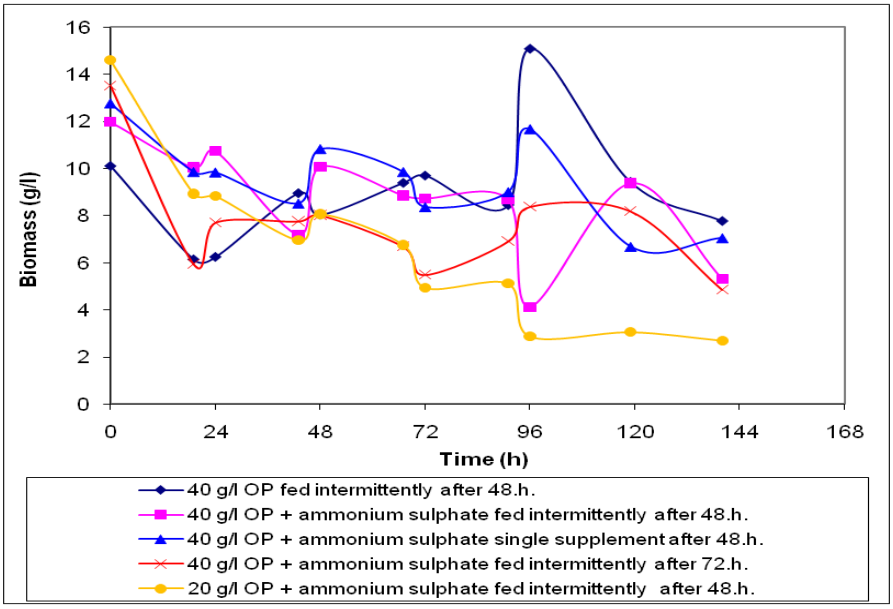


Figure 12.4. Biomass profiles of each fed batch fermentation.

PG activity and productivity values were maximum when 40 g/l OP + ammonium sulphate was fed as a single supplement after 48.h. When compared with the PG activity yield and productivity values of batch experiments performed with WOP and EOP in Section 10.2, fed-batch fermentation increased the yield 60% and 54% more than that of WOP and EOP, respectively. PG productivity value was also increased with the fed-batch fermentation namely 44% and 67% for WOP and EOP, respectively (Table 12.1).

Table 12.1. Yield and productivities of fed batch and batch fermentations.

Feeding type	**PG activity yield coefficient ($Y_{P/S}$) (U activity / mg substrate)**	**Productivity (U / ml.h)**
40 g/l WOP fed intermittently after 48.h.	18.080	0.742
40 g/l WOP + ammonium sulphate fed intermittently after	22.398	0.944
40 g/l WOP + ammonium sulphate single supplement after	27.006	1.334
40 g/l WOP + ammonium sulphate fed intermittently after	19.337	0.681
20 g/l WOP + ammonium sulphate fed intermittently after	17.604	0.785
Batch (with EOP)	**12.560**	**0.440**
Batch (with WOP)	**10.774**	**0.751**

Fungal fed-batch fermentations are very common in literature. In a study performed by Tang et al. (2011), the fed-batch fermentationof *G. lucidum* was successfully scaled-up from 7.5- to 200-LSTR based on the integrated scale-up criterion. The *Aspergillus niger* strain was grown inbatch, continuous (chemostat) and fed-batch cultivations in order to study the production of the extracellular enzyme glucoamylase under different growth conditions in a study performed by Pedersen et al. (2000). Furthermore the relationship between hyphal vacuolation, fragmentation and citric acid production by *Aspergillus niger*, was investigated in batch and fed-batch culture by Papagianni et al. (1999). Karimi et al. (2005) studied the fed-batch cultivation of *Mucor indicus* in dilute-acid lignocellulosic hydrolyzate for ethanol production and found that filamentous growth, which is regarded as the main obstacle to

large-scale cultivation of*M. indicus*, was avoided in the fed-batch experiments. Additionally Bodizs et al. (2007) implemented oxygen control by manipulating the substratefeed rate, i.e. the rate of oxygen consumption in *Aspergillus oryzae* fermentation. Also the effect of fed-batch process strategy on broth viscosity, and productivity of an industriallyrelevant recombinant enzyme (glucoamylase) was evaluated by Bhargava et al. (2003) and found that more carbon addition in pulse-fed fermentations led to increased enzyme productivity by as much as 75%. This finding was compatible with the current study indicating that fed-batch fermentation strategy increases the enzyme productivity dramatically.For the bioprocessing industry this kind of a simple process modificationwhich is likely to cost very little to be implemented in mostproduction facilities, has the potential to substantially increase productivity.

Besides, the pictures from the 1 ml sample from corresponding time point representing each fed batch experiment were taken and given in Figure 12.5. It was observed that morphology was in the form of small pellets for all the conditions. But at the optimum condition for maximum PG activity, where 40 g/l OP + ammonium sulphate was fed as a single supplement after 48.h, pellets were more homogeneous during the fermentation.

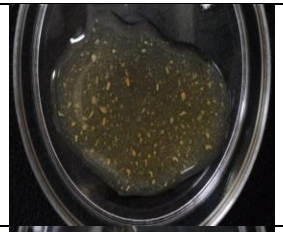
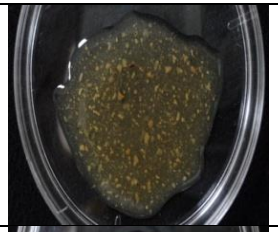
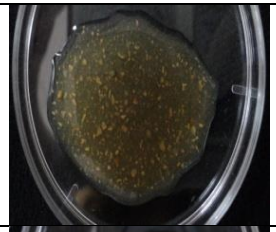
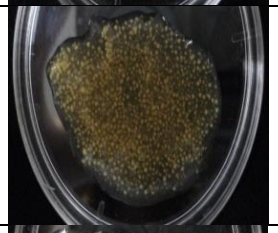
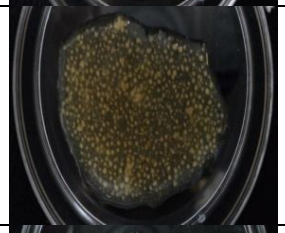
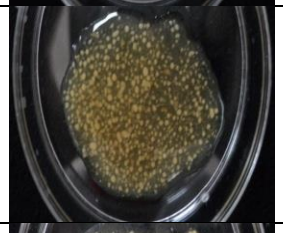
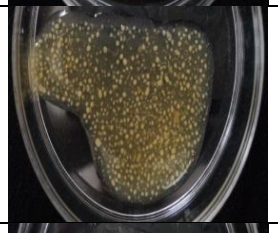
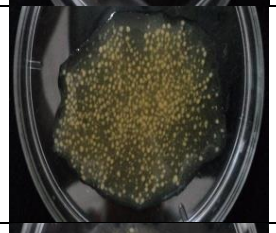
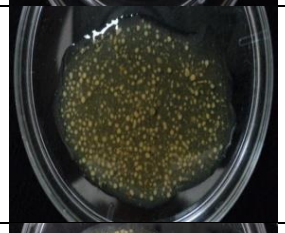
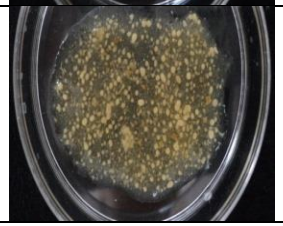
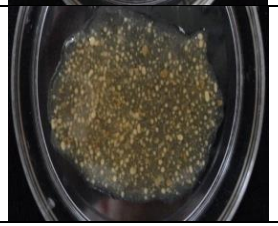
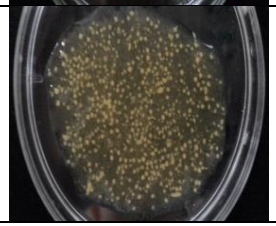
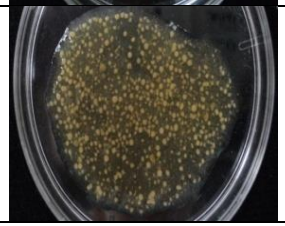
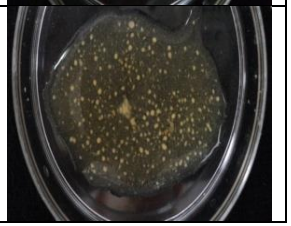
	40 g/l OP fed intermittently after 48.h.	40 g/l OP + ammonium sulphate fed intermittently after 48.h.	40 g/l OP + ammonium sulphate single supplement after 48.h.	40 g/l OP + ammonium sulphate fed intermittently after 72.h.	20 g/l OP + ammonium sulphate fed intermittently after 48.h.
24.h					
48.h					
72.h					
96.h					

Figure 12.5. Morphology of samples representing each fed-batch experiment.

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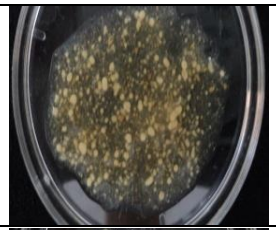
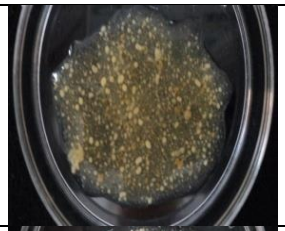
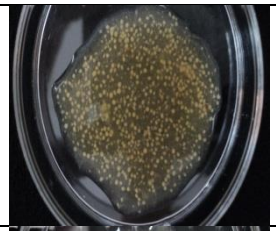
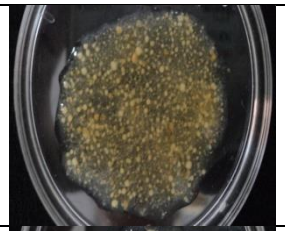
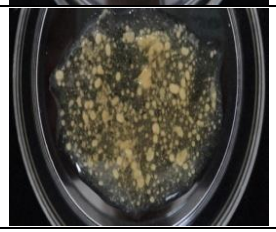
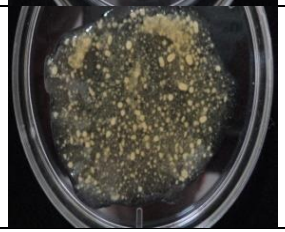
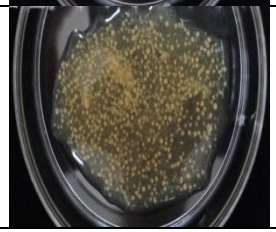
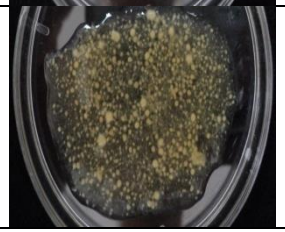
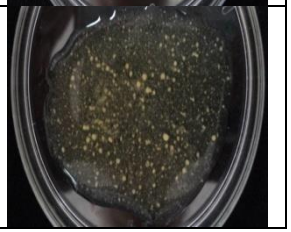
119.h					
140.h					

Figure 12.5. (cont.)

CHAPTER 13

PARTIAL CHARACTERIZATION OF CRUDE POLYGALACTURONASE ENZYME

The characterized PG enzyme was obtained from *A.sojae*mutant strain in bioreactor at the conditions of 0.5 vvm aeration rate, 600 rpm agitation rateand 30ºC fermentation temperature. Fermentation medium consisted of 40 g/lorange peel and 2.75 g/lammonium sulphatewith initial pH of 4.25, inoculation rate of $2.8x10^3$ spor/ml,and incubation time of 166 h. The effect of pH on the obtained crude PG enzyme activity and stability was characterized using the buffer solutions whose pH ranged between pH 3-12. The effect of temperature on the PG activity and stability was investigated between 25 - 80ºC.

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

The effect of pH on PG activity was determined by using various buffers ranging from3.0 to 12.0 as detailed in Section 5.15.1. As can be seen in Figure 13.1, the produced PGenzyme showed significant activity only at the acidic pH region (4.0 – 5.0). There was an activity difference of 7% between pH 4.8 and 5 and 32% between pH 4 and 5. Its activitywas very low at pH 6.0 and did not show any activity at the other pH values. Current crude enzyme preperation was found to be atypical acidic pectinase which shows its activity on the polygalacturonic acid under theacidic region of pH 4.0 to 5.0. This result confirmed the use of reaction mixture at pH4.8 in all of the PG activity assays conducted in this study. Sakamoto et al. (2002) also revealed that the PG enzyme produced from *Aspergillus niger*had optimum pH at the acidic region. Similarly, PG enzymes produced from *Fusarium moniliforme*showed optimum activity at pH 4.8 and 5.3 in the study performed by Niture and Pant (2004). In the study of Dinu et al. (2007), they determined that the purified PG produced by *Aspergillus niger*had optimum activity at pH 4.6 and lost its activity rapidly above pH 5.0 similar to the current study.

As far as it is known PG enzyme is generally used in the clarification of fruit juices in the industry. Therefore the PG enzyme produced in the current study may have potential to be used in the clarification of fruit juices as most of them have acidic pH values.

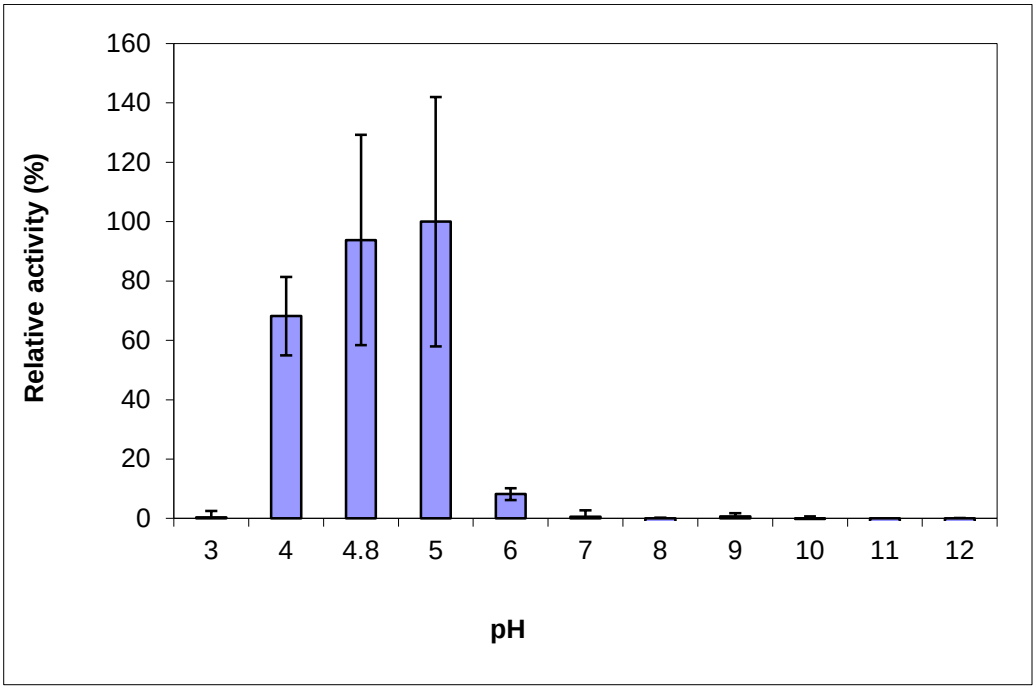


Figure 13.1. Effect of pH on the activity of PG

The stability of the mutant *Aspergillus sojae* PG was investigated in the pH rangeof 3.0 - 12.0. The residual activities (%) which remained after incubating the enzyme at various buffers (Section 5.15.1) for 2 hours at 30 °C were presented in Figure 13.2. Enzyme activity was measured by the standard activity method. The results of the PG stability study showed that the crude enzyme was stable in a broad pH range of 3.0 to11.0 by preserving at least 50% of its activity. After the 2 hours incubation of the enzyme at the current pH values, activity was increased at pH 3, 4, 5 ve 8 relative to the control (pH 4.8). Additionally, at pH 7, 9 and 11, PG enzyme preserved its activity quite good with a maximum loss of 17%. Maximum activity loss was observed at pH 12 with a 30% residual activity. Similar findings were reported by Thakur et al. (2010) in the study performed with PG produced by *Mucor circinelloides*. In that study they claimed that after 4 hours of incubation maximum stability was observed between pH 4.5 and

6.5. The crude PG enzyme of the current study obtained from mutant *Aspergillus sojae* was thought to serve better stability when compared to the crude PG enzyme produced by submerged fermentation from mutant *Aspergillus sojae* ATCC20235 which was reported to preserve its stability (more than 65%) only at pH 5.0 and6.0 (Tari et al., 2008). Consequently current crude PG enzyme preserved its stability at acidic and neutral environment and also partially at basal environment. Therefore it can be an advantage that should be considered in the packaging and storage of the possible end product.

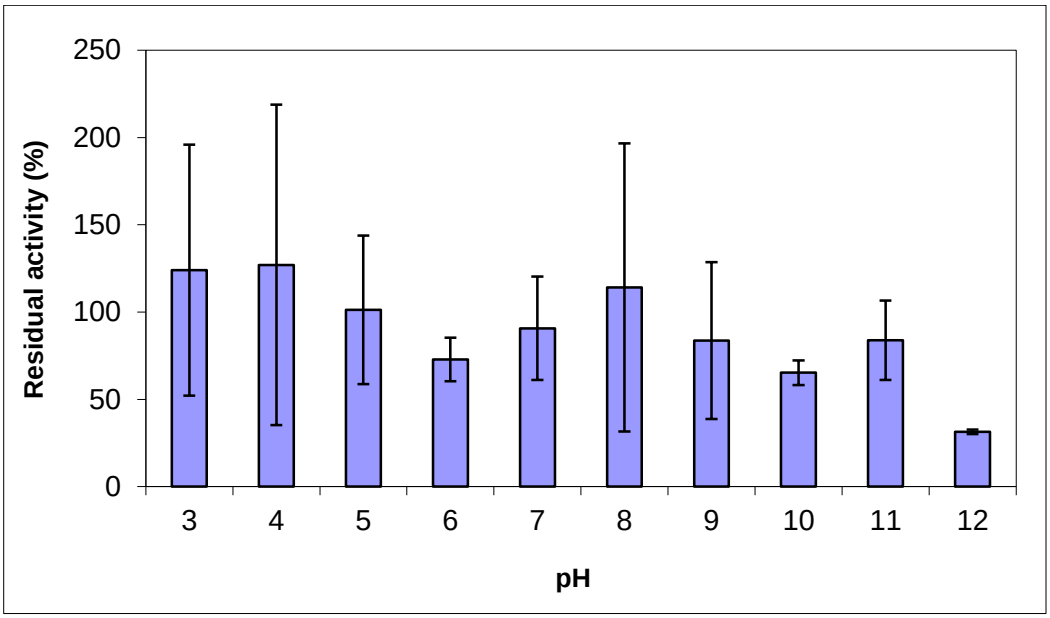


Figure 13.2. Effect of pH on the stability of PG

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

The effect of temperature on the crude PG activity was determined by the incubationof 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 13.3.The PG showed activity over a broad range of temperatures (25 – 80°C) increasing linearly up to 60°C.However, the optimum temperature for maximum PG activity was observed at 60°C,where it decreased sharply at 70 and 80°C. Optimum temperature of 60°C was also determined for exo-PG activity produced from *Bacillus* by Kobayashi et

al. (2001). Furthermoremost of the researchers determined the optimum temperatures of their exo-PGenzymes (fungal or bacterial) between 40 and 60°C (Dinu et al., 2007; Freitas et al., 2006; Kapoor et al., 2000) which is compatible with the current PG enzyme. The optimum temperature (60°C) of the mutant *Aspergillus sojae* PG is very suitable for fruit juice clarification applicationswhich are generally held between 30 – 50°C. But PG enzyme shouldn't be kept too long at the temperatures above 50°C, as it is not active above this temperature.

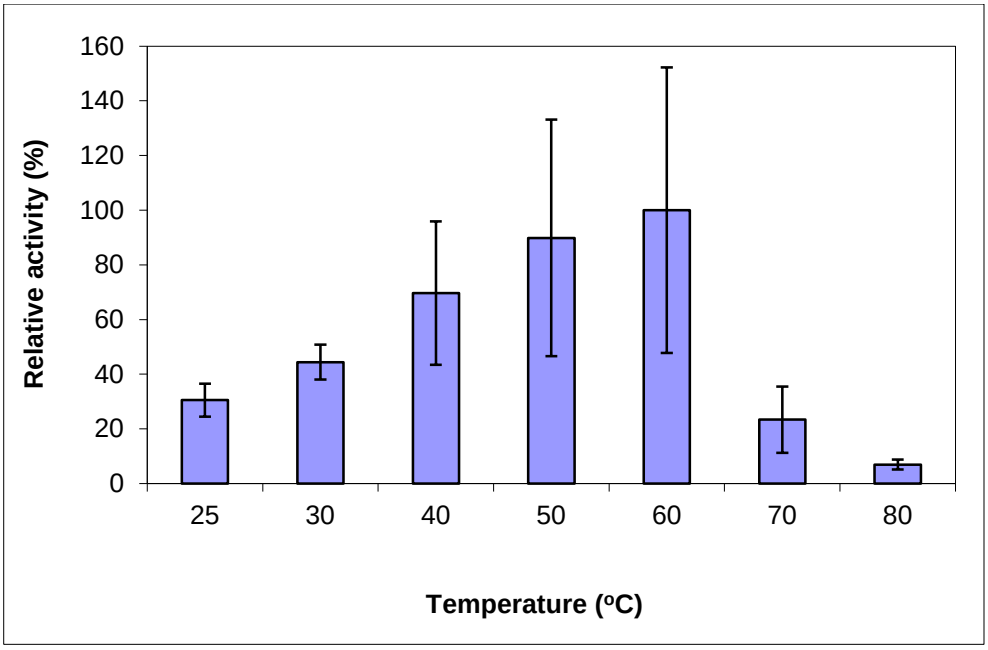


Figure 13.3. Effect of temperature on the activity of PG.

In order to investigate the thermostability of the PG, the enzyme was incubatedat various temperatures between 25– 80°C for 30 and 60 minutes. The residualactivities (%) after this incubation periods were plotted in Figure 13.4.As it can be seen in Figure 13.4, there was not any significant difference between30 and 60 minutes of incubation with respect to the thermostability of the enzyme. The mutant *Aspergillussojae* PG could preserve more than 60% activity between 25 and 40°C, bothfor 30 and 60 minutes of incubation. However, the enzyme could not present any thermostability above 40°C. This result indicated that the current PG has sufficient thermostabilityat the enzymatic depectinization of fruit juices held between 30–50°C. For example the process

conditions for the enzymatic clarification were optimized as 0.1% enzymeconcentration, at 40ºC for 120 minutes by Sin et al. (2006). However, the thermostability range of mutant *Aspergillus sojae* PGwas narrower when compared to the other studies of our group performed using *Aspergillus sojae* ATCC20235 (Tari et al., 2008).

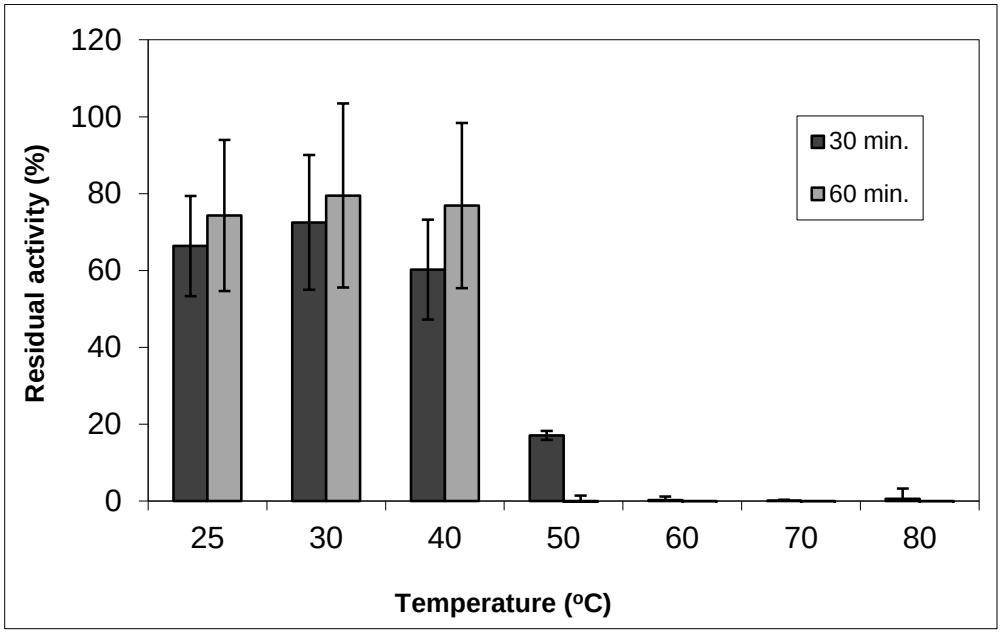


Figure 13.4. Effect of temperature on the stability of PG

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

In the industry the removal of the PG enzyme from the environment or its inactivation after it is used for the clarification process has a significant importance. Therefore determination of its thermal inactivation is essential.

In general, temperature is the most important variable in all biological systemsand in enzymatic processes as well. Enzymes are known to be complex labile proteinsand biocatalysis occurs under non-natural conditions, where the native properties of theenzymes can be significantly altered. Therefore it is not surprising that the temperature has a profound impact not only in enzyme activity but also on its stability. As a general rule, as the temperature increases, the reaction rate of the catalyzed chemical reaction increases, where the rate of the enzyme inactivation

increases,as well. The thermalinactivation of the enzyme is a result of weakening of the intermolecular forces that areresponsible for the 3D structure of the enzyme. The thermal inactivation causes areduction in catalytic capacity of the enzyme. Therefore, the knowledge on the thermalinactivation of the enzyme is critical to properly evaluate the performance of theenzyme under the process conditions (Illanes et al., 2008).

The inactivation of pectolytic enzymes is assumed to follow first order kinetics (Naidu and Panda, 2003). In fact the first-order deactivation rate constants were calculatedfrom the slope of semi logarithmic plot of residual activity versus time (Figure 13.5) andpresented in Table 13.1 for the temperatures of 30, 40, 50 and 60 °C. The lower k_d (deactivation rate constant) valuesat 30°C and 40°C supported the enzyme stability at 30°C and 40°C, andits easy inactivationafter 50°C.

Half-life values of the enzyme were estimated from the calculated k_d values using the Equation 5.17 and given in Table 13.1. The half-life dramatically reduced from 182.41 min. at 30 °C to 2.01 min at 60°C.

The inactivation energy (E_d) of crude mutant *A. sojae* polygalacturonase was determinedas 148.99 kJ $mol^{-1}$from the slopes of the linear curve plotted by 1/T versus ln (k_d) usingEquation 5.18 (Figure 13.6). The partially purified PG of *A. sojae* ATCC 20235 was found to have a higher E_d of 286.2 kJ mol^{-1}(Dogan and Tari, 2008), but a similar E_d of 152 kJ $mol^{-1}$was obtained with the crude PG of the same strain (Tariet al., 2008). These findings revealed that the current PG holds potential use in the applications below 50°C; however a further purification may enhance some of itsbiochemical properties.

Tablo 13.1. Thermal inactivation kinetic parameters of *Aspergillus sojae* crude exo-polygalacturonase enzyme.

T (ºC)	k_d (min^{-1})	$t_{1/2}$ (min)	T (ºK)
30	0.004	182.407	303
40	0.004	157.534	313
50	0.307	2.258	323
60	0.345	2.007	333

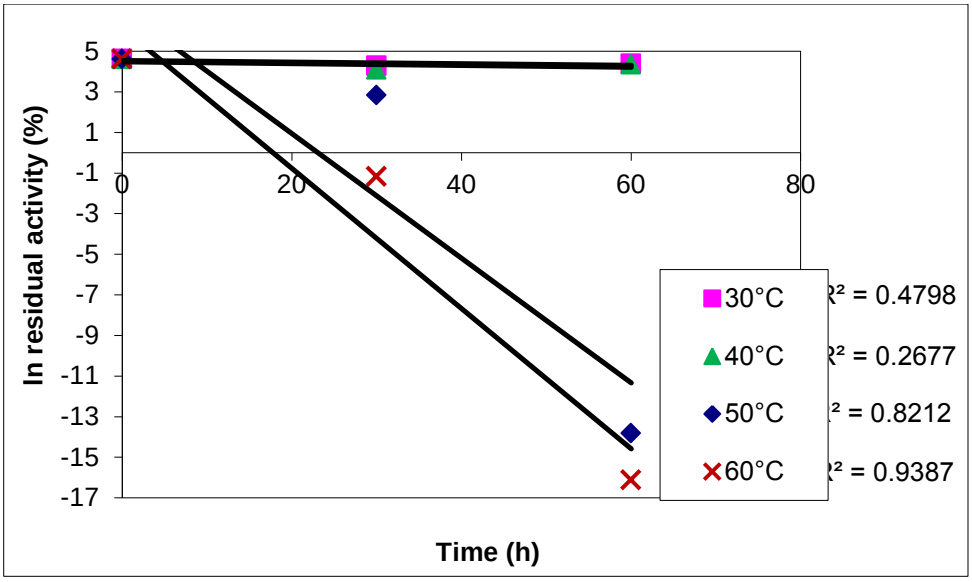


Figure 13.5. First-order plot of the effect of thermal denaturation of mutant *A. sojae* polygalacturonase.

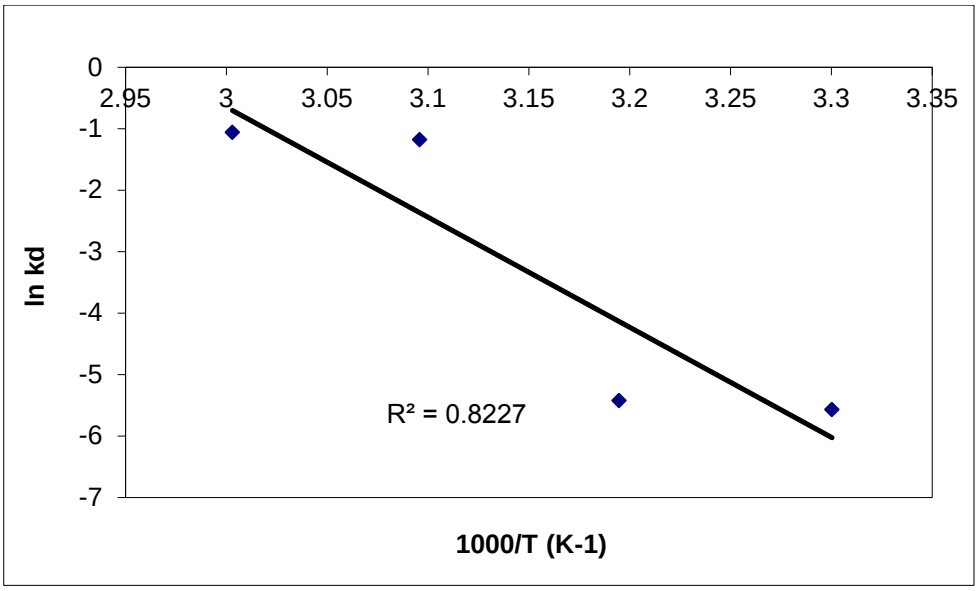


Figure 13.6. Arrhenius plots for the determination of thermal inactivation energy of mutant *A.sojae* polygalacturonase.

13.4. Estimation of Thermodynamic Parameters during Inactivation of Polygalacturonase

The changes in the enthalpy (ΔH*), free energy of inactivation (ΔG*) and entropy (ΔS*) for the thermalinactivation of the polygalacturonase were calculated according to the Equation 5.20, 5.21 and 5.22 by the transition state theory (Naidu and Panda, 2003). Positive ΔH* values were obtained with the investigated temperatures indicating the endothermic nature of theinactivation reaction. The free energy of inactivation (ΔG*) decreased slightly as thetemperature increased. This means that less energy was needed for inactivation of theenzyme at 50°C and 60°C, which was in accordance with the thermal stability results given inSection 13.2.Naidu and Panda (2003) predicted that entropy values provide informationregarding the degree of solvation and very likely the degree of compactness of proteinmolecule. The increase in entropy indicates opening up of the enzyme structure (Bhattiet al., 2006). As can be seen in Table 13.2, ΔS* did not change significantly with theincreasing temperature, which means that thermal deactivation did not imply any relevant variationin the enzyme tertiary structure. Moreover, Ortega et al. (2004) stated that largeactivation enthalpy is the characteristic of protein denaturation reaction. However, ΔH* values did not vary with the increasing temperature, supporting the idea of enzymeunfolding may

not be the rate-determining step for the irreversible thermal inactivation of PG under the conditions assayed.

Tablo 13.2. Thermodynamic parameters of crude polygalacturonase enzyme during inactivation.

T (°K)	ΔH^* (kJ mol^{-1})	ΔG^* (kJ mol^{-1})	ΔS^* (kJ $mol^{-1}K^{-1}$)
303	146.476	88.292	0.192
313	146.393	90.909	0.177
323	146.309	82.497	0.198
333	146.226	84.809	0.184

13.5. Molecular Weight Investigation of Polygalacturonase for WOP and EOP

The SDS-PAGE profiling of the crude enzymes produced by both of the substrate types at 60 g/l concentration, producing the highest PG activity was performed inorder to see if same enzymeswere produced. As it is seen from Figure 13.7 for the two types of substrates similar enzymes with the same molecular weight was produced. Two bands were seen in the profile at approximately 32 kDa and 42 kDa molecular weight. 42 kDa was compatible with the molecular weight of purified endo-polygalacturonase from *Aspergillus awamori* (41 kDa) determined by Nagai et al.(2000). Gadre et al 2003 also found that the endo-polygalacturonase from *Mucor flavus* has 40 kDa molecular weight. In one of the studies performed by Buga et al 2010, partially purified polygalacturonase from *Aspergillus niger* also showed two bands proposing an endo and exo PG with apparent molecular weights of 35 and 40 kDa, respectively. Therefore according to this study our bands with 32 kDa and 42 kDa may also belong to endo and exo PG, respectively.

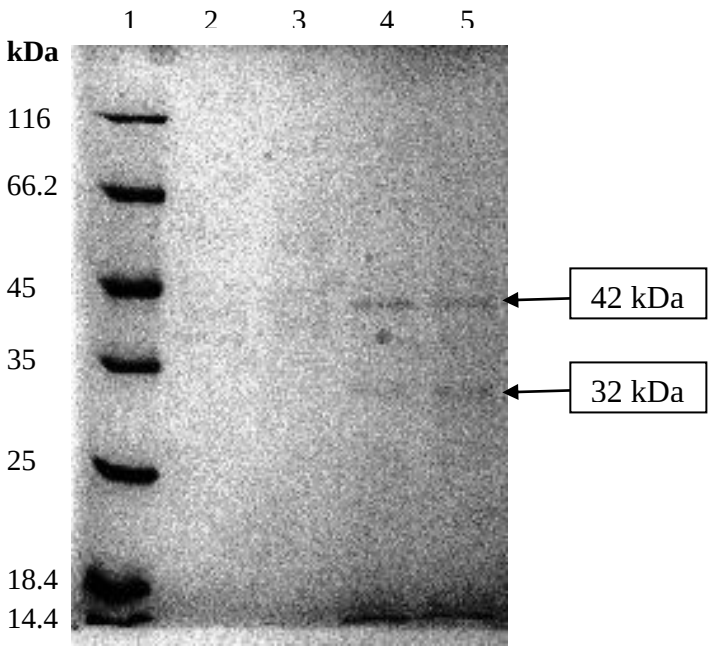


Figure 13.7. SDS-PAGE of crude enzymes produced by whole orange peel and extracted orange peel (10µl sample) 1: marker, 2: commercial pectinase 1 from *Aspergillus niger*, 3: commercial pectinase 2 from *Aspergillus niger*, 4: crude enzyme produced with 60 g/l whole orange peel, 5: crude enzyme produced with 60 g/l extracted orange peel.

CHAPTER 14

CONCLUSION

Nowadays the most significant demand of the bioindustry is the maintanance of the process under optimum conditions, in order to increase the productivity (Koutinas et al 2003). For this purpose the relationship between the growth of filamentous fungal cells and the expression of their products needs to be investigated for the optimization of industrial fungal processes. However the complex structure of the submerged fungal fermentations makes the kinetic parameter estimations difficult due to the problems like settling of mycelial clumps, less uniform distribution of biomass and additionally the use of heterogenous medium. In this study, it was aimed to define the kinetic parameters of *Aspergillus sojae* mutant strain in submerged fermentation by using a complex medium, orange peel. Initially the fermentation medium was optimized using experimental design tools and then the fermentation parameters were optimized in 1l scale serial bioreactor system for the first time. The kinetic parameters were evaluated by using these optimized conditions and the problems encountered in kinetic parameters determination study was tried to be solved. The important findings of the study were summarized below.

- In the determination of process parameters (section 6.2), the optimized fermentation conditions were, 600 rpm agitation speed, uncontrolled pH and 1.5 vvm aeration rate. But as it was seen in section 6.3., 0.5 vvm aeration rate also gave similar PG activity value at 20 l scale. Therefore due to the low cost and energy saving advantage, 0.5 vvm can also be used in future experiments. The media formulation used in sections 6.2 and 6.3 resulted in higher PG activity values than the more simple media formulation used in section 6.4, but as this media is more economical it can also be used if the main purpose is to perform a cost effective fermentation.
- According to the results of the 1 l scale bioreactor study, the highest enzyme activity (108.87 U/ml) was obtained with 40 g/l orange peel concentration at the end of the fermentation.
- Estimation of the concentration of biomass in fermentation broths of *A. sojae* could not be performed by spectrophotometric turbidity measurements on

homogenized sample of broths as there was no exact correlation between OD measurements and direct dry weight measurements. Also calculation of OD and dry weight data by discarding the substrate effect with sample blank did not give proper specific growth rate results.

- Additionally PG activity and biomass was found to be affected by the state of orange peel in shake flask experiments and the PG values obtained with whole orange peel (227.47 U/ml) was higher than that obtained with extracted orange peel (153.07 U/ml).
- Also at the end of the fermentation performed to compare the effect of substrate concentration and its state, it was found that with EOP, maximum PG activity (71.19 U/ml) was achieved at 40 g/l orange peel concentration. Finally WOP resulted in 35% more PG activity and 71% more biomass compared to that of EOP probably due to the complex structure of WOP.
- The biomass values of the WOP experiment was calculated by using sample blanks of the defined sample times in order to eliminate the problems of biomass determination in fungal fermentations like the growth of the strain on the solid particle. According to the kinetic parameters of both of the substrate states, the growth of *Aspergillus sojae* mutant strain in EOP was faster than that of WOP. As a result WOP produced higher PG activity and biomass when compared to EOP, which may be due to the complex structure of WOP. The composition of EOP was thought to be simple because of the lack of pectin or cellulosic compounds which were removed from the medium during extraction procedure.
- According to the results of the bioreactor study performed with the higher WOP concentrations (20, 25, 30, 40, 50, and 60 g/l) maximum PG activity (249.49 U/ml) was achieved with 60 g/l OP concentration at the end of fermentation (139.h) which was quite higher than our previous findings. According to the results of the Lineweaver Burke plot the maximum specific growth rate of *Aspergillus sojae* mutant strain was estimated as 0.068 h^{-1} with naturallogarithmic approach which was very low compared to literature. Lower saturation constant values (K_s) were calculated by Hanes Woolf plot (17.38 g/l and 57.29 g/l for naturallogarithmic and cubic root approaches, respectively) than that of Lineweaver Burke plot (152.61 g/l) which indicated higher affinity to WOP.

- Fed-batch experiments revealed that, fed-batch fermentation technique increased the PG production yield 60% and 54% more than that of batch WOP and EOP, respectively. Productivity value was also increased with the fed-batch fermentation 44% and 67% for WOP and EOP, respectively. While maximum PG activity (186.78 U/ml) was obtained when 40 g/l WOP + ammonium sulphate was fed as a single supplement after 48.h, maximum biomass (15 g/l) was achieved when 40 g/l WOP was fed intermittently after 48.h.
- According to the biochemical characterization results, the produced crude PGenzyme showed significant activity only at the acidic pH region (4.0 – 5.0)and it was stable in a broad pH range of 3.0 to11.0 by preserving at least 50% of its activity. Optimum temperature for maximum PG activity was observed at 60°C, however the enzyme could not present any thermostability above 40°C. The inactivation energy (E_d) of crude mutant *A. sojae* polygalacturonase was determined as 148.99 kJ mol^{-1}.
- SDS-PAGE profiles for the WOP and EOP states gave the same bands. Two bands were seen in the profile at approximately 32 kDa and 42 kDa molecular weight. Finally it can be said that extracted orange peel can be used as substrate in further studies to determine the kinetic growth parameters but the PG activity needs to be increased by optimization.

Overall the current study serves a starting point for the further studies in terms of biomass determination which is an essential parameter in the estimation of kinetic variables and scale up of the process.

REFERENCES

Abd-Aziz, S.; Fernandez, C.C.; Salleh, M.M.; Illias, R.M.; Hassan, M. A. Effect of Agitation and Aeration Rates on Chitinase Production Using *Trichoderma virens* UKM1 in 2-l Stirred Tank Reactor. *Appl Biochem Biotechnol.***2008**, 150, 193–204.

Ahamed, A.; Vermette, P. Enhanced Enzyme Production from Mixed Cultures of Trichoderma reesei RUT-C30 and *Aspergillus niger* LMA Grown as Fed Batch in a Stirred Tank Bioreactor. *Biochemical Engineering Journal***2008**, 42, 41–46.

Alkorta, I.; Garbisu, C.; Llama, M.J.; Serra, J.L. Industrial Applications of Pectic Enzymes: a Review. *Process Biochemistry***1998**, 33, 21-28.

Amanullah A.; Christensen L.H.; Hansen K.; Nienow A.W., Thomas C.R. Dependence of Morphology on Agitation Intensity in Fed-Batch Cultures of *Aspergillus oryzae* and Its Implications for Recombinant Protein Production. *Biotechnology and Bioengineering***2002**, 77, 815-826.

Ardestani, F. Survey of the Nutrient Utilization and Cell Growth Kinetic with Verhulst, Contois and Exponential Models for *Penicillium brevicompactum* ATCC 16024 in Batch Bioreactor. *World Applied Sciences Journal***2012**, 16, 135-140.

Aynsley M.; Ward A.C.; Wright A.R. A Mathematical Model for the Growth of Mycelial Fungi in Submerged Culture. *Biotechnology and Bioengineering***1990**, 35, 820-830.

Bai, Z.H.; Zhang, H.X.; Qi, H.Y.; Peng, X.W.; Li, B.J. Pectinase Production by *Aspergillus niger* Using Wastewater in Solid State Fermentation for Eliciting Plant Disease Resistance. *Bioresource Technology***2004**, 95, 49–52.

Bailey, M.J. Effect of Temperature on Polygalacturonase Production by *Aspergillus niger*. *Enzyme Microb. Technol*. **1990**, 12, 622-624.

Banerjee, U.C.; Chisti, Y.; Moo-Young, M. Spectrophotometric Determination of Mycelial Biomass. *Biotechnology Techniques***1993**, 7, 313-316.

Belmessikh, A.; Boukhalfa, H.; Mechakra-Maza, A.; Gheribi-Aoulmi, Z.; Amrane A. Statistical Optimization of Culture Medium for Neutral Protease Production by *Aspergillus oryzae*. Comparative study between solid and submerged fermentations on tomato pomace. *Journal of the Taiwan Institute of Chemical Engineers***2013**, 44, 377–385.

Bettin, F.; Osório da Rosa, L.; Montanari, Q.; Calloni, R.; Gaio, T.A.; Malvessi E.; Moura da Silveira, M.; Dillon, A.J.P. Growth Kinetics, Production, and Characterization of Extracellular Laccases from *Pleurotus sajor-caju* PS-2001. *Process Biochemistry***2011**, 46, 758–764.

Bhargava, S.; Nandakumar, M.P.; Roy, A.; Wenger, K.S.; Marten, M.R. Pulsed Feeding During Fed-Batch Fungal Fermentation Leads to Reduced Viscosity without Detrimentally Affecting Protein Expression. *Biotechnology and Bioengineering* **2003**, 81, 341-347.

Bhatti, H.N.; Muhammad, A.; Nawaz, A.R.; Sheikh, M.A. Studies on Kinetics and Thermostability of a Novel Acid Invertase from *Fusarium solani*. *J. Agric. Food Chem*. **2006**, 54, 4617-4623.

Blandino, A.; Dravillas, K.; Cantero, D.; Pandiella, S.S.; Webb, C. Utilisation of Whole Wheat Flour for the Production of Extracellular Pectinases by Some Fungal Strains. *Process Biochemistry***2001**, 37, 497–503.

Blandino, A.; Iqbalsyah, T.; Pandiella, S.S.; Cantero, D.; Webb, C. Polygalacturonase Production by *Aspergillus awamori* on Wheat in Solid-State Fermentation. *Appl Microbiol Biotechnol.***2002**, 58, 164–169.

Bodizs, L.; Titica, M.; Faria, N.; Srinivasan, B.; Dochain, D.; Bonvin, D. Oxygen control for an industrial pilot-scale fed-batch filamentous fungal fermentation. *Journal of Process Control*. **2007**, 17, 595–606.

Boswell, G.P.; Jacobs, H.; Davidson, F.A.; Gadd, G.M.; Ritz, K. Growth and Function of Fungal Mycelia in Heterogeneous Environments. *Bulletin of Mathematical Biology***2003**, 65, 447–477.

Buga, M.L., Ibrahim, S. and Nok, A.J. Partially Purified Polygalacturonase from *Aspergillus niger* (SA6). *African Journal of Biotechnology***2010**, 52, 8944-8954.

Casas Lopez, J.L.; Sanchez Perez, J.A.; Fernandez Sevilla, J.M.; Rodriguez Porcel E.M.; Chisti, Y. Pellet Morphology, Culture Rheology and Lovastatin Production in Cultures of *Aspergillus terreus*. *Journal of Biotechnology***2005**, 116, 61–77.

Chang, P-K.; Matsushima, K.; Takahashi, T.; Yu, J.; Abe, K.; Bhatnaga, D.; Yuan, G-F.; Koyama, Y.; Cleveland, T.E. Understanding Nonaflatoxigenicity of *Aspergillus sojae:* a Windfall of Aflatoxin Biosynthesis Research. *Appl Microbiol Biotechnol*. **2007**, 76, 977–984.

Chang, P-K., Ehrlich, K.C., and Hua, S.S.T. Cladal Relatedness among Aspergillus oryzae Isolates and *Aspergillus flavus* S and L Morphotype Isolates. *International Journal of Food Microbiology*. **2006**, 108, 172-177.

Chiang, C-C.; Chiang, B-H. Processing Characteristics of Submerged Fermentation of *Antrodia cinnamomea* in Airlift Bioreactor. *Biochemical Engineering Journal*. **2013**, 73, 65– 71.

Chien, H-C.R.; Lin, L-L.; Chao, S-H.; Chen, C-C.; Wang, W-C.; Shaw, C-Y.; Tsai, Y-C.; Hu, H-Y.; Hsu, W-H. Purification, Characterization, and Genetic Analysis of a Leucine Aminopeptidase from *Aspergillus sojae*. *Biochimica et Biophysica Acta*. **2002**, 1576, 119–126

Couri, S.; Pinto, G.A.S.; de Senna, L.F.; Martelli, H.L. Influence of Metal Ions on Pellet Morphology and Polygalacturonase Synthesis by *Aspergillus niger* 3T5B8. *Brazilian Journal of Microbiology*. **2003**, 34, 16-21.

Cui, Y.Q.; van der Lans, R. G. J. M.; Giuseppin, M. L. F.; Luyben, K. C. A. M. Influence of Fermentation Conditions and Scale on the Submerged Fermentation of *Aspergillus awamori*. *Enzyme and Microbial Technology*. **1998a**, 23, 157–167.

Cui, Y.Q.; Ouwehand, J.N.W.; van der Lans, R.G.J.M.; Giuseppin, M.L.F.; Luyben, K.C.A.M. Aspects of the Use of Complex Media for Submerged Fermentation of *Aspergillus awamori*. *Enzyme and Microbial Technology*. **1998b**, 23, 168–177.

Cunha, F.M.; Esperanca, M.N.; Zangirolami, T.C.; Badino, A.C.; Farinas, C.S. Sequential Solid-State and Submerged Cultivation of *Aspergillus niger* on Sugarcane Bagasse for the Production of Cellulase. *Bioresource Technology* **2012**, 112, 270–274.

De Nicolás-Santiago, S.; Regalado-González, C.; García-Almendárez, B.; Fernández, F.; Téllez-Jurado, A.; Huerta-Ochoa, S. Physiological, Morphological, and Mannanase Production Studies on *Aspergillus niger* uam-gs1 Mutants. *Electron J. Biotechnol.* **2006**, 9, 51-60.

Dhillon, S.S.; Gill, R.K.; Gill, S.S.; Singh, M. Studies on the Utilization of Citrus Peel for Pectinase Production Using Fungus *Aspergillus niger*. *International Journal of Environmental Studies.* **2004**, 61, 199–210.

Díaz-Montaño, D.M. Continuous Agave Juice Fermentation for Producing Bioethanol, Biomass Now - Sustainable Growth and Use. Dr. Miodrag Darko Matovic (Ed.), 2013, Chapter 8, ISBN: 978-953-51-1105-4, InTech, DOI: 10.5772/55923. Available from: http://www.intechopen.com/books/biomass-now-sustainable-growth-and-use/continuous-agave-juice-fermentation-for producing-bioethanol.

Dinu, D.; Nechifor, M.T.; Stoian, G.; Costache, M.; Dinischiotu, A. Enzymes with New Biochemical Properties in the Pectinolytic Complex Produced by *Aspergillus niger* MIUG 16. *J. Biotechnol*. **2007**, 131, 128-137.

Djekrif-Dakhmouche, S.; Gheribi-Aoulmi, Z.; Meraihi, Z.; Bennamoun, L. Application of a Statistical Design to the Optimization of Culture Medium for α-amylase Production by *Aspergillus niger* ATCC 16404 Grown on Orange Waste Powder. *Journal of Food Engineering***2006**, 73, 190–197.

Dogan, N.; Tari, C. Characterization of Three-phase Partitioned Exo-Polygalacturonase from *Aspergillus sojae* with Unique Properties. *Biochemical Engineering Journal***2008**, 39, 43-50.

Dorn, G.; Rivera, W. Kinetics of Fungal Growth and Phosphatase Formation in *Aspergillus nidulans. Journal of Bacteriology***1966**, 92, 1618-1622.

Dubois, M.; Gilles, K.A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem*. **1956**, 28, 350-356.

El-Enshasy, H.A. Filamentous Fungal Cultures - Process Characteristics, Products, and Applications. *In Bioprocessing for Value-Added Products from Renewable Resources*, 1st ed.; Yang, S-T., Ed.; Elsevier B.V.: Oxford, 2007; pp. 225-261.

El-Enshasy, H.; Kleine, J.; Rinas, U. Agitation Effects on Morphology and Protein Productive Fractions of Filamentous and Pelleted Growth Forms of Recombinant *Aspergillus niger*. *Process Biochemistry* **2006**, 41, 2103–2112.

El-Sheekh, M.M.; Ismail, A-m.S.; El-Abd, M.A.; Hegazy, E.M.; El-Diwany, A.I. Effective Technological Pectinases by *Aspergillus carneus* NRC1 Utilizing the Egyptian Orange Juice Industry Scraps. *International Biodeterioration & Biodegradation* **2009**, 63, 12–18.

Erjavec, J.; Kos, J.; Ravnikar, M.; Dreo, T.; Sabotic, J. Review: Proteins of Higher Fungi – from Forest to Application. *Trends in Biotechnology* **2012**, 30, 1-15.

Fang, Q-H.; Zhong J-J. Effect of Initial pH on Production of Ganoderic Acid and Polysaccharide by Submerged Fermentation of *Ganoderma lucidum. Process Biochemistry* **2002**, 37, 769–774.

Favela-Torres, E.; Cordova-Lopez, J.; Garda-Rivero, M.; Gutirrrez-Rojas, M. Kinetics of Growth of *Aspergillus niger* during Submerged, Agar Surface and Solid State Fermentations. *Process Biochemistry* **1998**, 33, 103-107.

Fawole, O.B.; Odunfa, S.A. Some Factors Affecting Production of Pectic Enzymes by *Aspergillus niger*. *International Biodeterioration & Biodegradation* **2003**, 52, 223 – 227.

Feng, Y-L.; Li, W-Q.; Wu, X-Q.; Cheng, J-W.; Ma, S-Y. Statistical Optimization of Media for Mycelial Growth and Exo-Polysaccharide Production by Lentinus edodes and a Kinetic Model Study of Two Growth Morphologies. *Biochemical Engineering Journal* **2010**, 49, 104–112.

Fontana, R.C.; da Silveira, M.M. Production of Polygalacturonases by *Aspergillus oryzae* in Stirred Tank and Internal- and External-Loop Airlift Reactors. *Bioresource Technology* **2012**, 123, 157–163.

Freitas, P.; Martin, N.; Silva, D.; Silva, R.; Gomes, E. Production and Partial Characterization of Polygalacturonases Produced by Thermophilic *Monascus* sp. N8 and by Thermotolerant *Aspergillus* sp. N12 on Solid-state Fermentation. *Braz. J. Microbiol.* **2006**, 37, 302-306.

Fu, C-C.; Su, C-H.; Nair, G.R.; Sung, Y-S.; Wu, W-T. Estimation of Fungal Biomass and Lipid Production by Morphological Characteristics of *Mucor rouxii*. *Journal of Bioscience and Bioengineering* **2010**, 110, 367–371.

Gabelle, J-C.; Jourdier, E.; Licht, R.B.; BenChaabane, F.; Henaut, I.; Morchain, J.; Augier, F. Impact of Rheology on the Mass Transfer Coefficient During the Growth Phase of *Trichoderma reesei* in Stirred Bioreactors. *Chemical Engineering Science* **2012**, 75, 408–417.

Gadre et al 2003 Gadre, R.V., Driessche, G.V., Beeumen, J.V., Bhat, M.K. Purification, Characterisation and Mode of Action of an Endo-Polygalacturonase from the Psychrophilic Fungus *Mucor flavus*. *Enzyme and Microbial Technology* **2003**, 32, 321–330.

Garcia, D.; Ramos, A.J.; Sanchis, V.; Marín, S. Modelling Mould Growth under Suboptimal Environmental Conditions and Inoculum Size. *Food Microbiology* **2010**, 27, 909-917.

Göğüş, N.; Tari, C.; Oncü, S.; Unluturk, S.; Tokatli, F. Relationship between Morphology, Rheology and Pectinase Production by *Aspergillus sojae* ATCC 20225 in Submerged Cultures. *Biochem. Eng. J.* **2006**, 32, 171-178.

Gomes, J.; Zeni, J.; Cence, K.; Toniazzo, G.; Treichel, H.; Valduga, E. Evaluation of Production and Characterization of Polygalacturonase by *Aspergillus niger* ATCC 9642. *Food and Bioproducts Processing* **2011**, 89, 281–287.

Goudar, C.T.; Strevett, K.A. Estimating Growth Kinetics of *Penicillium chrysogenum* by Nonlinear Regression. *Biochemical Engineering Journal* **1998**, 191-199.

Gummadi, S.N.; Panda, T. Purification and Biochemical Properties of Microbial Pectinases - A Review. *Process Biochemistry* **2003**, 38, 987-996.

Gummadi, S.N.; Manoj, N.; Kumar, D.S. Structural and Biochemical Properties of Pectinases. *In Industrial Enzymes*; Polaina, J., MacCabe, A.P., Eds.; Springer: Heidelberg, 2007; pp. 99-115.

Illanes, A.; Altamirano, C.; Lorena, W. Homogeneous Enzyme Kinetics, *In Enzyme Biocatalysis Principles and Applications*; Illanes, A., Ed.; Springer: Dordrecht, 2008; pp. 107-154.

Jacob, N.; Poorna, C.A.; Prema, P. Short Communication Purification and Partial Characterization of Polygalacturonase from *Streptomyces lydicus*. *Bioresource Technology* **2008**, 99, 6697–6701.

Jayani, R.S.; Saxena, S.; Gupta, R. Microbial Pectinolytic Enzymes: A Review. *Process Biochem.* **2005**, 40, 2931-2944.

Junker, B.H.; Hesse, M.; Burgess, B.; Masurekar, P.; Connors, N.; Seeley, A. Early Phase Process Scale-Up Challenges for Fungal and Filamentous Bacterial Cultures. *Applied Biochemistry and Biotechnology* **2004**, 119, 241-277.

Kant, S.; Vohra, A.; Gupta, R. Purification and Physicochemical Properties of Polygalacturonase from *Aspergillus niger* MTCC 3323. *Protein Expression and Purification* **2013**, 87, 11–16.

Kapoor, M.; Beg, Q.K.; Bhushan, B.; Dadhich, K.S.; Hoondal, G.S. Production and Partial Purification and Characterization of a Thermo-alkali Stable Polygalacturonase from *Bacillus* sp. MG-cp-2. *Process Biochem*. **2000**, 36, 467-473.

Karimi, K.; Brandberg, T.; Edebo, L.; Taherzadeh, M.J. Fed-batch Cultivation of *Mucor indicus* in Dilute-Acid Lignocellulosic Hydrolyzate for Ethanol Production. *Biotechnology Letters*. **2005**, 27, 1395–1400.

Kashyap et al., 2001; Kashyap, D.R.; Vohra, P.K.; Chopra, S.; Tewari, S. Applications of Pectinases in the Commercial Sector: A Review. *Bioresource Technology* **2001**, 77, 215-227.

Kelly, S.; Grimm, L.H.; Bending, C.; Hempel, D.C.; Krull, R. Effects of Fluid Dynamic Induced Shear Stress on Fungal Growth and Morphology. *Process Biochemistry* **2006**, 41, 2113-2117.

Khairnar, Y.; Krishna, V.K.; Boraste, A.; Gupta, N.; Trivedi, S.; Patil, P.; Gupta, G.; Gupta, M.; Jhadav, A.; Mujapara, A.; Joshi, B.; Mishra, D. Study of Pectinase Production in Submerged Fermentation using Different Strains of *Aspergillus niger*. *International Journal of Microbiology Research* **2009**, 1, 13-17.

Khamseh, A.A.G.; Miccio, Michele. Comparison of Batch, Fed-Batch and Continuous Well-Mixed Reactors for Enzymatic Hydrolysis of Orange Peel Wastes. *Process Biochemistry* **2012**, 47, 1588–1594.

Kim, S.W.; Hwang, H.J.; Xu, C.P.; Choi, J.W.; Yun, J.W. Effect of Aeration and Agitation on the Production of Mycelia Biomass and Exopolysaccharides in an Enthomopathogenic Fungus *Paecilomyces sinclairii*. *Letters in Applied Microbiology* **2003**, 36, 321–326.

Kimura, I.; Sasahara, H.; Tajima, S. Purification and Characterization of Two Xylanases and an Arabinofuranosidase from *Aspergillus sojae. Journal of Fermentation and Bioengineering* **1995**, 80, 334-339.

Kimura, I.; Yoshioka, N.; Tajima, S. Purification and Characterization of a β-glucosidase with β-xylosidase Activity from *Aspergillus sojae. Journal of Bioscience and Bioengineering* **1999**, 87, 538-541.

Kobayashi, T.; Higaki, N.; Yajima, N.; Suzumatsu, A.; Hagihara, H.; Kawal, S.; Ito, S. Purification and Properties of a Galacturonic Acid-releasing Exopolygalacturonase from a Strain of *Bacillus*. *Biosci. Biotechnol. Biochem.* **2001**, 65, 842-847.

Krishna, C., Solid-state Fermentation Systems-An Overview. *Crit. Rev. Biotechnol*. **2005**, 25, 1-30.

Krull, R.; Wucherpfennig, T.; Esfandabadi, M.E.; Walisko, R.; Melzer, G.; Hempel, D.C.; Kampen, I.; Kwade, A.; Wittmann, C. Characterization and Control of Fungal Morphology for Improved Production Performance in Biotechnology. *Journal of Biotechnology* **2013**, 163, 112– 123.

Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, 227, 680-685.

Lara-Marquez, A.; Zavala-Paramo, M.G.; Lopez-Romero, E.; Camacho H.C. Biotechnological Potential of Pectinolytic Complexes of Fungi. *Biotechnol Lett*. **2011**, 33, 859–868.

Larios, G.; Garcia, J.M.; Huitron, C. Endo- Polygalacturonase Production from Untreated Lemon Peel by *Aspergillus* sp. Ch-y-1043. *Biotechnology Letters* **1989**, 11, 729-734.

Li, Y.; Lin, J.; Meng, D.; Lu, J.; Gu, G.; Mao, Z. Effect of pH, Cultivation Time and Substrate Concentration on the Endoxylanase Production by *Aspergillus awamori* ZH-26 under Submerged Fermentation Using Central Composite Rotary Design. *Food Technol. Biotechnol*. **2006**, 44, 473–477.

Lin, E-S.; Chen, Y-H. Factors Affecting Mycelial Biomass and Exopolysaccharide Production in Submerged Cultivation of *Antrodia cinnamomea* using Complex Media. *Bioresource Technology* **2007**, 98, 2511–2517.

Lin, P-J.; Scholz, A.; Krull, R. Effect of Volumetric Power Input by Aeration and Agitation on Pellet Morphology and Product Formation of *Aspergillus niger*. *Biochemical Engineering Journal* **2010**, 49, 213–220.

Lopez-Isunza, F.; Larralde-Corona, C.P.; Viniegra-Gonzalez, G. Mass Transfer and Growth Kinetics in Filamentous Fungi. *Chemical Engineering Science* **1997**, 52, 2629-2639.

Maldonado, M.C.; Saad, A.M.S. Production of Pectinesterase and Polygalacturonase by *Aspergillus niger* in Submerged and Solid State Systems. *J. Ind. Microbiol. Biotechnol*. **1998**, 20, 34-38.

Malvessi, E.; da Silveira, M.M. Influence of Medium Composition and pH on the Production of Polygalacturonases by *Aspergillus oryzae*. *Brazilian Archives of Biology and Technology* **2004**, 47, 693-702.

Mamma, D.; Kourtoglou, E.; Christakopoulos, P. Fungal Multienzyme Production on Industrial By-products of the Citrus-processing Industry. *Bioresource Technology* **2008**, 99, 2373-2383.

Marin, S.; Ramos, A.J.; Sanchis, V. Comparison of Methods for the Assessment of Growth of Food Spoilage Moulds in Solid Substrates. *International Journal of Food Microbiology* **2005**, 99, 329– 341.

Martínez-Trujillo, A.; Aranda, J.S.; Gómez-Sánchez, C.; Trejo-Aguilar, B.; Aguilar-Osorio, G. Constitutive and Inducible Pectinolytic Enzymes from *Aspergillus flavipes* fp-500 and their Modulation by pH and Carbon Source. *Brazilian Journal of Microbiology* **2009**, 40, 40-47.

Matsushima, K.; Yashiro, K.; Hanya, Y.; Abe, K.; Yabe, K.; Hamasaki, T. Absence of Aflatoxin Biosynthesis in Koji Mold (*Aspergillus sojae*). *Appl. Microbiol. Biotechnol*. **2001**, 55, 771-776.

Mohamed, S.A.; Farid, N.M.; Hossiny, E.N.; Bassuiny, R.I. Biochemical Characterization of an Extracellular Polygalacturonase from *Trichoderma harzianum*. *Journal of Biotechnology* **2006**, 127, 54-64.

Mojsov, K. Experimental Investigations of Submerged Fermentation and Synthesis of Pectinolytic Enzymes by *Aspergillus niger*: Effect of Inoculum Size and Age of Spores. *Applied Technologies & Innovations* **2010**, 2, 40-46.

Murad, H.A.; Foda, M.S. Production of Yeast Wastes Polygalacturonase on Dairy Wastes. *Bioresource Technology* **1992**, 41, 247-250.

Nagai, M.; Katsuragi, T.; Terashita, T.; Yoshikawa, K.; Sakai, T. Purification and Characterization of an Endo-Polygalacturonase from *Aspergillus awamori*. *Biosci. Biotechnol. Biochem*. **2000**, 64, 1729-1732.

Naidu, G.S.N.; Panda, T. Studies on pH and Thermal Deactivation of Pectolytic Enzymes from *Aspergillus niger*. *Biochemical Engineering Journal* **2003**, 16, 57–67.

Najafpour, G.D. Growth Kinetics, In Biochemical Engineering and Biotechnology; Najafpour, G.D., Ed.; Elsevier, Chapter 5; 2007; pp. 81-141.

Nakkeeran, E.; Gowthaman, M.K.; Umesh-Kumar, S.; Subramanian, R. Techno-economic Analysis of Processes for *Aspergillus carbonarius* Polygalacturonase Production. *Journal of Bioscience and Bioengineering* **2012**, 113, 634–640.

Nitayavardhana, S.; Issarapayup, K.; Pavasant, P.; Khanal, S.K. Production of Protein-rich Fungal Biomass in an Airlift Bioreactor using Vinasse as Substrate. *Bioresource Technology* **2013**, 133, 301–306.

Niture, S.K.; Pant, A. Purification and Biochemical Characterization of Polygalacturonase II Produced in Semi-Solid Medium by a Strain of *Fusarium moniliforme*. *Microbiological Research* **2004**, 159, 305–314.

Oncu, S.; Tari, C; Unluturk, S. Effect of Various Process Parameters on Morphology, Rheology, and Polygalacturonase Production by *Aspergillus sojae* in a Batch Bioreactor. *Biotechnol. Prog*. **2007**, 23, 836-845.

Ortega, N.; de Diego, S.; Perez-Mateos, M.; Busto, M.D. Kinetic Properties and Thermal Behaviour of Polygalacturonase used in Fruit Juice Clarification. *Food Chemistry* **2004**, 88, 209-217.

Öztürk, B.; Cekmecelioglu, D.; Ogel, Z.B. Optimal Conditions for Enhanced Betamannanase Production by Recombinant *Aspergillus sojae*. *Journal of Molecular Catalysis B: Enzymatic* **2010**, 64, 135-139.

Panda, T.; Naidu, G.; Sinha, J. Multiresponse Analysis of Microbiological Parameters Affecting the Production of Pectolytic Enzymes by *Aspergillus niger*: *A Statistical View. Process Biochem.***1999**, 35, 187-195.

Papagianni, M.; Nokes, S.E.; Filer, K. Submerged and Solid-state Phytase Fermentation by *Aspergillus niger*: Effects of Agitation and Medium Viscosity on Phytase Production, Fungal Morphology and Inoculum Performance. *Food Technol. Biotechnol.* **2001**, 39, 319-326.

Papagianni, M. Fungal Morphology and Metabolite Production in Submerged Mycelial Processes. *Biotechnology Advances* **2004**, 22, 189–259.

Papagianni, M.; Nokes, S.E.; Filer, K. Production of Phytase by *Aspergillus niger* in Submerged and Solid-state Fermentation. *Process Biochem*. **1999**, 35, 397-402.

Park, J.P.; Kim, S.W.; Hwang, H.J.; Yun, J.W. Optimization of Submerged Culture Conditions for the Mycelia Growth and Exo-Biopolymer Production by *Cordyceps militaris*. *Letters in Applied Microbiology* **2001**, 33, 76-81.

Pazouki, M.; Panda, T. Understanding the Morphology of Fungi. *Bioprocess Engineering* **2000**, 22, 127-143.

Pedersen, H.; Beyer, M.; Nielsen, J. Glucoamylase Production in Batch, Chemostat and Fed-Batch Cultivations by an Industrial Strain of *Aspergillus niger*. *Appl Microbiol Biotechnol.* **2000**, 53, 272-277.

Pedrolli et al., 2009) Pedrolli, D.B.; Carmona, E.C. Purification and Characterization of the Exopolygalacturonase Produced by *Aspergillus giganteus* in Submerged Cultures. *J. Ind. Microbiol. Biotechnol.* **2010**, 37, 567–573.

Pollard, D.J.; Kirschner, T.F.; Hunt, G.R.; Tong, I-T.; Stieber, R.; Salmon, P.M. Scale Up of a Viscous Fungal Fermentation: Application of Scale-Up Criteria With Regime Analysis and Operating Boundary Conditions. *Biotechnology and Bioengineering* **2007**, 96, 307-317.

Posch, A.E.; Spadiut, O.; Herwig, C. A Novel Method for Fast and Statistically Verified Morphological Characterization of Filamentous Fungi. *Fungal Genetics and Biology* **2012**, 49, 499–510.

Potumarthi, R.; Subhakar, C.; Jetty, A. Alkaline Protease Production by Submerged Fermentation in Stirred Tank Reactor using *Bacillus licheniformis* NCIM-2042: Effect of Aeration and Agitation Regimes. *Biochemical Engineering Journal* **2007**, 34, 185–192.

Prosser, J.1.; Tough, A.J. Growth Mechanisms and Growth Kinetics of Filamentous Microorganisms. *Critical Reviews in Biotechnology* **1991**, 10, 253-274.

Puri, M.; Banerjee, A.; Banerjee, U.C. Optimization of Process Parameters for the Production of Naringinase by *Aspergillus niger* MTCC 1344. *Process Biochemistry* **2005**, 40, 195–201.

Rangarajan, V.; Rajasekharan, M.; Ravichandran, R.; Sriganesh, K.; Vaitheeswaran, V. Pectinase Production from Orange Peel Extract and Dried Orange Peel Solid as Substrates using *Aspergillus niger*. *Int. J. Biotechnol. Biochem.* **2010**, 6, 445-453.

Rocha-Valadez, J.A.; Estrada, M.; Galindo, E.; Serrano-Carreon, L. From shake flasks to stirred fermentors: Scale-up of an Extractive Fermentation Process for 6-pentyl-a-pyrone Production by *Trichoderma harzianum* using Volumetric Power Input. *Process Biochemistry* **2006**, 41, 1347–1352.

Rodriguez Porcel, E.M.; Casas Lopez, J.L.; Sanchez Perez, J.A.; Fernandez Sevilla, J.M.; Chisti, Y. Effects of Pellet Morphology on Broth Rheology in Fermentations of *Aspergillus terreus. Biochemical Engineering Journal* **2005**, 26, 139–144.

Rodríguez-Fernández, D.E.; Rodríguez-León, J.A.; de Carvalho, J.C.; Sturm, W.; Soccol, C.R. The Behavior of Kinetic Parameters in Production of Pectinase and Xylanase by Solid-State Fermentation. *Bioresource Technology* **2011**, 102, 10657–10662.

Rodriguez-Leon, J.A.; Soccol, C.R.; Pandey, A.; Roddguez, D.E. Factors Affecting Solid-state Fermentation. *In Current Developments in Solid-state Fermentation*; Pandey, A., Larroche, C., Soccol C.R., Eds.; Springer: Delhi, 2008; pp. 230-252.

Sakamoto, T.; Bonnin, E.; Quemener, B.; Thibault, J-F. Purification and Characterisation of Two Exo-polygalacturonases from *Aspergillus niger* Able to Degrade Xylogalacturonan and Acetylated Homogalacturonan. *Biochimica et Biophysica Acta* **2002**, 1572, 10–18.

San Jose, CA. Global industrial enzymes market to reach US$3.74 billion by 2015, according to a new report by Global Industry Analysts, Inc. PreWeb ebook; 2011, pp.1-3.

Sangeetha, P.T.; Ramesh, M.N.; Prapulla, S.G. Production of Fructosyl Transferase by *Aspergillus oryzae* CFR 202 in Solid-state Fermentation using Agricultural Byproducts. Appl. *Microbiol. Biotechnol*. **2004**, 65, 530-537.

Sardjono; Zhu, Y.; Knol, W. Comparison of Fermentation Profiles between Lupine and Soybean by Aspergillus oryzae and *Aspergillus sojae* in Solid-State Culture Systems. *J. Agric. Food Chem*. **1998**, 46, 3376-3380.

Schoustra, S.; Punzalan, D. Correlation of Mycelial Growth Rate with Other Phenotypic Characters in Evolved Genotypes of *Aspergillus nidulans*. *Fungal Biology* **2012**, 116, 630-636.

Scotti, C.T.; Vergoignan, C.; Feron, G.; Durand, A. Glucosamine Measurement as Indirect Method for Biomass Estimation of Cunninghamella Elegans Grown in Solid State Cultivation Conditions. *Biochemical Engineering Journal* **2001**, 7, 1–5.

Shabrukova, N.V.; Shestakova, L.M.; Zainetdinova, D.R.; Gamayurova, V.S. Research of Acid Hydrolyses of Chitin-Glucan and Chitosan-Glucan Complexes. *Chemistry and Computational Simulation.Butlerov Communications*. **2002**, 2, 57–59.

Shih, I-L.; Chou, B-W.; Chen, C-C.; Wu, J-Y.; Hsieh, C. Study of Mycelial Growth and Bioactive Polysaccharide Production in Batch and Fed-Batch Culture of *Grifola frondosa*. *Bioresource Technology* **2008**, 99, 785–793.

Shuler, M.L.; Kargi, F. Bioprocess Engineering Basic Concepts, 2nd ed.; Prentice Hall:Upper Saddle River, 2002; pp. 57.

Silva, D.; Martins, E.; Silva, R.; Gomes, E. Pectinase Production by *Penicillium viridicatum* RFC3 by Solid State Fermentation Using Agricultural Wastes and Agro-industrial By-products. *Braz. J. Microbiol*. **2002**, 33, 318-324.

Sin, H.N.; Yusof, S.; Sheikh Abdul Hamid, N.; Abd. Rahman, R. Optimization of Enzymatic Clarification of Sapodilla Juice Using Response Surface Methodology. *Journal of Food Engineering* **2006**, 73, 313–319.

Sinha, J.; Bae, J.T.; Park, J.P.; Song, C.H.; Yun, J.W. Effect of Substrate Concentration on Broth Rheology and Fungal Morphology During Exo–biopolymer Production by *Paecilomyces japonica* in a Batch Bioreactor. *Enzyme and Microbial Technology* **2001**, 29, 392–399.

Souza, J.V.B.; Silva, E.S.; Maia, M.L.S.; Teixeira, M.F.S. Screening of Fungal Strains for Pectinolytic Activity: Endopolygalacturonase Production by *Peacilomyces clavisporus* 2A.UMIDA.1. *Process Biochemistry* **2003**, 39, 455-458.

Stanbury, P.F.; Whitaker, A. Principles of Fermentation Technology, Pergamon Press Ltd., Oxford, 1987.

Subramaniyam, R.; Vimala, R. Solid State and Submerged Fermentation for the Production of Bioactive Substances: A Comparative Study. *International Journal of Science and Nature* **2012**, 3, 480-486.

Takahashi, T.; Hatamoto, O.; Koyama, Y.; Abe, K. Efficient Gene Disruption in the Koji-mold *Aspergillus sojae* using a Novel Variation of the Positive-Negative Method. *Mol Gen Genomics* **2004**, 272, 344–352.

Tang, Y-J.; Zhong, J-J. Role of Oxygen Supply in Submerged Fermentation of *Ganoderma lucidum* for Production of Ganoderma Polysaccharide and Ganoderic acid. *Enzyme and Microbial Technology* **2003**, 32, 478–484.

Tang, Y-J.; Zhang, W.; Liu, R-S.; Zhu, L-W.; Zhong, J-J. Scale-up Study on the Fed-batch Fermentation of *Ganoderma lucidum* for the Hyperproduction of Ganoderic acid and Ganoderma Polysaccharides. *Process Biochemistry* **2011**, 46, 404–408.

Tari, C.; Göğüş, N.; Tokatlı, F. Optimization of Biomass, Pellet Size and Polygalacturonase Production by *Aspergillus sojae* ATCC 20235 using Response Surface Methodology. *Enzym. Microb. Tech.* **2007**, 40, 1108-1116.

Tari, C.; Dogan, N; Gogus, N. Biochemical and Thermal Characterization of Crude Exo-polygalacturonase Produced by *Aspergillus sojae*. *Food Chemistry* **2008**, 111, 824-829.

Thakur, A.; Pahwa, R.; Singh, S.; Gupta, R. Production, Purification and Characterization of Polygalacturonase from *Mucor circinelloides* ITCC 6025. *Enzyme Research* [Online] **2010**, 2010, 1-7.

Trinci, A.P.J. A Kinetic Study of the Growth of *Aspergillus nidulans* and Other Fungi.*J. gen. Microbiol.* **1969**, 57, 11-24.

Martinez-Trujillo, A.; Aranda, J.S.; Gómez-Sánchez, C.; Trejo-Aguilar, B.; Aguilar-Osorio, G. Constitutive and Inducible Pectinolytic Enzymes from *Aspergillus flavipes* Fp-500 and their Modulation by pH and Carbon Source. *Brazilian Journal of Microbiology* **2009**, 40, 40-47.

Ustok, F.I.; Tari, C.; Gogus, N. Solid-state Production of Polygalacturonase by *Aspergillus sojae* ATCC 20235. *J. Biotechnol*. **2007**, 127, 322-334.

Vaidyanathan, S.; Harvey, L.M.; McNeil, B. Deconvolution of Near-infrared Spectral Information for Monitoring Mycelial Biomass and Other Key Analytes in a Submerged Fungal Bioprocess. *Analytica Chimica Acta* **2001**, 428, 41–59.

Waites Michael J., Morgan Neil L., Rockey John S. and Higton Gary. Industrial Microbiology: An Introduction, Backwell Science, 2001.

Wang, L.; Yang, S-T. Solid State Fermentation and Its Applications. *In Bioprocessing for Value-Added Products from Renewable Resources*, 1st ed.; Yang, S-T., Ed.; Elsevier B.V.: Oxford, 2007; pp: 465-489.

Ward, O.P. Production of Recombinant Proteins by Filamentous Fungi. *Biotechnology Advances* **2012**, 30, 1119–1139.

Weuster-Botz, D.; Experimental Design for Fermentation Media Development: Statistical Design or Global Random Search? *Journal of Bioscience and Bioengineering* **2000**, 90, 473-483.

Yingyi, D.; Lan, W.; Hongzhang, C. Digital Image Analysis and Fractal-based Kinetic Modelling for Fungal Biomass Determination in Solid-state Fermentation. *Biochemical Engineering Journal* **2012**, 67, 60–67.

Zhou, J-M.; Ge, X-Y.; Zhang, W-G. Improvement of polygalacturonase production at high temperature by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisiae. Bioresource Technology* **2011**, 102, 10085–10088.

Znidarsic, P.; Komel, R.; Pavko, A. Studies of a Pelleted Growth form of *Rhizopus nigricans* as a Biocatalyst for Progesterone 11a-hydroxylation. *Journal of Biotechnology* **1998**, 60, 207–216.

APPENDIX A

ANOVA TABLES

Table A.1. ANOVA table of screening process for PG activity.

Source	Sum of squares	df	Mean squares	F- value	p-value
Model	11364.07	9	1262.67	4.83	0.005
A-Complex C sources	10739.78	1	10739.78	41.12	<< 0.001
B-Add. C sources	118.92	1	118.92	0.45	0.511
C-Incubation time	110.94	2	55.47	0.21	0.811
AB	161.91	1	161.91	0.61	0.444
AC	116.89	2	58.44	0.22	0.802
BC	115.60	2	57.80	0.22	0.804
Residual	3656.51	14	261.17		
Lack of fit	641.61	2	320.80	1.27	0.314
Pure error	3014.90	12	251.24		
Cor Total	15020.58	23			

Table A.2.ANOVA table of pre-optimization process for PG activity.

Source	**Sum of squares**	**df**	**Mean squares**	**F-value**	**p-value**
Model	19685.28	7	2812.18	4.79	0.0021
A-Orange peel	15763.36	1	15763.36	26.87	< 0.0001
B-Maltrin	1440.85	1	1440.85	2.46	0.1313
C-Glucose	440.85	1	440.85	0.75	0.3954
AB	293.77	1	293.77	0.50	0.4866
AC	332.44	1	332.44	0.57	0.4595
BC	490.37	1	490.37	0.84	0.3705
CD	923.63	1	923.63	1.57	0.2227
Residual	12905.47	22	586.61		
Lack of fit	10706.85	17	629.81	1.43	0.3678
Pure	2198.62	5	439.72		
Cor Total	32590.74	29			

Table A.3.ANOVA table of optimization processes for PG activity.

Source	**Sum of squares**	**df**	**Mean squares**	**F-value**	**p-value**
Model	5358.56	4	1339.64	18.42	<<0.001
A-Orange peel	124.23	1	124.23	1.70	0.228
B-Maltrin	614.08	1	614.08	8.44	0.020
AB	1517.69	1	1517.69	20.87	0.002
A^2	3102.55	1	3102.55	42.66	<<0.001
Residual	581.75	8	72.71		
Lack of fit	483.59	4	120.89	4.92	0.076
Pure error	98.15	4	24.53		
Cor Total	5940.32	12			

APPENDIX B

STANDARD CURVES

Table B.1.Standard curve for PG activity calculations.

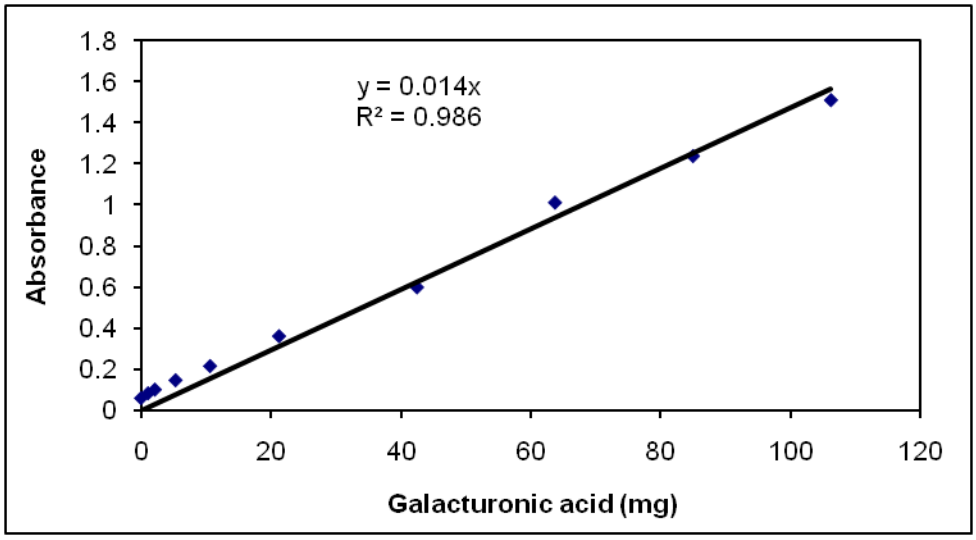


Table B.2.Standard curve for total carbohydrate calculations.

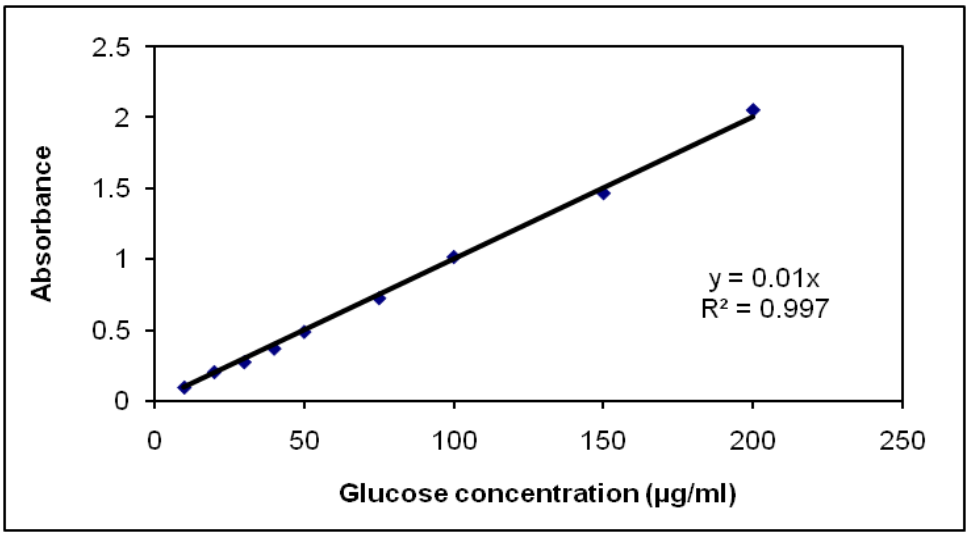
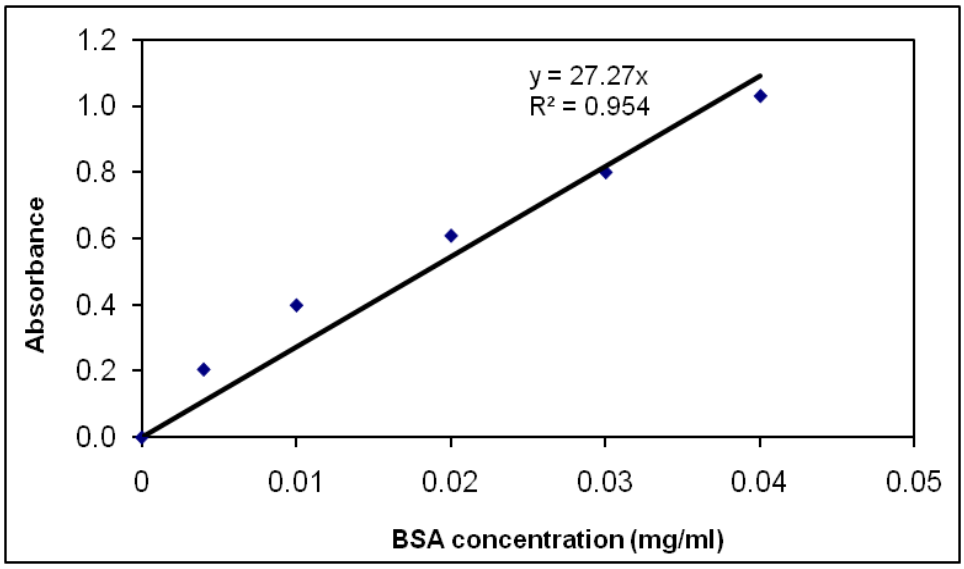


Table B.3.Standard curve for total protein calculations.



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Tokatli,F., Tarı,C., Unluturk, M.S., Göğüş, N. Modelling of Polygalacturonaseenzyme activity and biomass production by *Aspergillus sojae* ATCC 20235. *Journal of Industrial Microbiology and Biotechnology*2009, 36, 1139-1148.

Tarı, C., Dogan, N., Gogus, N. Biochemical and thermal characterization ofexo-polygalacturonase produced by *Aspergillus sojae*. *Food Chemistry*2008, 111, 824-829.

Tarı,C., Gogus,N., Tokatlı,F. Optimization of biomass, Pellet Size andPolygalacturaonase prodcution by *Aspergillus sojae* ATCC 20235 Using Response SurfaceMethodology. *Enzyme and Microbial Technology*2007, 40, 1108-1116.

Ustok,F.I. Tarı,C. Gogus,N. Solid state production of polygalacturonase by*Aspergillus sojae* ATCC 20235. *Journal of Biotechnology*2007, 127, 322-334.

Göğüş,N. Tarı,C., Ünlütürk, S., Öncü, S., Tokatlı,F. Relationship between morhpology, rheology and polygalacturonase production by *Aspergillus sojae* ATCC20235 in submerged cultures. *Biochemical Engineering Journal*2006, 32, 171-178.