INVESTIGATION OF THE EFFECTS OF DISSOLVED OXYGEN CONCENTRATION, AERATION AND AGITATION ON THE MORPHOLOGY AND RHEOLOGY IN SUBMERGED FUNGAL FERMENTATION

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ABSTRACT

INVESTIGATION OF THE EFFECTS OF DISSOLVED OXYGEN CONCENTRATION, AERATION AND AGITATION ON THE MORPHOLOGY AND RHEOLOGY IN SUBMERGED FUNGAL FERMENTATION

The effects of pH, agitation speed, dissolved oxygen tension (DOT) and aeration, significant in common fungal fermentations, on the production of polygalacturonase (PG) enzyme and their relation to morphology and broth rheology were investigated in a batch bioreactor using *Aspergillus sojae* which has no available literature report on the pectinase production.

All four factors were effective on the response parameters under study. An uncontrolled pH increased biomass and PG activity by 27% and 38%, respectively compared to controlled pH (pH 6). pH did not significantly affect the broth rheology but created an impact on the pellet morphology. Similarly, the maximum biomass obtained at 500 rpm and at 30h was 3.27 and 3.67 times more than at 200 and 350 rpm, respectively. The maximum enzyme productivity of 0.149 U ml^{-1} h^{-1} was obtained at 200 rpm. Non – Newtonian and pseudoplastic broth rheology was observed at 500 rpm agitation speed. Furthermore, a DOT range of 30-50% was essential for maximum biomass formation, whereas only 10% DOT was required for maximum PG synthesis. Non – Newtonian shear thickening behavior ($n>1.0$) was depicted at DOT levels of 10% and 30%, whereas, non-Newtonian shear thinning behavior ($n<1.0$) was dominant at 50% DOT. When 2.5 l/min aeration experiment was investigated detaily; it was determined that at about 21st hour, polygalacturonase production approaches its maximum (1.49 U) and pellets are smaller, high in number. At 48th hour; polygalacturonase production declines to zero, biomass reaches its maximum and pellets are big (average pellet size is 1.94±0.58 mm) and fluffy with compact centers. At the end of fermentation (96.hour), fermentation medium is close to Newtonian.

The overall fermentation duration (50-70h) was considerably shorter as opposed to common fungal fermentations revealing the economic feasibility of this particular process. As a result this study not only introduced a new strain with a potential of producing a highly commercially significant enzyme but also provided certain parameters significant in the design and mathematical modelling of fungal bioprocesses.

ÖZET

SIVI ORTAM KÜF FERMENTASYONUNDA MORFOLOJİYE VE REOLOJİYE ETKİ EDEN ÇÖZÜNMÜŞ OKSİJEN KONSANTRASYONUNUN (DO), HAVALANDIRMANIN VE KARIŞTIRMA HIZININ ETKİSİNİN İNCELENMESİ

Birçok küf fermentasyonunda önemli olan pH, karıştırma hızı, çözünmüş oksijen konsantrasyonu (DOT) ve havalandırmanın poligalakturonaz (PG) enzimi üretimine etkisi ve bu faktörlerin morfoloji ve ortam reolojisiyle olan ilişkisi, literatürde pektinaz enzimi ürettiğine dair mevcut bir bilgi olmayan *Aspergillus sojae* suşu kullanılarak kesikli biyoreaktörde incelenmiştir.

Bu çalışmada bütün faktörlerin cevap parametreleri üzerinde etkin olduğu ortaya konmuştur. Kontrolsüz pH, kontrollü pH'ya oranla biyomas ve PG aktivitesini sırasıyla %27 ve %38 yükseltmiştir. pH ortam reolojisin önemli ölçüde etkilememiştir fakat pellet morfolojisi üzerinde büyük bir etki yaratmıştır. Benzer şekilde, 500 rpm'de elde edilen maksimum biyomas 200 ve 350 rpm'den sırasıyla 3.27 ve 3.67 kat daha fazladır. Maksimum enzim üretkenliği 200 rpm'de elde edilmiştir. 500 rpm karıştırma hızında Non – Newtonian ve pseudoplastik ortam reolojisi gözlenmiştir. Bunlardan başka; maksimum biyomas oluşumu için %10 – 30 DOT oranı gereklidir. Oysa maksimum PG sentezi için %10 DOT yeterlidir. %10 ve %30 DOT'da Non – Newtonian shear thickening ($n>1.0$) davranış görülmesine rağmen %50 DOT'da Non – Newtonian shear – thinning ($n<1.0$) davranış baskındır. 2.5 L/dak havalandırma deneyi detaylı olarak incelendiğinde; 21. saatte PG üretiminin maksimuma ulaştığı (1.49 U) ve pelletlerin nispeten küçük, sayılarının yüksek olduğu görülmüştür. 48. saatte ise PG üretimi sıfıra düşer, biyomas maksimuma ulaşır ve pelletler büyük, merkezleri sıkı, kenarları tüylü olur. Fermentasyon sonunda ortam Newtonian'a yakındır.

Genel olarak küf fermentasyonlarının aksine tüm fermentasyon süresi (50 – 70 saat) oldukça kısadır ve bu durum prosesin ekonomik fizibilitesini göz önüne serer. Sonuç olarak, bu çalışma sadece ticari öneme sahip bir enzim üretme potansiyeli olan yeni bir suşu literatüre kazandırmakla kalmayıp aynı zamanda küf biyoproseslerinin dizayn ve matematik modellemesinde önemi olan bazı önemli parametreleri de sağlamıştır.

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CHAPTER 1

INTRODUCTION

The metabolic performance of a microbial culture in the bioreactor depends strongly on complex interactions of the various operating conditions. For example, the agitation intensity, the microbial species being cultured, the nutrients type and supply determine the bulk rheology and cellular morphology.

The fungal culture exhibits two major morphologies observed as pellets which are spherical agglomerates of hyphaes or mycelia which is free mycelium dispersed throughout the culture medium (Pedersen et al. 1993) . These morphologies are very much determined by several environmental and genetic factors including type of organism, pH and composition of the media, inoculation ratio and type of the inoculum, agitation speed and aeration rate, feeding rate and genetic factors of the culture (Reichl et al. 1992, Pedersen et al. 1993, Li et al. 2000, Pazauki and Panda 2000) .

Rheology in turn affects supply of nutrients, specially oxygen, and the ease of mixing of the broth. Rheology–morphology relationships are particular relevant in fermentations involving filamentous fungi and bacteria. The specific growth morphology produced under given conditions depends on several factors including the fungal strain, the method of initiation of culture (e.g. spores, pellets, and dispersed mycelium), the nature of the growth medium, and the hydrodynamic regime in the bioreactor (Metz and Kossen 1977, Van Suijdam and Metz 1981). Excessive hydrodynamic shear stresses are known to damage mycelial hyphae and pellets, but much lower shear stresses are sufficient to influence growth morphology.

The cost of providing power to disperse gas and mix the contents of fermenters contributes significantly to overall process economics. The major factor affecting the power demand is the viscosity of the fermentation medium, and this can be substantially reduced by growing molds in the form of pellets rather than as filaments (Schügerl et al. 1983).

However, in fermentations where the mycelia form is dominant and, the cell growth and productivity is higher, the broth is much more viscous, resulting in heterogeneous stagnant non – mixed zone formations that are harder and more expensive to operate (Metz and Kossen 1977). Hence, the morphology of the culture,

which effects productivity and results into rheological changes of the broth, needs to be controlled. Therefore, the relationship between morphology and rheology and the factors influencing them have to be fully investigated. Such knowledge can be very valuable in the optimization and design of common industrial fungal fermentations.

With this perspective, polygalacturonase which attracts the most attention among the pectinases due to its wide application areas is considered as a model product produced by a new organism *Aspergillus sojae,* which was not considered for this purpose so far to unveil the common industrial problem facing fungal fermentations. To best of our knowledge there is no available literature on the polygalacturonase production of this organism in batch bioreactors, therefore this study will be one of initial studies using this organism in the production of a highly valuable product and be a new reference point to the microbiology area and enzyme industry. Also there is no literature that we know about on the investigation of the relationship of morphology and rheology to polygalacturonase production using other organisms. Hence, it will close the gap in the literature to some degree which has been lacking so far. It is also reported that pectinases, as one of upcoming enzymes in commercial sector, contribute to almost 25 % of the global enzyme sales and its contribution is estimated to increase further by the year 2009 (Jayani et al. 2005). In order to meet this high demand, it is of great importance to produce this enzyme in a cost effective and productive way which makes this study extra novel.

Therefore in this study, the effect of process parameters significant to fungal fermentations such as pH, agitation speed, dissolved oxygen concentration and aeration which are regarded in the literature and industry, on pellet morphology, broth rheology and polygalacturonase production were investigated in submerged fermentation of *Aspergillus sojae.*

The outcome of this study will provide significant informations which can be used in the batch production of this product and in the design of the subsequent downstream process. Besides, it will also provide useful informations that can be used in scale up processes and mathematical modeling of common fungal fermentations

CHAPTER 2

FUNGAL MORPHOLOGY AND PECTINASE ENZYME

2.1 Fungi

Fungi play such a dominant role in human society that it could be readily argued that they are the most important biotechnologically useful organisms. The use of fungi for the production of commercially important products has increased rapidly over the past half century (Wainwright 1992, Papagianni 2004).

Fungi are increasingly being evaluated for industrial uses that are outside the scope of the fermentation industry, the traditional mainstay of fungal biotechnology. The fermentation industry without doubt provides the largest and economically most important use of fungi in biotechnology (Wainwright 1992). The widespread use of fungi in biotechnology stems from the unique ability of fungi to economically produce many different types of products, including some of the most important antibiotics, commodity chemicals and commercial enzymes.

Fungi generally exhibit correct glycosylation/posttransition modification of proteins, are often classified as GRAS (generally regarded as safe) by the FDA (Food and Drug Administration) (Cho et al. 2002). Because of their rapid growth rate and high protein content, fungi provide an ideal source of protein. Fungal protein can be produced in large amounts in a relatively small area, using cheap waste products as nutrient source.

Fungi are strict obligate chemoheterotrophs, therefore energy-rich substrates are required for fungi to cover their energy and biomass requirements. They usually use up carbohydrates as the main carbon source so as to get energy and synthesize the cellular material. Generally all fungi can use nitrate but they use nitrogen mainly in the form of ammonium. In the laboratory conditions, defined media including sugars such as glucose and sucrose or on polymers such as cellulose can be used to grow fungi. Moreover, complex media like potato-dextrose agar and vegetable based media can also be used for fungi growth (Wainwright 1992).

There are some 200 species of *Aspergillus*, found throughout the world growing on a vast array of substrates. *Aspergilli* are ubiquitous members of the air mycoflora and

so frequently occur as contaminant of culture media. *Aspergillus* species produce numerous extracellular enzymes many of which are of great importance in biotechnology (Wainwright 1992).

2.2 Fungal Morphology

Fungal morphology is often considered as one of the key parameters in industrial production and characterization of mycelial morphology is important for physiological and engineering studies of filamentous fermentation used in the design and operation of such fermentation (Pazouki and Panda 2000).

Most industrially important fungi are either single-celled yeasts or grow as filaments called hyphae (0.5 μm to 1.0 mm in diameter) and can aggregate to form a mycelium (Wainwright 1992).

2.2.1 Growth of Fungi

Filamentous fungi are morphologically complex microorganisms, exhibiting different structural forms throughout their life cycles. The basic vegetative structure of growth consists of a tubular filament known as hypha that originates from the germination of a single reproductive spore. As the hypha continues to grow, it frequently branches repeatedly to form a mass of hyphal filaments referred to as mycelium.

When grown in submerged culture, these fungi exhibit different morphological forms, ranging from dispersed mycelia filaments to densely interwoven mycelial masses referred to as pellets. They are very much determined by several environmental and genetic factors. These are;

- type of the strain,
- pH, temperature and composition of the media,
- inoculation ratio,
- type of the inoculum,
- agitation speed, aeration rate,
- feeding rate and
- genetic factors of the culture

Moreover, morphology affects oxygen transfer, nutrient consumption, and viscosity and, thus, mixing of the medium (Reichl et al. 1992).

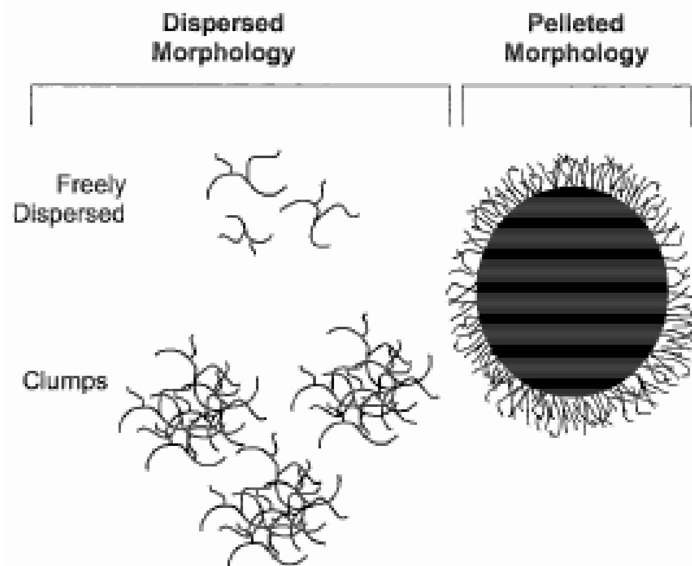


Figure 2.1. Schematic representation of fungal morphologies in suspension culture. (Source: Li et al. 2000)

2.2.2 Pellet Morphology

In industrial applications pellet morphology is usually preferred in fermentations and in downstream processing due to the non viscous rheology of the broth (Atkingson and Daoud 1976, Zhaou et al. 2000). In such fermentations, the mass transfer of oxygen and nutrients is considerably better and the subsequent separation of the pellets from the medium is simpler (Reichl et al. 1992). Since agitation and aeration is also much easier in such a system, the power input therefore the operating cost is lower. Also, the slime – forming tendencies of the fungus are eliminated (Zhaou et al. 2000).

In case, the limitation of nutrients and oxygen may cause the death of pellet interiors or cause the pellet interiors to become anaerobic and have lower yield. Therefore, control of the formation of small uniform pellets is a prerequisite for industrial applications to ensure adequate mass and heat transfer or metabolite production (Zhaou et al. 2000).

However, in fermentations where the mycelia form is dominant and, the cell growth and productivity is higher, the broth is much more viscous, resulting in heterogeneous stagnant non – mixed zone formations that are harder and more

expensive to operate (Metz and Kossen 1977). Also, filamentous forms may wrap around impellers, foul agitation blades, and block spargers (Zhaou et al. 2000).

In the literature several mechanisms have been given for pellet formation; agglomeration of hyphae, agglomeration of spores and hyphae, agglomeration of solid particles and hyphae, while generally the influence of pellet-pellet and pellet-wall interactions was considered to be important for pellet formation. Most common classification of pellet formation is;

a) Coagulating type; the spores coagulate in the early stages of development, germinate gradually, and aggregate with other small agglomerates and ungerminated spores to form pellets.
b) Noncoagulating pellet formation; one spore grows out to form one pellet.

The type of pellet formation changes from strain to strain used. Pellets of *Aspergillus niger* were formed from agglomerates of up to 500 spores, whereas *Penicillium chrysogenum* has noncoagulative pellet formation.

Pellets are spherical or ellipsoidal masses of hyphae with variable internal structure and can be classified in three groups;

a) Fluffy loose pellets; these pellets have a compact center and a much looser outer zone.
b) Compact smooth pellets; the whole pellet is compact and the outside of the pellet is smooth.
c) Hollow smooth pellets; the center of the pellet is hollow owing to autolysis and the outside is smooth.

The structural properties strongly depend on the culture conditions (Metz and Kossen 1977).

Wittler et al. (1986) proposed the existence of four regions. The outer region consists of viable hyphae and surrounds a layer of hyphae showing signs of autolysis. In hollow pellets, a third layer is found containing hyphae with irregular wall structure, while the center of the pellet contains no recognizable mycelia. The density of hyphae within pellets is of significance for diffusion of nutrients and oxygen to the mycelial biomass, with consequent effects on grown, particularly at the center of compact pellets.

Application of mycelial aggregates to metabolite production depends upon obtaining uniform pellets of a desired size. This is not easily accomplished, since many factors influence pellet formation. These are;

- inoculum size, type and age,

- genetic factors and ability to produce bioflocculants,
- medium composition,
- biosynthesis or addition of polymers, surfactants and chelators,
- shear forces,
- temperature, pH and pressure,
- medium viscosity (Papagianni 2004).

Industrial applications of the growth of molds in the form of pellets are; citric acid production, production of mushroom mycelium in submerged culture, production of penicillin, giberillic acid, itaconic acid, cellulose, D-mannitol and protein production on cellulose waste.

Many parameters affect the morphology of fungi during the process of fermentation, among them speed of agitation, specific growth rate, dissolved oxygen, number of spores per liter of fermentation broth are important and should be considered when higher yield is desired in the process. It is, therefore, of considerable importance to understand the mechanism underlying the morphology of the cell, its growth and product formation by filamentous fungi. Such knowledge may be used in the optimization of the microbial process (Pazouki and Panda 2000).

2.3 Pectinase Enzyme

The biotechnological potential of pectinolytic enzymes from microorganisms has drawn a great deal of attention world wide because of their myriad applications (Patil and Dayanand 2006). They are widely used for the production of broth primary (organic acids and enzymes for the food, textile, and paper industries) and secondary (antibiotics) metabolites. The fungus *Aspergillus* is an important organism for the industrial production of many enzymes and organic acids, e.g. amyloglucosidase and gluconic acid and pectinases (Hellendoorn et al. 1998).

Pectinases are one of the upcoming enzymes of fruit and textile industries. These enzymes break down complex polysaccharides of plant tissues into simpler molecules like galacturonic acids. The most important enzymes of the pectinase complex are polygalacturonase (PG), pectinlyase (PL) and pectinesterase (PE). The best-known microbial producers of pectinase are different species of *Aspergillus* fungi (Debing et al. 2006). The role of acidic pectinases in bringing down the cloudiness and bitterness

of fruit juices is well established. Recently, there have been a good number of reports on the application of alkaline pectinases in the textile industry for the retting and degumming of fiber crops, production of good quality paper, fermentation of coffee and tea, oil extractions and treatment of pectic waste water (Kashyap et al. 2001).

These enzymes are, primarily, responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells. Pectinases are now an integral part of fruit juice and textile industries as well as having various biotechnological applications (Kashyap et al. 2001). They help reduction in viscosity of fruit pulp which in turn helps filtration, clarification of fruit juices and wood preservation (Gikas and Livingston 1998).

CHAPTER 3

RHEOLOGY OF FERMENTATION BROTHS

An important feature of a batch fermentation process is the change in rheological behavior during fermentation due to the changing of substrate composition, biomass concentration and morphology of the microorganism (Cho et al. 2002). It is well known that the morphology of mycelium cultures has a strong influence on the rheological properties of the broth and shear can greatly affect the pellet morphology and productivity. To control fermenter performance, it is necessary to know how the operating conditions influence the rheological properties of the fermentation broths. As these depend on the morphology of the fungus, the relationship between rheology and morphology is vitally important (Pazouki and Panda 2000).

Rheological properties are affected by several factors like; temperature, shear rate, measuring conditions, time, pressure, homogeneity, previous history and composition of the fluid, additives and finally special characteristics of dispersions and emulsions (aggregation, attraction between particles in a dispersed phase, shape of particles, stability of dispersed phase) (Brookfield Viscometer Manual 2000).

3.1 The Use of Rheological Measurements

Rheology is a simple analysis that is being more and more applied to determine the behavior of solutions, suspensions and mixtures. It is a field which tends to combine continuum mechanics with ideas obtained by considering the microstructure of the fluid under study.

The experiences of thousands of people who have made viscosity measurements show that much useful behavioral and predictive information for various products can be obtained, as well as knowledge of the effects of processing, formulation changes, aging phenomena, etc.

A frequent reason for the measurement of rheological properties can be found in the area of quality control, where raw materials must be consistent from batch to batch. For this purpose, flow behavior is an indirect measure of product consistency and quality.

Another reason for making flow behavior studies is that a direct assessment of processability can be obtained. For example, a high viscosity liquid requires more power to pump than a low viscosity one. Knowing its rheological behavior, therefore, is useful when designing pumping and piping systems. It has been suggested that rheology is the most sensitive method for material characterization because flow behavior is responsive to properties such as molecular weight and molecular weight distribution. Rheological measurements are also useful in following the course of a chemical reaction. Such measurements can be employed as a quality check during production or to monitor and/or control a process.

Rheological measurements allow the study of chemical, mechanical, and thermal treatments, the effects of additives, or the course of a curing reaction. They are also a way to predict and control a host of product properties, end use performance and material behavior (Brookfield Viscometer Manual 2000). Rheological properties play a major role in describing heat transfer or in the design, evaluation and modeling of continuous treatment and its measurements are useful to determine behavioral and predictive information for various products, as well as knowledge of the effect of processing, formulation changes and aging phenomena. Therefore, it is necessary to have theoretical knowledge as related to rheological aspects (Wan Nik et al. 2005).

3.2 Viscosity

The fundamental parameter, obtained in the rheological study of fluid foods, is viscosity. Viscosity is a measure of a fluid's resistance to flow. In other words, viscosity is the measure of the internal friction of a fluid. This friction becomes apparent when a layer of fluid is made to move in relation to another layer. The greater the friction, the greater the amount of force required to cause this movement, which is called 'shear'. Shearing occurs whenever the fluid is physically moved or distributed, as in pouring, spreading, spraying, mixing etc. Highly viscous fluids, therefore, require more force to move than less viscous materials (Brookfield Viscometer Manual 2000).

Isaac Newton defined viscosity like this:

$$\frac{F}{A} = \eta \frac{dv}{dx} \tag{3.1}$$

$\eta =$ viscosity (cp)

$\frac{dv}{dx}$ = velocity gradient = 'shear rate' = γ (1/s)

$\frac{F}{A}$ = 'shear stress' = τ (dyn/cm^2)

So,

$$\eta = \text{viscosity} = \frac{\tau}{\gamma} = \frac{\text{shearstress}}{\text{shearrate}} \quad (3.2)$$

Fluid behavior is classified according to the observed behavior between the shear stress and shear rate into two main types. Newtonian fluids are defined as those exhibiting a direct proportionally between shear stress and shear rate while for the non-Newtonian fluids, the relation between the shear stress and shear rate is non linear.

3.2.1 Newtonian Fluids

A Newtonian fluid is represented graphically in Figure 3.1 and 3.2. Figure 3.1 shows that the relationship between shear stress (τ) and shear rate (γ) is a straight line. Figure 3.2 shows that the fluid's viscosity remains constant as the shear rate is varied. Typical Newtonian fluids include water and thin motor oils.

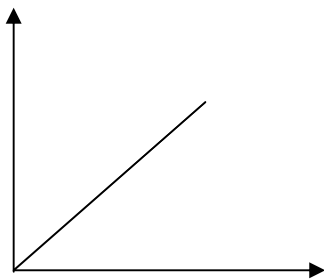


Figure 3.1 Shear stress-Shear Rate Graph

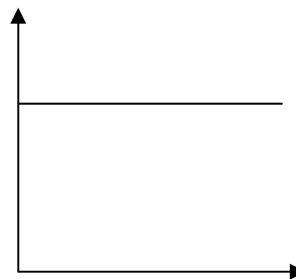


Figure 3.2 Viscosity-Shear Rate Graph

These graphs mean that at a given temperature the viscosity of a Newtonian fluid will remain constant regardless of which Viscometer model, spindle or speed you use to measure it (Brookfield Viscometer Manual 2000).

3.2.2 Non – Newtonian Fluids

A non – Newtonian fluid is broadly defined as one for which the relationship τ / γ is not constant. In other words, when the shear rate is varied, the shear stress doesn't vary in the same proportion (or even necessarily in the same direction). The viscosity of such fluids will therefore change as the shear rate is varied. Thus, the experimental parameters of Viscometer model, spindle and speed all have an effect on the measured viscosity of a non – Newtonian fluid. This measured viscosity is called the 'apparent viscosity' of the fluid and is accurate only when explicit experimental parameters are furnished and adhered to.

There are several types of non – Newtonian flow behavior, characterized by the way a fluid's viscosity changes in response to variations in shear rate. The most common types of non – Newtonian fluids you may encounter include;

1. *Pseudoplastic:* This type of fluid displays a decreasing viscosity with an increasing shear rate. The most common of the non – Newtonian fluids, pseudo – plastics include paints, emulsions, and dispersions of many types. This type of flow behavior is sometimes called 'shear – thinning'. An easily understanding model is to imagine that in the moment o turning the spindle in the sample will be destroyed, and the molecule formation will be orientated more parallel to the spindle surface. So the hindering of the spindle rotation will decrease. The faster the rotation will become, the more the structure is destroyed and the less the structure of molecules slide in together, the lower the viscosity will be.
2. *Dilatant:* Increasing viscosity with an increase in shear rate characterizes the dilatant fluid. Although rarer than pseudoplasticity, dilatancy is frequently observed in fluids containing high levels of deflocculated solids, such as clay slurries, candy compounds, corn starch in water, and sand/water mixtures. Dilatancy is also referred to as 'shear – thickening' flow behavior.
3. *Plastic:* This type of fluid behaves as a solid under static conditions. A certain amount of stress must be applied to the fluid before any flow is induced; this stress is called the 'yield stress'. Tomato catsup is a good example of this type of fluid; its yield value often makes it refuse to pour

from the bottle until the bottle is shaken or struck, allowing the catsup to gush freely. Once the yield value is exceeded and flow begins, plastic fluids may display Newtonian, pseudoplastic, or dilatant flow characteristics.

4. *Thixotropy:* A thixotrophic fluid undergoes a decrease in viscosity with time, while it is subjected to a constant shear rate.
5. *Rheopexy:* This is essentially the opposite of thixotropic behavior, in that the fluid's viscosity increases with time as it is sheared at a constant rate (Brookfield Viscometer Manual 2000).

3.3 Broth Rheology

The behavior of fermentation broth is of considerable importance with reference to cellular kinetics and in the engineering design of fermenters. A knowledge of the flow behavior of the broth serves as a prerequisite for an understanding of the heat and mass transfer involved in process design, scale up and operations. Relatively little information is available to describe fermentation broth rheology or the time changes in rheological behavior as the fermentation proceeds (Pazouki and Panda 2000).

Fermentation broths containing mycelial cells frequently exhibit a pseudoplastic non-Newtonian rheological behaviour, which can be described by the power-law model. This behaviour exerts a profound effect on the bioreactor performance, affecting mixing pattern, power requirement, heat and mass transfer processes, decrease in productivity and/or the production of undesirable metabolites. The increase in the broth apparent viscosity during aerobic fermentations can be partially compensated by increments in the operating conditions, in order to maintain adequate volumetric oxygen transfer rate (k_La) values (Olsvik et al. 1993, Badino et al. 2001).

Generally, particle dispersion shows high non-Newtonian behaviour even though the medium is a Newtonian liquid. And the deviation from the Newtonian flow behaviour becomes strong, concentrated suspensions often show gradual or abrupt increase in the shear viscosity. Continuous or discontinuous shear thickening affects actual process or decreases productivity. Therefore, it is necessary to investigate the microstructural changes in the suspension under flow field. By doing so, we can understand the flow characteristics of concentrated particle suspensions and somehow prevent severe fracture problems in practical processes (So et al. 2001).

The broth rheology change during the time course of batch fermentations is influenced by several factors like mycelial concentrations, the morphology of the organism and the production of extracellular material by organism.

The factors influencing the rheological properties of the fermentation broth are biomass concentration, specific growth rate, mixing qualities (impeller speed and working volume) and dissolved oxygen concentration.

The effect of the specific growth rate on broth rheology has been strongly influenced by the DO concentration in the broth. Higher influence of a change in the DO concentration on the viscosity of the broth has been found at the lowest growth rate and the minimum DO concentration gives a simple description of the structure. The morphology of the culture has been found to increase with cell concentrations (Pazouki and Panda 2000).

CHAPTER 4

INFLUENCE OF PROCESS PARAMETERS ON THE FUNGAL MORPHOLOGY AND BROTH RHEOLOGY

4.1 Inoculum

Among the factors that determine the morphology and the general course of fungal fermentations, the amount, type (spore or vegetative) and age of the inoculum are of prime importance. High concentrations of the spore inoculum give filamentous mycelium, while low concentrations produce pellets (Pazouki and Panda 2000, Papagianni 2004).

The size of the pellets, depend generally on the spore inoculum size, while the structure of pellets depend mainly on cultivation temperature (Papagianni 2004).

For consistency of fungal fermentations it is important to maintain a uniform inoculum, because once a mycelial structure has formed, it remains a discrete entity for long periods, extending and growing older.

Inoculum size can also influence growth, culture morphology, antibiotic production, and enzyme content of mycelia (Paul et al. 1993).

4.2 Media Composition

The media used in submerged industrial fermentations favor both growth and product formation at high yields. Fermentation media can be chemically defined (synthetic) or complex. For the industrial production of enzymes and antibiotics, complex media with a solid substrate are often used and the use of complex media in submerged fermentations of filamentous fungi has been shown to influence the morphology and growth kinetics.

Fungi require water, molecular oxygen, an organic source of carbon and energy, a source of nitrogen other than molecular nitrogen and several other elements. At least 13 elements are essential for growth, namely oxygen, carbon, hydrogen, nitrogen, phosphorus, potassium, sulfur, magnesium, manganese, iron, zinc, copper and molybdenum. The first eight are needed in relatively large quantities (macronutrients) and the latter five are required in small amounts (micronutrients) (Papagianni 2004).

From the literature, it is known that, high nitrogen concentrations give compact smooth pellets; low concentrations give very loose and fluffy pellets and ammonium sulfate in the medium caused pellet growth. Also, an inverse relationship existed between glucose concentration and the number of pellets. In this case, however, glucose concentration has very little effect on level of biomass produced (Metz and Kossen 1977, Pazouki and Panda 2000).

A significant change in morphology is often observed when the composition of the growth medium is varied.

4.3 Culture pH

The pH of culture broth is one of the most critical environmental parameters affecting growth and activity in submerged cultures. pH of the growth medium plays an important role in the process of pellet formation, especially in the coagulation of spores. It is suggested that surface properties of the spores that are influenced by pH are responsible for the coagulation. Fungi can grow over a wide range of pH. Most tolerate a pH range from 4 to 9 but grow and sporulate maximally near neutral pH.

pH effects are often investigated using the same microorganism in flask experiments with different initial pH values. Initial medium pH may affect cell membrane function, cell morphology and structure, the solubility of salts, the ionic state of substrates, the uptake of various nutrients, and product biosynthesis. In general, cells can only grow within a certain pH range, and metabolite formation is also often affected by pH (Metz and Kossen 1977, Fang and Zhong 2002, Papagianni 2004, Shu and Lung 2004).

The high concentrations of ions such as phosphate that are required to achieve some measure of pH stability often appreciably influence the biological activity being measured (e.g., growth and enzyme activity). Also, the authors concluded that the surface properties of spores were influenced by pH (Papagianni 2004).

4.4 Temperature

Studies of temperature effects on growth and metabolite production are few with respect to filamentous fungi. Closed culture systems are highly unsuitable for the elucidation of temperature effects on fungal growth. Although temperature is an environmental parameter that is easy to control, changes in temperature produce

simultaneous changes in other culture variables. An increase in incubation temperature within physiological ranges enhances the growth rate and Q_{10} values of 2 and 30 are common in the literature. [Q_{10} is the factor by which the death rate increases for a 10°C increase in temperature.]

Dissolved oxygen tension is also temperature dependent and varies inversely with increasing temperature. Similarly, nutritional and pH requirements for growth may be influenced by the temperature. Also, pellet formation and pellet size distributions are influenced by temperature (Papagianni 2004).

4.5 Aeration and Dissolved Oxygen Tension

On the basis of present information it appears that most fungi require molecular oxygen to grow. Fungi can grow over very wide ranges of oxygen tensions and it is known that increasing the rate of oxygen transfer can enhance the rate of product formation (Papagianni 2004, Dhanasekharan et al. 2005).

Oxygen tension in the growth medium is of course very important for the oxygen supply of the pellets. Oxygen diffuses into the pellet and is consumed. Owing to the limited solubility of oxygen, the amount present is very low so that oxygen depletion in the center of the pellets can occur very easily. This will cause autolysis of the cells and eventually the formation of a hollow center. So, activity in terms of oxygen consumption must be located on the surface of the pellet.

Metz and Kossen (1977) found for *Aspergillus niger* that aeration with air produced fluffy loose pellets. These pellets became hollow at diameters greater than 1.75 mm. Aeration with gas containing 50% oxygen produced a much denser growth at pellet wall, owing to a much shorter, thicker, and more branched character of the hyphae.

High dissolved oxygen tension intended to assist oxygen transfer into pellets might be maintained through increased impeller speed and additional air flow within stirred tank systems. However, the increasing shear near the impeller limits the formation of surface growth by ‘clipping off’ hyphae, so limiting the growth of the annular region of the pellet by preventing the extension of surface hyphae. The disruption of whole pellets might also occur (Cox and Thomas 1992).

It is well known that, particularly in the case of fungal microorganisms, as the biomass concentration increases, there is a considerable reduction in the absorption coefficient. The interaction between the oxygen consumption and supply rates will determine the exact shape of the dissolved oxygen concentration. Industrial aerobic processes usually attempt to operate in such a way that the full capacity of the fermenter aeration system for oxygen transfer is utilized. This means that at some point in the batch fermentation, the oxygen concentration will be very close to zero. It is thus most important to appreciate any factor which might disturb the oxygen concentration profile and possibly cause a period of supply limitation (Brown and Zainudeen 1978).

Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product/by-product and oxygen (Mantzouridou et al. 2002).

The oxygen supply into the broths constituents one of the decisive factors of cultivated microorganisms' growth and can play an important role in the scale-up and economy of aerobic biosynthesis systems. The aeration efficiency depends on oxygen solubilization and diffusion rate into the broths, respectively on the bioreactor capacity to satisfy the oxygen demand of microbial population. However, the dissolved oxygen in the broths is limited by its consumption rate on cells or the oxygen uptake rate, as well as its oxygen transfer rate.

The oxygen mass transfer can be described and analyzed by means of mass transfer can be described and analyzed by means of mass transfer coefficient, k_{La}. It represents the most important parameter implied on the design and operation of mixing-sparging equipment of the bioreactors. The k_{La} values are affected by a lot of factors such as;

- agitation,
- air flow rate,
- air pressure,
- temperature,
- fluid characteristics (density, viscosity, surface tension, etc.),
- presence of antifoam agents,
- geometrical and operational characteristics of the vessel,
- media composition, type, concentration
- microorganisms morphology,

- biocatalysts properties (Ozbek and Gayik 2001, Galaction et al. 2004, Bandaiphet and Prasertsan 2006).

In a mechanically agitated tank, one frequently used method to overcome oxygen limitation is simply to increase impeller power, thus increasing oxygen mass-transfer rate. However, an increase in impeller power can also affect cells by changing their morphology of fragmentation behavior (Cho et al. 2002).

In submerged fermentations, oxygen in gas bubbles diffuses toward the liquid film where it dissolves at the gas-liquid interface. The dissolved oxygen, then, diffuses through the liquid film that surrounds the gas bubble before entering the continuous liquid bulk where it is made available to microorganisms. k_{La} accounts for the total surface area between the gas and the liquid phase (a), and the overall convective mass transfer coefficient (K_L). The latter considers the mass transfer resistance on both sides of the gas-liquid interface. Outside the gas and the liquid films, the gaseous and dissolved oxygen concentrations are considered homogeneous. Several methods, such as sulphite oxidation, the dynamic method or overall gas balance are currently employed to measure or estimate k_{La}. Moreover, the dissolved oxygen concentration, required by most methods for k_{La} evaluation, can be easily measured in this culture system using a dissolved oxygen probe (Thibault et al. 2000).

4.6 Agitation

In submerged fermentations, agitation is important for good mixing and mass and heat transfer, and homogenization. In aerobic processes, mixing is required to ensure sufficient oxygen transfer throughout the vessel (Papagianni 2004).

Whether pellets are formed or not is to large extent determined by agitation. The first effect of agitation that is important is the dispersion of spore agglomerates. Strong agitation can prevent the formation of pellets for strains with a coagulative pellet formation. For noncoagulative pellet formation, pellet formation only occurs under strong agitation conditions. Increase of aeration rate causes filamentous growth. Mechanical agitation causes development of mycelium around the impeller.

Agitation also influences the structure and survival of pellets once they have been formed. Especially, agitation affects the size of the pellets. The general tendency in the literature is that more agitation gives smaller and more compact pellets. Hollow

smooth pellets especially develop under conditions of strong agitation. Strong agitation can cause break-up of pellets also, the pellet size decreases with increasing agitation intensity (Metz and Kossen 1977). Also, strong agitation results in a higher dissolved oxygen tension and more branching of hyphae. So, for a growing system or fermentation, the pellets subjected to a stronger agitation can be expected to have a higher tensile strength (Cui et al. 1997).

In the case of a conventional stirred tank bioreactor, the vessel can be divided into two regions. The region around the impeller represents a zone of high energy dissipation and, hence, good mass and heat transfer. The remainder of the fermentation volume may be subjected to an inadequate supply of oxygen because of poor mixing. In large fermenters, mass transfer gradients across the vessel become significant and this can affect mycelial growth and product formation. Agitation applied during fermentation creates shear forces which can affect microorganisms in several ways, e.g., damage to cell structure, morphological changes, as well as variations in growth rate and product formation (Papagianni 2004).

The agitation conditions in fungal fermentations require great attention. Together with the total power input, the choice of impeller geometry, position, and number determines the mechanical forces that might affect microorganisms in a fermentor, therefore influencing growth or production and possibly changing the morphology and differentiation of filamentous species (Jüsten et al. 1998). Potential damage to microorganisms can limit the impeller speed or power input and consequently the oxygen and nutrient transfer capability of a fermenter, and ultimately the volumetric productivity. Furthermore, changes in morphology can alter the viscosity of filamentous fermentation broths, with additional effects on mixing and mass transfer (Jüsten et al. 1996).

The spacing impellers are critical to the process results. When impeller spacing is too far apart, dead zones occur. These dead zones contribute to the starvation of the micro-organisms which turn leads to a reduction in yield and productivity (Kaufman et al. 1997). It was suggested that the difference in productivity can be due to a much higher specific power input and oxygen transfer rate attained by the Rushton turbine, and due to the higher shear stress produced in the impeller region of the high-speed propeller (Jüsten et al. 1998).

Baffles are very important in the majority of fermentation processes. Baffles reduce the inefficient tangential velocity component of all impellers to produce a more

efficient radial or axial flow. The placement of baffles is not trivial. Positioning and design of baffles along with the placement, type and design of heating coils has a significant impact on the flow patterns and thus, on the overall process results (Kaufman et al. 1997).

However, all these factors can be controlled and optimized, agitation induced fragmentation, apart from growth, is considered to be one of the most important factors influencing mycelial morphology (especially in the design, operation and scale-up of fungal fermentations) (Amanullah et al. 2000).

CHAPTER 5

MATERIALS AND METHODS

5.1. Materials

5.1.1 Microorganism

Aspergillus sojae ATCC 20235 was purchased in the lyophilized form, from Procochem Inc., and international distributor of ATCC (American Type of Culture Collection) in Europe.

5.1.2 Chemicals

Table 5.1 Chemicals used

NO	CHEMICAL	CODE
1	Ammonium heptamolybdate-tetrahydrate	Merck 1.01182
2	Antifoam	Mazu DF 2105
3	Bacteriological agar	Oxoid LP0011
4	Brain Heart Infusion Agar (BHI)	Fluka 70138
5	Copper(II)sulphate-5-hydrate	Riedel-De Haën 12849
6	Corn steep liquor	Sigma C4648
7	D-Galacturonic acid	Fluka 48280
8	D-Glucose	AppliChem A3666
9	di-Sodium hydrogen arsenate heptahydrate	Fluka 71625
10	Ethanol	Riedel-De Haën 32221
11	Folin-Ciocateu’s phenol reagent	Merck 1.09001
12	Formaldehyde	Fluka 47630

(cont. on next page)

Table 5.1 Chemicals used (cont.)

NO	CHEMICAL	CODE
13	Glacial acetic acid	Riedel-De Haën 27225
14	Glycerol	Sigma G5516
15	Iron(II)suplhate heptahydrate	Riedel-De Haën 12354
16	K_2HPO_4	Riedel-De Haën 04243
17	KCl	Riedel-De Haën 31248
18	Malt Extract	BD 218630
19	Maltrin	Cargill Starch&Sweeteners
20	Manganese(II)sulphate monohydrate	Riedel-De Haën 13255
21	Monosodium phosphate	Riedel-De Haën 04270
22	Molasses	Pakmaya Kemalpasa Uretim Tesisi
23	$MgSO_4.7H_2O$	Merck 1.05886
24	NaCl	Riedel-De Haën 13423
25	NaOH	Merck 1.06462
26	Na_2SO_4	Carla Erba 483007
27	Peptone	Acumedia 7182A
28	Phenol	Sigma P5566
29	Polygalacturonic acid	Sigma P3850
30	Potassium sodium tartarate tetrahydrate	Sigma S6170
31	Protein standard, 2mg BSA	Sigma P5619-25VL
32	Sodium carbonate anhydrous	AppliChem A3900
33	Sodium dihydrogen phosphate monohydrate	Fluka 71507
34	Sodium phosphate di basic dihydrate	Riedel-De Haën 04272
35	Sulphuric acid	Merck 1.00713
36	Yeast Extract	Fluka 70161
37	Tween80	Merck 8.22187

5.2 Methods

5.2.1 Spore Production

The propagation of the cultures was done on Yeast Malt Extract (YME) agar slant medium containing, malt extract at 10 g/l, yeast extract at 4 g/l, glucose at 4 g/l and agar at 20 g/l concentrations, incubated at 30^0C until well sporulation (1 week). Stock cultures of these strains were prepared with 20% glycerol water and stored at -80^oC.

The spore suspensions used as inoculum were obtained on molasses agar slants containing glycerol (45 g/l), peptone (18 g/l), molasses (45 g/l), NaCl (5 g/l), $FeSO_4.7H_2O$ (15 mg/l), KH_2PO_4 (60 mg/l), $MgSO_4$ (50 mg/l), $CuSO_4.5H_2O$ (12 mg/l), $MnSO_4.H_2O$ (15 mg/l) and agar (20 mg/l). Initially, the frozen stock cultures were inoculated on YME agar and incubated for one week at 30^oC for activation. After this period a single isolate of the strain was inoculated on molasses slant and incubated for another week at the same temperature. Following the incubation period, 5 ml of 0.02% (v/v) tween80 solution was added into each slant and harvested into empty sterile falcon tube and tested for sterility, viability and spore count. The spore counts were performed using Thoma bright line hemacytometer (Marienfield,Germany). The suspensions were stored at 4^oC and used as inoculum for the fermentation process. The initial spore counts and viability counts were recorded.

5.2.2 Production Medium and Fermentation

The fermentations were performed in a New Brunswick BioFlo 3000 (NJ, USA) stirred tank reactor with agitation, aeration, temperature, pH and dissolved oxygen control. Experiments were carried out at 30^oC in a 6.6L capacity culture vessel with 4L culture medium for 96 hours.

Agitation was provided with two sets of a standard six-blade rushton impeller (diameter: 6 cm, length: 1.5 cm, width: 2 cm) and four baffle plates (width: 1.5 cm, length: 20 cm) equipped in the bioreactor in order to favour turbulence and to prevent vortex formation. The geometry of the used fermenter is given in Figure 5.1.

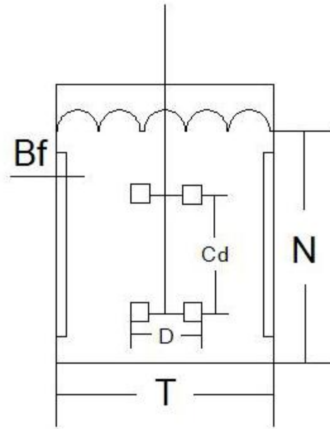


Figure 5.1 Geometry of the fermenter used

Where;

B_f = 1.5 cm

C_d = 10.8 cm

D= 3.3 cm

T= 40 cm

N= 32 cm

The fermentation media composition was prepared with corn steep liquor (5 g/l), peptone (5 g/l), maltrin 50 (75 g/l), disodium phosphate (3.2 g/l), monosodium phosphate (3.3 g/l) and glucose (0.75g/l) in the amount of 4L. First, they were solved in 2000 ml distilled water in erlenmayer, then 200 µL antifoam was added and the pH was adjusted to 6.1. Media was transferred to fermenter, in the fermenter 2000 ml distilled water was added and again the pH was adjusted to 6.1. The bioreactor and all its parts were sterilized by autoclave along with the medium (except glucose) at 121°C for 50 minutes and glucose (3g/10ml) which was already autoclaved was fed to the fermenter.

The fermentation media was prepared a day before the run, autoclaved and hooked up to the main fermenter unit after cooling. The bioreactor was turned on and the pH was calibrated. In order to polarize the DO probe the unit was turned on with the water supply, the agitation was set to 100 rpm, temperature to 30°C, DO and pH to PID and run for overnight. Next day after connecting acid, base and antifoam pumps, the units were set to 100 rpm and 2.5 L/min aeration for at least1 hour to saturate the media

with 100% dissolved oxygen. Once the 100% saturation was reached the DO probe was calibrated and operating parameters were set up to desired values.

Temperature was controlled by a microprocessor based controller. The media temperature is sensed by a Resistance Temperature Detector (RTD) submerged in the thermowell.

The dissolved oxygen concentration was monitored by means of an autoclavable polarographic oxygen electrode (Mettler Toledo, USA) on line. The control was maintained by the P-I-D (proportional/integral/derivative) controller by changing the speed of agitation and/or the percentage of oxygen in aeration.

Air was introduced into the media through a 0.2 µm pore size cellulose filter by using a ring sparger. The flow rate was controlled automatically by the thermal mass flow controller.

The pH was monitored by means of a sterilizable liquid – filled pH electrode (Mettler Toledo, USA) and controlled at desired values using automatic control equipment (P-I-D) driving a peristaltic pump delivering sterile 6N NaOH or 10% H_2SO_4 when required.

Foam is controlled by the antifoam probe. The controller operates the antifoam addition pump that adds chemical defoamer (Mazu DF 2105, USA) into the vessel.

The inoculum amount into fermenter was $2x10^6$ total spore. Depending on the spore count obtained from the spore suspension of the frozen culture, the inoculum was suspended with additional 10 ml sterile water and transferred through sterile syringe into the fermenter.

Sterility was checked on the original spore suspension as well as on the fermentation media after inoculation. Also during the run, samples were taken for sterility check on BHI plates.

Samples were taken periodically and assayed for enzyme activity, biomass determination and substrate utilization. Enzyme activity and carbohydrate content were determined on supernatant obtained by centrifugation of the broth at 5000 rpm for 15 minutes at 4^oC. Fungal morphology and broth rheology were investigated during and at the end of the fermentation period (at 96 hours).

5.2.3 Enzyme Assay

PG (polygalacturonase) activity was assayed according to the procedure given by Panda et al. (1999) by using 2.4 g/l of polygalacturonic acid as substrate at pH 6.6 and 26°C. The amount of substrate and enzymes used were 0.4 and 0.086 ml respectively. The absorbance was read on Varian Cary Bio 100 UV-Visible spectrophotometer at 500 nm. In this study, one unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 μmol of galacturonic acid per unit volume of culture filtrate per unit time at standard assay conditions. Galacturonic acid was used as standard for calibration curve of PGase activity. Calibration curve was prepared using 50, 100, 200, 300, 400, 500 μl concentrations of the stock solution containing 500 nmol galacturonic acid in 500 μl.

$$\text{Activity (U/ml)} = (\text{mg of galacturonic acid} / 212.12) \times (1/20) \times (1/0.086) \qquad (5.1)$$

Where, 212.12 is the molecular weight of galacturonic acid (mg/mole), 20 is the reaction time (min) and 0.086 is the amount of enzyme in the reaction mixture (ml).

5.2.4 Protein and Total Carbohydrate Assays

The total protein contents of the samples were determined according to the method described by Lowry et al. (1951). Protein standard calibration curve was prepared in the range of 0-300 μg/ml with the protein standard Bovine Serum Albumin. The absorbance was read at 750 nm using Varian Cary Bio 100 UV-Visible spectrophotometer.

The total carbohydrate contents of the samples were determined by the phenol – sulfuric acid method. Carbohydrate standard calibration curve was prepared in the range of 10-40 μg/ml with D-glucose as the standard. The absorbance was read at 490 nm using Varian Cary Bio 100 UV-Visible spectrophotometer (DuBois et al. 1956).

5.2.5 Biomass Determination

The biomass expressed as dry cell weight (g/l) was determined by means of gravimetric method. The fermentation broth (25ml) which was taken periodically during fermentation was filtered through the pre-weight Whatman No.1 filter paper, followed by drying to constant weight at 37°C for approximately 24 hours.

5.2.6 Rheological Measurements

Rheological properties of fermentation broth were determined by using rotational viscometer (Brookfield DV II + Pro, Brookfield Engineering Lab. Inc., MA, USA) which was equipped with a Rheocalc32 program. This program has an auto range feature which shows in screen display that complete viscosity range which can be measured at any shear rate for a specific spindle geometry. The principle of operation of DV II + Pro is to drive a spindle (which is immersed in the test fluid) through a calibrated spring. The viscous drag of the fluid against the spindle was measured by the spring deflection. Spring deflection was measured with a rotary transducer for a given spindle geometry and speed (Brookfield Viscometer Manual, 2000). An increase in viscosity was detected by an increase in deflection of the spring which allowed to measure a variety of viscosity ranges. Temperature was adjusted to 30°C for all measurements.

Before use, the viscometer (accuracy, $\pm 1\%$ of full - scale range; repeatability, $\pm 2\%$ full–scale range) is calibrated with 100 cP Brookfield silicone viscosity standard at 25°C. A correction factor was calculated.

The viscometer was equipped with a cylinder spindle (600 ml of sample volume) which was used for the samples taken at the end of the fermentation period (at 96 h). These spindles provide a defined spindle geometry for calculating shear stress and shear rate values as well as viscosity. Because their defined geometry facilitates mathematical analysis, cylindrical spindles are particularly valuable when measuring non-Newtonian fluids. The results obtained can be converted into viscosity functions by a mathematical procedure described below:

$$\text{Shear Rate (sec}^{-1}\text{)}: \ \gamma = \frac{2\omega R_c^{2} R_b^{2}}{X^2 \left(R_c^{2} - R_b^{2}\right)} \quad (5.2)$$

Shear Stress (dynes/cm^2): $\tau = \frac{M}{2\pi R_b^{2L}}$ (5.3)

Viscosity (poise): $\eta = \frac{\tau}{\gamma}$ (5.4)

Where;

ω = angular velocity of spindle (rad/sec)

$\omega = [(2\pi/60).N]$, N=RPM (5.5)

R_c = radius of container (cm)

R_b = radius of spindle (cm)

X = radius at which shear rate is being calculated

M = torque input by instrument

L = effective length of spindle

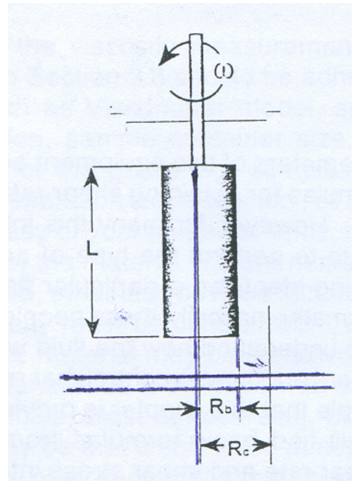


Figure 5.2 Geometry of cylindrical spindle

(Source: Brookfield Viscometer Manual 2000)

Table 5.2 Specifications of Cylindrical Spindle

(Source: Brookfield Viscometer Manual 2000)

Spindle	Radius (cm) ½ C	Effective Length (cm)	Actual Length (cm) D
LV1	0.9421	7.493	6.510

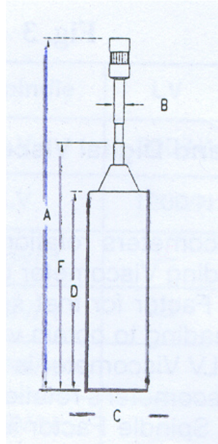


Figure 5.3 Dimensions of cylindrical spindle
(Source: Brookfield Viscometer Manual 2000)

Table 5.3 Dimensions of Cylindrical Spindle
(Source: Brookfield Viscometer Manual 2000)

Spindle	A (mm)	B (mm)	C (Diameter) (mm)	D (mm)	F (mm)
LV1	115	3.2	18.84	65.1	80.97

On the other hand, UL adapter attachment was used for the samples taken periodically during fermentation period because the sample amount which could be taken during the fermentation was 25 ml for rheological measurements. UL adapter features coaxial – cylinder geometry with a removable polyethylene end cap for outer cylinder. With the end cap in place, the adapter holds a sample volume of 16.0 ml. Temperature is controlled using a thermal water bath with precision of $\pm 2\,^{o}$C by a temperature controller (temperature accuracy of $\pm 1\%$) (Brookfield Viscometer Manual 2000).

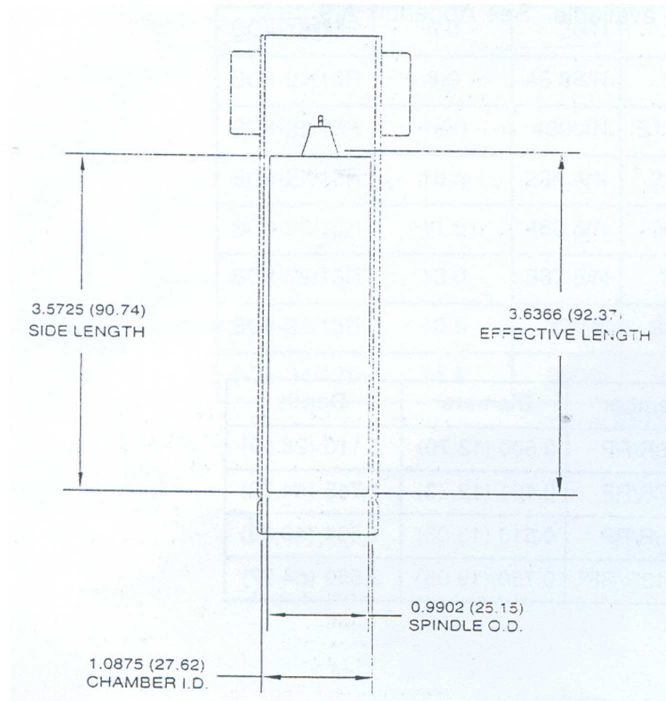


Figure 5.4 Geometry of ULA spindle

(Source: Brookfield Viscometer Manual 2000)

Table 5.4 Dimensions of ULA Spindle

(Source: Brookfield Viscometer Manual 2000)

Spindle	Spindle Effective Length (mm)	Actual Length (mm)	Diameter (mm)	Chamber Inside Diameter (mm)
ULA	92.39	90.74	25.15	27.62

But in these samples which were measured by UL adapter, the size of pellets was generally of the same order of magnitude as the annulus of the UL adapter. In order to overcome this measurement problem, the measured viscosity of fermentation broth was assumed to be a suspension and corrected by using a mathematical model developed by Metzner (1985).

The model for estimating viscosity of dilute suspensions was based on volume fraction of the suspended solids (pellet) (ϕ) and the relative viscosity of the suspension, given in Equation 5.6 and 5.7, where η was the viscosity of the suspension and η_s was the viscosity of the continuous phase:

$$\eta_r = \frac{\eta}{\eta_s} \qquad (5.6)$$

$$\eta_r = \left[1 - \left(\frac{\varphi}{A}\right)\right]^{-2} \qquad (5.7)$$

In equation 5.6, A was in the range of 0.44 and 0.68 for the crystal and spherical shape particles. For this purpose, the volume and mass of the each sample containing spherical pellets was initially measured and the bulk density of the suspension (fermentation broth) was calculated. Then the sample was filtered and mass and volume measurements were repeated for the filtrate (clear broth). Volume fractions (ϕ) (v/v) were calculated from these measurements. After, the viscosity of the clear broth, η_s, was measured by using viscometer and suspension viscosity η was calculated from equation 5.6.

All the shear data were then analyzed according to the power law (Eqn. 5.8) and Herschel-Bulkley model (Eqn. 5.9) (Rao 1999).

$$\tau = K\gamma^n \qquad (5.8)$$

$$\tau = \tau_0 + K\dot{\gamma}^n \qquad (5.9)$$

where, τ_0, τ, γ are the yield stress, shear stress and the shear rate respectively. The constants, K and n represent the consistency index ($Pa.s^n$) and the flow behavior index. Taking logarithm on both sides of Equation 5.8 and 5.9, yields K and n values. Since extra force (τ_0) was needed to start the flow in the fermentations where the number of pellets were very high, equation 5.9 was preferred to explain the rheological behavior of the broth.

5.2.7 Morphological Measurements

In order to investigate the pellet morphology; 1 g of fermentation broth was transferred to a Petri-dish and fixed with an equal volume of fixative (13ml of 40% formaldehyde, 5ml glacial acetic acid, 200 ml of 50% ethanol) (Park et al. 2002).

Pellet morphology was characterized by using image analysis (Cox and Thomas 1992). Pellet particles were analyzed for determination of the number of pellet per given volume, pellet diameter and pellet size distribution by image analysis.

At the same time, individual pellet images were captured with a eurocam (Euromax, Holland) mounted on a phase contrast microscope (Novex, Holland) after the pellet sample was air dried on a slide. The eurocam image was monitored and captured by Grabbee program. Normally, a magnification of 4 was used. This magnification was user selected to ensure optimal visualization of pellet detail for any given sample to preserve the measurement and characterization accuracy, while keeping the number of pellets per measured field high to maintain the speed of analysis (Cox and Thomas 1992).

Image analysis was performed with a software package Image – Pro Plus 4.5.1. (Media Cybernetics Inc., Silver Spring, MD, USA). The size of the pellet was quantified using the diameter corresponding to a circular area equivalent to the pellet projected area (Lopez et al. 2005).

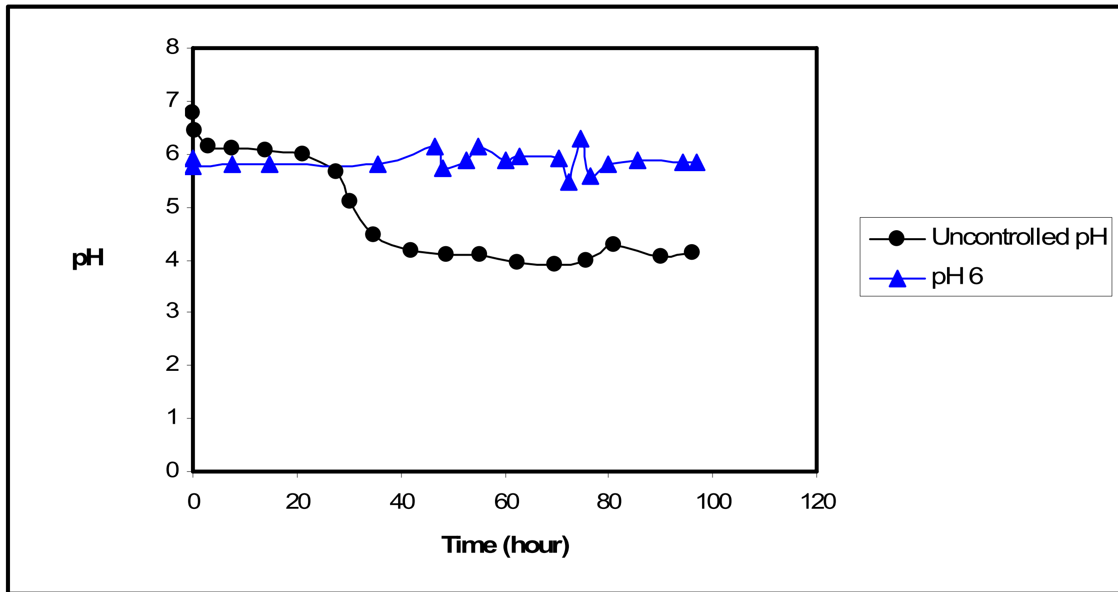
CHAPTER 6

RESULTS AND DISCUSSION

6.1 Effect of Medium pH on Biomass, Polygalacturonase, Pellet Morphology and Broth Rheology

The pH of the medium which is an important parameter is often a neglected environmental factor in fungal fermentations (Papagianni 2004). However, initial pH has profound effect on cell membrane function, cell morphology and structure, solubility of salts, the ionic state of substrates, the uptake of various nutrients and product biosynthesis (Fang and Zhong 2002). Also the effect of pH on growth and biosynthesis of products varies with different microorganism, operational conditions and medium composition. Often the optimum pH for growth is different from the optimum pH for maximum product formation demanding in some occasions two-stage operating conditions where in first stage the pH is adjusted for maximum growth and in second stage for maximum product formation. Besides, the pH inducing pellet type of growth might be different from the pH inducing the mycelial type of growth encountered in fungal fermentations. In general the tendency of the mycelium to form pellets increases as the pH value of the culture raises (Papagianni 2004). This is explained due to the change of the surface properties of the spores because of the pH (Carlsen et al. 1995). Therefore, it is vital to investigate the effect of this key parameter on the polygalacturonase production of a new strain (*Aspergillus sojae*) where there is lack of information in the literature and investigate its relation to morphology and broth rheology.

With this goal, two types of fermentations were run at constant pH of 6 and under un-controlled conditions (initial pH was adjusted to 6 but afterwards not controlled). The fermentation conditions were such that dissolved oxygen tension (DOT) was controlled at 50 % saturation through cascading the agitation speed between the ranges of 200-500 rpm at constant airflow of 2.5 L/min.



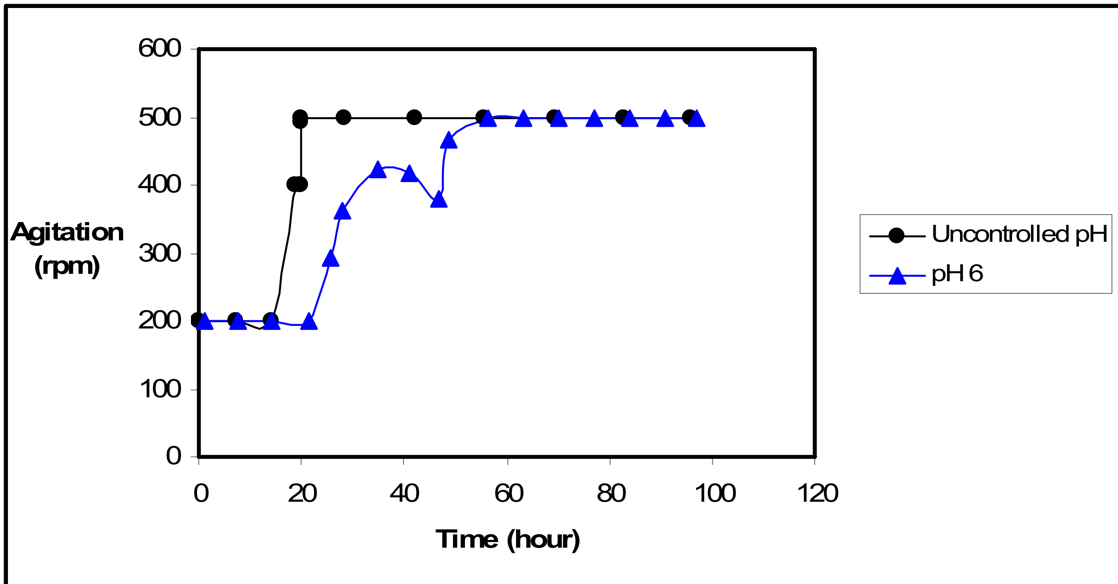
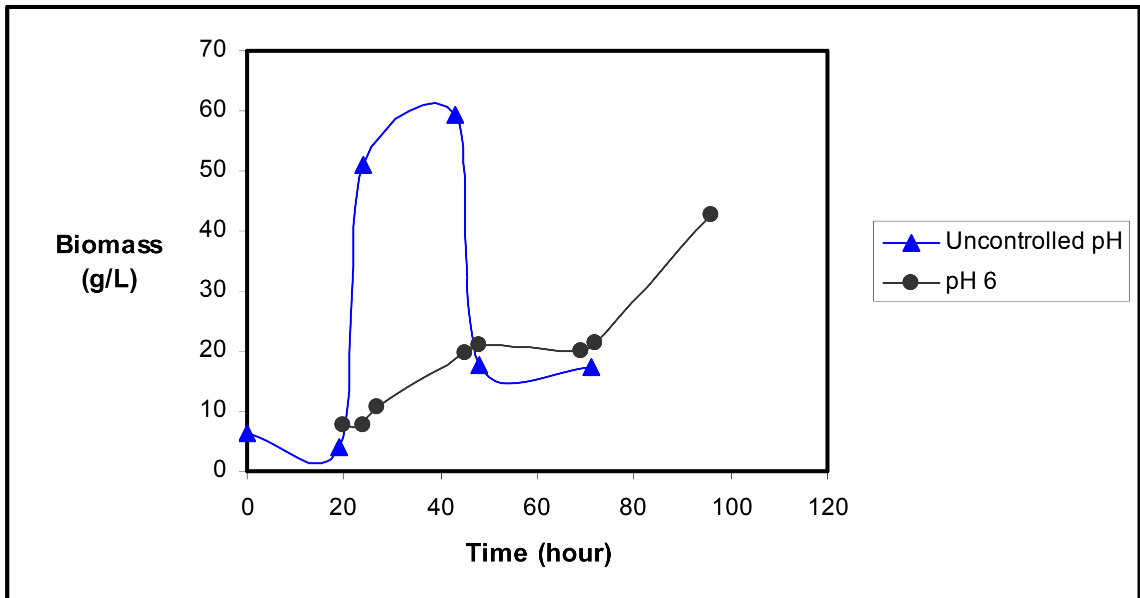


Figure 6.1. Profiles of a) pH b) agitation of uncontrolled and constant pH 6 experiments conducted at 30°C, 2.5 L/min air flow rate and cascaded between 200-500 rpm agitation speed.

The corresponding profiles of pH and agitation speed are presented in Figure 6.1.(a-b), whereas the profiles of biomass and carbohydrate utilization are presented in Figure 6.2.(a-b).



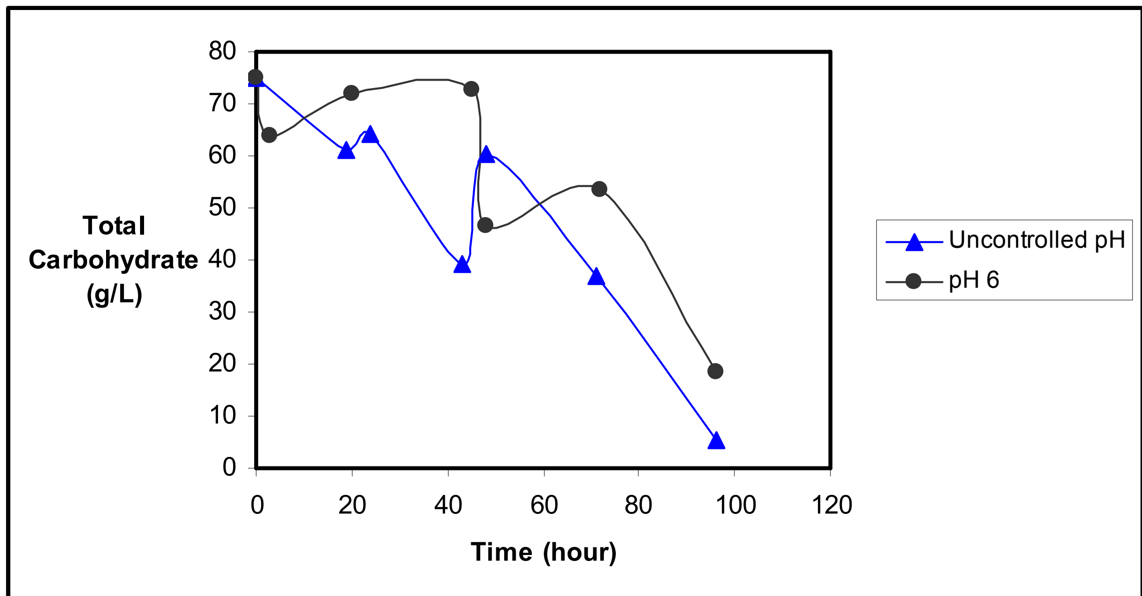


Figure 6.2. Profiles of a) biomass b) carbohydrate utilization of uncontrolled and constant pH 6 experiments conducted at 30°C, 2.5 L/min air flow rate and cascaded between 200-500 rpm agitation speed.

The un-controlled pH profile (Fig. 6.1.a) dropped to pH 4 at the end of 96 hours. Comparing this profile with biomass formation and carbohydrate utilization profiles (Fig. 6.2.a and Fig. 6.2.b), following conclusions can be withdrawn: (1) Uncontrolled pH resulted in a better and faster carbohydrate utilization as a result of better solubility of the nutrient compounds in the broth due to pH effect; (2) Cell membrane

permeability was affected because of low pH and nutrient transport into the cells was more efficient.

In fact maximum biomass which was 4 times higher compared to controlled pH experiment was achieved at 40 hours. The reason for the low biomass (at constant pH 6) was explained with the small compact and very dense smooth pellet structure (Table 6.1) possibly causing nutrient and oxygen limitations to the interior of the pellets reducing the growth.

Table 6.1. The Effect of pH on Rheology and Morphology at 30°C, 2.5 L/min Air Flow Rate and Cascaded Between 200-500 rpm Agitation Speed.

	Un controlled pH	pH = 6.0
Viscosity (cP) (at 73.38 1/s)		
Suspension	3.20	4.20
Filtrate	1.04	1.79
Pellet volume fraction	0.26	0.20
Pellet number/1 g media	92	74
Pellet morphology	Small pellets with dense core with a fluffy region surrounding the core	Small and compact smooth very dense pellets
Average pellet size (mm)	1.69±0.48	1.95±0.46

The overall biomass profile was such that, controlled pH (pH 6) resulted into a gradual increase whereas uncontrolled pH resulted into biomass decrease after 45 hours. This could be attributed partly to the pH changes encountered during this period but also to the high agitation speed observed at 500 rpm in order to maintain the dissolved oxygen tension at 50 % (Fig. 6.1.b) exhibiting shear effect and possibly mechanical damage to the cells. Hence, the decrease in DOT was faster in uncontrolled pH experiments as a result of faster growth increasing the oxygen consumption rate, leading to an earlier start of the cascade. The combination of all these factors could have contributed to the smaller size and higher number of pellet formation (Table 6.1, Fig. 6.3 and Fig. A.1.) observed in case of uncontrolled pH experiments.

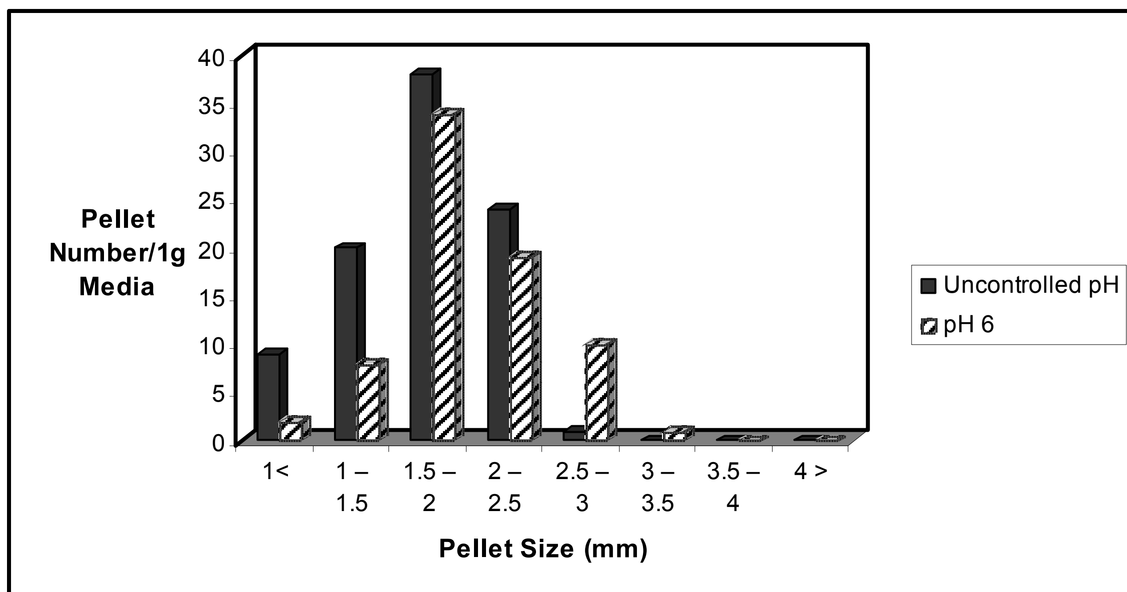


Figure 6.3. Pellet size distribution profiles of uncontrolled and constant pH 6 experiments conducted at 30°C, 2.5 L/min air flow rate and cascaded between 200-500 rpm agitation speed.

Hence, this observation pointed out some critical informations on the mechanism of pellet formation, which was due to chipping off and fragmentation of the existing pellets. However, this can not be attributed solely in case of controlled pH experiments where larger and fewer pellets were formed.

The effect of pH on morphology and its relation to rheology was also investigated (Table 6.1). Pellet volume fraction and number of pellet formation were comparable; the variation among the suspension viscosities was not extremely significant in both cases in spite of slightly higher (4.2 cP) suspension viscosity when pH was set to 6. This was due to the filtrate viscosity which was found to be faintly higher, imposing an effect on the suspension viscosity calculations. The difference between the filtrate viscosities could also be attributed to the production and secretion of some unknown metabolites into the medium by this microorganism. Overall, pH did not significantly affect the suspension viscosity, average pellet size, pellet number and pellet size distribution (Fig. 6.3) but created an impact on the pellet morphology. Morphology varied between the pellet form with a hairy region surrounding a dense core to compact, smooth and very dense pellet structure.

The polygalacturonase profiles (Fig. 6.4) for the two experiments were similar in profile but differed in quantities. The maximum polygalacturonase activity was achieved around 45 hours for both cases. The un-controlled pH experiments resulted into 38 % more activity compared to the controlled one. This could be related to the higher biomass and higher number of pellets obtained.

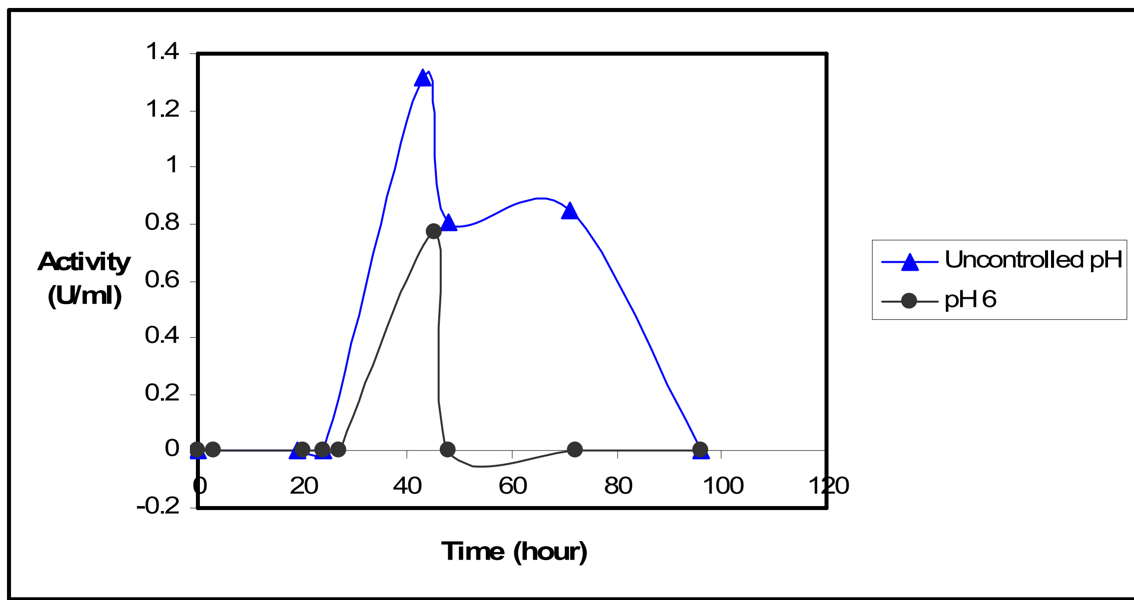


Figure 6.4. Polygalacturonase profiles of uncontrolled and constant pH 6 experiments conducted at 30°C, 2.5 L/min air flow rate and cascaded between 200-500 rpm agitation speed.

However in both experiments the activity declined after this period and no activity could be detected at the end of the fermentation (96 h). Explanations for this can be either this organism produced proteases that hydrolyzed the enzyme or the medium pH was not suitable to maintain the enzyme stable. Since the proteolytic activity was not determined, the former statement could not be justified but it is known to be very common among this type of organisms. This microorganism (*Aspergillus sojae)* is considered as GRAS (generally considered as safe) and very popular in Koji fermentation of a Japanese food in solid state fermentation of soya sources (Bennet 2001, Cho et al. 2002). Therefore, it is very likely that it might produce high amount of proteases. However this issue will be exploited in the future experiments.

As a conclusion, it is recommended to run the fermentation under uncontrolled pH conditions and stop the run around 50 hours in order to recover the

polygalacturonase enzyme. This is actually a positive outcome since not only the operational costs will be reduced due to shorter run but also the acid and base consumption will be eliminated, therefore reducing the overall material cost. On the basis, the forthcoming experiments were run under uncontrolled pH conditions.

Since there is no literature either on this organism or on the submerged polygalacturonase, no direct comparisons could be made. However there are several literatures investigating the effect of pH on morphology and on the fermentation of various products produced by various fungal organisms (Mattey 1992, Papagianni 1994, Fang and Zhong 2002). For example it is reported by a study performed by Carlsen et al. (1995), that cultivation of *Aspergillus oryzae* in stirred tank was strongly influenced by the medium pH, where below pH 2.5, mycelium grew heavily vacuolated and cells appeared swollen, resulting in poor growth. However at pH 4.0-5.0 they observed both pellets and freely dispersed hyphal elements. Similarly, in the same study the amylase production had a sharp maximum at pH 6, in contrast to the growth rate which had a broad maximum between pH 3.0 and 7.0. In summary, all these observations revealed, that the effect of pH as a key parameter needs to be investigated for each and every single strain under study.

6.2 Effect of Agitation Speed on Biomass, Polygalacturonase, Pellet Morphology and Broth Rheology

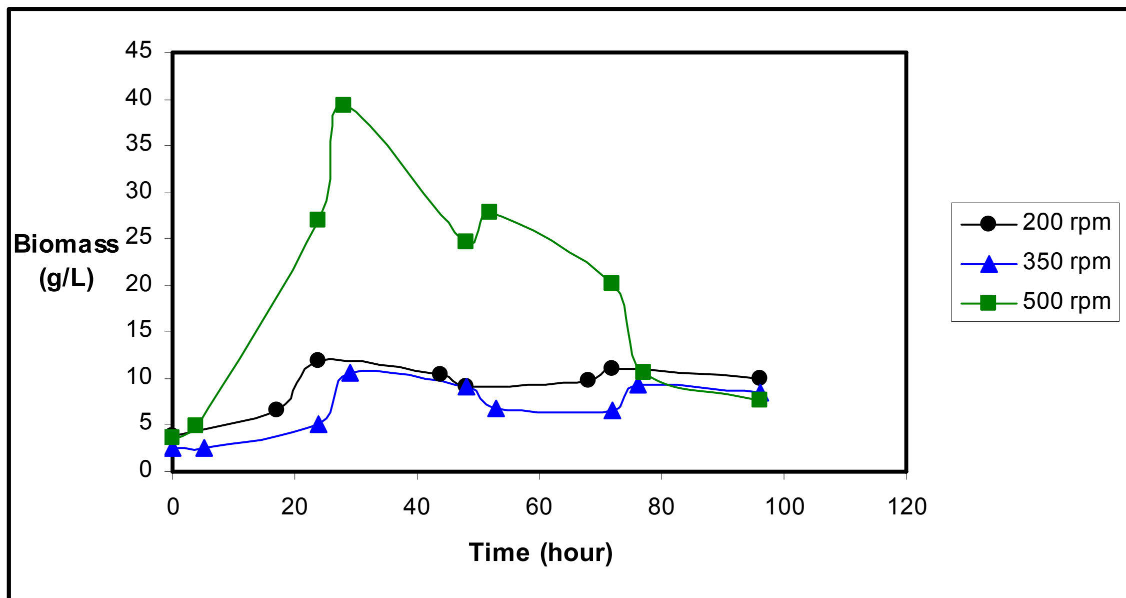
Agitation, another key factor playing significant role in submerged fermentation is mainly responsible from good mixing, mass and heat transfer and maintaining homogeneous chemical and physical condition of the entire broth (Mantzouridou et al. 2002). Especially in aerobic processes, mixing is essential to ensure sufficient oxygen transfer throughout the vessel (Papagianni 2004). It is reported by number of researchers that agitation creates shear forces, which affect microorganisms in several ways, causing morphological changes, variation in their growth and product formation, and damage even the cell structure (Jüsten et al. 1998, Papagianni et al. 1998, Mantzouridou et al. 2002). For example agitation induced fragmentation of fungal cultures, apart from growth is considered to be one of the most important factors influencing mycelial morphology, especially in design, operation and scale-up of fungal fermentations (Amanullah et al. 2000). Moreover, potential damage to the cells can

limit the impeller speed or power input and therefore the oxygen and nutrient transfer capability of a bioreactor, which ultimately will affect the volumetric productivity. In fact, optimum conditions of agitation for each culture will be determined besides their physiological state, by the resistance of the hyphae or pellets to mechanical forces (Paul et al. 1994, Papagianni et al. 1999).

Hence, it is essential to determine the effect of such an important parameter, in order to establish the correct correlations between morphology, rheology and polygalacturonase production for this particular study.

Based on the discussions above, fermentation runs at three different but constant agitation speeds of 200 (tip speed 0.628 m/s), 350 (tip speed 1.099 m/s) and 500 rpm (tip speed 1.57 m/s) and constant air flow (2.5 l/min), under uncontrolled pH conditions were performed. All the other fermentation conditions were the same as described in Materials and Methods.

The corresponding profiles of biomass and pH are presented in Fig 6.5.(a-b) and DOT and carbohydrate are presented in Fig. 6.6.(a-b) from which it can be seen that the growth was considerably faster at 500 rpm compared to the other agitation speeds. The maximum biomass obtained at this speed was 3.27 times and 3.67 times more than 200 and 350 rpm (at 30h), respectively.



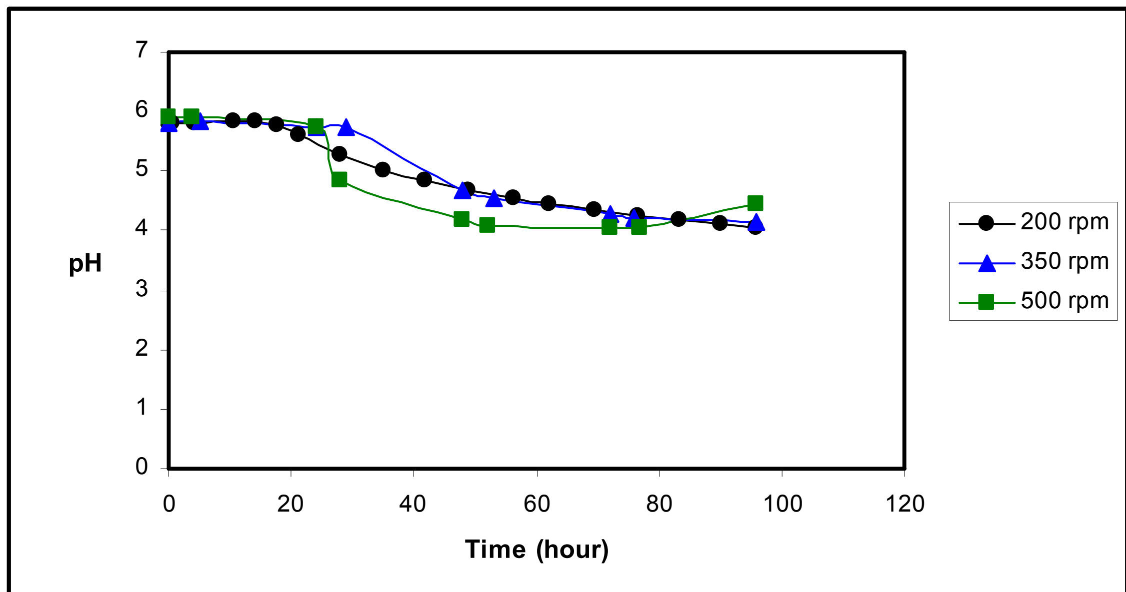
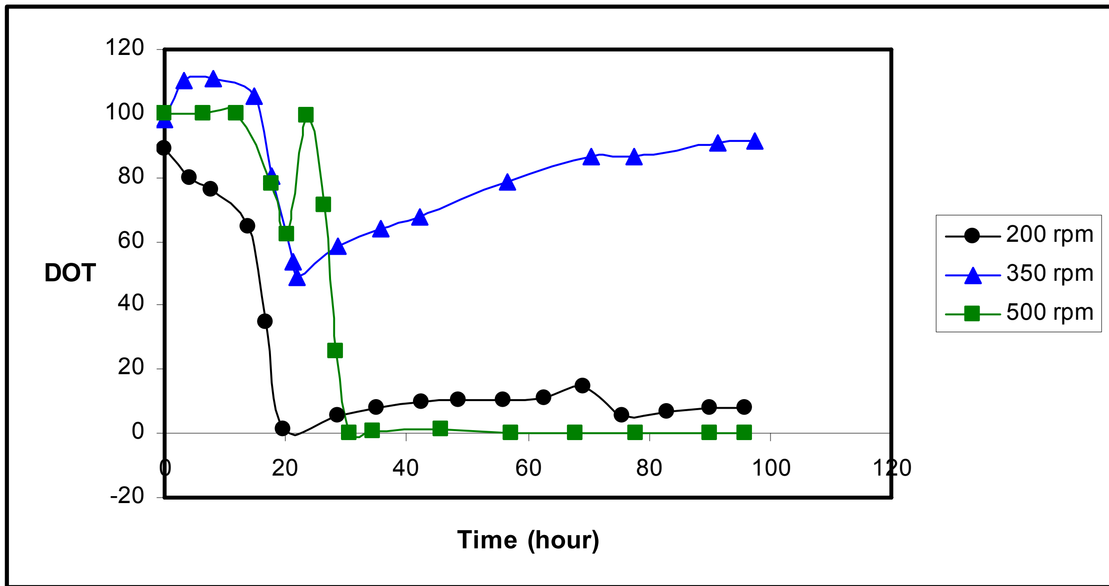


Figure 6.5. Profiles of a) biomass b) pH of experiments conducted at different agitation speed of 200, 350 and 500 rpm at 30°C, 2.5 L/min air flow rate.

For all three runs the differences in pH profiles were encountered mainly during the lag and growth phases, reaching the final pH of 4– 4.5 at the stationary and death phases.



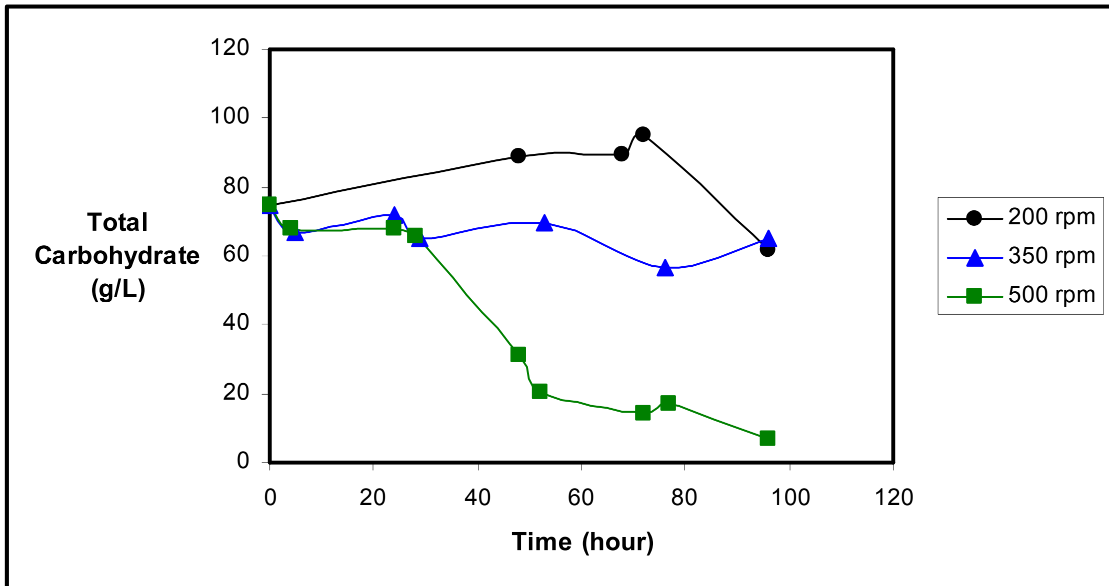


Figure 6.6. Profiles of a) DOT b) carbohydrate utilization of experiments conducted at different agitation speed of 200, 350 and 500 rpm at 30°C, 2.5 L/min air flow rate.

The slow growth at 200 rpm could be related to insufficient oxygen supply, which dropped to 0% saturation at 16 hours, increased to 10 % after 34 hours and remained at this level until the cessation of the run.

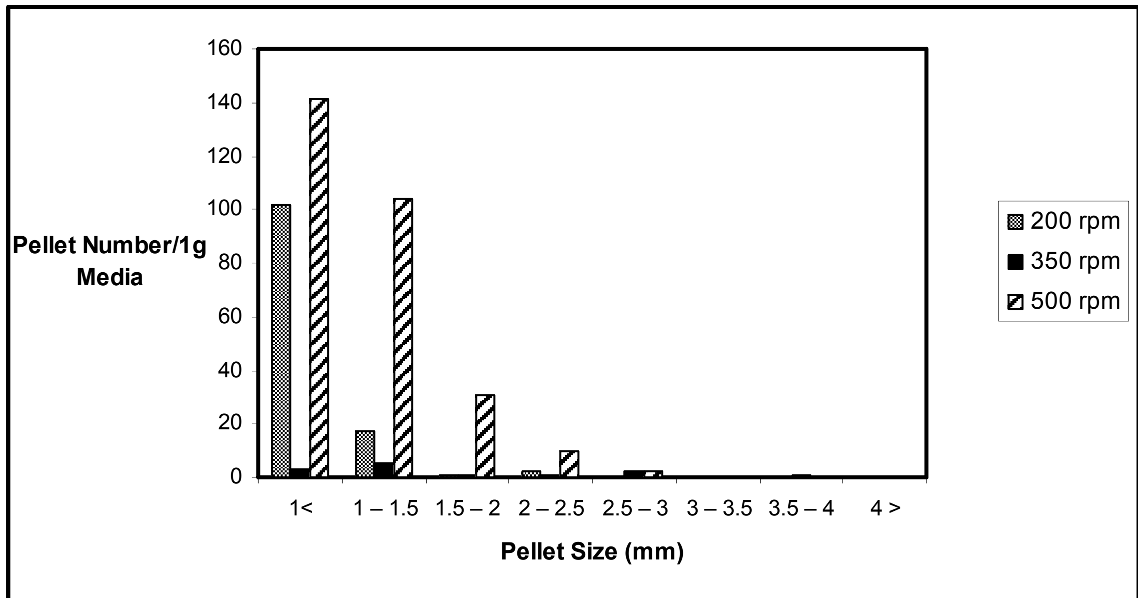
This finding was also supported by the carbohydrate utilization profile which was slower at this agitation speed (Fig. 6.6.b). At both 200 and 350 rpm carbohydrate was not utilized completely and remained at high levels. Because of faster biomass

formation carbohydrate consumption was almost complete at 500 rpm. The DOT profile at this speed was very well correlated with both carbohydrate and biomass profiles (Fig 6.6.a, Fig 6.6.b and Fig 6.5.a).

The oxygen supply could have been a limiting factor (DOT was 0%) at 500 rpm since oxygen transfer rate was almost equal to oxygen consumption rate. Although DOT level was not the limited factor at 350 rpm (since it was above 50 % until the end of fermentation), some other factors might have contributed to the slow growth discussed above. Hence, this brings up the discussions on the effect of agitation speed on the morphology of the culture and consequently on the broth rheology.

As it can be seen from the pellet size distribution (Fig 6.7.a) very small compact spherical pellets with an average size of 0.71±0.35 mm were obtained at 200 rpm with narrow size distribution. However, at 500 rpm small fluffy pellets with an average size of 1.08±0.42 mm with wide pellet size distribution was obtained (Fig B.1.).

Also the mechanism for the pellet formation at this speed as discussed in previous section (6.1) was due to chipping off as can be observed from the pellet size profile taken during the entire run (Fig 6.7.b and Fig 6.8.). As can be observed from this profile the pellets were formed within 24 hours and the perimeter and core diameter were reduced over the run pointing out that in addition to the chipping off, the breakage of pellets might have occurred as well. This explains also the wider size distribution observed in this particular run.



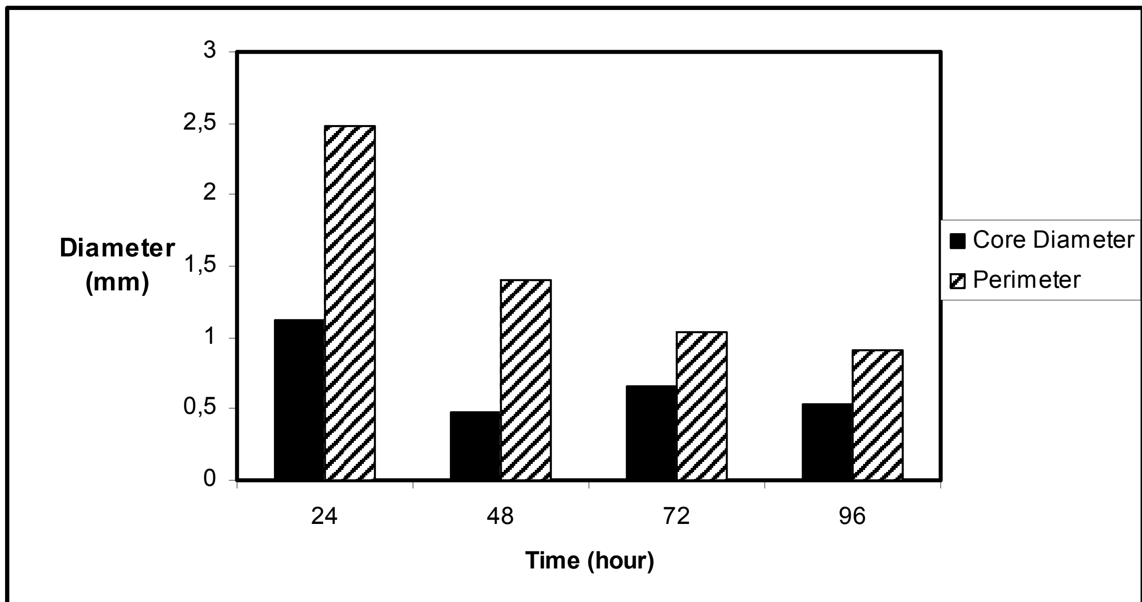


Figure 6.7. Profiles of a) pellet size distribution of experiments conducted at different agitation speed of 200, 350 and 500 rpm at 30°C, 2.5 L/min air flow rate b) pellet size profiles during the course of fermentation conducted at 500 rpm.

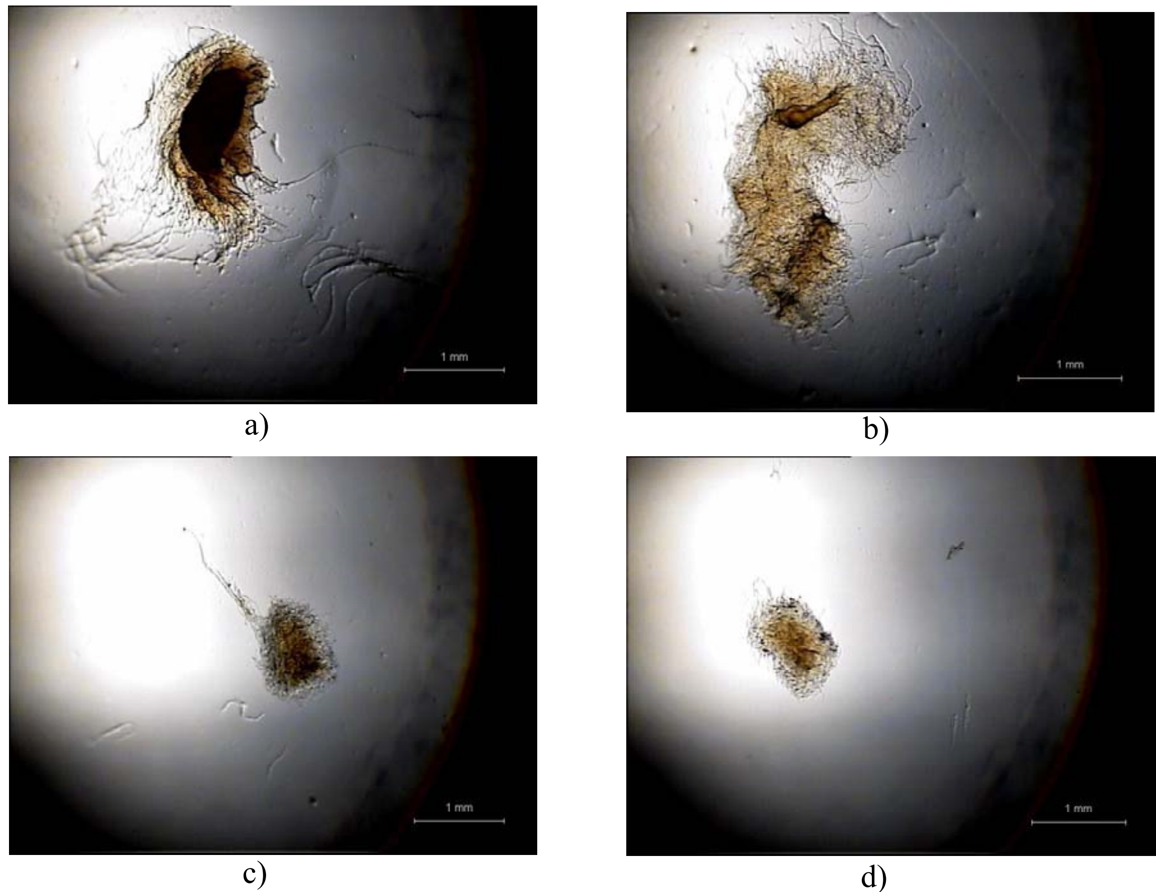


Figure 6.8. Pellet morphologies at a) 24.h, b) 48.h, c) 72.h, d) 96.h

The current observation was in accordance with many reports in the literature, reporting two main mechanisms of pellet size changes: (1) Chipping off pellicles from the surface of pellets and reducing the diameter and (2) Direct brake up of the pellet structure due to higher hydrodynamic loads (Nielsen et al. 1995, Papagianni 2004). Considering the morphology at 350 rpm, few big pellets with compact centers with an average size of 1.70±0.93 mm were obtained (Fig B.1.). In fact, this explains that there might have been diffusion mass transfer resistances in the interior of the pellets with respect to oxygen and nutrients. Hence, the DOT level and carbohydrate levels were high, whereas the biomass formation was low (Fig 6.6.a, Fig 6.6.b and Fig 6.5.a).

Furthermore, the mechanism of pellet formation in this case was mostly due to agglomeration of small pellets into bigger ones induced by agitation. Also this is reported in the literature by many researchers (supporting our findings) that in stirred tanks agitated up to 300 rpm, the hydrodynamic shear regimens are comparable and permit stable existence of pellets up to 2.3 mm in diameter (Porcel et al. 2005).

Overall one can draw the following final conclusions that low agitation speed (200 rpm) was not sufficient to fulfill the high demand of oxygen. which inturn affects the carbohydrate metabolism of the culture, resulting in low biomass and small pellets. On the other hand a medium level of agitation (350 rpm) induced bigger pellet formations with compact centers facing both oxygen and nutrient mass transfer resistance, resulting in low biomass. However, high agitation speed (500 rpm) was also not adequate in supplying the necessary oxygen for the fast carbon metabolism due to faster growth. Chipping off (erosion) and breakage (outright rupture) mechanism resulted in small sized pellets with wide distribution.

As it is well known, biomass concentration and morphology affects the rheological properties of the fermentation broth. These effects on the rheology can be interpreted using the biomass concentration profile (Fig. 6.5.a) and viscosity data (Fig. 6.9) along with corresponding consistency index (K) and flow behavior index (n) (Table 6.2).

Table 6.2. Rheological Parameters of the Broths Obtained in Fermentations Conducted at Different Agitation Speeds of 200, 350 and 500 rpm at 30°C, 2.5 L/min Air Flow Rate.

	200 rpm	**350 rpm**	**500 rpm**
Consistency Index (K) (Pa.s^{n})	0.005	0.045	0.1215
Flow Behavior Index (n)	1.9805	0.9567	0.5033

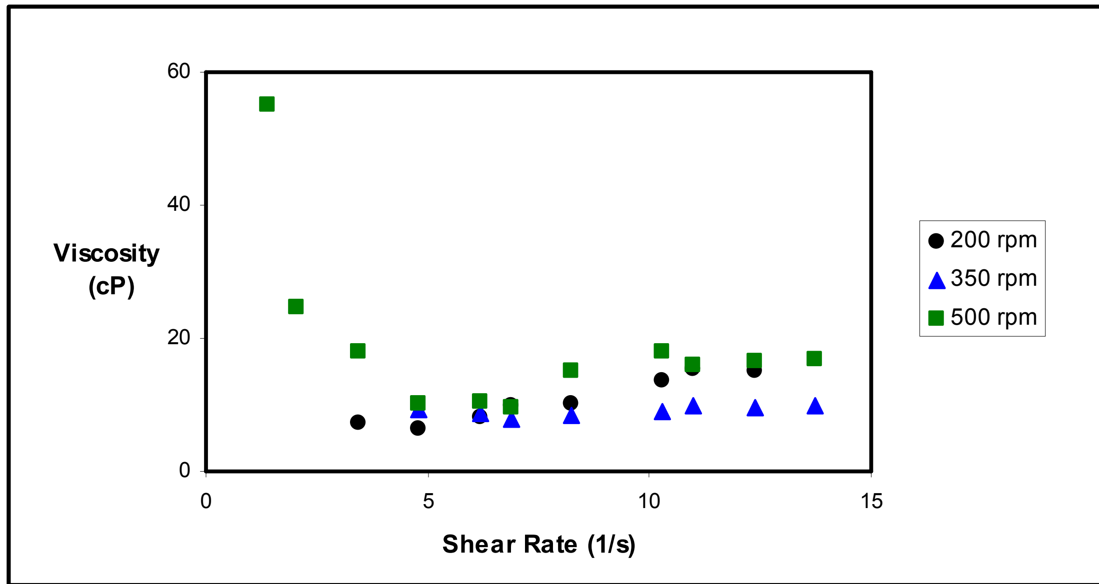


Figure 6.9. Changes in the viscosity of broths at different shear rates obtained at the end of fermentations carried out at 200, 350 and 500 rpm, 2.5 L/min air flow rate.

Non – Newtonian and pseudoplastic (shear thinning, n<1) broth rheology was observed in the case of 500 rpm agitation speed. Conversely, broth rheology exhibited dilatant (shear thickening, n>1) behavior at the lower agitation rate (200 rpm). On the other hand, at the medium agitation speed (350 rpm) the broth was close to newtonian (n~1.0).

These findings reveal some important features of broth rheology: (1) At an agitation speed of 500 rpm, the K-value (Table 6.2) and biomass concentration (Fig. 6.5.a) were the highest with an average pellet size of 1.08 ± 0.42 mm (Fig. 6.7.a). At a low intensity agitation of 200 rpm, the biomass concentration was the lowest where the pellet size was the smallest (0.71 ±0.35 mm). This suggests that the K-value of broths is sensitive to both pellet size and biomass concentration, which is also supported by the study of Lopez et al. (2005). They reported a sensitivity of K-value of broths to pellet size but found little effect of biomass concentration on the rheology. (2) The small pellets formed at the low agitation speed produced broths with n-value exceeding unity, i.e. shear thickenning behavior. Broths of the containing comparably larger pellets obtained at high intensity of agitation had a shear thinning behavior (i.e. n<1). On the other hand, at agitation speed of 350 rpm, formation of large pellets that are low in number did not affect the broth rheology significantly, Newtonian type of flow behavior

was observed (n~1.0). (3) The apparent viscosity calculated at the estimated shear rate of 13.74 s^{-1} (Eqn. 5.8) for each agitation intensity (200, 350 and 500 rpm) was found to be 15.72, 9.75 and 16.95 cP, respectively. Generally, because of the shear thickening and shear thinning behavior, the broths produced at the highest and lowest agitation rates were more viscous than the one obtained at the intermediate agitation speed where Newtonian type flow behavior was observed.

Applying regression analysis to experimental data obtained at the end of fermentation (96 h), following relationship was found with a high correlation coefficient (R^2=1) between K-value and the biomass concentration of the broths produced at different agitation speed:

$$Y = a X^2 - bX + c \quad (6.1)$$

where; Y is the consistency index (K), X is the maximum biomass concentration (on dry basis, g/L), and a, b and c are the constants obtained from regression analysis (Table 6.3).

Table 6.3. Constants obtained from regression analysis

a	b	c	R^2
105.2	-49.8	5.9	1.0

The consistency index varies with biomass concentration to the power of 2. This is in good agreement with the data cited in the literature. The reported values of consistency index (K) for the broths that have power-law like behavior varies with biomass concentration, typically to the power of 0.3-3.3 (Papagianni 2004).

Maximum polygalacturonase activity (Fig.6.10) was observed at 200 rpm, demonstrating an inverse relationship with biomass formation. This was prevailing that enzyme synthesis was not growth associated but a secondary product.

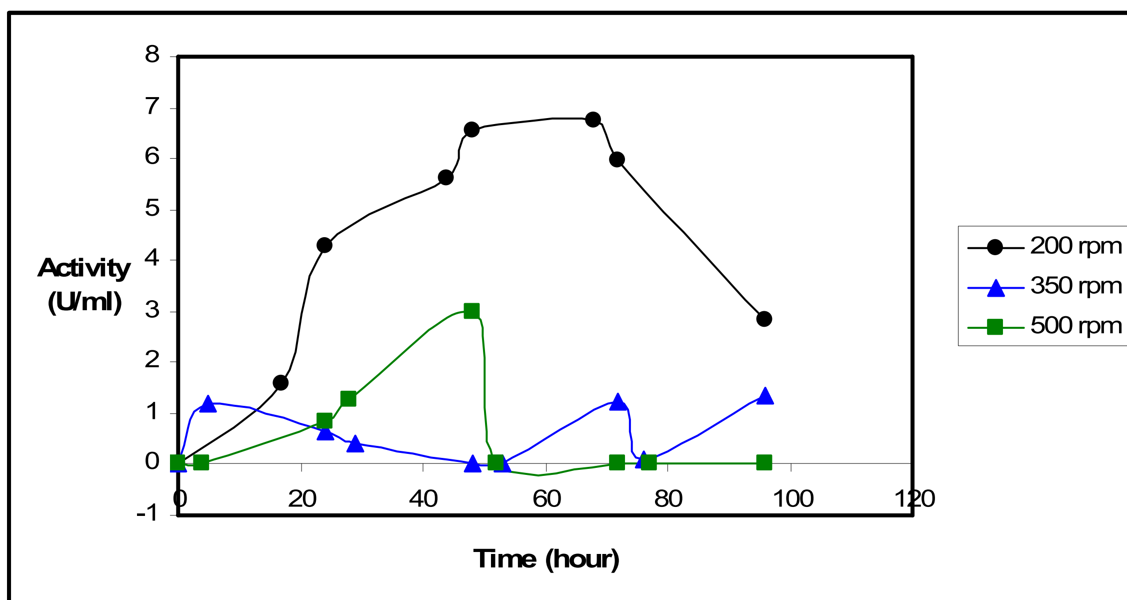


Figure 6.10. Polygalacturonase profiles of experiments conducted at different agitation speed of 200, 350 and 500 rpm at 30°C, 2.5 L/min air flow rate.

Similar to pH effect maximum enzyme synthesis was achieved at 45 to 50 hours. Based on this time point (50 h) the maximum enzyme productivities were 0.1341, 0.059 and 0.027 U mL^{-1} h^{-1} for 200, 350 and 500 rpm, respectively. As with the pH effect, the enzyme was degraded after 70 hours at both 200 and 350 rpm, and after 50 hours at 500 rpm. Similarly, proteolysis could be stated as one of the reason for this degradation as discussed before.

Overall if the primary goal is to produce the enzyme in high yields, it is recommended to apply an agitation speed of 200 rpm with an incubation period of 50 or at most 70 hours. This would not only reduce the operating cost due to shorter fermentation time but also reduce the power input. Besides, these conditions would also result into a pellet morphology easing the operation (during the fermentation run) and due to less biomass formation the subsequent downstream process.

As a conclusion this study not only provided the preferred conditions for maximum polygalacturonase production but also provided numerical data on morphological (pellet size and number) and rheological (K, n) parameters which can be used in mathematical model development studies or scale up processes of common fungal fermentations.

6.3 Effect of Dissolve Oxygen Tension on Biomass, Polygalacturonase, Pellet Morphology and Broth Rheology

It is reported through a number of studies that filamentous fungi are susceptible to regulation by the dissolved oxygen tension of the medium in the production of various metabolites (Kubicek et al. 1980, Wang and Inoue 2001, Wang et al. 2005). In aerobic fermentations the supply of oxygen is often the rate limiting step and satisfying oxygen demands can often constitute a large proportion of the operating and capital cost of industrial scale fermentations (Papagianni 2004). Supplying oxygen to aerobic cells has always been a significant challenge to fermentation technologists. The problem arises from the fact that oxygen is poorly soluble in water. Under oxygen limitation, the metabolic rate of the microorganisms decreases significantly and the culture may respond adversely to the resulting stress (Lee 1992). The extreme stress conditions may inhibit the growth and activity of microorganisms by blocking nutrient transfer and consumption of cells during growth (Bandaiphet and Prasertsan 2006).

In general, oxygen transfer into microbial cells will strongly affect the product formation through the changes in metabolic fluxes which ultimately will influence the metabolic pathways (Çalik et al. 2000). Hence, oxygen transfer in a bioreactor depends on microorganism physiology and bioreactor efficiency. The interaction between oxygen consumption and supply therefore will determine the level of dissolved oxygen concentration in the broth. Industrial aerobic processes usually attempt to operate so that the full capacity of the fermenter aeration system for oxygen transfer is utilized. But this still can not prevent the condition where the oxygen concentration drops close to zero at some point during the fermentation run. That is why; oxygen supply into the broth constitutes one of the decisive factors of cultivated microorganism growth which plays an important role in the scale up and economy of aerobic biosynthesis systems.

As discussed above, since oxygen plays a detrimental role in the fungal fermentations, it was anticipated to determine the critical DOT level for maximum polygalacturonase production and its effect on the morphology and broth rheology.

Therefore three different saturation levels (10, 30 and 50%) were set by the bioreactor and were controlled through cascading the agitation speed between ranges of

200-500 rpm at constant aeration rate of 2.5 l/min. All other conditions were the same as described in the Materials and Methods (5.2.2.).

The effect of DOT could also be investigated by cascading air flow at certain ranges. However, agitation is more effective in providing better mixing and braking up air bubbles (Cho et al. 2002). Therefore it was believed that cascading agitation would have better control on the DOT and being more efficient in supplying oxygen to the cells. Furthermore, this attempt is closer to industrial application (Cho et al. 2002). Based on the discussions in previous section agitation has also more influence on morphology and rheology compared to direct air supply. This was another reason why cascading agitation rather than air was chosen in this study.

As can be seen from the profiles of agitation (Fig. 6.11.), cascading first started with experiments where DOT was set to 50%, followed by 30% and then 10%. The system increased the agitation automatically between the prescribed ranges in order to keep the desired DOT levels. It was anticipated that, the sooner the drop in the DOT, the sooner would cascading start and the effect of agitation would prevail on the response parameters. The highest DOT (50%) required higher agitation levels compared to the low set DOT, which started cascading later with lower agitation speeds. All these combinations affected the response parameters at different levels.

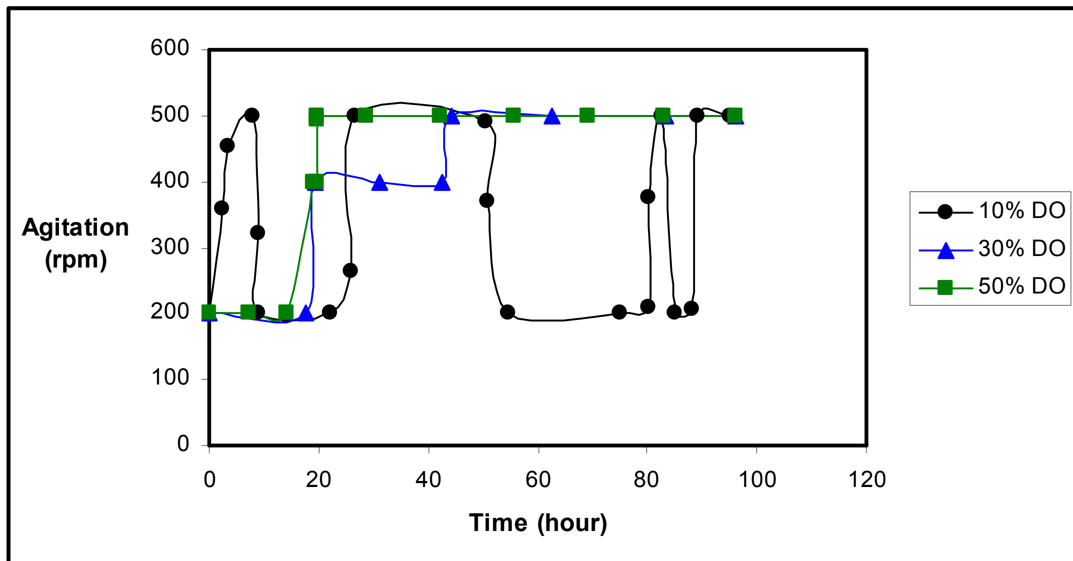
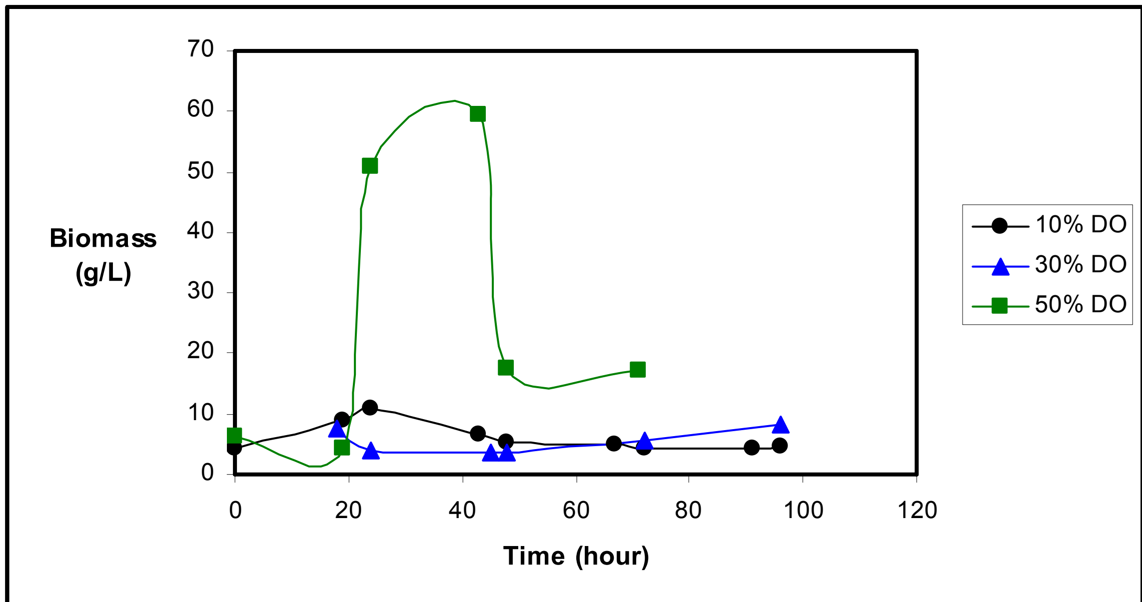


Figure 6.11. Agitation speed profiles of experiments conducted at different dissolved oxygen tension (DOT) 10%, 30% and 50% at 30°C, 2.5 L/min air flow rate.

At 50 % DOT the biomass formation was 6 times higher than at 30% and 10 % DOT at 45 hours of fermentation revealing the oxygen dependency of carbon metabolism (Fig. 6.12.a). This was also justified by the carbohydrate profiles (Fig 6.12.b), where carbohydrates were almost completely utilized at the end of fermentation. This profile also indicated that oxygen was utilized efficiently in this particular run. However the same could not be observed for the other two set points (30% and 10% DOT). For these two runs the biomass formation was very low and there was a high possibility of oxygen limitation as can be observed from the carbohydrate profiles (not completely utilized). A significant difference in the biomass formation and carbohydrate utilization rates of these two runs could not be detected. Therefore the critical DOT level with respect to biomass formation was suggested to be in the ranges of 30-50 % DOT. Since 40 % was not checked, an exact level at this point could not be recommended.



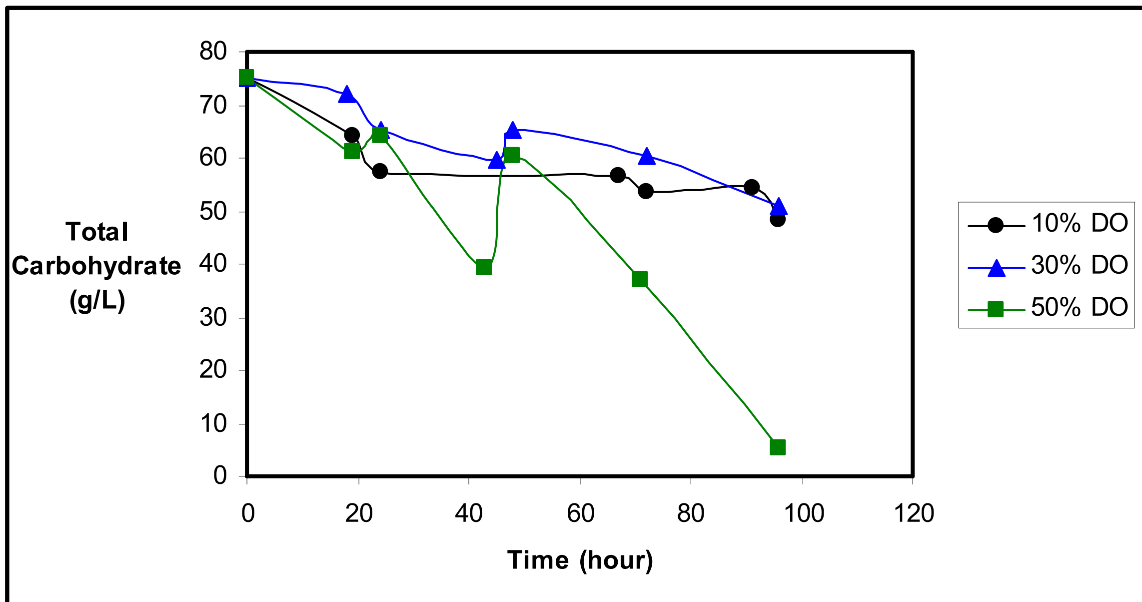


Figure 6.12. Profiles of a) biomass b) carbohydrate utilization of experiments conducted at different dissolved oxygen tension (DOT) 10%, 30% and 50% at 30°C, 2.5 L/min air flow rate.

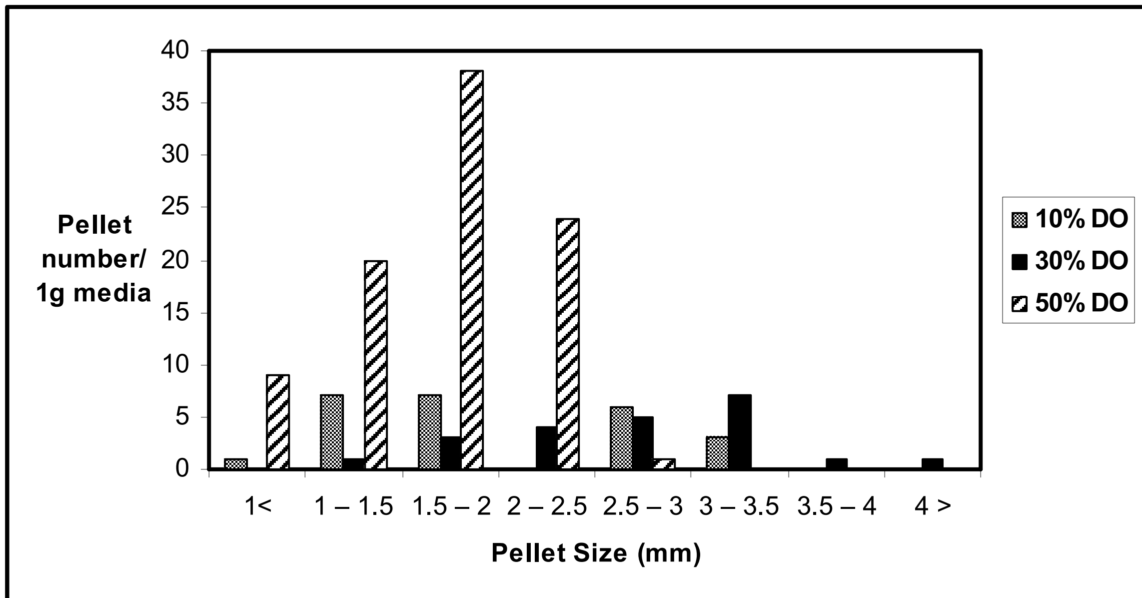
The effect of cascading in order to keep the certain DOT levels, exhibited a profound effect on the pellets sizes and distributions as can be observed in Fig. 6.13. (a-b). At all three DOT set points a wide size distribution with different pellet number and sizes was observed. The average pellet sizes were 1.98±0.75mm, 2.80±0.87 and 1.70±0.48 mm for 10%, 30% and 50 % DOT, respectively.

Similarly, there was significant difference in the pellet structure with big fluffy pellets for 30 %, medium sized hairy pellets for 10 % and small compact smooth pellets for 50% being obtained (Fig. C.1.).

Furthermore the number of the pellets in 1 g of medium varied considerably among the three set points, indicating critical information about the mechanism of pellet formation. This difference can be mainly attributed to the cascading effect through the agitation applied.

The mechanism for the 50% DOT could be related to the chipping off and direct breakage of the pellets, which increased the number of pellets but reduced their sizes due to shear effect at higher agitation speed. This was in accordance with the visual observations of the pellets as described above. The mechanism for the other two was similar but with few differences. Here for both set points agglomeration due to the lower agitation speeds were in place, increasing their sizes but reducing the numbers. This was more pronounced for set point of 30 % compared to 10 %, where in case of 10 % DOT, oxygen limitation occurred in the medium, preventing pellet growth. This was also justified with the hairy fluffy structure that did not exhibit interior diffusion limitation otherwise (Fig. 6.13.b). However, in case of 30 % DOT in addition to the agglomeration forming larger pellets, diffusion limitation of oxygen to the interior of the pellet was observed (Fig 6.14).

As a result it was concluded that DOT set point was effective in determining the size, numbers and morphology of the pellets of *Aspergillus sojae* under the conditions studied.



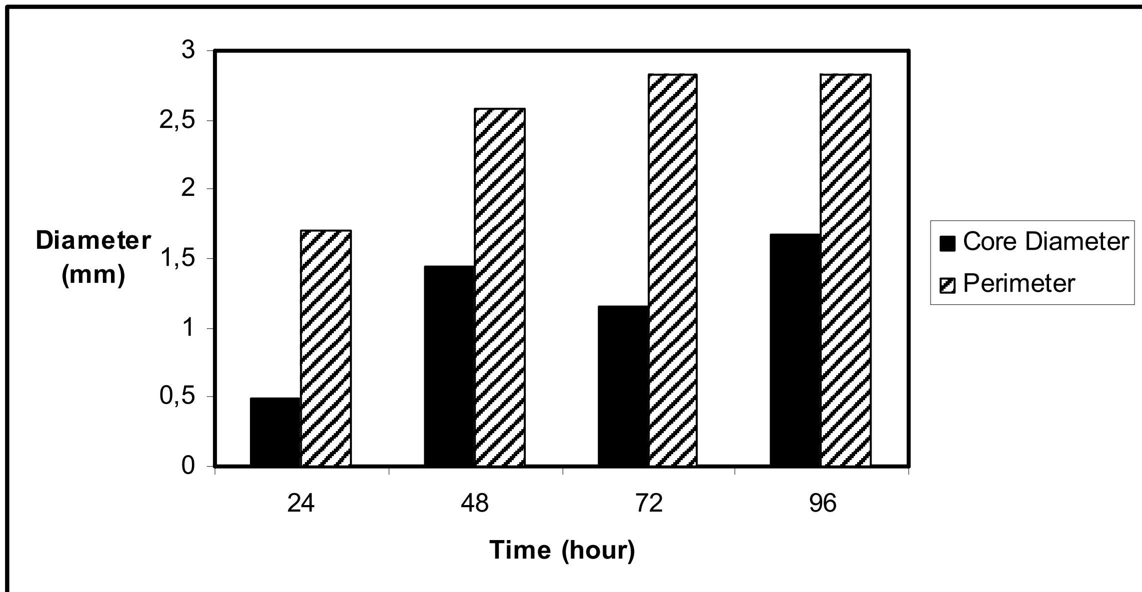


Figure 6.13. Profiles of a) pellet size distribution of experiments conducted at different dissolved oxygen tension (DOT) 10%, 30% and 50%, b) pellet sizes during the course of fermentation run at 10% DOT conducted at 30°C, 2.5 L/min air flow rate.

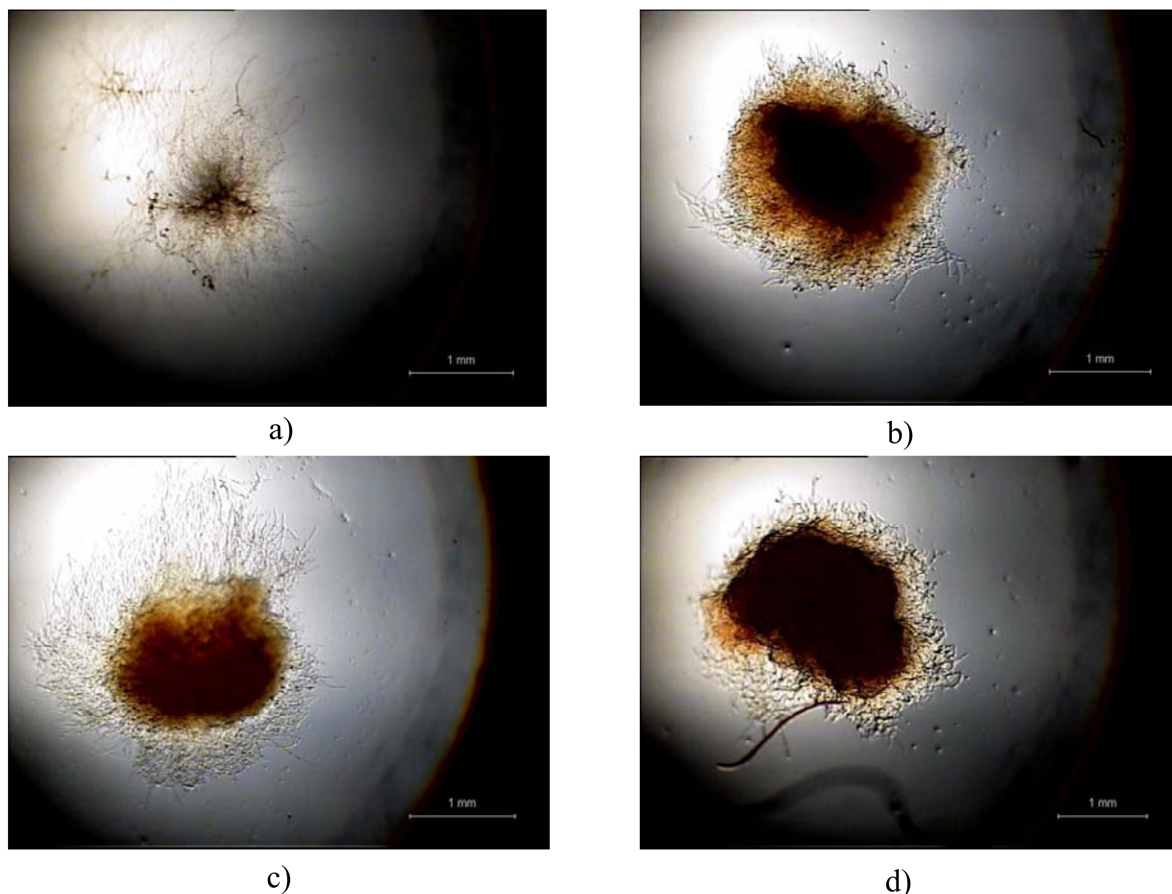


Figure 6.14. Pellet morphologies at a) 24.h b) 48.h c) 72.h d) 96.h

There are various studies in the literature contradicting with the effect of DOT on the pellet morphology and on the production of the product of interest. For example, Gomez et al. (1988) found that no difference in morphology for pellets or filaments of *Aspergillus niger* could be related to DOT levels, although the production of citric acid was enhanced by increasing DOT at different fermentation stages.

Similarly, Carter and Bull (1971) found out that the morphology of *Aspergillus nidulans* was unaffected by DOT levels. Also no direct relationship between DOT and the morphology of *Penicillum* was determined by a study of Van Suijdam and Metz (1981).

On the contrary, in a study of Higashiyama et al. (1999), the fungal morphology of *Mortiella alpina* in the process of arachidonic acid production appeared to be very sensitive to DOT. Similarly, in the study conducted by McIntyre et al. (2002), oxygen was found to be the overriding effecter of morphological development of the dimorphic fungus *Mucor circinelloides*.

With this respect the current study will be a new reference point in highlighting the effect of DOT on the morphology of a new strain (*Aspergillus sojae)* and contribute to the literature.

Considering the rheology that is a function of the morphology, different rheological behaviour was observed (Fig. 6.15, Table 6.4).

Table 6.4. Rheological Parameters of the Broths Obtained in Fermentations Conducted at Different Dissolved Oxygen Tension (DOT) of 10%, 30% and 50% at 30°C, 2.5 L/min Air Flow Rate.

	10% DO	**30% DO**	**50% DO**
Consistency Index (K) (Pa.s^n)	0.002	0.0035	0.117
Flow Behavior Index (n)	1.9712	1.9552	0.6317

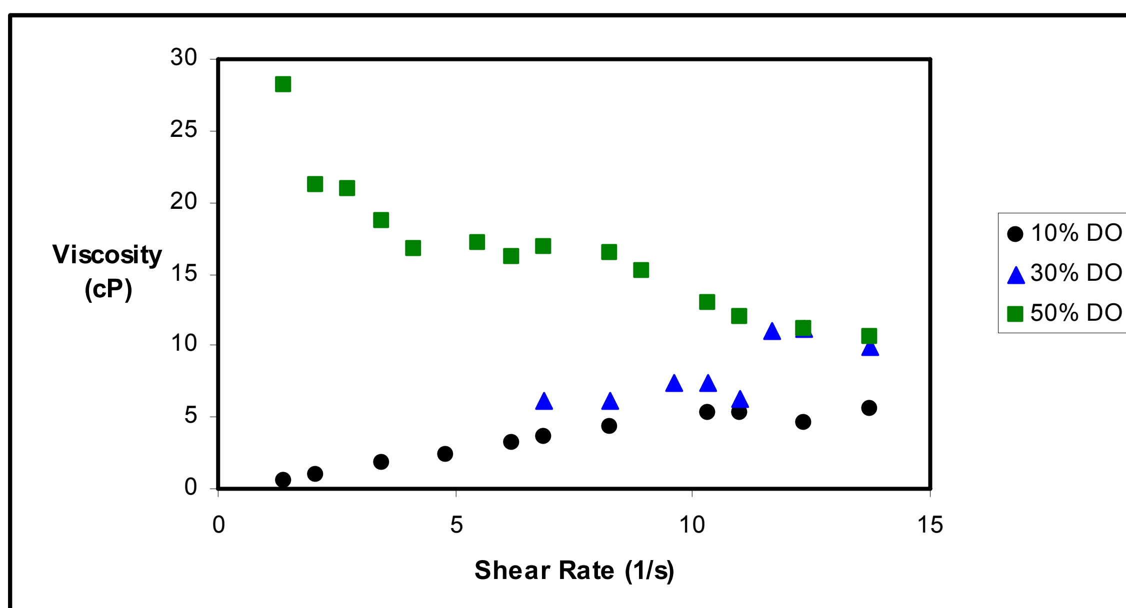


Figure 6.15. Changes in the viscosity of broths at different shear rates obtained at the end of fermentations carried out at 10 %, 30 % and 50 % DO, 2.5 L/min air flow rate.

Non-Newtonian shear thickening behavior (n>1.0) was depicted in the case of fermentation where the DOT level was kept at 10% and 30%. On the other hand, when the DOT level was increased to 50%, non-Newtonian shear thinning behavior (n<1.0) was dominant.

Using higher DOT level produced smaller and compact spherical pellets whereas large and fluffy pellets were obtained at 10% and 30% DOT. Despite some debates in the literature about the effect of DOT on the morphology and the rheology of the fermentation broth, it is appeared that *Aspergillus sojae* morphology was sensitive to DOT in the process of polygalacturonase production and that the rheology of the broth was directly affected by the morphology.

The effect of different DOT set points on polygalacturonase activity is presented in Fig. 6.16. In contrast to biomass formation highest enzyme activity was observed at 10% DOT confirming the inverse relation ship between biomass and enzyme production. Obviously, at this DOT level (10%) the resources were directed toward enzyme synthesis rather than biomass formation. This could be partly related to the enzyme synthesis metabolisms that oxygen was not a limiting step for this process and also to the morphology of the culture where medium sized pellets with hairy region could make better use of the necessary oxygen and nutrient available in the medium. The maximum enzyme activity achieved at 65 hours at 10 % DOT was 4 and 6 times higher than at 30 % and 50% DOT, respectively. Again a degradation of the enzyme was accounted after this time point for all three set points was due to possible proteolysis as discussed before.

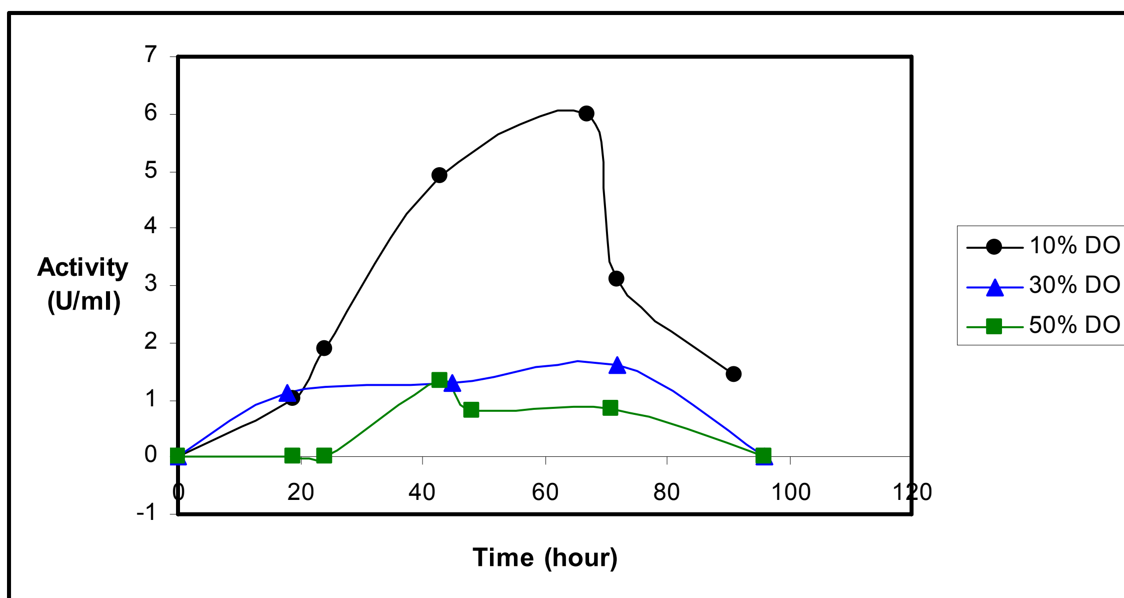


Figure 6.16. Polygalacturonase profiles of experiments conducted at different dissolved oxygen tension (DOT) 10%, 30% and 50% at 30°C, 2.5 L/min air flow rate.

Therefore, on the basis of these findings, if the primary goal is to produce the polygalacturonase enzyme, then it is recommended to set the DOT to 10% which not only will maximize the product yield but also reduce the power input due to delayed cascading process, eventually being reflected in the low operational cost. Also it is highly advisable to run the fermentation run until 65 hours in order to avoid the degradation of the product. Compared to general long runs in fungal fermentations, which are usually around 96 hours or longer, the duration of this fermentation is relatively shorter; therefore it is economically quite feasible. The final pH of all three runs was around pH 4 similar to the other runs discussed before pointing out that this enzyme might not be stable at this pH. However this issue remains a task to perform in the future characterization studies of this enzyme.

A direct relationship between the DOT levels and the production of citric acid by *Aspergillus niger*, penicillin production by *Penicillium chrysogenum* and arachidonic acid production by *Mortiella alpina* was established in a number of studies (Gomez et al. 1988, Higashiyama et al. 1999). Also in the current study a critical DOT level (50 %) for biomass formation different from the critical level (10 %) for maximum polygalacturonase production was determined.

6.4 Effect of 2.5 L/min Aeration on Biomass, Polygalacturonase, Pellet Morphology and Broth Rheology

Most fungi require molecular oxygen to grow. Fungi can grow over very wide ranges of oxygen tensions and it is known that aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product/by-product and oxygen (Mantzouridou et al. 2002).

Also, aeration results in better mixing of the fermentation broth, thus helping to maintain a concentration gradient between the interior and the exterior of the cell. This concentration gradient works in both directions; through better diffusion it helps maintain a satisfactory supply of sugars and other nutrients to the cells, while it facilitates the removal of gases and other by-products of catabolism from the microenvironment of the cells.

The aeration efficiency depends on oxygen solubilization and diffusion rate into the broths, respectively on the bioreactor capacity to satisfy the oxygen demand of microbial population (Bandaiphet and Prasertsan 2006). However, the dissolved oxygen in the broths is limited by its consumption rate on cells or the oxygen uptake rate, as well as its oxygen transfer rate.

The oxygen supply into the broths constituents one of the decisive factors of cultivated microorganisms' growth and can play an important role in the scale-up and economy of aerobic biosynthesis systems. Therefore, it is important to investigate the effect of this key parameter during a fermentation time (96 hours) on the polygalacturonase production of *Aspergillus sojae.*

Consequently, one fermentation was carried out at 2.5 L/min aeration, 200 rpm and 30% DOT level and observed detailed morphology, rheology and other parameters during 96 hours. This fermentation can be analyzed in 3 main phases. The first phase which is the combination of lag and exponential phase continues until 21st hour. At this phase pH starts to fall down due to assimilation of the carbon and nitrogen sources, DOT reaches its minimum (zero) and biomass increases (Fig. 6.17., Fig. 6.18(a-b) and Fig 6.19(a-b)).

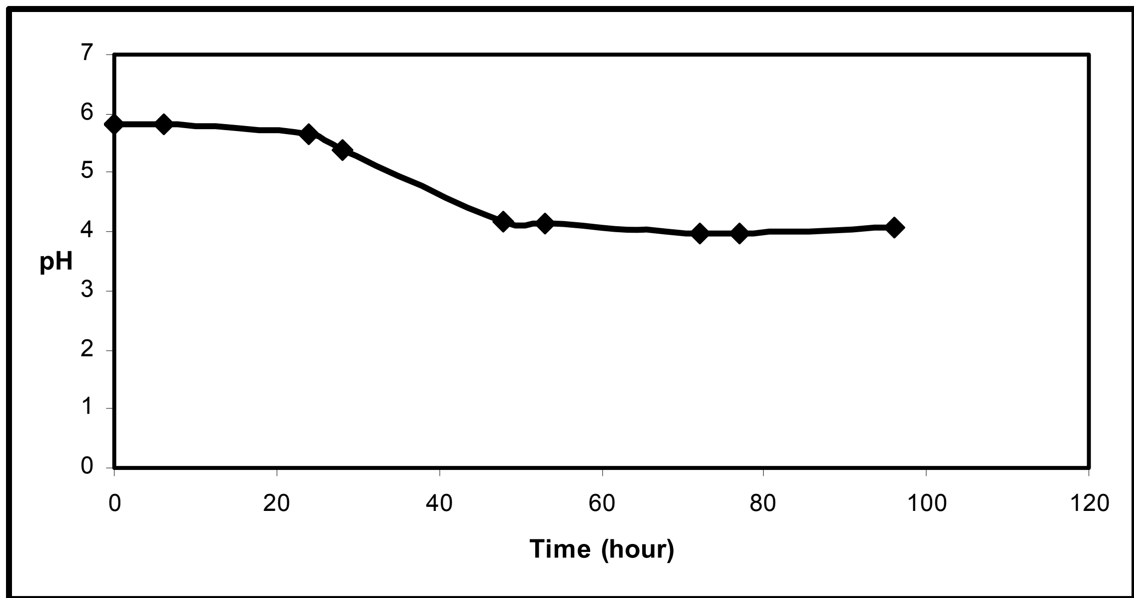
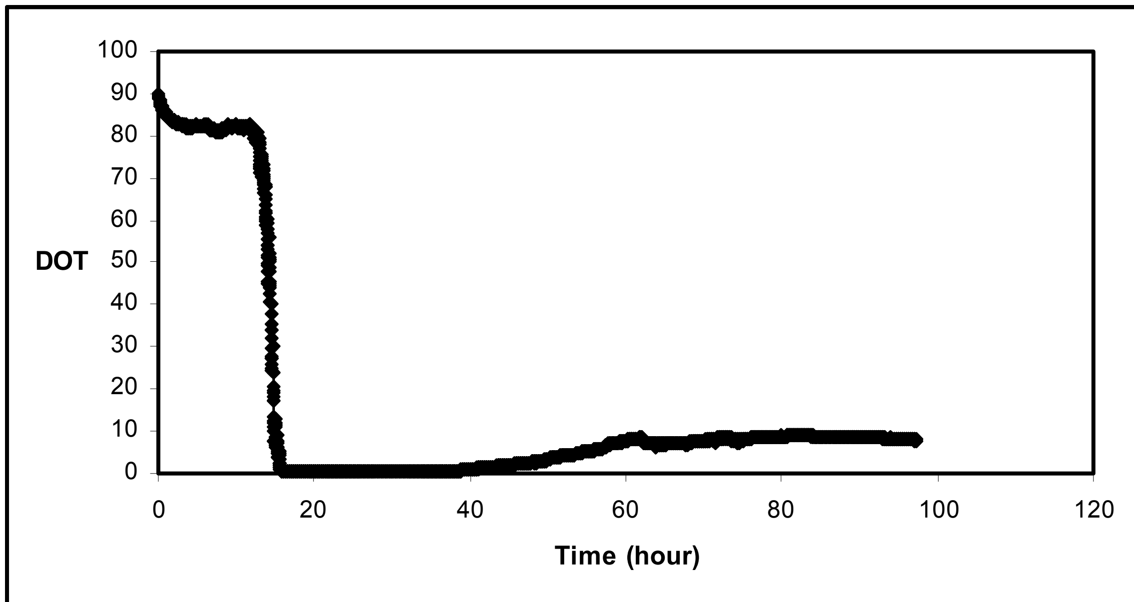


Figure 6.17. pH profile of 2.5 L/min aeration experiment conducted at 30°C and 200 rpm agitation speed.

During this period polygalacturonase production approaches its maximum (1.49U) (Fig. 6.19.a) Oxygen is used mainly for enzyme and biomass production, indicating a growth associated product formation. Pellets on the other hand are smaller and high in number with a narrow size distribution (Fig. 6.20).



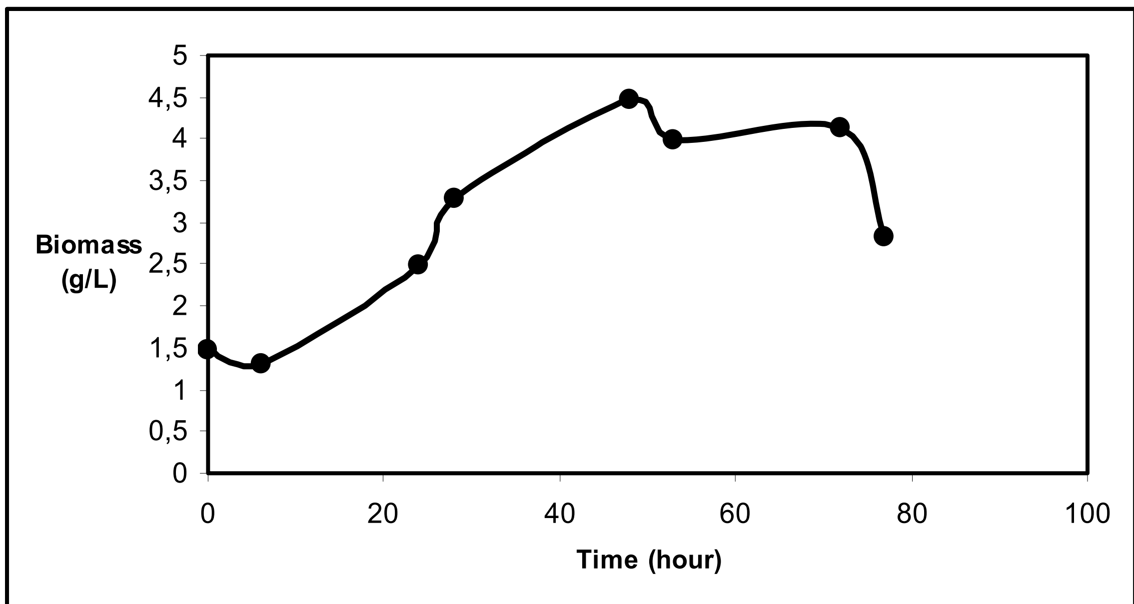
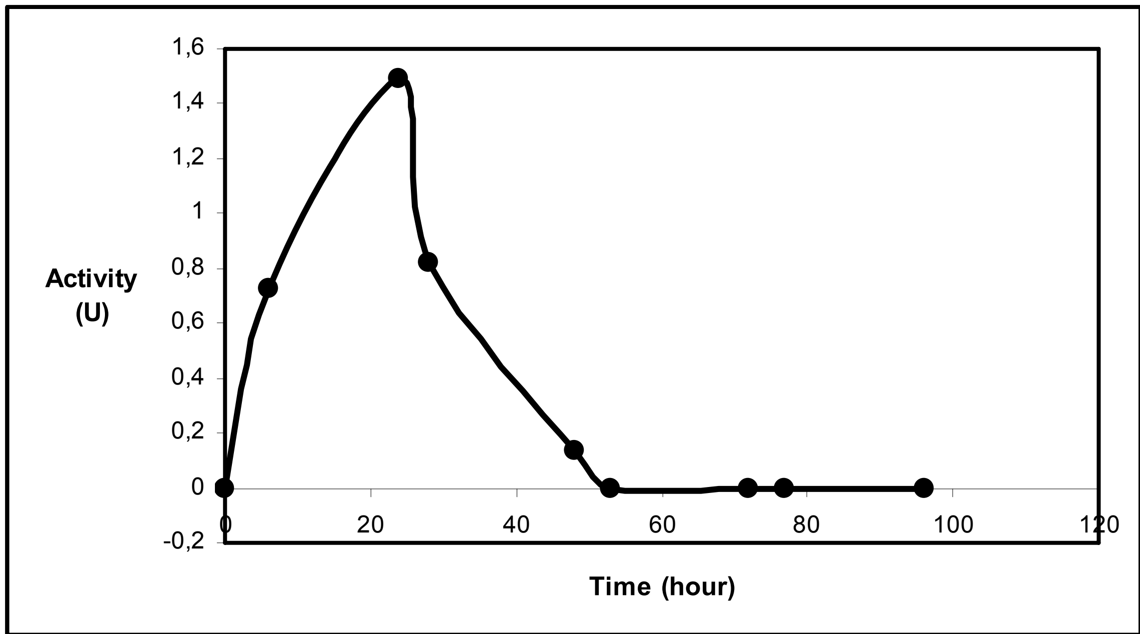


Figure 6.18. Profiles of a) dissolved oxygen tension b) biomass of 2.5 L/min aeration experiment conducted at 30°C and 200 rpm agitation speed.



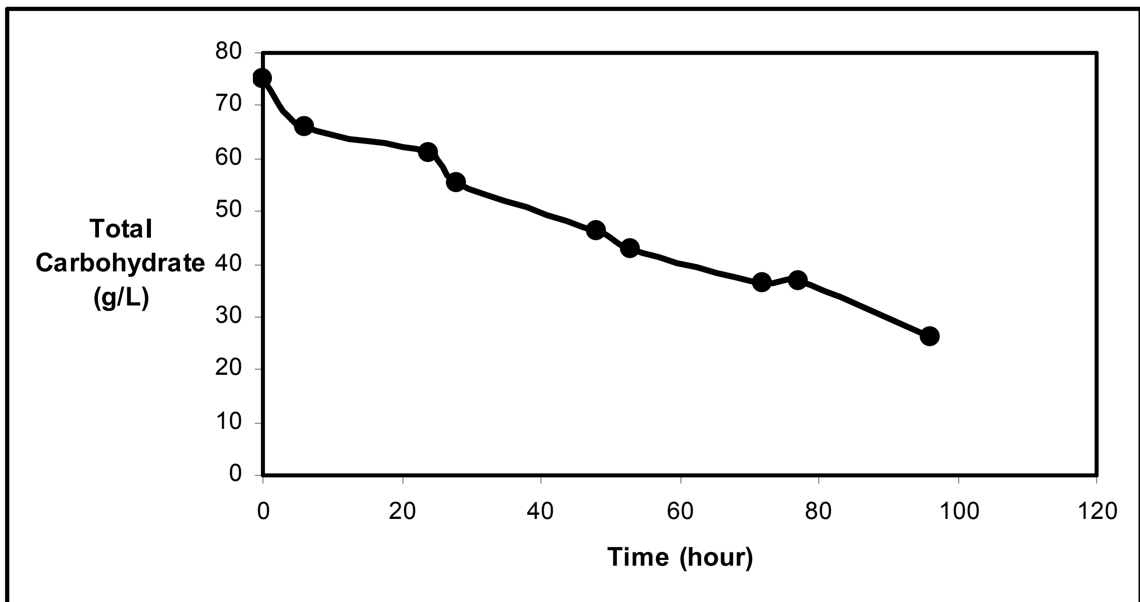
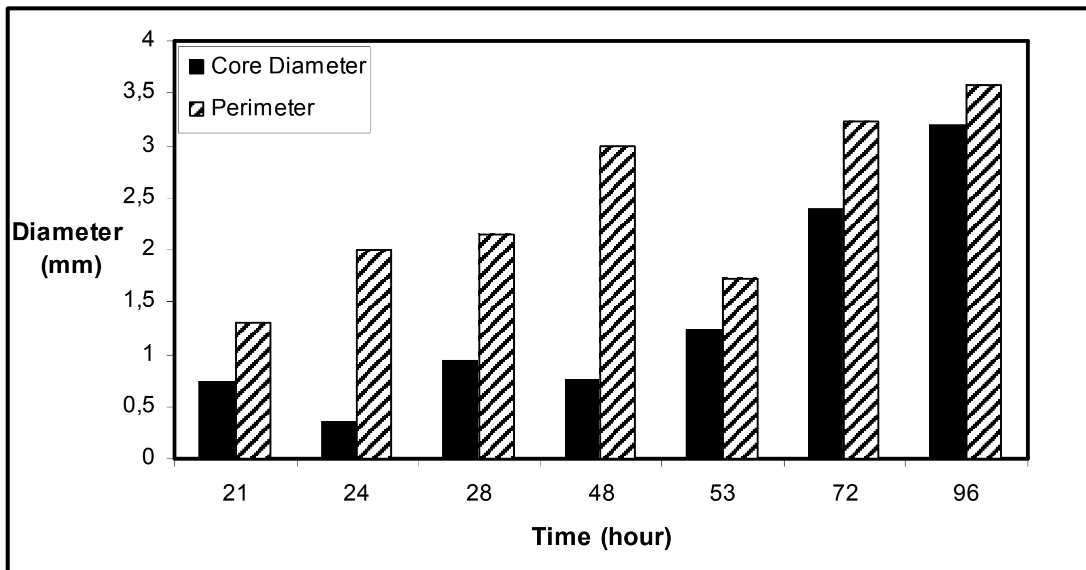


Figure 6.19. Profiles of a) polygalacturonase production b) carbohydrate utilization of 2.5 L/min aeration experiment conducted at 30°C and 200 rpm agitation speed.

Second phase, covering the late exponential phase and stationary phase is between 21st hour and 48th hours. During this period polygalacturonase synthesis decreases, the metabolic activities are towards biomass formation which are mainly in the form of pellets.



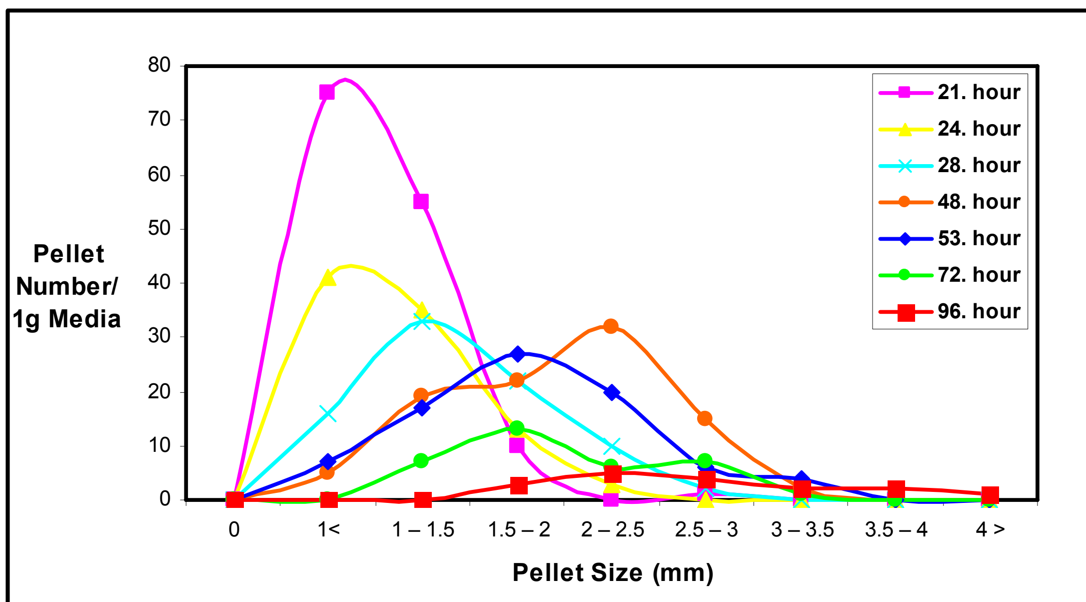


Figure 6.20. Profiles of a) growth of pellet during fermentation b) pellet size distributions of 2.5 L/min aeration experiment conducted at 30°C and 200 rpm agitation speed.

As can be seen from figure 6.20a, pellet perimeter increases with a decrease in the core diameter indicating a hairy structure. This is also visualized from the pictures taken with the camera as shown in figure 6.21. The hairy structure induces the oxygen metabolism towards more biomass formation due to easier oxygen uptake.

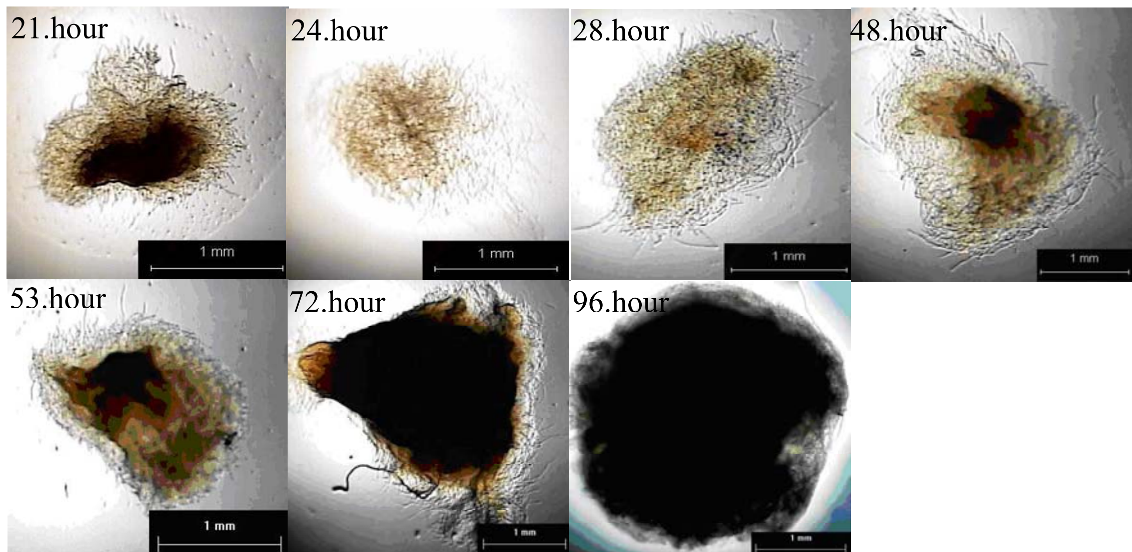


Figure 6.21. Microscope pictures of pellets during fermentation

When we compare pH profile with carbohydrate utilization and biomass formation profiles we can conclude that fast carbohydrate utilization occurs which could be a result of better solubility of the nutrient compounds in the broth due to the pH effect or it can be because of the cell membrane permeability which was affected because of low pH and transport into the cells was more efficient as stated in section 6.1. The latter phenomena can be supported with biomass formation profile where highest biomass formation was achieved at 48. hour during the course of fermentation. At 48th hour pellets were big and fluffy with compact centers and near to normal size distribution.

After this period which is followed with the late stationary and early death phases, the perimeter increases slowly with a faster increase in the core diameter. This is explained with the fact, that with the constant agitation in the absence of growth the current hairy region is incorporated into compact pellets. This phenomena is best observed between 48 and 53 hours. In fact during the death face oxygen utilization is at minimum resulting to an increased DOT in the medium. However, the activity declined after this period and no activity could be detected at the end of fermentation. Few explanations for this can be stated such as; the organism might produce proteases that can hydrolyze the enzyme or the medium pH is not suitable to maintain the enzyme stable.

As discussed above all the factors affecting the morphology will indirectly effect the broth rheology since these depend on each other. The behavior of fermentation broth is of considerable importance with reference to cellular kinetics and in the engineering design of fermenters. A knowledge of the flow behavior of the broth serves as a prerequisite for an understanding of the heat and mass transfer involved in process design, scale up and operations.

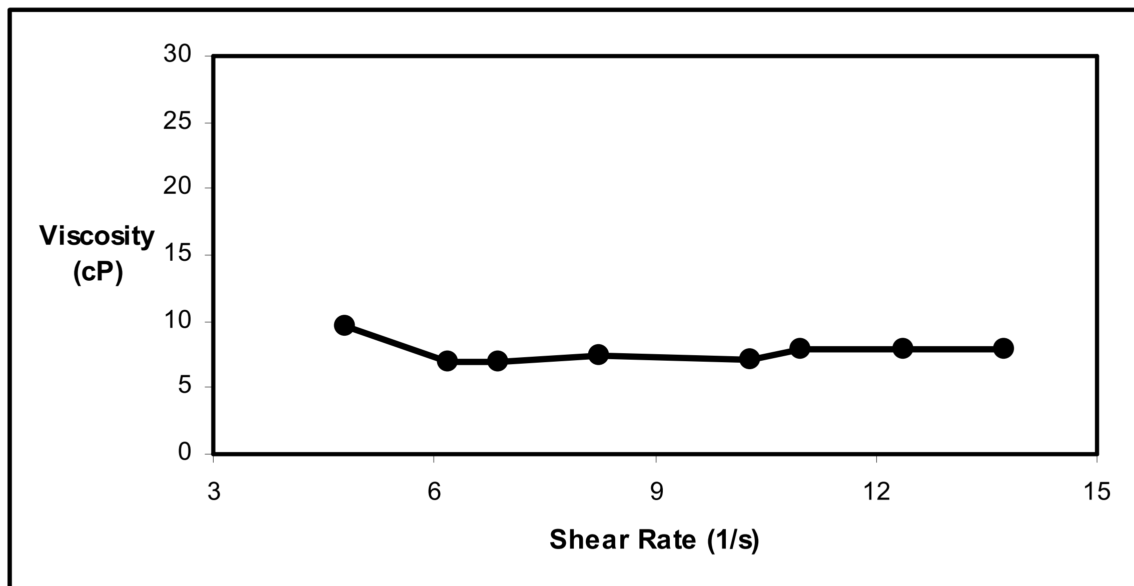


Figure 6.22. The effect of 2.5 L/min aeration on broth rheology

As we can see from Figure 6.22, fermentation medium is slightly non-Newtonian. When we apply Herschel-Bulkley model K and n values were found to be 0.035 $Pa.s^n$ and 0.93, respectively. Since n is very close to 1, we can assume that the fermentation medium at the end of 96th hour is close to Newtonian. This behavior assists the biomass and morphology which the pellets are small in amount, fluffy and large at 96th hour.

As a conclusion of this run, it is recommended to stop the run around 50 hours in order to recover the polygalacturonase enzyme. This is actually a positive outcome since the operational costs will be reduced due to shorter run.

CHAPTER 7

CONCLUSIONS

The current study demonstrated that pH, agitation speed, aeration and DOT levels are critical key parameters influencing the morphology, broth rheology, and polygalacturonase production of *Aspergillus sojae* in a batch bioreactor.

This study did not only provide novel information about the production conditions of polygalacturonase by a new strain, *Aspergillus sojae*, known mainly in solid state fermentation of the popular koji food, but also provided new numerical data on morphological (pellet size and number) and rheological (K, n) parameters that can be used in the mathematical modeling and scaling up processes of common fungal fermentations.

To date, no reports are available in the literature regarding the fermentation conditions for *Aspergillus sojae* in bioreactor. Therefore, this study will serve as a base line of the initial studies in this field. Through these experiments, pH did not significantly affect the broth rheology but created an impact on the pellet morphology. Uncontrolled pH increased biomass and polygalacturonase activity. So, it is recommended to run the fermentation under uncontrolled pH conditions and stop the run around 50 hours in order to recover the polygalacturonase enzyme. This is actually a positive outcome since not only the operational costs will be reduced due to shorter run but also the acid and base consumption will be eliminated and therefore reducing the overall material cost. Maximum biomass and Non-Newtonian pseudoplastic rheology was obtained at 500 rpm. On the other hand, maximum enzyme production and dilatant behavior was observed at 200 rpm. Newtonian behavior was observed at 350 rpm. If the primary goal is to produce the enzyme in high yields, according to our results, it is recommended to apply an agitation speed of 200 rpm with an incubation period of 50 or at most 70 hours. This would not only reduce the operating cost due to shorter fermentation time but also reduce the power input. Besides, these conditions would also result into a pellet morphology easing the operation (during the fermentation run) and due to less biomass formation the subsequent downstream process. The results showed that, a DOT range of 30-50% was essential for maximum biomass formation but 10% DOT was required for maximum PG synthesis. At all three conditions, the broth was Non-Newtonian. Therefore, based on these findings, if the primary goal is to produce

the polygalacturonase enzyme than it is recommended to set the DOT to 10% which not only will maximize the product yield but also reduce the power input due to delayed cascading process, eventually being reflected in the low operational cost.

These data can be used further in the overall process design and the subsequent down stream process and in the future optimization studies of these parameters can be studied.

REFERENCES

Amanullah, A., Justen, P., Davies, A., Paul, G.C., Nienow, A.W., Thomas, C.R. 2000. "Agitation induced mycelial fragmentation of *Aspergillus oryzae* and *Penicillium chrysogenum*" *Biochemical Engineering Journal.* Vol. 5, pp. 109-114.

Atkingson, B. and Daoud, L.S. 1976. "Microbial Flogs and Flocculation in Fermentation Process Engineering" *Adv. Biochem. Eng*. Vol. 4, pp. 41-124.

Badino Jr., A.C., Facciotti, M.C.R., Schmidell, W. 2001. "Volumetric oxygen transfer coefficients (k_La) in batch cultivations involving non-Newtonian broths" *Biochemical Engineering Journal.* Vol. 8, pp. 111-119.

Bandaiphet, C. and Prasertsan, P. 2006. "Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, k_La in exopolysaccharide production from *Enterobacter cloacae* WD7" *Carbohydrate Polymers.* Vol. 66, pp. 216-228.

Benett, J.W. 2001. "*Aspergillus* and Koji: History, Practice and Molecular Biology" *Society of Industrial Enzymes News*. Vol. 51, pp. 65-71.

Brookfield Viscometer Manual. 2000. *More Solution to Sticky Problems: A guide to Getting More from Your Brookfield Viscometer,* (Middleboro, USA), pp. 1-50.

Brown, D.E. and Zainudeen, M.A. 1978. "Effect of inoculum size on the aeration pattern of batch cultures of a fungal microorganism" *Biotechnology and Bioengineering.* Vol. 20, pp. 1045-1061.

Carlsen, M., Spöhr, A.B., Nielsen, J., Villadsen, J. 1995. "Morphology and physiology of an α-amylase producing strain of *Aspergillus oryzae* during batch cultivations" *Biotechnol. Bioeng.* Vol. 49, pp. 266-276.

Carter, B.L.A. and Bull, A.T. 1971. “The effect of oxygen tension in the medium on the morphology and growth kinetics of *Aspergillus nidulans” J. Gen. Microbiol.* Vol. 65, pp. 265-273.

Cho, Y.J., Hwang, H.J., Kim, S.W., Song, C.H., Yun, J.W. 2002. “Effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by *Paecilomyces sinclairii* in batch bioreactor” *Journal of Biotechnology.* Vol. 95, pp. 13-23.

Cox, P.W. and Thomas, C.R. 1992. “Classification and Measurement of Fungal Pellets by Automated Image Analysis” *Biotechnol. Bioeng.* Vol. 39, pp. 945-952.

Cui, Y.Q., van der Lans, R.G.J.M., Luyben, K.Ch.A.M. 1997. “Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation” *Biotechnology and Bioengineering*. Vol. 57, pp. 745–790.

Çalik, P., Çalik, G., Özdamar, T.H. 2000. “Oxygen-transfer strategy and its regulation effects in serine alkaline protease production by *Bacillus licheniformis” Biotechnology and Bioengineering.* Vol. 69, No. 3, pp. 301-311.

Debing, J., Peijun, L., Stagnitti, F., Xianzhe, X., Li, L., 2006. “Pectinase production by solid fermentation from *Aspergillus niger* by a new prescription experiment” *Ecotoxicology and Environmental Safet.,* Vol. 64, pp. 244-250.

Dhanasekharan, K.M., Sanyal, J., Jain, A., Haidari, A. 2005. “A generalized approach to model oxygen transfer in bioreactors using population balances and computational fluid dynamics” *Chemical Engineering Science.* Vol. 60, pp. 213-218.

DuBois, M., Gilles K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. “Colorimetric method for determination of sugars and related substances” *Anal. Chem.* Vol.28, No.3, pp. 350-356.

Fang, Q.H. and Zhong, J.J. 2002. “Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*” *Process Biochemistry.* Vol. 37 pp. 769-774.

Galaction, A.-I., Cascaval, D., Oniscu C., Turnea, M. 2004. “Prediction of oxygen mass transfer coefficients in stirred bioreactors for bacteria, yeasts and fungus broths” *Biochemical Engineering Journal.* Vol.20, pp. 85-94.

Gikas, P. and Livingston, A.G. 1998. “Use of specific ATP concentration and specific oxygen uptake rate to determine parameters of a structured model of biomass growth” *Enzyme and Microbial Technology.* Vol. 22, pp. 500-510.

Gomez, R., Schnabel, I., Garrido, J. 1988. ”Pellet growth and citric acid yield of *Aspergillus niger* 110” *Enzyme Microb. Technol.* Vol. 10, pp. 188-191.

Hellendoorn, L., Mulder, H., Van den Heuvel, J.C., Ottengraf, S.P.P. 1998. “Intrinsic kinetic parameters of the pellet forming fungus *Aspergillus awamori*” *Biotechnol. Bioeng.* Vol. 58, pp. 478-485.

Higashiyama, K., Murakami, K., Tsujimura, H., Matsumoto, N., Fujikawa, S. 1999. “Effects of dissolved oxygen on the morphology of an arachidonic acid production by *Mortiella alpina* 1S-4” *Biotechnol. Bioeng.* Vol. 63, pp. 442-448.

Jayani, R.S., Saxena, S., Gupta, R. 2005. “Microbial Pectinolytic Enzymes: A review” *Process Biochem.* Vol. 40, No. 9, pp. 2931-2944.

Jüsten, P., Paul, G.C., Nienow, A.W., Thomas, C.R. 1996. “Dependence of mycelial morphology on impeller type and agitation intensity” *Biotechnology and Bioengineering.* Vol. 52, pp. 672-684.

Jüsten, P., Paul, G.C., Nienow, A.W., Thomas, C.R. 1998. “Dependence of *Penicillium chrysogenum* growth, morphology, vacuolation, and productivity in fed-batch fermentations on impeller type and agitation intensity” *Biotechnology Bioengineering.* Vol. 59, pp. 762-775.

Kashyap, D.R., Vohra, P.K., Chopra, S., Tewari, R. 2001. "Applications of pectinases in the commercial sector: a review" *Bioresource Technology,* Vol. 77, No. 3, pp. 215-227.

Kaufman, P., Kubera, P., Post, T. 1997. "Fermentation: Critical process phenomena and new technology developments that affect yield and productivity" *Pharmaceutical Engineering.* pp. 24-30.

Kubicek, C.P., Tehentgruber, O., Housam, E-K., Röhr, M. 1980. „Regulation of citric acid production oxygen: effect of dissolved oxygen tension on adenylate levels and respiration in *Aspergillus niger*" *Eur. J. Appl. Microbiol. Biotechnol.* Vol. 9, pp. 101-115.

Lee, JM., 1992. *Biochemical engineering*, (Englewood Cliffs, NJ: Prentice- Hall), p. 240.

Li, Z.J., Shukla, V., Fordyce, A.P., Pedersen, A.G., Wenger, K.S., Marten, M.R. 2000. "Fungal Morphology and Fragmentation Behavior in a Fed-Batch *Aspergillus oryzae* Fermentation at the Production Scale" *Biotechnol. Bioeng.* Vol. 70, pp. 300-312.

Lopez, L.C., Perez, J.A.S., Sevilla, J.M.F., Porcel, E.M.J., Chisti, Y. 2005. "Pellet Morphology, Culture Rheology and Lovastatin Production in Cultures of *Aspergillus terreus"* J. *Biotechnol*. Vol. 116, pp. 61-77.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, J.R. 1951. "Protein measurement with the folin phenol reagent" *J. Biol. Chem.* Vol.193, pp. 265-275.

Mantzouridou, F., Roukas, T., Kotzekidou, P. 2002. "Effect of the aeration rate and agitation speed on β-carotene production and morphology of *Blakeslea trispora* in a stirred tank reactor: mathematical modeling" *Biochemical Engineering Journal.* Vol. 10, pp. 123-135.

Mattey, M. 1992. “The production of organic acids” *CRC Crit. Rev. Biotechnol.* Vol.12, pp. 87-132.

McIntyre, M., Breum, J., Arnau, J., Nielsen, J. 2002. “Growth physiology and dimorphism of *Mucor circinelloides* (syn. *racemosus)* during submerged batch cultivation” *Appl. Microbiol. Biotechnol.* Vol. 58, pp. 495-502.

Metz, B. and Kossen, N.W.F. 1977. “The Growth of Molds in the Form of Pellets, A literature review” *Biotechnol. Bioeng.* Vol. 19, pp. 781-799.

Metzner, A.B. 1985. “Rheology of Suspensions in Polymeric Liquid” *Society Rheology.* Vol. 29, No. 6, pp. 739-775.

Nielsen, J., Johansen, C.L., Jacobsen, M., Krabben, P., Villadsen, J. 1995. ”Pellet formation and fragmentation in submerged cultures of *Penicillum chrysogenum* and its relation to penicilin production” *Biotechnol. Prog.* Vol. 11, pp. 93-98.

Olsvik, E., Tucker, K.G., Thomas, C.R., Kristiansen, B. 1993. “Correlation of *Aspergillus niger* broth rheological properties with biomass concentration and the shape of mycelial aggregates” *Biotechnology and Bioengineering.* Vol. 42, pp. 1046-1052.

Ozbek, B. and Gayik, S. 2001. “The studies on the oxygen mass transfer coefficient in a bioreactor” *Process Biochemistry.* Vol. 36, pp. 729-741.

Panda, T., Naidu, G.S.N., Sinha, J. 1999. “Multiresponse Analysis of Microbiological Parameters Affecting the Production of Pectolytic Enzymes by *Aspergillus niger:* a statistical view” *Process Biochem*. Vol. 35, pp.187-195.

Papagianni, M., Mattey, M., Kristiansen, B. 1994. ”Morphology and citric acid production of *Aspergillus niger* PM1” *Biotechnol. Lett.* Vol. 16, pp. 929-934.

Papagianni, M., Mattey, M., Kristiansen, B. 1998. ”Citric acid production and morphology of *Aspergillus niger* as functions of the mixing intensity in a stirred tank and a tubular loop bioreactor” *Biochem. Eng. J.* Vol. 2, pp. 197-205.

Papagianni, M., Mattey, M., Kristiansen, B. 1999. ”Hyphal vacuolation and fragmentation in batch and fed-batch culture of *Aspergillus niger* and its relation to citric acid production” *Process Biochem.* Vol. 35, pp. 359-366.

Papagianni, M. 2004. “Fungal morphology and metabolite production in submerged mycelial processes” *Biotechnology Advances.* Vol. 22, pp. 189-259.

Park, J.P., Kim, Y.M., Kim, S.W., Hwang, H.J., Cho, Y.J., Lee, Y.S., Song, C.H., Yun, J.W. 2002. “Effect of aeration rate on the mycelial morphology and exo-biopolymer production in *Cordyceps militaris*” *Process Biochemistry.* Vol. 37, pp. 1257-1262.

Patil, S.R. and Dayanand, A. 2006. “Optimization of process for the production of fungal pectinases from deseeded sunflower head in submerged and solid-state conditions” *Bioresource Technology.* Vol. 97, pp. 2340-2344.

Paul, G.C., Kent, C.A., Thomas, C.R. 1993. “Viability testing and characterization of germination of fungal spores by automatic image analysis” *Biotechnology and Bioengineering.* Vol. 42, pp. 11-23.

Paul, G.C., Kent, C.A., Thomas, C.R. 1994. “Hyphal vacuolation and fragmentation in *Penicillum chrysogenum*” *Biotechnol. Bioeng.* Vol. 44, pp. 655-660.

Pazauki, M. and Panda, T. 2000. “Understanding the Morphology of Fungi” *Bioprocess Eng.* Vol. 22, pp. 127-143.

Pedersen, A.G., Bundgaard-Nielsen, M., Nielsen, J., Villadsen, J., Hassager, O. 1993. “Rheological Characterization of Media Containing *Penicillium chrysogenum*” *Biotechnology and Bioengineering.* Vol. 41, pp. 162.

Porcel, E.M.R., Lopez, J.L.C., Perez, J.A.S., Sevilla, J.M.F., Chisti, Y. 2005. "Effects of pellet morphology on broth rheology in fermentations of *Aspergillus terreus*" *Biochem. Eng. J.* Vol. 26, pp. 139-144.

Rao, M.A., 1999. *Rheology of fluid and semisolid foods: Principles and Applications,* (Maryland, USA).

Reichl, U., King, R., Gilles, E.D. 1992. "Characterization of Pellet Morphology during Submerged Growth of *Streptomyces tendae* by Image Analysis" *Biotechnol. Bioeng.* Vol. 39, pp. 164-170.

Schügerl, K., Wittler, R., Lorenz, T. 1983. "The use of molds in pellet form" *Trends in Biotechnology.* Vol. 1, No. 4, pp. 120-123.

Shu, C.H. and Lung, M.Y. 2004. "Effect of pH on the production and molecular weight distribution of exopolysaccharide by *Antrodia camphorate* in batch cultures" *Process Biochemistry.* Vol. 39 pp. 931-937.

So, J.H., Yang, S.M., Hyun, J.C. 2001. "Microstructure evolution and rheological responses of hard sphere suspensions" *Chemical Engineering Science.* Vol. 56 pp. 2967-2977.

Thibault, J., Pouliot, K., Agosin, E., Perez-Correa, R. 2000. "Reassessment of the estimation of dissolved oxygen concentration profile and K_La in solid-state fermentation" *Process Biochemistry.* Vol. 36, pp. 9-18.

Van Suijdam, J.C. and Metz, B. 1981. "Influence of engineering variables on the morphology of filamentous molds" *Biotechnol. Bioeng*. Vol. 23, pp. 111–148.

Wainwright, M., 1992. *An Introduction to Fungal Biotechnology,* (John Wiley, New York).

Wang, F., Du, G.C., Li, Y., Chen, J. 2005. "Effects of dissolved oxygen concentration and two-stage oxygen supply strategy on the production of γ-CGTase by *Bacillus macorous*" *Process Biochemistry.* Vol. 40, pp. 3468-3473.

Wang, Y. and Inoue, Y. 2001. "Effect of dissolved oxygen concentration in the fermentation medium on transformation of the carbon sources during the biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxypropionate) by *Alcaligenes latus" International Journal of Biological Macromolecules.* Vol. 28, pp. 235-243.

Wan Nik, W.B., Ani, F.N., Masjuki, H.H., Eng Giap S.G. 2005. "Rheology of bio-edible oils according to several rheological models and its potential as hydraulic fluid" *Industrial Crops and Products.* Vol. 22 pp. 249-255.

Wittler, R., Baumgartl, H., Lubbers, D.W., Schugerl, K. 1986. "Investigations of oxygen transfer into *Penicillium chrysogenum* pellets by microprobe measurement" *Biotechnol. Bioeng.* Vol. 28, pp. 1024-1031.

Zhaou, Y., Du, J., Tsao, G.T. 2000. "Mycelial Pellet Formation by *Rhizopus oryzae* ATCC 20344" *Appl Biochem. Biotechnol.* Vol. 84-86, pp. 779-789.

APPENDIX A

EFFECT OF pH ON PELLET MORPHOLOGY

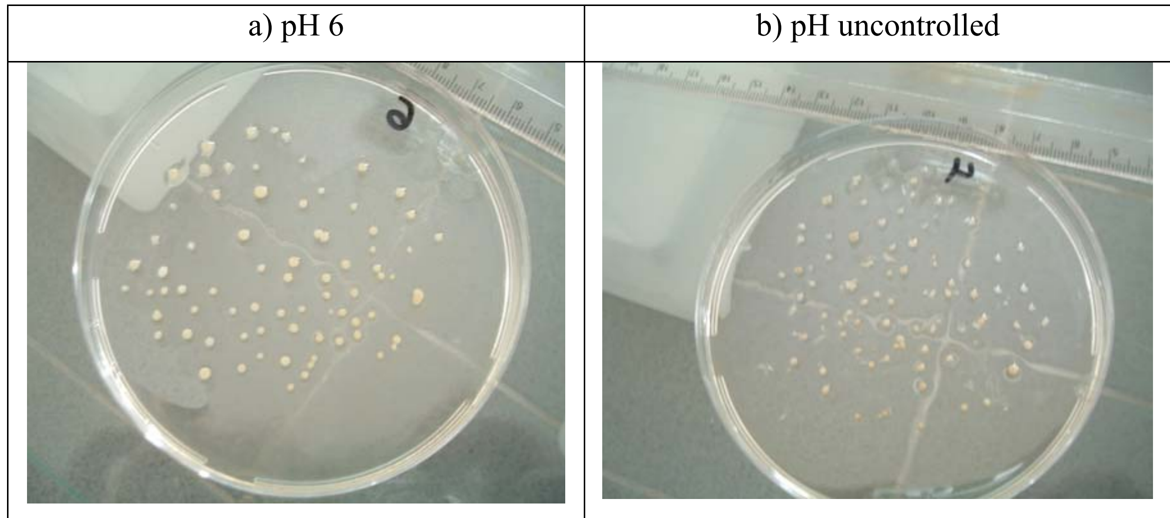


Figure A.1. Pellet morphology when pH was a) kept at 6 b) uncontrolled in 1g media

APPENDIX B

EFFECT OF AGITATION SPEED ON PELLET MORPHOLOGY

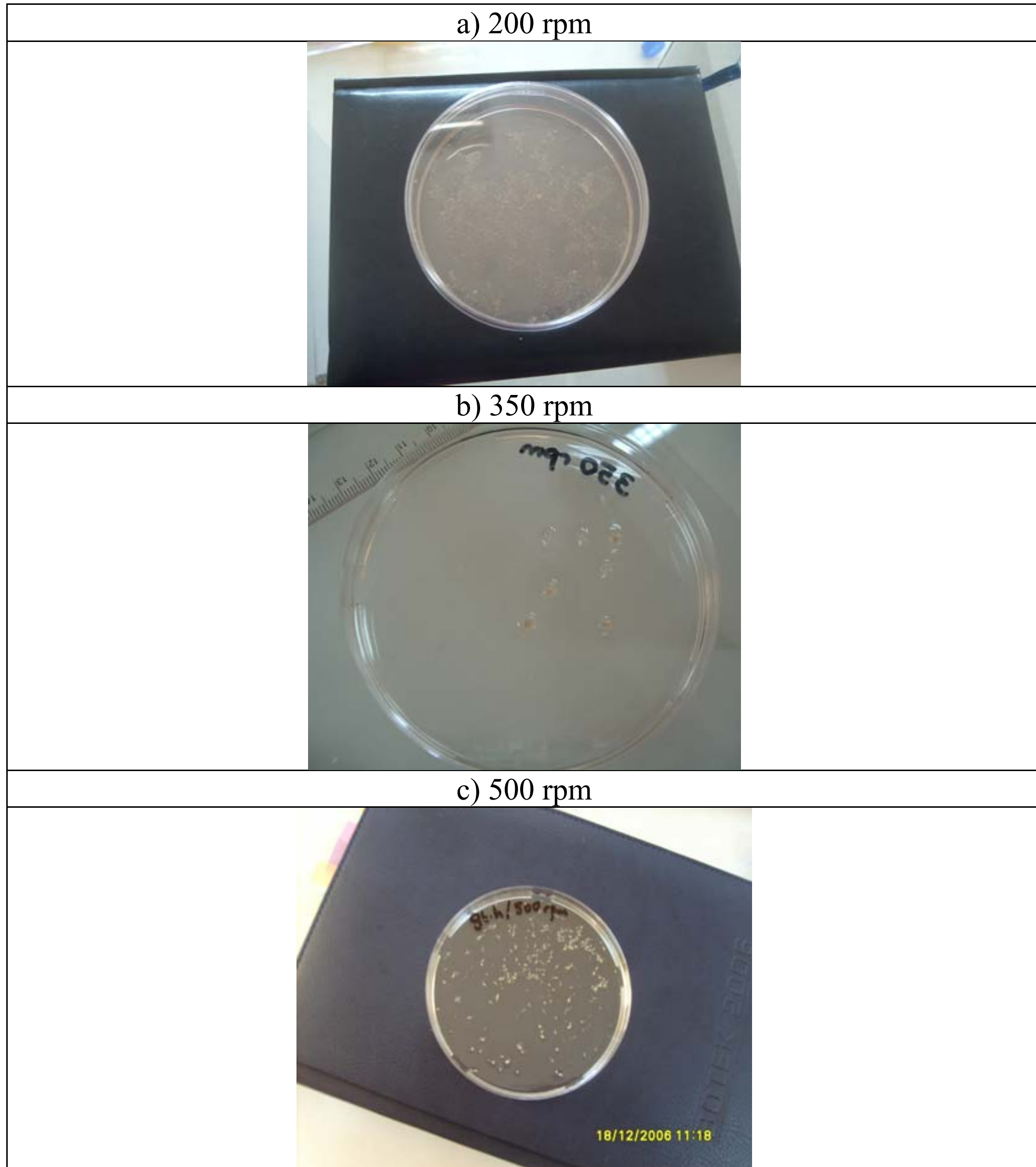


Figure B.1. Pellet morphology when agitation was kept at a) 200 rpm b) 350 rpm c) 500 rpm

APPENDIX C

EFFECT OF DISSOLVED OXYGEN TENSION ON PELLET MORPHOLOGY

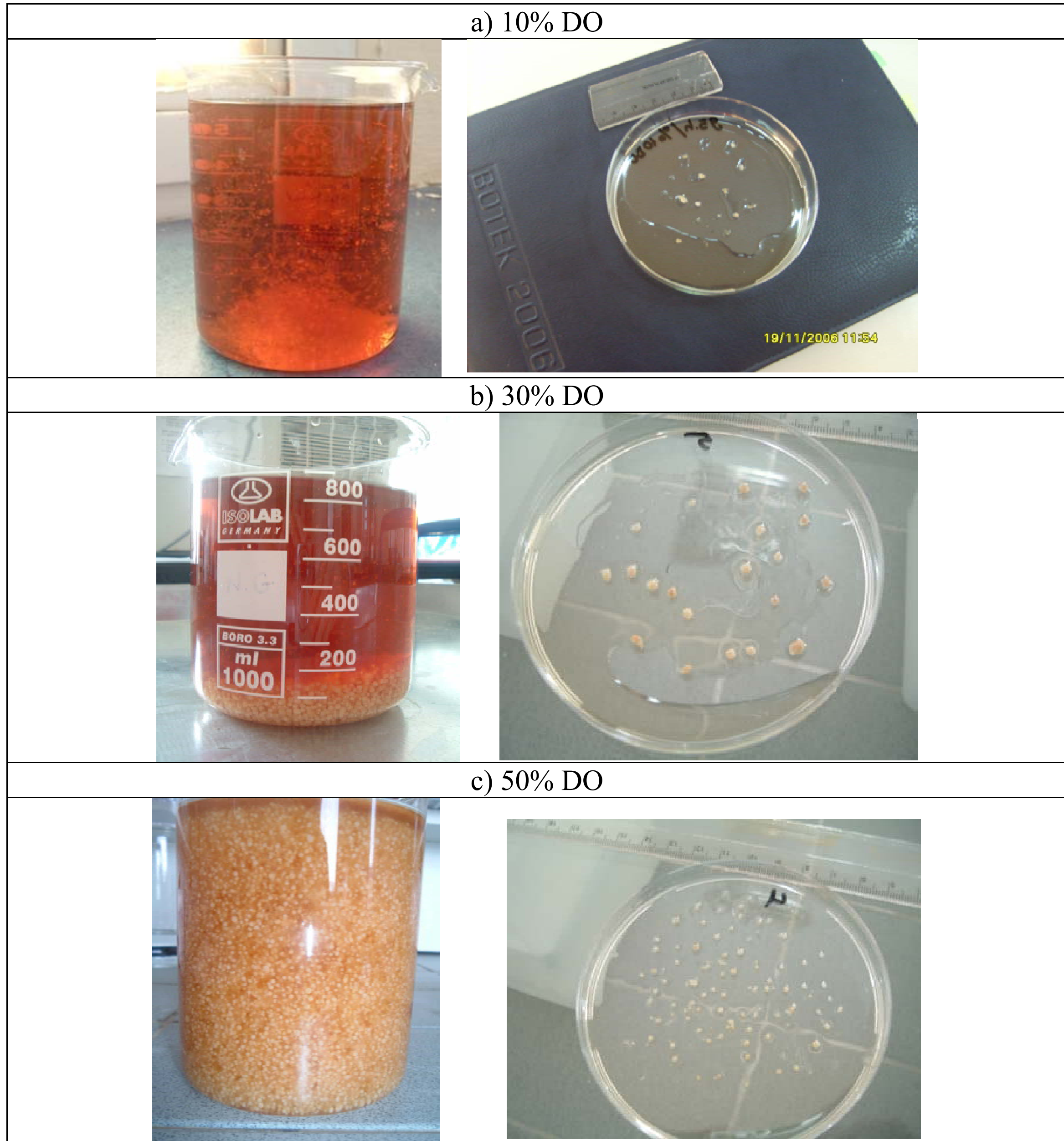


Figure C.1. Pellet morphology when dissolved oxygen was kept at a) 10% b) 30% c) 50%