IUFoST 2006
DOI: 10.1051/IUFoST:20060271

VARIOUS FACTORS AFFECTING THE PELLET MORPHOLOGY, BROTH REOLOGY AND PECTINASE ENZYME PRODUCTION IN SUBMERGED FERMENTATION OF ASPERGILLUS SOJAE

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ABSTRACT

Deep tank fermentations are widely used in the production of enzymes, antibiotics and organic acids, which have many applications in the food, medicine, pharmaceutical, chemical and textile industry (Mitard and Riba, 1987). The diverse range of commercially exploited fungal products, which have large contribution to the global economy is expanding enormously. However, their filamentous growth characteristic creates a number of process engineering problems attributed to the morphological change accounted during the fermentation process in large scales. This effects the maximum product and productivity of an enzyme. In this study, pectinase enyzme production was taken as a model for submerged fermentation. For this purpose, *Aspergillus sojae* strain which is not well known in the literature, was taken as a model organism and subjected to fermentation at 30^{o}C for 96 hours to produce pectinase enzyme of commercial value. Composition of media, inoculation ratio and type, temperature and aeration rate was kept constant during fermentation. Experiments were conducted according to different dissolved oxygen concentrations, agitation speeds and pH in order to investigate the effects of these parameters on rheology, morphology and activity. As a result of these experiments, it was observed that small pellets gave higher activities under the conditions of low agitation speed, high dissolved oxygen concentrations and uncontrolled pH.

Key words: Pellet morphology, broth rheology, pectinase enzyme, *Aspergillus sojae*

INTRODUCTION

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., 1991; Pedersen et al., 1993; Li et al., 2000; Pazauki and Panda, 2000).

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. 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, dispersed mycelium), the nature of the growth medium, and the hydrodynamic regime in the bioreactor (Metz and Kossen, 1977; 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.

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., 1991). Since agitation and aeration is also much easier in such a system, the power input therefore the operating cost is lower.

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.

The effects of aeration rate, agitation speed and dissolved oxygen concentration on pectinase production are important factors affecting successful progress of fermentation. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product and oxygen (Martin and Bailey 1985; Olsvik and Kristiansen 1992; Rau et al. 1992; Cui et al. 1998; Sinha et al. 2001; Mantzouridou et al. 2002). Agitation is also an important parameter for adequate mixing, mass and heat transfer. Agitation creates shear forces, causing morphological changes, variation in their growth and product formation, and also damage to the cell structure (Taguchi et al. 1968; Martin and Bailey 1985; McNeil and Kristiansen 1987; Smith and Lilly 1990; Pfefferle et al. 2000; Mantzouridou et al. 2002; Park et al. 2002b).

Oxygen transfer limitations occur commonly in bioreactors, leading to decreased performance. Due to the poor solubility of oxygen in aqueous solutions, the dissolved oxygen (DO) supply in aerobic fermentations is usually viewed as a limiting step for product

IUFoST 2006
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formation (Lai et al., 2002). When oxygen is limited, the metabolic rate of the microorganisms decreases significantly and the culture may respond adversely to the resulting stress (Lee, 1992).

The objective of this work is to examine the effect of dissolved oxygen, pH and agitation speed on the pellet morphology, broth rheology and pectinase production in submerged fermentation of *Aspergillus sojae* by keeping aeration rate, temperature, media composition, inoculation ratio and type constant.

MATERIALS AND METHOD

Microorganism

Aspergillus sojae ATCC 20235 was purchased from Procochem Inc., an international distributor of ATCC (American Type of Culture Collection) in Europe. The propagation of this culture was done on 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^{o}C until well sporulated (1 week). Stock cultures of this strain were prepared with 20 % glycerol water and stored at -80^{o}C.

Growth Medium

For the seed medium, molasses media formulation (Glycerol (45 g/l), Pepton (18 g/l), Mollasses (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), Agar (20 g/l)) was used. Initially, the frozen stock cultures were inoculated on YME agar and incubated for one week at 30^{o}C 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 0.01 % (v/v) tween 80 solution was added into the 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^{o}C and used as inoculum for the fermentation process.

Production Medium and Fermentation

The fermentation was carried out in a 5 L bioreactor (New Brunswick BioFlo 3000, NJ, USA) with a working volume of 4 L. The fermenter was equipped with two sets of a standard six-blade rushton impeller and four baffle plates. (The distance between two impellers is 108 mm and the distance to bottom impeller is 39 mm)

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) prepared in the amount of 4L.

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.

The pH of the fermenter medium was maintained by using automatic control equipment, which added 6N NaOH of 10% H_2SO_4 when pH deviated from the desired value.

When DO, rpm and pH were adjusted to desired values, the fermentation was started at 30°C by keeping aeration rate at 2.5 vvm. During this period, samples taken periodically were assayed for enzyme activity. Enzyme activity was determined on supernatant obtained after the centrifugation of the broth at 5000 rpm for 15 minutes. At the end of the fermentation period (at 96 hours) fungal morphology and broth rheology were investigated.

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. 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 (Sigma, St. Louis, MO) was used as standard for calibration curve of PGase activity.

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

Rheological and Morphological Measurements

Rheological properties of fermentation broth were determined by using concentric cylinder viscometer (Brookfield DV II + Pro, Brookfield Engineering Lab. Inc., MA, USA) equipped with a cylindrical spindle (cylinder diameter 18.84 mm, length 115 mm, beaker diameter 86.30 mm and 600 ml of sample volume). The cylindrical spindle geometry was used for the samples taken at the end of the fermentation period (at 96 h). On the other hand, small sample and UL adapter attachments are used for the samples taken periodically during fermentation period. But in these samples the size of pellets was generally of the same order of magnitude as the annulus of the UL and small sample 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 2

IUFoST 2006
DOI: 10.1051/IUFoST:20060271

and 3, where η was the viscosity of the suspension and η_s was the viscosity of the continuous phase (Metzner, 1985):

$$\eta_r = \frac{\eta}{\eta_s} \tag{2}$$

$$\eta_r = \left[1 - \left(\frac{\phi}{A}\right)\right]^{-2} \tag{3}$$

In equation 3, 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 3.

Pellet morphology (pellet number and size) was characterized by using image analysis (Cox and Thomas, 1992). Pellet particles were analyzed for determination of the number of pellet per given volume and pellet size by fixing the samples with a fixative (13ml of 40% formaldehyde, 5ml glacial acetic acid, 200 ml of 50% ethanol) (Park *et al.,* 2002).

The image was captured with a eurocam (Euromax, Holland) mounted on a phase contrast microscope (Novex, Holland). Image analysis was performed with the software package Image – Pro Plus 4.5.1. (Media Cybernetics Inc., Silver Spring, MD, USA). The size of the pellet was quantified using the diameter corresponding to a circular area equivalent to the pellet projected area (Lopez *et al.,* 2005).

RESULTS AND DISCUSSION

In this study, the effects of pH, dissolved oxygen concentration (DO) and agitation speed on broth rheology, pellet morphology and activity were investigated. At all experiments, media composition, temperature, inoculation ratio and type, aeration rate and starting parameters were kept the same.

The effect of pH

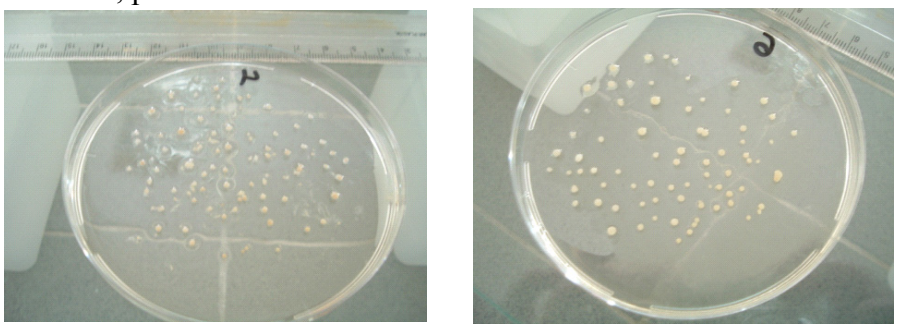
In order to investigate the effect of pH, two experiments were conducted. In the first experiment pH was kept at 6 and in the second one pH was not controlled. In both experiments DO was kept at 50%, through cascading the agitation speed between 200 and 500 rpm. In the first experiment, it was not possible to measure the viscosity of the broth by using spindle geometry so that rheological measurement was conducted with UL adapter, filtrate and suspension viscosity values were calculated according to Metzner model (Eqn. 3) and comparison was made for two experiments.

DOI: 10.1051/IUFoST:20060271

Table 1. The effect of pH on rheology, morphology and enzyme activity

	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	94	74
Enzyme Activity (U)		
at 50 h	7.34	3.97
at 96 h	4.64	0
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
Biomass dry wieght (g/L) at 96 h	8.75	11.62

It was observed that the suspension viscosity was higher when pH was set to 6 (Table 1). Although pellet volume fraction and number of pellet formation was not high, the filtrate viscosity was found to be slightly higher in the medium where pH was kept at 6.0, which imposed an effect on the suspension viscosity calculations. This could be attributed to the production and secretion of some metabolites into the medium by this micro-organism. This might have had also an influence on the enzyme activity. The enzyme was deactivated at the end of the fermentation period when pH was kept at 6. On the other hand, the activity was found to be higher for the uncontrolled pH medium and the maximum activity was reached at 45 hour in both fermentations. When pH gets lower (acidic medium) nutrients are better and can be easily used by microorganisms and bigger pellets are formed. For this reason enzyme activity is higher when pH is lower. Additionally, pH did not significantly affect the average pellet size, pellet number and pellet size distribution (data not given) but created a higher impact on the morphology (pellets with a hairy region surrounding a dense core or compact, smooth and very dense pellets) (Fig 1). In the fermentation run where pH was not controlled, at the end of 96. h, pH of the medium decreased to 4.0



a) pH uncontrolled b) pH=6

Figure 1. Pellet morphologies when a) pH was uncontrolled and b) pH was at 6 in 1g of broth

IUFoST 2006
DOI: 10.1051/IUFoST:20060271

The Effect of Dissolved Oxygen (DO) Concentration

In order to study the effect of dissolved oxygen concentration (at 30% and 50 %) same experimental conditions as described in materials and methods were applied, with only the difference where pH of the medium was adjusted initialy to pH 6 and afterwards not controlled..), Agitation speed was cascaded between 200-500 rpm in order to keep the dissolved oxygen concentration at 30 and 50 % . The results are given in figure 2, 3 and 4.

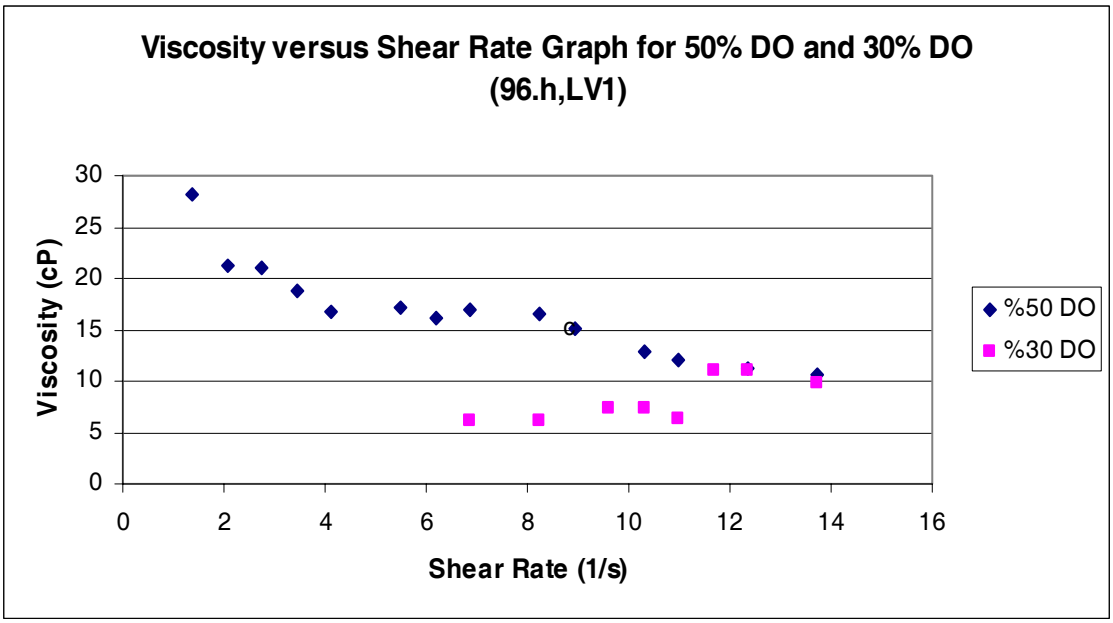


Figure 2. The effect of DO on rheology (viscosity as a function of shear rate)

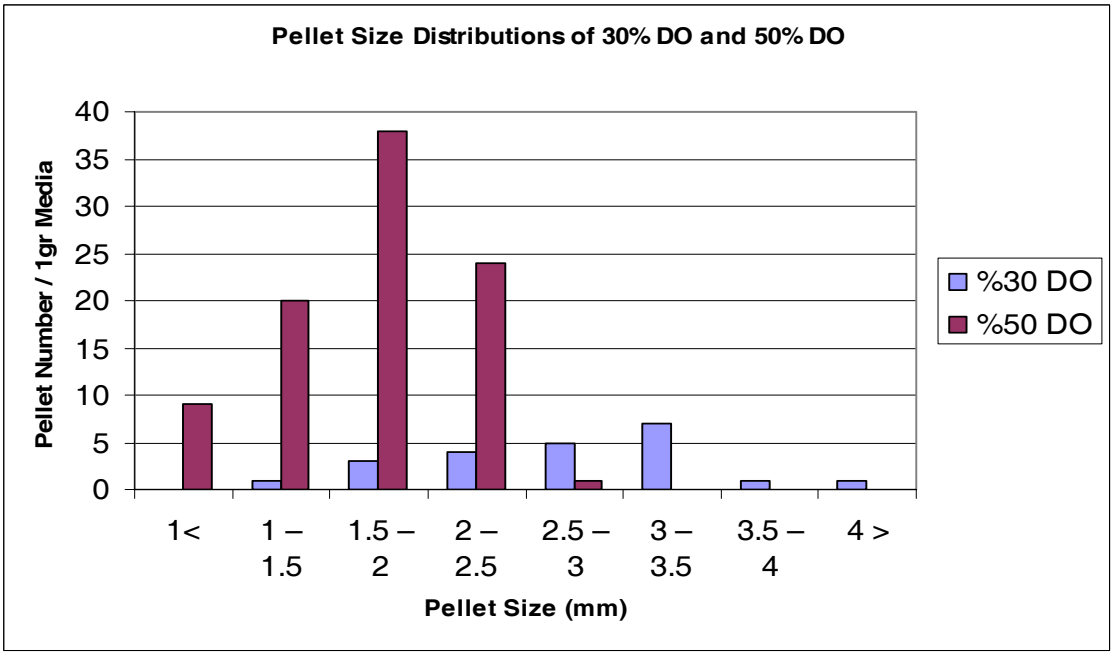


Figure 3. The effect of DO on pellet size distribution

Non-Newtonian behavior was observed for both DO concentrations. While medium at 30% DO was exhibiting non-Newtonian and dilatant behavior, 50% DO medium was showing non–Newtonian and pseudoplastic behavior (Fig.2). Flow behavior index (n) and consistency index (K, Pa.s) values for 30% DO medium and 50% DO medium were found to be 1.88 and 0.0039 Pa.s, 0.63 and 0.12 Pa.s, respectively. Dilatancy (i.e. n>1) might be due to adhesion of hairy and large pellets to form big clumps exhibiting high resistance to increased shear rates or sedimentation of heavy particles during viscosity measurements (Fig.4). The average pellet size for 30 % DO and 50% DO medium was found to be 2.8±0.87 mm and 1.69±0.48 mm, respectively. Higher DO concentration resulted in small pellet formation with a high pellet number (Fig. 3). The maximum activities were 7.34 U and 6.26 U for 50% DO medium and 30 % DO medium, respectively. In summary, large pellet formation, lower viscosity and enzyme activity were observed in 30% DO concentration medium. On the other hand, high DO concentration medium (50%) assisted to form small pellets of high in numbers which resulted in higher enzyme activity.

a) 30% DO b) 50% DO

Figure 4. Pellet morphologies of a) 30% DO and b) 50% DO in 600 ml beaker

The Effect of Agitation Speed

In order to investigate the effect of agitation speed on the enzyme activity, pellet morphology and rheology, fermentations were carried out at 350 rpm and at 200 rpm agitation speeds. All the other fermentation conditions were kept the same (inoculation rate-2x10^6 total spore, 50% DO, uncontrolled pH , aeration rate-2.5 vvm).

Table 2. The effect of agitation on morphology and enzyme activity

Agitation speed	200 rpm	300 rpm
Pellet number/1 g media	122	13
Maximum enzyme activity (U)	6.73	4.39
Pellet morphology	Small pellets	Large and loose pellets
Average pellet size (mm)	0.70±0.34	1.70±0.90
Biomass dry weight (g/L) at 96 h	8.2	6.75

IUFoST 2006
DOI: 10.1051/IUFoST:20060271

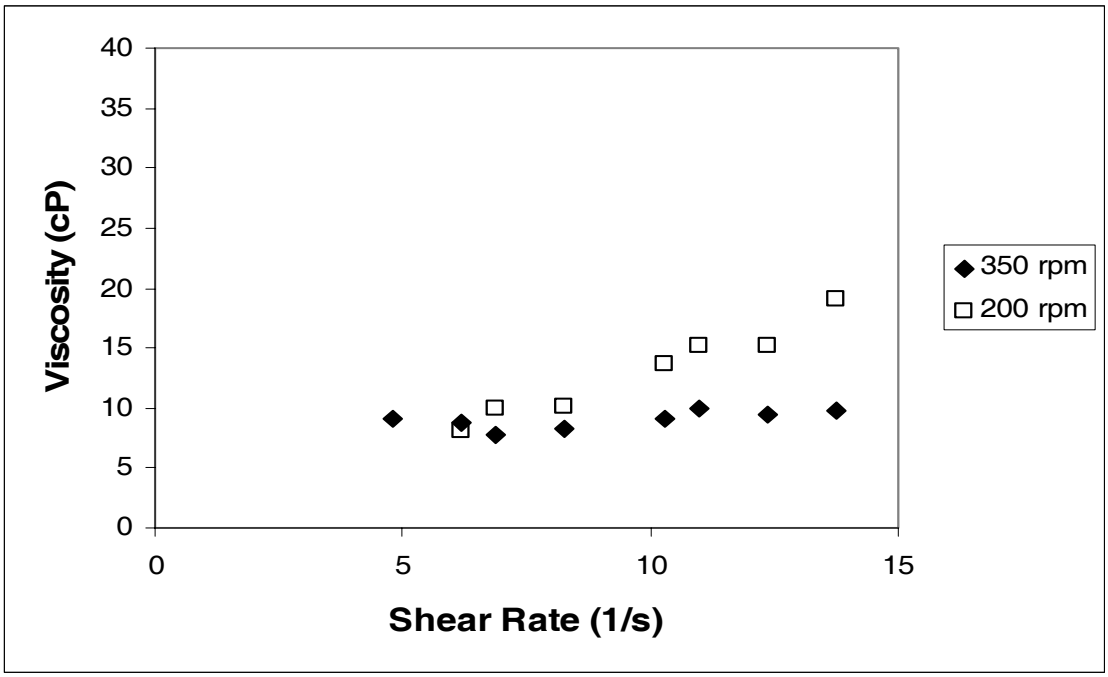


Figure 5. The effect of agitation speed on broth rheology

Non–Newtonian and dilatant behavior (n=1.98 and K=0.0054 Pa.s) (Fig.5) and, small pellet formation was observed at 200 rpm (Table 2).Additionally, pellet number per 1 g media was found to be very high at 200 rpm. Small pellets had a tendency to stick together to form a flock massiveness when the shear rate was increased. This caused a big resistance to flow and resulted in dilatant behavior with increased shear rates. On the other hand, Newtonian type of behavior was detected at 350 rpm. A mixture of loose, large and dense pellets and free filaments were observed in this medium. In many reports, it is claimed that high agitation rate creates higher shear forces and reduces agglomeration of hyphal elements and reducing both pellet diameter and the concentration of pellets (Papagianni, 2004). Although the number of pellets in the culture agitated at 350 rpm was low as indicated in these reports, *A. sojae* formed loose and large pellets quite the opposite of literature data. In addition, the biomass dry weight of the culture having loose and large pellets was in the same order of magnitude with the culture having small pellets. This may be explained by increased oxygen transfer rate due to high agitation in Newtonian medium which promoted the growth of pellets. Moreover, maximum enzyme activity was found to be 6.73 U and 4.39 U at 200 rpm and 350 rpm, respectively. Consequently, the cultures of small pellets yielded higher levels of enzyme activity than did the cultures composed mainly of free filaments and loose pellets.

CONCLUSION

It was found that pellet morphology, broth rheology and pectinase enzyme production from *Aspergillus sojae* depended on the pH, dissolved oxygen concentration and agitation speed. When small dense pellets were formed, the pectinase production was the highest.

This work is a preliminary study to understand and obtain more information about the fermentation conditions of this strain used the first time for pectinase production in a

submerged fermentation. The optimization conditions and oxygen transfer phenomena need to be examined in more detailed in future studies.

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