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Bioprocess Development for the Production of α-amylase by *Bacillus amyloliquefaciens* in Batch and Fed-Batch Cultures

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Abstract: In the present study, the influence of aeration on α-amylase production kinetics by *Bacillus amyloliquefaciens* was studied in both shake flask and bioreactor. In the first optimisation step in shake flask cultures, different cultivations were conducted to study the effect of aeration on the kinetics of cell growth and amylase production by changing the shape of flask (i.e., baffled and non baffled flask), working volume of fermentation media and shaking intensity. In shake flask cultures, the maximal enzyme production of about 2200 μkat L^{-1} was obtained in baffled Erlenmeyer flask agitated at 200 rpm and 10% working volume. Further improvement in enzyme production was obtained by scaling up the cultivation process to stirred tank bioreactor. Cultivations were carried out in 3 L stirred tank bioreactor, Bioflow III (New Brunswick, USA), at aeration rate of 1 vv m^{-1} and constant pH of 6.0. For batch cultures in bioreactor, both volumetric and specific productions were increased and the maximal volumetric enzyme production reached 3600 μkat L^{-1}. Moreover, the cultivation in fed-batch culture with intermittent addition of soluble starch increased the enzyme production significantly reaching about 5300 μkat L^{-1} after 34 h.

Key words: α-amylase, *Bacillus amyloliquefaciens*, batch cultivation, fed-batch cultivation

INTRODUCTION

Nowadays, there are many enzymes produced by microorganisms that are routinely used in industrial processes. The global market for industrial enzymes is estimated at $2 billion in 2004 and is expected to rise at an Average Annual Growth Rate (AAGR) of 3.3% to $2.4 billion in 2009. However, carbohydrates, proteases and lipases are the main industrial interesting enzymes. The global market for starch processing enzymes is around US $156 million and the cost of the enzymes used in the liquefaction process represents 24% of the total process cost (Crabb and Mitchinson, 1997). Therefore, any improvement in enzyme production yield, thermostability or activity will have a direct impact in the process performance, economics and feasibility.

α-amylase (1,4-α-D-glucan-glucanhydrolase, EC 3.2.1.1,) is a classical calcium-containing enzyme which belongs to the endo-amylases family and catalyze the cleavage of α-D-(1-4) glycosidic bonds in starch and related carbohydrates with retention of the α-anomeric configuration in the products (Kandra, 2003). Therefore, this enzyme is essential for the conversion of starch into oligosaccharides (McMahon *et al*., 1999). For industrial applications, α-amylase used extensively in starch liquefaction, paper industries, food industries, pharmaceutical and sugar industries (Nigam and Singh, 1995; Ikram-Ul-Haq *et al*., 2003; Reilly, 2007). The industrial production of this enzyme carried out mainly in submerged culture using different types of microorganisms (Mørkeberg *et al*., 1995; Mc Mahon *et al*., 1997; Ray, 2004; Balkan and Ertan, 2005). Among bacterial strains, *Bacillus subtilis*, *B. stearothermophilus*, *B. amyloliquefaciens* and *B. licheniformis* and some other species belongs to

Bacilli are the main industrial bacterial amylase producer (Bajpai and Sharma, 1989; Nigam and Singh, 1995; Hillier *et al*., 1996; Milner *et al*., 1997; Beshay and El Enshasy, 2002; Carmelo *et al*., 2002; Ikram-ul-Haq *et al*., 2005; Mitsuiki *et al*., 2005). The production process is usually carried out in submerged cultures in batch or fed-batch mode and requires high oxygen level. The oxygen transfer in shake flask is usually critical factor in the production of both primary and secondary metabolites (Clark *et al*., 1995; Gibbs and Seviour, 1996; El Enshasy *et al*., 1999). However, different factors have been reported for their significant effect on the oxygen transfer in shake flasks. These include: flask shape and size, shaking frequency, shaking eccentricity, filling volume, surface properties of flask material (hydrophilic and hydrophobic properties of surface) and physico-chemical properties of the fermentation broth such as viscosity, oxygen solubility and diffusivity (Henzler and Schedel, 1991; Büchs, 2001; Maier and Büchs, 2001; El Enshasy *et al*., 2000). In the present study, the influence of aeration on the kinetics of cell growth and amylase production was studied in shake flask by changing the shape of flask, working volume of fermentation media in and agitation speed of shaker. Further improvement in the production process was achieved by scaling up the process to stirred tank bioreactor level in both batch and fed-batch cultivation mode by intermittent addition of starch.

MATERIALS AND METHODS

Microorganism and Culture Media

Bacillus amyloliquefacients NRRL B-14396, obtained from the Northern Regional Research Laboratory, US. Department. of Agriculture, Peoria, Illinois, was used for α-amylase production. This stain was kept frozen at -80°C in an LB medium containing 50% ($v.v^{-1}$) glycerol solution. Before use, the cells were propagated twice in LB agar medium. LB medium with a composition in ($g\ L^{-1}$) of beef extract, 5.0; peptone, 10.0; yeast extract, 5.0 and NaCl, 5.0 and agar, 20.0 was used for medium activation. The pH was adjusted to 7.0 before sterilization. LB broth was used for inoculum preparation for enzyme production medium. The production medium (pH 7.0) for fermentation study was composed of ($g\ L^{-1}$): $(NH_4)_3PO_4$, 5.0; yeast extract, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; sodium citrate, 0.1; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.1; $MnSO_4.H_2O$, 0.1 and soluble starch, 10.0.

Cultivation Conditions

Unless otherwise mentioned, cells were grown at 30°C on a rotary shaker in 250 mL Erlenmeyer flasks containing 50 mL of fermentation medium. The inoculum was in the form of 3 mL of cell suspension (of 1 OD at 600 nm) obtained from growing cells in the LB medium for 24 hrs. In case of shaken flaks cultures, unless otherwise mentioned, cultivations were done in a rotary incubatory shaker at 30°C (Infors Co.; Switzerland). In bioreactor experiments, cultivation was carried out in a 3 L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with a working volume of 2 L. Agitation was performed using a three 4-bladed rushton turbine impellers ($d_{i(\text{impeller diameter})} = 65$ mm; $d_{t(\text{tank diameter})} = 135$ mm, $d_i d_t^{-1} = 0.48$) at 600 rpm. Aeration was performed by filtered sterile air at the rate of [1 vv m^{-1}]. Dissolved oxygen concentrations were analyzed by polarographic electrode (Ingold, Germany). Foam was suppressed, when necessary, by the addition of silicon antifoam reagent (Sigma, USA).

Analytical Methods

At different time intervals, 2 and 10 mL samples were taken at different times during cell cultivation in case of shake flasks and bioreactor cultures, respectively. After sampling, cell density was determined immediately using a spectrophotometer (Novaspec II, Pharmacia Biotech, Sweden). Cell dry weight was also determined again after drying of double washed cells in an oven at 110°C for a constant weight. One A_{600} unit was equivalent to 0.235 g (cell dry weight L^{-1}). Cells were centrifuged at 5°C and 5000 rpm. The supernatant was frozen at -20°C and left for analysis. α-amylase activity

was determined according to the method of Bernfeld (Stellmach, 1988). 0.5 mL of properly diluted enzyme in 0.05 M acetate buffer pH 4.9 was incubated for 3 min at 25°C with 0.5 mL of 1% starch solution. The reaction was stopped by addition of 1 mL. of colour developing agent containing dinitrosalicylic acid. The tube containing the mixture was heated for 5 min in a boiling water bath and cooled in ice bath immediately to stop the colour developing reaction. After the addition of 10 mL. of distilled water, the optical density of the solution was determined at 540 nm. The enzymatic activity is expressed by Katal unit which is defined as the amount of activity that produce one mole of reducing group per second (Florkin and Stotz, 1973). Maltose, ranging from 0.2-2 mg mL^{-1}, was used as standard.

RESULTS AND DISCUSSION

Effect of Aeration on α-amylase Production

This study was undertaken to investigate the effect of aeration on the kinetics of cell growth and amylase production. Three different methods for varying the dissolved oxygen in shake flask were applied:

Effect of Agitation Speed

Cells were incubated in an incubatory shaker in 250 Erlenmeyer flask containing 50 mL culture (20% working volume) at different agitation speeds ranging from 50-250 rpm to give different levels of dissolved oxygen content. As shown in Fig. 1, both of cell growth and enzyme production increased significantly with the increase of agitation speed. The maximal cell dry weight of 2 g L^{-1} and enzyme

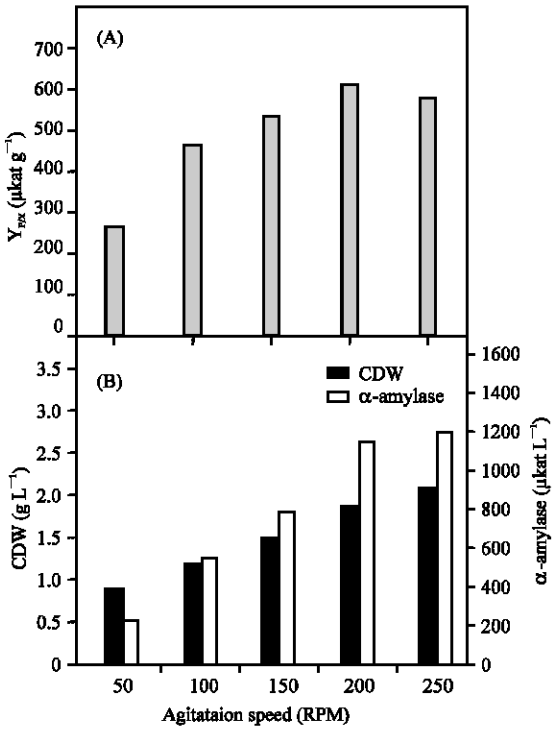

Fig. 1: Effect of different agitation speeds on the cell growth and α-amylase production by *B. amyloliquifaciens*

production of about 1200 μkat L^{-1} was obtained in 250 rpm agitated culture. On the other hand, the specific enzyme production ($Y_{p/x}$) which reflects the cell productivity increased gradually upon the increase of agitation speed from 50-200 rpm and reached its maximal value of about 605 μkat g^{-1} in 200 rpm agitated culture and slightly decreased in cultures agitated at higher speed. This indicates that the increase in enzyme production by agitation is a result of better mixing and better oxygen availability which increases cell productivity rather than only the increase in cell mass. A higher agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium. It might, therefore, have contributed to the higher growth and better enzyme production in many bacillus species (Feng *et al*., 2003).

Effect of Medium Working Volume

Medium working volume in shake flask is also one of the main factors affecting the dissolved oxygen content. As medium working volume increase, the DO content decreased. Thus, in this study different medium volume in shake flaks were applied ranged from 5% up to 50%. The results in Fig. 2 indicates that the increase of working volume from 5-10% showed positive effect on both cell growth and enzyme production and increased the specific production which indicates also the higher production efficiency of the cell. The maximal volumetric and specific enzyme production at 10% working volume was 1750 μkat L^{-1} and 921 μkat g^{-1}, respectively. Further increase in medium working volume beyond 15% resulted in significant decrease in cell mass concomitant with reduction in both volumetric and specific enzyme production. However, the increase in medium volume decreases the mixing inside the flask and oxygen availability. Such decrease in DO shows usually negative effect on the production of several primary and secondary metabolites (El Enshasy *et al*., 2000). However, the obtained results in this experiment support the hypothesis of high oxygen requirement for α-amylase production.

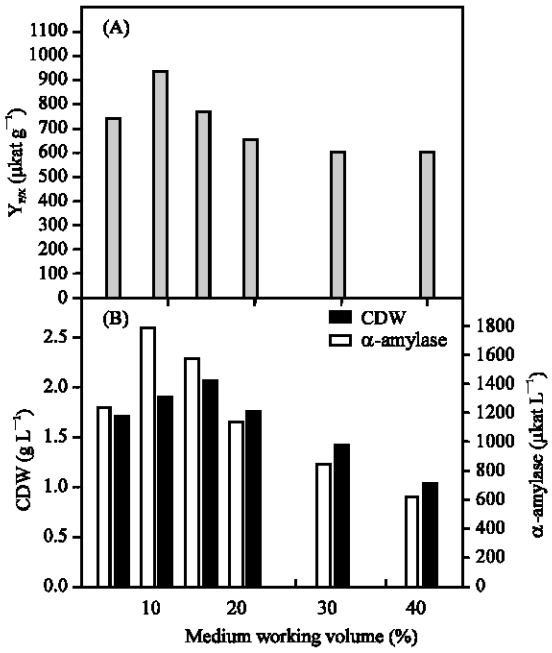

Fig. 2: Effect of different medium working volume in 250 mL Erlenmeyer flask on the cell growth and α-amylase production by *B. amyloliquifaciens*

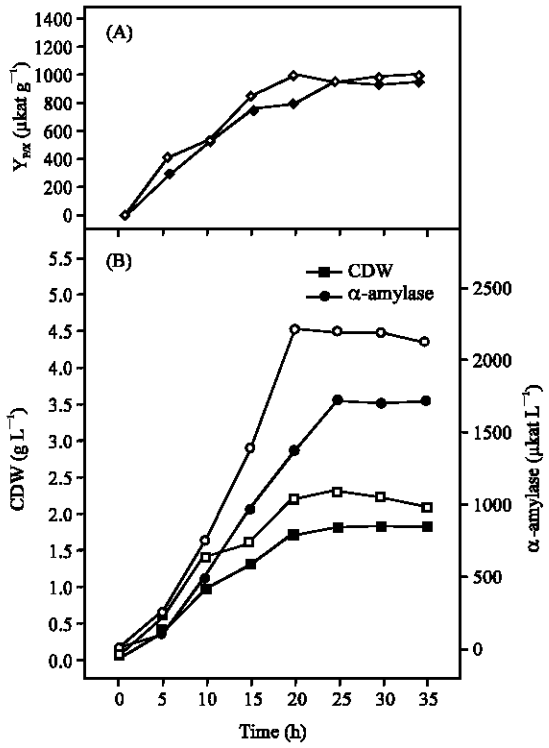

Fig. 3: Effect of shape of cultivation vessel on the kinetics of cell growth and α-amylase production by *B. amyloliquefaciens*. Opened and closed symbol represent baffled and non-baffled flasks, respectively

Effect of Shape of Cultivation Vessel (Baffled and Non-baffled Erlenmeyer Flask)

For further investigation for the relation between mixing/oxygen availability and enzyme production in shake flask level, two types of shake flasks (baffled and non-baffled Erlenmeyer flasks) were used. The results in Fig. 3 indicated that, when the non-baffled flasks were replaced by shake flasks equipped with two baffles, a significant increase in both cell growth and volumetric enzyme production by about 27% after 25 h cultivation when the cells entered the stationary phase. This indicates that, the increase in volumetric enzyme production was a result of higher cell growth rather than the increase in cell productivity. The specific enzyme production in both cultures was around 1000 μkat g^{-1}. It have been reported that if high oxygen transfer capacities in shaking flasks are required, the introduction of baffles is a good solution. This provides high oxygen transfer. Moreover, the higher level of hydromechanical stress caused by baffles may be an additional advantage in process requires shear and mixing for growth and product excretion (Büchs, 2001). The increase of cell productivity and growth in baffled flaks was also reported for the production of glucose oxidase enzyme by *A. niger* (El Enshasy *et al*., 1999). Baffled flasks not only increase the oxygen transfer inside the vessel but also become important in case of enzyme excretion and improve cell excretion capacity.

Bioreactor Cultivations

Batch Cultivations

For further development of the production process and transfer the production to higher production scale, cultivations were carried out in 3 L stirred tank bioreactor at 600 rpm and aeration

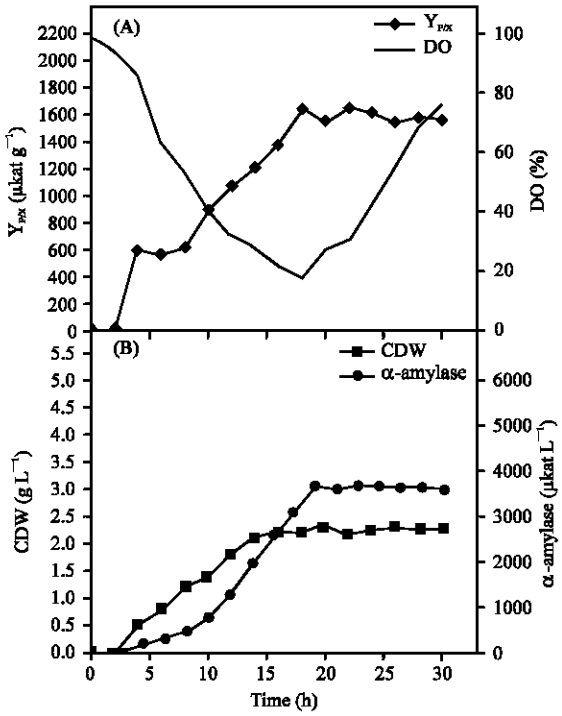

Fig. 4: Kinetics of cell growth and α-amylase production by *B. amyloliquefaciens* in stirred tank bioreactor in batch culture

rate of 1 vv min^{-1}. The results in Fig. 4 indicated that cells grew exponentially with specific growth rate of 0.116 h^{-1} reaching the maximal growth of 2.1 g L^{-1} after 14 h. On the other hand, the maximal production of about 3600 µkat L^{-1} was obtained after 18 h. This was about 54% higher than the best optimized shaken flask cultures. On the other hand, the specific enzyme production in bioreactor cultures was about 1600 µkat g^{-1} which is also about 60% higher than the maximal value obtained in shake flasks. This indicates that the better oxygenation and mixing in stirred tank bioreactor increase the cell productivity and as shown in Fig. 4a there was no oxygen limitation in culture and the minimal value was 20% saturation as cells entered the stationary phase. The data of dissolved oxygen in culture (Fig. 4a) indicated that during the growth phase, the DO value decrease as a result of high oxygen consumption and cell activity and increased again gradually as cells entered the stationary phase. As shown, in Fig. 4 the α-amylase production carried out mainly during the logarithmic growth phase and not during the stationary phase. However, the parallel relation between the curves of α-amylase production and cell growth was also observed in different bacterial strains such as *B. licheniformis* (Rothstein *et al.*, 1986) and *Micrococcus varians* (Adeleye, 1990). Thus, fed-batch cultivation was designed to extend exponential growth phase and to increase the enzyme production accordingly.

Fed-batch Cultivations

Based on the data of batch cultivation, fed-batch cultivation was designed with intermittent addition of starch at different time intervals. After batch cultivation for 16 h, starch was added in form of 200 mL containing 20 g soluble starch, the addition time was calculated from the batch cultivation when cell entered the stationary phase and DO start to increase. The second pulse addition of starch was done after 26 h with the same starch concentration. As shown in Fig. 5, cells grew exponentially and reached 4.3 g L^{-1} after 36 h. This increase in cell mass resulted also in a significant increase in

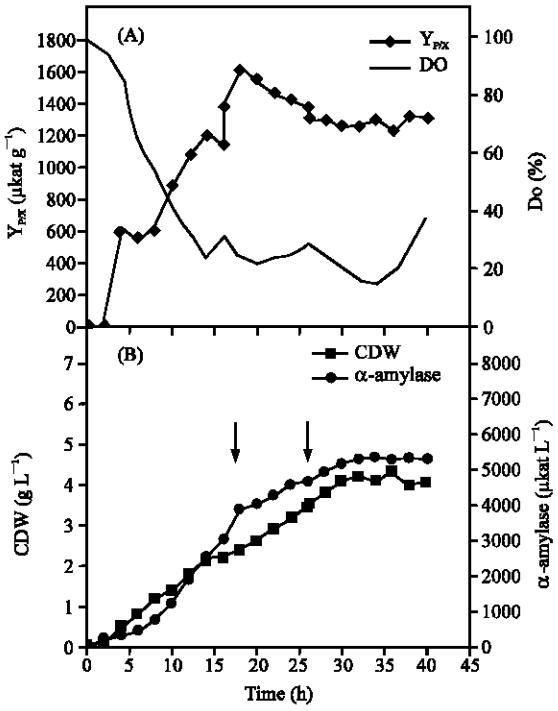

Fig. 5: Kinetics of cell growth and α-amylase production by *B. amyloliquefaciens* in stirred tank bioreactor in fed-batch culture. Arrows show the time of starch addition

enzyme production and α-amylase volumetric production reached its maximal of 5300 µkat L^{-1} after 34 h. This value is about 55% higher than those obtained in batch culture. It is also worthy to note that, no oxygen limitation was observed during fed-batch cultivation. The cell growth and enzyme production curves were parallel during growth phase. Thus, extending cell growth increased the enzyme production. Huang *et al.* (2004) showed also that a significant increase in α-amylase production by *Bacillus subtilis* was achieved using fed-batch cultivation strategy using glucose as substrate. The maximal enzyme production was 41.4 U mL^{-1} (about 690 µkat L^{-1}). This value is lower that those obtained in our study. This may due to they used glucose, as main substrate instead of starch in their study. However, glucose was found to be repressive substrate for amylase production by *B. subtilis* free cells (Duran-Paramo *et al.*, 2000). However, the α-amylase production was mediated by the presence of starch in cultivation medium (Tonkova, 1991). Therefore, the increase enzyme production in fed-batch culture was not only due to extended cell growth phase but also due to the better induction of enzyme by intermittent starch addition.

CONCLUSIONS

This study demonstrated the importance of aeration on the kinetics of cell growth and α-amylase production. The production in shake flask increased significantly by decreasing working volume, increasing agitation speed and the use of baffled flask. In bioreactor level, where agitation and aeration are better than shake flask, the enzyme production increased significantly. Thus, aeration is one of the main bottle neck(s) for the bioprocess improvement for industrial α-amylase production by *B. amyloliquefaciens*. Further improvement in production process was achieved by using fed-batch cultivation strategy with mono-feeding of starch. The enzyme production was further increased due

extended cell growth and better enzyme induction. Thus, intermittent addition of starch during exponential growth phase was found to be good cultivation strategy to increase α-amylase production.

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