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α-Amylase Production by *Bacillus amyloliquefaciens* Using Agro Wastes as Feed Stock

Dhanya Gangadharan, K. Madhavan Nampoothiri and Ashok Pandey*

Biotechnology Division, National Institute for Interdisciplinary Science and Technology (NIIST), CSIR, 695 019 Trivandrum, India

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Summary

The productivity of enzyme fermentations depends critically on maintaining a high oxygen transfer rate to satisfy the optimal oxygen demand of the microorganism for product formation. Among the several factors that affect oxygen transfer rates in a fermentor are the air flow rate and agitation. The production of α-amylase by *Bacillus amyloliquefaciens* was performed in 600-mL and 5-litre fermentor with a working volume of 300 mL and 3 L, respectively. The experiments indicated a requirement of high rates of aeration to enhance the enzyme yield. The biomass yield and productivity of the enzyme were found to have a linear relationship with the air flow rate, and the highest productivity was observed at 1.0 vvm. A maximum productivity of 41.4 U/(mL·h) was obtained after 14 h of fermentation in 600-mL fermentor system and a comparable productivity of 40 U/(mL·h) was obtained after 12 h in the 5-litre fermentor.

Key words: α-amylase, *Bacillus amyloliquefaciens*, fermentor, bioreactor, aeration, submerged fermentation

Introduction

Bacillus species are used as bacterial workhorses in industrial microbial cultivations for the production of a variety of enzymes as well as fine biochemicals and antibiotics. The capacity of various *Bacillus* strains to produce and secrete large quantities (20–25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. Indeed, they produce about 60 % of the commercially available enzymes (*1–3*). The production of enzymes on a large scale is mostly carried out by batch fermentation in stirred tank bioreactors. Expanding a fermentation process from a laboratory scale unit to a commercial one is a challenge due to the difficulty in assessing the factors affecting the scale-up process during the cultivation. As a result, many large-scale fermentation processes give a lower yield than is expected in the laboratory. Increased production of enzyme in a scale-up process is achieved by optimizing the relation between the microorganism and its environment, and the major factors found to play major roles include the type of fermentation, medium composition, dissolved oxygen tension, aeration, agitation, pH regulation and fermentation temperature (*4*). The results of the production of fermentation products aerobically in shake flasks usually cannot be extrapolated to indicate possible performance in a fermentor as both physical and biological factors at play are quite different in a fermentor and in a shake flask.

Among the major types of reactors for submerged fermentation, the continuous stirred tank reactor (CSTR), which provides a high k_La (volumetric mass transfer coefficient), is commonly used in many of the bioprocesses as it allows efficient contact of three phases, *i.e.* gas, liquid medium and solid cells. Gas under pressure is supplied to the sparger, the size of the gas bubbles and their dispersion throughout the tank are critical for reactor performance. Agitating the fermentation broth normally satisfies the oxygen demand of a fermentation process. Among other factors having an impact on the operating

*Corresponding author; Phone: ++91 471 251 5366; Fax: ++91 471 249 1712; E-mail: madhavan85@hotmail.com

conditions during fermentation in bioreactors are agitation and mixing. Agitation is important for adequate mixing, mass transfer and heat transfer. It is beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate and product/by-product (*5*). Multiple impellers on a single shaft with appropriate combination and spacing have been suggested as optimum. In such cases, mixing and mass transfer are dependent on the gas flow rate, type of agitator, its speed and the properties of liquids. Power consumption per impeller decreases with an increase in the number of impellers and this increases the uniformity of energy dissipation (*6*). The dissolved oxygen (DO) concentration becomes a limiting nutrient in the processes of high oxygen demand (*7*). The supply of oxygen can be the controlling step in industrial bioprocesses and in the scale-up of aerobic biosynthesis systems (*8–10*). The oxygen transfer rate could be affected by several factors, such as geometry and characteristics of the vessels, liquid properties (viscosity, superficial tension, *etc.*), the dissipated energy in the fluid, biocatalyst properties, concentration, and morphology of microorganisms, and it also depends on the air flow rate, the stirrer speed, mixing, *etc.* Mechanically agitated aerated vessels are widely used rather than vessels with aeration only, which can be inadequate to promote the liquid turbulence necessary for small air bubble generation. Although agitation can maintain the dissolved oxygen in the fermentor available, the inappropriate speed of agitation results in poor oxygen transfer, especially in highly viscous broths. The production of amylase by *Bacillus amyloliquefaciens* is generally believed to be an aerobic process. The aeration rate needed to maintain an adequate DO level is often of the order of one volume of air per volume of fermentor per minute (vvm) at the laboratory scale with a gas hold-up as high as 20 % (*11*).

The industrial demand for most of the enzymes is met by the production using submerged fermentation (SmF). The utilization of agroresidues for the production of enzymes has gained renewed interest as it solves solid waste disposal problem and also produces less wastewater (*12*). Industrial fermentation processes are usually conducted as a batch or fed-batch system. As cultivation systems change with respect to time, studies of the transient behaviour in batch cultures are helpful in understanding the dynamics of the system. The production studies on α-amylase by *B. amyloliquefaciens* ATCC 23842 through solid-state (SSF) and submerged (SmF) fermentations at flask level have proven an effective utilization of agroresidual resources (*13,14*). The fermentation process with complex agroresidue as a substrate requires more adequate levels of aeration and agitation than a synthetic medium to facilitate optimum and uniform mass transfer due to particulate and fibrous nature of the substrate. The present study intends to investigate the effect of aeration rate on α-amylase production by *Bacillus amyloliquefaciens* in 600-mL and 5-litre bioreactors.

Materials and Methods

Microorganism and enzyme production

Bacillus amyloliquefaciens ATCC 23842 was used in the present study. The strain was grown on nutrient agar (Hi-Media, Mumbai, India) slants at 37 °C for 24 h and subcultured every two weeks. The medium for enzyme production was composed of 12.5 % (by mass per volume) of wheat bran and groundnut oil cake (1:1) supplemented with $MgSO_4$ 0.05 M, NH_4NO_3 0.2 M, KH_2PO_4 0.05 M and $CaCl_2$ 0.0275 M. Inoculum of 2 % ($2 \cdot 10^9$ CFU/mL of 18-hour culture) was used and the fermentation was carried out for 48 h at 37 °C. Based on the extracellular yield of α-amylase, sugar utilization and biomass formation, the efficiency of fermentation was assessed.

α-Amylase production in a 600-mL fermentor

Parallel fermentor system (600 mL, Infors HT, Bottmingen, Switzerland) with a working volume of 300 mL and a working volume to space ratio of 1:2 was used to study the effect of aeration on α-amylase production by *B. amyloliquefaciens.* The fermentor system was equipped with dissolved oxygen (DO) probe, pH electrode and two Rushton-type impellers fitted with six blades. Four peristaltic pumps were plugged for acid, base, antifoam and feed mode. Agitation was controlled by a stirrer and a control base driven by magnetic stirrer. The aeration system was an air inlet through a ring sparger with air-flow meter and filter. The pH was maintained at 7.0 using 0.8 M HCl and 0.8 M NaOH and coconut oil was used as antifoam agent. The agitation was set at 300 rpm and DO was maintained at 100 %. The effect of aeration on α-amylase production was studied at 0.2 (bioreactor A), 0.5 (bioreactor B) and 1.0 vvm (bioreactor C). Fermentation was carried out for 48 h with sampling at regular intervals.

α-Amylase production in 5-litre stirred tank bioreactor

The 5-litre stirred tank bioreactor (Biostat® B-5, B. Braun Biotech-Sartorius, Melsungen, Germany) is a baffled cylindrical acrylic vessel with a working volume of 3 L and a working volume to space ratio of 1:1.66, having an internal diameter of 160 mm and height of 250 mm with dual impellers mounted on the shaft. The baffles with a width of 12 mm were placed perpendicular to the vessel. The system was equipped with six-bladed Rushton turbine impeller for agitation with a diameter of 64 mm, blade height of 13 mm and width of 19 mm. The spacing between the impellers was maintained at 110 mm and the lower impeller was located at a distance of 80 mm from the bottom of the vessel. The sparger was located at a distance of 5 mm from the bottom of the vessel, through which air was sparged to the tank. The ring sparger was 52 mm in diameter and had 16 symmetrically drilled holes of 1 mm in diameter. The flow rate of sparged air was fixed at 1.5 vvm. The fermentation was carried out at 37 °C and monitored by temperature probe which was controlled by circulating the chilled water. Foaming in the fermentation broth was monitored by a ceramic-coated antifoam probe and coconut oil was used as antifoaming agent. The DO was maintained at 100 % saturation level, which was continuously monitored by a sterilized polarographic electrode (Mettler-Toledo InPro® 6000 Series, Greifensee, Switzerland). The DO electrode was calibrated by a two-point calibration method between 0 and 100 % oxygen saturation. The impeller rotation speed was maintained at

400 rpm. Fermentation was carried out for 48 h and the samples were withdrawn at 6-hour intervals to check the enzyme production, biomass and sugar utilization level.

Analytical methods

The reducing sugar was estimated by the method of Miller (*15*). The Folin-Lowry method (*16*) was used for the estimation of total soluble protein. The method described by DuBois *et al.* (*17*) was used for the estimation of total carbohydrates. Bacterial biomass estimation was done by determining the *N*-acetylglucosamine released by the acid hydrolysis of peptidoglycan present in the cell wall of the bacteria (*18*). Glucosamine (Sigma-Aldrich, St. Louis, MO, USA) was used as the standard and the biomass was expressed in terms of milligram of *N*-acetylglucosamine released per gram of dry fermented substrate (mg/g). The amylase activity was determined by the method of Okolo *et al.* (*19*).

Results and Discussion

Production of α-amylase in 600-mL fermentor

The growth rate at different levels of aeration was analysed by determining the biomass produced at 0.2, 0.5 and 1.0 vvm. Bioreactor A with an aeration rate of 0.2 vvm produced a biomass of (9.0±0.1) mg/mL and a drastic increase to (28.3±0.8) mg/mL was observed at 1.0 vvm, while 0.5 vvm resulted in (14.3±0.52) mg/mL biomass (Fig. 1). The highest biomass was obtained when the aeration rate was maintained at 1.0 vvm. The analysis of growth pattern in all three bioreactors showed that the log phase was initiated at 14 h in bioreactor C with the highest aeration, while log phase was initiated at 16 h in bioreactor B and was delayed up to 20 h in bioreactor A with the lowest rate of aeration (Fig. 1). The biomass reached maximum in the post-logarithmic phase and was observed at 42 h, beyond which stationary phase was obtained. The enzyme yield showed a linear increase with increased levels of aeration and biomass, thus proving a growth-dependent pattern of α-amylase production. High enzyme titers ((997±3.4) U/mL) were obtained at 42 h of fermentation in bioreactor C, which were comparable with the enzyme yield of 1060 U/mL obtained in flask level experiments. Bioreactors A ((310±2.3) U/mL) and B ((611±1.7) U/mL) with lower levels of aeration resulted in comparatively lower titres of enzyme, indicating the profound effect of aeration on biomass and thus enzyme production (Fig. 1). Milner *et al.* (*11*) have reported α-amylase production by *B. amyloliquefaciens* as highly aerobic and requiring high aeration rate for the production. The investigation of optimal culture conditions for maximum production of α-amylase by *B. macerans* observed that aeration increased the enzyme yields with simultaneous decrease in the required fermentation time (*20*). Model-based method was used for the estimation of $k_L a$ (volumetric oxygen transfer coefficient) in the cultivation of *B. amyloliquefaciens* in batch reactors for the production of alkaline proteases. It has been understood that oxygen transfer rate is one of the crucial parameters which determines the cell growth and alkaline protease production by *B. amyloliquefaciens* (*21*).

Microorganisms can grow under a variety of physical, chemical and nutritional conditions. In a suitable nutrient medium, organisms extract nutrients from the medium and convert them into biological compounds (*22*). The rate of substrate utilization and protein yield showed that they were directly related to the growth of biomass. Minimum residual sugar (0.9 mg/mL) observed in bioreactor C (Fig. 2) and higher residual sugars observed at the end of fermentation in bioreactors A ((3.6±0.21) mg/mL) and B ((2.0±0.11) mg/mL) indicated comparatively lower utilization of sugars by the microorganisms at lower rates of aeration due to lower growth. The protein levels were proportional to the level of substrate utilized in all three bioreactors even though maximum protein yield (16 mg/mL) was obtained in bioreactor C (Fig. 2) with the highest rate of aeration. Lower levels of protein in bioreactors A ((7±0.12) mg/mL) and B ((10.4±0.24) mg/mL) could be correlated with the lower substrate utilization and biomass formation at lower levels of aeration (Fig. 2).

Oxygen transfer can often be very crucial during fermentation due to its low solubility in the medium (*23*), and hence the productivity and biomass yield (biomass produced/total sugars consumed) were compared at various levels of aeration. It was evident from the results that the biomass yield was the highest in bioreactor C

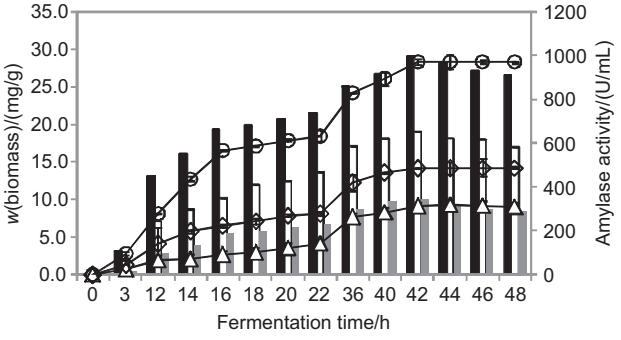

Fig. 1. α-Amylase and biomass production by *B. amyloliquefaciens* at various levels of aeration rate in the parallel fermentor system. Biomass (■) and α-amylase (○) levels at 1.0 vvm in bioreactor C; biomass (□) and α-amylase (◇) levels at 0.5 vvm in bioreactor B; biomass (■) and α-amylase (△) levels at 0.2 vvm in bioreactor A

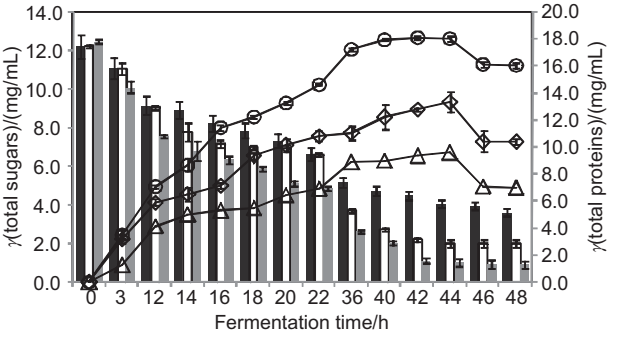

Fig. 2. Substrate utilization plot of *B. amyloliquefaciens* at various aeration rates in parallel fermentor system. Substrate utilization (■) and total protein (○) levels at 1.0 vvm in bioreactor C; substrate utilization (□) and total protein (◇) levels at 0.5 vvm in bioreactor B; substrate utilization (■) and total protein (△) levels at 0.2 vvm in bioreactor A

with the highest aeration (Fig. 3). The yield reached maximum at 16 h and remained constant throughout the stationary phase. Biomass yield at 0.2 vvm aeration was 0.6 mg/g at the initial growth phase and reached 1.1 mg/g in the stationary phase. At 0.5 vvm, it increased to 1.03 mg/g in the initial growth phase and reached a maximum of 1.38 mg/g. Maximum yield (2.4 mg/g) was obtained at 1.0 vvm aeration. The productivity of α-amylase was maximum (41.4 U/(mL·h)) at the beginning of the log phase with maximum aeration during the fermentation period of 14 h. High rate of production was observed throughout the log phase in all the bioreactors and gradually decreased towards the end of the log and stationary phases. Maximum obtained productivity was 11.75 in bioreactor A and 22.7 U/(mL·h) in bioreactor B.

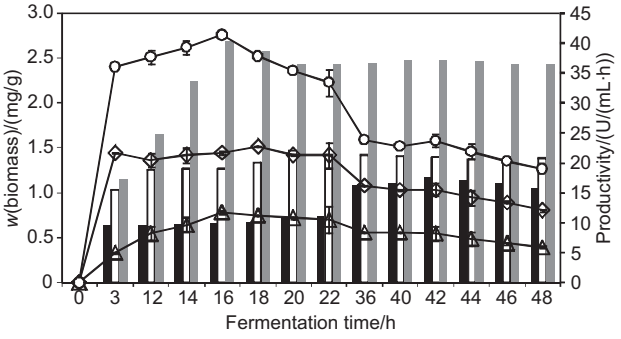

Fig. 3. Comparison of productivity with biomass yield at different rates of aeration in parallel fermentor system. Productivity (■) and biomass yield (○) levels at 1.0 vvm in bioreactor C; productivity (□) and biomass yield (◇) levels at 0.5 vvm in bioreactor B; productivity (▒) and biomass yield (△) levels at 0.2 vvm in bioreactor A

Production of α-amylase in 5-litre fermentor

The α-amylase production in 5-litre Biostat B-5 fermentor was studied at high rate of aeration of 1.5 vvm and impeller speed of 400 rpm. With the large volume of air, intense agitation and rich medium, foaming becomes a problem, thus the agitation level was maintained at 400 rpm. The analysis of enzyme and biomass production at equal time intervals showed maximum enzyme ((1112±3.4) U/mL) and biomass production (32 mg/mL) after 42 h of fermentation. In an earlier report (*24*), the production of α-amylase was studied in a 7.5--litre fermentor with a working volume of 3 L by amplified variants of *B. subtilis* and *B. amyloliquefaciens*. The study demonstrated maximum production of the product and growth rate at high aeration rate (3 vvm) and agitation speed (300 rpm). Syu and Chen (*25*) described the production of α-amylase by *B. amyloliquefaciens* in a 1.7-litre fermentor at high rates of aeration. The residual sugar obtained at the end of the fermentation was (0.8± 0.01) mg/mL (data not shown), which denoted efficient utilization of substrate for growth and α-amylase production.

In the scale-up of aerobic processes, it is often observed that the biomass yield on the carbon/energy source is decreased. In an *E. coli*-based recombinant protein process, the biomass yield on glucose and the maximum cell density dropped by about 20 % when scaled up from 3 L to 9 m^3 (*26*). In the present study, the productivity of α-amylase (40 U/(mL·h) and the biomass yield (2.5 mg/g) were the highest at 12 h of fermentation (Fig. 4). The maximum biomass yield and productivity values in the 600-mL fermentor at an aeration rate of 1 vvm were comparable but obtained only after 14 h of fermentation. The biomass yield remained constant throughout the post-logarithmic and stationary phases indicating efficient substrate utilization by the microorganism until it reached the stationary phase. Hessleink (*27*) reported that the catalytic activities of an organism could be fully utilized if the oxygen levels in the immediate vicinity of the cells were maintained through aeration and agitation. Higher levels of agitation could also cause disruption of free cells in the reactor by shear forces and the formation of vortex could result in poor mass transfer and thus reduced enzyme titres (*28,29*).

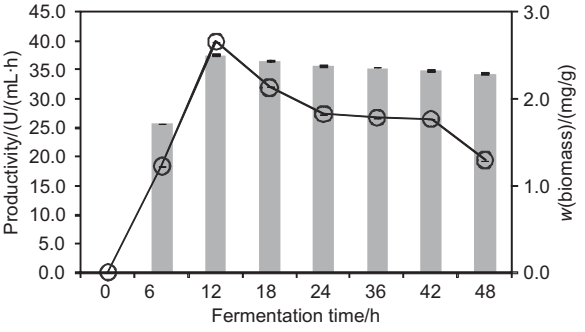

Fig. 4. α-Amylase productivity (▒) and biomass yield (○) by *B. amyloliquefaciens* at 1.0 vvm in a 5-litre fermentor

Conclusion

The physical and biological factors in a fermentation process have an important role in enhancing the enzyme yields. The present study demonstrates the importance of aeration and agitation on biomass yield and enzyme production in a scale-up process. The enzyme production profile clearly demonstrates a growth-related pattern. This study also encourages the effective utilization of agroresidual substrates for large scale industrial processes.

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