



INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES (Int. J. of Pharm. Life Sci.)

α -Amylase Production by *Bacillus amyloliquefaciens* using Agro wastes

Pushpendra Singh^{1*}, Ravindra Singh¹, Paras Gupta² and Arvind Kumar Shrivastava²

1, Department of Biological sciences, M.G.C.G.V., Chitrakoot, Satna, (MP) - India

2, United Institute of Pharmacy, Naini, Allahabad, (UP) - India

Abstract

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 42.5 U/(mL·h) was obtained after 14 h of fermentation in 600-mL fermentor system and a comparable productivity of 41.2 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 (20–25g/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 kLa (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 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

* Corresponding Author

E.mail: pushpendra_singhbiotech@yahoo.co.in

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.

Material and Methods

Microorganism and production of enzyme

Bacillus amyloliquefaciens 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 MgSO₄ 0.05 M, NH₄NO₃ 0.2 M, KH₂PO₄ 0.05 M and CaCl₂ 0.0275 M. Inoculum of 2 % (2·10⁹ 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. *a-Amylase production in a 600-mL fermentor* Parallel fermentor system 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.

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. 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 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. 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 kLa (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 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 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. 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 with the highest aeration. 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 (42.5 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.

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 m³ (26). In the present study, the productivity of α -amylase (41.2 U/(mL·h) and the biomass yield (2.5 mg/g) were the highest at 12 h of fermentation. 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).

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.

Acknowledement

The authors are very grateful to Department of Biological sciences M.G.C.G.V. Chitrakoot (M.P.).

References

1. C.R. Harwood, *Bacillus subtilis* and its relatives: Molecular biological and industrial workhorses, *Trends Biotechnol.* 10 (1992) 247–256.
2. E. Ferrari, A.S. Jarnagin, B.F. Schmidt: Commercial Production of Extracellular Enzymes. In: *Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics*, A.L. Sonenheim, J.A. Hoch, R. Losick (Eds.), American Society for Microbiology Press, Washington DC, USA (1993) pp. 917–937.
3. M. Schallmeyer, A. Singh, O.P. Ward, Developments in the use of *Bacillus* species for industrial production, *Can. J. Microbiol.* 50 (2004) 1–17.
4. M. Thiry, D. Cingolani, Optimizing scale-up fermentation processes, *Trends Biotechnol.* 20 (2002) 103–105.
5. J. Kongkiattikajorn, A. Rodmui, Y. Dandusitapun, Effect of agitation rate on batch fermentation of mixture culture of yeasts during ethanol production from mixed glucose and xylose, *Thai J. Biotechnol.* 5 (2007) 1–4.
6. A.W. Nienow, M.D. Lilly, Power drawn by multiple impellers in sparged agitated vessel, *Biotechnol. Bioeng.* 21 (1979) 2341–2345.
7. Y.M. Lo, C.H. Hsu, S.T. Yang, D.B. Min, Oxygen transfer characteristics of a centrifugal, packed-bed reactor during viscous xanthan fermentation, *Bioprocess. Biosyst. Eng.* 24 (2001) 187–193.
8. W.A. Al-Masry, Effect of scale-up on average shear rates for aerated non-Newtonian liquids in external loop airlift reactors, *Biotechnol. Bioeng.* 62 (1999) 494–498.
9. M. Elibol, D. Ozer, Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*, *Process Biochem.* 36 (2000) 325–329.
10. D. Weuster-Botz, E. Hünekes, A. Hartbrich, Scale-up and application of a cyclone reactor for fermentation processes, *Bioprocess Eng.* 18 (1998) 433–438.
11. J.A. Milner, D.J. Martin, A. Smith, Oxygen transfer conditions in the production of alpha-amylase by *Bacillus amyloliquefaciens*, *Enzyme Microb. Technol.* 18 (1996) 507–512.
12. A. Pandey, Solid-state fermentation, *Biochem. Eng. J.* 13 (2003) 81–84.
13. D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, A. Pandey, Solid culturing of *Bacillus amyloliquefaciens* for alpha amylase production, *Food Technol. Biotechnol.* 44 (2006) 269–274.
14. D. Gangadharan, S. Sivaramakrishnan, K.M. Nampoothiri, R.K. Sukumaran, A. Pandey, Response surface methodology for the optimization of alpha amylase production by *Bacillus amyloliquefaciens*, *Bioresour. Technol.* 99 (2008) 4597–4602.
15. G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* 31 (1959) 426–429.
16. O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
17. M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350–356.
18. Y. Sakurai, T.H. Lee, H. Shiota, On the convenient method of the glucosamine estimation in koji, *Agr. Biol. Chem.* 41 (1977) 619–624.
19. B.N. Okolo, L.I. Ezeogu, C.N. Mba, Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources, *J. Sci. Food Agric.* 69 (1995) 109–115.

20. H.S. Daniels, G.L. Stahly, The effect of aeration on amylase production by *Bacillus macerans*, *J. Bacteriol.* 52 (1946) 351–352.
21. R. Sachidanandham, I.A. Ranjit Kumar, M.R.V. Krishnan, K. Jayaraman, Mathematical model based estimation of volumetric oxygen transfer coefficient(s) in the production of proteolytic enzymes in *Bacillus amyloliquefaciens*, *Bioprocess Eng.* 21 (1999) 319–322.
22. S.K. Shin, K.K. Kim, Effect of elevated pressure on α -amylase and protease synthesis by *Bacillus subtilis*, *J. Ind. Eng. Chem.* 20 (1996) 91–96.
23. E.S. Bylinkina, V.V. Birukov, The problem of scaleup in antibiotic biosynthesis, *Proceedings of the 4th International Fermentation Symposium, Fermentation Technology Today*, G. Terui (Ed.), The Society of Fermentation Technology, Osaka, Japan (1972) pp. 105–115.
24. O. El-Tayeb, F. Mohammad, A. Hashem, M. Aboulwafa, Optimization of the industrial production of bacterial alpha amylase in Egypt. IV. Fermentor production and characterization of the enzyme of two strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*, *Afr. J. Biotechnol.* 7 (2007) 4521–4536.
25. M.Y. Syu, Y. Chen, A study on the α -amylase fermentation performed by *Bacillus amyloliquefaciens*, *Chem. Eng. J.* 65 (1997)237–247.
26. F. Bylund, E. Collet, S.O. Enfors, G. Larsson, Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation, *Bioprocess Eng.* 18 (1998)171–180.
27. P.G.M. Hessleink: *Oxygen and Gas-Liquid Mass Transfer in Operational Modes of Bioreactors*, Butterworths/Heinemann,Oxford, UK (1992) pp. 9–39.
28. V.B. Shukla, U. Parasu Veera, P.R. Kulkarni, A.B. Pandit,Scale-up of biotransformation process in stirred tank reactor using dual impeller bioreactor, *Biochem. Eng. J.* 8 (2001)19–29.
29. R. Potumarthi, C. Subhakar, A. Jetty, Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes, *Biochem. Eng. J.* 34 (2007) 185–192.
30. D.Gangadharn,alfa amylase production by *B. amyloliquefacience* food technol. *Biotechnol* 49(3)336-340(2011).

How to cite this article

Singh P., Singh R., Gupta P. and Shrivastava A.K. (2014). α -amylase production by *Bacillus amyloliquefaciens* using agro wastes. *Int. J. Pharm. Life Sci.*, 5(1):3265-3269.

Source of Support: Nil; Conflict of Interest: None declared

Received: 11.11.13; Revised: 19.12.13; Accepted:21.12.13