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PRODUCTION OF ALPHA AMYLASE FROM A RANDOMLY INDUCED MUTANT STRAIN OF *BACILLUS AMYLOLIQUEFACIENS* AND ITS APPLICATION AS A DESIZER IN TEXTILE INDUSTRY

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Abstract

The present study is concerned with the improvement of *Bacillus amyloliquefaciens* strain UNG-16 for alpha amylase production. The bacterial culture was exposed to UV irradiation at 1.6×10^2 J/m^2/S for 15-60 min. However, UV induced viables did not give improved alpha amylase production; therefore chemical mutation using ethyl methane sulphonate (EMS 50-300 µl/ml) was undertaken for 10-60 min. The mutant *B. amyloliquefaciens* EMS-6 gave 102.78±2.22 U/ml/min enzyme activity which was 1.4 fold higher than the parental strain. In stirred fermentor, the incubation period was reduced from 72 to 48 h after inoculation. The production of alpha amylase was found to be maximal when the 60% volume, 2.0 vvm air supply and 400 rpm agitation rate was maintained during the fermentation period. The incubation temperature (37ºC) and size of inoculum (8.0 %) were also optimized. A 100% desizing of grey fabric (or starch removal) was obtained with 200-250 enzyme units at pH 6.5 at 60ºC in 1 h.

Introduction

The production of microbial alpha amylase by bacteria is dependent on the type of strain, composition of medium, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermostability (Prescott & Dunn's, 1987). The enzyme is extensively used in many industries including starch liquefaction, brewing, food, paper, textile and pharmaceuticals (Arican, 2008). These uses have placed stress on increasing alpha amylase production and search for an efficient fermentation process. Highly active alpha amylase is required for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent strain of microorganism for alpha amylase production. *Bacillus* species such as *Bacillus subtilis* and *B. amyloliquefaciens* are the organisms of choice for enzyme production (Qirang & Zhao, 1994; Haq *et al*., 2005). The mutant strains of *Bacillus* showed better ability to produce alpha amylase, which can be derived by random mutagenesis. Different chemical agents such as nitrous acids or ethyl methane sulphonate (EMS) can be used for the mutation of bacteria (Bin *et al*., 1999). Extensive screening is required for the isolation of a mutant with increased alpha amylase production.

Higher titers of the enzyme alpha amylase production by *Bacillus amyloliquefaciens* were obtained by submerged fermentation using stirred fermentor. In addition, both complex and synthetic culture media were examined to improve enzyme production rate. In a complex medium, which supports faster growth rates, enzyme production occurred only when the growth rate declined, principally in the stationary phase (Nigam & Singh, 1995). By contrast, in a synthetic culture medium with lactose as the carbon source supported much lower growth rates and the enzyme formation occurred simultaneously with cell growth. The repression of enzyme formation during rapid growth may be due to catabolite repression (Ghasemi *et al*., 2007). High aeration rates were found to be

essential for better yield of enzyme, however foaming problems resulted (Philip *et al*., 1988). On the basis of inexpensive raw materials and glucose as a main carbon source, the optimal parameters for alpha amylase production were agitation rate, constant air flow and cultivation (Sivaramakrishnan *et al*., 2006).

The present study describes enhanced alpha amylase production by the bacterium *Bacillus amyloliquefaciens* UNG-16 was improved through mutation by both radiations (UV) and chemicals (EMS). The screening and selection of a hyper-producer strain of alpha amylase was undertaken by both dye method and shake flask technique. The optimization of the cultural conditions and nutritional requirements for enzyme production were carried out in a stirred fermentor of 7.5 L total capacity. The crude enzyme was then evaluated for desizing operation the grey fabric in textile industry.

Materials and Methods

Organism and culture maintenance: The bacterial culture *Bacillus amyloliquefaciens* UNG-16 was obtained from the available culture-bank of IIB. It was maintained on nutrient agar medium containing (g/l); nutrient broth 8.0, agar 20.0, pH 7.0. The slants were incubated at 37°C for maximum growth and stored at 4°C in a cold cabinet (Model: MPR-1410, SANYO, Japan).

Inoculum preparation: Vegetative inoculum was used in the present studies. Fifty millilitre of inoculum medium containing nutrient broth 8.0 g/l, pH 7.0 was transferred to a 250 ml conical flask and cotton plugged. It was sterilized in an autoclave at 15 lbs/in^2 pressure (121°C) for 15 min. After cooling to room temperature, a loopful of bacteria was aseptically transferred to it. The flask was rotated at 200 rpm (37°C) in a rotary shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) for 24 h.

Shake flask fermentation: The optimization of alpha amylase fermentation was carried out using submerged technique in 250 ml Erlenmeyer flasks. Fifty mililitre of the fermentation medium (M4 optimized) was transferred to the individual flasks and cotton plugged. The flasks were sterilized in an autoclave at 121°C for 15 min and cooled to room temperature. Each flask was inoculated with 2.0 ml of the bacterial inoculum. The flasks were placed in the rotary shaking incubator (200 rpm) at 37°C for 48-96 h. After the incubation, the fermented broth was centrifuged in a centrifuge machine (Model: D-37520, Osterodeam-Harz, Germany) at 5,631×g for 15 min.

Fermentor studies: A glass stirred fermentor of 7.5 L capacity with working volume of 4.5 L was employed for alpha amylase fermentation. All the culture media were sterilized in autoclave and cooled at room temperature. The vegetative inoculum was transferred to the production medium at a rate of 8% (v/v) based on total working volume of the fermentation medium. The incubation temperature was maintained at 37°C through out the fermentation period. Agitation speed of the stirred was kept at 200 rpm and aeration rate was maintained at 1.5 l/l/min. Silicone oil was used to control the foaming during fermentation.

Fermentation media: Following fermentation media (g/l) were used for alpha amylase fermentation (Saito & Yamamoto, 1974; Kelly *et al*., 1997).

M1: Lactose 10.0, peptone 5.0, yeast extract 2.5, KCl 1.0, $MgCl_2$ 0.2, $CaCl_2$ 0.25, $FeSO_4$ 0.001, $MnSO_4$ 0.0005, pH 7.0.

M2: Lactose 7.5, peptone 5.0, yeast extract 3.5, KCl 1.0, $CaCl_2$ 0.25, $MgCl_2$ 0.2, $MnSO_4$ 0.001, $FeSO_4$ 0.0005, KH_2PO_4 1.0, K_2HPO_4 2.0, pH 7.0.

M3: Starch 10.0, K_2HPO_4 9.0, KH_2PO_4 2.0, $(NH_4)_2SO_4$ 5.0, sodium citrate 1.0, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.01, $FeSO_4.7H_2O$ 0.045, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$ 0.0005, pH 7.0.

M4: Soluble starch 10.0, nutrient broth 8.0, $(NH_4)_2SO_4$ 2.25, $(NH_4)_2HPO_4$ 1.0, NaCl 0.85, $MgSO_4.7H_2O$ 0.25, $CaCl_2$ 0.2 prepared in 0.02 M phosphate buffer, pH 7.0.

Culture improvement: The bacterial culture (24 h old) was prepared in nutrient broth medium and centrifuged aseptically at 8,782×g for 15 min. The bacterial cells were resuspended in 50 ml of saline water and diluted up to 10^6 times.

Ultraviolet (UV) irradiation: Ten millilitre of the diluted suspension was transferred in a sterilized Petri plate (180°C for 2 h). The Petri plate was placed under a UV lamp (emitting the energy of 1.6×10^2 J/m^2/S) for 15-60 min. After pre-determined time intervals, 0.5 ml of the bacterial suspension was transferred to the petriplates containing nutrient starch agar medium. The plates were placed in a cooled incubator (Model: MIR-153, Sanyo, Japan) at 37°C for 24-48 h.(Buchanan & Gibbons, 1974).

Ethyl methane sulphonate (EMS) treatment: Five millilitre of Ethyl methane sulphonate (EMS 50-300 µl/ml) was transferred to individual sterilized centrifuged tubes containing 5.0 ml of bacterial suspension. The tubes were placed to room temperature for different time intervals (10-60 min). After the time interval, the tubes were centrifuged at 8,782×g rpm for 15 min. The supernatant was discarded to remove the EMS from the bacterial cells. Ten millilitre of saline water was added to each of the tubes. The tubes were re-centrifuged for the removal of traces of EMS from bacterial cells. This process was repeated three times. After washing the cells, 10 ml of sterilized saline water was added to each tube to prepare bacterial suspension. Approximately 0.5 ml of the suspension was transferred to the Petri plates containing nutrient starch agar medium and incubated at 37°C for 24 h.

Enzyme assay: Alpha amylase was estimated according to the method of Rick & Stegbauer (1974). One millilitre of enzyme extract was added to a test tube containing 1.0 ml of 1.0 % soluble starch solution, pH 7.0. The mixture was incubated at 60ºC for 10 min. After the incubation, 1.0 ml of DNS reagent was added to each of the tubes. The tubes were placed in boiling water for 5 min and cooled to room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The optical density (OD) of reaction mixture was determined at 546 nm using a spectrophotometer.

One unit of activity is equivalent to that amount of enzyme, which in 10 min librates reducing group from 1.0 % Lintner's soluble starch corresponding to 1.0 mg maltose hydrate.

Statistical analysis: Treatment effects were compared by the method of Snedecor & Cochran (1980). Post-hoc multiple comparison tests were applied under one-way analysis of variance (ANOVA). Significance has been presented in the form of probability (<p>) values.

Desizing with alpha amylase: A stiffed piece of grey fabric having maximum starch was used in present study. Equal size (5×5inch) of fabric piece was weighed on electric balance. The cloth strip was then dipped in 100 ml of enzymatic solution (pH 6.5) and then placed in incubator at 60^0C for 1.0 h. The cloth strip was washed with tap water and then oven dried. After drying the cloth strip was weighed again. The % removal of starch be calculated by applying the following formula:

$$\text{Desizing (\%)} = \frac{\text{Wt. of starch removed by enzyme}}{\text{Total starch present on the fabric strip}}$$

Total starch was calculated by hydrolyzing the starch with 0.1 N H_2SO_4.

Results

Strain improvement after randomly induced mutagenesis with UV and EMS: The bacterial cells (*Bacillus amyloliquefaciens* UNG-16) were exposed to UV irradiation for 15-60 min at 1.6×102 J/m2/S. The mutants were picked up from the nutrient broth agar plates having at least 90 % death rate. A total of 18 colonies were selected on the basis of clear zones due to starch hydrolysis. UV irradiation did not end up in a high yielding mutant strain of *B. amyloliquefaciens*; rather alpha amylase production was decreased when compared to the parental strain. Only one mutant i.e., UV-14 gave 76.05±2.90 U/ml/min enzyme activity, a little higher than the parent (73.26±1.44 U/ml/min) was insignificant. As UV mutagenesis gave mutants with decreased level of alpha amylase activity, therefore chemical mutation using ethyl methane sulphonate (EMS) was undertaken.

The mutant strains of *B. amyloliquefaciens* UNG-16 developed after exposure to ethyl methane sulphate (EMS) were screened for alpha amylase activity in shake flasks. The dose of EMS ranged from 50-300 µg/ml for 10-60 min. The mutants were picked up from the nutrient agar plates having at least 90 % death rate. A total of 31 colonies were selected on the basis of larger and clear zones of starch hydrolysis. Sixteen mutant strains gave less enzyme activity compared to the parental strain, however 15 others gave relatively better activity. Among these, 3 mutant strains gave amylolytic activity in the range of 80-90 U/ml/min, 6 gave from 90-100 U/ml/min while only 2 mutants produced enzyme over 100 U/ml/min. The mutant strain of *B. amyloliquefaciens* EMS-6 gave 102.78±2.22 U/ml/min alpha amylase activity which was around 1.4 fold higher than the parental strain.

Fermentor studies for alpha amylase production: The selection of fermentation medium for alpha amylase production by the mutant strain of *Bacillus amyloliquefaciens* EMS-6 in the stirred fermentor was investigated. For each media, the fermentation was carried out from 24-72 h after the inoculation and results have been depicted in Fig. 1. Among the four different media (M1, M2, M3, M4) investigated, M4 gave maximum alpha amylase activity (62.5 U/ml/min), 48 h after incubation. The enzyme activity declined after the optimal incubation period, which was reduced to 52.2 U/ml/min at an incubation period of 72 h. All other fermentation media gave relatively lower enzyme activity.

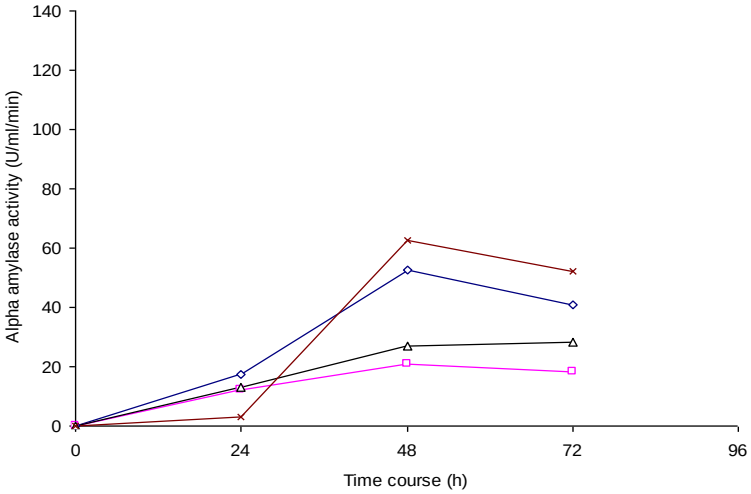

Fig. 1. Evaluation of different fermentation media (-◊- M1, -□- M2, -Δ- M3, -×- M4) for alpha amylase production by the mutant strain of *B. amyloliquefaciens* EMS-6 in stirred fermentor. Incubation temperature 37ºC, pH 7.0, agitation rate 200 rpm, aeration 1.5 vvm.

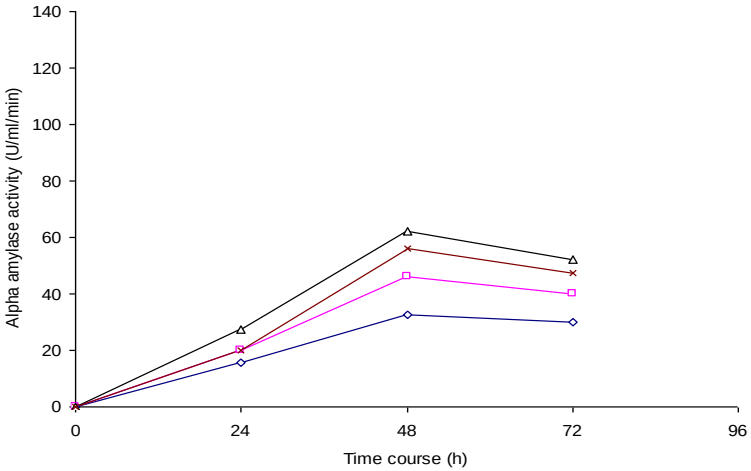

Fig. 2. Effect of different initial pH (-◊- 6, -□- 6.5, -Δ- 7, -×- 7.5) on alpha amylase production by the mutant strain of *B. amyloliquefaciens* EMS-6 in stirred fermentor. Incubation temperature 37ºC, agitation rate 200 rpm, aeration 1.5 vvm.

Effect of different initial pH (6.0, 6.5, 7.0, 7.5) on alpha amylase production by the mutant strain of *Bacillus amyloliquefaciens* EMS-6 was investigated in stirred fermentor. The results are highlighted in Fig. 2. The fermentations were carried out from 24-72 h after the inoculation. The enzyme activity was found less (15.5 U/ml/min) at pH 6.0 and 24 h after incubation which increased at 48 h of fermentation when the pH was further increased. The maximum alpha amylase activity (62.3 U/ml/min) was however obtained at pH 7.0 and 48 h after the inoculation. An enzyme activity of 47.3 U/ml/min was recorded at pH 7.5 when fermentation was run for 72 h. So, a pH value of 7.0 was optimized for alpha amylase production and subsequent parameters.

The effect of different incubation temperature (35, 37, 40, 43ºC) on alpha amylase production by the mutant strain of *Bacillus amyloliquefaciens* EMS-6 in stirred fermentor is shown in Fig. 3. The fermentations were carried out from 24-72 h after the inoculation. At 35ºC, the enzyme activity was not found encouraging at all the rates examined. A better activity was however noticed when the incubation temperature of the medium was adjusted at 37ºC. At this optimal temperature, 52.5 U/ml/min alpha amylase production was obtained 24 h after the incubation. The enzyme activity was increased as the fermentation period was further increased. The maximum alpha amylase production (60.9 U/ml/min) was at 37ºC in 48 h. When the temperature was increased up to 43ºC, the enzyme activity markedly declined.

The effect of size of inoculum (6, 8, 10%, v/v) on alpha amylase production by the mutant strain of *Bacillus amyloliquefaciens* EMS-6 was studied in stirred fermentor. All the fermentations were carried out from 24-72 h after the inoculation. The results are shown in Fig. 4. The enzyme activity was found less (22.4 U/ml/min) at 2 % inoculum and 24 h after incubation and it was increased when the size of inoculum was further increased. The maximum alpha amylase activity (82.1 U/ml/min) was however obtained at an inoculum of 8% and 48 h after the inoculation. At other inoculum level such as 10%, the enzyme activity markedly declined at all the rates examined. The minimum enzyme activity (45.9 U/ml/min) was recorded at 10% inoculum when fermentation was run for 72 h.

The fermentation of enzyme was carried out at 200, 300, 400 and 500 rpm for 24-72 h., (Fig. 5a). The production of enzyme following growth of the organism was found to be the maximal (96.5 U/ml/min) as the agitation intensity of the fermentor was maintained at 400 rpm. Further increase in the agitation intensity resulted in the decreased production of biomass as well as alpha amylase by the bacterium. At 200-300 rpm, both the production of biomass and enzyme were extremely low. The enzyme activity remained very low (51.2 U/ml/min) when air supply was maintained at 1.5 vvm, 24 h after incubation. The production of enzyme following growth of the organism was found optimal (108.42 U/ml/min) when the air supply to the fermentation medium was maintained at 2 vvm (Fig. 5b). Further increase in the air decreased the production of alpha amylase by the bacterial culture. At 2.5 vvm of air supply, the enzyme production declined to 79 U/ml/min, 72 h after the inoculation.

Desizing operation with alpha amylase: The effect of enzyme concentration on the desizing of grey fabric by alpha amylase showed that the concentration of the enzyme varied from 25-350 U/ml/min., the desizing (removal of starch) of the cloth was increased with increase in the concentration of enzyme and found optimum with 200 to 225 units(Fig. 6). Further increase in the concentration of enzyme did not reveal any significant effect on the desizing of grey fabric.

Discussion

In the present study, *Bacillus amyloliquefaciens* strain UNG-16 was improved for alpha amylase production. UV irradiation did not end up in a high yielding mutant strain of *B. amyloliquefaciens*; rather alpha amylase production was decreased. So, chemical mutation of the bacterial strain using ethyl methane sulphonate (EMS) was undertaken. The mutant *B. amyloliquefaciens* EMS-6 gave 102.78±2.22 U/ml/min alpha amylase which was around 1.4 fold higher than the parental strain. Many workers have employed random mutagenesis for alpha amylase production by exposing the cultures with UV or chemicals like EMS and nitrous acid (Markkanen & Suihko, 1974; Haq *et al*., 2007).

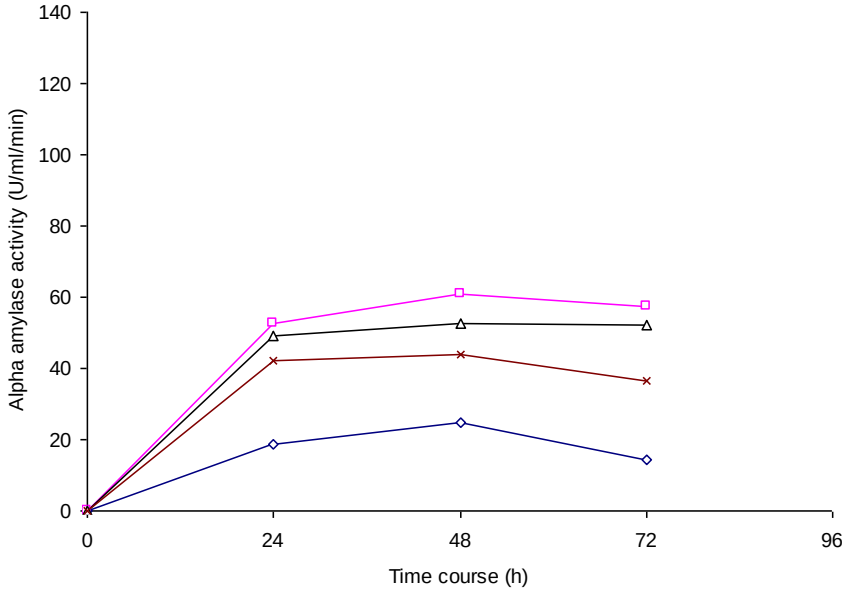

Fig. 3. Effect of different incubation temperature (-◊- 35ºC, -□- 37ºC, -Δ- 40ºC, -×- 43ºC) on alpha amylase production by the mutant strain of *B. amyloliquefaciens* EMS-6 in stirred fermentor. Initial pH 7.0, agitation rate 200 rpm, aeration 1.5 vvm.

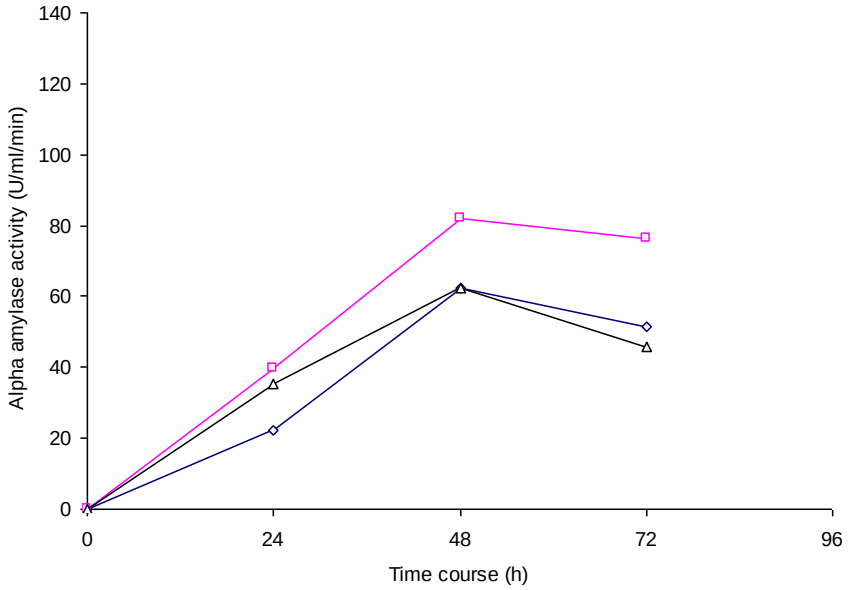

Fig. 4. Effect of inoculum size (-◊- 6%, -□- 8%, -Δ- 10%) on alpha amylase production by the mutant strain of *B. amyloliquefaciens* EMS-6 in stirred fermentor. Incubation temperature 37ºC, initial pH 7.0, agitation rate 200 rpm, aeration 1.5 vvm.

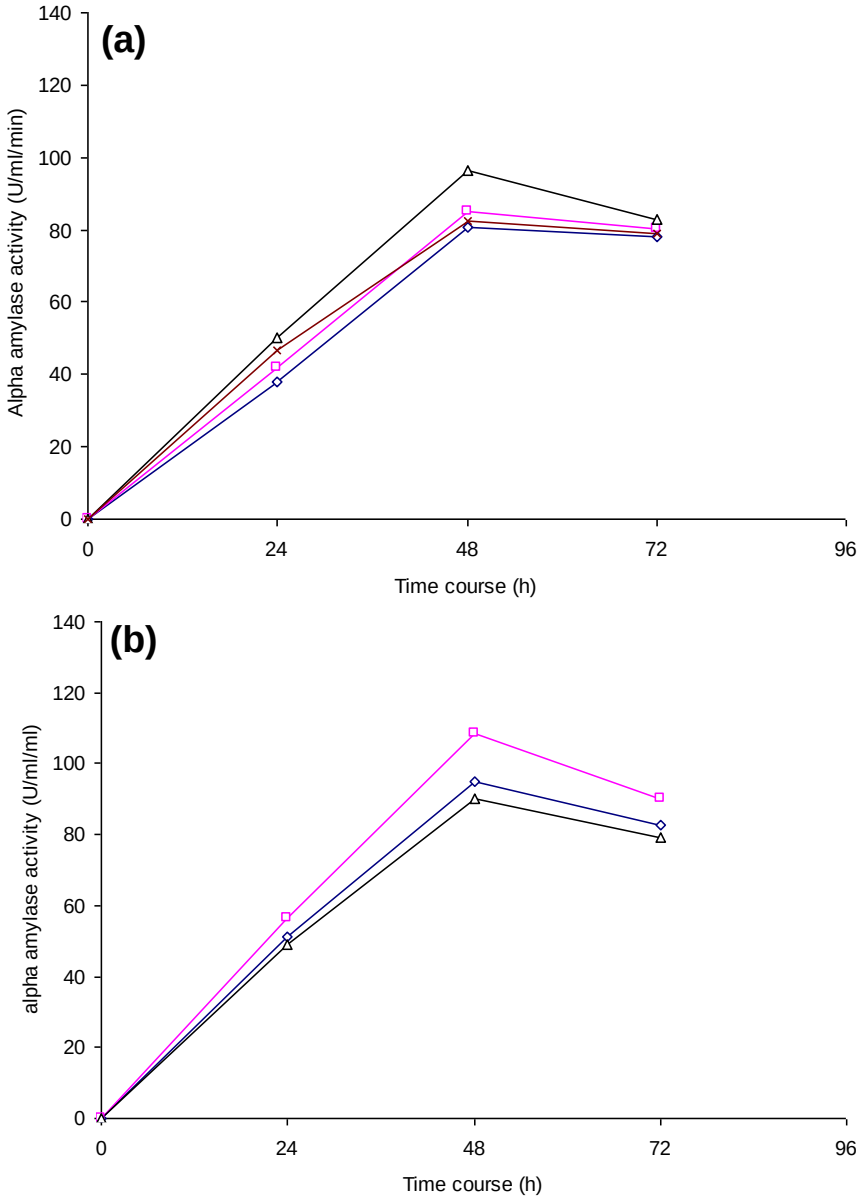

Fig. 5. Effect of, a) agitation (-◊- 200rpm, -□- 300rpm, -Δ- 400rpm, -×- 500rpm) and, b) aeration (-◊- 1.5vvm, -□- 2vvm, -Δ- 2.5vvm) rates on alpha amylase production by the mutant strain of *B. amyloliquefaciens* EMS-6 in stirred fermentor. Incubation temperature 37ºC, initial pH 7.0.

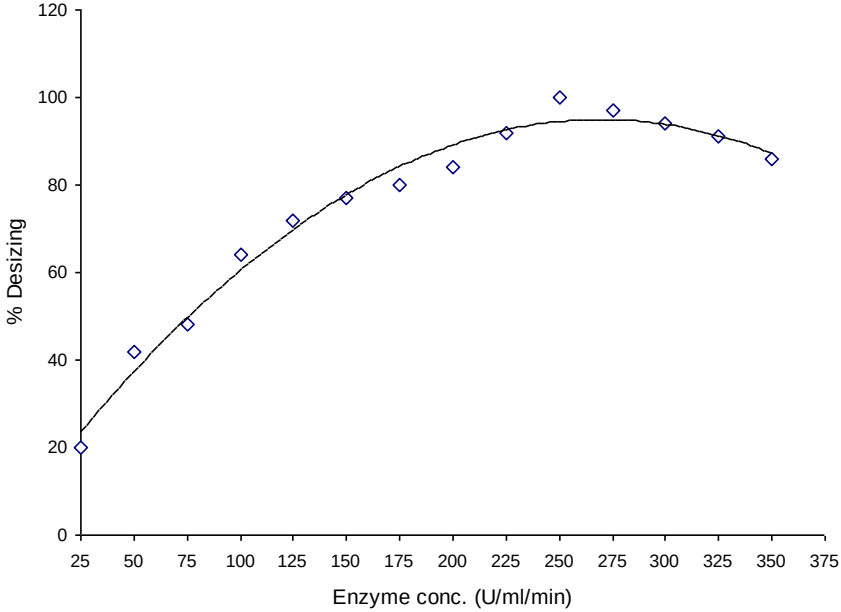

Fig. 6. Desizing with different concentrations of alpha amylase. Temperature 60ºC, incubation time 1 h.

The scale up studies for alpha amylase production was carried out in a glass fermentor of 7.5 L capacity. Selection of suitable fermentation medium is very essential for optimum production of alpha amylase. In the present study, M4 medium was found to be best for maximum production of alpha amylase. The M2 medium gave relatively less production of alpha amylase. It might be due to the fact that this medium contained wheat bran that made hindrance in the agitation, aeration and hence in the productivity of alpha amylase. Carbon source affects amylase formation not only the mode but also with the velocity with which carbon source metabolized in the bacterial cells. It might be due to these carbon sources were easily available for the growth of the microorganism so that the biosynthesis of the inducible alpha amylase was not initiated by the microorganism (Satyanarayana *et al*., 2004).

The production of alpha amylase following growth of bacteria was found to be highly significant ($p<0.05$) when the initial pH of the fermentation medium was maintained at 7.5 in the stirred fermentor. Jin *et al*., (1998) have described the optimum production of alpha amylase at pH 8.0-9.5 by the bacterium used. However, in the present study pH 7.5 was optimized. It might be due to enzyme produced by this strain was quite stable at pH 7.5. At acidic pH the production of the enzyme was highly insignificant.

In the present study, the production of alpha amylase following growth of the organism was found to be optimum as the fermentation medium was incubated at 40ºC. Further increase in the incubation temperature reduced the production of alpha amylase. At 60ºC the production of alpha amylase was greatly inhibited. It might be due to the fact that at high temperature, the moisture contents in the fermentation conditions became reduced. With the reduction of moisture contents, the growth of the organism in the fermentation medium was decreased that resulted in the decreased production of alpha amylase (Boesel *et al*., 2006).

The size of Inoculum plays an important role in the fermentation of enzymes (Lin *et al*., 1998). The different inoculum levels were tested for the production of alpha amylase. The production of enzyme was increased with increase in the size of inoculum and found to be optimal at 8% inoculum level. As, the inoculum level was further increased, the production of the enzyme was gradually inhibited. It might be due to the fact that inoculum level at high concentration, the bacteria grow rapidly and the nutrients essential for the growth of bacteria were consumed at the initial stages that resulted in the accumulation of other by products in the fermentation medium. Thus, the production of alpha amylase was affected at higher concentration of inoculum. The insignificance of the result at low level of the inoculum might be due to the fact that the organism grew slowly and the time period for the bacteria to reach in the stationary phase was increased (Tsurikova *et al*., 2002).

The supply of oxygen is very essential for the aerobic fermentation. The oxygen dissolved in the medium becomes available to the organism for growth. The production of enzyme following growth of the organism was found to be the maximal (96.5 U/ml/min) as the agitation intensity of the fermentor was maintained at 400 rpm. Further increase in the agitation intensity resulted in the decreased production of biomass as well as alpha amylase by the bacterium. At 200-300 rpm, both the production of biomass and enzyme were extremely low. Thus, agitation intensity 400 rpm was adequate and selected for maximum accumulation of alpha amylase in the stirred fermentor. The production of enzyme reached maximal as the air supply was maintained at 1.0l/l/min. at low level of air supply, the productivity of enzyme was greatly inhibited. It might be due to the reduction of growth of microorganism at low level of air supply and hence in enzyme production (Lee *et al*., 2008).

The desizing of grey fabric with crude amylase is very promising in textile industry. The effect of different concentrations of enzyme was evaluated for the desizing of grey fabric cloth by alpha amylase. It was observed that the desizing of fabric increased with increase in enzyme concentration up to certain limit. But as the concentration of the enzyme increased than the optimum level, it showed adverse effects on the desizing of the fabric. It might be due to the fact that appropriate enzyme to substrate ratio is essential to obtain optimal results (Allan *et al*., 1997).

Conclusion

In the present study, mutant *B. licheniformis* EMS-6 gave 102.78 U/ml/min enzyme production which was 1.4 fold higher than the parental strain. The medium containing (g/l) soluble starch 10.0, nutrient broth 8.0, $(NH_4)_2SO_4$ 2.25, $(NH_4)_2HPO_4$ 1.0, NaCl 0.85, $MgSO_4.7H_2O$ 0.25, $CaCl_2$ 0.2 prepared in 0.02 M phosphate buffer, pH 7.0 supported a maximal enzyme production of 112.9 U/ml/min. The incubation period was notably reduced from 72 to 48 h in stirred fermentor. The rates of agitation (400 rpm) and aeration (2.0 vvm) were also optimized. A 100 % desizing of grey fabric was achieved with 200-225 units of enzyme at pH 6.5 (60ºC) in 1 h.

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References

Allan, S., B. Torbenvedel and B.F. Henrik. 1997. Recombinant alpha amylase mutants and their use in textile desizing, starch liquefaction and washing. *Journal of Applied Microbiology*, 12: 205-210.

Arikan, B. 2008. Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. *Bioresource Technology*, 99: 3071–3076.

Bin, G., X. Laisu, D. Youfang and L. Yanquan. 1999. Screening of alpha amylase high-producing strains from *Bacillus subtilis. Journal of Zhejiang* 23: 88-92.

Boesel, L.F., H.S. Azevedo and R.L. Reis. 2006. Incorporation of alpha-amylase enzyme and a bioactive filler into hydrophilic, partially degradable, and bioactive cements (HDBCs) as a new approach to tailor simultaneously their degradation and bioactive behavior. *Biomacromolecules*, 7: 2600-2609.

Buchanan, R.E. and S.D. Gibbons. 1974. *Bergey's Manual of determinative Bacteriology*, 8th Ed. Baltimore. pp. 529-550.

Ghasemi, A., K. Khajeh and B. Ranjbar. 2007. Stabilization of *Bacillus licheniformis* alpha-amylase by specific antibody which recognizes the N-terminal fragment of the enzyme. *International Journal of Biology and Macromolecules*, 41: 162-167.

Haq, I., H. Ashraf, S. Ali and M.A. Qadeer. 2007. Kinetic characterization of extracellular alpha-amylase from a derepressed mutant of *Bacillus licheniformis*. *Applied Biochemistry and Biotechnology*, 141: 251-264.

Haq, I., H. Ashraf, M.A. Qadeer and J. Iqbal. 2005. Pearl millet, a source of alpha amylase production by *Bacillus licheniformis. Bioresource Technology*, 96: 1201-1204.

Jin, F., L. Xianzhen, Z. Chunzi, S. Qiao, W. Hua, L. Ziqiang, Z. Xuezhong, W. Xiaoxia and C. Yuhua. 1998. A thermostable alpha amylase from *Bacillus* sp-JF strain. *Journal of General and Applied Microbiology*, 38: 293-302.

Kelly, C.T., M.A. Tigue, E.M. Doyle and W.M. Fogarty. 1997. Raw starch degrading alkaline amylase of *Bacillus* sp. *Journal of Industrial Microbiology*, 15: 446-448.

Lee, C.K., Q.T. Le, Y.H. Kim, J.H. Shim, S.J. Lee, J.H. Park, K.P. Lee, S.H. Song, J.H. Auh, S.J. Lee and K.H. Park. 2008. Enzymatic synthesis and properties of highly branched rice starch amylose and amylopectin cluster. *Journal of Agriculture and Food Chemistry*, 56: 126-131.

Lin, L.L., C.C. Chyau and W.H. Hsu. 1998. Production and properties of a raw-starch-degrading amylase from the thermophilic and alkalophilic *Bacillus* species. TS-23. *Biotechnology and Applied Bichemistry*, 28: 61-68.

Markkanen, P.H. and M.L. Suihko. 1974. The use of UV-radiation in the improvement of enzyme production by *Bacillus subtilis*. *Chemistry Letters*, 4: 89-92.

Nigam, P. and D. Singh. 1995. Enzymes and microbial system involved in starch processing enzyme. *Enzyme and Microbial Technology*, 17: 770-778.

Philip, J.B., E.H. Ronald and W.T. Martin. 1988. Purification and properties of a raw commercial thermostable *Bacillus stearothermosphilus* alpha-amylase. *Food Biotechnology*, 2: 67-80.

Prescott, S. and A. Dunn's. 1987. *Industrial Microbiology*. 4th Ed. CBS Publishers and Distributors, New Delhi, India. pp. 550-565.

Qirang, J. and W. Zhao. 1994. Selection and breeding of a high productivity strain of alpha-amylase from multi resistant mutant of *Bacillus. Wuxi Qinggongyo Xueguan Xuebae*, 13: 21-26.

Rick, W. and H.P. Stegbauer. 1974. Alpha amylase measurement of reducing groups. In: *Methods of enzymetic analysis*, (Ed.): H.V. Bergmeyer, 2nd Ed., Vol. 2, Academic Press, New Dehli, India.

Saito, N. and K. Yamamoto. 1974. Regularity factors affecting α-amylase production in *Bacillus licheniformis*. *Journal of Biotechnology*, 64: 848-856.

Satyanarayana, T., S.M. Noorwez, S. Kumar, J.L. Rao, M. Ezhilvannan and P. Kaur. 2004. Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. *Biochemical Society Transactions*, 32: 276-278.

Sivaramakrishnan, S., D. Gangadharan, K.M. Nampoothiri, C.R. Soccol and A. Pandey. 2006. α-Amylase from microbial sources - an overview on recent developments. *Food Technology and Biotechnology*, 44: 173-184.

Snedecor, G.W. and W.G. Cochran. 1980. *Statistical Methods*, 7th Ed. Iowa State University Press, Iowa, USA .

Tsurikova, N.V., L.I. Nefedova, E.V. Kostyleva, V.I. Zvenigorodskii, V.G. Iasinovskiĭ, T.A. Voeĭkova and A.P. Sinitsyn. 2002. Preparation of an active strain of *Bacillus licheniformis* producer of thermostable alpha-amylase. *Prikle Biokhimical Mikrobiology* 38: 502-508.

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