

MUTAGENESIS OF *BACILLUS LICHENIFORMIS* THROUGH ETHYL METHANESULFONATE FOR ALPHA AMYLASE PRODUCTION

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Abstract

The present study is concerned with the improvement of *Bacillus licheniformis* strain GCB-30^{UCM} for alpha amylase production in 250 ml Erlenmeyer flasks. Chemical mutation using ethyl methanesulfonate (EMS 50-300 µ/ml) was undertaken for 10-60 min. Twenty eight isolates were selected on the basis of clear zones of starch hydrolysis. Only one isolate designated as *B. licheniformis* EMS-200⁴⁰ gave 102.78±2.01 U/ml/min enzyme activity. The enzyme production was found to be maximal when fermentation medium containing (g/l) lactose 10.0, bactopectone 14.0, yeast extract 6.0, KCl 1.0, CaCl₂ 0.25, MgCl₂ 0.2, MnSO₄ 0.001, FeSO₄ 0.0005, pH 6.5 was incubated at 37°C for 72 h. The volume of medium (50 ml) and size of inoculum (4.0 %) were also optimized. The optimal enzyme activity was determined as a function of buffer pH (7.0) and temperature (60°C).

Introduction

Alpha amylase (*EC 3.2.1.1*), an extracellular enzyme degrades α-1,4-glucosidic linkages of starch and related substrates in an endo-fashion producing oligosaccharides including maltose, glucose and alpha limit dextrins. The enzyme is used particularly in starch liquefaction, brewing, textile, pharmaceuticals, paper, detergents, drugs, toxic wastes removal and oil drilling (Ajayi & Fagade, 2003). The enzyme production is largely dependent on the type of strain, medium composition, cell growth, initial pH and thermostability (Qirang & Zhao, 1994; Haq *et al.*, 2002, 2005). Highly active α-amylase is preferred for the conversion of starch into oligosaccharides. So, it is worthwhile to select a potent microbial strain for α-amylase production. For this, *Bacillus* species such as *B. subtilis* and *B. licheniformis* have been the organisms of choice (Sivaramakrishnan *et al.*, 2006). Traditional mutation and selection techniques have been exploited for the improvement of bacterial cultures. Chemicals such as nitrous acid or nitrosomonas have been commonly used (Prescott & Dunn's, 1987). However, the optimal growth of bacterial culture and subsequent α-amylase production is greatly affected by the cultivation method and supplementation of essential nutrients (Fogarty *et al.*, 1999).

The enzyme is being produced both by solid-state and submerged fermentation techniques (Babu & Satyanarayana, 1995; Ashraf *et al.*, 2001). Submerged fermentation holds a tremendous potential for enzyme production particularly where crude fermented product may directly be used as an enzyme source. The addition of substrates to a batch process under controlled conditions has been reported for prolonged growth and increasing the enzyme production (Lulko *et al.*, 2007; Markkanan 2008). Further work is still needed on the optimization of fermentation conditions for α-amylase production by a chemically treated mutant strain of *B. licheniformis*. In the present study, a bacterial strain of *B. licheniformis* GCB-30^{UCM} was improved for α-amylase production using ethyl methanesulfonate (EMS) as an inducing mutagen. Screening and selection of a hyper-

producer was performed by both dye method and shake flask technique. Cultural conditions such as temperature, pH, volume of medium, incubation period and size of inoculum for enzyme production by the mutant were optimized to gain an insight of the batch fermentation process. The enzyme (as a function of temperature and buffer pH) was also characterized.

Materials and Methods

Organism and culture maintenance: *Bacillus licheniformis* GCB-30^{UCM} was obtained from the available stock culture of IIB and maintained on nutrient agar medium containing (g/L); nutrient broth 8.0, agar 20, 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: Fifty millilitre of medium containing nutrient broth 8.0 g/l, pH 7.0 was transferred to a 250 ml Erlenmeyer flask and cotton plugged. It was sterilized in an autoclave at 15 lbs/in² pressure (121°C) for 15 min. After cooling, a loopful of bacterial culture was aseptically transferred and rotated at 200 rpm (37°C) in a shaking incubator (Model: 10X400.XX2.C, SANYO Gallenkamp, PLC, UK) for 24 h.

Fermentation media, techniques and critical phases: Microbial fermentations were carried out using submerged technique in 250 ml Erlenmeyer flasks. Fifty millilitre of the fermentation medium (M4 optimized) was transferred to the individual flasks and cotton plugged. The flasks were sterilized and cooled at room temperature. Each flask was inoculated with 2.0 ml of the bacterial inoculum (1.365×10^7 CFU) and placed in the shaking incubator (200 rpm) at 37°C for 48-96 h. After incubation, the fermented broth was spun at 6000×g for 15 min., in a centrifuge (Model: D-37520, Osterodeam-Harz, Germany). All the experiments were run parallel in triplicates. Following media were evaluated,

M1: Starch soluble 40, (NH₄)₂HPO₄ 5.0, yeast extract 5.0, Sodium citrate 2.0, MgSO₄·7H₂O 0.5, CaCl₂ 0.08, pH 7.0 (Saito & Yamamoto, 1974).

M2: Starch 20, KCl 1.0, MgCl₂ 0.2, NaHPO₄ 5.4, Na₂HPO₄ 7.0, CaCl₂ 0.25, FeSO₄ 0.005, MnSO₄ 0.001, pH 7.0 (Hensely *et al.*, 1980).

M3: Starch 10.0, nutrient broth 10.0, (NH₄)₂SO₄ 2.0, NaCl 1.0, MgSO₄·7H₂O 2.0, CaCl₂ 2.0, pH 7.0.

M4: Lactose 10.0, bactopectone 14.0, yeast extract 6.0, KCl 1.0, CaCl₂ 0.25, MgCl₂ 0.2, MnSO₄ 0.001, FeSO₄ 0.0005, pH 7.0 (Kelly *et al.*, 1997).

M5: Starch 10.0, FeCl₃ 5.0, yeast extract 6.0, peptone 0.35, Sodium citrate 2.0, CaCl₂ 0.08, pH 7.0 (Tan & Tsay, 1985).

Culture improvement through EMS treatment and selection: Five millilitre of EMS (50-300 µg/ml) was transferred to individual sterilized centrifuged tubes containing 5.0 ml of bacterial suspension and placed at 30°C for 10-60 min. After which the tubes were centrifuged at 6000×g for 15 min. The supernatant was discarded to remove the EMS. The pellet was washed thrice with saline water to remove traces of EMS from bacterial cells. The suspension was made in 10 ml of sterilized phosphate buffer (pH 7.2). Approximately 0.5 ml of the suspension was transferred to the Petri plates containing nutrient starch agar (NSA) medium. The plates were rotated clockwise and counter clockwise and incubated at

40°C for 24 h. The bacterial colonies showing bigger zones of starch hydrolysis compared to the parental strain were picked up and transferred to the NSA slants.

Enzyme assay: One milliliter of enzyme extract was added to a test tube containing 1.0 ml of 1.0 % soluble starch, pH 7.0 (prepared in acetate buffer). The mixture was incubated at 60°C for 10 min. Then 1.0 ml of DNS reagent was added to each of the test tubes. The tubes were placed in boiling water for 5 min., and cooled at room temperature. The contents of test tubes were diluted up to 10 ml with distilled water. The absorbance of reaction mixture was determined at 546 nm on a spectrophotometer after Rick & Stegbauer (1974).

One unit enzyme: One unit of α -amylase is defined as the amount of enzyme that liberates 1.0 mg of reducing sugar as maltose per min., under the defined assay conditions.

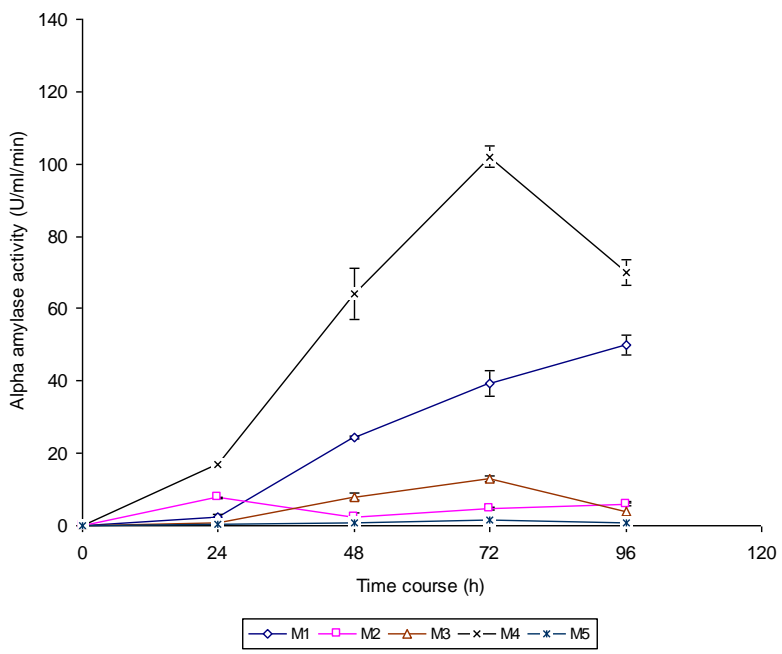
Enzyme characterization: The enzyme was characterized as a function of pH and temperature as described by Mamo & Gessesse (1999) and Shahhoseini *et al.*, (2005).

Statistical analysis: Treatment effects were compared by the method of Snedecor & Cochran (1980). Significance has been presented in the form of probability ($p \leq 0.05$) values.

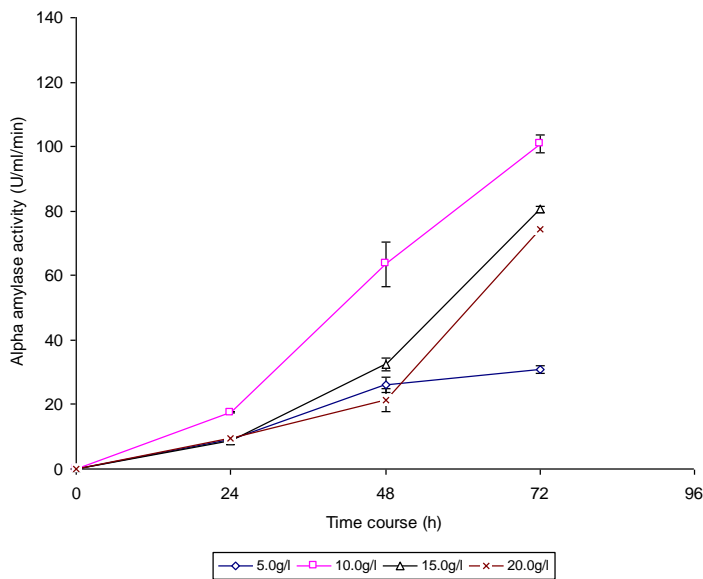
Results and Discussion

Twenty-eight isolates of parental strain *Bacillus licheniformis* GCB-30^{UCM} (73.98 ± 1.76 U/ml/min) were screened for enhanced α -amylase production in shake flasks. Sixteen isolates showed less enzyme production compared to the parental strain; however 12 others gave relatively better amylase production. Among these, 6 isolates gave enzyme production in the range of 75.0-85.0 U/ml/min., 5 gave from 86.0-95.0 U/ml/min while only 1 isolate produced 102.78 ± 2.01 U/ml/min α -amylase, so it was selected for further studies. This mutant strain was designated as *B. licheniformis* EMS-200⁴⁰. Workers have employed random mutagenesis for α -amylase production by exposing the cultures with chemicals like EMS (Markkanen & Suihko, 1974; Jin *et al.*, 1998).

The evaluation of a suitable medium is critical for a successful fermentation process by microbes. The selection of fermentation medium (M1, M2, M3, M4, M5) for α -amylase production by the mutant strain of *B. licheniformis* EMS-200⁴⁰ was investigated. The results are depicted in Fig. 1a. Fermentations were carried out from 24-96 h. The medium M4 gave maximum α -amylase production 72 h after incubation. It might be due to the fact that this medium provided adequate amount of essential nutrients required for microbial growth and subsequently enzyme production. The effect of different lactose concentrations (5.0, 10.0, 15.0 & 20.0 g/l) on α -amylase production was also studied and Fig. 1b shows the results. The maximum enzyme activity (102.9 ± 1.92 U/ml/min) was obtained in fermentation flasks containing 10.0 g/l lactose and 72 h after the inoculation. Synthetic culture medium with lactose as the carbon source supported a lower growth rate with simultaneous production of enzyme (Hewitt & Solomons, 1996). Further increase in incubation period decreased the production of α -amylase. It might be due the depletion of nutrition, accumulation of harmful by-products in the culture medium and proteolysis of α -amylase and microorganism (Chamber *et al.*, 1999). All the other lactose concentrations gave relatively less enzyme production. It may be due the over growth of bacterial cells or catabolite repression (Tan & Tsay, 1985; Sivaramakrishnan *et al.*, 2006).



(a) Selection of fermentation medium



(b) Lactose concentration

Fig. 1. Alpha amylase production by the mutant strain of *B. licheniformis* EMS-200⁴⁰. Incubation temperature 37°C, initial pH 7.0, agitation rate 200 rpm. Y-error bars indicate standard deviation (\pm sd) among the three parallel replicates.

Initial pH is one of the critical parameters which correlate with the microbial growth directly. The effect of different initial pH (6.0, 6.5, 7.0 & 7.5) on α -amylase production by the mutant *B. licheniformis* EMS-200⁴⁰ was undertaken from 24-72 h after the inoculation. The results are given in Fig. 2a. The maximum α -amylase activity (112.5 ± 4.94 U/ml/min) was obtained when initial pH of the medium was kept at 6.5. It might be due to enzyme produced by the strain was quite stable at this pH. In contrast to the present findings, Ivanova *et al.*, (2001) achieved the optimal α -amylase production at a pH range of 8.0-9.5. All the other initial pH gave comparatively less enzyme production. The different volumes of fermentation medium i.e., 25, 50 and 75 ml in 250 ml Erlenmeyer flasks were studied for 24-72 h after inoculation. The results are given in Fig. 2b. Maximum α -amylase activity (110.9 ± 3.12 U/ml/min) was found in flasks containing 50 ml of the medium at an optimal incubation period of 72 h. As the volume of the medium was increased the production of α -amylase was inhibited. It might be due to the fact that the higher level of the volume makes hindrance in the proper agitation and hence air supply that resulted in the decreased production of α -amylase. However, Riaz *et al.* (2003) achieved the optimal α -amylase production with 25 ml volume.

Fig. 2c reveals the effect of incubation temperature (30, 37, 50°C) on α -amylase production by the mutant strain of *B. licheniformis* EMS-200⁴⁰ from 24-72 h after the inoculation. Maximum α -amylase production was noticed when the incubation temperature of the medium was adjusted at 37°C. It might be due to the fact that 37°C is the optimal temperature of growth of bacterial culture and subsequently for enzyme production. In addition, high temperature might have reduced the moisture contents of the fermentation medium and growth of the organism resulting in the decreased enzyme production (Markkanen & Suihko, 1974). The effect of size of inoculum (2.0, 4.0, 6.0 & 10.0 %) on α -amylase production by the mutant strain was also evaluated. The results are shown in Fig. 2d. The maximum α -amylase activity (112.5 ± 2.01 U/ml/min) was obtained at an inoculum level of 4.0% and 72 h after the inoculation. All the other inoculum levels gave comparatively less α -amylase production. It might be due to the fact that the bacteria grew rapidly and the nutrients essential for the growth were consumed at the initial stages of fermentation which resulted in the accumulation of other by products. The less α -amylase production at low level of the inoculum might be due to the fact that the organism grew slowly (Babu & Satyanarayana, 1995; Lulko *et al.*, 2007).

The effect of the addition of nitrogen sources such as bactopectone and yeast extract individually and in combination (bactopectone+yeast extract) on α -amylase production was also investigated (Fig. 3a). The addition of bactopectone or yeast extract as sole nitrogen source gave less α -amylase production. However, maximum α -amylase activity was achieved in the cultural medium supplemented with bactopectone+yeast extract at a ratio of 1.4:0.6 (Fig. 3b). It might be due to the fact that the bactopectone+yeast extract provided adequate concentration of nitrogen for the growth of microorganism and hence enzyme formation.

The optimal activity of α -amylase was determined as a function of pH and temperature (Mamo & Gessesse, 1999). The effect of different temperatures (30, 40, 50, 60, 70 & 80°C) and buffer pH (6.0, 6.5, 7.0, 7.5 & 8.0) on α -amylase activity by the mutant strain of *B. licheniformis* EMS-200⁴⁰ was investigated (Fig. 4a and 4b). The maximum enzyme activity (114.5 ± 1.06 U/ml/min) was observed at a temperature of 60°C and buffer pH of 7.0 (neutral). Any change in temperature and pH values other than the optimal decreased the α -amylase activity. The present work is in accordance with the findings reported earlier (Haq *et al.*, 2002; Shahhoseini *et al.*, 2005).

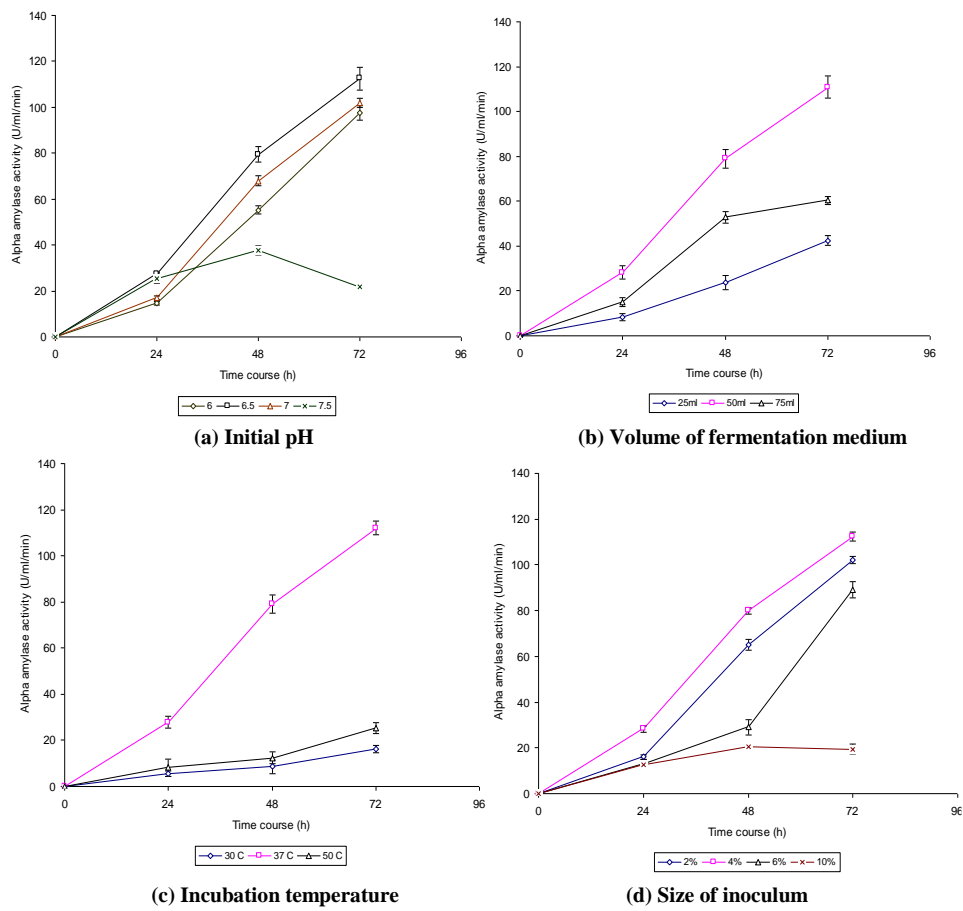


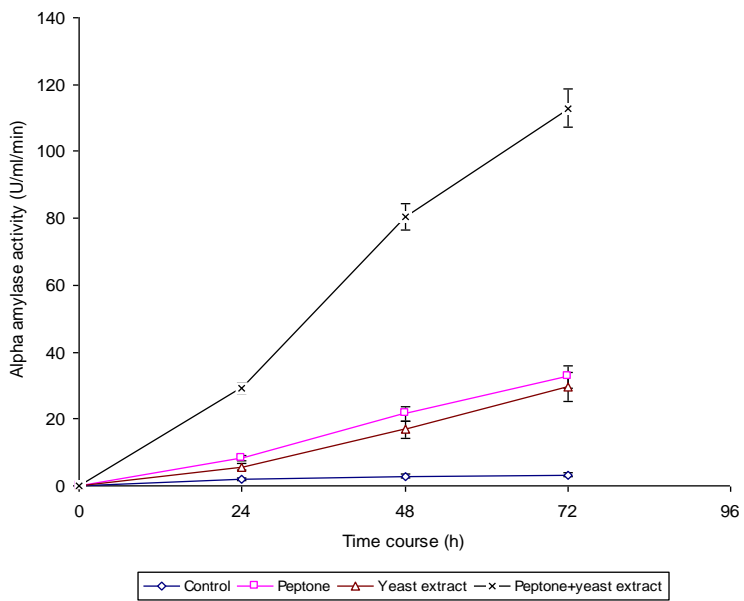
Fig. 2. Optimizations for alpha amylase production by the mutant strain of *B. licheniformis* EMS-200⁴⁰. Lactose concentration 10 g/l, agitation 200 rpm. Y-error bars indicate standard deviation (\pm sd) among the three parallel replicates.

Conclusion

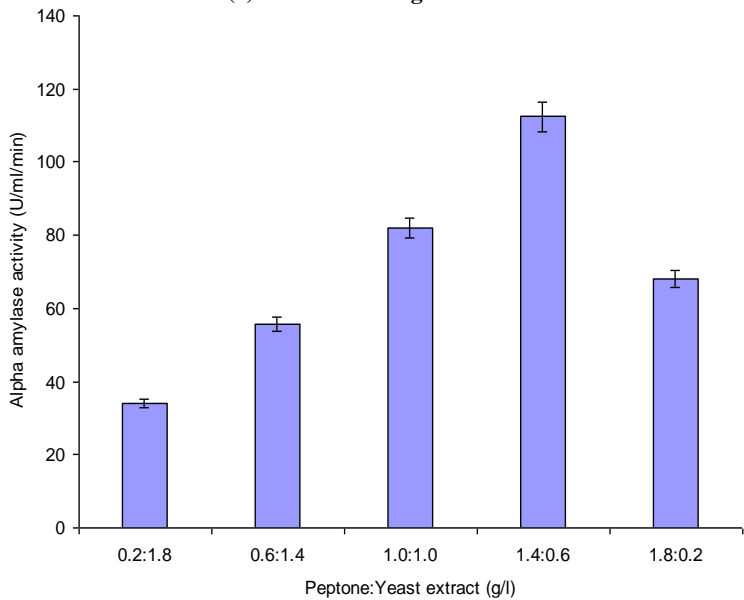
In the present study, a mutant *Bacillus licheniformis* EMS was developed by ethyl methanesulfonate (EMS). M4 contained (g/l) lactose 10.0, bactopectone 14.0, yeast extract 6.0, KCl 1.0, CaCl₂ 0.25, MgCl₂ 0.2, MnSO₄ 0.001, FeSO₄ 0.0005 was optimized as the basal fermentation medium. The optimal conditions were pH 6.5, temperature 37°C and incubation period 72 h. However, further investigation on the improvement of *Bacillus licheniformis* through M-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for scale up studies is needed.

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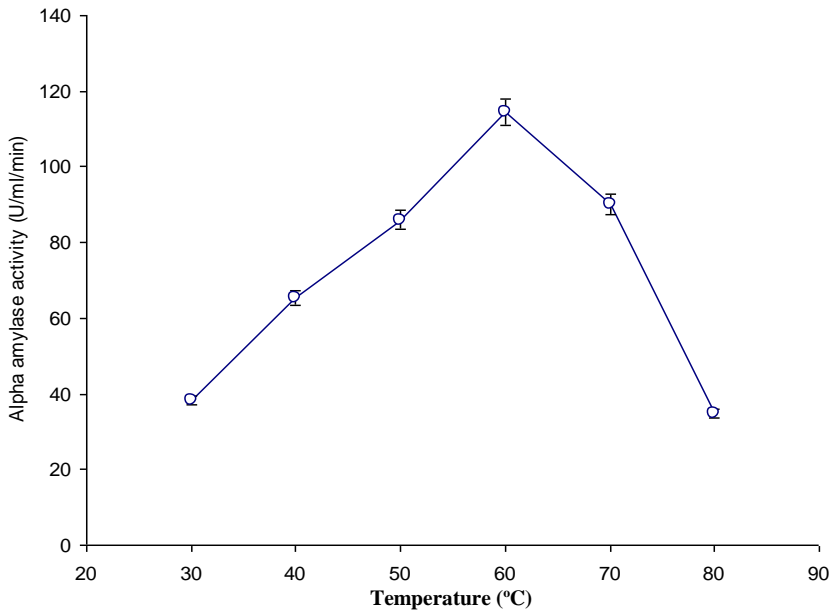


(a) Different nitrogen sources

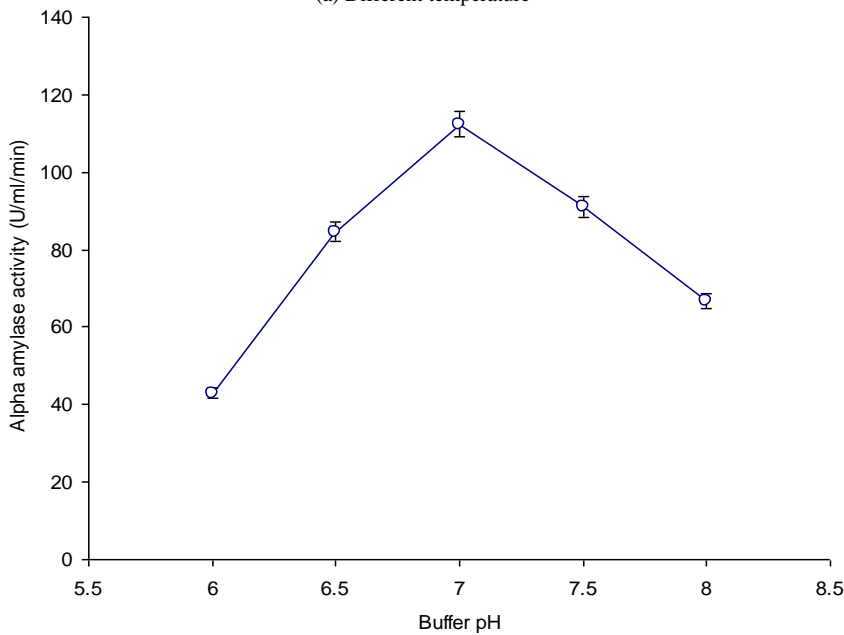


(b) Partial replacement of bacto-peptone with yeast extract

Fig. 3. Effect of different nitrogen sources on alpha amylase production by the mutant strain of *B. licheniformis* EMS-200⁴⁰. Incubation temperature 37°C, initial pH 7.0, agitation rate 200 rpm, lactose concentration 10 g/l. Y-error bars indicate standard deviation (\pm sd) among the three parallel replicates.



(a) Different temperature



(b) Different buffer pH

Fig. 4. Characterization of alpha amylase activity by the mutant strain of *B. licheniformis* EMS-200⁴⁰.

References

- Ajayi, A.O. and O.E. Fagade. 2003. Utilization of corn starch as substrate for β -amylase by *Bacillus* spp. *Afr. J. Bio. Res.*, 6: 37-42.
- Ashraf, H., I. Haq, M.A. Qadeer and J. Iqbal. 2001. Screening of *Bacillus licheniformis* mutants for improved production of alpha amylase. *Pak. J. Bot.*, 33: 517-525.
- Babu, K.P. and T. Satyanarayana. 1995. Alpha amylase production by thermophilic *Bacillus coagulans* in solid-state fermentation. *Proc. Biochem.*, 30: 305-309.
- Chamber, R., E. Haddaoui, M.F. Petitglatran, O. Lindy and M. Sarvas. 1999. *Bacillus subtilis* α -amylase. The rate limiting step of secretion is growth phase in dependent. *FEMS Microbiol. Lett.*, 173: 127-131.
- Fogarty, W.M., E.M. Doyle and C.T. Kelly. 1999 Comparison of the action pattern of two high maltose forming alpha-amylase on linear malto-oligosaccharides. *Enz. Microbial Technol.*, 25: 330-335.
- Haq, I., N. Riaz, H. Ashraf and M.A. Qadeer. 2002. Effect of inorganic salts on the production of α -amylase by *Bacillus subtilis*. *Ind. J. Plant Sci.*, 2: 115-119.
- Haq, I., N. Shamim, H. Ashraf, S. Ali and M.A. Qadeer. 2005. Effect of surfactants on the biosynthesis of alpha amylase by *Bacillus subtilis* GCBM-25. *Pak. J. Bot.*, 37: 373-379.
- Hensely, D.E., K.L. Smiley, A. Boundy and A.A. Lagoda. 1980. Beta-amylase production by *Bacillus polymyxa* on a corn steep salts medium. *Appl. Environ. Microbiol.*, 39: 678-680.
- Hewitt, C.J. and G.L. Solomons. 1996. The production of alpha-amylase by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium. *Biotechnol. Lett.*, 14: 321-327.
- Ivanova, V., D. Yankov, L. Kabaivanova and D. Pashkkoulov. 2001. Simultaneous biosynthesis and purification of two extra cellular *Bacillus* α -amylases. *J. Biochem. Eng.*, 8: 61-81.
- 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. *J. Gen. Appl. Microbiol.*, 38: 293-302.
- Kelly, C.T., M.A. Tigue, E.M. Doyle and M.M. Fogarty. 1997. Raw starch degrading alkaline amylase of *Bacillus* sp. *J. Ind. Microbiol.*, 15: 446-448.
- Lulko, A., J.W. Veenig, G. Buist, W.K. Smits, E.J. Blom, A.C. Beekman, S. Brown and O.P. Kuipers. 2007. Production and screening stress caused by over-expression of heterogeneous α -amylase pools to inhibition of sporulation of prolong motile phase in *Bacillus subtilis*. *Appl. Environ. Microbiol.*, 73: 5354-5362.
- Mamo, G. and A. Gessesse. 1999. Effect of cultivation conditions on growth and α -amylase production by a thermophilic *Bacillus* species. *Lett. Appl. Microbiol.*, 29: 61-65.
- Markkanan, P.H. 2008. Influence of alternation of aeration and temperature on production of α -amylase by *Bacillus subtilis*. *J. Biotechnol.*, 25: 863-865.
- Markkanen, P.H. and M.L. Suihko. 1974. The use of UV-radiation in the improvement of enzyme production by *Bacillus subtilis*. *Fin. Chem. Lett.*, 4: 89-92.
- Prescott, S. and A. Dunn's. 1987. *Industrial Microbiology*. 4th Ed. CBS Publishers and Distributors, New Delhi, India.
- Qirang, J. and W. Zhao. 1994. Selection and breeding of a high productivity strain of alpha amylase from multi resistant mutant of *Bacillus*. *Wux. Qing. Xue. Xu.*, 13: 21-26.
- Riaz, N., I. Haq and M.A. Qadeer. 2003. Characterization of α -amylase by *Bacillus subtilis*. *Int. J. Ag. Biol.*, 5: 249-252.
- Rick, W. and H.P. Stegbauer. 1974. Alpha amylase measurement of reducing groups. In: *Methods of enzymatic 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*. *J. Biotechnol.*, 64: 848-856.

- Shahhoseini, M., A.A. Ziaee, A.A. Pourbabal, N. Ghaemi and N. Declerck. 2005. A natural variant of *Bacillus licheniformis* α -amylase isolated from flour mill wastewaters sheds light on the origin of high thermostability. *J. Appl. Microbiol.*, 98: 24-32.
- 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 Technol. Biotechnol.*, 44: 173-184.
- Snedecor, G.W. and W.G. Cochran. 1980. *Statistical Methods*, 7th Ed. Iowa State University Press, Iowa, USA.
- Tan, J.A. and S.S. Tsay. 1985. Extracellular thermostable α -amylase from *Bacillus licheniformis* B7. *Zhong. Min. Wei. Sheng. Za. Zhi.*, 18: 44-53.

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