

Biosynthesis of Alpha Amylase by Chemically Treated Mutant of *Bacillus subtilis*

Ikram-ul-Haq, Shafia Rani, Hamad Ashraf and M. A. Qadeer
Biotechnology Research Laboratory,
Department of Botany, Government College, Lahore, Pakistan

Abstract: The present study is concerned with the selection of hyper-producer strain of *Bacillus subtilis* and optimization of the cultural conditions for the production of alpha amylase in shake flask. The UV irradiated strain of *Bacillus subtilis* was treated with NTG for different intervals of time (5-60 min). One hundred mutant strains were isolated and tested for the production of alpha amylase and *Bacillus subtilis* GCBCM-25 gave maximum production of enzyme (2210 U/ml). The optimum conditions for the production of alpha amylase were, NaNO₃ as nitrogen source, pH 7.5, phosphate buffer as diluent and 4mM CaCl₂. The treatment of NTG for 25 min was found to be suitable for the production of best mutant of *Bacillus*. The production of alpha amylase was significantly increased with the addition of NaNO₃ and 4 mM CaCl₂ to the fermentation medium.

Key words: Biosynthesis, alpha amylase, *Bacillus subtilis*, chemical treatment, NTG

Introduction

The production of alpha amylase by mutant strain of *Bacillus subtilis* is well known (Qirang and Zhao, 1994). The parental strain can be treated with N-methyl-N-nitro-N-nitroguanidine (NTG). After screening the hyper producing mutants of *Bacillus* can be detected. (Eugenia *et al.*, 1992; Guan *et al.*, 1997; Akpan *et al.*, 1999). Different nitrogen sources have been used for enhancement in the production of alpha amylase (Akher *et al.*, 1973; Pedersen and Nielsen., 2000). The pH of the fermentation medium have very critical role in the production of alpha amylase by the bacterium. The organism can grow in acidic medium (Lealem and Gashe, 1994) or basic medium (Fogerty *et al.*, 1994) or at neutral pH (Castro *et al.*, 1999). The production of alpha amylase is Ca⁺² dependent. There was significantly increase in the production of alpha amylase by *Bacillus licheniformis* by the addition of Ca⁺² ion in the fermentation medium (Allan *et al.*, 1997).

The present study is concerned with the selection of the hyper-producing mutant for alpha amylase production and optimization of the cultural conditions for the production of alpha amylase by selected mutant culture.

Materials and Methods

The UV treated strain of *Bacillus subtilis* GCBU-20 was taken from Biotechnology Laboratory, Department of Botany, Govt. College, Lahore in 1999. The strain was maintained on the nutrients starch agar slopes. The fermentation technique was as reported by Haq *et al.* (1997). The UV treated strain of *Bacillus* was treated with N-methyl-N-nitro-N-nitroso guanidine (NTG) according to the method reported by Bailey and Markkanen (1975). Alpha amylase estimation was carried out according to the method of Fisher and Stein (1961). One unit of activity is equivalent to that amount of enzymes, which 3 minutes liberates reducing group from 1% Linters soluble starch corresponding to 1 mg maltose hydrate.

Results

Screening of mutants: The *Bacillus subtilis* GCBU-20 was treated with NTG for 5-60 minutes. One hundred cultures were isolated and tested for the production of alpha amylase. Of all the mutant cultures tested, *Bacillus subtilis* GCBCM-25 gave maximum production of alpha amylase (2210 U/ml) (Table 1). The complete death of bacteria was observed after 55 min of NTG treatment. However the mutant strain *Bacillus subtilis* GCBCM-25-2 that gave maximum production of alpha amylase was selected for further studies.

Effect of different nitrogen sources: The inorganic salts on the basis of 0.2% nitrogen were added in the fermentation medium. The maximum production of alpha amylase was achieved in the culture medium containing sodium nitrate. Other nitrogen sources

such as ammonium nitrate, ammonium sulphate, ammonium chloride, urea and ammonium carbonate gave 1776, 2198, 2100, 1250 and 1070 U/ml respectively (Table 2). The different concentrations of sodium nitrate were also evaluated for the production of alpha amylase (Fig. 1). 0.2% sodium nitrate on the basis of nitrogen was found to be optimum for the production of alpha amylase. Further increase in the nitrogen resulted decrease in the production of alpha amylase. However 0.2% nitrogen in the form of sodium nitrate was selected.

Effect of different diluents: The data of Table 3 shows the effect of different diluents on the production of alpha amylase by *Bacillus subtilis* GCBCM-25. The maximum production of enzyme (2328 U/ml) was obtained in the medium containing phosphate buffer. Other diluents such as tap water, citrate buffer and acetate buffer gave insignificant results.

Effect of pH: The data of Fig. 2 shows the effect of different pH on the production of alpha amylase by *Bacillus subtilis* GCBCM-25. The maximum production of alpha amylase was achieved when the pH of the medium was adjusted to 7.5. As the pH of the medium was increased or decreased from 7.5 there was gradual reduction in the enzyme formation. However, pH 7.5 was optimized for the production of alpha amylase.

Effect of CaCl₂ concentrations: The CaCl₂ at 1.0-7.0 mM level were evaluated for the production of enzyme (Fig. 1). Maximum production of alpha amylase was observed in the medium containing 4.0mM CaCl₂. With the increase in the concentration of calcium ion there was slightly reduction of alpha amylase. When calcium ion was not added in the medium, the results were insignificant.

Discussion

The mutant strains of *Bacillus* species have better ability for the production of alpha amylase (Qirong and Zhao, 1994). In this study UV irradiated strain of *Bacillus* was treated with NTG for different intervals of time. One hundred mutant cultures of *Bacillus* were isolated by observing bigger zone of hydrolysis of starch in the petri plates and tested for alpha amylase production. Among all the strains tested, *Bacillus subtilis* GCBCM-25 gave maximum production of alpha amylase. The complete death of bacteria was observed after 55 min of NTG treatment. This may be due to high dose of mutagenic agents have inhibitory effect on the growth of bacteria (Gardner *et al.*, 1991). The optimization of cultural conditions is very essentials for the enhanced production of alpha amylase. Inorganic nitrogen sources have inducory effect on the production of alpha amylase (Akher *et al.*, 1973). Among different nitrogen sources evaluated, sodium nitrate was found to be the best inducer of alpha amylase. Pedersen and Nielson (2000) have reported the influence of

Table 1: Screening of *Bacillus subtilis* mutants after NTG treatments

Treatment time (min)	NO. of survivals	Range of alpha amylase(U/ml)
5	20	110-1200
10	16	200-1914
15	13	1237-1237
20	12	990-2000
25	10	1485-2210
30	10	870-2077
35	8	872-2100
40	5	496-1988
45	4	1432-1783
50	2	880-1230
55	Nil	Nil
60	Nil	Nil

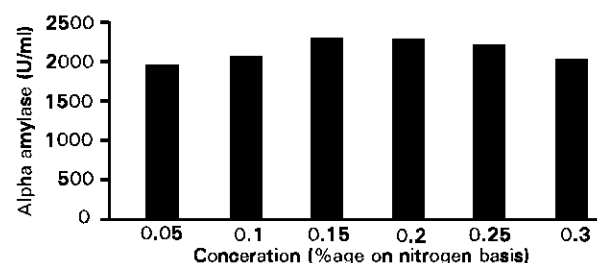
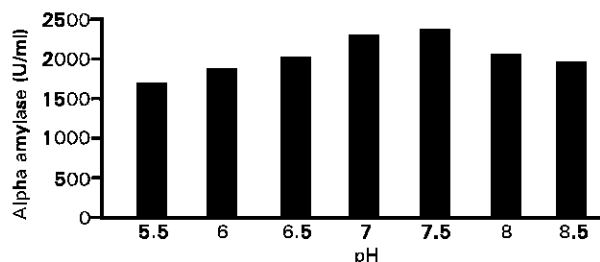
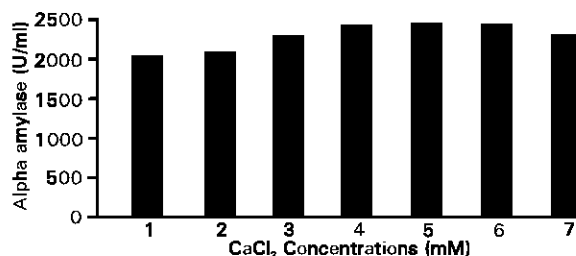
pH = 7.5 Temperature = 40°C

Table 2: Effect of different nitrogen sources on the production of alpha amylase by *Bacillus subtilis* GCBCM-25-2

Nitrogen sources	Alpha amylase (U/ml)
(NH ₄) ₂ SO ₄	2198
NH ₄ NO ₃	1776
NH ₄ Cl	2100
NaNO ₃	2280
Urea	1250
NH ₂ CO ₃	1070

Table 3: Effect of different diluents on the production of alpha amylase by *Bacillus subtilis* GCBCM-25-2

Diluents	Alpha amylase (U/ml)
Tap water	2030
Distilled water	2030
Phosphate buffer	2328
Citrate buffer	1832
Acetate buffer	1800
Citrate phosphate buffer	1931

Fig. 1: Effect of different concentrations of sodium nitrate on the production of alpha amylase by *Bacillus subtilis* GCBCM-25-2Fig. 2: Effect of different pH on the production of alpha amylase by *Bacillus subtilis* GCBCM-25-2Fig. 3: Effect of different concentrations of CaCl₂ on the production of alpha amylase by *Bacillus subtilis* GCBCM-25-2

nitrogen sources on alpha amylase production. They reported that nitrate was inferior to ammonium ion as nitrogen source. Young *et al.* (1995) used NaCl in the fermentation medium for sodium ion which stabilize the conformation of alpha amylase and increase the efficiency of the enzyme. This work is economically more significant than the work of Pedersen and Nielson (2000) and Young *et al.* (1995) because sodium nitrate provided sodium ion in the culture medium and acted as nitrogen source.

The production of alpha amylase is very sensitive to initial pH of the fermentation medium. In this pH between 7.5-8.0 was found to be best for the production of alpha amylase. In acidic medium the results were insignificant. It may be due to the bacteria required slightly alkaline pH for the production of enzyme. The production of alpha amylase is very sensitive to the diluents. Among all the diluents tested, phosphate buffer was found to be the best diluent for the production of alpha amylase. It may be due to the phosphate ions increase the permeability of the cells for the extraction of extra cellular alpha amylase. This finding is in accordance with the work reported by Prescott and Dunn (1987). The stability of alpha amylase is calcium dependent as reported by Kennedy and White (1979). In this study different amounts of CaCl₂ were evaluated. The 4mM CaCl₂ was found to be optimum for the production of alpha amylase. It may be due to that calcium ion was best binder, stabilizer and activator of alpha amylase. So the efficiency of the enzyme was enhanced as long as the calcium ion was present in the medium. This finding is in accordance with the work reported by Suisheng *et al.* (1997).

References

- Akher, M., M. A. Leithy, M. K. Massafy and S. A. Kasim, 1973. Optimal conditions of the production of bacterial amylase. Zentralbl. Bakteriologie, Parasitenkunde, Infektionskrankheiten Hygiene, 2, 128: 483-90.
- Akpan, I., M. O. Mankole and A. M. Adesemowo, 1999. A rapid plate culture method for screening of alpha amylase producing microorganisms. Biotechnol. Techniques, 13: 411-413.
- Allan, S., B. Torbenvedel and B. F. Henrick, 1997. Recombinant alpha amylase mutants and their use in textile desizing, starch liquification and washing. PTC. Int. Appl., 12: 205-210.
- Bailey, M. J. and P. H. Markkanen, 1975. Use of mutagenic agents in improvement of α -amylase Production by *Bacillus subtilis* J. Appl. Chem. Biotechnol., 25: 73-79.
- Castro, G. R., M. D. Baigori and F. Sineriz, 1999. Studies on alpha amylase production by *Bacillus licheniformis* Mir. 61. Acta Biotechnologica, 19: 263-272.
- Eugenia, D., M. Doina and R. O. Rom, 1992. *Bacillus subtilis* mutant with high production potential for amylolytic enzyme. Mendenicopschi Gheorghe, 101: 113.
- Fisher, E. M. and Stein, 1961. Biochemical Prep., 8: 27.
- Fogarty, W. M., M. A. Doyle, M. C. Tighe and C. T. Kelly, 1994. Production studies on the alkaline amylases by *Bacillus* sp. J. Agric. Chem., 19: 487, 643, 769, 853.

- Gardner, J. E., J. E. Simmons and D. P. Snustad, 1991. Principal of Genetic. 8th Edition. Jhon Wiley and Sons, Inc., 304-305.
- Guan, B., X. Laisu, D. Youfang and L. Yanquan, 1997. Screening of alpha amylase high producing strains from *Bacillus subtilis*. Nongye Daxue Xuebao, 23: 88-92.
- Haq, I., H. Ashraf, S. Ali and M. A. Qadeer, 1997. Submerged fermentation of alpha amylase by *Bacillus licheniformis* GCB-36. Biologia., 43:39-45.
- Kennedy, J. F. and C. A. White, 1979. Stability and kinetic properties of magnetic immobilized alpha amylase. Starch/Starke., 31; 375-381.
- Lealem, F. and B. A. Gashe, 1994. Amylase production by a gram positive bacterium isolated from fermenting. J. Appl. Bacteriol., 77: 348-352.
- Pedersen, H. and J. Nielsen, 2000. The influence of nitrogen sources on the alpha amylase productivity of *Aspergillus oryzae* in continuous cultures. Applied Microbiology and Biotechnology, 53: 278-281.
- Prescott and Dunn, 1987. Industrial microbiology. 4th ed. CBS Publishers and Distributors, New Delhi, India, pp: 550-565.
- Qirong, 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 Xuebao, 13: 21-26.
- Suisheng, Z., H. Quansheng and Z. Linixiang, 1997. Study on activity of *Bacillus subtilis* alpha amylase. J. Jaiyuan Gongye Daxue Xuebao., 28: 22-27.
- Young, C. S., J. Youngming and W. Yan, 1995. The influence of different factors on the confirmation and activity of alpha amylase from *Bacillus subtilis*. Shengwu Huaxu Zazhi., 11: 205-209.