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Characterisation of Amidohydrolytic Activity of *Bacillus megaterium* in Submerged Fermentation

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Abstract. Cultural conditions for the production of penicillin amidohydrolase by *Bacillus megaterium* 5B were investigated in shake flasks. The extra-cellular amidohydrolytic activity of the strain after 24 h of incubation was 37 u/ml. The enzyme production was found to be affected by different carbon sources at different concentrations in the fermentation medium. The most suitable carbon source was sucrose at the concentration of 0.3% (w/v). The enzyme activity reached the maximum level (70 u/ml) with a cell mass of 3.1 g/l in 25 ml of the fermentation medium contained in 250 ml flask at pH 7 after 24 h of incubation.

Keywords: penicillin amidohydrolase, *Bacillus megaterium*, submerged fermentation

Introduction

Penicillin amidohydrolase is the enzyme that cleaves the acyl side chain of penicillin. This enzyme is used widely in pharmaceutical industries for the production of 6-aminopenicillanic acid (6-APA), which is processed further during the manufacturing of various clinically important semi-synthetic penicillins (Bruggink *et al.,* 1998). Penicillin amidohydrolase catalyses the hydrolysis of penicillin to 6-APA, which is widely distributed among bacteria, fungi and actinomycetes (Bernard *et al.,* 2002; Babu and Panda, 1991). Bacteria, like *Escherichia coli* and *Bacillus megaterium,* are known to produce penicillin amidohydrolase. The penicillin amidohydrolase activity of *B. megaterium* in the fermentation broth has been reported by Son *et al.* (1982) and Fumian *et al.* (1996) in the range of 11.0 and 45.0 iu/ml, respectively. Most of the reported work has been on the intracellular penicillin amidohydrolase activity of *E. coli,* despite the fact that it involves expensive cell disruption for the enzyme recovery (Isebel *et al.,* 1994; Sudhakaran *et al.,* 1991). Therefore, the present work deals with the optimization of cultural conditions in shake flasks for the production of extra-cellular penicillin amidohydrolase by the selected strain of *B. megaterium* 5B.

Materials and Methods

Microorganism. The strain of *B. megaterium* 5B was provided by the Pakistan Type Culture Collection (PTCC), Biotechnology and Food Research Centre, PCSIR Laboratories Complex, Lahore, Pakistan. The cultures were maintained on nutrient agar slants at 4 °C and revived after every 2 weeks.

Fermentation technique. The 20 h old vegetative inoculum was developed in 250 ml flasks containing 25 ml of nutrient medium (g/l): yeast extract, 2.0; peptone, 5.0; sodium chloride 5.0 (Oxoid). Fermentation was carried out at 37 °C for 24 h in shake flasks. The fermentation medium consisted of (g/1): glucose, 2.0; polypeptone, 5; yeast extract, 5; K_2HPO_4,1; $MgSO_4$. $7H_2O$, 0.2; and benzyl penicillin, 0.1 at pH 7.0 (Sunaga *et al.,* 1976). The inoculum was transferred aseptically into 25 ml fermentation medium in 250 ml shake flasks at the rate of 3% v/v. After optimization of carbon source, glucose was replaced by sucrose at the rate of 0.3% (w/v) in the fermentation medium for further studies.

Assay of penicillin amidohydrolase. The penicillin amidohydrolase activity was determined by measuring 6-APA liberated from benzyl penicillin according to the ninhydrin method (Nam and Ryu, 1979). One unit of penicillin amidohydrolase activity was defined as the enzyme that formed 1 µmole of 6-APA per min under the procedure conditions.

Determination of cell mass. Cell mass was obtained from the culture broth by centrifugation at 10,000 rpm and dried at 105 °C to constant mass (Kim *et al.,* 1981).

Standard solutions. ***Phosphate buffer (0.5 mol/l, pH 7.0).*** Phosphate buffer was prepared by mixing 24.3 g potassium dihydrogen phosphate and 56.0 g di-potassium hydrogen phosphate in 1 litre of distilled water. Lower or higher pH values were obtained by adding hydrochloric acid (5 mol/l) or sodium hydroxide (5 mol/l).

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Citrate buffer. Citrate buffer was prepared by dissolving 1 g of citric acid in 50 ml of sodium hydroxide (0.8 mol/l).

Ninhydrin solution. 0.5 gram of ninhydrin was dissolved in 100 ml of acetone.

Results and Discussion

Effect of carbon source on the production of enzyme. The composition of culture medium is known to influence the propagation of bacterial cultures and synthesis of enzymes or metabolites. Carbon sources and other growth factors are reported to particularly influence the fermentation pattern (Roshan and Ganapathi, 1960). It is known that some strains of B. *megaterium* produce penicillin amidohydrolase in appreciable quantities using different carbohydrate sources (Yang, *et al.*, 2003). Sunaga *et al.* (1976) have also evaluated different carbon sources and found 0.3% glycerol to be the best carbon source for enzyme production by alkalophillic *Bacillus* species. Five different carbon sources, namely, glucose, sucrose, starch, maltose and fructose were incorporated into the medium for maximum production of the enzyme by *B. megaterium* 5B. All the carbon sources were supplemented at the rate of 0.1%, 0.2% and 0.3%. Cell growth and enzyme activity was recorded after 24 h of fermentation. The experimental data so obtained are summarized in Table 1. All the sugars tested supported the cell growth. However, the most suitable carbohydrate source for enzyme production was found to be sucrose at the concentration of 0.3% (w/v). Glucose at a concentration of 0.3% also enhanced the enzyme production considerably, though lesser than in in the presence of sucrose. In the subsequent experiments, therefore, 0.3% sucrose was used as the carbon source; pH of the medium was adjusted to 7.0.

Table 1. Effect of carbon sources on cell growth and penicillin amidohydrolase production

Carbon source	Concentration (%, w/v)	Cell growth (g/l)	Activity (u/ml)
Glucose	0.1	1.7	26
	0.2	1.9	37
	0.3	2.2	55
Sucrose	0.1	2.4	52
	0.2	2.9	60
	0.3	3.1	70
Starch	0.1	1.7	36
	0.2	1.9	40
	0.3	2.0	49
Maltose	0.1	0.9	10
	0.2	1.5	17
	0.3	1.8	32
Fructose	0.1	0.9	11
	0.2	1.3	16
	0.3	1.5	21

Effect of pH on enzyme synthesis. The initial pH of the fermentation medium influenced significantly the synthesis and stability of the enzyme, as the extra-cellular release of the enzyme depended on the pH gradient on bacterial cell wall. In order to study this effect, initial pH of the fermentation medium was adjusted to 5, 6, 7 and 8. The maximum enzyme production was attained at pH 7.0 (Fig. 1). It is also evident that in acidic and slightly basic conditions, the synthesis of enzyme by *B. megaterium* was less than at the neutral pH. The decrease in enzyme activity above pH 7 may be due to

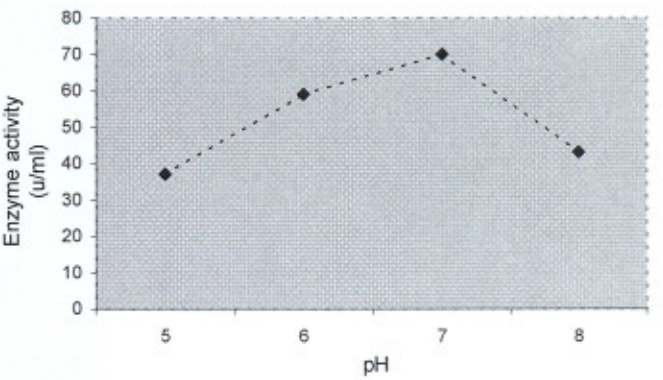

Fig.1. Effect of initial pH of the fermentation medium on the production of penicillin amidohydrolase.

alkaline degradation of penicillin amidohydrolase (Sunaga *et al.*, 1976). Meesschaert *et al.* (1991) have reported pH 8.0 to 8.5 to be optimum for enzyme production by some strains, whereas Nam and Ryu (1979) have reported pH 7.0 to 7.5 as the optimum for enzyme production.

Effect of aeration during fermentation. The supply of oxygen in aerobic fermentation is of importance since the supply of oxygen to the culture should be greater than the rate of consumption by the growing culture. The supply of oxygen in shake flask studies was varied by changing the volume of the culture medium (5-30 ml) in 250 ml conical flasks (Fig. 2). It was noted that increase in the culture medium volume resulted in increase in the enzyme activity. Similar observations were reported by Vojtisek and Slezak (1975) and Qadeer *et al.* (1992). Nam and Ryu (1979) also reported that an increase in the culture medium volume in shake flasks (low aeration) contributed to an increase in enzyme production. This increase was, however, limited only upto 100 ml in 500 ml flasks, while volume increase beyond that decreased aeration rate, further resulting in lower enzyme production. This result is consistent

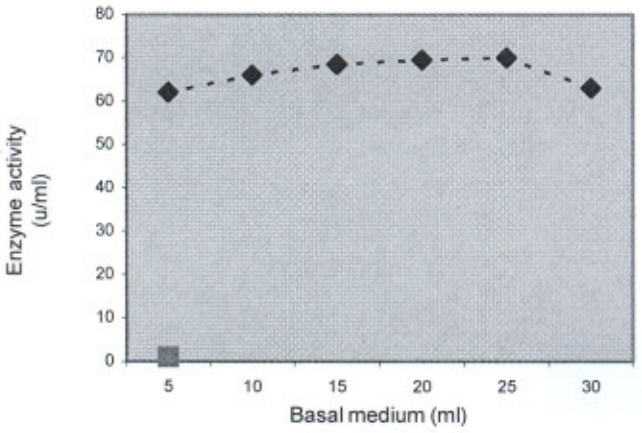

Fig.2. Effect of aeration on penicillin amidohydrolase production by *Bacillus megaterium* in shake flasks.

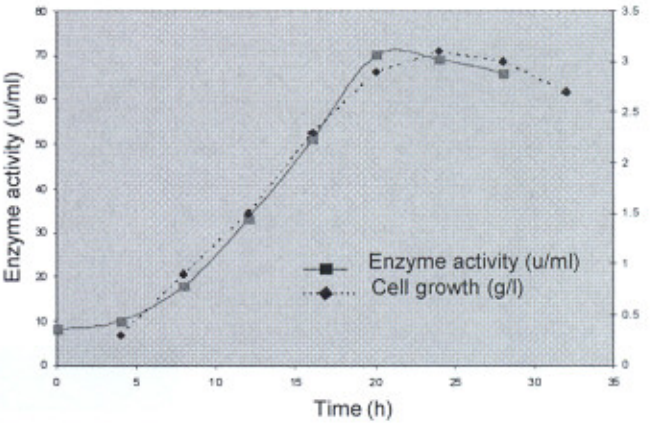

Fig.3. Time course of penicillin amidohydrolase activity and cell growth of *Bacillus megaterium* in shake flasks.

with the hypothesis of Lockhart and Squires (1963) that although sufficient oxygen must be provided to permit adequate metabolism, aeration above a certain level may induce an oxidation-reduction potential that may be unsuitable for enzyme formation or inhibit the function of essential sulfhydryl groups of the enzyme.

Effect of incubation temperature on the production of enzyme. Table 2 shows the biomass growth of *B. megaterium* 5B cultured at three different temperatures (35, 37 and 40 °C). The

Table 2. Effect of different temperatures on the production of amidohydrolase penicillin by *Bacillus megaterium* 5B

Incubation temperature (°C)	Cell mass (g/l)	Enzyme activity (u/ml)
35	2.7	62
37	3.1	70
40	2.0	59

cell mass after 24 h incubation was 2.7 mg/l at 35 °C. With the increase in temperature, the cell mass increased upto 3.1 g/l, yielding 70 u/ml of enzyme activity at 37 °C. Further increase in temperature resulted in the decrease of cell mass (2.0 g/l) as well as enzyme activity (59 u/ml). Similar temperature dependent behaviour was noted in the case of some strains of *E. coli*, which showed the maximum enzyme activity at 25 and 27 °C (Marancenbaum and Park, 1979).

Time course influence on cell growth and enzyme production in shake flask. Rate of fermentation was studied in 250 ml shake flasks. The enzyme production initiated slowly after the inoculation and increased gradually up to 8 h. A drastic increase was found between 8 and 24 h of incubation, which shows that the production of enzyme was related to cell growth. The cell growth was also monitored during the course of enzyme production. The cell mass at zero hour of incubation was 0.3 mg/ml. It increased exponentially during the course of fermentation and reached the maximum after 24 h of fermentation. Further incubation resulted in decrease in cell growth as well as the enzyme activity (Fig.3). Sunaga *et al.* (1976) reported that maximum enzyme activity occurred after 20 to 24 h of incubation of *B. megaterium* cultures. Similar growth behaviour of *B. megaterium* during the production of penicillin amidohydrolase in 500 ml shake flasks was reported by Son *et al.* (1982).

Conclusion

From the above studies it is concluded that the strain 5B of *B. megaterium* produced maximum activity (70 u/ml) of penicillin amidohydolase in 25 ml of the fermentation medium (pH 7.0) containing 3.1 g/l of sucrose after 24 h of incubation at 37 °C.

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