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PRODUCTION OF BACITRACIN BY *BACILLUS LICHENIFORMIS*

M.A. Qadeer, O. Younus, Syed Rehan Ashfaq and F.Z. Khan*

PCSIR Laboratories, Lahore-16

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The production of antibiotic bacitracin was studied in starch-glucose-soybean meal medium by *Bacillus licheniformis* PCSIR-72 in shake flasks. The effect of the replacement of (i) soybean meal by sunflower or pharmamedia and (ii) glucose by mannose, sucrose, lactose or beet molasses was investigated. The production of the antibiotic, however, was found to be maximum in the presence of soybean meal and sucrose or mannose. The pH near neutral was optimum for maximum bacitracin production. The effect of aeration by changing the volume (25-100 ml) of the basal medium in 250 ml flask was studied. The antibiotic titre was maximum in flasks containing 25 ml basal medium. Scaled up production of antibiotic in 10 litre glass-stainless steel fermenter was very fast and reached maximum in 28 hr. after inoculation instead of 44 hr. in shake flasks.

Key words: Bacitracin, *Bacillus licheniformis*, Antibiotic.

INTRODUCTION

The antibiotic bacitracin is commercially produced by thermophilic bacteria such as *Bacillus subtilis* and *B. licheniformis*. Bacitracin, being very active against many gram positive and few gram negative bacteria, finds industrial applications as supplement in poultry feed as it increases feed efficiency and reduces the incidence of infections diseases [1-4]. The first approach to bacitracin production was the surface culture method [5-6]. Currently the antibiotic is manufactured using submerged culture and a strain of the *Licheniformis* group of *Bacillus subtilis* [7]. Several studies have been carried out on the medium composition for optimum yield of the antibiotic [8-10]. Regulatory inter-relationship between the primary and secondary metabolites of bacterial cultures for antibiotic formation has also been studied by many workers [11-13].

The present study describes the synthesis of antibiotic by *B. licheniformis* in shake flasks using starch-glucose-soybean meal medium. The evaluation of other nitrogenous sources such as sunflower meal or pharmamedia, by replacing soybean meal, was also investigated. Replacement of glucose by other sugars such as sucrose, lactose, mannose or beet molasses in the fermentation medium and their effects on antibiotic formation was studied.

MATERIALS AND METHODS

Organism. B. licheniformis PCSIR-72, derived from NRRL B-1001 after UV treatments, was used for the production of antibiotic bacitracin. The culture was maintained on tryptone-glucose-Y.E.– agar medium consisting of (g/1) tryptone, 5.0; glucose, 1.0; yeast extract, 2.5; and agar 15.0 (pH 7.0). All the media were sterilized at 121° for 15 min. The culture slants were incubated at 35-37° for 48 hr and then kept in a refrigerator for further use.

Inoculum preparation. The bacterial cell suspension (10^6 cells/ml) was prepared by scrapping growth aseptically from 48 hrs old agar slants. 6 % v/v of the cell suspension was used as an inoculum for shake flask studies.

For scaled up studies in 10 litres glass-stainless steel fermenter, however, a 20-hr old vegetative inoculum was developed at 35-37° as shake culture (Orbital Shaker Model MK III, L.H. Engineering Col. Ltd., U.K) in a 250 ml conical flask, containing 50 ml medium.

Fermentation procedure. Several culture media were screened for bacitracin formation in 250 ml shake flasks containing 25 ml basal medium (Table 1). The shake cultures were incubated at 37° for 45 hr. The initial pH of all the media was adjusted to 7.0.

Scaled-up studies in 10 L glass-stainless steel fermenter, using medium (M_4), were carried out. The working volume of the fermenter was 5 litres. The basal medium was sterilized in autoclave at 121° for 30 min. and aseptically transferred to the fermenter which had been sterilized by steaming for 45 min. at 121°. The fermentation medium was inoculated with 6 % v/v of 20 hr. old inoculum. The rate of agitation and aeration was 200 rpm and L/L/min., respectively. Peanut oil was used as antifoaming agent. The temperature was maintained at 37° by circulating water through the coils. Air was sterilized by passing through glass wool tube.

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*Faculty of Pharmacy, Punjab University, Lahore.

Table 1. Composition of culture media.

Medium	M_1(g/L)	M_2(g/L)	M_3(g/L)	M_4(g/L)	M_4a(g/L)	M_5(g/L)	M_6(g/L)	M_7(g/L)
Soybean meal	45.00	–	–	50.00	50.00	–	30.00	30.00
Sunflower meal	–	–	74.00	–	–	–	30.00	30.00
Pharmamedia	–	–	–	–	5.00	60.00	–	–
Glucose	–	–	–	5.00	10.00	–	5.00	–
Starch	10.00	–	10.00	10.00	6.00	–	10.00	10.00
Cal. pantothenate	–	–	2.5-5.0	–	–	–	–	5.00
Yeast extract	3.00	–	–	–	–	–	–	–
Sodium chloride	0.01	–	–	–	–	–	–	–
L-Glutamic acid	–	20.00	–	–	–	–	–	–
Citric acid	–	1.00	–	–	–	–	–	–
$MnSO_4.H_2O$	–	0.01	–	0.01	–	–	0.01	–
$MgCl_2$-H_2O	–	0.20	0.015	–	–	–	–	0.02
Na_2SO_4	–	0.50	–	–	–	–	–	–
$CaCl_2$-$2H_2O$	–	0.01	–	–	–	–	–	–
$FeSO_4$-$7H_2O$	–	0.01	–	–	–	–	–	–
NaH_2PO_4-$2H_2O$	–	2.00	–	–	–	–	–	–
KCl	–	0.5-	–	–	–	–	–	–
K_2HPO_4	1.00	–	–	–	–	–	–	–
$(NH_4)_2SO_4$	2.00	–	–	–	–	–	–	–
$CaCO_3$	5.00	–	5.00	–	–	–	–	–
$MgSO_4$-$7H_2O$	0.20	–	–	1.00	1.00	–	1.0	–

Note: All the experiments were run in triplicate and average potencies were taken.

Antibiotic activity. The fermented mash was centrifuged at 4,000 rpm and clear supernatent was used for antibiotic activity by the agar diffusion method [14] using *Micrococcus luteus* CN 5537, kindly supplied by Wellcome Laboratories, Karachi.

RESULTS AND DISCUSSION

Screening of culture media. The composition of culture medium has great influence on the synthesis of microbial end-products. Fig. 1 shows the synthesis of antibiotic in different culture media. Medium M_4, containing soybean meal, gave highest bacitracin yield (47.50 i.u/ml). The antibiotic activity in the fermented mash was determined 44-48 hr. after inoculation. Soybean meal replacement by sunflower meal (M_3) gave lesser yield of antibiotic(38.0 i.u/ml). Partial replacement of soybean meal with sunflower meal (M_6) also did not show any increase in the antibiotic production. However, the incorporation of calcium pantothenate in the basal medium (M_7) slightly increased the production of bacitracin (42.4 i.u/ml). The use of only Pharmamedia [cottonseed meal] (M_5) gave

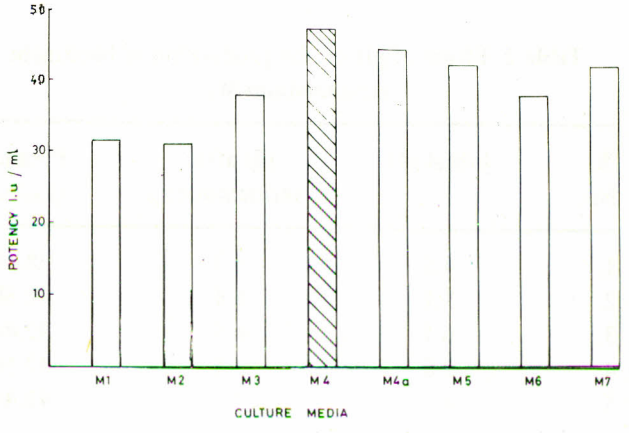

Fig. 1. Screening of culture media for the production of bacitracin.

encouraging results for antibiotic production (42.4 i.u/ml). Pharmamedia, therefore, seems to provide all the nutrients needed for antibiotic synthesis and cell formation. Thus nitrogenous sources, providing various amino acids, are most suitable for synthesizing polypeptide antibiotic bacitracin. This is in accordance with the reports made earlier [12, 15-17]. The variation in the amount of bacitra-

cin produced in different media may be due to the alteration in the amino acid pool which is believed to influence the biosynthesis of bacitracin [15].

The antibiotic production by *B. licheniformis* was also studied in the presence of different levels of Pharmamedia as the sole nitrogen source ranging from 0.1-0.6 % w/v (Fig. 2). The optimum level of nitrogen was found to be 0.3 % w/v and further increase in its concentration did not show any improvement in antibiotic formation (Fig. 2).

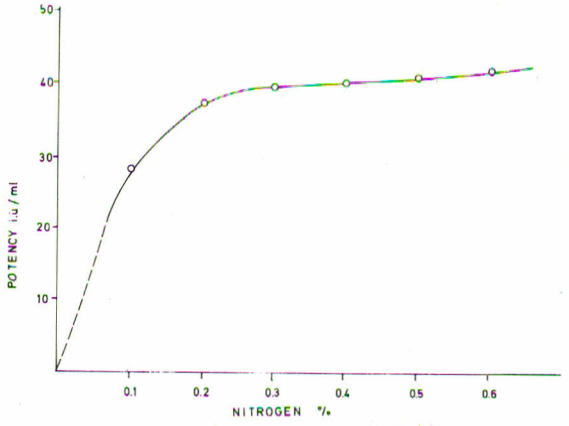

Fig. 2. Effect of various concentrations of pharmamedia on the production of bacitracin.

Effect of pH. The pH of the fermentation medium, in general, has great influence on the production of microbial metabolites. Table-2 shows the effect of varying initial pH of the fermentation medium (M_4) on the biosynthesis of bacitracin by *Bacillus licheniformis*. The initial pH ranged from 4.5-8.0 and it was not controlled during fermentation. It was observed that 45 hours after inoculation, the final pH of the basal medium in all experiments was about neutral. The bacitracin formation was lower when the initial pH of the medium was 4.5 (28.40 i.u/ml) and it was increased with the increase in the initial pH. The production of bacitracin was maximum (47.5 i.u/ml) when the initial pH of the medium was 7.0. Thus pH near neutral was found to be optimum for the biosynthesis of bacitracin. At pH 8.0, however, the production of bacitracin was decreased. These observations are different from the results obtained by Hendlin [18]. The worker observed that the bacterium *Bacillus subtilis* was incapable of producing antibiotic at pH 4.5. However, optimum bacitracin titre was obtained at pH near neutral. The inhibition of bacitracin production at low pH is believed to be due to the inhibitory effect on the enzyme activities [19].

Table 2. Effect of pH on the production of bacitracin using medium M_4.

S. No.	Initial pH	pH after fermentation	Potency (i.u/ml)
1.	4.5	6.3	28.40
2.	5.1	6.4	42.30
3.	6.1	6.5	42.44
4.	7.0	7.5	47.50
5.	8.1	7.2	42.44

Effect of carbohydrates. The production of bacitracin by *B. licheniformis* was investigated in the presence of different carbon sources, replacing glucose and starch in medium M_4. The sugar level was kept at 0.5 % w/v in all the experiments (Table 3). The antibiotic activity was maximum in the presence of starch. The basal medium without any sugar added gave 26.00 i.u/ml of bacitracin. If follows therefore that soybean meal also provides nutrients both for bacterial growth and antibiotic formation. The addition of sugar, however, increases the synthesis of bacitracin.

Table 3. Effect of organic carbon sources on the production of bacitracin in medium M_4.

Carbon sources (0.5 %)	Potency (i.u/ml)
Glucose	31.50
Sucrose	31.50
Starch	35.60
Lactose	22.25
d-mannose	31.15
Beet molasses	26.00
Blank (without sugar)	26.00

The optimum level of the carbohydrates was also determined by varying the concentration of starch, glucose and sucrose in the medium M_4, for antibiotic formation (Fig. 3). The sugar level ranged from 0.1-1.5 % w/v. The production of bacitracin was maximum when the sugar level was 0.5 % w/v and decreased with increase in the concentration of all the carbohydrates. The reduction in the bacitracin production is probably due to the long duration of low pH produced by sugar metabolism [20]. Also the production of many antibiotics including bacitracin is supposed to be controlled by catabolites repression by glucose [19].

Effect of metal ion. Metal ions play an important role in the metabolism of cell. It is believed that metal ions play

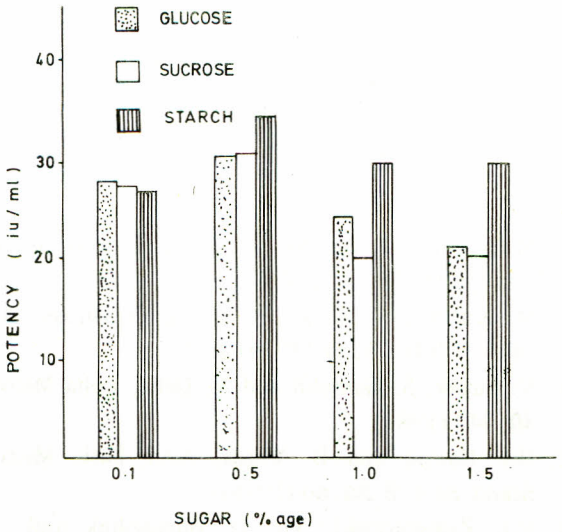

Fig. 3. Effect of various concentrations of organic carbon sources on the production of bacitracin in medium M_4.

a vital role in the utilization of chemical energy by enzyme bacitracin synthetase [21]. Table 4 shows the effect of the addition of magnesium ion on the biosynthesis of

Table 4. Effect of Mg^{+2} ion on bacitracin production using medium M_4.

S. No.	Mg^{+2} ion ($mg\ l^{-1}$)	Potency (i.u/ml)
1.	00.0	24.24
2.	50.0	46.14
3.	100.0	46.14
4.	200.0	46.14
5.	250.0	46.14

bacitracin. The control culture without adding this metal ion was also run in parallel. The amount of Mg^{+2} ion added was 50-250 mgl^{-1}. The activity of antibiotic bacitracin in fermentation medium, without adding Mg^{+2}, was 24.24 i.u/ml. The concentration of Mg^{+2} ion which gave the highest titre of bacitracin was found to be 50 mgl^{-1}. Further increase in the Mg^{+2} ions, however, did not show any stimulatory effect on the production of bacitracin. Thus the presence of this metal ion is essential for bacitracin

Effect of aeration. The availability of oxygen to the microbes during fermentation plays an important role in the synthesis of secondary metabolites. The effect of aeration on bacitracin production in Erlenmeyer flasks was investigated by varying the volume of the medium M_4 from 25-100 ml, while maintaining the other conditions constant (Fig. 4). The supply of oxygen (aeration) was

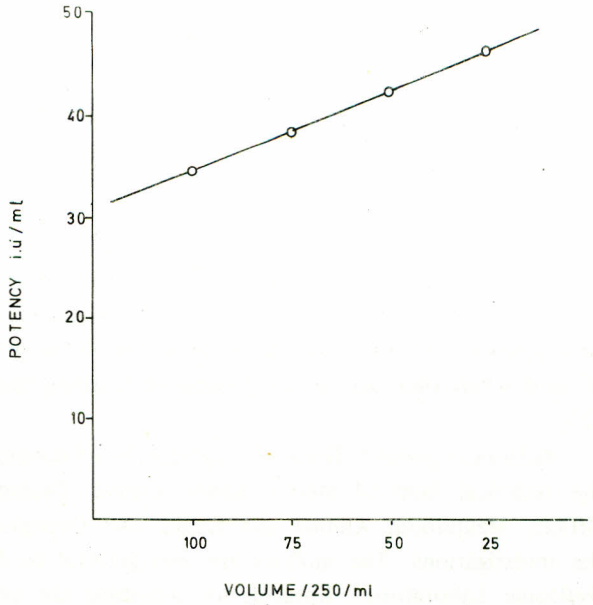

Fig. 4. Effect of aeration on the production of bacitracin using medium M_4.

affected by increasing the volume of basal medium in the flask. Since the agitation (or mixing) of the culture medium was decreased with increase in the volume of medium in the flasks, the amount of antibiotic titre was maximum in flasks containing 25 ml medium (46.29 i.u/ml) and it decreased with increase in the volume of the basal medium. Hendlin [18] has reported that variation in the volume of medium only affected the rate of antibiotic production but exercised no significant effect on bacitracin yield.

Production of bacitracin in stirred fermenter. The production of antibiotic bacitracin, after shake flask studies, was carried out in 10 L glass-stainless steel fermenter using medium M_4. The fermenter was run for 50 hr and the activity of antibiotic and pH changes during fermentation were determined (Fig. 5). The rate of antibiotic

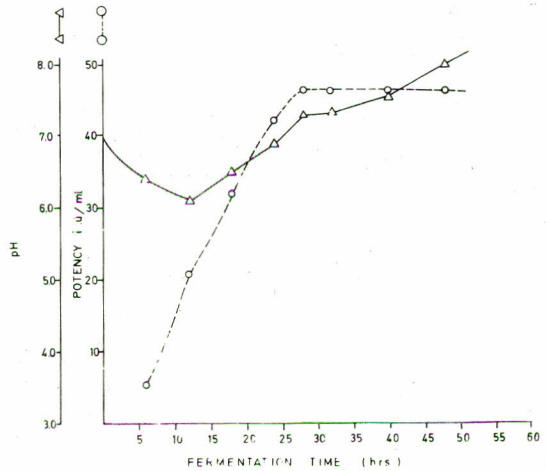

Fig. 5. Production of bacitracin by *B. licheniformis* – PCSIR-72 in 10 litres glass-stainless steel fermenter.

formation in the fermenter was very fast as compared with shake flask experiments described earlier. It was due to better aeration and agitation, and the antibiotic titre obtained in the fermenter was maximum in 28-30 hr in comparison with shake flasks where maximum activity was obtained 44-46 hrs after incoluation. The initial decline in the pH observed may be due to the organic acids produced by glucose metabolism. Similar reports have been made earlier [22]. Bacitracin was produced mainly during the later stages of growth or during the post-logarithmic phase of cells when they are in the process of forming spores [23].

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REFERENCES

1. P.N. Koturnanoy and N.K. Kapustin, Tr. Beloruss, Sel'skokhoz. Akad., **90**, 26 (1971). (Chem. Abstr., **79**, 121789d (1973).
2. M.V. Shalak, Tr. Beloruss, Sel'skokhoz Akad., **90**, 42 (1971), (Chem. Abstr., **79**, 121709x (1973).
3. L. Salvik, Agrochemia. **13**(6), 175 (1973), (Chem. Abstr., **78**, 96190w (1973).
4. J. Smejkal, M. Vavrova, L. Slavik, Z. Frydrych. Fleischwritschaft, **53**(1), 91 (1973), (Chem. Abstr. **78**, 96190W (1973).
5. H.S. Anker, B.A. Johnson, J. Goldberg, F.L. Melency, J. Bact., **55**, 249 (1948).
6. W.L. Keko, R.R. Benett and F.C. Arzberger, U.S. Pat . **2**, 627 494 (1953).
7. S.C. Harvey, Remington Pharm. Sci., **16**, 1144 (1980).
8. F. Smekal, P. Franstiska, V. Vladimir, Czech., **175**, 992 (CL-C12) D 9/22). 3 pp (1979).
9. M. Kurima, S. Eisceke, K. Rojoj, O. Hiroshi, Japan, 7446, 079 (Cl. 12d), (1974), p. 3.
10. M. Kurima, S. Eiscke, K. Rajoj, O. Hiroshi, Japan, 7446, 079 (Cl. 12d), (1974), p. 3.
11. V. Supek, S. Gamulin and V. Delic, Folia Microbiol, **30**, 342 (1985).
12. H.I. Haavik and B. Vessia, Acta, Path. Microbiol. Scand. Sect. B., **86**, 60 (1978).
13. V.I. Zvingorodski, Yu. D. Bugouchuk and D.A. Kuzovunikova, Antibiotiki, **26** (9), 647-51 (1981).
14. H.I. Haavik and S. Thomassen, J. Gen. Microbiol. **76**, 451 (1973).
15. H.I. Haavik, Folia, Microbiol. **24**, 234 (1979).
16. H.I. Haavik, FEMS Microbiol. Lett. **10** (2), 111 (1981).
17. H.I. Haavik, O. Froyshov, Pept. Antibiot. Biosynth. Funct. Pap Mect. J., Ed: Klein Kauf, Horst, Von Doehren; Hans de gruyter. 155 (1982).
18. D. Hendlin, Arch. Biochem. **24**, 435 (1949).
19. A.L. Demain, Lloydia, **31**, 395 (1968).
20. H.I. Haavik, J. Gen. Microbiol. **84**, 321 (1974)
21. O. Froyshuv, A. Mathesen, H.I. Haavik, J. Gen. Microbiol. **117**, 163 (1980).
22. H.I. Haavik, J. Gen. Microbiol **84**, 226 (1974)
23. R.W. Bernlohr, G.D. Nuvelli, Arch. Biochem. Biophy, **103**, 94 (1963).