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BIOSYNTHESIS OF BACITRACIN IN SOLID-STATE FERMENTATION BY *BACILLUS LICHENIFORMIS* USING DEFATTED OIL SEED CAKES AS SUBSTRATE

KALSOOM FARZANA[+], SYED NISAR HUSSAIN SHAH, FAROOQ BASHIR BUTT* AND SATTAR BUKHSH AWAN**

Department of Pharmacy, B.Z. University, Multan, Pakistan
**Punjab Pharmacy Council, Lahore, Pakistan*
***Department of Pharmaceutics, Gomal University, D.I. Khan, Pakistan*

Bacitracin is being imported in Pakistan involving substantial amount of foreign exchange for its incorporation in poultry feed. The cheap raw material for its production is readily available and cheap such as soybean meal, sunflower meal, wheat bran etc. Thus development of this technology in our country would result in saving a reasonable amount of foreign exchange by exploiting indigenous resources.

The present study is concerned with the biosynthesis of antibiotic bacitracin in solid-state fermentation by *Bacillus licheniformis* on laboratory scale using defatted oil seed cakes of agricultural bye-products as starting material for maximum production of the antibiotic Bacitracin.

In solid-state fermentation, wheat bran, soybean meal, sunflower meal, rice hulls and their different combinations were used. The antibiotic activity, 48 hours after inoculation was 4375 i.u / gm when only soybean was used. However, maximum titre 4820 i.u / gm of antibiotic was obtained using wheat bran and soybean meal in ratio of 1:3.

Keywords: Biosynthesis, antibiotics, bacitracin, fermenter, inoculation, defatted oil seed.

INTRODUCTION

Bacitracin consists of one or more of the antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* and by *Bacillus subtilis* var Tracy and yielding on hydrolysis the amino acids L-cysteine, D-glutamic acid, L-histidine, D-phenylalanine, L-lysine, L-isoleucine, L-leucine, D-ornithine and DL-aspartic acid (BP 2002).

Bacitracin is produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* and functions as an inhibitor of cell wall biosynthesis (Azevedo *et al.,* 1993). Bacitracin is a peptide antibiotic nonribosomally produced by *Bacillus licheniformis* (Ohki *et al.,* 2003).

Different types of bacitracin like A, A1, B, C, D, E, F, F1, F2, F3 and G have been isolated. The most potent antibiotic is Bacitracin A, whereas Bacitracin B & C are less potent and the rest posses very little antibacterial activity. This antibiotic is most effective against Gram +ve and a few Gram –ve species of bacteria. It is almost exclusively used as a topical preparation in the treatment of infections (Brunner, 1965).

Bacillus licheniformis, a bacitracin producer, has an ABC transporter system which is hypothesized to pump out bacitracin for self-protection (Podlesek, 1995).

It is also widely used as supplement in poultry nutrition to control infection, increase the growth promotion and effect the activity and synthesis of certain liver enzymes in laying hens (Shalak, 1971; Rybinska, 1977, Smekal, 1979). There seems not to be any problems with residues, bacterial resistance, or effects on the environment when bacitracin is used at nutritional levels up to 100 PPM (Rosen, 1980).

MATERIALS AND METHODS

Organism

Bacillus licheniformis was used for the production of antibiotic bacitracin.

The culture was maintained on tryptone-glucose – Y.E agar medium.

Table 1
Tryptone-glucose-Y.E agar medium

Tryptone	5 gm/litre
D-glucose	1 gm/litre
Yeast extract	2.5 gm/litre
Agar	15 gm/litre

pH of the medium was adjusted to 7.0 after dissolving all the ingredients in distilled water except agar which was added at the end. The medium was poured into test tubes and sterilized at 121^{o}C for 15 minutes. The medium of test tubes was allowed to congeal in slanting position. Slants thus formed were inoculated with Bacillus licheniformis and incubated at 37^{o}C for 48 hours and then kept in refrigerator for experimental work.

[+]*Correspondence*

Inoculum preparation

The bacterial growth was aseptically scrapped from 48 hours old agar slants and transferred to 50 ml sterilized basal medium (Table-2) in 250 ml conical flask and shaken on rotary shaker at 150 rpm for 24 hours at 37°C. The vegetative culture thus obtained was used for inoculation into fermentation medium.10% v/v inoculum was used in this study as given in the following table-2.

Table 2
Basal media

Peptone	10.0 gm/litre	7.0 pH
Glucose	5.0 "	"
Beef extract	5.0 "	"
Sodium chloride	2.5 "	"
$MnCl_2$	0.167 "	"

Fermentation media for bacitracin production

Fermentation media used for the production of antibiotic bacitracin by *Bacillus licheniformis* is given in the following table-3.

Table 3
Fermentation media

Citric Acid	1.0 gm/litre
Glucose	0.5 "
KH_2PO_4	0.5 "
K_2HPO_4	0.5 "
$MgSO_4.7H_2O$	0.2 "
$MnSO_4.4H_2O$	0.01 "
$FeSO_4.7H_2O$	0.01 "
Soybean/or sunflower meal or wheat bran or rice hulls etc.	45.0 "

The medium was sterilized at 121°C for 15 minutes at pH 7, distilled water was used in the preparation of media. These mixtures in different ratio were also used in some experiments.

Fermentation technique (Method)

10 gm of the substrate was taken in 250 ml of conical flask. It was wetted by 10 ml of distilled water previously adjusted to pH 7 or phosphate buffer of pH 7 was used. Medium (table 3) was autoclaved at 121°C for 15 minutes, it was allowed to cool and then was inoculated with 1ml of seed culture. After inoculation, the flasks were shaken well and then incubated at 37°C for 48 hours. At the end of fermentation period, the fermented material was soaked in N/100 HCl for 1hour and then centrifuged. The supernatant layer was assayed for calculation of antibiotic activity.

Rate of production of bacitracin by *Bacillus licheniformis* in wheat bran by solid-state fermentation is given in the following table 4.

Table 4
Rate of production of bacitracin
by *Bacillus licheniformis* in wheat bran

No. of observation	Fermentation period (Hours)	Potency (i.u/gm)
1	12	140.9
2	24	402.6
3	36	2214.0
4	48	3134.0
5	60	2852.8

Effect of different oil seed cakes on the production of bacitracin

Bacitracin consists of a group of closely related peptides. Thus effect of different defatted oil seed cakes as a source of amino acids, vitamins, minerals and sugars were investigated as in table-5.

Assay

The activity of the antibiotic bacitracin present in the fermented material was determined by agar diffusion method. The composition of the Nutrient agar medium is given as in table 6.

Table 6
Nutrient agar media

Beef extract	1.0 gm/litre
Yeast extract	2.0
Sodium chloride	5.0
Peptone	5.0
Agar	15.0

The pH of the medium was adjusted to 7.0 before the addition of agar. The medium was sterilized at 121°C for 15

Table 5
Effect of different substrate on the production of bacitracin by solid-state fermentation

Substrate	Quantity (gm/flask)	Incubation temp.	Duration (hrs.)	Potency (i.u/gm)
Soybean meal	10	37°C	48	4375
Wheat bran	10	37°C	48	3134
Sunflower meal	10	37°C	48	1615
Rice hulls	10	37°C	48	475

minutes. The nutrient agar medium was used for bioassay. About 4ml of melted assay medium which was previously inoculated with the pre-determined concentration of test organism i.e. *Micrococcus luteus* (CN5537), was spread uniformly over the first layer and was allowed to congeal. After setting the second layer, four wells (of 0.8 cm in diameter) were made in the plates, aseptically with stainless steel borer of uniform edge and size. Standard solution (45 i.u/ml) of bacitracin was prepared by dissolving 65.2mg of Zn bacitracin in 100 ml of N/100 HCl. The dilution of standard solution was made in N/100 HCl.

Two opposite holes were filled with working standard of 1:4 dilution (S_1, S_2) and the remaining two were filled with sample (T_1, T_2) using 1 cc insulin syringe. About 0.12 ml solution was poured in each well. The plates were incubated for 24 hours at 37^{o}C. Clear zones of inhibition were developed both by standards and samples.

Diameter of zones of inhibition were measured and compared with the known standard. The potency of the sample was determined by the following formulae:

1. Difference due to dose; $E = ½ (T_2+S_2) – (T_1+S_1)$
2. Difference due to sample; $F = ½ (T_2+T_1) – (S_2+S_1)$
3. Log ratio of doses; $I = \log 4 = 0.602$
4. Slope; $B = E / I$
5. Potency ratio = Antilog of M; where $M = F / B$
6. Potency of sample = Antilog of M x Potency of standard

RESULTS AND DISCUSSION

The production of antibiotic by solid state fermentation involves less consumption of energy compared to stirred fermenters where continuous aeration, agitation and control of foaming are necessary.

The rate of production was determined by using wheat bran as solid substrate. The antibiotic activity was determined after every 12 hours during the course of fermentation (table 4).The antibiotic activity reached maximum (3134 i.u/gm), 48hours after inoculation. Further increases in fermentation period resulted in decline of bacitracin activity. It may be attributed to inhibition of “Bacitracin Synthetase” enzyme by bacitracin itself by feedback mechanism.

Rate of fermentation by solid-state method was faster than that in shake-flask but slower than that in stirred fermenter. Data of table 5 show that synthesis of bacitracin was maximum in of soybean meal (4375 i.u/gm) while amount of bacitracin produced in sunflower meal was 1615 i.u/gm. wheat bran also gave good antibiotic titre i.e. 3134 i.u/gm but rice hulls only produced 475 i.u/gm. The reason of low antibiotic production by rice hulls may be of its being poor source of carbon and nitrogen while soybean and wheat bran are ideal substrate providing all the nutrients required by *Bacillus licheniformis.*

Better yield of bacitracin by soybean meal and wheat may also be due to the porosity of the substrate, hence better supply of oxygen to the culture resulted in greater antibiotic yield.

Organic and inorganic contents are considered as an indicator of rich nitrogen source of media (Varvel, 1994).

The conditions like pH, temperature, aeration, different ratio of substrates as nitrogen sources and other parameters were optimized.

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