ORIGINAL ARTICLE

EFFECT OF PARTIAL REPLACEMENT OF DIFFERENT DEFATTED OIL SEED CAKES AS SUBSTRATE IN BIOSYNTHESIS OF BACITRACIN IN SOLID-STATE FERMENTATION BY *BACILLUS LICHENIFORMIS*

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ABSTRACT

The present study was conducted to ascertain the effect of partial replacement of different defatted oil seed cakes as substrate i.e. sunflower meal, rice hulls and soybean meal, in biosynthesis of Bacitracin in Solid-State Fermentation by *Bacillus licheniformis* on laboratory scale.

In solid-state fermentation, wheat bran, soybean meal, sunflower meal, rice hulls and their different combinations were used. The antibiotic activity was determined at various intervals and recorded 48 hours gave maximum yield, 4375 i.u/gm when only soybean was used. However, maximum titre 4820 i.u / gm of antibiotic were obtained when wheat bran and soybean meal was in ratio of 1:3. The raw material for its production is readily available and cheap such as soybean meal, sunflower meal and wheat bran. Thus development of this technology in our country would result in utilizing our own resources in Pakistan.

Keywords: *Bacillus licheniformis*, 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 (B.P., 2002). The effects of controlling the dissolved oxygen tension at 0.01, 0.02 and 0.05 atm by the use of oxygen-enriched aeration were investigated during growth and bacitracin production by *Bacillus licheniformis* ATCC 10716. Up to a 2.35-fold increase in the final antibiotic yield and a 4-fold increase in the rate of bacitracin synthesis were observed in response to O_2-enriched aeration (Flickinger and Perlman, 1979). Biosynthesis of bacitracin performed successfully in an airlift bioreactor by *B. licheniformis* in submerged aerobic cultivation. It was also observed that power requirements in airlift bioreactors were 17-64% lower than mechanically agitated system, depending on aeration rate (Gavrilescu and Roman, 2004). Biosynthesis of bacitracin in solid-state fermentation by *B. licheniformis* on laboratory scale was prepared by using defatted oil soybean seed cakes, wheat bran, sunflower meal, rice hulls and various combinations. The activity was found maximum at 30 and 48 hours, with defatted soybean meal, soybean meal, sunflower meal and wheat bran (Farzana *et al*., 2004; Farzana *et al*., 2005).

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

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

It is also widely used as supplement in poultry nutrition to control infection, increase the growth promotion and affect the activity and synthesis of certain liver enzymes in laying hens (Rybinska, 1977; Grady and Greenwood, 1997). 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). The present study was designed to investigate the bacitracin production biosynthetically for incorporation in poultry feed to get foreign exchange.

MATERIALS AND METHODS

The present study was conducted to ascertain the effect of partial replacement of different defatted oil seed cakes as substrate i.e. sunflower meal, rice hulls and soybean meal etc., (agricultural bye-products as starting material for maximum production) in biosynthesis of bacitracin in Solid-State Fermentation by *B. licheniformis* on laboratory scale.

The culture was maintained on Tryptone-glucose – Yeast Extract agar medium (table 1) 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°C for 15 minutes. The medium of test tubes was allowed to congeal in slanting position. Slants thus formed were inoculated with *B. licheniformis* and incubated at 37°C for 48 hours and then kept in refrigerator for experimental work.

Inoculum preparation

The bacterial growth was aseptically scrapped from 48 hours old agar slants and transferred to 50 ml sterilized basal medium (table 1) in 250 ml conical flask and shaken on rotary shaker (like orbital shaker) at 150 rpm for 24 hours at 37°C. The vegetative culture thus obtained was used for inoculation into fermentation media, 10% v/v inoculum was used in this study.

Fermentation media for bacitracin production

Fermentation media used for the production of antibiotic Bacitracin by *B. licheniformis* is given in the (table 1).

Table 1: Composition of various media used in study

Composition of 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
Composition of Basal Medium	
Peptone	10 gm/litre
Glucose	5 gm/litre
Beef extract	5 gm/litre
Sodium Chloride	205 gm/litre
Manganese Chloride	0.167 gm/litre
Composition of Nutrient Agar	
Beef Extract	1 gm/litre
Yeast Extract	2 gm/litre
Sodium Chloride	5 gm/litre
Peptone	5 gm/litre
Agar	15 gm/litre
Composition of Fermentation media	
Citric Acid	1.0 gm/litre
Glucose	0.5 gm/litre
KH_2PO_4	0.5 gm/litre
K_2HPO_4	0.5 gm/litre
$MgSO_4.7H_2O$	0.2 gm/litre
$MnSO_4.4H_2O$	0.01 gm/litre
$FeSO_4.7H_2O$	0.1 gm/litre
Soybean or Sunflower meal or Wheat bran or Rice hulls etc	45.0 gm/litre

The media 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 experiments.

Fermentation technique (method)

Ten gm. of the substrate was taken in 250 ml of conical flask. It was wetted with 10 ml of distilled water previously adjusted to pH 7 or phosphate buffer of pH 7. Medium (table 1) 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 *B. licheniformis* in wheat bran by solid-state fermentation is given in the table 2.

Effect of partial replacement of wheat bran by different oil seed cakes

a. *Sunflower meal:* The data of the following table 1 shows the effect of replacing wheat bran by different amounts of defatted sunflower meal. The ratios of wheat bran: Sunflower meals were 10:0, 3:1, 1:1, 1:3 and 0:10. The amount of total substrate for fermentation was kept 10.0 gm/flask and incubated at 37°C for 48 hours. The antibiotic activity in the control flask containing wheat bran inoculated with culture was 3134 i.u/gm.

b. *Rice hulls:* The production of bacitracin in wheat bran substrate partially replaced with rice hulls in different ratio was studied, as shown in the table 1. The control experiment of 100% wheat bran was also run parallel which gave antibiotic titre 3134 i.u/gm.

c. *Soybean meal*: The following table 1 shows the effect of replacing wheat bran by different amounts of soybean meal on the production of antibiotic. The ratios of wheat bran to soybean meal were 10:1, 3:1, 1:1, 1:3 and 0:10. The amount of substrate for fermentation was kept 10 gm/ flask and fermentation media inoculated with culture after addition of 10 ml distilled water having pH 7 incubated at 37°C for 48 hours. The antibiotic activity in the control cultures containing wheat bran only was 3134 i.u/gm.

Assay

The activity of the antibiotic Bacitracin present in the fermented material was determined by agar diffusion method. The composition of the Nutrient agar media is given as in the table 1.

The pH of the medium was adjusted to 7.0 before the addition of agar. The medium was sterilized at 121ºC for 15 minutes. The nutrient agar medium was used for bioassay. Approximate 20ml of the medium was aseptically poured in the sterile petri-plates and was allowed to solidify. Then, 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 holes of diameter, 0.8cm 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 (S1, S2) and the remaining two were filled with sample to be determined of 1:4 dilution (T1, T2) using 1 cc insulin syringe. 0.12 ml solution was poured in each digged hole. The plates were then very carefully placed in incubator for 24 hours at 37°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 (T2+S2) – (T1+S1)
2. Difference due to sample; F = ½ (T2+T1) – (S2+S1)
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 as compared to stirred fermenters where continuous aeration, agitation and control of foaming are quite necessary.

The rate of production was determined by using wheat bran as solid substrate. The antibiotic activity was determined after every 12hours during the course of fermentation (tables 2 and 3). The antibiotic activity reached maximum (3134 i.u/gm), 48 hours after inoculation. Further increases in fermentation period resulted in decline of bacitracin yield. It may be attributed to inhibition of "Bacitracin Synthetase" enzyme by bacitracin itself by feedback mechanism. Our results are comparable with the previous studies, where the rate of fermentation by solid-state method was faster than that in shake-flask but slower than that in stirred fermentor (Gavrilescu and Roman, 2004; Farzana *et al*., 2004).

Table 2: Production of antibiotic at different interval time

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

The partial replacement of wheat bran by sunflower meal did not show any increase in the antibiotic formation. The presence of only defatted sunflower meal, however, showed a significant decline in the antibiotic production i.e. 1615 i.u/gm. It could be due to decrease in porosity of the sunflower meal as compared to wheat bran. Partial replacement of wheat bran by the sunflower meal gradually decreased the porosity of the substrate hence the supply of oxygen to the culture was decreased. Due to porosity of wheat bran, nutritional contents are readily available to the culture for the biosynthesis of bacitracin, so the higher antibiotic titre obtained in a flask containing wheat bran only.

Whereas in comparison to previous biosynthesis study in solid-state fermentation by *B. licheniformis* by using defatted oil seed cakes found more effective substrate

Table 3: Comparative activity of various media after 48 hours.

S. No.	Wheat bran (gm/flask)	Sunflower meal (gm/flask)	Incubation at 37ºC for hours	Potency for Sunflower meal (i.u/gm)	Potency for Rice hulls (i.u/gm)	Potency for Soybean meal (i.u/gm)
1	13.0	0.0	48	3134	3134	3134
2	7.50	2.5	48	2683	2282	2817
3	5.0	5.0	48	1989	906	3705
4	2.5	7.5	48	1736	683	4820
5	0.0	10.0	48	1615	475	4375

than single soybean meal (Farzana *et al.*, 2005).

Replacement of wheat bran by rice hulls greatly influenced the production of bacitracin and antibiotic production was decreased with gradual increase of rice hulls. Potency achieved by rice hulls is very low. The low antibiotic formation in rice hull was may be due to composition of the rice hulls. It may be due to poor nutritional content so poor supply of amino acids, sugars, minerals and vitamins, resulted in decreased production of bacitracin. Low antibiotic formation could also be due to low nitrogenous content and higher ash content of the rice hulls. In optimizing biosynthetic conditions like pH, temperature, aeration, different ratio of substrates as nitrogen sources and other parameters were studied (Shabbir *et al*., 2001). It is clear that medium composition and supply of oxygen are very important parameters affecting the antibiotic production.

Moreover higher yield by the combination mixture of wheat bran and soybean meal could be due to porosity and richness of the substrates in amino acids, vitamins, minerals, sugars as compared to other defatted oil seed cakes. These results are in accordance with the result reported by Qadeer and co-workers (1991). The present study indicated that combination of wheat and soybean meal are potentially economical, efficient and proved better substrate even for industrial production of bacitracin.

ACKNOWLEDGEMENT

We are very thankful to PCSIR Laboratories Lahore for providing valuable guidance and solving intricate problems encountered during this research work.

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