

Biosynthesis of Bacitracin in Stirred Fermenter by *Bacillus Licheniformis* Using Defatted Oil Seed Cakes as Substrate

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Abstract

Bacitracin is being imported in India involving substantial amount of foreign exchange for its incorporation in poultry feed. The raw material for its production is readily available and cheap such as soybean meal, sunflower meal & wheat bran. Thus development of this technology in this country would result in saving a reasonable amount of foreign exchange by utilizing our resources. The present study is concerned with the biosynthesis of antibiotic Bacitracin by *Bacillus licheniformis* on laboratory to scale up studies in Stirred Fermenter using defatted oil seed cakes of agricultural by-products as starting material for maximum production of the antibiotic Bacitracin. In stirred fermenter, antibiotic formation reached maximum (342 i.u. ml⁻¹), 30 hours after inoculation at 37 °C using different natural media such as defatted soybean meal, glucose and metal ions. 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 4540 i.u/g when only soybean was used.

Keywords: Antibiotics, Bacitracin, Defatted oil seed, Fermenter, Inoculation

1. Introduction and Literature Survey

Bacitracin is derived from cultures of *Bacillus subtilis* (Tracey). It is a white to pale buff, hygroscopic powder, odorless or having a slight odor. It is freely soluble in water; insoluble in acetone, chloroform, and ether. While soluble in alcohol, methanol, and glacial acetic acid, there is some insoluble residue. It is precipitated from its solutions and inactivated by many of the heavy metals.

The molecular formula is: C₆₆H₁₀₃N₁₇O₁₆S. *Bacitracin* is comprised of a polypeptide complex and *Bacitracin A* is the major component in this complex. The molecular weight of *Bacitracin A* is 1422.71.

Bacitracin consists of one or more of the antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* var Tracy and yields the Amino acids L-cysteine, D-glutamic acid, L-histidine, D-phenylalanine, L-lysine, L-isoleucine, L-leucine, D-ornithine and DL-Aspartic acid on hydrolysis (BP 2002) and functions as an inhibitor of cell wall biosynthesis (Azevedo, 1993). *Bacitracin* of other micro-organism is an antibiotic as well as non-ribosomally produced by *Bacillus licheniformis* (Ohki, 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* and *C* are less potent and the rest possess a very little antibacterial activity. This antibiotic is the 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 is a bacterium that is commonly found in soil and bird feathers. Birds that tend to stay on the ground more than the air (i.e. sparrows) and on the water (i.e. ducks) are common carriers of this bacterium; it is mostly found around the bird's chest area and back plumage.

B. licheniformis is part of the *subtilis* group along with *Bacillus subtilis* and *Bacillus pumilus*. These bacteria are commonly known to cause food poisoning and food spoilage. *B. licheniformis* also is known for contaminating dairy products. Food borne outbreaks usually involve cases of cooked meats and vegetables, raw milk, and industrially produced baby food contaminated with *B. licheniformis*.

This bacterium, although detrimental, can be modified to become useful. Researchers are trying to turn bird feathers into a nutritious livestock feed by fermenting non-digestible proteins on bird feathers with *B. licheniformis*. There is also research about the possibility that *B. licheniformis* causes changes in color in birds' feathers; this will provide information on the evolution of molting. Also, cultures of *B. licheniformis* are made to retain its protease, which is in turn used in laundry detergent.

Bacillus licheniformis, a Bacitracin producer, has an ABC transporter system which is hypothesized to pump out Bacitracin for self-protection (Podlesek, 1995). Bacitracin holds considerable importance. It is also widely used as supplement in poultry nutrition. Its addition to the feed increases feed efficiency and the incidence of infectious diseases are greatly reduced (Shalak, 1971; Smekal, et al., 1979). Zinc Bacitracin and Bacitracin methyl disalicylate (feed grade) are widely used for growth promotion. Addition of Bacitracin to the feed may affect the activity and synthesis of certain liver enzymes (Rybinska, 1977) and increase the level of proteases and amylases in the digestive tract of laying hens.

2. Materials and Methods

2.1 Organism

Bacillus licheniformis (ATCC 9945, 10716, 11945, 11946 and 14580- PCSIR 89 locally isolate) were collected from National Chemical Laboratory (N.C.L) in Pune-India and used for the production of antibiotic Bacitracin.

2.2 Gram Staining

Gram staining was carried out before Inoculum Preparation to ensure whether collected *Bacillus licheniformis* from N.C.L was pure.

2.3 Inoculum Preparation

The bacterial growth was aseptically scrapped from 48 hours old cultures lants and transferred to 50 ml sterilized basal medium (Table 1) in 250 ml conical flask and then shaken on rotary shaker at 150 rpm for 24 hours at 37 °C. The vegetative culture thus obtained was used for inoculation into fermentation media. 4% v/v inoculum was used in this study.

2.4 Fermentation Media for Bacitracin Production

Fermentation media used for the production of Bacitracin by *Bacillus licheniformis* is given in Table 2.

3. Fermentation Technique (Method)

10 g 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 2) 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 1 hour 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 3.

3.1 Effect of Different Oil Seed Cake 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 4.

The production of Bacitracin on laboratory scale was carried out in 30-L Glass Stainless Steel Fermenter (B.E. Marubishi – MSJ – N2, Japan). It was connected with bioprocess operator-MSSD-1 and bioprocess controller-MEDIAC-93. The basal medium was sterilized inside the fermenter automatically. Temperature, pH, agitation and foaming were controlled automatically. The fermentation medium (Table 2) which gave the best results in shake flasks was used. The fermenter was run for 48 hours. The antibiotic activity during fermentation was determined from time to time.

Fermentation phenomenon can be occurred in simple condition. A rotary shaker is required. The fermentation medium would be a flask which is plugged by cotton. Note that transfer of inoculum (basal) medium to fermentation flask must be done in absolute sterilized environment.

3.2 Assay

The activity of the antibiotic Bacitracin present in the fermented material was determined by agar diffusion method (Table 5).

The pH of the medium was adjusted to 7.0 with 1N NaOH/HCl before the addition of agar. The medium was sterilized at 121 °C for 15 minutes. Approximately 20 ml of the medium was aseptically poured into the sterile Petri-plates and allowed to solidify. Then, 4 ml of melted assay medium which was previously inoculated with the pre-determined concentration of test organisms i.e. *Staphylococcus aureus* and *Escherichia coli* were spread uniformly over the first layer and were allowed to congeal. After setting the second layer, four holes 0.8 cm of diameter were made in the plates aseptically with stainless steel borer of uniform edge and size.

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 micropipette. 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. Diameters 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): $E = \frac{1}{2} (T2 + S2) - (T1 + S1)$
2. Difference due to sample (F): $F = \frac{1}{2} (T2 + T1) - (S2 + S1)$
3. Log ratio of doses (I): $I = \log 4 = 0.602$
4. Slope (B): $B = E / I$
5. Potency ratio = Antilog of M, where $M = F / B$
6. Potency of sample = Antilog of M x Potency of standard

Where

S1 = Standard High (in concentration)

S2 = Standard Low (in concentration)

T1 = Test High

T2 = Test Low

3.3 Units of Bacitracin

One unit of Bacitracin activity is the amount of antibiotic in 0.2 ml of culture supernatant broth that will cause a 1 mm inhibition zone outside the cylinder (Bernlohr, & Novelli, 1960). One unit of Bacitracin is equivalent to 26 µg of USP standard (Harvey, 1980). The USP Unit of Bacitracin is the Bacitracin activity exhibited by the weight of USP Bacitracin Reference Standard indicated on the label of the Standard. It has a potency of not less than 40 USP Units of Bacitracinmg-1 (Nichols, 2000).

3.4 Isolation of Bacitracin by Centrifugation

The fermented broth was centrifuged at 10,000 rpm for 15 minutes in a refrigerated centrifuge at 4 °C in order to remove cells and solid suspended particles. The clear supernatant solution was used for the isolation of antibiotic.

3.5 Partitioning

Physical separation may be used for isolation, purification, identification and determination of organic compounds. It is, of course, generally a two-phase process, and can be classified into solid-liquid, liquid-liquid, gas-liquid and gas-solid systems. An exception is electrophoresis, which takes place in a single phase. Applications of physical separation may cut across these categories- for example; phase titrations may result in separation of a solid or a liquid phase from the initial liquid phase. Similarly, some of the factors associated with physical separation have implications for figurative separation.

4. 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 3). The antibiotic activity reached maximum (3287 i.u/g), 48 hours 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.

Data of Table 4 shows that synthesis of bacitracin was maximum in of soybean meal (4540 i.u/g) while amount of bacitracin produced in sunflower meal was 1330 i.u/g. wheat bran also gave good antibiotic titre i.e. 3287 i.u/g but rice hulls only produced 562 i.u/g. 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*.

4.1 Screening of Culture Media

The composition of the basal medium (Table 1) greatly influence the production of antibiotics. Replacing soybean meal with sunflower meal and/or wheat bran of same quantity (Table 2) in fermenting medium was used for the screening purpose. The nutritional studies were carried out.

The antibiotic activity in the fermented broth was determined, 44-48 hours after inoculation with 4% v/v bacterial cell suspension obtained from the slant surface. Of the medium tested, soybean meal medium (Table 2) gave the best results of antibiotic titer.

K₂HPO₄ and KH₂PO₄ were used as buffering agents, MnSO₄·7H₂O and MgSO₄·4H₂O as co-factors of enzymes while FeSO₄·7H₂O was used to assist the action of Manganese ion. Addition of citric acid leads to the formation of soluble coordinate complex with the metal ion thus making them available to the microorganism at adequate time (Haavik, 1976).

Organic and inorganic matter content is considered as an indicator of rich resources of media for Nitrogen source (Varvel, 1994). The conditions like pH, temperature, aeration, different ratio of substrates as nitrogen sources and other parameters were optimized (Shabbir, 2001).

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Table 1. Composition of basal medium

Materials	Content (g /L)
Peptone	10.0
Glucose	5.0
Beef Extract	5.0
Sodium Chloride	2.5
Manganese Chloride	0.167

Table 2. Composition of fermentation media

Materials	Content (g/L)
Citric Acid	1.0
Glucose	0.5
KH_2PO_4	0.5
K_2HPO_4	0.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.01
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
Soybean meal/Sunflower/or Wheat bran	45.0

The media was sterilized at 121 °C for 15 minutes at pH 7. All media were prepared in distilled water.

Table 3. Rate of production of bacitracin by *Bacillus licheniformis* in wheat bran

No. of observation	Fermentation period (Hours)	Potency (i.u/g)
1	12	135.0
2	24	411.0
3	36	2001.0
4	48	3287.0
5	60	3000.0
6	72	2133.0

Table 4. Effect of different substrate on the production of bacitracin by solid-state fermentation

Substrate	Quantity (g/flask)	Incubation temp.	Duration (hrs.)	Potency (i.u/g)
Soybean meal	10	37°C	48	4540
Wheat bran	10	37°C	48	3287
Sunflower meal	10	37°C	48	1330
Rice hulls	10	37°C	48	562

Table 5. Composition of nutrient agar media

Materials	Content (g/L)
Beef extract	1.0
Yeast extract	2.0
Sodium Chloride	5.0
Peptone	5.0
Agar	25.0

Note: Nutrient broth powder can be used instead of beef extract, yeast extract, Sodium Chloride and peptone, because it contains all mentioned components. It will be sufficient to dissolve Nutrient broth and agar powder in distilled water and sterilize it.