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BIOSYNTHESIS OF BACITRACIN IN STIRRED FERMENTER BY *BACILLUS LICHENIFORMIS* USING DEFATTED OIL SEED CAKES AS SUBSTRATE

Kalsoom Farzana[1,*], Syed Nisar Hussain Shah[1], Farooq Bashir Butt[2] and Sattar Bukhsh[3]
[1]Department of Pharmacy, Bahauddin Zakariya University, Multan.
[2]Punjab Pharmacy Council, Gulshan Iqbal, Lahore.
[3]Department of Pharmaceutics, Gomal University, D.I. Khan.

Abstract: 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 bye-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 37ºC using different natural media such as defatted soybean meal, glucose and metal ions.
Bacitracin is being imported in Pakistan 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 our country would result in saving a reasonable amount of foreign exchange by utilizing our resources.

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

INTRODUCTION

Antibiotics are substances, produced by micro-organisms, which in low concentration and destroy or inhibit the growth of other species of micro-organisms. The term has been extended to include chemically related and derived substances which are produced wholly or partly by chemical synthesis.

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 posses 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, a Bacitracin producer, has an ABC transporter system which is hypothesized to pump out Bacitracin for self-protection [Podlesek 1995]. Bacitracin holds considerable importance.

* Author for correspondence.

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 and 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.

MATERIALS AND METHODS

ORGANISM

Bacillus licheniformis (ATCC 9945, 10716, 11945, 11946 and 14580-PCSIR 89 locally isolate) were used for the production of antibiotic Bacitracin.

The culture was maintained on Tryptone-glucose – Y. E. agar medium (Table 1).

Table 1: Composition of Tryptone-glucose Y. E. agar medium.

Materials	Content (g l^{-1})
Tryptone	5.0
D-glucose	1.0
Yeast Extract	2.5
Agar	15.0

pH of the medium was adjusted to 7.0 with 1N NaOH/HCl 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.

INOCULUM PREPARATION

The bacterial growth was aseptically scrapped from 48 hours old culture slants and transferred to 50 ml sterilized basal medium (Table 2) in 250 ml conical flask and then shaken on rotary shaker at 150 rpm for 24 hours at 37^{o} C. The vegetative culture thus obtained was used for inoculation into fermentation media. 4% v/v inoculum was used in this study.

Table 2: Composition of basal medium.

Materials	Content (g l^{-1})
Peptone	10.0
Glucose	5.0
Beef Extract	5.0
Sodium Chloride	2.5
Manganese Chloride	0.167

FERMENTATION MEDIA FOR BACITRACIN PRODUCTION

Fermentation media used for the production of Bacitracin by *Bacillus licheniformis* is given in Table 3.

Table 3: Composition of fermentation media.

Materials	Content (g l^{-1})
Citric Acid	1.0
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 meal/Sunflower/or Wheat bran	45.0

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

FERMENTATION TECHNIQUE (METHOD)

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 3) 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.

Table 4: Production of Bacitracin in stir-fermenter using Soybean meal medium (Table 3).

Observations	Fermentation Time (hrs.)	pH	Potency (i.u. ml^{-1})
1	6	6.6	1.53
2	12	6.2	3.15
3	18	6.5	4.36
4	24	6.95	177.38
5	30	7.6	342.00
6	36	7.65	338.00
7	42	7.9	333.80
8	48	8.0	327.00

The antibiotic activity was found to be 342.00 i.u. ml^{-1} after 30 hours inoculation and further incubation resulted in the reduction of antibiotic production (Table 4). It may be due to toxic effects of Bacitracin on the *Bacillus licheniformis* and exhaustion of nutrients in the basal medium. Moreover, potent forms of the antibiotic are converted to less potent forms with passage of time. Under mild alkaline conditions Bacitracin A and B were oxidized to less potent Bacitracin as reported by Craig and Konigsberg [1957]. The conversion involves oxidative deamination of thiazoline ring to ketothiazole. The deterioration of Bacitracin may also result due to the presence of tautomeric and resonating structure and can undergoe to series of changes to less active forms.

The pH of the fermentation medium was first reduced to 6.2 after 12 hours of inoculation and then it started increasing and reached 8.0 after 48 hours due to liberation of ammonium compounds. The decrease in pH after first 12 hours could be due to the production of organic acids.

ASSAY

The activity of the antibiotic Bacitracin present in the fermented material was determined by agar diffusion method (Table5).

Table 5 : Composition of Nutrient agar media

Material	Content (g l^{-1})
Beef extract	1.0
Yeast extract	2.0
Sodium Chloride	5.0
Peptone	5.0
Agar	15.0

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, 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 8 cm of diameter were made in the plates aseptically with stainless steel borer of uniform edge and size.

Standard solution (45 i.u. ml^{-1}) of Bacitracin was prepared by dissolving 65.2 mg of Zn Bacitracin in 100 ml of N per 100 ml HCl. The dilution of standard solution was made in N per 100 ml 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. Diameters of zones of inhibition were measured and compared with the known standard.

The potency of the sample was determined by the following formulae:

Difference due to dose

$$E = ½ (T2 + S2) – (T1 + S1)$$

Difference due to sample

$$F = ½ (T2 + T1) – (S2 + S1)$$

Log ratio of doses

$$I = \log 4 = 0.602$$

Slope

$$B = E / I$$

Potency ratio = Antilog of M, where $M = F / B$

Potency of sample = Antilog of M x Potency of standard

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 1mm inhibition zone outside the cylinder [Bernlohr and 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 Bacitracin mg^{-1} [Nichols 2000].

RESULTS AND DISCUSSION

Defatted oil seed cakes are good substrate as a source of carbon and providing amino acids, sugars, minerals and vitamins necessary for the growth of microorganism and as well as for the production of secondary metabolites by fermentation.

SCREENING OF CULTURE MEDIA

The composition of the basal medium (Table 2) greatly influence the production of antibiotics. Replacing soybean meal with sunflower meal and/or wheat bran of same quantity (Table 3) in fermenting medium were 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 3) gave the best results of antibiotic titer.

The antibiotic activity was found 342.00 i.u. ml^{-1} after 30 hours inoculation and further incubation resulted in the reduction of antibiotic production (Table 4) during this study.

K_2HPO_4 and KH_2PO_4 were used as buffering agents, $MnSO_4.7H_2O$ and $MgSO_4.\ 4H_2O$ as co-factors of enzymes while $FeSO_4.7H_2O$ 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 micro-organism 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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