Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Suppl 4, 2011

Research Article

ISOLATION, PURIFICATION AND CHARACTERIZATION OF BACITRACIN FROM *BACILLUS SP.*

SATPAL SINGH BISHT*, B.PRAVEEN, AMRITA PANDA AND V.RAJAKUMAR

Department of Biotechnology, Roland Institute of Pharmaceutical Sciences Berhampur-760010, Orissa India. Email: sps.bisht@gmail.com

Received: 2 May 2011, Revised and Accepted: 6 June 2011

ABSTRACT

Bacterial cultures of bacillus species capable of producing bacitracin were isolated from local habitats (garden soil, timber yard soil and sea water) and screened for the production of bacitracin on nutrient media against test organisms viz: *Micrococcus luteus,*(ATCC 9341) *& Staphylococcus aureus* (ATCC 13565).Optimization of culture conditions and media composition for bacitracin production was done by investigating the effect of different nitrogen sources (tryptone, meat extract, yeast extract), carbon sources (maltose, glucose, fructose, sucrose, lactose) pH (3.6, 4.6, 5.6, 6.6), time(6hrs,12hrs,16hrs,24hrs,48hrs 72hrs), temperature (4°C,15°C,25°C,37°C,48°C) on the growth of isolates. The isolated strains were subjected to solid state and submerged fermentation on different production media containing Soyabean meal & Wheat bran. The bacitracin was extracted by butanol-ether solvent system and purified by thin layer chromatography and characterized by SDS-PAGE.

Keyword: Bacitracin, SDS-PAGE, Antimicrobial activity

INTRODUCTION

The word "antibiotic" is derived from Greek term antibiosis, which literally means "Against life". It can be purified from microbial fermentation and modified chemically or enzymatically for basic research [1, 2]. Several hundred naturally produced antibiotics have been purified, but only a few have been sufficiently non-toxic to be of use in medical practices. Those that are currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Streptomyces, Cephalosporium, Micromonospora* and *Bacillus* [3]. More than 5000 different antibiotics have been isolated from cultures of bacteria, fungi and plant cells, 60% of them are contributed by the genus *Streptomyces* [4, 5].

Most of the peptide antibiotics produced by bacilli are active against gram-positive bacteria; however, compounds such as polymyxin, colistin and circulin exhibit activity almost exclusively upon gram-negative bacteria, whereas bacillomycin, mycobacillin and fungistatin are effective against molds and yeasts [6]. The production of 167 peptide antibiotics from *Bacillus subtilis* and *Bacillus brevis* was reported by [7] of this total, 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*. Bacitracin affects protein synthesis, cell wall synthesis and membrane functions. Studies on antibiotics and antibiotics with enzyme combination have been made by [8, 9]. It is exclusively used as topic antibiotic in the treatment of infection and often supplemented with antibiotics such as Polymyxin-B and Neomycin to make broad spectrum combination preparation .Bacitracin is an important feed supplement for number of animal species. It improves the weight gain and feed efficiency when added in concentration of 5-100 ppm.

MATERIALS AND METHODS

Isolation and screening of *Bacillus sp.*

Soil samples obtained from different sources such as Garden Soil, Timber yard Soil, Sea water, etc., were subjected to 10 fold serial dilution and aliquots(0.1 ml) were plated on Nutrient agar plates and incubated for 24hrs at optimal conditions [10]. The isolated strains were kept in slant cultures at 4°C.

Characterization of isolated strains

The isolated strains were studied for morphological, cultural and biochemical characteristics. The morphology was identified by high resolution microscope [11].

Screening of *Bacillus sp*. against Micrococcus and Staphylococcus

Bacillus sp. was inoculated into nutrient broth and incubated at 37°C for 24hrs. The culture was centrifuged at 6000rpm for 10 min. Supernatant was collected and filtered by Whatman filter paper aseptically. Then it was heated for 10 minutes at 90°C and cooled. Antimicrobial activity of the supernatant was determined by cup plate method .The test organisms *Micrococcus luteus,*(ATCC 9341) *and Staphylococcus aureus* (ATCC 13565) were inoculated on nutrient agar plates and the wells were loaded with 70,80,90 and 100 µl of the supernatant, incubated at 37°C for 24hrs and zone of inhibition was measured.

Submerged Fermentation

The submerged fermentation was carried out with soybean meal in 250 ml flask. 50 ml of the fermentation medium was autoclaved at 121^{0}C for 15 minutes. The medium was inoculated with 20 hrs of old vegetative inoculum (5%v/v).After inoculation the flasks were incubated at 37^{0}C on an orbital shaker with 150rpm.After 48 hrs of incubation, the fermentation broth was centrifuged at 10,000 rpm for 15 minutes and supernatants were assayed for bacitracin potency.

Solid state Fermentation

Solid state fermentation was carried out in one litre conical flasks. Wheat bran was used as solid substrate for the production of antibiotic from *Bacillus subtilis.*30 g of substrate was moistened with 15-20 ml of phosphate buffer at pH-7.The flasks were sterilized, inoculated and incubated at 37^{0}C for 48 hrs. The antibiotic was extracted by adding 300ml of N/100 HCl solution to fermented substrate. The suspension was squeezed through muslin cloth and then centrifuged at 10,000rpm at 4^{0}C for 15 minutes. The supernatant was collected and used for assay.

Bacitracin Assay

The collected supernatant was tested for antimicrobial activity against the test pathogens viz: *Micrococcus luteus* (ATCC 9341) *and Staphylococcus aureus* (ATCC 13565) on nutrient agar plates incubated at 37^{0}C for 24hrs by measuring the inhibition zones.

Purification of antibiotic by solvent extraction method

An equal volume of butanol-ether solution is added to aqueous concentrate in a separating funnel and shaken vigorously. concentrated Hcl is added drop by drop until the mixture is separated into layers immediately after shaking. The upper layer becomes darker as acid is added owing to the removal of impurities from the aqueous chamber. The pH of aqueous layer is maintained between 3-4.The lower aqueous layer which contains the active material is decanted and the extraction is repeated. The extraction is repeated five times allowed to stand for ten minutes and pH is tested. The lower layer is decanted and extracted five times with peroxide-free ether. 44 ml of the extract was obtained from submerged fermentation and 142 ml from solid state fermentation. The final aqueous layer is distilled under reduced pressure until all

butanol and ether has been removed. The volume of the extracts is 13 ml and 33ml for submerged and solid state fermentation respectively. After distillation the solution is brought to pH 7 with sodium bicarbonate. The neutralized solution is then lyophilized, which is a yellowish powder.100 mg of MgO is added to 10ml of faintly acidic or neutral solution of concentrated bacitracin. This mixture is stirred and after several hours storage in refrigerator it is filtered cold. The filtrate was found alkaline and was neutralized with Hcl and MgO treatment. The process was repeated until the activity of MgO is negligible. The first formed precipitate was discarded as it does not exhibit antibiotic activity. The filtrate was neutralized with sodium bicarbonate and salicylic acid was then added for complete precipitation. The precipitate was collected and dried, repeatedly washed with peroxide free ether to remove excess of salicylic acid. It is grounded in funnel and washed again with peroxide-free ether, dried in vacuum at room temperature over phosphoric anhydride. The salicylate was dissolved by percolating 0.05 N HCl and used for assay.

Media optimization

Optimization of culture conditions and media composition for bacitracin production was done by studying the effect of different nitrogen sources (Leucine, Glycine, Aspargine, Tyrosine and Methionine) carbon sources (Acetic acid, Glycerol, Maltose and Lactose) pH (5, 6,7,8,9.), temperature(30^0C, 37^0C and 50^0C), time (24, 48, 72 and 96 hours) on the growth of isolates.

Idetification of Bacitracin in the given sample using thin layer chromatography

Silica gel was prepared with distilled water in 1 : 2 ratio and immediately transferred over the glass slide uniformly, air dried and kept in the oven at 105°C for 30 min. The solvent was prepared with chloroform and methanol in the ratio of 90:10.The reference line was drawn on the silica gel plate at a distance of 1.5 cm from the lower end. Two spots were marked on the end and the test samples were applied on the spots (10-20µl) the silica gel plate was suspended in the solvent. The plate was taken out when the migration of the solvent nearly equals to 70% of the length of the plate.

To estimate the molecular weight of Bacitracin using SDS-PAGE

The molecular size was analyzed using SDS-PAGE as per [12] with minor modifications. The resolving gel with 12% and stacking gel 5% with a constant voltage of 100 for 6 hrs, stained with coomassie brilliant blue and followed by the documentation of electrophorogram.

RESULTS & DISCUSSION

The present study revealed that the isolated strain ***Bacillus sp***.was found to be irregular, Gram+ve rods with lobate margin (Table-1,2&3) & tested positive for glucose and mannitol fermentation, nitrate reduction (Table-4) exhibited antimicrobial activity against the test organisms(Table-5&6), The Bacitracin production by *Bacillus subtilis* was growth associated reaching maximum after 45hrs.The production of the Bacitracin is significantly influenced by different carbon and nitrogen sources. The Bacitracin production in the presence of maltose was found to be optimum. Among the nitrogen sources aspargine was found to be the most suitable nitrogen source. The antibiotic showed maximum activity at pH 8.0 and at temperature 37^0C.The extracted Bacitracin was identified by thin layer chromatography and its Rf value is found to be 0.26cm. The electrophoregram revealed the size of the bacitracin is approximately 1.4kDa.

Table 1: Morphological characters of *Bacillus subtilis* obtained from garden soil

S.No	Form	Margin	Elevation	Texture	Appearance	Pigment
1.	Rhizoid	Filamentous	Flat	Moist	Transparent	White
2	Irregular	Lobate	Flat	Moist	Transparent	White
3.	Irregular	Lobate	Flat	Dry	Transparent	White

Table 2: Morphological characters of *Bacillus subtilis* obtained from Timber yard soil

S.No:	Form	Margin	Elevation	Texture	Appearance	Pigment
1	Irregular	Lobate	Flat	Dry	Transparent	White
2	Irregular	Lobate	Flat	Dry	Transparent	White

Table 3: Morphological characters of *Bacillus subtilis* obtained from Sea water

.S.No	Form	Margin	Elevation	Texture	Appearance	Pigment
1.	Irregular	Lobate	Flat	Dry	Transparent	White
2	Irregular	Filamentous	Flat	Dry	Transparent	White
3.	Irregular	Lobate	Flat	Dry	Transparent	White

During optimization of various parameters it was observed that the maximum antibiotic production was obtained after 45 hours of incubation. Although significant amount was found after 3-5 days of incubation period however after that it started declining. Maximum antibiotic production was obtained at pH 8 and significant amount of antibiotic was also produced at pH 5.Maximum antibiotic production was obtained when maltose was used as carbon source. The antibiotic production was at maximum when aspargine was used as nitrogen source. Next best source was found to be tyrosine and methionine, and no activity was observed when NaNO3 and NH4Cl were used as nitrogen sources. However significant antibacterial activity was also achieved when lactose, acetic acid, glycerol and glucose were employed as carbon sources.

The antibiotic activity at different temperatures was dependent on test organism. Best activity was observed at 37^0c for both staphylococcus and Micrococcus. Against *Micrococcus luteus* considerably good activity was achieved even at 50^0C.In the recent years, the emphasis has been laid on extremophiles for their potential use in the production of antibiotics because of various adaptive mechanisms and strategies that help them to survive and function in brutal circumstances. The ability to access antibiotic activities from the extreme of environmental spectrum rather than conventional domains is very much needed to develop industrial applications.. The present study was carried out to exploit the microbial diversity and enzymatic potential of the thermophilic bacteria from different habitats. The isolated *Bacillus* sp. based on its characterization could be a useful source for Bacitracin production and has the potential for industrial applications.

Table 4: Biochemical tests

Tests	Result
Gram staining	+ve rods
Mannitol fermentation	+ve
Glucose fermentation	+ve
Catalase	-ve
Lactose fermentation	-ve
Nitrate reduction test	+ve

Table 5: Antimicrobial assay of Bacitracin from solid state fermentation

Test organism	Zone of inhibition (mm)
Staphylococcus aureus	14
Micrococcus luteus	12

Table 6: Antimicrobial assay of Bacitracin from submerged fermentation

Test organism	Zone of inhibition (mm)
Staphylococcus aureus	25
Micrococcus luteus	21

REFERENCES

1. Robbers EJ, Marilyn SK and Varro TE. Antibiotics. In : Pharmacognosy and Pharmacobiotechnology,University School of Pharmacy and pharmaceutical Sciences,1996. p.219- 220.
2. De Mondena JA, Guttierrez SAJ, Falchini R.A, Gallazo JL, Hughes DE, Bailey JE and Martin JF. Intracellular expression of vitreoscilla haemoglobin improves cephalosporin C production by *Acremonium chrysogenum*. Biotech. 1993;11: 926-929.
3. Zinsser H. Antimicrobial Agents. In: Zinsser Microbiology. (Eds.): Zinsser H, Joklik WK, Willett HP, Amos DB and Wilfert C. Prentice Hall International, UK ;1988.p. 128- 160.
4. Todar, K. Antimicrobial Agents used in the treatment of infectious disease. Department of Bacteriology University of Wisconsin-Madison; 2002.
5. Claus GW and Balckwill D. Antibiotic Evaluation by Kirby-Bauer Method. In: Understanding Microbes: A Laboratory Textbook for Microbiology; 1989. p. 405.
6. Katz E and Demain AL. The peptide antibiotics of Bacillus; chemistry, biogenesis, and possible functions. Bacteriol. Rev. 1977; 41: 449-474.
7. Berdy J. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv. Appl. Microbiol. 1974;18: 309-406.
8. Jyothi P, Kiran K J, Radhika M. Antimicrobial studies of selected antibiotics and their combination with enzymes . International Journal of Pharmacy and Pharmaceutical Sciences.2010;2(3).
9. Neeraj M and Behal K K. Antimicrobial activity of some species against selected microbes. International Journal of Pharmacy and Pharmaceutical Sciences.2010;2(3).
10. Cappucino&Sherman.In:MicrobiologyLabaratoryManual,7th ed,Pearsoneducation,India; 2007.
11. William ST,Goodfellow M & Alderson G.Genus *Streptomyces*.In:Bergeys Manual of Systematic Bacteriology.Williams & Wilkins Co., Baltimore,USA; 1989.
12. Sambrook & Russel.In:Molecular Cloning Labaratory Manual,3rd ed,Coldspring Harbour Laboratory press,Cold spring Harbour,Newyork; 2001.