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Biosynthesis and optimization of bacitracin by mutant *Bacillus licheniformis* using submerged fermentation

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The bacitracin is an important antibiotic, which is being used in various biomedical fields. The bacitracin with zinc salt is used in poultry feed to promote growth and to minimize disease incidences. The present study was concerned with the low-cost production of bacitracin using mutant *Bacillus licheniformis* employing submerged fermentation. Mutant strain was developed from parent culture of *B. licheniformis* by UV irradiations for different time periods (5 to 40 min). The maximum bacitracin (235 IU/mL) produced by 25 min exposure culture was selected and designated as *B. licheniformis* PCSIR-410-(5). Culture and fermentation conditions of mutant strain were investigated to improve the yield. Maximum antibiotic (301 IU/mL) production was observed in a soybean meal containing a medium. The highest antibiotic yield was achieved using 24 h old 10% inoculums with 20% dissolved oxygen. While optimizing the conditions it was found that higher levels of antibiotic *i.e.* 168, 112, 208 and 208 IU/mL were measured at 400 rpm agitation with pH 7.0 at 35°C after 48 h. The result suggested that by using these optimizing conditions for production process by a mutant, *B. licheniformis* PCSIR-410-(5) and simple techniques for the recovery process, the cost of antibiotic can be decreased to a significant level. The mutated strain could be applied to produce antibiotic at industrial scale to obtain maximum yield.

Keywords: Bacitracin, fermentation, agitation, soybean meal, *Bacillus licheniformis*

Introduction

The genus *Bacillus* produces various classes of antibiotics, including bacitracin. Of the latter category, bacitracin A is the dominant commercial product. *Bacillus* antibiotics are generally produced in the early stages of sporulation[1]. Eppelmann *et al*[2] demonstrated the transfer of the bacitracin biosynthetic gene cluster from *Bacillus licheniformis* to the engineered host *Bacillus subtilis* and the biosynthesis of bacitracin in high levels. Bacitracin ($C_{66}H_{103}N_{17}O_{16}S$) is one of the important polypeptide antibiotics that have cyclic rings in their structures. The antibiotic is very active against Gram-positive and few Gram-negative bacteria. It is commonly used in pharmaceutical, animal, and poultry feed industries. Its importance has stimulated various scientists to undertake intensive investigations for increasing its production to meet the commercial demands. Bacitracin was first discovered in 1943 when a culture of *B. licheniformis* isolated from wounds of a seven years old American girl, Margaret Tracey was found to produce the antibiotic[3]. Bacitracin is widely used as metallopeptidase antibiotic produced by *Bacillus subtilis* and *B. licheniformis* with a potent bactericidal activity directed against Gram-positive and Gram-negative bacteria[4]. Biosynthesis of bacitracin was first reported by surface culture method. Relatively, recently several investigators have studied different parameters affecting the antibiotic production. As the process is being understood more thoroughly, ways leading to efficient and economically feasible production strategies are being identical too. For instance, Baig *et al*[5] studied the effect of different amino acids on production of the bacitracin by a mutant strain of *B. licheniformis* and found that L-leucine gave the highest increase in yield of bacitracin. By using submerged fermentation in flasks, different media were investigated for the production of bacitracin[6]. El-Syed *et al*[7] investigated the controlled trial of evaluating the clinical efficacy of an antimicrobial lozenge containing bacitracin using a validated mucositis scoring system on prophylaxis of irradiation associated mucositis in

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conventionally treated patient with head and neck cancer. Simultaneous identification and quantitative determination of neomycin sulfate, polymixin B sulfate, zinc bacitracin and methyl and propyl hydroxybenzoates in ophthalmic ointments by thin layer chromatography was reported previously[8]. It has been reported that zinc combined with bacitracin to increase the weight gain, the rate of growth and feed consumption efficiency feed the chicks. In the past, the metal binding property of different congeners of bacitracin has also been studied with a potentiometer and spectroscopic techniques[9]. The aim of present study was to optimize the parameters to produce maximum bacitracin by mutated *B. licheniformis* PCSIR-410-(5).

Materials and Methods

Microorganisms

Bacillus licheniformis PCSIR-410-(5) derived from parent culture *Bacillus licheniformis* PCSIR-410 which was obtained from Institute of Food Biotechnology, Pakistan Council of Scientific and Industrial Research Laboratories, Complex, Ferozpur Road, Lahore, Pakistan. The culture was UV irradiated to obtain mutant. This multan strain was used for bacitracin fermentation. In this study, *Micrococcus luteus* was used for the bioassay of bacitracin by a fermented broth of bacitracin and by zinc bacitracin. *M. luteus* supplied by Welcome Laboratories, Karachi, Pakistan was used as the test organism.

Maintenance of Microorganisms

Both *B. licheniformis* PCSIR-410-(5) and *M. luteus* were maintained on nutrient agar slants (Difco Laboratories, UK) consisting of Lab-Lemco powder, (1 g/L); yeast extract, (2 g/L); peptone, (5 g/L); NaCl, (5 g/L); agar, (15 g/L); (pH 7.0). Inoculated slants were incubated at 36 ± 0.5°C for 48 h in an incubator (Eyela, Japan). Slants were then stored in the refrigerator for further use. The viable cultures were revived every week.

Mutagenesis

Cell suspensions of parent culture of *B. licheniformis* PCSIR-410 were prepared from 24 h old agar slant by suspending one loop of the culture in 10 mL sterilized saline and then diluting this by mixing its 1 mL with 9 mL saline. Similarly, seven more dilutions were prepared. For UV irradiation, a method of Baig *et al*[5] was adopted. The cell suspensions were then transferred to a sterilized Petri dish and placed under UV lamp (DESAGA SARSTEDT GARUPPE) at 254 nm for different time intervals (5 - 40 min) at 6 cm height. Samples were taken, inoculated on nutrient agar and incubated at 37°C for 24 h. Bacitracin production was checked in comparison with control (cell without exposure). For this, bacitracin was determined using bioassay technique[10].

Inoculum development for Bacitracin Production

The inoculum was prepared according to the method of Baig *et al*[5]. Inoculum development for the bacitracin fermentation was started by inoculation of *B. licheniformis* PCSIR-410-(5) in 100 mL of nutrient broth medium (Difco Laboratories UK) in cotton-plugged, 250 mL Erlenmeyer flask pre-autoclaved at 121°C for 15 min in an autoclave. This flask was incubated on a rotary shaker (Eyela, Japan) at 36 ± 0.5°C for 24 h at 150 rpm. This culture was then inoculated into 1000 mL of fermentation medium.

Inoculum Medium

Nutrient broth (Oxide; CM1) was used as inoculum medium. A loop full of *B. licheniformis* PCSIR-410-(5) culture was added to 25 mL nutrient broth in a 250 mL Erlenmeyer shake flask and incubated at 35°C in a rotary shaker for 24 h. For small scale fermentation such as flask level and benchtop fermenters were used whereas for large-scale 10 L and 30 L fermenters were prepared.

Fermentation Medium

Wheat bran, rice hulls, sunflower, soybean meal and cotton seeds were used as substrates. Fermentation medium prepared as the following composition: a substrate (45 g/L), starch (5 g/L), citric acid (1 g/L), and calcium carbonates (4 g/L). The pH of the medium was maintained at 7.0 ± 0.2. Nutrient agar medium (Oxide; CM001) was used as assay medium.

Fermentation Technique

The submerged fermentation technique was employed to investigate the production of antibiotic bacitracin in shaking flasks, 2 L glass jar fermenter, 10 L and 30 L stainless steel fermenters. Fermentation technique for bacitracin production was used according to the method of Baig *et al*[5].

Shake Flasks

Bacitracin fermentation was carried out by submerged fermentation in 1 L Erlenmeyer cotton

plugged flasks containing 100 mL of the fermentation medium with each substrate for the maximum bacitracin production in flasks. Different inoculum sizes such as 4, 6, 8, 10, 12 and 14% were used. Similarly, effects of the age of inoculums (16 h, 24 h, 32 h, 40 h and 48 h old) were studied. To observe the effect of temperature, fermentations were carried out at 25, 30, 35, 40, 45 and 50°C in six different runs. To optimize the fermentation time for bacitracin production, incubation periods were carried out for 24, 36, 48, 60, and 72 h. The flasks were sterilized in an autoclave at 15 $lbs/inch^2$ at 121°C for 15 min. The medium was cooled to room temperature and inoculated by a given inoculants. The flasks were placed on a rotary shaker for incubation at different temperatures, at different agitation speeds and for different time periods.

Bacitracin Production in Fermenter in Repeated Batch Cycles

Two liters glass stainless steel aerobic fermentor was used for the maximum production of bacitracin. To observe the effect of aeration, different dissolved oxygen levels (8, 12, 16, 20, 24 and 28%) were maintained in six different runs of the fermenter. Six different agitation speeds (100, 200, 300, 400, 500 and 600 rpm) were used to optimize agitation in six runs. Fermenters containing 1.5 fermentation medium consisting of soybean meal as a substrate were sterilized in an autoclave at 20 $lbs/inch^2$ at 121°C for 20 min. Freshly prepared antifoam (10% silicon oil), 1 N NaOH and 1 N H_2SO_4 were autoclaved in washed bottles. Fermenters were cooled at room temperature. The 10% inoculum of 24 h old was inoculated in the medium. Six different pH values 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 were adjusted for bacitracin production.

Subsequently, bacitracin production was carried out in 10 L and 30 L jar glass mechanically agitated fermenter (MBF Eyela, Japan). The bacitracin production medium was identical to that which was used for the development of inoculums. The fermentation medium was automatically sterilized and controlled by bioprocess operator (MSSD-1) and bioprocess controller (MDAC-S3). The fermentation parameters such as pH, the speed of agitator, temperature, and addition of acid /alkali each and antifoam was controlled, while dissolved oxygen tension was monitored by bioprocess controller (MDIA-S3) installed in control panel of fermenters.

Recovery of Bacitracin from Fermented Broth

Following techniques were employed for the recovery of bacitracin from the fermented broth of *B. licheniformis* and it was finally recovered in its stable form as zinc bacitracin. Recovery of bacitracin was conducted according to the method of Baig *et al*[5].

Isolation of Bacitracin by Precipitation with Zinc Chloride

A number of experiments were carried out for isolation of bacitracin from the fermentation broth by precipitation technique. The fermented broth (500 mL) was centrifuged at 10000 rpm for 15 min at 5°C to remove proteinaceous material like unused and spent soybean meal. The pH of the clear supernatant was adjusted to 3.0 - 4.5 with 1 N H_2SO_4 and $ZnCl_2$ 0.5, 1.0, 2.0, 3.0 and 4.0 g/100 mL was added in the acidified broths. The broths were then shaken for about 15 min at 25, 30, 35, 40 and 45°C. The pH of broths was kept as 2.5, 3.0, 3.5, 4.0 and 4.5 for the precipitation of zinc bacitracin. The fine powder of $CaCO_3$ (0.4, 0.5, 0.6, 0.7 and 0.8 g/100 mL) was mixed as a carrier. The precipitated zinc bacitracin was dried in an oven at 37°C.

Separation of Bacitracin by Solvent Extraction Technique

This procedure involved filtering the fermented medium to remove solid material extracting the bacitracin from the resulting filtrate with n-butanol. The fermented broth (200 mL) was centrifuged 10,000 rpm for 15 min at 5°C to remove proteinaceous material like unused and spent soybean meal. This clear supernatant was transferred to a 500 mL Erlenmeyer flask. A washed magnetic bar was put in a flask. One hundred mililitre of n-butanol was added and the mixture was shaken under magnetic shaker for about one hour at room temperature (25°C) and at 200 rpm. The bacitracin was extracted into the n-butanol layer leaving impurities in the aqueous phase. The contents of the funnel were allowed to stand till the two layers separated from each other. The organic phase was separated and the process was again repeated with the aqueous solution twice taking 50 mL of n-butanol each time. The total volume of the organic phase obtained from the above process was 200 mL. Distilled water (50 mL) was added to n-butanol and adjusted its pH to 3.5 with 1 N HCl. The contents were shaken for about half an hour at room temperature under magnetic shaker. At this pH, the antibiotic was transferred into the aqueous phase. The whole broth was allowed to stand and separate into two layers. The volume of the aqueous concentrate obtained was 48 mL afterward adding 4.0 g of $ZnCl_2$ then raising the pH to neutral stabilized the bacitracin. Fine powder of $CaCO_3$ (1.0 g) as a carrier was mixed and then the mixture was evaporated. Contents were dried to get solid zinc bacitracin.

Bioassay of Bacitracin

Fermented broth was centrifuged for 15 min at 10,000 rpm and the supernatant was used for bioassay. Five standards of concentrations i.e., 15, 30, 45, 60 and 75 IU/mL were prepared. For culture suspension, a loop full of *Micrococcus luteus* culture from nutrient agar slant was transferred in Erlenmeyer flask containing nutrient broth and incubated for 24 h on a rotary shaker at 150 rpm and 36°C temperature. Sterilized and cooled to 35°C, nutrient agar was inoculated with 1% test organism culture and poured onto Petri plates. Nutrient agar plates were then allowed to solidify and 4 wells were made in each Petri plate with a sterilized borer. Cell free 40 µL each of fermented broth and standards were poured in the wells. Three plates were used for each assay solution. On each plate, 2 alternate wells were filled with reference concentrations and other 2 wells with assay solutions. Plates were incubated for 24 h at 36°C. The inhibition zones were then measured with Vernier caliper and with graduated scale. The activity of bacitracin was determined according to Aftab *et al*[11].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03). To analyze the impact of various parameters on bacitracin production, one-way analysis of variance and Dunnett's multiple comparison tests with a probability level of 5% as the minimal criterion of significance were used. Data are presented as mean ± SEM.

Results

Mutagenic Effect of UV Exposure on Bacitracin Production by *B. licheniformis*-PCSIR-410

Mutant strains were developed from parent culture of *B. licheniformis* PCSIR-410 by UV irradiations for different time periods ranging from 5 - 40 min. Colonies were picked up from Petri plates for each exposure time and were screened for bacitracin production. The control culture was run in parallel. In the present study, the parent strain PCSIR-410 produced 127 IU/mL of the bacitracin, whereas different mutant strains yielded from 68 to 235 IU/mL at 48 h of incubation (Table 1). The mutant colony picked up after UV exposure for 25 min showed maximum antibiotic, bacitracin producing potential up to 235 IU/mL was selected and designated as *B. licheniformis* PCSIR-410-(5). The mutant strain with maximum production was used in further studies.

Hyperproduction of Bacitracin

The production of bacitracin antibiotic by *B. licheniformis* is greatly influenced by the solid substrate. The production of antibiotic was studied by using different substrates like wheat bran, rice hulls, soybean meal, and sunflower and cottonseed meal in different 5 runs containing 0.45% of each substrate in 1 L shake flasks. Maximum antibiotic (301 IU/mL) production was observed in soybean meal containing a medium. Bacitracin produced in wheat bran and rice hulls medium was found very low as compared to soybean meal. Sunflower meal and cotton seed meal containing media gave better results than wheat bran

Table 1 — Mutagenic effect of UV exposure time on bacitracin production by different strains of *Bacillus licheniformis*-PCSIR-410

Sr. No.	UV exposure time (min)	Bacterial strains[¥]	Diameter (mm) of inhibition zones[¶]	Bacitracin activity (IU/mL)[¶]
1	(Control) 0	PCSIR-410	43 ± 0.47	127 ± 7.25
2	5	(1)	43 ± 0.00	126 ± 0.00
3	10	(2)	40 ± 0.47	88 ± 5.20 [†††, §§§]
4	15	(3)	41 ± 0.0	99 ± 0.00 [††, §§,]
5	20	(4)	44 ± 0.47	144 ± 8.50 [†, §, ‡‡‡, βββ]
6	25	(5)*	48 ± 0.00	235 ± 0.00 [†††, §§§, ‡‡‡, βββ, €€€]
7	30	(6)	40 ± 0.47	88 ± 5.20 [†††, §§§,ββ, , €€€, ααα]
8	35	(7)	38 ± 0.00	68 ± 0.00 [†††, §§§, ‡‡, βββ, €€€, ααα, ≠]
9	40	(6)	38 ± 0.00	68 ± 0.00 [†††, §§§ ‡‡‡, βββ, , €€€, ααα, ≠]

Keys: [¥]Digit in parentheses indicates the dilution number of *Bacillus licheniformis*–PCSIR-410 that yielded conspicuous growth following a given UV exposure and was selected for further studies. [¶]values are means of three replicates ± SEM; *This strain yielded highest bacitracin. It was used in subsequent experiments; [†]Significance difference between Sr. No. 1 and 2 to 9; [§]Significance difference between Sr. No. 2 and 3 to 9; [‡]Significance difference between Sr. No.3 and 4 to 9; [β]Significance difference between Sr. No. 4 and 5 to 9; [€]Significance difference between Sr. No. 5 and 6 to 9; [α]Significance difference between Sr. No. 6 and 7 to 9; [≠]Significance difference between Sr. No. 7 and 8 to 9. Single symbol = $p \leq 0.05$; double symbols = $p \leq 0.01$; triple symbols = $p \leq 0.001$.

and rice hulls containing media but still less than the medium having soybean meal. These results showed that medium containing substrate soybean meal has more potential for bacitracin production (Table 2).

Effect of Inoculums Size on the Production of Bacitracin

The production of antibiotic was studied by using six sizes of inoculums (4, 6, 8, 10, 12 and 14%) in six different runs. Maximum antibiotic production (208 IU/mL) was found with 10% inoculum. Bacitracin production with 4, 6 and 8% was determined very low as compared to 10% inoculum. Bacitracin produced with 12% inoculum was higher than 4, 6 and 14% inoculum while lower than 8% and 10%. These results showed that by increasing inoculums sizes more than 10% decreased the bacitracin production (Table 3).

Effect of Dissolved Oxygen on the Production of Bacitracin

To study the effect of aeration on the production of bacitracin six different levels (8, 12, 16, 20, 24 and 28%) of dissolved oxygen were used. Maximum bacitracin produced (301 IU/mL) at 20% dissolved oxygen. These results showed that by increasing the quantity of dissolved oxygen from 8% to 20% bacitracin production increased while more increased dissolved oxygen from 20% to 28%, production decreased (Table 3).

Effect of Age of Inoculums on the Production of Bacitracin

Bacitracin production was studied by using inoculums of five different ages in five different runs. Maximum bacitracin production (235 IU/mL) achieved by using 24 h old inoculum. It was determined that by using inoculum older than 24 h, bacitracin production decreased. The 16 h old inoculum produced more bacitracin than inoculums of 32, 40 and 48 h old inoculum but lower from 24 h old (Table 3).

Effect of Agitation Speed on the Production of Bacitracin

Six different agitation speeds 100, 200, 300, 400, 500 and 600 rpm were used to find out the effect of agitation on the production of bacitracin. Maximum bacitracin (168 IU/mL) was produced at 400 rpm agitation (Table 3). It was investigated that by increasing the agitation rate from 100 to 400 rpm bacitracin production increased but by further increasing the agitation rate higher than 400 rpm, decreased the bacitracin production.

Effect of pH on the Production of Bacitracin

The effect of pH was studied on the production of antibiotic bacitracin. Production was carried on different pH (5.5, 6.0, 6.5, 7.0, 7.5 and 8.0) values. Maximum bacitracin production 112 IU/mL was recorded at pH 7.0 (Table 3). The bacitracin production in acidic medium was less as compared to neutral medium. Furthermore, bacitracin production decreased in basic medium.

Effect of Temperature on the Production of Bacitracin

Production of bacitracin was studied on different temperatures values (25, 30, 35, 40, 45 and 50°C). The maximum production (208 IU/mL) was measured at 35°C (Table 3). It was cleared that bacitracin produced lower at low temperature. Furthermore by increasing the temperature more than 35°C bacitracin production decreased.

Effect of Fermentation Period on the Production of Bacitracin

Production of bacitracin was studied for different time periods (24, 36, 48, 60 and 72 h). The results revealed that maximum production (208 IU/mL) of antibiotic bacitracin was obtained after 48 h. Table 3 showed that increasing or decreasing fermentation period than 48 h bacitracin production decreased.

Optimization of $ZnCl_2$ Concentration for Separation of Zinc Bacitracin

The separation of zinc bacitracin was studied by four different concentrations of $ZnCl_2$. The maximum concentration of zinc bacitracin (209 IU/mL) was

Table 2 — Bacitracin production by *Bacillus licheniformis* PCSIR-410-(5) isolate in different solid substrates

Sr. No.	Substrates	Diameter (mm) of inhibition zones[a]	Bacitracin activity (IU /mL)[a]
1	Sunflower meal	48 ± 0.47	236 ± 13.70
2	Soybean meal	50 ± 0.00	301 ± 0.00 ††
3	Cotton meal	47 ± 0.47	209 ± 12.27 §§§,
4	Wheat bran	45 ± 0.47	163 ± 9.44††, §§§, ‡,
5	Rice hulls	40 ± 0.47	88 ± 5.20†††, §§§, ‡‡‡, ββ,

Keys: [b]Values are means of three replicates ± SEM; †Significance difference between Sr. No. 1 and 2 to 5; §Significance difference between Sr. No. 2 and 3 to 5; ‡Significance difference between Sr. No. 3 and 4 to 5; βSignificance difference between Sr. No. 4 and 5. Single symbol = $p \leq 0.05$; Double symbols = $p \leq 0.01$; Triple symbols = $p \leq 0.001$

achieved by using $ZnCl_2$ (1.0 g/100 mL fermented broth) as given in Table 4. By using below and above the optimum concentration it was less.

Optimization of $CaCO_3$ Concentration for Separation of Zinc Bacitracin

The separation of zinc bacitracin was carried out by four different concentrations of $CaCO_3$. The maximum concentration of zinc bacitracin (162 IU /mL) was achieved by using $CaCO_3$ (0.5 g/100 mL fermented broth) as shown in Table 4. By using below and above the optimum concentration it was less.

Optimization of pH for Separation of Zinc Bacitracin

The separation of zinc bacitracin was determined at four different pH values. The maximum concentration of zinc bacitracin (165 IU/mL) was achieved at pH 3.5 (Table 4). Below and above the optimum pH, it was less.

Optimization of Zinc Concentration for Separation of Zinc Bacitracin

The separation of zinc bacitracin was investigated at four different temperature values. The maximum concentration of zinc bacitracin (162 IU/mL) was achieved at 37°C (Table 4). Below and above the optimum temperature, it was less.

Production of the Bacitracin on Large Scale

Bacitracin producing a mutant strain of *B. licheniformis* PCSIR-410-(5) was used for these studies. Bacitracin was produced in the submerged fermentation technique was employed to investigate

Table 3 — Effect of different factors on the production of bacitracin by *Bacillus licheniformis* PCSIR-410-(5) with soybean meal

Factors / Sl. No	1	2	3	4	5	6
Inoculum size (%)	4	6	8	10	12	14
Diameter of inhibited zones (mm)[a]	40 ± 0.00	41 ± 0.47	43 ± 0.47	47 ± 0.00	42 ± 0.47	39 ± 0.00
Bacitracin activity (IU/mL)[a]	87 ± 0.00	99 ± 5.90	127 ± 7.32[†††, §§]	208 ± 0.00[†††, §§, ‡‡‡]	112 ± 6.37[††, βββ]	97 ± 0.00[§, ‡‡, βββ, €€€]
Inoculum age (h)	16	24	32	40	48	
Diameter of inhibited zones (mm)[a]	45 ± 0.47	48 ± 0.00	40 ± 0.47	38 ± 0.00	37.5 ± 0.00	
Bacitracin activity (IU/mL)[a]	162 ± 9.44	235 ± 0.00	88 ± 5.20	68 ± 0.00	64 ± 0.00	
pH	5.5	6.0	6.5	7.0	7.5	8.0
Diameter of inhibited zones (mm)[a]	39 ± 0.00	40 ± 0.47	41 ± 0.47	42 ± 0.47	38 ± 0.00	38 ± 0.00
Bacitracin activity (IU/mL)[a]	77 ± 0.00	88 ± 5.20	01 ± 11.58[†]	112 ± 6.37[††, §]	68 ± 0.00[‡‡, βββ]	68 ± 0.00[‡‡, βββ]
Temperature (°C)	25	30	35	40	45	50
Diameter of inhibited zones (mm)[a]	40 ± 0.00	42 ± 0.94	47 ± 0.00	40 ± 0.47	38 ± 0.00	38 ± 0.00
Bacitracin activity (IU/mL)[a]	87 ± 0.00	114 ± 13.24[†]	208 ± 0.00[†††, §§§]	88 ± 5.20[§,‡‡‡]	68 ± 0.00[§§, ‡‡‡]	68 ± 0.00[§§,‡‡‡]
Dissolved oxygen (D.O. %)	8	12	16	20	24	28
Diameter of inhibited zones (mm)[a]	40 ± 0.00	43 ± 0.47	45 ± 0.47	50 ± 0.00	39 ± 0.47	39 ± 0.00
Bacitracin activity (IU/mL)[a]	87 ± 0.00	127 ± 7.3[†††]	162 ± 9.44[†††, §§,]	301 ± 0.00[†††, §§, ‡‡‡]	77 ± 4.48[§§§, ‡‡‡, βββ]	77 ± 0.00[§§§, ‡‡‡, βββ]
Fermentation time (h)	24	36	48	60	72	
Diameter of inhibited zones (mm)[a]	39 ± 0.20	40 ± 0.47	43 ± 0.94	39 ± 0.00	38 ± 0.00	
Bacitracin activity (IU/mL)[a]	77 ± 4.48	98 ± 5.90	129 ± 14.92[†††, §§§]	77 ± 0.00[‡‡‡]	68 ± 0.00[§,‡‡‡]	
Agitation speed (rpm)	100	200	300	400	500	600
Diameter of inhibited zones (mm)[a]	38 ± 0.00	39 ± 0.47	41 ± 0.47	45 ± 0.00	40 ± 0.47	38 ± 0.00
Bacitracin activity (IU/mL)[a]	68 ± 0.00	77 ± 4.48	101 ± 11.58[†††,§]	162 ± 0.00[†††,§§§, ‡‡‡]	88 ± 5.20[βββ]	68 ± 0.00[‡‡‡, βββ]

Keys: [a]Values are means of three replicates ± SEM; [†]significance difference between Sr. No. 1 and 2 to 6; [§]significance difference between Sr. No. 2 and 3 to 6; [‡]significance difference between Sr. No. 3 and 4 to 6; [β]significance difference between Sr. No. 4 and 5 to 6; [€]significance difference between Sr. No. 5 and 6. Single symbol = $p < 0.05$; double symbols = $p < 0.01$; triple symbols = $p < 0.001$.

the production of antibiotic bacitracin in shaken flasks, 2 L, 10 L glass jar fermenters and 30 L stainless steel computerized fermenters. Of all these fermenters, maximum bacitracin (95 IU/mL) produced in 2 L glass jar fermenter in comparison to bacitracin produced (94 IU/mL) and (78 IU/mL) in 10 L and 30 L fermenters, respectively. The reason for these variations seemed to be that in 2 L and 10 L fermenters, physical conditions were easy to control as compared to 30 L fermenter. The results were shown in (Table 5).

Recovery of Bacitracin by Different Techniques

Using different techniques such as precipitation with zinc chloride, by solvent extraction, and by ion exchange column chromatography, bacitracin was recovered. Maximum bacitracin isolation (78.2 ± 2.69%) achieved by precipitation with zinc chloride while by solvent extraction 74.4 ± 1.97% was obtained (Table 6).

Table 4 — Optimization of $ZnCl_2$, $CaCo_3$ concentrations, pH and temperature values for bacitracin separation

Factors — Sl. No	1	2	3	4	5
$ZnCl_2$ (g/100 mL)	0.5	1.0	2.0	3.0	4.0
Diameter of inhibited zones (mm) [a]	44 ± 0.94	47 ± 0.47	45 ± 0.47	43 ± 0.94	41 ± 0.47
Concentrations of Zn bacitracin (IU/mL) [a]	146 ± 16.80	209 ± 12.27†	162 ± 9.44	129 ± 14.92§§	144 ± 8.50§
$CaCO_3$ (g/100 mL)	0.4	0.5	0.6	0.7	0.8
Diameter of inhibited zones (mm) [a]	41 ± 0.47	45 ± 0.00	44 ± 0.47	38 ± 0.00	40 ± 0.00
Concentrations of Zn bacitracin (IU/mL) [a]	112 ± 6.37	162 ± 0.00$^{\dagger\dagger\dagger, \S}$	144 ± 8.50†††	68 ± 0.00$^{\dagger\dagger, \S\S\S, \ddagger\ddagger\ddagger}$	87 ± 0.00$^{\S\S\S, \ddagger\ddagger\ddagger, \beta}$
pH values	2.5	3.0	3.5	4.0	4.5
Diameter of inhibited zones (mm) [a]	40 ± 0.47	39 ± 0.00	45 ± 0.94	41 ± 0.94	38 ± 0.00
Concentrations of Zn bacitracin (IU/mL) [a]	88 ± 5.20	77 ± 0.00	165 ± 19.39$^{\dagger\dagger, \S\S\S}$	101 ± 11.56‡‡	68 ± 0.00‡‡‡
Temperature (°C)	25	30	35	40	45
Diameter of inhibited zones (mm) [a]	38 ± 0.00	44 ± 0.94	45 ± 0.00	40 ± 0.47	39 ± 0.47
Concentrations of Zn bacitracin (IU/mL) [a]	68 ± 0.00	146 ± 16.80†††	162 ± 0.00†††	88 ± 5.20$^{\S\S, \ddagger\ddagger}$	77 ± 4.48$^{\S\S\S, \ddagger\ddagger}$

Keys: [a]Values are means of three replicates ± SEM; †significance difference between Sr. No.1 and 2 to 5; §Significance difference between Sr. No. 2 and 3 to 5; ‡Significance difference between Sr. No. 3 and 4 to 5; $^{\beta}$Significance difference between Sr. No. 4 and 5; Single symbol= $p < 0.05$; Double symbols = $p < 0.01$; Triple symbols = $p < 0.001$

Table 5 — Bacitracin production from different fermenters by precipitation technique

Fermented broth samples	Fermented used	Dried zinc bacitracin (g/l) [a]	Diameter of inhibited zones (mm) [a]	Activity of Zinc bacitracin (IU/mL) [a]
1	2 L	59 ± 1.13	41 ± 0.93	99 ± 12.77
2		59 ± 1.37	40 ± 0.91	93 ± 6.82
3		59 ± 0.89	41 ± 0.46	98 ± 5.62
4		60 ± 0.67	40 ± 0.77	93 ± 8.77
5		58 ± 0.59	40 ± 0.61	93 ± 6.82
6		57 ± 0.43	40 ± 0.61	93 ± 6.80
Mean		58.7 ± 0.85	40.3 ± 0.72	94.8 ± 7.93
1	10 L	58 ± 1.01	39 ± 0.48	77 ± 4.48
2		58 ± 0.98	40 ± 0.46	88 ± 5.20
3		56 ± 0.25	41 ± 0.40	99 ± 5.90
4		62 ± 1.12	42 ± 0.37	112 ± 6.37
5		63 ± 1.20	42 ± 0.00	112 ± 0.00
6		57 ± 0.91	39 ± 0.47	77 ± 4.40
Mean		59 ± 0.91	40.5 ± 0.36	94.2 ± 7.93
1	30 L	60 ± 0.78	38 ± 0.00	68 ± 0.00
2		58 ± 0.71	38 ± 0.00	68 ± 0.00
3		56 ± 1.31	41 ± 0.47	99 ± 5.90
4		62 ± 0.89	42 ± 0.47	112 ± 9.20
5		63 ± 0.86	39 ± 0.47	77 ± 4.48
6		58 ± 0.69	38 ± 0.00	68 ± 0.00
Mean		59.5 ± 0.87	39 ± 0.24	82 ± 3.26

Key: [a]Values are means of six replicates ± SEM; Single factor analysis of variances: No significant difference was found

Table 6 — Comparison of techniques used for the recovery of bacitracin fermented broth of *B. licheniformis*-410-(5) through different techniques on large scale

Recovery methods	Activity of bacitracin (IU/mL)	Mean ± Standard deviation			
		Dried zinc bacitracin (g/l) [a]	Diameter of inhibited zones (mm) [a]	Activity of zinc bacitracin (IU/mL)[a]	Percentage recovery (%)[a]
Precipitation with zinc chloride	208	60 ± 1.02	45 ± 2.80	162 ± 5.66	78.2 ± 2.69
Solvent extraction technique	208	55 ± 1.02	44.6 ± 0.22	154.4 ± 4.16	74.4 ± 1.97

Keys: [a]Values are means of six replicates ± SEM; Single factor analysis of variances: No significant difference was found

Weighing of Dried Recovered Zinc Bacitracin

The quantity of zinc bacitracin, isolated by different techniques was weighed. Maximum zinc bacitracin (60 ± 1.02 g/L) gained by precipitation by zinc chloride while the quantity of zinc bacitracin isolated by solvent extraction was 55 ± 1.02 g/L (Table 6).

Discussion

Ultraviolet irradiation (254 nm) for 25 min yielded a mutant strain *B. licheniformis* PCSIR-410-(5) that yielded the highest amount of the bacitracin. It gave 85% more production as compared to parent culture. This much elevation is quite significant. As in earlier studies, the UV mutants have been reported to give higher than the control yield but not comparable to the increase recorded in this study. For example, Baig *et al*[5] reported that UV irradiation produced 10% more bacitracin by a mutant strain than parent culture.

The production of bacitracin antibiotic by *B. licheniformis* is greatly influenced by the solid substrate. Medium containing soybean meal produced maximum bacitracin than other substrates. These results were in agreement with Smekal *et al*[12] who described that maximum bacitracin produced by *B. licheniformis* while employing a medium that contained soybean meal, starch, glucose, $MgSO_4$, and $MnSO_4$. Soybean meal is a rich in protein and nitrogen source and bacitracin seems to be dependent on excess nitrogen for its higher production. Wheat bran and rice hulls are deficient in proteins, therefore, gave poor results for bacitracin productions. Sunflower meal and cotton seed meal have better proteins or nitrogen values than wheat bran and rice hulls but still less than soybean meal. Proper inoculum is vital for cost-effective fermentations[13]. Effect of different inoculum sizes were evaluated for the bacitracin production. Maximum bacitracin produced with 10% inoculum. At low inoculum sizes, the numbers of cells are not sufficient to utilize the essential amount of substrate to produce the product. At high concentration of inoculum anaerobic conditions might have prevailed following tremendous growth of the organism leading to nutritional imbalance and less production of bacitracin. The 20% of dissolved oxygen was found optimal for bacitracin production. These results are similar with Flickinger and Perlman[14], who studied the physiological effects of controlling the dissolved oxygen tensions at 0.01, 0.02 and 0.05 atmosphere by the use of oxygen enriched aeration, during and noted up to 2.35 fold increase in the final bacitracin yield and a 4-fold increase in the rate of bacitracin synthesis in response to oxygen enriched aeration. These authors described that oxygen enrichment of the aeration decreased medium carbohydrate uptake and the maximum specific growth rate of *B. licheniformis.* Such cultures might stimulate conditions that would occur if the carbon source were fed slowly, as is often employed to optimize antibiotic production. In the present study, maximum bacitracin production (235 IU/mL) was achieved when 24 h old inoculum was used. Six different agitation speeds 100, 200, 300, 400, 500 and 600 rpm were used to evaluate the effect of aeration on the production of bacitracin. Maximum bacitracin (168 IU/mL) was produced at 400 rpm agitation.

The effect of pH on the production of bacitracin was studied and it was found, neutral pH gave maximum yield. These results are in good agreement with previous studies[15,16]. They have shown that if buffer capacity of a medium is carefully manipulated, a marked increase in the antibiotic titer can be obtained. Both high and low pH values inhibit bacitracin synthesis. Haavik[17] suggested that the inhibition of the bacitracin production at low pH is due to the inhibitory effect on the enzyme activities.

Maximum yield of bacitracin was obtained at 35°C. These results are similar to Egorov *et al*[18] and Zarei[19] They studied the effect of temperature on the

synthesis in a liquid medium, at temperatures ranging from 30-55°C and found 50°C as optimum temperature. Hendlin[15] studied the effect of temperature on the rate of bacitracin production. Maximum titer was obtained at 1 - 2 days, 3 - 4 days, and 4 - 5 days at 37°C, 28°C and 24°C, respectively. Thus, showing the rate of bacitracin production varied directly with the incubation temperature.

Maximum bacitracin production was recorded after 48 h of incubation by *B. licheniformis* PCSIR-410-(5). These results are in good agreement with that of Zarei[19] and Haavik[20] and who also obtained the maximum titer of bacitracin after 48 of incubation. It might be due to the toxic effect of bacitracin on *Bacillus licheniformis* and exhaustion of nutrients in basal medium. A feedback mechanism also may inhibit further production of bacitracin. Moreover with the passage of fermentation period the potent forms of bacitracin oxidize to less potent form under mild alkaline conditions[21]. This conversion involves oxidative deamination of thiozoline ring to ketothiozole. It also might be due to the fact that there has been depletion of the nutrient and production of non-target products such as proteases in the fermentation medium[22].

Conclusion

Bacitracin is being used in the treatment of many human diseases like bacterial endocarditis, oesteomyelitis, bacteremia, meningitis and intracranical infections, surgical infections, infections of eye and ear, chronic sinusitis, *Clostridium difficile* associated disease, gastrointestinal infections, necrotic enteritis and topical treatment. The cost can be reduced significantly if the parameters of bacitracin production optimized. In this study, different parameters were optimized for maximum bacitracin production.

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