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Optimization of Bacitracin Production by *Bacillus licheniformis* B5

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Abstract: Production of the polypeptide antibiotic bacitracin by a newly isolated *Bacillus licheniformis* B5 strain, using a laboratory medium was optimized. A medium of the following conditions was obtained: 1.5% glycerol as carbon source, 0.05% glutamic acid as nitrogen source, 1.43% inorganic phosphate, 0.0025% magnesium sulfate, with an initial pH of 7.5. Culture was incubated at 37°C for 48 h. The bacitracin productivity was about 174.2 units mL^{-1}. Bacitracin antibiotic was also produced using immobilized cells of the B5 isolate in different immobilization supports (7.5% polyacrylamide, 4% sodium alginate beads and 2% agar). It was found that the highest bacitracin production rate (192 units mL^{-1}) was obtained when using 7.5% polyacrylamide gel as an immobilization material.

Key words: Polypeptide antibiotic, bacitracin, *Bacillus*, immobilized cells

INTRODUCTION

Bacitracin is one of the most important polypeptide antibiotics; it is produced by some strains of *Bacillus licheniformis* and *Bacillus subtilis* and functions as an inhibitor of the cell wall biosynthesis. It is a potent antibiotic used clinically in combination with other antimicrobial drugs (Cao and Helmann, 2002). Bacitracin inhibits preferentially the growth of *Streptococcus pyogenes* and *Staphylococcus aureus* (Bass *et al*., 1997; Skaar *et al*., 1994). Bacitracin is composed of 12 amino acid residues and it is produced non-ribosomally by the thiotemplate mechanism (Eppelmann *et al*., 2001).

In addition to its use as a drug, bacitracin is widely used in the form of diagnosis disks selectively inhibiting the growth of β-hemolytic type A *Streptococcus* (Skaar *et al*., 1994) and as an additive for animal feedstuffs (Smith, 1996).

Fermentation media used for the production of bacitracin by *Bacillus* cells were either natural, like soya bean extract and beef extract, or minimal media containing different carbon and nitrogen sources like amino acids, carbohydrates (glucose and sucrose) and some organic acids (Rabinovitch and Filho, 1982; Hanlon and Hodges, 1981). Biosynthesis of bacitracin in solid-state fermentation by *Bacillus licheniformis* using defatted oil seed cakes of agricultural by-products was recently reported (Farzana *et al*., 2005).

Immobilization of *Bacillus* cells for the production of bacitracin was rarely reported. Morikawa *et al*. (1980) reported the production of the polypeptide antibiotic by polyacrylamide immobilized *B. licheniformis* cells. Bacitracin from *B. licheniformis* strain B5 was previously isolated and characterized according to its physical and chemical properties (Ouled Haddar *et al*., 2002). In this artical, we reported the optimization of some culture conditions for the production of bacitracin by *B. licheniformis* B5 cells, as well as the immobilization of the above cells in different matrices to increase their ability to produce bacitracin.

MATERIALS AND METHODS

Bacterial strains and growth conditions: *Bacillus licheniformis* B5 isolated from Iraqi soil and identified by biochemical tests (Ouled Haddar *et al*., 2002), was used for the production of bacitracin. The test organism *Micrococcus luteus* ATCC 8553 was provided by Ibnu-Sina Center for Drug Research, Baghdad.

Bacterial inoculum was prepared by growing the bacteria during 48 h, at 37°C on nutrient broth, to reach an absorbance at 430 nm of about 0.5. Inoculum represented 10% of the production medium. Basal medium containing the following components (g L^{-1}) was used: Na_2HPO_4, 10.75; KH_2PO_4, 3.55; NH_4Cl, 0.5; $MgSO_4$, 0.025; $MnCl_2$ $4H_2O$, 0.0025; $FeSO_4$ $7H_2O$, 0.00275 and $CaCl_2$ $6H_2O$, 0.015, pH was adjusted to 7.0. Aliquots (50 mL) of the basal medium were dispensed in 250 mL Erlenmeyer flasks, sterilized and incubated for 24 h, after being inoculated, under shaking (150 rpm) at 37°C. Modifications in medium

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composition are cited later. To optimize culture conditions, the effect of different carbon sources, nitrogen sources, Mg^{2+} concentrations, phosphate concentrations, different pH and different temperatures was studied.

Bacitracin assay: The level of bacitracin in the culture filtrate was assayed according to a previously cited method (Hanlon *et al*., 1982) using *Micrococcus luteus* ATCC 8553 as test organism and Mueller Hinton agar plates. Standard bacitracin disks (10 units) and powder (66.000 units g^{-1}) were purchased from Oxoid and Sigma, respectively.

Cell immobilization: *B. licheniformis* B5 cells were collected by centrifugation after growing them for 30 h in the optimized medium, washed twice with normal saline and resuspended in the same medium to reach 250 mg wet cells mL^{-1}. Bacterial cells were immobilized in 7.5% polyacrylamide gel, as described by Morikawa *et al.* (1980). In order to immobilize bacterial cells in 4% Na alginate, 10 mL of bacterial suspension were mixed with 40 mL of previously sterilized Na alginate solution (5%). Entrapment was carried out by dropping the mixture into 0.1 M sterilized and pre-cooled $CaCl_2$ solution. The resulting beads were collected and washed several times with $CaCl_2$ solution to remove unbound cells. Bacterial cells were also immobilized in agar as follow: 10 mL of bacterial suspension was mixed with 40 mL of previously sterilized and cooled to 40°C agar-agar solution (2.5%). The mixture was poured in Petri dishes and cut into small pieces (about 3 mm^3). Immobilized cells were washed with sterilized normal saline. Immobilized cells were added to production medium at a concentration of 25 mL per 50 mL medium and were incubated at 37°C for 48 h with shaking at 150 rpm.

RESULTS

Effect of carbon source: The effect of different carbon sources (glucose, sucrose, glycerol, citric acid, soluble starch and date juice) at a final concentration of 0.5% (w/v) or (v/v) on bacitracin production by *B. licheniformis* B5 was evaluated. Results shown in Table 1, indicated that bacitracin production was highly increased (89.5 units mL) when using glycerol as carbon source in the minimal medium. The antibacterial activity was about 84, 76.4 and 74.2 units mL^{-1}, in cultures containing sucrose, glucose and citric acid, respectively. Since glycerol was selected as the best carbon source, the effect of different concentrations of glycerol on the production level was studied. Bacitracin concentration increased to reach 98.5 units mL^{-1} as the concentration of glycerol was increased to 1.5%. At higher concentrations (2 and 2.5%), a slight decrease in bacitracin level was observed; it attained 95 and 93.3 units mL^{-1}, respectively (Table 2).

Table 1: Effect of different carbon sources on bacitracin production by *B. licheniformis* B5 cells

Carbon source (0.5%)	Bacitracin concentration (units mL^{-1})
Glucose	76.4
Sucrose	85.0
Glycerol	89.5
Citric acid	74.2
Soluble starch	82.0
Date juice	83.5

Table 2: Effect of different concentrations of glycerol on bacitracin production by *B. licheniformis* B5 cells

Glycerol (%)	Bacitracin concentration (units mL^{-1})
0.5	88.3
1	88.3
1.5	98.5
2	95.0
2.5	93.3
0.5	88.3

Table 3: Effect of different nitrogen sources on bacitracin production by *B. licheniformis* B5 cells

Nitrogen source (0.1%)	Bacitracin concentration (units mL^{-1})
Na_2NO_3	109.05
NH_4Cl	105.00
Glutamic acid	126.00
Alanine	91.69

Table 4: Effect of different concentrations of glutamic acid on bacitracin production by *B. licheniformis* B5 cells

Glutamic acid concentration (%)	Bacitracin concentration (units mL^{-1})
0.025	121.1
0.05	134.3
0.1	130.5

Effect of nitrogen source: The effect of two organic nitrogen sources (Glutamic acid and alanine) as well as the effect of two inorganic ones (NH_4Cl and Na_2NO_3) at a concentration of 0.1% on the production of bacitracin by *B. licheniformis* B5 was examined. As shown in Table 3, a considerable increase in bacitracin productivity (126 units mL^{-1}) was seen in Glu-containing culture. Lower and closer levels were obtained with the other nitrogen sources; they reached 109.5, 105 and 91.69 units mL^{-1} with Na_2NO_3, NH_4Cl and alanine, respectively. In Table 4, the effect of three concentrations of glutamic acid on the production of bacitracin was shown. Maximum antibiotic activity (134.3 units mL^{-1}) was found for glutamic acid at a concentration of 0.05%. A slight decline was observed at 0.025 and 0.1% of glutamic acid.

Effect of magnesium sulfate: In an attempt to determine the optimum Mg^{2+} concentration for the production of bacitracin by *B. licheniformis* B5, five different concentrations of $MgSO_4$ (from 0.01 to 0.1 g L^{-1}) were

Table 5: Effect of different concentrations of $MgSO_4$ on bacitracin production by *B. licheniformis* B5 cells

$MgSO_4$ concentration (%)	Bacitracin concentration (units mL^{-1})
0.01	119.5
0.025	140.2
0.05	114.0
0.075	42.4
0.1	0.0

Table 6: Effect of different concentrations of inorganic phosphate on bacitracin production by *B. licheniformis* B5 cells

Inorganic phosphate concentration (%)	Bacitracin concentration (units mL^{-1})
14.3	145.3
7.15	121.0
3.6	80.2
1.6	80.5

tested. It was found that the addition of 0.025 g L^{-1} of $MgSO_4$ increased the production of the antibiotic to 140.2 units mL^{-1}. A considerable decrease in bacitracin level was seen with higher $MgSO_4$ concentrations. Bacitracin secretion was completely inhibited in the presence of 0.1 g L^{-1} of $MgSO_4$ (Table 5).

Effect of inorganic phosphate: The effect of different concentrations of inorganic phosphate (in the form of Na_2HPO_4 and KH_2PO_4) on the production of bacitracin was studied. The results showed a gradual decrease in bacitracin activity with the decrease of phosphate concentration. Maximum bacitracin production (145.3 units mL^{-1}) was obtained in the culture containing 14.3 g L^{-1} phosphate, while it reached half the value when phosphate was added at 1.8 g L^{-1} (Table 6).

Effect of initial pH: The results illustrated in Table 7 indicated that maximum bacitracin production was obtained when the initial pH of the medium was adjusted to 7.0 or to 7.5; the resulted activity reached 140.2 and 142.2 units mL^{-1}, respectively. Moreover, in lower pH values, bacitracin activity was lower and no growth was observed in alkaline pH value.

Effect of temperature: In Table 8, the effect of temperature on the production of bacitracin is shown. Bacitracin was found to be optimally produced between 37 and 40°C, where it reached 141.5 units mL^{-1}. Lower level (88.8 units mL^{-1}) was obtained at 45°C.

Monitoring bacitracin production: The optimum incubation period for bacitracin production was determined by following the activity during 72 h of incubation in the optimized conditions. Results presented in Fig. 1 showed that bacitracin production started during the first 6 h, it increased after to reach a maximum level (174.2 units mL^{-1}) after 48 h of incubation. Elongation of incubation period was accompanied with a constant rate of production.

Table 7: Effect of initial pH on bacitracin production by *B. licheniformis* B5 cells

pH	Bacitracin concentration (units mL^{-1})
6	119.0
6.5	120.5
7	140.2
7.5	142.2
8	135.0
9	0.0

Table 8: Effect of temperature on bacitracin production by *B. licheniformis* B5 cells

Temperature (°C)	Bacitracin concentration (units mL^{-1})
30	134.2
37	141.5
40	141.5
45	88.8

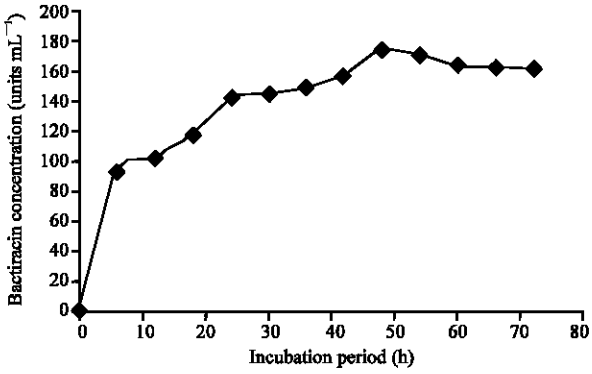

Fig. 1: Monitoring bacitracin production by *B. licheniformis* B5 cells during 72 h of incubation

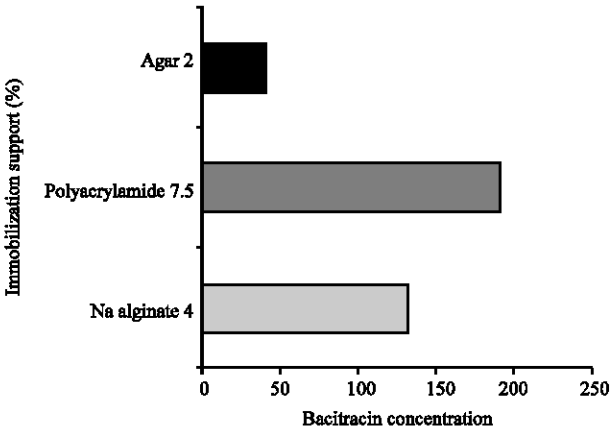

Fig. 2: Effect of different immobilization supports on bacitracin production by *B. licheniformis* B5 cells

Bacitracin production by immobilized cells: The ability of *B. licheniformis* B5 cells to produce bacitracin while being immobilized in different supports was investigated. According to the results presented in Fig. 2, the use of 7.5% polyacrylamide gave a maximum and a considerable amount of bacitracin (192 units mL^{-1}).

Sodium alginate-immobilized cells produced lower activity (131.5 units mL^{-1}), whereas agar-immobilized bacterial cells gave a very low level of bacitracin.

DISCUSSION

In this study, optimization of the cultural conditions for the production of bacitracin by *B. licheniformis* B5 was presented. Glycerol was found to highly induce bacitracin production. The production of antibiotics was generally activated when easily consumed carbon sources are exhausted from the production medium (Iwai and Omura, 1982). Additionally, glycerol was found to increase the growth rate of *B. licheniformis* cells (Hanlon and Hodges, 1981). Moreover, the addition of 1% glucose to a minimal medium was found to be responsible for the delay of bacitracin secretion to 24 h of incubation, while citric acid as sole carbon source induced early the synthesis of the polypeptide (20 h), this was probably due to the rapid consumption of glucose giving rise to the synthesis of organic acids that decrease pH of the culture (Haavik, 1974a). To avoid this, lowering glucose concentration and adjusting pH using calcium carbonate are recommended. The results obtained after optimization of glycerol concentration are in great agreement with that reported by Rachid (1999), where glycerol at 1.5% was suitable for the production of the polypeptide antibiotic gramicidin S by *B. brevis*. It was reported that the effect of easily degradable carbon sources is controlled by catabolite repression of enzymatic processes; their presence at high concentration decreased or inhibited the enzymatic synthesis of the polypeptide antibiotic (Haavik, 1974b).

Nitrogen source was also an important factor for the production of antibiotics. It was stated that both Glutamic acid and Alanine as sources of nitrogen in a basal medium enhanced the production of the antibiotic by *B. licheniformis* (Haavik, 1974a). Glutamic acid at a concentration of 0.01M in a minimal medium gave a maximal rate of production of the same antibiotic by *B. licheniformis* (Hanlon and Hodges, 1981). Amino acids are considered as rapidly consumed sources; since the nitrogen on their molecules is present in reduced form, in addition, they are very important members of the microbial metabolism; they are the building blocks of proteins and polypeptides (Egorov, 1985). With the increase of amino acid concentration, the production decreased, possibly because of the competition between amino acid molecules on membrane receptors to penetrate in the bacterial cells (Al-Khafaji, 1990).

Magnesium ions play a vital role in bacitracin biosynthesis, it was reported that Mg^{2+} are key elements in the activation of amino acids into aminoacyladenylates throughout the biosynthesis process via bacitracin synthetase enzyme complex (Froyshov *et al*., 1980). The total inhibition of bacitracin biosynthesis by the high levels of $MgSO_4$ is due to its toxic effect on bacterial growth (Al-Khafaji, 1990).

On the other hand, inorganic phosphate was found to enhance bacitracin synthesis by *B. licheniformis;* it had an important role to play in maintaining medium pH, thus minimizing the occurring variations (Al-Khafaji, 1990; Haavik, 1974c).

Most of the reported studies stated that the optimum pH for bacitracin synthesis by *Bacillus* sp. bacteria was between 7.0 and 7.5 (Hanlon *et al*., 1982; Flickinger and Perlman, 1979). The synthesis of bacitracin is affected by either acidic or alkaline conditions in which the bacitracin synthesis responsible enzymes, as well as the primary metabolism enzymes could be inhibited (Smith, 1996).

Usually, bacitracin antibiotic is commercially produced by *B. licheniformis* at 37°C (Crueger and Crueger, 1984). Temperature affects the production process of many metabolites such as antibiotics through affecting microbial growth rate as well as the velocity of enzymatic processes of the metabolism (Nester *et al*., 2001).

Generally, antibiotic biosynthesis processes are subdivided into two important phases, the trophophase which is related to logarithmic phase of bacterial growth, the production of antibiotics started within its second half, while in the idiophase, parallel to the stationary phase of growth, maximum production of antibiotics occurred (Sajidi and Ali, 1987). It was also reported, that antibiotic biosynthesis started usually at the end of the log phase and at the beginning of the stationary one (Katz and Demain, 1977).

In the cell immobilization experiment, bacitracin was considerably produced by polyacrylamide-entrapped cells. As a result of its small pore size, its stability and its high ability to entrap microbial cells, polyacrylamide gel is considered as the best immobilization support to preserve antibiotic-producing *Bacillus* cells (Hall and Loughlin, 2000). The low level of bacitracin obtained from alginate-immobilized cells, may be due to the weak structure of alginate beads, furthermore, alginate beads might melt in the presence of phosphate ions (Leenen, 2000). In addition, agar-immobilized cells produced lower level of bacitracin since agar structure is not strong enough to maintain bacterial cells, allowing them to grow in the medium; in addition, it has a high melting tendency, rendering it not suitable for large-scale production (Hall and Loughlin, 2000).

In conclusion, the isolated strain *B. licheniformis* B5 produce bacitracin efficiently when grown on an optimized medium composed of less complex carbon and nitrogen sources (glycerol and glutamic acid, respectively). Elevated antibiotic activity (192 units mL^{-1}) was obtained by polyacrylamide-immobilized bacterial cells, which may be used for industrial bacitracin production.

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