OPTIMIZATION OF DIFFERENT PARAMETERS FOR THE PRODUCTION OF BACITRACIN IN SYNTHETIC MEDIUM BY *BACILLUS LICHENIFORMIS* MUTANT STRAIN UV-MN-HN-8

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ABSTRACT The present study is focused on the optimization of different parameters for the production of bacitracin using synthetic medium by *Bacillus licheniformis* UV-MN-HN-8. Maximum bacitracin production was achieved at 37°C. Optimization of incubation time reveals that bacitracin production reached its maximum limit after 48 hours of incubation. At neutral pH, bacitracin production was maximum. Effect of the addition of metal ions, phosphate sources, organic acids, and phosphate sources, inoculum age and size, temperature, pH, incubation time and aeration indicate that Ni^{+2}, KH_2PO_4, lactic acid, 20 hours old 6% inoculum, 37ºC, pH 7, 48 hours of incubation time and 25 ml medium gave higher yields of bacitracin ((51.3±1.29 IU/ml). On the basis of kinetic variables, notably $Y_{p/s}$ (IU/g substrate), $Y_{p/x}$ (IU/g cells), $Y_{x/s}$ (g/g), $Y_{p/s}$ mutant strain *B. licheniformis* UV-MN-HN-8 was found to be producing 2.33 fold more bacitracin than wild type after optimization of different parameters.

Key words: Bacitracin, *Bacillus licheniformis*, synthetic medium, optimization, production

INTRODUCTION

Bacitracin ($C_{66}H_{103}N_{17}O_{16}S$) is a branched cyclic dodecyclpeptide complex produced by *Bacillus licheniformis* and *Bacillus subtilis* (Azevedo *et al.,* 1993; Ishihara *et al.,* 2002) and is synthesized non-ribosomally by the large multienzyme complex BacABC (Konz *et al.,* 1997). It was initially reported as a single-component compound, but was later identified as a mixture of more than 50 different closely related congeners (Kang *et al.,* 2001). Bacitracin consists of a mixture of structurally similar polypeptides from 12 amino acids. It is most commonly used in complex with zinc that seems to stabilize the antibiotic complex (Ikai *et al.,* 1992). It is most commonly used in complex with zinc which seems to stabilize the antibiotic complex (Quinlan and Gutteridge, 1989). It is poorly absorbed from the gastrointestinal tract (Donoso *et al.,* 1970) as well as from skin and mucosal surfaces. Absorbed bacitracin is excreted by glomerular filtration (Prescott and Baggot, 1993).

Bacitracin was first discovered in 1943 and named after a culture of *Bacillus* and the last name of a seven-year-old American girl, Margaret Tracey, *Bacillus* was isolated from her wounds (Johson *et al.,* 1945). The compound has bactericidal effect on gram positive but little activity against gram-negative organisms (Prescott and Baggot, 1993). It is one of the most important antibiotic used in human medicine, topical application and used after surgical operations (Maciver *et al.,* 2006; Katz and Fisher, 1987) and one most commonly used in animal and poultry feed which increases feed efficiency and reduce infectious diseases (Hampson *et al.,* 2002). Despite its widespresd use, bacitracin resistance is still scarce (Ming and Epperson, 2002).

Biosynthesis was first reported by surface culture method (Flickinger and Perlman, 1979). Influence and development of the culture medium (Ganchev and Kozhuharova, 1984), effect of different amino acids (Haavik, 1981), effect of inter-relationships between the primary and secondary metabolites (Supek, *et al.,* 1985), development of solid-phase method (Lee *et al.,* 1996) and industrial aerobic parameters in real time (Iztok, *et al.,* 2000) has been studied on the biosynthesis of bacitracin.

Due to wide spread use of bacitracin, it is necessary to find out ways and measures to reduce the cost of this product. To achieve this, our focus was to utilize appropriate fermentation technology and optimization of adequate control of fermentation processes that could minimize the product costs that allows microbial factories to yield higher titers of bacitracin.

MATERIALS AND METHODS

Bacillus licheniformis strain: The cultures of *Bacillus licheniformis* isolated from soil samples and poultry dropping collected from local habitat were subjected to different mutagens. The survived strains obtained were tested for bacitracin production (Data not shown). The *Bacillus licheniformis* UV-MN-HN-8 strain that gave maximum bacitracin titer was selected for optimization of

cultural conditions and was designated as *Bacillus licheniformis* UV-MN-HN-8. This strain was maintained on nutrient agar slants having composition (g/L); Lab-Lemco powder; 1 g, Yeast extract; 2 g, Peptone; 5 g, Sodium chloride; 5 g, Agar; 15 g.

Optimization of nutritional parameters

Inoculum preparation: The inoculum was developed in 250 ml conical flask containing 25 ml medium having composition (g/L); peptone 10, Glucose 5, Beef extract 5, NaCl 2.5, $MnCl_2$ 0.7. The flask was incubated overnight at 37°C at 200 rpm. The 0.3 ml (6%) from the overnight culture was used to inoculate the 50 ml LB medium in 250 ml flask and incubated at 37°C for 6-7 hours in rotary shaker at speed of 250 rpm until O.D 600 reached at 1.5.

Effect of divalent ions: The effect of metal ions like Ba^{+2}, Co^{+2}, Cu^{+2}, Hg^{+2}, Sn^{+2}, Sr^{+2} and Zn^{+2} on the production of bacitracin production by *Bacillus licheniformis* wild (GP-40) and mutant (UV-MN-HN-8) strains were investigated. Two different ion concentrations (1×10^{-5} and 5×10^{-5}) were used. The effect of different metal ions was carried out at 37ºC, agitation speed 200 rpm and initial pH 7.0 for 48 hours.

Effect of inoculum age: Effect of age of inoculum on bacitracin production was also determined. Vegetative inoculum was developed in 250 ml flask by using rotary shaker. A 5% inoculum of different ages (08, 12, 16, 20, 24, 28, 32 and 36 hours) was examined on the mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 for the production of bacitracin in a 250 ml flask at 200 rpm after 48 hours at pH 7.0 at 37°C.

Effect of size of inoculum: The effect of 20 hour old inoculum of varying sizes (2, 4, 6, 8 and 10%) on bacitracin production by the mutant as well as the wild strain was employed to inoculate 25 ml M_8 medium in 250 ml shake flask was studied. The flasks were placed on rotary shaker for 48 hours at 200 rpm at pH 7.0 and temperature 37°C.

Effect of organic acids: Different organic acids (tartaric acid, oxalic acid, lactic acid and gluconic acid) were added in the M_8 medium separately to study their effect on the production of bacitracin. For this purpose, citric acid was not added in the M_8 medium. Each organic acid was added in the range of 0.5 gL^{-1} to 1.5 gL^{-1}. The effect of different organic acids was investigated at 37ºC, agitation speed 200 rpm and initial pH 7.0.

Effect of phosphate sources: Effect of different phosphate sources (K_2HPO_4, KH_2PO_4, $NH_4H_2PO_4$ and Na_2HPO_4) was observed for bacitracin production by both wild strain *B. licheniformis* GP-40 and mutant strain *B. licheniformis* UV-MN-HN-8. Different concentrations (0.1%, 0.2%, 0.3% and 0.4 %) were added in the culture medium (50 ml M_8 medium) at 37°C for 48 hours at 200 rpm.

Effect of temperature: The effect of different incubation temperatures (28, 30, 32, 35, 37, 40 42, 45 and 47°C) on the production of bacitracin by wild strain *B. licheniformis* GP-40 and mutant strain *B. licheniformis* UV-MN-HN-8 was carried out in 250 ml shake flask containing 25 ml M_8 medium inoculated with 24 hours old, 6% inoculum at 200 rpm for 48 hours.

Effect of pH: The effect of the different initial pH on the bacitracin production was also investigated using 25 ml medium, at 37°C, 24 hours old, 6% inoculum at 200 rpm in a 200 ml flask. Effect of different values of initial pH (4-10) of M_8 medium was studied on the production of antibiotic for mutant and wild strains.

Effect of incubation time: Different times of incubation (06, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, 78, 84, 90 and 96 hours) were employed to obtain the maximum bacitracin activity. Twenty five milliliter of M_8 medium was inoculated with 24 hours old, 6% inoculum at 37ºC in 200 ml conical flask for different time intervals for wild strain *B. licheniformis* GP-40 and mutant strain *B. licheniformis* UV-MN-HN-8.

Effect of aeration: The effect of different volumes of fermentation medium on bacitracin production by mutant as well as wild strain in 250 ml flasks was investigated at 200 rpm at 37ºC. The volume of fermentation medium used were 5, 10, 20, 30, 40 and 50%.

Statistical analysis MStat C software was used to do the statistical analysis (Rajoka *et al.,* 1997).

Standard stock solution: Zinc bacitracin (70 IU/mg) was purchased from Sigma. The bacitracin standard was weighed accurately to give 45 IU/ml concentration using N/100 HCl as diluent. The stock solution was stored at 4ºC.

Centrifugation: Twenty milliliter of cell suspension of culture broth was centrifuged in a pre-weighed falcon tube at 10,000 rpm for 15 minutes. The cell mass was washed twice with distilled water and dried at 105°C till constant weight was achieved.

Dry cell mass: Dry cell mass of *Bacillus licheniformis* was determined by the methods of Suzuki *et al*. (1976).

Antibiotic assay: The activity of the extracts was analyzed by agar diffusion method (William, 1977). The fermented broth was centrifuged at 4000 rpm and clear supernatant was used for antibiotic activity using *Micrococcus luteus* as test organism.

The potency of the sample was calculated by the following formula:

i) Difference due to doses:

$$E = ½ [(T2+S2) – (T1+S1)]$$

ii) Difference due to sample:
F = ½ [(T2+T1) – (S1+S2)]
iii) Log ratio of doses:
I = log 4
iv) Slope:
B = E/I
M = F/B
v) Potency ratio:
Antilog M
vi) Potency of sample = Potency of standard x antilog M = X units/ml
where
S2 = Standard High (in concentration)
S1 = Standard Low (in concentration)
T2 = Test High
T1 = Test Low

RESULTS

Isolation of *Bacillus licheniformis* strains: *Bacillus licheniformis* designated as GP-40 was isolated from soil and poultry droppings. The bacitracin activity of this strain was determined by measuring the zone of inhibition of *Micrococcus luteus* that came out to be 21±0.72 IU/ml.

Composition of the medium: A synthetic medium containing L-Glutamic acid, 20; L-Alanine, 0.2; Citric acid, 1; $NaH_2PO_4.H2O$, 2; KCl, 0.5; Na_2SO_4, 0.5; $MgCl_2.6H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01 was used in this study (Haavik, 1974).

Optimization of fermentation parameters: Various parameters were optimized during this study to obtain better bacitracin production. These parameters are discussed below.

(i) Effect of divalent ions: Among all the divalent ions investigated, Ni^{+2} gave maximum bacitracin production at higher concentration (5 x 10^{-5}) for both mutant strain (31±1.13 IU/ml) and wild strain (19±0.63 IU/ml) (Figure 1). Other ions Co^{+2}, Cu^{+2}, Sn^{+2}, Zn^{+2}, Hg^{+2} and Ba^{+2} gave maximum bacitracin yield at lower concentration and produced 27±1.03 IU/ml, 23±1.31 IU/ml 29±0.83 IU/ml, 26±0.69 IU/ml, 25±0.86 IU/ml and 20±1.17 IU/ml respectively for mutant strain and 12±0.43 IU/ml, 17±0.67 IU/ml, 16±0.75 IU/ml, 14±0.53 IU/ml, 13±0.58 IU/ml and 09±0.37 IU/ml respectively for wild type (Figure 1). These values are statistically significant at probability level of ≤ 0.05. The maximum bacitracin production was achieved (29±1.37 IU/ml) when Sr^{+2} were added at the concentration of 1x10^{-5}. Similarly, better bacitracin yield (31±1.26 IU/ml) was obtained when Ni^{+2}, was added at higher concentration (5x10^{-5}).

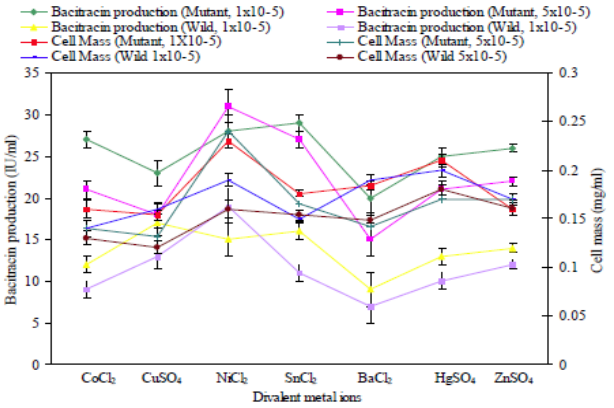

Figure 1. Effect of addition of different divalent ions on the production of bacitracin by mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 in shake flask

(ii) Effect of inoculum age: Maximum production of bacitracin (36±0.94 IU/ml) was produced by *B. licheniformis* UV-MN-HN-8 when 20 hours old inoculum was used (Figure 2). Further increase or decrease in inoculum size reduced the antibiotic production and reached 18±0.76 IU/ml and 28±1.07 IU/ml when 6 and 36 hours old inoculum was used. Similarly, wild type strain also produced maximum bacitracin (20±0.74 IU/ml) when 20 hours old inoculum was used. Bacitracin production decreased with the increase or decrease of inoculum age and reached 09±0.32 IU/ml and 18±0.48 IU/ml when 6 and 36 hours old inoculum was used. Cell

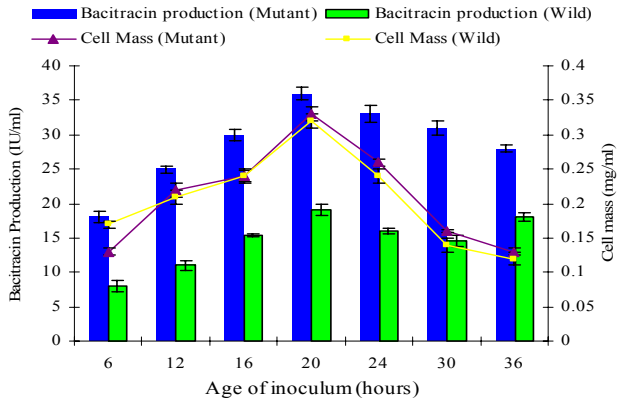

Figure 2. Effect of age of inoculum on the production of *B. licheniformis* UV-MN-HN-8 and parental strain *B. licheniformis* GP-40 in shake flask.

mass production for mutant and wild strains were calculated to be 0.328±0.021 mg/ml and 0.308±0.052 mg/ml respectively.

(iii) Effect of size of inoculum: Maximum bacitracin production (39±1.19 IU/ml) was achieved by mutant strain *B. licheniformis* UV-MN-HN-8 when 6% inoculum

was used (Figure 3). No significant increase in the bacitracin production was achieved when more than 6% inoculum was used. Maximum bacitracin production (21±0.67 IU/ml) was also observed for parent strain *B. licheniformis* GP-40. Further increase in the inoculum size did not have any significant increase on the production of bacitracin (Figure 3). Cell mass production for mutant and wild strains when 6% inoculum was used, calculated to be 0.337±0.039 mg/ml and 0.315±0.044mg/ml respectively.

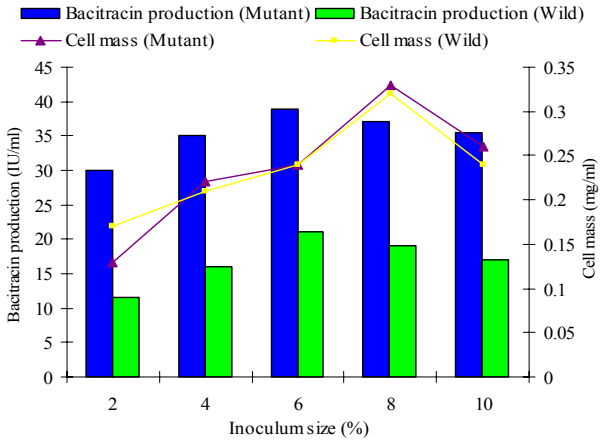

Figure 3. Effect of inoculum size on the production of *B. licheniformis* UV-MN-HN-8 and parental strain *B. licheniformis* GP-40 in shake flask.

(iv) Effect of phosphate sources: Maximum bacitracin production (43.3±1.49 IU/ml) was observed for mutant strain when 0.2% KH_2PO_4 was added in the production medium (Figure 4). Similarly, maximum bacitracin production (21±0.73 IU/ml) for wild strain was observed when 0.2% KH_2PO_4 was added in the medium. Production of bacitracin remained almost same after the addition of different concentrations of $NH_4H_2PO_4$ (0.1%, 0.2% and 0.3%) and was calculated to be 33±1.21 IU/ml, 35±0.97 IU/ml and 31±0.88 IU/ml for mutant strain and 19±0.63 IU/ml, 17±0.59 IU/ml and 17.3±0.71 IU/ml for wild strain. These values are statistically significant at probability level of ≤ 0.05. The cell mass production of mutant as well of wild strains varied slightly after the addition of different phosphate salts in the medium but production of bacitracin was significantly different (Figure 4).

(v) Effect of organic acids: Among these organic acids, addition of lactic acid produced maximum amount of bacitracin (45±1.65 IU/ml) at the concentration of 1.0 gL^{-1} (Figure 5). Increase or decrease in the concentration of lactic acid, reduced the production of bacitracin. At concentration 0.5 gL^{-1} and 1.5 gL^{-1}, bacitracin production

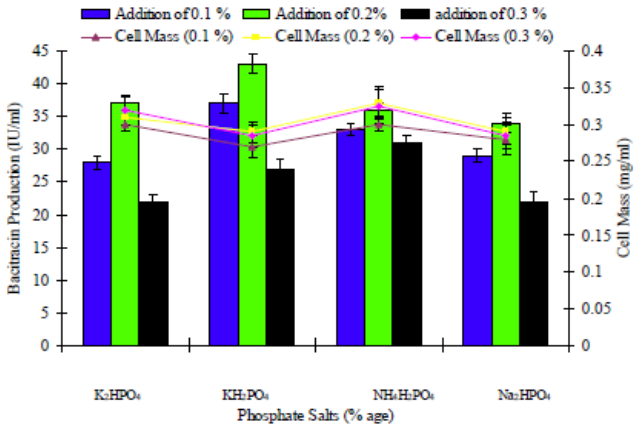

Figure 4. Effect of addition of different phosphate salts on the production of bacitracin by mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 in shake flask

was 31±0.94 IU/ml and 34±0.88 IU/ml respectively. Bacitracin production of 25±0.78 IU/ml, 36±0.98 IU/ml and 20±0.73 IU/ml was obtained when 0.5 gL^{-1}, 1.0 gL^{-1} and 1.5 gL^{-1} tartaric acid was added in the medium respectively. Similarly, 19±0.93 IU/ml, 28±1.42 IU/ml

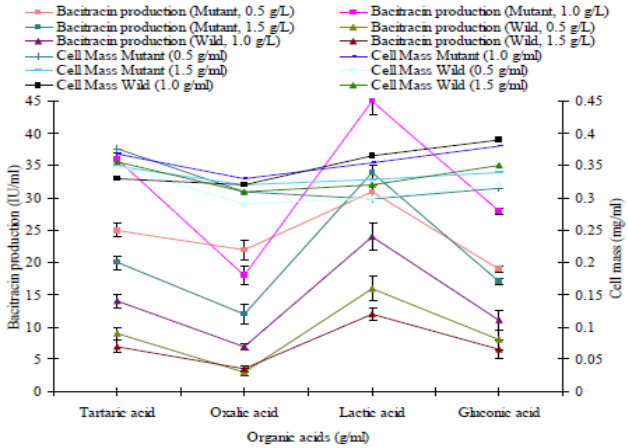

Figure 5. Effect of addition of different organic acids on the production of bacitracin by mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 in shake flask

and 17±0.57 IU/ml of bacitracin was obtained when 0.5 gL^{-1}, 1.0 gL^{-1} and 1.5 gL^{-1} of gluconic acid was added in the medium. The values obtained are statistically significant at $P \leq 0.05$. Lowest production of bacitracin was obtained when oxalic acid at the concentration of 1.5 gL^{-1} was added in the culture medium and was estimated to be 12±0.35 IU/ml. For wild strain, maximum bacitracin production was obtained when 1.0 gL^{-1} of lactic acid was added in the culture medium. Minimum bacitracin production (3±0.08 IU/ml) was obtained for

wild type when 0.5 gL^{-1} of oxalic acid was added in the medium.

(vi) Effect of temperature: It was observed that at 37°C maximum bacitracin yield of 47.6±1.78 IU/ml and 23±1.34 IU/ml was obtained for mutant and wild stain respectively. At temperature 28°C, 29±0.98 IU/ml and 9±0.46 IU/ml bacitracin was produced for mutant and wild strain respectively. When temperature was increased to 45°C, 18±1.12 IU/ml and 07±0.45 IU/ml bacitracin was produced by mutant and wild strain respectively. These values are statistically significant at $P \leq 0.05$. Dry cell mass at 37°C was 0.335±0.019 mg/ml and 0.315±0.017 mg/ml for mutant and wild strain respectively (Figure 6).

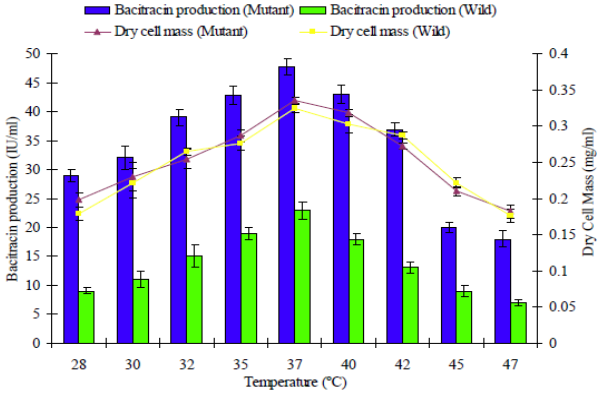

Figure 6. Effect of temperature on the production of bacitracin by *B. licheniformis* UVMN-4 HN-8 and wild strain *B. licheniformis* GP-40 in shake flask.

(vii) Effect of pH: Mutant strain produced maximum bacitracin (48±1.87 IU/ml) at pH 7.0. Bacitracin production was dropped sharply and reached 20±0.58 IU/ml at pH 10. The bacitracin synthesis was greatly reduced at pH 4 (24.9±1.22 IU/ml) (Figure 7). Similarly, for wild strain, maximum production of bacitracin (27±0.84 IU/ml) was obtained at neutral pH. Cell mass production for mutant and wild strains were calculated to be 0.31±0.026 mg/ml and 0.295±0.041 mg/ml respectively.

(viii) Effect of incubation time: Maximum bacitracin activity (49±1.43 IU/ml) was obtained after 48 hours of inoculation by mutant strain (Figure 8). In first 24 hours there was a more or less progressive increase in the production of bacitracin with the time, but after 24 hours of inoculation, an abrupt increase in the antibiotic formation was observed (18±0.89-52±1.74 IU/ml) that continued till 48 hours incubation. After 48 hours, however, the bacitracin production was declined steadily

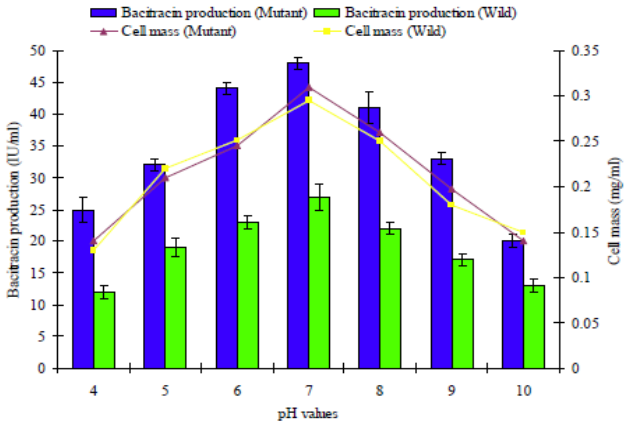

Figure 7. Effect of pH on the production of *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 in shake flask.

with time and reached 26±1.57 IU/ml after 96 hours incubation. It may be due to the inhibitory effect of produced antibiotic. Maximum production by parent strain *B. licheniformis* GP-40 was also achieved after 48 hours of incubation (26±1.05) with abrupt increase in bacitracin production after 24 hours.

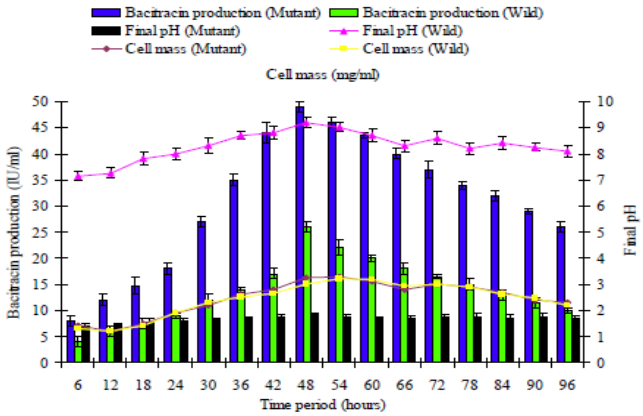

Figure 8. Effect of incubation time on the production of bacitracin by mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 in shake flask

(ix) Effect of aeration: The adequate supply of oxygen for aerobic fermentation is very essential for the propagation of microbial cultures and synthesis of metabolites. The dissolved oxygen in the medium becomes available to the cultures for growth and metabolism. The maximum bacitracin production (51.3±1.29 IU/ml) was obtained when 10% volume of fermentation medium was used i.e., 25 ml/250 ml flask. Further increase in the volume of medium resulted in the decreased bacitracin production and reached 38±1.25 IU/ml and 27±0.87 IU/ml when 5% and 50% medium was used respectively (Figure 9). The maximum bacitracin production (22±0.77 IU/ml) for wild strain was

also obtained when 10% (25 ml) fermentation medium was used in 250 ml flask. Like mutant strain, bacitracin production for wild strain also decreased when volume of the medium was increased or decreased and estimated to be 14±0.46 IU/ml and 9±0.32 IU/ml when 5% and 50% medium was used. These values are statistically significant at probability level of ≤ 0.05. The dry cell mass was calculated to be 0.31±0.012 mg/ml and 0.29±0.027 mg/ml for mutant and wild strains respectively when 10% volume of the medium was used.

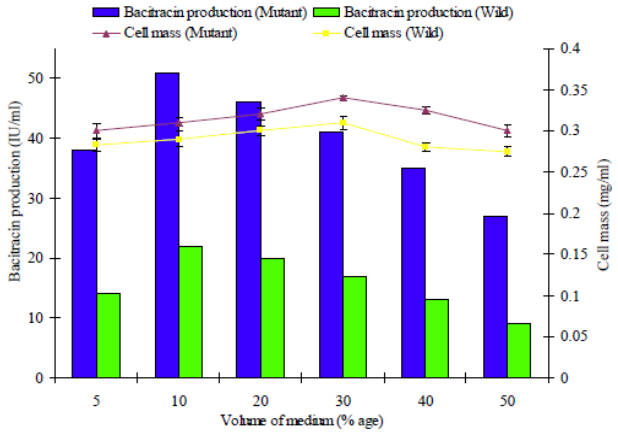

Figure 9. Effect of volume of the medium on the production of bacitracin by mutant strain *B. licheniformis* UV-MN-HN-8 and parental strain *B. licheniformis* GP-40 in shake flask.

Table 1. Comparative substrate consumption parameters of *Bacillus licheniformis* wild strain (GP-40) and *Bacillus licheniformis* mutant strain (UV-MN-HN-8) following optimization of different parameters in the synthetic medium M_8.

Kinetic parameters	Parental strain (*B. licheniformis*)	Mutant strain (*B. licheniformis* UV-MN-HN-8)
$Y_{p/s}$ (IU/g substrate)	21±0.72^{ab} IU/ml	49±1.43^{ac} IU/ml
$Y_{p/x}$ (IU/g cells)	60.86±1.42^{de} IU/ml	151±2.14^{ab} IU/ml
$Y_{x/s}$ (g/g)	0.70±0.07^{cd} IU/ml	0.72±0.09^{cd} IU/ml
Q_p (IU/m/h)	12.55±0.31^{ae} IU/ml	37.75±0.92^{ad} IU/ml
Q_s (g/l/h)	1.90±0.09^{ad} IU/ml	3.10±0.1^{ab} IU/ml
Q_x (g/l/h)	1.54±0.076^{ae} IU/ml	1.97±0.069^{ac} IU/ml

Each value is a mean of three replicates. Values followed by different letters differ significantly at $p \leq 0.05$.

$Y_{p/s}$= enzyme produced/g substrate consumed. $Y_{p/x}$= enzyme produced/g cell mass formation. $Y_{x/s}$= g cells produced/g substrate consumed. Q_p= enzyme produced/l/h. Q_s= g substrate consumed/l/h. Q_x= g cell mass formation/l/h.

Kinetic parameters of mutant strain: The mutant strain was compared with the parental strain for the productions of bacitracin. There was significant enhancement in the antibiotic production by the mutant strain. It is clear from the table 1 that the yield of the antibiotic per gram substrate consumption and per gram cell mass formation was about 2.5 times more than the parental strain. The values of Qp, Qs and Qx by the parental and mutant strain were 12.55±0.31, 1.90±0.09, 1.54±0.076 and 37.75±0.92, 3.10±0.1, 1.97±0.069 respectively. Each value is an average of three replicates.

DISCUSSION

In this work, we have explained the optimization of mutant strain of *Bacillus licheniformis* UV-MN-HN-8 that was earlier mutated by using different mutagens. Synthetic media containing all essential minerals, carbon and nitrogen sources was used as production media (Haavik, 1974). The higher yields of bacitracin in M_8 medium obtained due to the presence of L-glutamic acid that is a constituent of bacitracin that is entered in the cell and converted to its optical isomer for the synthesis of bacitracin. The addition of organic salts like citric acid helped in the formation of soluble salt arrangement after combining with metal ions, thus making them available to the microorganisms. The result obtained is in agreement with the observations of Snoke (1961). The addition of organic salts like citric acid helped in the formation of soluble salt arrangement after combining with metal ions, thus making them available to the microorganisms. Data indicate that the production of bacitracin is maximum i.e 47.6±1.78 IU/ml at temperature 37°C (Figure 1). 18±1.12 IU/ml for mutant strain and 09±0.46 IU/ml and 07±0.45 IU/ml for wild strain. This phenomenon may be due to the fact that rate of all metabolic processes increased by increase in temperature but these metabolic processes decreased after certain temperature limits. The results obtained in this study were also demonstrated by other workers (Cohen, 1957; Freeny and Allen, 1958; Zorn, 1961; Sandov *et al.,* 1978; Bushra *et al.,* 2007).

The effect of divalent metal ions on bacitracin production was also investigated. Maximum bacitracin production was obtained when Ni^{+2} was added in the medium (Figure 1). Similarly better bacitracin yield (31±1.13 IU/ml) was obtained when Sn^{+2} was added. Froyshov *et al.,* (1980) studied the influence of divalent metal ions (Mg^{+2}, Mn^{+2}, Fe^{+2} or Co^{+2}) on the activity of the bacitracin of *Bacillus licheniformis* ATCC 10716 and proposed that complexes between bacitracin and metal ions exert feedback control on the synthetase. It was proposed by Froyshov *et al.,* (1980) that L-isomers of the bacitracin constituent amino acid, D-glutamic acid, D-phenylalanine, D-aspartic acid supported the synthesis of bacitracin in the presence of divalent metal ions on

bacitracin activity and showed that these metal ions are important for bacitracin activity.

Maximum bacitracin production (36±0.94 IU/ml) was obtained when 20 hours old inoculum was used. Further increase or decrease in inoculum size reduced the antibiotic production. It could be due to the fact that cells of a younger inoculum were explained to be in a more active state in terms of multiplication, whereas an older inoculum could be partially or fully induced to product formation (Neves *et al.,* 2000; Lopes *et al.,* 2002). As far as size of inoculum is concerned, maximum bacitracin production (39±1.19 IU/ml) was produced when 6% inoculum was used. Further increase in the inoculum size did not have any significant increase on the production of bacitracin (Figure 3). It might be due to the reason that it consumed majority of the substrate for growth and metabolic processes, hence antibiotic synthesis decreased.

The addition of phosphate (K_2HPO_4, KH_2PO_4, $NH_4H_2PO_4$ and Na_2HPO_4) in the production medium showed that maximum production of bacitracin (43.3±1.49 IU/ml) was obtained when 0.2% KH_2PO4 was added in the medium (Figure 4). Haavik (1974a) studied the role of different inorganic phosphate that addition of these phosphates have no affect on bacitracin production but excessive addition of these phosphate binder bacitracin production.

Effect of organic acids also plays an important role in the bacitracin production. Addition of lactic acid plays a vital role in bacitracin production and maximum bacitracin production (45±1.65 IU/ml) was obtained (Figure 5). Similar results were obtained by Haavik (1974) and Supek *et al.,* (1985).

The incubation temperature also has a profound effect on the bacitracin production (Figure 6). Data indicate the production of bacitracin over a wide rage of temperature by both wild type strain *B. licheniformis* GP-40 and mutant strain *B. licheniformis* UV-MN-HN-8. At temperature 37°C, maximum production of bacitracin was obtained by wild and mutant strains i.e. 47.6±1.78 IU/ml and 23±1.34 IU/ml respectively. By increasing or decreasing the temperature, the bacitracin production dropped sharply and at temperature 28°C and 47°C, the bacitracin production dropped to 29±0.98 IU/ml and 18±1.12 IU/ml for mutant strain and 09±0.46 IU/ml and 07±0.45 IU/ml for wild strain. . This phenomenon may be due to the fact that rate of all metabolic processes increased by increase in temperature but these metabolic processes decreased after certain temperature limits and high temperature has inhibitory effect on the growth of microorganism. The results obtained in this study were also demonstrated by other workers (Cohen, 1957; Freeny and Allen, 1958; Zorn, 1961; Sandov *et al.,* 1978). They have shown that maximum bacitracin titers were obtained at temperatures 30°C-37°C. However, Egorov *et al*. (1985) and Kitada *et al*. (1987) have reported maximum bacitracin yields in *Bacillus licheniformis* 28KA at temperature 55°C and 50°C-60°C respectively. Our results were also in agreement with the result obtained by Bushra *et al*. (2007) that indicated that maximum polypeptide antibiotic produced by *Bacillus subtilis* that was active against *Micrococuss leutus* was obtained at temperature 37ºC. Haddar *et al*. (2007) obtained maximum bacitracin production of *Bacillus licheniformis* B5 at temperatures 37ºC-40ºC.

Maximum bacitracin production by mutant strain *B. licheniformis* UV-MN-HN-8 (48±1.87 IU/ml) was obtained at pH 7.0, both high and low pH values inhibit bacitracin production (Figure 7). Change in pH might affect the basal metabolism of the organism that resulted in decreased growth and low bacitracin production. It may also alter the structure and function of the antibiotic and thus retard its activity. Ionization of inorganic salts may also have adverse effect on their solubility due to change in pH that in turn will not be available to the microorganisms. This is in accordance with the results obtained by Hendlin (1949), Haavik (1974a) and Haddar *et al.,* (2007) who reported that both high and low pH values inhibit bacitracin synthesis.

Time period is also important factor in determination of maximum metabolite production. Maximum bacitracin production was obtained after 48 hours of incubation (Figure 8). For the first 24 hours, there was a less progressive increase in the bacitracin production (8±0.48 to 18±0.89 IU/ml). After 24 hours, an abrupt increase was observed that continued till 48 hours and reached 52±1.74 IU/ml. It is evident from the results that time course of antibiotic production plays a very critical role in antibiotic synthesis (Sztajar and Maliszewska, 1988). It might be due the growth phase, organisms entered in the stationary phase of growth. The decreased antibiotic production might also be due to the exhaustion of the nutrients and production of metabolic byproducts (inhibitors) in the fermenting medium (Martinez *et al.,* 1993). After 48 hours, however, bacitracin production was declined and reached 29±0.92 IU/ml after 96 hours of incubation. A similar relationship between time course and antibiotic production was observed by Haavik (1979). Awais *et al.,* (2008) also showed maximum bacitracin production by mutant strain *B. pumilus* after 48 hours incubation at 37°C. The maximum bacitracin production (51.3±1.29 IU/ml) was obtained when 10% volume of fermentation medium was used i.e., 25 ml/250 ml flask. Further increase in the volume of medium resulted in the decreased bacitracin production and reached 27±0.87 IU/ml when 50% medium was used respectively (Figure 4.9). It might be due to the improper agitation and inadequate aeration which consequently decreased enzyme production (Martinez *et al.,* 1993). At low level (12.5 ml) of the volume of the fermentation medium both wild (14±0.46 IU/ml) and mutant (8.7±0.2) strains showed insignificant

production of enzyme. It might be due to the insufficient supply of nutrients for the growth of bacteria and hence enzyme formation (Vanot *et al.,* 2002).

Among the kinetic parameters, the values for $Y_{p/s}$, $Y_{p/x}$ and $Y_{x/s}$ after optimization of different parameters clearly indicates the significant improved production of bacitracin by mutant strain over the parental strain. The maximum growth in terms of rate for cell mass formation (Q_x) was only marginally different for two strains. Similar, findings have previously been reported by Pirt *et al.,* (1975). However, present report is several folds (≈2.33) improved bacitracin production over wild type.

The findings of the present study led to conclude that bacitracin production by mutant strain *Bacillus licheniformis* UV-MN-HN-8 can be enhanced by optimizing the different parameters during shake flask studies.

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