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ENHANCED PRODUCTION OF BACITRACIN BY A MUTANT STRAIN *BACILLUS LICHENIFORMIS* UV-MN-HN-8 (ENHANCED BACITRACIN PRODUCTION BY MUTAGENESIS)

MUHAMMAD NAUMAN AFTAB[1]*, IKRAM-UL-HAQ[1] AND SHAHJAHAN BAIG[2]

[1]*Institute of Industrial Biotechnology, GC University, Lahore, Pakistan*
[2]*Biotechnology and Food Research Center, PCSIR Laboratories Complex, Lahore, Pakistan*

Abstract

The present study is focused on the improvement of *Bacillus licheniformis* through random mutagenesis to obtain mutant having enhanced production of bacitracin. Many isolates of *Bacillus licheniformis* were isolated and the isolate GP-40 produced maximum bacitracin production (16±0.72 IU/mL). Treatment of *Bacillus licheniformis* GP-40 with ultraviolet (UV) radiations increased bacitracin production to 29±0.69 IU/mL. Similarly, treatment of vegetative cells of GP-40 with chemicals like N-methyl N'-nitro N-nitroso guanidine (MNNG) and Nitrous acid (HNO_2) increased bacitracin production to 35±1.35 IU/mL and 29±0.89 IU/mL respectively. Studies regarding the combined effect of UV and chemical treatment on parental cells exhibited significantly higher titers of bacitracin with maximum bacitracin production reached to 47.6±0.92 IU/mL. An increase of 2.97 fold production of bacitracin in comparison to wild type was observed. Mutant strain was highly stable and produced consistent yield of bacitracin even after 15 generations. On the basis of kinetic variables, notably $\mu(h^{-1})_{max}$, $Y_{p/x}$, q_p, Q_p and Q_x mutant strain *B. licheniformis* UV-MN-HN-8 was found to be a hyperproducer of bacitracin.

Introduction

Bacitracin ($C_{66}H_{103}N_{17}0_{16}S$) is a branched cyclic dodecyclpeptide produced by *Bacillus licheniformis* and some strains of *Bacillus subtilis* (Ishihara *et al*., 2002). It is synthesized non-ribosomally by the large multienzyme complex BacABC (Katz & Fisher, 1987). It was initially reported as a single-component compound, but was later identified as a mixture of more than 50 different closely related peptides (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). Bacitracin was first discovered in 1943 and named after a culture of *Bacillus* and the last name of a 7 year old American girl, Margaret Tracey, from whose wounds the *Bacillus* was isolated (Johnson *et al*., 1945). The compound has bactericidal effect on gram-positive bacteria but little activity against gram-negative organisms (Prescott & Baggot, 1993). Bacitracin has wide spread application in pharmaceutical for the treatment of chronic sinusitis, *Clostridium difficile* associated disease, necrotic enteritis, topical antibiotic (Nicole *et al*., 2007; Brad *et al*., 2008) feed additive and reduction of diseases in poultry (Hampson *et al*., 2002; Miles *et al*., 2006). Despite its widespread use, bacitracin resistance is still scarce (Ming & Epperson, 2002). It has also been reported that bacitracin has no negative impact on human health.

Previous workers have improved the bacitracin yields of *Bacillus licheniformis* by treating its vegetative cells with UV and chemical mutagens. Vegetative cells of *Bacillus licheniformis* were treated with UV irradiation and then cultured on medium containing Fe^{+2} ions and pantothenic acid (Liyong *et al*., 1988). Bacterial cells were treated with 0.5 M ethyl methanesulphonate for 3 h and cultured in a medium containing soybean meal,

sucrose and mineral salts (Paleckova & Smekal, 1981). Vegetative cells of *Bacillus licheniformis* were exposed with N-methyl-N'-nitro-N-nitrosoguanidine by which bacitracin production was drastically increased (Lukin *et al*., 1986). Better yield of bacitracin was obtained by treatment of cells with MNNG and was suggested that blocking of alternative pathways of intermediates could increase the bacitracin production (Delic *et al*., 1970).

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 random mutagenesis using physical (UV) and chemicals (MNNG and HNO_2) to induce positive mutations in microbial DNA for enhanced production of bacitracin. By use of mutagens we have developed the hyper producing bacitracin strain *Bacillus licheniformis* UV-MN-HN-8. Appropriate fermentation technology and optimization of adequate control of fermentation processes could further minimize the product costs and allowed the small microbial factories to yield higher titers of bacitracin.

Material and Methods

Isolation, identification and screening: The cultures of bacitracin producing strains were isolated from different soil and milk samples collected from local habitat. All the samples were collected in sterile containers. Different strains were isolated from the above samples prepared in saline water by making serial dilutions by plate method (Warcup, 1950). Identification of isolated bacitracin producing species was carried out by studying morphological, cultural, physiological and biochemical characters on the basis of Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). Screening of the identified isolates was carried out on the basis of bacitracin production (IU/mL) and was determined by agar diffusion method after William (1977) in terms of bacitracin activity produced by using submerged fermentation in 250 mL flasks containing screening medium.

Mutagenesis and isolation of hyper producer of bacitracin: In this study both physical (UV) and chemical (MNNG and HNO_2) mutagens were employed in systematic manner to obtain mutants that gave higher bacitracin production. For UV irradiation, method of Parekh *et al*., (2000) was adopted. For chemical mutagenesis using MNNG and HHO_2 method of Delic *et al*., (1970) was followed. The parental strain *B. licheniformis* GP-40 was treated with all the three mutagens (UV, MNNG and HHO_2) consecutively. First the parental strain was subjected to UV irradiation ($1.2x10^2$ $J/m^2/s$) for 20 minutes. The mutant strain that produced maximum bacitracin was subjected to 1.5 mg/mL MNNG for 20 minutes. After this treatment, mutant strains obtained were screened for the bacitracin titer. The strain that yielded the better bacitracin production was then treated with 0.06 M HNO_2 for 15 minutes. The survivals that produced better yield of bacitracin than wild type was selected.

Inoculum and screening medium: 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 500 mL flask and incubated at 37ºC for 6-7 hours in rotary shaker at 250 rpm until O.D 600 reached at 1.5.

Bioassay of bacitracin: The activity of the extracts was analyzed by agar diffusion method (William, 1977). LB agar medium was autoclaved and 20 mL of the medium was poured aseptically in the sterile petri plates and allowed to congeal. In the mean while, sterilized LB agar medium (assay medium) having 50-60°C temperature was inoculated with predetermined concentration of *Micrococcus luteus* (CN-5537) by using broth culture prepared by the inoculation of *Micrococcus luteus*. Four mililiter of the inoculated melted assay medium was spreaded uniformLy over the first layer and allowed to solidify. The plates were refrigerated at 4°C and used according to the need. At the time of assay the plates were taken out from the refrigerator and 4 wells of 0.8 cm diameter were made in each plate aseptically with sterilized stainless steel bores of uniform edge and size. The pieces of agar from the digged wells were picked and removed with the help of sterilized loop. The two opposite wells were filled with the working standard of 1:4 dilutions and marked as S_1 and S_2, respectively. The remaining two were filled with the sample whose potency was to be determined in the same dilution (1:4) and marked T_1 and T_2 respectively. One hundred and twenty micro liter bacitracin standards as well as samples were poured with the help of micropipette in the digged holes. The plates were then placed carefully (to avoid spreading of solution due to tilting of the plates) in incubator for 18-24 hours at temperature 37°C. Clear zones of inhibition were developed and diameter of zones of inhibition were measured and compared with the known standard.

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

The difference due to doses (E) is calculated by subtracting the (T_1+S_1) from ½ (T_2+S_2). The difference due to sample (F) is calculated by subtracting the (S_1+S_2) from ½ (T_2+T_1). The log ratio of doses (I) was taken as log 4. Slope (M) was calculated by dividing the (F) with (B) where (B) was calculated by dividing (E) with (I). Potency ratio was measured by taking the antilog of M. Potency of sample was measured by multiplication of potency of standard with antilog M, where S_2 = Standard High (in concentration), S_1 = Standard Low (in concentration), T_2 = Test High, T_1 = Test Low. The units determined were represented in X units/mL.

Kinetic studies: Kinetic parameters for batch fermentation experiments were determined according to the methods described by Pirt (1975) and Lawford & Roseau (1993). The following parameters of kinetics were studied. Dry cell mass of *Bacillus licheniformis* was determined by the methods of Suzuki *et al*., (1976). The values of specific growth rate was measured by dividing the values of plot of ln(X) by the time of fermentation. Product yield co-efficient $Y_{p/x}$ was measured by dividing the values of 'dP' by the values of 'dX'. The volumetric rates for product formation (Q_p) was determined by dividing the values obtained from the maximum slopes in plots of bacitracin produced by the time of fermentation. The volumetric rates for biomass formation (Q_x) was measured by dividing the values of maximum slope in plot of cell mass by the incubation time. The specific rate constant for bacitracin formation (q_p) was determined by multiplying the values obtained for μ with the values obtained for $Y_{p/x}$.

Table 1. A: General characteristics of the isolates of *B. licheniformis*. Identification of *B. licheniformis* GP-40 on the basis of B: Carbohydrate fermentation test, C: Biochemical tests.

A

S. No.	Characteristics	Results for all isolated cultures
1.	Shape on agar plates	Large, spreading, rough surface, hairy outgrowth
2.	Shape on agar slants	Growth abundant, rough, opaque, adherent, spreading with hairy out growths
3.	Gram staining	+ve
4.	Endospore formation	+ve
5.	Motility	+ve

B

S. No.	Characteristics	Results for *B. licheniformis* GP-40
1.	Sucrose	+ve
2.	Lactose	+ve
3.	Glucose	+ve
4.	Rhamnose	-ve
5.	Raffinose	+ve
6.	Galactose	-ve
7.	Mannitol	+ve

C

S. No.	Test	Characteristics of *B. licheniformis*	Results for *B. licheniformis* GP40
1.	Indole	-ve	-ve
2.	Methyl red	-ve	-ve
3.	Urease	+ve	+ve
4.	Gelatin liquefaction	+ve	+ve
5.	Growth in NaCl	Good growth up to 7% NaCl	Good growth in 7% NaCl
6.	Hugh and Leifson test	Fermentative	Fermentative
7.	Voges Proskauer test	-ve	-ve
8.	Catalase test	+ve	+ve
9.	KCN test	-ve	-ve
10.	Simon citrate	+ve	+ve
11.	Reduction of nitrate to nitrite	+ve	+ve

Results and Discussion

A total of 87 bacitracin producing strains were isolated from different samples of soil and milk by the method of Warcup (1950). These isolates were identified to be *Bacillus licheniformis* on the basis of morphological characteristics (Table 1A). It has been reported earlier that bacitracin was mainly produced by *Bacillus licheniformis* (Gavrilescu & Roman, 2004; Murphy *et al*., 2007). Screening for the isolate that produce maximum bacitracin was carried out on the basis of bacitracin production. The bacitracin activity was determined by agar diffusion method after William (1977) by measuring the zone of inhibition of test strain *Micrococcus luteus*. Maximum bacitracin was produced by *Bacillus licheniformis* designated as GP 40 and was calculated to be 16±0.72 IU/mL. This strain (GP 40) was further confirmed on the basis of carbohydrate fermentation and biochemical tests (Table 1B and C).

Table 2. Effect of UV, MNNG and HNO_2 on bacitracin production.

Mutagen	Concentration	Time of treatment (min)	Total number of screened survivors	Over-producing clones of bacitracin	Maximum production of bacitracin by mutant
UV	$1.2x10^2$ J/m^2/s	5-30	135	22 (16.3 %)	GP-UV-15 26±0.69 IU/mL
MNNG	1.0-2.0 mg/mL	5-30	295	37 (12.5 %)	GP-MNNG-28 33±0.79 IU/mL
HNO_2	0.04-0.08 M	2-60	367	51 (13.8 %)	GP-HN-23 29±0.89 IU/mL

In the strategy adopted to improve *Bacillus licheniformis* strains, classical mutagenesis was applied to such an extent that the frequency of desired mutation was maximized. Therefore, amongst various mutagens causing multiplicity of mutations (Parekh *et al*., 2000), UV and nitrous acid were employed in addition to MNNG. The vegetative cells of wild type strain *B. licheniformis* GP-40 was irradiated by UV light. Out of 135 mutant strains, only 22 (16.3%) mutant strains were selected that gave increased bacitracin production as compared to wild type (16±0.72 IU/mL). Maximum bacitracin production (26±0.69 IU/mL) was observed for mutant strain designated as GP-UV-15. The work is in accordance with the findings of Liyong *et al*., (1988) who treated vegetative cells of *Bacillus licheniformis* by UV irradiation to improve the production of bacitracin. Wild cells of *B. licheniformis* were exposed to 5-30 minutes with different concentrations (1.0-2.0 mg/mL) of MNNG. A total of 295 mutant strains were obtained after different time of exposure. Survivors were screened for bacitracin production. It was observed that only 37 (12.5%) mutant strains gave increased production of bacitracin in comparison with wild type. Out of these *Bacillus licheniformis* GP-MNNG-28 produced the best yield of bacitracin (33±0.79 IU/mL) (Table 2). Likewise, out of 367 mutant strains obtained only 51 (13.8%) colonies produce higher bacitracin yield than parental strain when cells were treated with 0.04-0.08 M HNO_2 respectively for different time intervals (Table 1). Maximum bacitracin production (29±0.89 IU/mL) was obtained by *Bacillus licheniformis* GP-HN-23. Furthermore, mutagenesis studies showed that all mutagens (UV, MNNG and HNO_2) helped to develop mutant strains that gave better yield of bacitracin than the wild strain. An increased production of bacitracin was demonstrated after exposure of *B. licheniformis* cells to chemical mutagen (Paleckova & Smekal, 1981). The parental strain *B. licheniformis* GP-40 was also treated with all the three mutagens (UV, MNNG and HHO_2) consecutively. Mutant strain GP-UV-15 (26±0.69 mg/mL) obtained after UV treatment (Table 2) was subjected to 1.5 mg/mL MNNG for 20 minutes. Survivals were tested and maximum bacitracin production (37±1.35 IU/mL) obtained from mutant strain was designated as *B. licheniformis* UV-MN-5. The vegetative cells of this strain were further treated with 0.06 M HNO_2 for 15 minutes. Survivals were tested and better bacitracin production (47.6±0.92 IU/mL) was obtained by strain designated as *B. licheniformis* UV-MN-HN-8. The flow sheet diagram of this scheme and mutants obtained is shown in Fig. 1. Similar results regarding mutagenesis were reported by Calam (1964) and Adelberg *et al*., (1965).

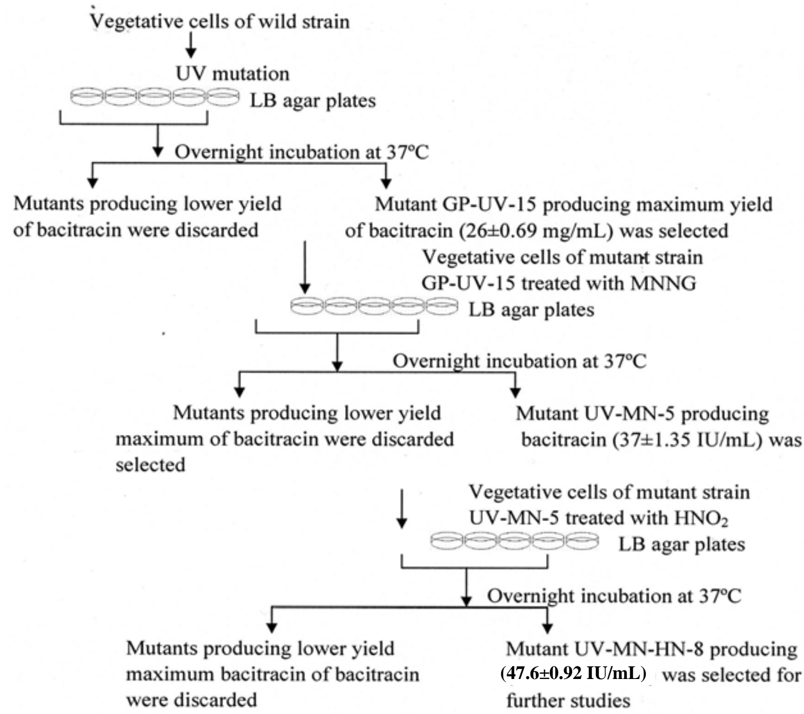

Fig. 1. Adopted strategy for screening of *B. licheniformis* UV-MN-HN-8 survivors that over produce bacitracin following mutations by UV, MNNG and HNO_2.

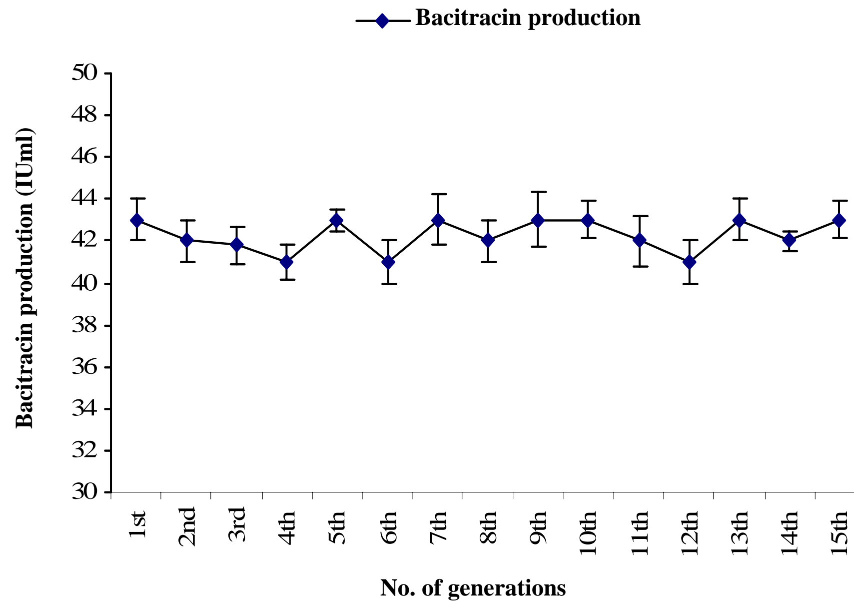

Fig. 2. Validation of the stability of the mutant strain *B. licheniformis* UV-MN-HN-8.

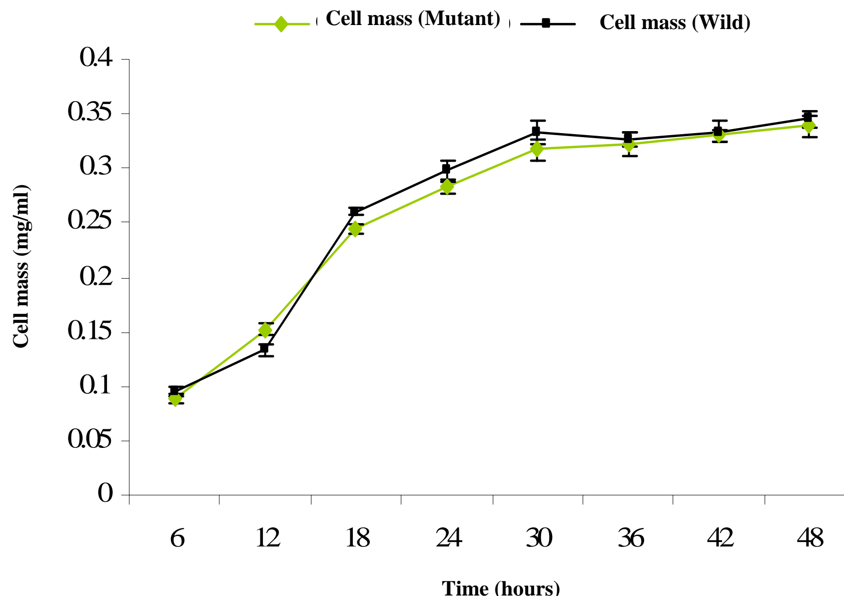

Fig. 3. Comparison of wild and mutant strain with respect to cell mass production.

Table 3. Comparison of wild and mutant strain by kinetic study.

Kinetic parameters	Parental strain (*B. licheniformis* GP-40)	Mutant strain (*B. licheniformis* UV-MN-HN-8)
$\mu(h^{-1})_{max}$	0.19 ± 0.017	0.196 ± 0.021
$Y_{p/x}$ (IU/g cells)	60.86 ± 2.42	151 ± 3.14
Q_p (IU/m//h)	12.55 ± 0.31	37.75 ± 1.33
Q_s (g/l/h)	1.90 ± 0.41	2.10 ± 0.28
Q_x (g/l/h)	1.34 ± 0.076	1.97 ± 0.069
q_p (g cells/h)	1.11 ± 0.16	1.2 ± 0.28

$Y_{p/x}$= antibiotic produced/g cell mass formation, Q_p= antibiotic produced/l/h, Q_s= g substrate consumed/l/h, Q_x= g cell mass formation/l/h, q_p = Unit product produced/g cells/h

The stability of mutant strain *B. licheniformis* UV-MN-HN-8 with respect to bacitracin production was also evaluated. It was observed that this mutant was highly stable upto 15 generations tested in a time period of about 6 months. It might be due to MNNG and HNO_2 that have caused permanent change in DNA sequence or stabilized the pre-induced UV mutation (Fig. 2). It has been observed that wild strain and their mutants showed similar general characters. The colonies of mutant strain showed distinct circular and opaque morphology similar to that of wild strain. No unusual phenotype was detected after mutation. No difference in the growth of both wild type and mutant strain was observed as assessed by measurement of biomass (Fig. 3). This result indicates that the enhancement of antibiotic production by the mutant strain is not because of an increase in growth but due to the enhancement in production of the antibiotic. Kinetic parameters studied clearly indicate

the difference between the parental strain *B. licheniformis* GP-40 and the mutant strain *B. licheniformis* UV-MN-HN-8 (Table 3). The parental and mutant strain showed 60.86±2.42 and 151±3.14 for $Y_{p/x}$ values indicating that specific enzyme yield is much higher for mutant strain as compared to parent strain. Antibiotic production rate (Q_p) for mutant strain was almost three times higher (37.75±1.33) than that of wild strain (12.55±0.31). As far cell mass formation was (Q_x) concerned, mutant strain has much faster rate 1.97±0.069 as compared to wild strain i.e 1.34±0.076. Kinetic parameters like $Y_{p/s}$, $Y_{p/x}$, Q_p and q_p for mutant strain was also studied by Haq *et al*., (2008).

Conclusion

Bacitracin producing *Bacillus licheniformis* isolated from soil sample was subjected to mutagens. Better yields of bacitracin was obtained from *Bacillus licheniformis* UV-MN-HN-8 that produced 47.6±0.92 IU/mL in comparison to 16±0.72 IU/mL produced by wild type. A 2.97 fold increased bacitracin was achieved by mutant strain after mutagen treatment. The mutant strain was stable and gave consistent production of bacitracin even after 15 generations. Kinetic parameters also revealed the hyper active ability for bacitracin production by mutant strain *Bacillus licheniformis* UV-MV-HN-8.

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