ABSTRACT

Fifty seven strains of *Bacillus licheniformis* were isolated from soil, milk and poultry droppings from different areas of Lahore. Pour plate method using TYE agar medium was used for the isolation of *B. licheniformis*. All the isolated cultures were screened for the bacitracin production by hole plate method using *Micrococcus luteus* as test strain. Strain *Bacillus licheniformis* GP-40 produced maximum bacitracin production (21±0.72 IU/mL) and was identified on the basis of physiological and biochemical tests. *Bacillus licheniformis* GP-40 was treated with ultraviolet (UV) radiations and chemical treatment by N-methyl N-nitro N-nitroso guanidine (MNNG) and nitrous acid (HNO_2) for improvement in bacitracin production. UV treatment of parental cells produced 87 mutants. Out of these mutants only 29 produced higher concentrations of bacitracin than wild type and maximum bacitracin production (29±0.69 IU/mL) was observed for mutant strain designated as GP-UV-15. When parental cells were treated with different concentrations of MNNG 53, 42, 57, 43, 59 and 41 mutants were obtained. Out of these mutants 9, 7, 8, 9, 8 and 7 mutants produced higher bacitracin titers. Maximum bacitracin production (35±1.35 IU/mL) was obtained from mutant strain designated as GP-MNNG-28. Similarly, parental cells were treated with different concentrations of HNO2. Out of 48, 63, 52, 57, 45, 49 and 53 mutant strains obtained, 8, 8, 9,8, 6 and 9 strains produced higher bacitracin yield. Maximum bacitracin (31±0.89 IU/mL) was produced by mutant strain designated as GP-HN-23. Studies regarding the combined effect of UV and chemical treatment on parental cells yield significantly higher titers of bacitracin with maximum bacitracin (43±1.21 IU/mL) produced by mutant strain designated as *B. licheniformis* UV-MN-HN-8. Mutant strain was highly stable and produced consistent yield of bacitracin. After mutagenesis, cultural conditions of the mutant strain *B. licheniformis* UV-MN-HN-8 as well as wild strain *B. licheniformis* GP-40 were optimized. Both strains were grown at different temperature values ranging from 28-47ºC. Maximum bacitracin production for wild (47.6±1.78 IU/mL) as well as for mutant strain (23±1.34 IU/mL) was obtained when temperature was maintained at 37ºC. The effect of pH on the production of bacitracin by *B. licheniformis* was also studied. *B. licheniformis* was grown on different pH values (4-10). Maximum bacitracin titers were obtained for wild (27±0.84 IU/mL) and mutant strain (48±1.87 IU/mL) when pH value of

fermentation medium was maintained at 7.0. Incubation time also plays a vital role in the bacitracin production. Maximum bacitracin production was achieved for wild (26±1.05 IU/mL) and mutant (49±1.43 IU/mL) strain after 48 hours of incubation. Maximum bacitracin production was achieved for wild (23±0.74 IU/mL) and mutant (49±1.15 IU/mL) strains when 20 hours old inoculum was used. Similarly, maximum bacitracin production for both wild strain (22.5±0.67 IU/mL) and mutant strain (50.3±1.89 IU/mL) was achieved when 6% inoculum was used. Agitation speed also influenced the bacitracin production. Wild and mutant strains produced highest yield of bacitracin i.e. 51.4±1.30 IU/mL and 21±0.85 IU/mL when agitation speed was kept at 200 rpm. Parameters like effect of addition of organic acids, nitrogen sources, divalent metal ions and phosphate salts were employed to enhance the bacitracin production in shake flask studies. Maximum bacitracin production obtained after optimizing all the parameters in shake flask studies was 53±1.79 IU/mL for mutant strain and 36±0.93 IU/mL for wild strain. For scale up studies, 2 L glass fermenter (working volume 1 L) was used for bacitracin production. Different parameters like incubation time, inoculum age, inoculum size, aeration, agitation and dissolved oxygen were optimized to further enhance the bacitracin production. The effect of incubation time on the bacitracin production in fermenter was carried out. Maximum bacitracin production was achieved after 30 hours of incubation i.e., 62±2.25 IU/mL and 44±1.32 IU/mL for mutant and wild strain respectively. Effect of inoculum age on the production of bacitracin by both mutant and wild type strains in fermenter was studied. Maximum bacitracin production of 63±1.53 IU/mL and 42±0.87 IU/mL was achieved for mutant and wild strain when 20 hours old inoculum was used. As far as inoculum size is concerned, maximum bacitracin production of 65±2.42 IU/mL and 45±0.86 IU/mL was achieved for mutant and wild strains respectively when 6% inoculum size was utilized. Similarly, effect of different rates of air supply (aeration) on bacitracin production was also studied. Maximum bacitracin production of 67±2.56 IU/mL and 48±1.47 IU/mL was obtained by mutant and wild strains when 1.25 L/L/min aeration was supplied in fermenter. Parameters like effect of agitation and dissolved oxygen were also employed to enhance the bacitracin production in fermenter studies. Maximum bacitracin production achieved after scale up studies in fermenter was 71±2.13 IU/mL and 50.5±1.76 IU/mL for mutant and wild

strains. An increase of 28±0.89 IU/mL of bacitracin by mutant strain *B. licheniformis* UVMN-HN-8 was obtained after optimizing different parameters in fermenter studies in comparison to shake flask studies. Bacitracin was extracted by the precipitation of metal ions. Parameters such as divalent metal ions (Zn+2), pH (7.0), temperature (60°C), $CaCO_3$ (3g/L) were studied to enhance the percentage recovery of the bacitracin. After optimization 69.4% (49.3±1.39 IU/mL) and 65% (32.7±1.13 IU/mL) Zn-bacitracin was recovered from the fermentation broth from the bacitracin produced by mutant strain *B. licheniformis* UV-MN-HN-8 and wild strain *B. licheniformis* GP-40 respectively. Characterization of the Zn-bacitracin was also performed. It was observed that, it is stable at wide range of pH, Temperature and salt concentration. Zn-bacitracin thus obtained was supplemented in the poultry feed to validate its efficacy as a growth promoter. Good results were obtained in comparison to imported Zn-bacitracin obtained from local market.