BIOSYNTHESIS OF Zn BACITRACIN BY *Bacillus licheniformis* UNDER SUBMERGED FERMENTATION USING WHEAT BRAN

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ABSTRACT

Bacitracin is a potent antibiotic which is used in combination with other antimicrobial agents. Bacitracin in the form of its zinc salt is used as feed additive, growth promoter and for reduction of diseases in poultry. In the present study, twenty five bacterial strains capable of producing bacitracin were isolated from milk samples by heat shock method. Among all bacterial strains, the isolate no. 21 was found to be the most potential strain; it was identified and designated as *Bacillus licheniformis*BCL-21. Different fermentation media were evaluated for antibiotic production under submerged fermentation in shake flasks. The medium M_1 gave higher antibiotic production (245.5 IU/ml) than M_2(186.0 IU/ml) and M_3(202.2 IU/ml). Different parameters were optimized in shake flask studies. The antibiotic production was 271.2±1.51 IU/ml by using 24 h old inoculum. Inoculum size was optimized to be 6.0 % ((274.0±1.89 IU/ml). The optimum pH was found to be 8.0 and bacitracin production was 245.5±0.58 IU/ml. At 37^{o}Ctemperature, production was295.0±1.34 IU/ml. Antibiotic activity was observed to be 283.9±1.43 IU/mlafter 48 h of incubation. The optimum agitation speed and working volume were observed to be 150 rpm(207.0±0.85 IU/ml) and 100 mL(232±1.29 IU/ml) respectively. Partial purification of bacitracin from fermentation broth was successfully done by precipitation method.

***Keywords*:** Zn bacitracin, Biosynthesis, Submerged fermentation, Optimization, Purification

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INTRODUCTION

Poultry industry in Pakistan has been developed as the associated part of agriculture and livestock. The development of poultry industry depends on adequate and reliable supply of good quality feed and feed additives (Prabakaran, 2003). Zinc bacitracin is such a feed additive which is used as a growth promoter and egg enhancer for broilers and layers respectively (Huyghebaert and Groote, 1997). As Pakistan is a developing country, it does not meet the expense for the massive import of antibiotics from foreign countries, so Zn bacitracin can be produced through microbial fermentation using low-cost agricultural and industrial residues like gram bran, maize bran, wheat bran, rice husk, rice bran and fruit wastes etc. (Farzana*et al.,* 2005).

Various Bacillus species are used to produce a number of valuable polypeptide antibiotics like tyrocidin, mycobacillin, polymyxin, colistin and Zinc bacitracin etc (Weinstein and Wagman, 2008). About 400 different peptide antibiotics have been isolated and discovered(Dubin*et al.,* 2005).Bacitracin is primarily active against gram positive bacteria and generally inactive against gram negative bacilli (Demain and Adrio, 2008).Bacitracin is used in the form of its salts like Zinc, manganese, sodium and methylene disalicylate salt or as the lignin Bacitracin complex (Singh and Ghosh, 2004). It is a stable antibiotic in the form of its Zn complex. Zinc is an active constituent which is used to improve the stability of Bacitracin molecule during feed processing. Other components of Zinc Bacitracin product are proteins, carbohydrates, minerals and peptides(Quinlan and Gutteridge, 1989).

The bacitracin antibiotic is used in feed for poultry, calves, lambs and animals in the concentration range of 5-100 ppm. Bacitrcain in combination with other antibiotics like Ploymyxin B and Neomycin has strong antimicrobial activities (Alcamo, 2003).Addition of Zn bacitracin to the chicken feed significantly enhances the weight gain, growth rate and feed utilization efficiency (Ogboko, 2010).Zn Bacitracin is also used in medicines but it has limited clinical uses. Commercial pharmaceutical Bacitracin Zn contains 4-10 % Zn.It is administered as topical antibiotic in the treatment of surgical infections. It can be used for the treatment of Staphylococcus Pneumonia, meningitis, chronic sinusitis and osteomyelitis. It is not absorbed by Gastro Intestinal tract, thus it is used in the treatment of gastrointestinal infections(Brad *et al.,* 2008).

Industrial production of bacitracin was started after 1950. Biosynthesis of Zn bacitracin by *Bacillus licheniformis* under submerged fermentation is considered highly desirable.In sub-merged fermentation, usually unwanted metabolites are not produced and purification of antibiotics takes place in easy way. SMF has many advantages over SSF that control of fermentation process is simpler and fermentation times can be reduced. Production costs can also be reduced as less labor is required (Espinosa and Webb, 2003).Various factors directly or indirectly affect the synthesis of peptide antibiotic Bacitracin. These factors are media composition for example; carbon and nitrogen sources, inorganic salts, inorganic phosphates, trace metals etc. and fermentation conditions like pH, temperature, aeration, agitation, fermentation time etc. (Chandrashekhara, 2010).

Purification of antibiotics from fermentation mesh is a determining aspect of manufacturing process which influences the economy of this process. A number of methods have been used for extraction and purification of bacitracin. Bacitracin can be isolated by precipitation method using divalent metal ions, ion exchange method, solvent extraction method etc(Singh and Ghosh, 2004).

MATERIALS AND METHODS

Isolation of *Bacillus licheniformis*

Different bacterial cultures capable of producing Zn Bacitracin were isolated from different milk samples by heat shock method. Bacterial strains were isolated on Trypton-Yeast Extract (TYE) agar medium and incubated at 37^{o}C for 24 hours. The composition of TYE agar medium was

Tryptone (5.0 g/L), Nutrient agar (15.0 g/L), Yeast Extract (2.5 g/L) and Glucose (1.0 g/L). Isolated bacterial colonies were identified on the basis of different morphological, physiological and biochemical tests according to Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 2000).

Preparation of inoculum

50 ml inoculum was prepared in 250 ml of cotton plugged conical flask and autoclaved at 121^{o}C for 15 minutes. The composition of inoculum was Peptone (10.0 g/L), Glucose (5.0 g/L), Beef extract (5.0 g/L), Sodium chloride (2.5 g/L) and Manganese chloride (0.167 g/L). The pH of inoculum was kept 7.0 before sterilization. After sterilization, it was allowed to cool at room temperature and inoculated with two loops full of the 48 hours old bacterial culture. The medium was kept in rotary shaker at 150 rpm for 24 hours at 37^{o}C.

Fermentation technique

Three different fermentation media M_1, M_2 and M_3 were screened for the Zn Bacitracin production (Table 1). Sub-merged fermentation was carried out in 250 ml conical flasks plugged with cotton buds, supplemented with wheat bran as a substrate and using 100 ml distilled water.The pH was adjusted to 7.0 before autoclaving. All media were sterilized inoculated with 10 % v/v of 24 hours old inoculum and incubated at 37^{o}C for 48 hours in rotary shaker. After fermentation, fermented material was centrifuged at 10,000 rpm for 15 min at 37^{o}C. The cell free supernatant was used for bacitracin assay to determine antibiotic potency.

Table 1: Composition of Fermentation Media

Sr. No.	Fermentation Media	Composition(g/L)
1	M_1	Wheat bran (150.0), L-Glutamic acid (5.0), Glucose (0.5), KH_2PO_4 (0.5), K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.2), $MnSO_4.H_2O$ (0.01), NaCl (0.01), $FeSO_4.7H_2O$ (0.01), $CUSO_4.7H_2O$ (0.01), $CaCl_2.2H_2O$ (0.015) (Bushra*et al.,* 2007)
2	M_2	Wheat bran (15.0), Citric acid (1.0), Glucose (0.5), KH_2PO_4(0.5), K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.2), $MnSO_4.H_2O$ (0.01), $FeSO_4.7H_2O$ (0.01) (Frazana*et al.,* 2005)
3	M_3	Wheat bran (15.0), L-Glutamic acid (20.0), Citric acid (1.0), $NaH_2PO_4.H_2O$ (2.0), KCl (0.5), $MgCl_2.6H_2O$ (0.2), Na_2SO_4(0.5), $FeSO_4.7H_2O$ (0.01), $MnSO_4.H_2O$ (0.01), $CaCl_2.2H_2O$ (0.01) (Aftab*et al.,* 2010)

Bio Assay of bacitracin

To determine the potency of antibiotic bacitracin, Agar Well Diffusion method was used (Farzana*et al.,* 2007). About one ml of autoclaved water was inoculated with test organism (Micrococcus luteus CN-5537) and spread on petri plate. Then sterilized nutrient agar

mediumwas poured over it and allowed to solidify. The medium contained Beef extract (1.0 g/L), Yeast extract (2.0) g/L, Sodium chloride (5.0 g/L), Peptone (5.0 g/L), Agar (15.0 g/L). After the medium was congealed, four wells of 0.8 cm diameter were made in plates with the help of sterilized borer of uniform edge and size. Standard solution of bacitracin was prepared by dissolving 65.2 mg of Zn bacitracin in 100 mL of N/100 HCl. The dilution of standard solution was made in N/100 HCl (Farzana*et al.,* 2005). About 100 µl of the culture filtrate whose potency was to be determined were poured in two opposite wells (T1, T2) in 1:4 dilutions.The remaining two opposite holes (S1, S2) were filled with standard Zn-Bacitracin in the same 1: 4 dilutions. The plates were carefully placed in incubator at 37^{o}C for 24 hours. Clear zones of inhibitions were developed and diameter of zones were measured and compared with the Bacitracin standard. The potency of sample was calculated by the following formula:

Difference due to dose $E = ½ [(T2 + S2) – (T1 + S1)]$

Difference due to sample $F = ½ [(T2 + T1) – (S2 + S1)]$

Log ratio of doses $I = \log 4 = 0.602$

Slope $B = E/I$

$M = F/B$

Potency ratio = Antilog of M

Potency of sample = Antilog of M × Potency of standard

Where, S2 = Standard High (in concentration)

S1 = Standard Low (in concentration)

T2 = Test High (in concentration)

T1 = Test Low (in concentration)

The units determined were expressed in X units/ml.

Optimization of fermentation conditions

For maximum Bacitracin production, different culture conditions such as pH, temperature, inoculum size, inoculum age, aeration, and agitation and incubation time were optimized. To determine the effects of initial pH, the pH of fermentation medium was varied from acidic to alkaline. The pH was changed from 5.0 to 9.0. The culture media was incubated at 30, 35, 37, 40 and 45^{o}C. Inoculum size was varied in the range of 2, 4, 6, 8 and 10 % in order to determine the optimum inoculum size. The effects of inoculum age were determined after 24, 48, 72, 96 and 120 hours. The fermentation media was incubated for different time courses like 24, 48, 72, 96 and 120 h at 37^{o}C to optimize the incubation time periods.The influence of oxygen supply to the fermentation medium was also optimized by changing the depth of fermentation medium from 50, 75, 100 and 125 to 150 ml in 250 ml of conical flask. The effect of agitation on antibiotic production was optimized by changing agitation speed from 110, 130, 150 and 170 to 190 rpm.

Partial purification of bacitracin

The partial purification of bacitracin from fermentation broth was carried out by precipitation method of Crisler and Weinberg (1963).

Statistical analysis

The results obtained were statistically analyzed using Minitab (version 15.0 INC., U.S.A.). The standard deviation was applied on graphs that statistically show the variations in data.

RESULTS AND DISCUSSION

Isolation of *Bacillus licheniformis* strains

25 bacterial strains were isolated from different milk samples by heat shock method. All bacterial strains were screened for the production of Bacitracin on three different fermentation media such as M_1, M_2 and M_3(Table 2).Based on the antibiotic potency of the individual strain, the most potential isolate with maximum antibiotic production (BCL-21:245.5 IU/ml) was selected for further use. Selected bacterial strain was identified in terms of various morphological, physiological and biochemical tests. This bacterial strain was identified to be *Bacillus licheniformis.*

Fermentation medium

The composition of fermentation media is detrimental for bacterial growth and antibiotic production. Thus, different fermentation media were screened in this study to optimize the bacitracin production. Out of all the fermentation media screened, BCL-21 gave maximum antibiotic production with M_1 (245.5 IU/ml) in contrast to other media such as M_2 (186.0 IU/ml) and M_3 (202.2 IU/ml) (Fig 1). Thus, BCL-21 with fermentation medium M_1 was selected to carry out further work. Wheat bran was used as a substrate to supplement the Bacitracin production.

Optimization of fermentation parameters

Various parameters were optimized to obtain better bacitracin production. These parameters are as below:

Effect of pH

It was evident from the results obtained that maximum bacitracin production (245.5±0.58 IU/ml) was observed at pH 8.0. Bacitracin production at pH 5.0, 6.0, 7.0 and 9.0 was 125.0±1.32, 178.8±0.61, 195.0±0.48 and 186.0±1.21 IU/ml respectively. Variations in the pH may cause changes in the ionization of nutrient molecule and reduce their availability to the organisms. Theresults revealed that optimal antibiotic production is obtained under neutral and slightly alkalineconditions (Lin *et al.,*2009).

Effect of inoculum age

Maximum antibiotic (271.2±1.51 IU/ml) was produced when 24 hours old inoculum was added into fermentation media. Further increase or decrease in inoculum age leads to reduced bacitracin activity. The antibiotic production at 48, 72, 96 and 120 hours old inoculum were 201.0±1.34, 148.0±1.07, 118.3±0.48 and 95.4±0.74 IU/ml respectively. This decline in antibiotic activity is possibly due to the accumulation of metabolites in older inoculum which causes repression of

cell growth. Old inoculum usually contains high proportion of spores leading to limitation of cell growth and metabolites formati

onwhereas cells of fresh inoculum are more active in terms of multiplication state(Lopes *et al.,* 2002).

Table 2: Screening of bacterial isolates on different fermentation media for Bacitracin production

Sr No.	Isolate No.	Antibiotic activity (IU/ml)			Sr No.	Isolate No.	Antibiotic activity (IU/ml)		
		M_1	M_2	M_3			M_1	M_2	M_3
1	BCL-01	110.0	89.9	102.5	13	BCL-13	158.4	162.0	146.8
2	BCL-02	125.0	110.6	143.8	14	BCL-14	120.7	105.0	135.6
3	BCL-03	98.9	80.2	78.6	15	BCL-15	121.3	115.5	102.9
4	BCL-04	167.0	132.6	120.0	16	BCL-16	144.0	167.0	156.7
5	BCL-05	145.8	165.8	125.4	17	BCL-17	110.0	105.5	121.1
6	BCL-06	136.0	105.0	95.5	18	BCL-18	78.8	80.0	124.0
7	BCL-07	155.9	175.8	155.5	19	BCL-19	154.0	189.9	176.2
8	BCL-08	80.8	98.0	102.5	20	BCL-20	72.5	96.8	104.6
9	BCL-09	189.0	175.0	140.0	**21**	**BCL-21**	**245.5**	202.2	186.0
10	BCL-10	112.0	132.0	121.3	22	BCL-22	184.0	176.5	130.8
11	BCL-11	151.5	146.0	178.8	23	BCL-23	135.7	127.6	166.6
12	BCL-12	77.8	88.0	124.4	24	BCL-24	186.6	192.8	174.8
13	BCL-13	158.4	162.0	146.8	25	BCL-25	89.7	114.8	130.0

Effect of inoculum age

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possibly due to the accumulation of metabolites in older inoculum which causes repression of cell growth. Old inoculum usually contains high proportion of spores leading to limitation of cell growth and metabolites formationwhereas cells of fresh inoculum are more active in terms of multiplication state(Lopes *et al.,* 2002).

Effect of inoculum size:

The maximum antibiotic production (274.0±1.89 IU/ml) was observed by using 6.0 % inoculum. The Bacitracin production was lower (139.5±0.92) when 2.0 % inoculum was used. Antibiotic activity at 4.0, 8.0 and 10.0 % inoculum sizes were 156.6±1.02, 238±1.31 and 209.7±0.24 IU/ml respectively.

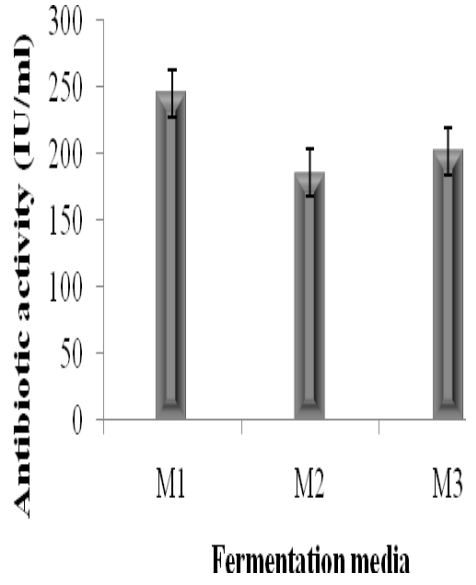

Fig 1: Screening of different fermentation media for the production of Bacitracin by *Bacillus licheniformis*

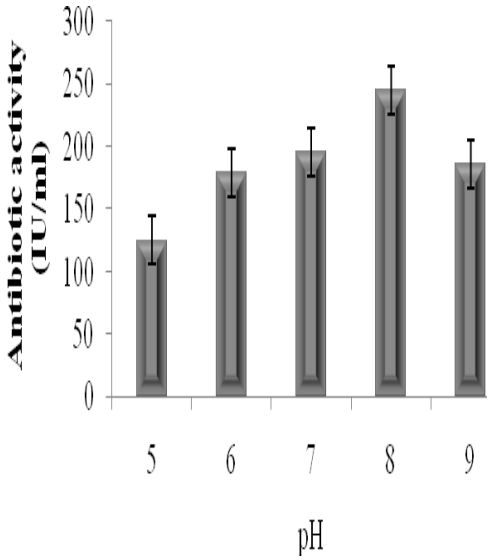

Fig 2: Effect of pH on the production of Bacitracin by *Bacillus licheniformis*

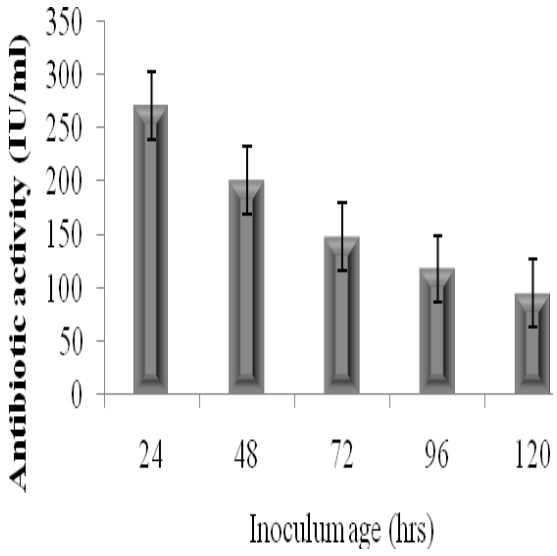

Fig 3: Effect of inoculum age on the production of Bacitracin by *Bacillus licheniformis*

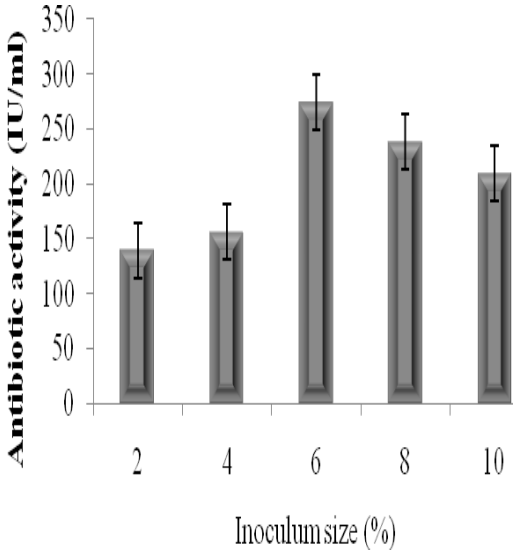

Fig 4: Effect of inoculum size on the of Bacitracin by *Bacillus licheniformis*

Effect of incubation temperature:

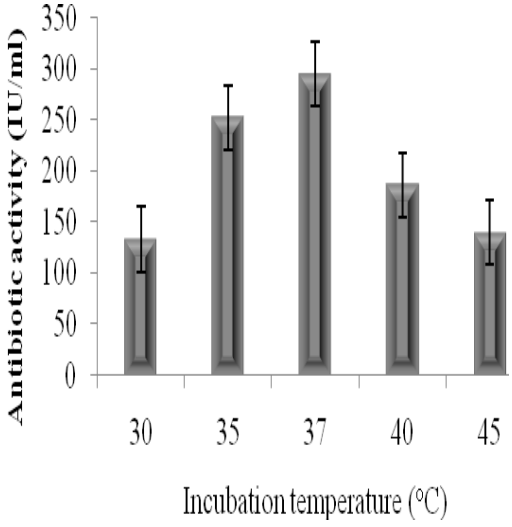

Fig 5: Effect of incubation temperature on the production of Bacitracin by *Bacillus licheniformis*

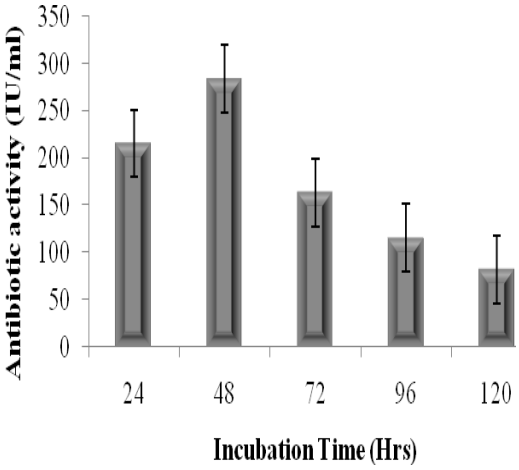

Fig6: Effect of incubation time on the production of Bacitracin by *Bacillus licheniformis*

Optimum level of bacitracin synthesisis associated with the adequate amount of bacteria production. At 6.0 % inoculum size, majority of the substrate is being utilized for growth and metabolic activities (Woolford, 2002).

The production of bacitracin was low at 30°C but it going to increase up to 37°C. It was observed that maximum bacitracin production was obtained at 37°C (295.0±1.34 IU/ml). Above 37°C there was no significant increase in the production of bacitracin. The bacitracin production at temperatures 30, 35, 40 and 45°C was 133.0±1.22, 252.7±1.87, 186.5±0.67 and 140.0±0.75 IU/ml respectively. Change in temperature above or below 37°C abruptly fall the bacitracin yield. Rise in temperature causes increased metabolic processes but after a certain limit, high temperature obstructs the microbial growth (Cladera-Oliver*aet al.,* 2004).

Effect of incubation time

The fermentation media was incubated for different times (24-120 hours) to investigate the optimum bacitracin activity. As the time interval increased from 0 to 24 h, bacitracin biosynthesis was also increased. After 24 hours, antibiotic production was 215.3±0.89. The maximum bacitracin production was obtained at 48 hours of incubation (283.9±1.43 IU/ml). After 48 hours, no increase was observed when incubation time was increased from 72 to 120 hours. This would be due to the entrance of organisms in the stationary phase, exhaustion of nutrients and synthesis of interfering by-products (Hanlon *et al.,* 1986). The antibiotic production at 72, 96 and 120 hours of incubation time was 163.4±1.05, 115.7±1.57 and 81.9±1.15 IU/ml respectively.

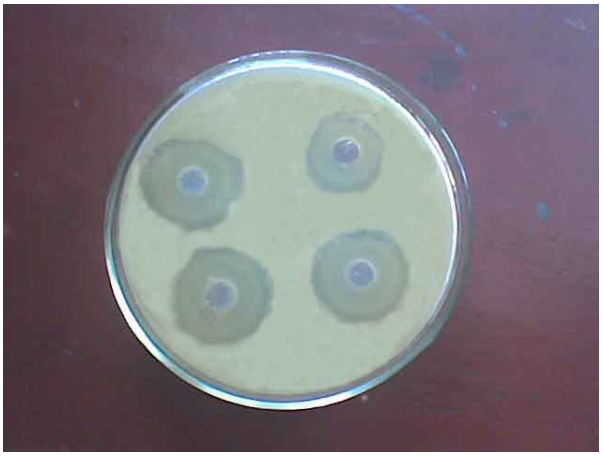

Fig 7: clear zones of inhibitions developed 48 hours of incubation.

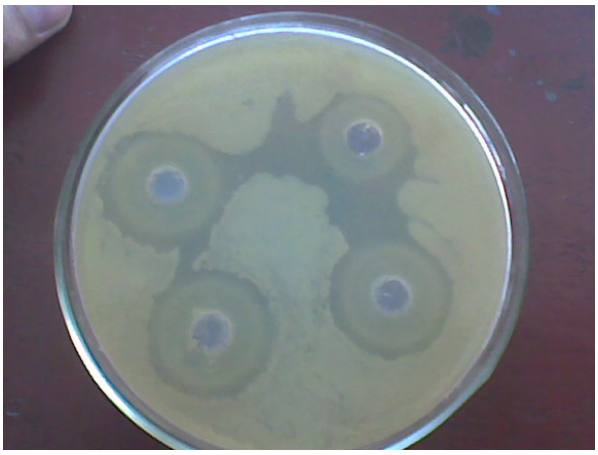

Fig 8: antimicrobial activity of *Bacillus* after *licheniformis* at 37°C.

Effect of aeration

The production of antibiotic was optimum (232±1.29 IU/ml) in flasks containing 100 ml fermentation medium.Supply of oxygen has influential effect in aerobic fermentation. At this level, provision of oxygen was enough for optimum bacitracin production. When volume of medium was further increased (125ml to 150 ml), the production gone to decrease from 175.0±0.77 to 105.8±1.22 IU/ml respectively. The antibiotic production at 50 ml and 75 ml was 110.9±1.35 and 185.5±0.96 IU/ml respectively. At this level, provision of oxygen was enough for optimum bacitracin production.

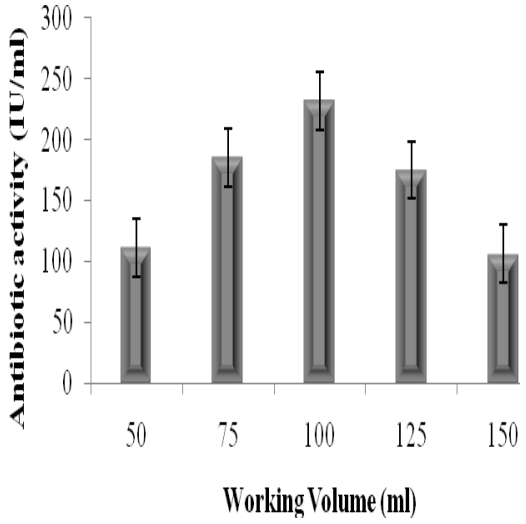

Fig9: Effect of aeration on the production Bacitracin by *Bacillus licheniformis*

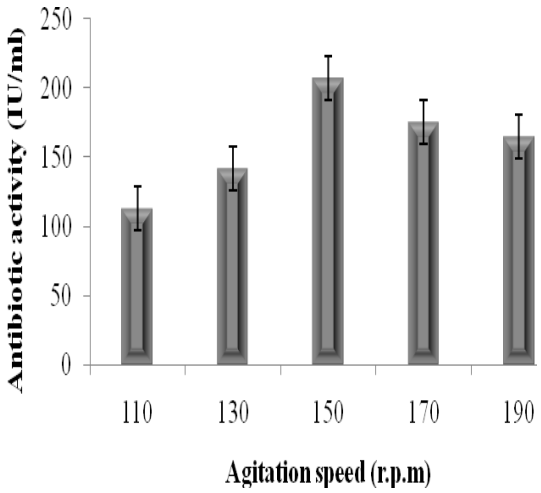

Fig10: Effect of agitation speed on the of production of Bacitracin by *Bacillus licheniformis*

Effect of agitation

Effect of agitation on bacitracin production by *Bacillus licheniformis* (BCL-21) in sub-merged fermentation was studied. The agitation rate was varied from 110 to 190 rpm. The maximum production (207.0±0.85 IU/ml) was observed at 150 rpm. At agitation speed of 110 rpm, bacitracin production was very lower (113.0±0.29 IU/ml). The biosynthesis of Bacitracin at other agitation speeds like 130, 170 and 190 rpm was 142.0±0.12, 175.1±0.71 and 165±1.91 IU/ml respectively. Proper agitation leads to adequate aeration of culture medium. Agitation also causes appropriate mixing of nutrients which resulted in optimum bacitracin production(Hanlon *et al.,* 1986).

Purification of bacitracin from fermentation broth

The antibiotic produced in culture medium was successfully isolated and purified. Water soluble Zn salt (preferably Zinc Chloride) was added keeping the pH of the medium lower. Then pH was allowed to rise and Calcium Carbonate was added which resulted in the formation of precipitates. The medium was centrifuged and cake of Zn-BC was oven dried. Zinc Chloride and Calcium Carbonate provide stability and strength to the antibiotic and also increases its yield. The results obtained showed that 200 m L of fermentation broth contained 1.07 g of dried Zinc Bacitracin.

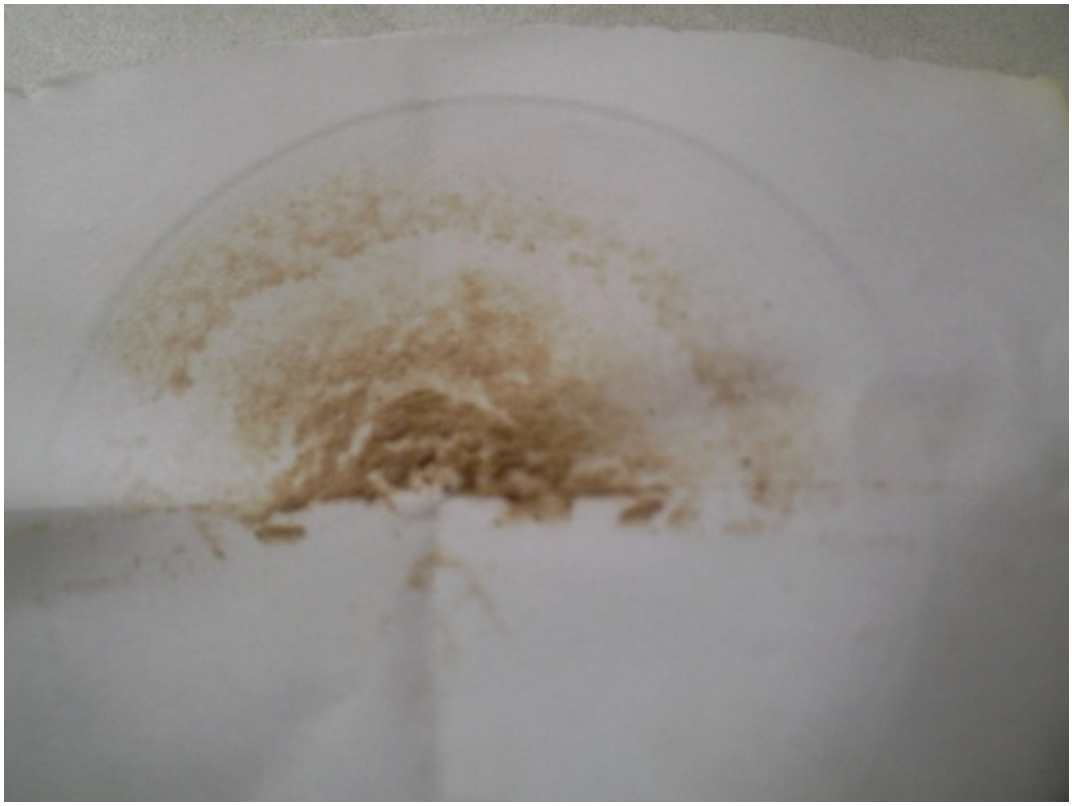

Fig 11: Partial purification of Zn Bacitracin

CONCLUSION

In present study, locally isolated culture (BCL-21) of *Bacillus licheniformis* was found to be the best producer of Bacitracin among all other isolates. The medium M_1 gave higher antibiotic productionthan M_2 and M_3. Wheat bran was used as a substrate in all three culture media. It has been proved to be cheapest raw material. The parametric studies showed that optimization of culture conditions have triggering effects on the production of antibiotics. Culture conditions such as inoculum size (6.0 %), inoculum age (24 h), pH (8.0), temperature (37^{o}C), incubation time (48 h), agitation speed (150 rpm) and working volume (100 ml) were optimized for bacitracin production. Partial purification of bacitracin from fermentation broth was successfully done by precipitation method.

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