

PRODUCTION AND ANTIMICROBIAL POTENTIAL OF A BROAD SPECTRUM ANTIMICROBIAL PROTEIN FROM A NEW STRAIN OF *BACILLUS AMYLOLIQUEFACIENS* MBL27

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ABSTRACT

The production of broad spectrum antimicrobial protein (AMP) by *Bacillus amyloliquefaciens* MBL27 and the influence of cultivation conditions, nutritional requirements and growth kinetics on the production of AMP were studied. The synthesis of AMP in a medium containing 1.0% glucose as carbon source and 0.25% triammonium citrate as nitrogen source was maximum at late exponential growth phase when the culture was incubated at 30°C at pH 7.0 and it remained almost stable through out the stationary growth phase. The protein produced has an activity of 6900 AU/ml against *Staphylococcus aureus*, 2300 AU/ml against *Escherichia coli* and 1400 AU/ml against *Pseudomonas aeruginosa*. This AMP exhibited inhibitory activity towards a broad spectrum of wound and clinical pathogens and spoilage microorganisms including both gram positive and gram negative organisms. This antibacterial protein was found to have an apparent molecular mass of 34 kDa from the clearance zone observed in a polyacrylamide gel.

Keywords: *Bacillus amyloliquefaciens*, Antimicrobial protein, Antibacterial activity, Growth media

INTRODUCTION

Antimicrobial proteins (AMPs) are ubiquitous in nature where they play important roles in host defence and microbial control. The widespread resistance of bacterial pathogens to conventional antibiotics has prompted renewed attention in the use of alternative natural microbial inhibitors such as AMPs as potential substitutes for, or as additions to currently used antibiotics. Bacteriocins are a very diverse group of compounds in terms of form and functions. Bacteriocins are ribosomally synthesized antimicrobial peptides that inhibit or kill microorganisms that are usually, but not always closely related to the producer strain¹.

Although, bacteriocin activity is considered to be species specific, antimicrobial compounds produced by gram positive organisms having unique nature have greater spectra of activity and thus possibly have broader industrial applications. *Bacillus sp.* are a rich source of antimicrobial peptides². *B.subtilis* produces subtilin, subtilosin³, *B.licheniformis* produces lichenin⁴, *B.cereus* produces cerein⁵, *B.halodurans* produces haloduracin⁶ and *B.thuringiensis* produces thuricin⁷ that are most active against gram positive organisms. Some of the peptides produced by *Bacillus sp.*, mainly the lipopeptides, demonstrate antifungal properties⁸.

Like lactic acid bacteria (LAB) the genus *Bacillus* includes a variety of industrially important species which are "generally recognized as safe" (GRAS) and to use in both food and industry⁹⁻¹¹. The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas, especially the agri-food industry. Some bacteriocins inhibit food spoilage and pathogenic microorganisms, and they are therefore potentially useful as natural replacements for synthetic food preservatives. Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is one of the most intensively studied lantibiotics, due to its industrial application and potential for other uses¹². It is commercially produced by microbial cultivation and has been widely used as a food preservative in many countries.

In this study we have reported the isolation, identification and process optimization for the production of an antimicrobial protein from *Bacillus amyloliquefaciens* MBL27 and its broad spectrum antibacterial activity against a wide range of gram positive and gram negative organisms including pathogenic bacteria for therapeutic applications with a special focus on controlling wound pathogens.

MATERIALS AND METHODS

All the culture media were purchased from Himedia. Chitosan was purchased from SIGMA, USA. Catfish collagen was the kind gift from

the Department of Biotechnology, CLRI. Other chemicals and reagents used were of analytical grade (AR).

Microorganisms

The microorganism employed in this investigation was isolated from dairy unit wastes and checked for antimicrobial activity both by spot on lawn and well diffusion assay¹³. The bacterial isolate that showed highest antibacterial activity against broad range of both gram positive and gram negative pathogens was identified based on staining, biochemical, SEM analysis and 16S rDNA gene sequencing analysis. Phylogenetic data were obtained from BLAST algorithm (<http://www.ncbi.nlm.nih.gov>) using the software CLUSTAL W version 1.8 with the standard parameters. The indicator strains used in this study were obtained from different culture collection centres and clinical isolates from different hospitals and are listed in Table 1. The strains were kept frozen in 20% (v/v) glycerol at -20°C until use, and propagated in nutrient broth at 37°C for 24 h when needed.

Production media

Mineral salt (MS) medium of the following composition was prepared and autoclaved at 121°C and 15 psi for 20 min. The mineral salt medium used for AMP production contained (g l⁻¹): K₂HPO₄ 2; MgSO₄·7H₂O 0.2; MnSO₄ 0.2; tri ammonium citrate 2.5; glucose 10.0. Glucose was filter sterilized separately and added to the medium. The pH of the medium was adjusted to 7.0. Glucose utilization during the growth of the organism was evaluated by the phenol-sulphuric acid method¹⁴.

50 ml of medium was inoculated with 1% v/v inoculum of *Bacillus amyloliquefaciens* MBL27 containing 2.2 x 10⁶ cells/ml and incubated at 30°C and at 200 rpm for AMP production studies. After 24, 48 and 72 h of incubation, the cells were removed by centrifugation at 10,000 rpm for 10 min at 4°C. Inhibitory activity due to acidic metabolites were eliminated by adjusting the pH of cell free culture supernatant to 6.2-6.4 using 1N HCl and 1N NaOH. Inhibitory activity from hydrogen peroxide was eliminated by the addition of 5 mg/ml Catalase. It was then filter sterilized using sterile 0.22 µm syringe filter (Millipore, Bedford, MA, USA) and checked for antimicrobial activity.

Antimicrobial protein from culture supernatant was recovered by precipitation using 40% ammonium sulphate followed by centrifugation at 10,000 rpm for 15 min. This crude antimicrobial protein was dialysed, filter sterilized using sterile 0.22 µm syringe filter (Millipore, Bedford, MA, USA) and 0.02 ml of the supernatant was loaded to evaluate the activity.

Antimicrobial assay

Spot on lawn assay

Initial screening for antimicrobial activity was done by spot on lawn method. The producer organism was first spotted on a nutrient agar plate and incubated at 37°C for 24-48 h for bacterial growth. The indicator organism $\approx 10^5$ cells/ml was added to soft agar and overlaid on to the producer organism, incubated for 24 h at 37°C and observed for zone of bacterial inhibition.

Well diffusion assay

Organisms which showed positive results from spot on lawn method were checked for activity by well diffusion method. Mueller Hinton Agar (MHA) was used for well diffusion assay. Indicator strains of $\approx 10^5$ cells/ml was added to MHA and poured onto sterile plates. After solidification, wells of 0.5 cm diameter were created using well borer. 0.2 ml of pH adjusted culture supernatant and 0.02 ml of crude antimicrobial protein obtained after ammonium sulphate precipitation were loaded into the wells. After 24 h incubation, the plates were observed for zone of clearance.

All the experiments were done in triplicates.

Bacterial growth

Bacterial growth was determined spectrophotometrically by measuring the OD at 600 nm. 1.0 OD is equivalent to 0.4 mg/ml dry cell weight (DCW).

Quantification of antimicrobial activity

The antagonistic activity was expressed in terms of arbitrary units (AU/ml). To determine AU/ml, filter sterilized AMP were serially diluted and their activities checked by well diffusion assay.

One arbitrary unit (AU) against an individual indicator strain was defined as the reciprocal of the highest dilution that still produced a minimum detectable zone of inhibition and expressed as AU/ml. The minimum detectable zone of diameter was 1 mm beyond well diameter. Zone diameter was measured using an antibiotic zone measuring scale (HIMEDIA).

AMP production under different culture conditions

The production studies were carried out in 250 ml conical flasks containing 50 ml mineral salt medium. The medium was inoculated with 1% inoculum containing approximately 2.2×10^6 cells and incubated in an incubator shaker at 200 rpm at 30°C. Cell growth was monitored spectrophotometrically (A_{600}) and antimicrobial activity of the crude antibacterial protein was evaluated by the well diffusion method as described above.

To study the effect of initial pH on AMP production, the medium was adjusted to different pH 4, 5, 6, 7, 8, 9, 10, 11, 12 using 1N NaOH and 1N HCl. The effect of incubation temperature was studied by incubating the medium at different temperatures 25, 30, 37 and 45°C. To study the influence of carbon source, glucose was replaced with other carbohydrates such as fructose, sucrose, maltose, xylose, lactose, galactose, mannitol, glycerol and inositol. Different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) of the best carbohydrate were used in the medium to optimize maximal production of AMP. To evaluate the effect of different nitrogen sources on AMP production, tri ammonium citrate in the medium was replaced with different organic and inorganic nitrogen sources such as peptone, yeast extract, beef extract, tryptone, sodium nitrate, potassium nitrate, ammonium nitrate, ammonium chloride, ammonium acetate and ammonium sulphate. Different concentrations (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3%) of the best nitrogen sources and combination of both organic and inorganic nitrogen sources were also studied.

Molecular weight determination and activity detection

To find out the protein nature of the partially purified antibacterial protein, poly acrylamide gel electrophoresis was performed on to 12% gel both in the presence and absence of SDS using tris glycine buffer¹⁵. Molecular weight markers were also loaded onto the gel. Following electrophoresis at 50 mA, the gel was cut into two halves

vertically. One part was stained with Coomassie Brilliant Blue R-250 for visualization of the protein bands. The other part of the gel was washed thrice with sterile distilled water at one hour interval. Then it was placed aseptically in a sterile petri dish and covered with 20 ml soft agar containing $\approx 10^5$ cells/ml of *Staphylococcus aureus* as the indicator strain incubated at 37°C for 24 h and observed for zone of inhibition.

RESULTS AND DISCUSSION

A total of 72 isolates of bacteria were isolated from soil and dairy wastes, out of which 48 isolates showed antibacterial activity by spot on lawn method and 13 strains showed positive results by well diffusion method against three important test organisms *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Out of 13 strains, one strain was selected for further production studies due to its high inhibitory activities towards wide range of gram positive and gram negative organisms including clinical pathogens as listed in Table 1.

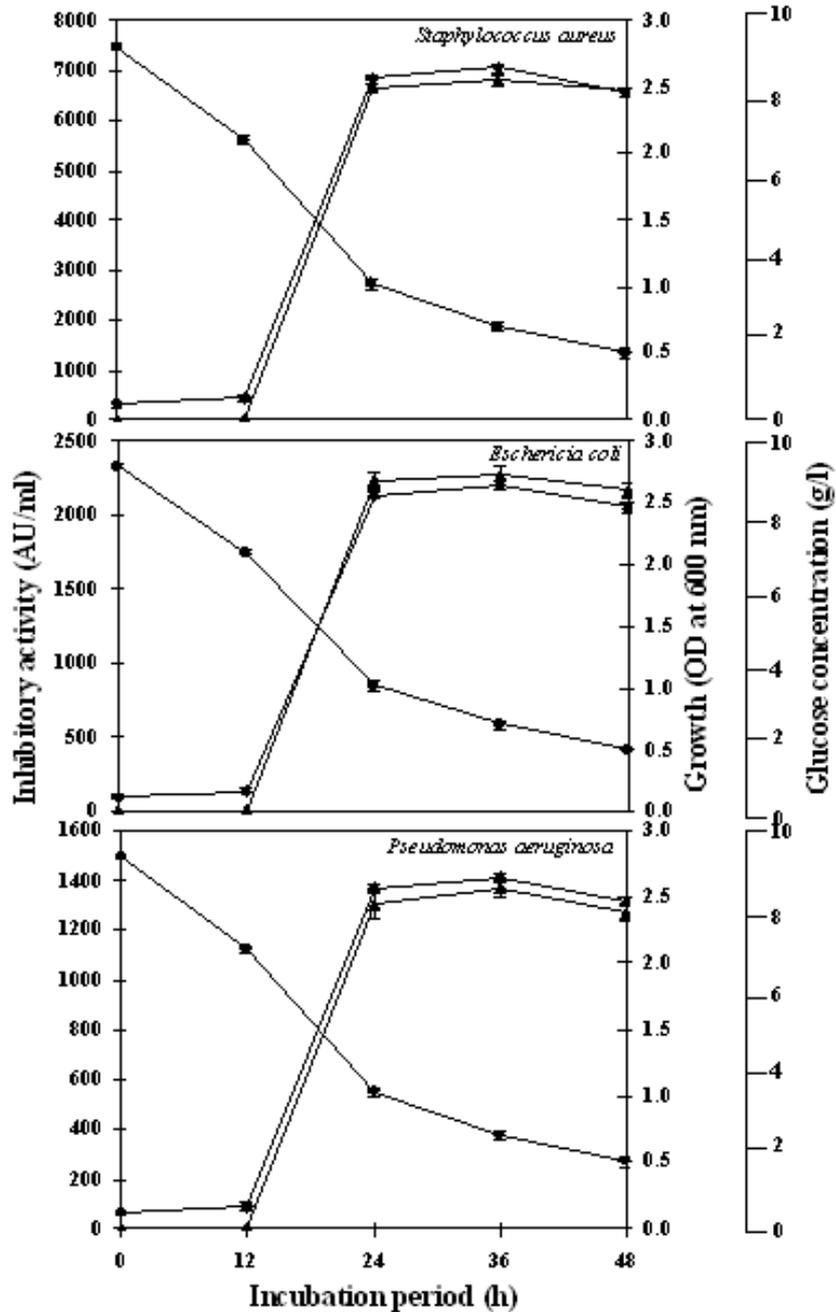
Table 1: Antimicrobial activity spectrum of AMP from *B.amyloliquefaciens* MBL27

Organisms	Zone of inhibition (mm)
<i>Escherichia coli</i> (MTCC)	22
<i>Escherichia coli</i> (urine)	21
<i>Escherichia coli</i> (soil)	22
<i>Staphylococcus aureus</i> (MTCC)	21
<i>Staphylococcus aureus</i> (MRSA-pus)	21
<i>Staphylococcus aureus</i> (OXA)	15
<i>Pseudomonas aeruginosa</i> (MTCC)	18
<i>Klebsiella</i> (MTCC)	29
<i>Klebsiella sp.</i> (blood)	18
<i>Bacillus sp.</i>	26
<i>Shigella sp.</i> (stool)	26
<i>Salmonella typhi</i> (blood)	25

This makes it a very important property to combat wound infection and promote wound healing. Most of the bacteriocins under *Bacillus* show a narrow spectrum of activity, only affecting closely related strains and species^{7,16,17}. Activity against gram negative bacteria is an unusual phenomenon and has been reported so far for thermophilin 81, produced by *Streptococcus thermophilus*¹⁸, bacteriocins produced by *L.paracasei subsp.paracasei* L126 and L134¹⁹, a bacteriocin produced by *L.lactis* KCA2386²⁰ and plantaricin 35d produced by *L.plantarum*²¹.

Based on the staining, morphological and biochemical characters, the isolated strain was identified as belonging to *Bacillus sp.* Using 16S rDNA analysis and subsequently from the BLAST the strain was identified as *Bacillus amyloliquefaciens* (percentage of identity = 99%) a closely related species to *Bacillus subtilis*. The organism was gram positive rod, catalase positive and grows under aerobic conditions. The ability of the organism to grow in 10% NaCl and its good growth at 30 to 40°C and no growth at 50°C are the important biochemical tests which differs it from *B.subtilis*. Starch hydrolysis ratio was also observed in the optimum range (4-4.6) which is characteristic for *B.amyloliquefaciens*. Carbohydrate fermentation results, acid without gas formation for arabinose, xylose, glucose, sucrose, mannitol and lactose also acted as a supportive evidence for the culture to be identified as *B.amyloliquefaciens*²². This strain was isolated from dairy unit wastes.

AMP production by *B.amyloliquefaciens* MBL27 was growth associated and biomass production was proportional to glucose utilization (Fig. 1) and it was maximal during the late exponential phase, retaining its activity throughout the stationary phase. The activity reached a maximum of 6800 AU/ml against *Staphylococcus aureus*, 2266 AU/ml against *Escherichia coli* and 1366 AU/ml against *Pseudomonas aeruginosa* after 36 h incubation and there was only a slight decrease in the activity even upto 10 days of incubation and minimum amount of activity was noticed upto 10 weeks of incubation against *Staphylococcus aureus* (2300 AU/ml). This is similar to bacteriocin from *Lactobacillus pentosus* ST151BR²³. This shows its high stability and resistance to proteolytic degradation.



▲ Inhibitory activity; ◆ Cell growth; ● Glucose concentration

Fig. 1: Growth kinetics and AMP production by *B.amyloliquefaciens* MBL27

Effect of shake and still culture conditions was studied by inoculating the cultures in the minimal medium under both shake and still culture conditions. Growth and activities were noticed in both the conditions but the rate of AMP production under shake culture conditions was comparatively higher indicating the supportive role of oxygen in AMP production.

Different concentrations of inoculum were added to the culture media to optimize the conditions for maximal production of AMP. It was found that growth and AMP production was minimal when 10^5 cells/ml were added. Bacterial growth and AMP production was maximal when 10^6 cells/ml were inoculated to the medium (Fig. 2).

When the count was increased beyond this level production and growth was decreased, which may be due to the exhaustion of nutrients.

Optimization of culture conditions, including temperature, pH, carbon and nitrogen influenced the synthesis of antimicrobial protein. Growth temperature plays an important role, because AMP production varies with temperature. Enhanced production of AMP was observed at 30°C with an activity of 6575 AU/ml for *Staphylococcus aureus* (Fig. 3). At 37°C growth was maximal, and activity was also close to that of 30°C. At 25°C and 45°C moderate activity was observed. Maximal AMP production was achieved at 30°C.

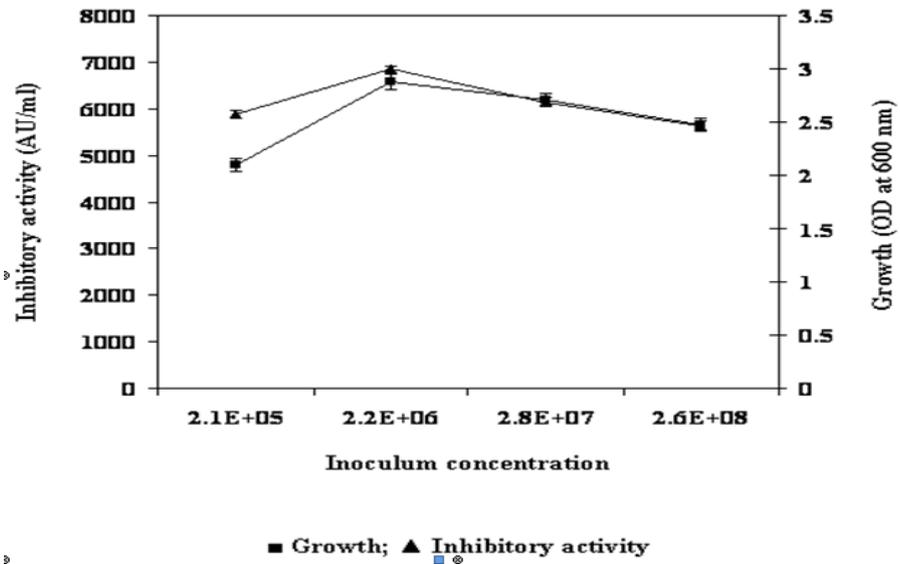


Fig. 2: Effect of inoculum concentration on cell growth and AMP production by *B.amyloliquefaciens* MBL27

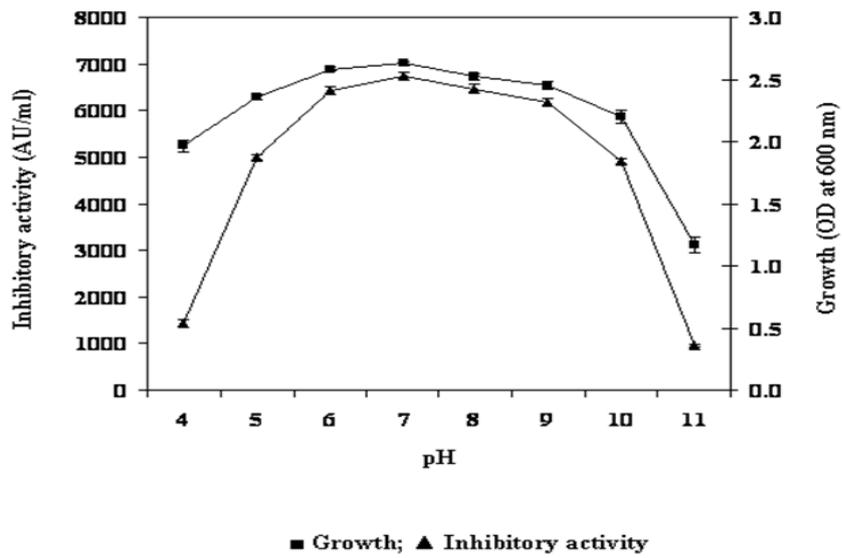


Fig. 3: Effect of temperature on cell growth and AMP production by *B.amyloliquefaciens* MBL27

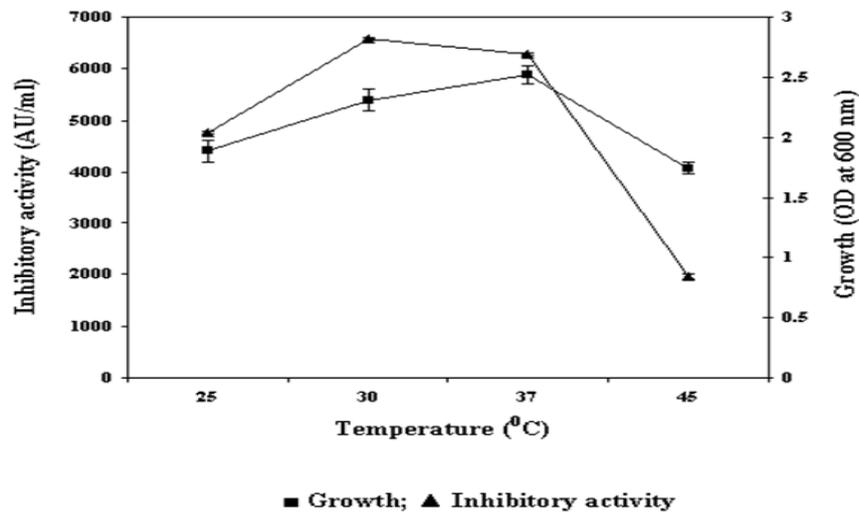


Fig. 4: Effect of initial pH on cell growth and AMP production by *B.amyloliquefaciens* MBL27

Similarly, Ogunbanwo *et al*²⁴ observed that bacteriocin production by *L.brevis* OG1 was maximal when the growth temperature was 30°C. Similar findings were also observed by Paynter *et al*²⁵ where plantaricin F production was maximal when *L.plantarum* BF001 was cultivated at 30°C.

The initial pH of the medium has a profound effect on the production of AMP. At pH 7.0 both growth and AMP activity were maximal, 6733 AU/ml against *Staphylococcus aureus*, 2133 AU/ml against *Escherichia coli* and 1233 AU/ml against *Pseudomonas aeruginosa*. Mi-Hee *et al*²⁶ also reported that micrococccin G05 production was maximized when the initial pH was between 7.0 and 9.0. At pH 4.0 the activity was slightly lower (1433 AU/ml against *Staphylococcus aureus*, 1200 AU/ml against *Escherichia coli* and 733 AU/ml against *Pseudomonas aeruginosa*). Activity of AMP was found in the efficient level upto pH 10. But at pH 11 bacterial growth and AMP production were affected (Fig. 4) as reported in the case of bacteriocin produced by *B. subtilis* LFE-1, *B.firmus* H20-1 and *B.licheniformis* T6-5²⁷.

The effect of carbon source on AMP production was studied by using different carbon sources in MS medium at a concentration of 10 g/l. Maximal activity of 6750 AU/ml against *Staphylococcus aureus* was observed with glucose as carbon source (Table 2).

Fructose, maltose, glycerol, sucrose and xylose also produced almost same level of activity though bacterial growth was reduced to some extent followed by lactose and galactose. Mannitol supported moderate activity. Inositol was a poor carbon source for bacterial growth.

Maximal AMP production of 6750 AU/ml was achieved when 1.0% glucose was used as carbon source (Fig. 5). The results also show that catabolite repression was not found in AMP production. Similar

results were reported by Ogunbanwo *et al*²⁴ where bacteriocin by *L.brevis* OG1 were synthesized in larger amounts when the medium was supplemented with 1.0% (w/v) glucose and by Todorov and Dicks²³ where *Lactobacillus pentosus* ST151 BR shows maximal activity of 6400 AU/ml when glucose was used at a concentration of 1.0% (w/v) in the production medium.

Table 2: Effect of different carbon sources on cell growth and AMP production by *B.amyloliquefaciens* MBL27

Carbon sources	Growth (OD at 600 nm)	Inhibitory activity (AU/ml) against <i>S. aureus</i>
Glucose	2.55 ± 0.05	6750 ± 150
Fructose	2.48 ± 0.12	6650 ± 50
Sucrose	1.67 ± 0.17	6550 ± 50
Xylose	1.73 ± 0.13	6600 ± 50
Maltose	2.39 ± 0.11	6650 ± 50
Lactose	1.85 ± 0.05	6200 ± 100
Galactose	1.63 ± 0.07	6250 ± 50
Mannitol	1.07 ± 0.07	5550 ± 150
Glycerol	1.08 ± 0.1	6550 ± 50
Inositol	0.39 ± 0.01	3550 ± 150

Table 3 shows the effect of nitrogen source on growth and AMP production. The medium was substituted with different nitrogen sources at a concentration of 2.5 g l⁻¹. Maximum activity of 6850 AU/ml was observed on addition of triammonium citrate. Peptone contributed to maximum activity of 6888 AU/ml. All other nitrogen sources used produced significant activity except potassium and sodium nitrate.

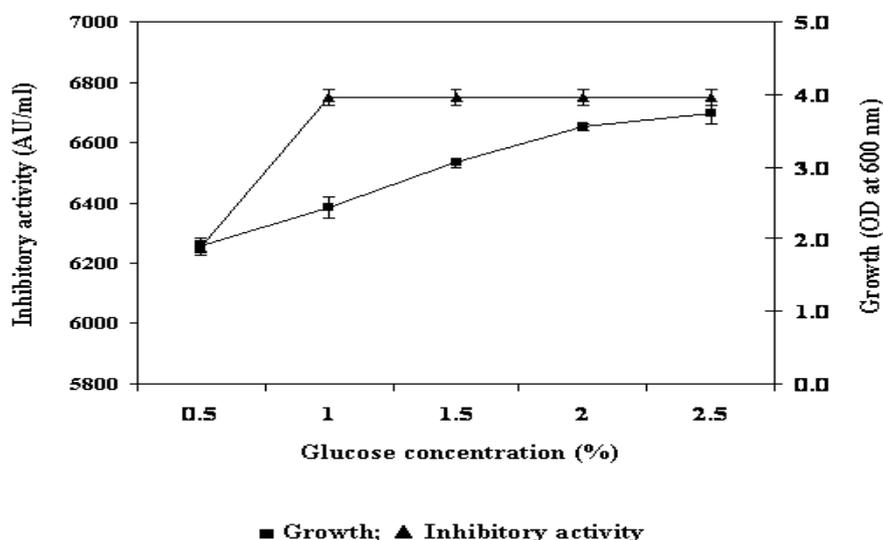


Fig. 5: Effect of glucose concentration on cell growth and AMP production by *B.amyloliquefaciens* MBL27

Table 3: Effect of various nitrogen sources on cell growth and AMP production by *B.amyloliquefaciens* MBL27

Nitrogen sources	Growth (OD at 600 nm)	Inhibitory activity (AU/ml) against <i>Staphylococcus aureus</i>
Peptone	2.97 ± 0.05	6888 ± 12.5
Tryptone	2.80 ± 0.01	6875 ± 25
Beef extract	2.67 ± 0.03	5975 ± 25
Yeast extract	2.56 ± 0.02	6550 ± 50
Triammonium citrate	2.62 ± 0.02	6850 ± 50
Ammonium acetate	2.41 ± 0.02	5650 ± 50
Ammonium sulphate	2.30 ± 0.04	6250 ± 50
Ammonium nitrate	2.25 ± 0.05	6588 ± 12.5
Ammonium chloride	2.15 ± 0.03	6013 ± 12.5
Sodium nitrate	0.60 ± 0.02	2875 ± 25
Potassium nitrate	0.41 ± 0.01	2875 ± 25

Similar findings were observed for micrococcin G05 production (256 AU/ml) by *Micrococcus* sp. G05 by supplementation of medium with 1.0% (w/v) tryptone²⁶, and for bacteriocin production by *Leuconostoc mesenteroides* subsp. *mesenteroides* strain ST33LD where the presence of tryptone as the nitrogen source resulted in maximal activity of 6400 AU/ml²⁸. The combination of peptone, tryptone, beef extract and yeast extract with triammonium citrate showed the same level of activity (6825-6850 AU/ml) to that of peptone as well as triammonium citrate after 36 h of incubation.

Variation in the concentrations of triammonium citrate on AMP production was studied. It was observed that maximum activity of 6888 AU/ml was obtained at 2.5 g/l and it was chosen for further experiments.

In order to improve the AMP yield further, the effect of magnesium sulphate concentration (0.005-0.03%) on growth and AMP activity was studied. Growth and AMP production was enhanced by the addition of magnesium sulphate at 0.02% concentration with an activity of 6650 AU/ml. Though there was not much variation in cell growth only moderate activity was observed at lower concentrations.

The antimicrobial substance produced by *B.amyloliquefaciens* MBL27 is a protein and precipitated readily by adding 40% saturation with about 98% recovery of the antimicrobial protein. The antimicrobial protein has an apparent molecular weight of 34 kDa. The antibacterial efficacy of the crude supernatant of *B.amyloliquefaciens* MBL27 was estimated to be 34 kDa on native PAGE (Fig. 6).

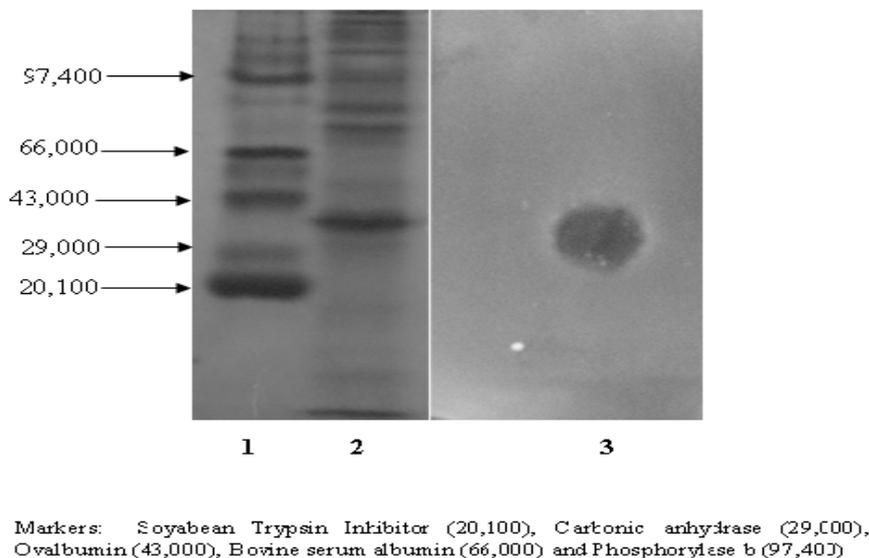


Fig. 6: Native PAGE and antimicrobial activity of partially purified AMP

Chitosan and Collagen-chitosan sheets are used as carriers for drug delivery for wound healing^{29,30}. In this investigation the effectiveness of the AMP obtained from *B.amyloliquefaciens* MBL27 in controlling wound pathogens was tested by incorporating the AMP in chitosan sheet and also in sheet prepared using mixtures of

chitosan and collagen. The AMP incorporated sheets of both the preparations showed antimicrobial activity against most important wound pathogens (Fig. 7). This study shows the prospects of using AMP from this bacterium as a potent wound healer.

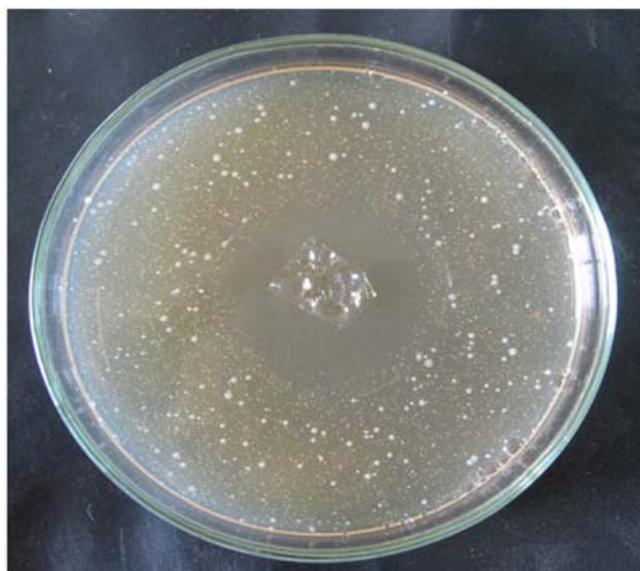


Fig. 7: Plate showing zone of clearance for AMP incorporated chitosan sheet against mixed culture

Thus the results of the present study shows that *B.amyloliquefaciens* MBL27 is a good producer of AMP with best inhibitory activity against wide range of pathogens.

CONCLUSION

The resistance developed by pathogens towards almost all conventional antibiotics have developed the urge to search for novel antibiotics of microbial origin. The present study indicated that *B.amyloliquefaciens* MBL27 produced the highest concentration of AMP with inhibitory activities against wide range of pathogens. This study suggests the potential feasibility of this strain to be explored for therapeutic applications including healing of chronic wounds. The broad spectrum of activity, the molecular weight and the kinetics of production differentiate it from AMPs and bacteriocins produced by other *Bacillus* sps.

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