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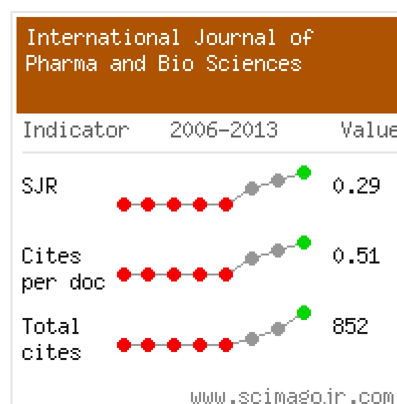

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STATISTICAL OPTIMISATION OF THE CRUDE MEDIA AND COMPONENTS FOR ENHANCED PRODUCTION OF BACTERIOCIN FROM *BACILLUS SPECIES* BY PLACKETT- BURMAN DESIGN.

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ABSTRACT

A *Bacillus* strain producing a bacteriocin-like substance was isolated from mangrove soil & identified by biochemical profiling and 16S rRNA sequencing. Phylogenetic analysis indicated that the strain has high sequence similarity with *Bacillus licheniformis.* The culture supernatant of *B. licheniformis* exhibited bacteriocin-like activity against Gram-positive and Gram negative bacteria and fungi. Enhancement of bacteriocin activity was tested using cheap crude sources like molasses, corn steep liquor and cheese whey. Corn steep liquor and cheese whey is rich nitrogen sources rather than carbon sources. Selection of the crude media sources was done by Students t-test where corn-steep liquor for *Bacillus licheniformis* was found to be significant. Optimization using Plackett-Burman design showed Sodium chloride, potassium nitrate, sugar,as the most important variables for bacterocin production in the first step of media. The stability study shows bacteriocin produced by *Bacillus licheniformis* was stable over a wide pH (3-11) and temperature [4-37 ^{0} C]. A low-molecular-weight bacteriocin-like protein from *B. licheniformis* exhibited a wide spectrum of antimicrobial activity. A 2-fold increase in the production of bacteriocin was achieved using the culture medium optimized through Plackett-Burman design.

KEY WORDS: ***Bacillus spp*, Bacteriocins, Characterisation, Medium Optimisation, Plackett-Burman design, Stability.**

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INTRODUCTION

Despite the critical need for new antibiotics to treat drug resistant infections and other infectious diseases of humans and animals, very few new antibiotics are being developed. At this point, a new antibiotic is required, which is active against resistant bacteria and fungi. Bacteriocins are antibacterial peptides or proteins produced by ribosomal synthesis that generally affect members of related genera or species (Kumar S. *et al* 2012). Bacteriocin genes are either chromosomally or plasmid encoded with resulting toxins employing a variety of killing mechanisms, including cytoplasmic membrane pore formation, cell wall interference, and nuclease activity (Osnat G. *et al* 2005). Bacteriocins from lactic acid bacteria (LAB) have been studied mostly because of their potential use as biopreservatives in food processing. Reports on factors affecting the production of bacteriocins are limited, except for Nisin, a well-studied bacteriocin, and a few other LAB bacteriocins .The production of bacteriocin from LAB is growth associated and is therefore influenced by growth conditions such as temperature and pH (Kayalvizhi N. *et al* 2008). Most of the species from genus *Bacillus* are also considered as industrially important bacteriocin producers and have a history of safe use and they are also GRAS status microorganisms (Lisboa P. *et al* 2006). Moreover, the ability of *B. subtilis* and close relatives to secrete 30 grams per litre proteins directly into the growth medium and their well-proven safety have made them prime candidates for the production of heterologous proteins. Several bacteriocins or bacteriocin-like substances (BLS) produced by the genus *Bacillus* have been reported. The best-characterized are subtilin of *B. subtilis and* megacin of *B. megaterium.* Although bacteriocins and bacteriocin-like substances are synthesized by *Bacillus sp*., the information on bacteriocin production by this genus is limited. We report here on broad spectrum active bacteriocin production by *Bacillus licheniformis* and the optimization of components in a medium for the production of bacteriocin (Abriouel H. *et al* 2010).

MATERIALS AND METHODS

Isolation of Bacillus spp.by preliminary bacteriocin activity

Bacteriocin activity was determined by agar well diffusion assay onto the test culture seeded Mueller Hinton agar plates with 50 µL of bacteriocin preparation. The plates were examined for clear zone of inhibition in the agar surrounding the well (Torkar. *et al* 2003). The bacteriocin-producing bacterial strain was isolated from mangrove soil and identified as *B. licheniformis*, based on its morphological and biochemical characteristics. In order to confirm its taxonomical identity, the 16S rRNA sequencing was carried out.

Table 1
Species and Sources of Standard Test Cultures.

Sr.no	TEST CULTURES	SOURCE
	Human pathogens	
1.	*Staphylococcus aureus*	MTCC 1144
2.	*Streptococcus pyogenes*	NCIM 2608
3.	*Escherichia coli*	NCIM 2641
4.	*Pseudomonas aeruginosa*	MTCC 2488
5.	*Klebsiella pneumonia*	MTCC 4032
6.	*Salmonella typhi*	MTCC432
7.	*Candida albicans*	MTCC 183
8.	*Corynebacterium diphtheria*	NCIM 2640
9.	*Malassezia furfur*	MTCC1374
	Fungal pathogens	
10.	*Asperjillus niger*	NCIM 902

Production and purification of Bacteriocin

An overnight culture of *B. licheniformis* isolated from mangrove soil was inoculated into the nutrient broth and incubated aerobically at 5,000 rpm for 72 hours. Cells were separated by centrifugation at 10,000 rpm for 30 min at 4^0C.The crude cell free extracts from selected *Bacillus species* were filter sterilized using 0.2 µm Acrodisc syringe membrane filters (Pall Corporation), and proteins were precipitated with ammonium sulphate at 65% saturation by constant stirring at 200 rpm for 16 h at 4^oC (Bizani. *et al.*2005). Precipitated proteins were pelleted by centrifugation at 10,000 rpm for 30 min at 4^0C,dissolved in 5 ml sterile phosphate-buffered saline (PBS) at pH 7.4,and butanol was added (1:4 butanol: sample ratio) for extraction of bacteriocin (Risoen. *et al* 2003). Butanol was evaporated and precipitates were dissolved in 2ml sterile phosphate buffer saline at pH 7.4. Following dissolution the extracts were filter sterilized using 0.2 µm Acrodisc syringe membrane filters and stored at 4^0C before use. These solutions were designated as Purified bacteriocin extracts. Samples were withdrawn before and after purification and were analysed for bacteriocin activity (AU ml^{-1}).

Determination of the MIC and arbitrary units (AU)

The microdilution method using 96 well microtitre plates described by the national assessment committee for clinical laboratory standards (NCCLS) was performed for the assessment of the minimal inhibitory concentration. For this microtitre plate assay, 100 µlitre of butanol extracts were serially diluted two folds in sterile. Mueller Hinton broth, with 0.2% triphenyl tetrazolium chloride (TTC) dye. 50 µlitre of 4 hr young test culture was added to respective wells. The results were observed after 4-5h and 24h and inhibition of growth was detected by Microtitre plate reader; BioTek, Powerwave XS .Medium control, extract controls and positive controls were also used for elimination of false positive results (Dusane. *et al* 2013). The bacteriocin activity was expressed in terms of arbitrary units (AU), as the maximum dilution that produced a clearly visible zone of inhibition. The unit of bacteriocin activity (AU) was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (Kayalvizhi N. *et al* 2008).

OPTIMIZATION STUDIES

Selection of crude source

Inoculation of the *Bacillus licheniformis* was done in the fermentation medium containing (g l^{-1}): peptone – 5.0, sodium chloride- 10.0, KNO_3- 3.0, sorbitol- 5.0 and the respective crude source (molasses, corn-steep liquor, cheese whey) and incubated aerobically at 5000 rpm for 72 hours. Purification was carried out by above method and arbitrary units were calculated by performing MIC for selection of best crude source. (Dusane. *et al* 2013).Statistical analysis was conducted using Students t-test. Students t test is a statistical method which is used for testing the hypotheses Student's t-test is used for , testing the hypotheses about the mean of a small sample drawn from a normally distributed population when the population standard deviation is unknown. Significance levels was set at P = 0.05.

Formulation of an Optimum Medium using Plackett-Burman Design

A fractional factorial design described by Dhouha G.*et.al*. 2011 was used for the optimisation of the medium for maximum bacteriocin production. The PB design has proved very effective and is widely used to identify significant variables with minimum of 'trails'. When more than five independent variables are to be investigated, the Plackett-Burman design may be used to find the most important variables in a system, which is than then optimised in the further studies.(H A Modi 2009). This technique allows for the evaluation of X-1 (7 in this study) variables by X (8 in this study) experiments. The incorporation of dummy variable into an experiment makes it possible to measure the variance of an effect (experimental error).

Table 2
A 8 run design of Plackett-burman matrix

	Variables.						
Trials	A	B	C	D	E	F	G
1	H	H	H	L	H	L	H
2	L	H	H	H	L	H	L
3	L	L	H	H	H	L	H
4	H	L	L	H	H	H	L
5	L	H	L	L	H	H	H
6	H	L	H	L	L	H	H
7	H	H	L	H	L	L	L
8	L	L	L	L	L	L	L

Table 3
High and low concentrations of the factors used for fermentation study

Sr.no	Variables	High concentration grams/litre	Low concentration grams/litre
1	Peptone	**10**	**2.5**
2	Sodium chloride	**20**	**5**
3	Potassium nitrate	**6**	**1.5**
4	Sugar	**10**	**2.5**
5	pH	**8**	**6**

The bacteriocin activity was calculated by agar well diffusion assay (Torkar. *et al* 2003), arbitrary units by MIC (Dusane. *et al* 2013) and Protein content by Folin- Lowry's method (Lowry H. *et.al* 1951). The stages in analysing the data are as follows: (Zulkuli M. *et.al* 2011)

1. Determination of the difference between the average of the High (H) and the low (L) responses for each independent variable and the dummy variable.
   The effect of an independent variable on the response is the difference between the average response for the four experiments at the high and the low level.
   Thus the effect of

$$A = \frac{2\,[\sum A\,(H) - \sum A\,(L)]}{8}$$

This value should be near to zero for the dummy variables.

2. Estimation of the mean square of each variable (the variance of the effect).

$$A = \frac{[\sum A\,(H) - \sum A\,(L)]^2}{8}$$

3. Calculation of experimental error by averaging the mean squares of the dummy effects of dummy variables. The effects of the dummy variables are calculated in the same way as the effects of the experimental variables. If there are no interactions and no errors in measuring the response, the effect shown by a dummy variable should be 0. If the effects in is not equal to 0, it is assumed to be a measure of the lack of experimental precision as well as analytical error in measuring the response.

Stability of Bacteriocin

To analyse thermal stability of the bacteriocin cell free supernatant were incubated at temperature ranging from 4^oC to 100^oC. Upon incubation for 1 hour the activity of the bacteriocins which are subjected to different temperature conditions was checked by agar well diffusion method against *Escherichia coli* NCIM 2641. The activity of bacteriocin at different pH values was estimated by adjusting the pH of the samples from 3.0 to 11.0. After the treatments the samples were tested for bacteriocin activity by agar well diffusion assay using *Escherichia coli* NCIM 2641 as test organism (Kayalvizhi N. *et al* 2008).

Characterisation of the bacteriocin compounds

Separation and Characterisation of the bacteriocin compounds was done using

analytical technique like Liquid Chromatography- Mass Spectroscopy. High performance liquid chromatography (HPLC) was carried on VARIAN Pro Star/ Dynamic System Model 210 equipped with a pump and water photodiode array detector with variable wavelength detector (100-800) nm. The column used was a Nucleosil C 18 column (VARIAN). Pump A was water and Pump B was formic acid.20 μl of sample dissolved in formic acid was injected and run time was 20 minutes with sampling interval of 0.20 sec. The mass spectra were obtained for separated components using VARIAN 500 MS-IT Mass Spectrometer coupled with APCI ionization mode.

RESULTS & DISCUSSIONS

In the present study we isolated a bacteriocin producing bacterial strain which exhibited broad spectrum antagonistic activity. Preliminary characterization of the strain was done by examining its morphological and biochemical properties. Furthermore, based on blast analysis of partial sequence of the 16S rRNA gene of this strain, its taxonomic identity was confirmed as *Bacillus licheniformis.*

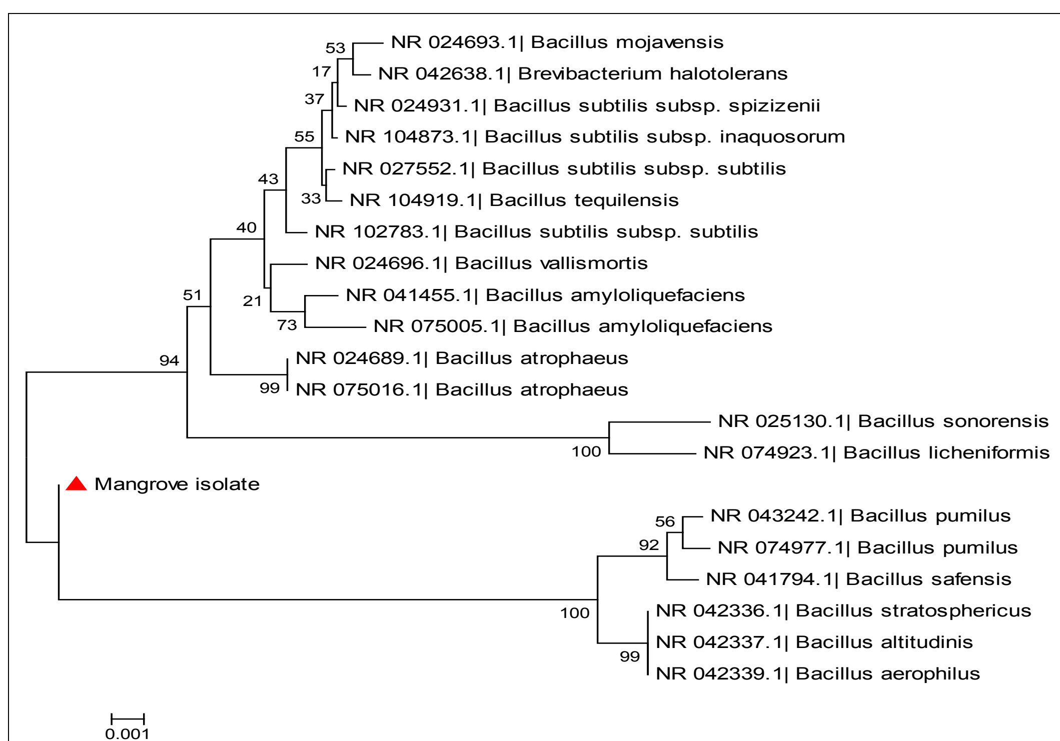

Figure 1
Phylogenetic tree for Mangrove isolate using partial 16S rRNA gene sequence

The natural habitat of these *Bacillus species* are the soil and the marine environment and the isolate was isolated from the mangrove soil which is also a type of marine environment. The culture supernatant of *Bacillus licheniformis* exhibited broad spectrum bacteriocin like activity against Gram-positive, Gram negative bacteria and fungi. The bacteriocin production and secretion was observed in the stationary phase, after 10 to 16 hour of bacterial population in fermentation broth. It is possible that sporulation process is required for bacteriocin production or at least a common regulatory pathway as reported for other bacteriocin compounds and hence bacteriocins are secondary metabolites (Syed I., *et . al* 2011).

Optimisation studies

For the enhancement of the bacteriocin activity using agricultural and industrial byproducts waste like molasses, corn steep liquor and cheese whey which provides different carbon and nitrogen sources were used for

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optimization studies. Since these crude sources are readily available and possess a high amount of carbon and nitrogen source these crude carbon sugars could be used for mass production instead of synthetic medium. Methods like strain improvement/modification, optimization of various process parameters and cost effective downstream processing can be employed for over production of bacteriocin. Another factor associated with cost is the fermentation media used for the production of bacteriocin. Almost 30% of the total production cost relies on synthetic media and nutritional supplements used for growth of microorganisms. Use of low cost media or agro-industrial byproducts as raw substrates (with least or no value) can make the fermentation process economically viable along with reduction in environmental pollution.Agro-industrial byproducts contains appropriate and sufficient amount of balance nutrients required for the growth and product formation, thus, can serve as fermentation medium (Bali V., *et.al* 2014). Upon analysis of the bacteriocin activity, it was found that corn-steep liquor profoundly affected the bacteriocin production by *Bacillus licheniformis* which was selected for further optimization on the basis of t-test. Also upon comparison of the fermentation medium (4000 AU/ml) to that of the fermentation medium with crude source (8000 AU/ml) there was increase in the activity observed for *Bacillus licheniformis*.

Table 4
Arbitrary units (AU/ml) of purified Bacteriocin Extract

Bacteriocin Extracts	Cheese Whey	Molasses	Corn steep liquor	Fermentation medium
B. licheniformis	-	4000	**8000**	4000

Production of bacteriocin is affected by different physical and chemical parameters including carbon and nitrogen sources, metal ions, temperature, pH, aeration etc. Effect of addition of industrial byproducts i.e. corn steep liquor was observed, which led to increased bacteriocin production. This may be due to presence 7.5% nitrogen and 2.5% reducing sugars (carbon) source , metal ions and salts in corn steep liquor (Bali V., *et.al* 2014). Cladera O *et al.,* findings implicate that the bacteriocin production was optimized and the maximum was achieved when the pH was between 6.5 and 7.5, when the producer was grown at a temperature between 26 and 37 ^{0}C and the cheese whey concentration in the growth medium was about 70 g/ L.

Optimization of the media components using Plackett-Burman Design

The Plackett-Burman design was used to evaluate the relative importance of five parameters for *B. licheniformis* bacteriocin production. Bacteriocin productivity, protein content, specific activity from the fermentation trials are given in Table 5.The protein content was considered for measuring the response.The amount of the bacteriocin activity produced in the eight different runs varied from 2000 to 8000 AU/ml while its specific activity ranged between 57to526 AU/mg for *B. licheniformis.*

Table 5
Tabulation of bacteriocin activity, protein content and specific activity

Bacteriocin from *B. licheniformis.*			
Trial	Antibacterial activity (AU/ml)	Protein content (mg)	Specific activity (AU/mg)
1	4000	20	200
2	8000	36	222.22
3	4000	20	200
4	4000	7.6	**526.31**
5	4000	11.4	350.87
6	2000	15.2	131.57
7	4000	70	57.14
8	2000	3.8	**526.31**

Comparison of specific activity and protein content in each trial was analysed and there was significant difference in the amount of protein content to its specific activity present in each trial. In case of *Bacillus licheniformis* (trial 4) there was high amount of protein and high amount of bacteriocin activity this means all the protein present are bacteriocin, in (trial 8) there is less amount of protein but higher bacteriocin activity this specifies that the bacteriocin activity is very potent, in (trial 6) there is more amount of protein and less amount of bacteriocin activity this means that there is inactivation of bacteriocin during production or purification. (Table 5) Upon calculating the effect and the mean square variance for each individual component the main variables affecting the bacteriocin activity of *B. licheniformis* were sodium chloride, potassium nitrate, sugar (crude source), as identified by the Plackett-Burman design. (Table: 6). Since the *Bacillus strain* was isolated from the mangrove soil and mangrove plants grow on the interface between land and water, their habitat, ecosystem and nutritional requirement are completely different than the other organisms and they posses high tolerance to increase salt content. The high saline condition is an important growth requirement which is evident by the optimization by Plackett-Burman design. Sodium Chloride was one of the important variable affecting bacteriocin activity and production. The level of bacteriocin by *B. licheniformis* with optimized medium was increased 2-fold (8000 AU /ml) as against the un optimized medium (4000 AU /ml). This strain were isolated from marine environment therefore NaCl maintains salinity for growth and production of bacteriocin. Optimised media composition: sodium chloride (20g/l), potassium nitrate (6g/l), sugar: (2.5g/l), pH 7 Metal ions are important components for any biological production process. Due to specific ionic and water binding capacity, these metal ions affect the bacterial metabolic activity. Eight fold increase in bacteriocin was observed when concentration of KNO_3 was increased to 2-5% from 0.2% in growth medium (Bali V., *et.al* 2014).

Table 6
Tabulated results of Plackett –Burman analysis calculation for bacteriocin from B. Licheniformis

	Peptone	NaCl	Dummy variable	KNO_3	Sugar	pH	Dummy variable
∑(High)	112.8	137.4	91.2	133.6	59	70.2	66.6
∑(low)	71.2	46.6	92.8	50.4	125	113.8	117.4
difference	41.6	90.8	-1.6	83.2	-66	-43.6	-50.8
Effect	10.4	22.7	-0.4	2.6	-16.5	-10.9	-12.7
Mean square	216.32	1030.58	0.32	865.28	544.5	237.62	322.68

$$\text{Mean square for Error} = \frac{0.32+322.68}{2} = 161.5$$

The effect for each variable was calculated and the interpretation of the effect shown is that

1. A negative sign means that going from low level to high level for a factor decreases the response.
2. A positive sign means that going from the low level to the high level increases the response.
3. In case of mangrove soil isolate sugar, pH, and dummy variables were shown to have negative effect and hence the concentration of these variables should not be increased as it decreases the response. Though the second dummy variable shows more, but average of both variables is very less as compared to other variables.

Stability of the Bacteriocin

Stability of the purified bacteriocins to various pH conditions and temperature conditions were determined. *B. licheniformis* was stable over a wide pH range (3-11) and showed maximum activity at pH 7. Bacteriocin of *B. licheniformis* was thermo tolerant but loss of activity was observed after incubation to higher temperature i.e. 100^0C.

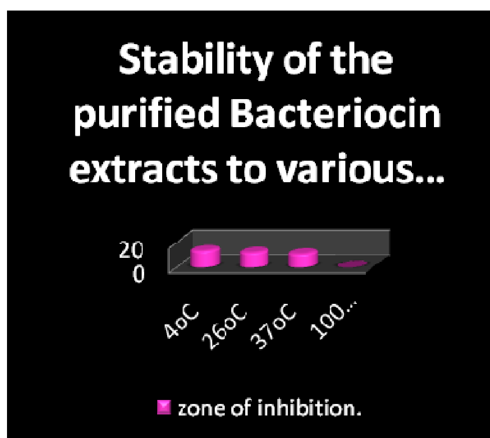


Figure 2
Graphical Representation of Stability of the purified Bacteriocin extracts to various temperature

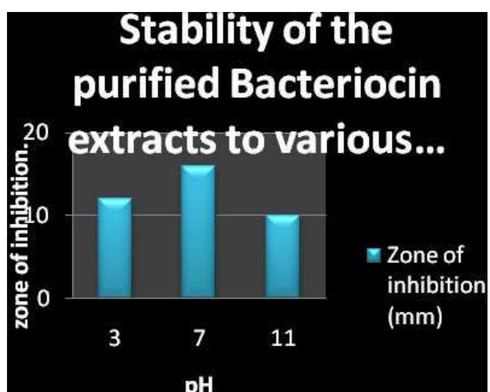
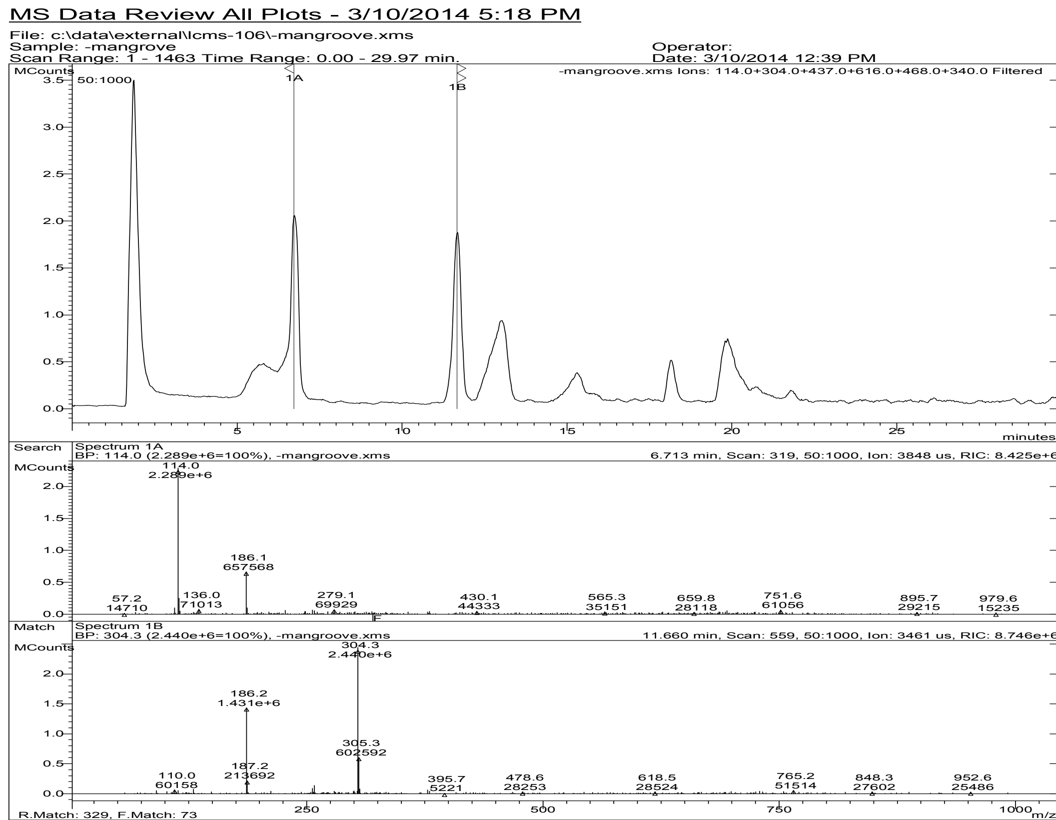


Figure 3
Stability of bacteriocin at various pH.

Characterisation of the bacteriocin compounds

Liquid Chromatography- Mass Spectroscopy suggested that *Bacillus licheniformis* itself produced a wide range of compounds with possible role in antagonism. Since the extracts are a mixture of metabolites with possible role in antagonism, purification by coloumn chromatography followed by antimicrobial assays of eluted fractions is desired to identify the compounds actually exhibiting the antimicrobial activity. For full identification of the structure, the amino acids of the polypeptide proteomics based study along with database search and Nuclear Mangnetic Resonance is beneficial.

Figure 4
LCMS chromatogram of bacteriocin from Bacillus licheniformis



CONCLUSION

During the present research work, the ability of *Bacillus licheniformis* for the production of bacteriocin was investigated. This strain was isolated from the mangrove soil. A bacteriocin with a broad spectrum of activity against Gram-positive bacterial pathogens, filamentous fungi and yeast suggested its potential clinical use. Statistical method facilitated optimization of cultural medium for the improved production of bacteriocin. Corn steep liquor is a crude optimised medium source which increased the bacteriocin of *Bacillus licheniformis.* Bacteriocin produced has stability at low temperature and is also stable at low and high pH. The bacteriocin isolated from the above *Bacillus sps*, would be useful in medical and food industry to control food borne infections or as a food preservative that may be used while food packaging.

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