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Isolation and Characterization of Bacteriocins like Antimicrobial Compound from *Lactobacillus delbrueckii* subsp *lactis*

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Abstract

Bacteriocins are naturally produced antimicrobial peptides that inhibits microorganism in narrow and broad range. *Lactobacillus delbrueckii* subsp *lactis* were isolated from yogurt and screened to produce bacteriocin like compound by Agar diffusion assay, further cultured in shake flask fermentation at 30^{o}C for mass production. Characterization of bacteriocins was done by UV spectrophotometric analysis. At different pH (6-9), incubation time (0-144 hours), temperature (20 – 40) and agitation (50 – 150 rpm) the production of the compound was characterized and checked by agar diffusion assay to perceive the size of zones of inhibition. The study aimed to optimize the growth condition for the enhanced production of bacteriocin by *Lactobacillus delbrueckii* subsp *lactis* using Response Surface Methodology (RSM). Maximum zones of inhibition were observed at pH 8, after 72 hours of incubation at 30^{o}C with an agitation of 100 rpm. The compound was centrifuged and purified using ammonium sulphate precipitation, dialysis and chromatographic techniques. Bacteriocins like polypeptide antimicrobial substance showed activity against Gram positive organisms like *Bacillus cereus* and *Staphylococcus aureus* that proved to be sensitive.

Keywords: Bacteriocins, *Lactobacillus delbrueckii* subsp *lactis*, Response surface methodology (RSM -CCD).

1. Introduction

Lactobacillus delbrueckii subsp *lactis* is a nonpathogenic member and lactic acid emanating bacteria that are largely used in dairy products production, especially in cheese-making and yogurt production. It has the characteristics of diminishing lactose intolerance, changing the intestinal milieu and improving immunity to stimulate physical health (Piard *et al.*, 1992). Recent studies in the field of bacteriocin produced by *L. delbrueckii* have validated that the bacteriocin has a broad spectrum in terms of inhibition on different bacteria and fungi. Lactic acid bacteria's bacteriocin is a kind of antibacterial polypeptide synthesized in the metabolism process (Jagannathan *et al.*, 2015; Larsen *et al.*, 1993; Pingitore *et al.*, 2007; Zhao *et al.*, 2015). There are reports revealing that the bacteriocin was extracted from *Lactobacillus bavaricus* (Larsen *et al.*, 1993); *Lactobacillus brevis* MTCC 7539 (Neha Gautam and Nivedita Sharma, 2009); south Indian special dosa (appam) batter (Pal *et al.*, 20); *Lactobacillus plantarum* (Ray *et al.*, 2009; Ravi Sankar *et al.*, 2012; Zhou *et al.*, 2014). Bacteriocin has an antibacterial effect on Gram-positive pathogens associated with bovine mastitis and helps in eliminating them (Klostermann *et al.*, 2010).

Optimization of cultural parameter is a vital facet in the field of food biotechnology and fermentation to recuperate product yield and trim down the process variability, as well as reducing processing time and costs. Due to the complexity of the culture media, prolonged growth time, and slow agitation for producing bacteriocin from *Lactobacillus delbrueckii* subsp *lactis*, it is practically impossible for a one factor one time to identify an optimum combination of culturing conditions using a predetermined number of experiments. Response Surface Methodology (RSM) is a tactic approach that can be used to analysis the effect of variables and to pursue the conditions for a multivariable system. The aim of this probe was to quest the optimal cultural condition for *Lactobacillus delbrueckii* for mass production by using the statistical tools (Shaileshkumar *et al.*, 2009; Siew *et al.*, 2013; Vidhyalakshmi *et al.*, 2016). Reports relating to the antibacterial properties of these organisms have been constrained.

In the present study, optimal cultural conditions were determined and the effects of these variables on the bacteriocin activity were assessed, using a response

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surface approach that includes statistical and plotting methods for experimental design and analysis.

2. Materials and Method

2.1. Sample Collection

Different bacterial samples were isolated from homemade yogurt.

2.2. Isolation of Bacteria

2.2.1. Isolation of Microorganism

The yogurt samples were brought to the laboratory and 1 gm of yogurt was serial diluted. Appropriate dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} were selected and replicates were maintained throughout the present study. After 24hrs of incubation the bacterial cultures were enumerated, isolated and inoculated in separate Petri plates and vials and stored in the refrigerator for further analysis. The Milk agar plates and slants were labelled properly and were kept undisturbed.

2.2.2. Identification of Microorganisms

2.2.2.1. Physical Identification

Phenotypic identifications were carried out on the basis of their color, colony formation, texture, etc. and they were isolated, stored and classified as 4 isolates.

2.2.2.2. Microscopic Observations

The 4 isolates were Gram stained and observed under the microscope for their morphological appearance. The structure of organisms was identified as bacilli as per Bergey's manual, 1976 and standard methods.

A motility test using semisolid agar tubes was carried out and the motile nature of the organism was observed along the line of inoculation.

2.2.2.3. Biochemical Tests

Identification of these organisms was carried out by conventional biochemical tests, like indole, Methyl red, carbohydrate fermentation, Voges Proskauer, catalase, citrate, urease, triple sugar iron, starch hydrolysis and oxidase test.

2.3. 16s rRNA Analysis

After the isolation and biochemical identification of organism, 16srRNA was performed and the sequence was submitted in EBI (http://www.ebi.ac.uk).

2.4. Bacteriocin Production by Lactobacillus Delbrueckii Subsp Lactis Species

2.4.1. Inoculum Preparation

From the isolated organism, *Lactobacillus delbrueckii* subsp *lactis* was identified, showing an antimicrobial activity. Hence, this organism was used as inoculums to produce bacteriocin like compound. The organism was inoculated in (De Man, Rogosa and Sharpe agar) MRS Broth and incubated 37^{o}C was used for the screening of antimicrobial substance.

2.4.2. Production Media

The cell suspensions were aseptically transferred to experimental flasks for the growth studies. The bacteriocin production was carried out in mineral medium containing: 2.0 mgl^{-1} NH_4 NO_3, 0.01 mgl^{-1} $CaCl_2$, 0.5 mgl^{-1} KH_2PO_4 , 1.0 mgl^{-1} K_2HPO_4 , 0.5 mgl^{-1} $MgSO_4$, 0.1 mgl^{-1} KCl and 0.06 mgl^{-1} yeast extract, respectively. For a large scale production of Bacteriocin like compound, a production medium was optimized and the organism was inoculated. After 72 hours of incubation, the cell free filtrate was analyzed for antimicrobial activity using spectrometry and further by Agar well diffusion method.

2.4.3. Protein Estimation

The protein estimation was carried out according to Lowry's method by using BSA as standard.

2.4.4. Determination of Bacteriocin Activity

A well diffusion assay procedure was used. Aliquots of 50 µl from each bacteriocin dilution were placed in the wells in plates seeded with the bioassay strain. The plates were incubated overnight at 30ºC for lactic acid bacteria indicators and the diameters of the inhibition zone were measured (Rammelsberg and Radler, 1990).

2.4.5. Determination of Bacteriocin titer

The titres of bacteriocin produced were calculated by two-fold serial dilutions of bacteriocin in saline solution and aliquots of 50 µl from each dilution were placed in wells in plates seeded with the bioassay strain. These plates were incubated at 37ºC for 18-24 h and examined for the presence of 2 mm or larger clear zones of inhibition around the wells. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU ml^{-1}) (Graciela *et al.*, 1995). One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

2.5. Response Surface Methodology RSM- Central Composite Design - CCD

Response surface methodology was used for optimization of bacteriocin production. The selection of parameters was selected from previous literature and the ranges were fixed using one factor at a time (OFAT). The combination of the media influences the production of bacteriocin along with the physical parameter. Table 1 represents the coded value and actual value for all the parameter used (Mandenius and Brundin, 2008)

Table 1. Representing the factors its Coded and Actual value

Factor	Low		High	
	Actual	Coded	Actual	Coded
pH (X_1)	6	-1	10	1
Temperature (X_2) ^oC	20	-1	40	1
Incubation time (X_4) h	0	-1	144	1
Agitation (X_4) rpm	50	-1	150	1

Equation $Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_4 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{44}X_4^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{14}X_1X_4 + a_{23}X_2X_3 + a_{24}X_2X_4 + a_{34}X_3X_4$

where Y is the predicted response; X_1, X_2, X_3, X_4, the independent variables, a0 the offset term, a_1, a_2, a_3, a_4, the coefficients of linear effects; a_{11}, a_{22}, a_{33}, a_{44}, coefficients of squared effect, a_{12}, a_{13}, a_{14}, a_{23}, a_{24}, a_{34}, coefficients of interaction terms.

2.6. Spectrophotometric Method (UV – VIS Spectrum)

The crude extra-cellular protein was estimated using spectrophotometer. It was analyzed using Varian Cary 300 UV-VIS spectrophotometer in a spectrum mode and the maximum activity was at a wavelength of 280 nm.

2.7. Gel Permeation Chromatography

Twenty-three 2ml of fraction were separated using Gel permeation method and further subjected to the UV –VIS spectroscopic analysis to 280 nm and the absorption was found maximum in 14 fraction.

2.8. SDS- PAGE (Polyacrylamide Gel Electrophoresis)

2.8.1. Slab gel electrophoresis

This method was performed to establish the molecular weight of the bacteriocin present in the *Lactobacillus delbrueckii* subsp *lactis* sample as it showed maximum protein content in the Lowry's method. The standard protein was kept as Bovine Serum Albumin (BSA). Based on the protein standard the molecular weight of the enzyme was estimated.

2.8.2. FTIR

The absorbance FT-IR spectra of the samples was documented using an FT-IR Perkin–Elmer spectrometer. The spectra were collected within a scanning range of 400-4000 cm^{-1}.

3. Results and Discussion

3.1. Isolation and Identification of Lactobacillus Delbrueckii Subsp Lactis

The organism was isolated from the yogurt and identified as *Lactobacillus delbrueckii subsp lactis* by biochemical and 16s rRNA sequencing. The sequence obtained after sequence is given below:

3.2. Aligned Data

>bi05 Lactobacillus lactis

CCTAAACAGTAGAAATATATTGAAAGCTGTGT
AAACTATGAAATCTCAATCTCTACCTGTAAATATT
CTAGCACTACTTGATAAGGACTGTTTTGTCATGCG
TAGAAACAAAAAACTTGTTCAAGGATCATACCAA
AATGAAGAGCCCTACAATTGTTAGACATAGACAT
CTAACGATTGTGGGGTATTTTTATGACCAAATATT
CATCTGAACAAAAAGTACAAATTGCTTCTGATTAT
CTTTACGGCAGAGACTCATACAATGGATTAAACC
AAAAGTATAATATGGCTGCGTCAATAATTCGTAC
GTGGGTGAAAGCCGCTGAACTTAATGGATTGGAA
CTCATCTTCAATCTATATCTTTTAACAATTGCTGTT
TTCCAAAGTCAATTCACCGCCAAAAGCTTCCAAG
CGATCGACTTCAGCCAGGTAGATTTGGCCAGCAA
TAGTCTGGTACTTGCCAGACGGGTATTGCTCAAA
GCTTAAGGCATCCCCTTCCTTGCTCTTCAAGATGC
CAAATTCTGCTTCTTCCTCATCTTTCATGACAAAG
GACACGACTTGCCCAGCAGCTAATTGCCAGAAGT
CCTGAATCTGCTTGATTTCTGCTGCTTTATCTTGA
ACTTCAAAGGGATCTTCTAATCCTCTTTCCTTAAG
CTGGCCCCGGCAGCGGCTGATATCATGCAAATAC
TCTGATCGATCAATTACTCTGTCAACGGCCTTCTT
CTTAACCTGCAGCAATGAGTCATCCCGGCCAATTT
CGTCAACAGTCAAATACAAGCTGAAGTCGTCATC
AGCACACAATAGCCGACCAACCGCGAATGAAGCT
GGATCTTCTTTCAGATGAACTTCGCACAAACCATC
TACAGTTGTATATTCAGTATCCCGCAACTTAGGAT
TGTCCAGCAACCATTCACCGGAGAAAGCGGCTAG
CCGCCGGAGTTGACAGACAGCAACATTTCCTGCG
AAAGGCTGGTACTTGCCAGAAGGATATGCCGCAA
AATTAACTTGCCCGGCAGAAATATCATCAATATA
TCCCCATTGCTGGTAATCTTCGCCTTCCAGGCAAA
AATATACAACCTGCCCGGGCAATAAGTCCATAAA
GCTTCCGACTGACTTGATTAAGGTAACTTTGTCTT
CCAGCTTGAAAGGATCTGCCAGTCCATTTTGCGTT
AGCAGCTTCCGCCGGCGCTGGATCTCTGCCAAAT
AATGCGATTGATCCAACAGCCGCGTGACCGCTGA
CTTTCTGATCAAGAGCAAGGAATCATCCCGGCCC
AGCTGATCCAAAGTCAAGTAGAGCTGCCAGCCGT
TTTCTTCAAACAAGAGGCGACCGAGTTCAAAACT
GTCCTGAGCAGCGTCTGTGTAGATTTCGTATAATT
TTTCCA

3.3. Identified as Lactobacillus Delbrueckii Subsp Lactis

The sequence was subjected to BLAST and the phylogenetic analysis was reported.

The isolated and confirmed strain was inoculated in the production media for enhanced production of Bacteriocin that can be used as a food preservative (Paul Cotter *et al.*, 2005); it also enhances the immunity of the food against invading pathogens. A database for bacteriocin was classified and created (Hammami *et al.*, 2010).

3.4. Optimization of Bacteriocin Production

Analysis was done to understand the effect of various parameters on the production of Bacteriocin. The ability of the productivity influences the factors that enhance the productivity of the component from the organism. When a single factor analysis was performed the influence of other factors is not understood over the other.

3.4.1. Effect of pH on Bacteriocin Production

The optimum pH for the antimicrobial production by *Lactobacillus delbrueckii subsp lactis* was 8, although a good activity was also achieved at pH 6-9.

3.4.2. Effect of Temperature on Bacteriocin Production

The optimum temperature for the antimicrobial production by *Lactobacillus delbrueckii subsp lactis* was 30^oC.

3.4.3. Effect of Incubation Time on Bacteriocin Production

The maximum antibiotic activity by *Lactobacillus delbrueckii subsp lactis* was achieved at 96 hours of incubation period.

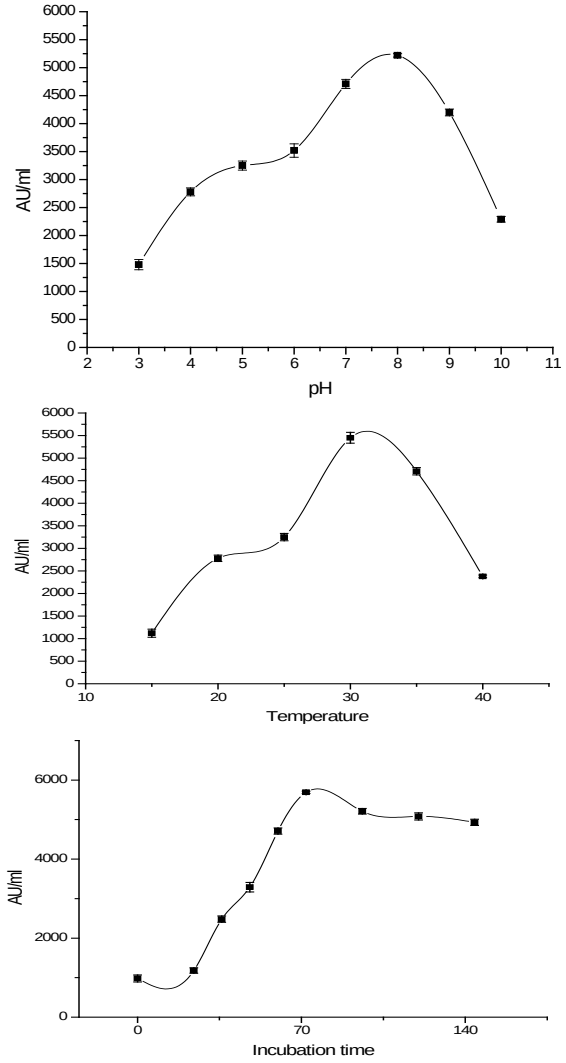

Figure 1. pH, temperature and incubation time with respect to enzyme activity

3.5. Response Surface Methodology

The four-parameter pH (X_1), temperature(X_2), Incubation time (X_3) and Agitation (X_4) were used to Design of Experiment (DOE) using Design expert version 7.0.0 software. After the analysis was performed, the values were loaded into the software again and analysis of variance (ANOVA) was observed. The maximum experimental value 5980AU/ml while the predicted value was estimated at 5646.6 AU/ml, a close correlation with each other. The model was significant and the lack of fit showed non-significance, assign that the design is functional with polynomial equation. The coefficient of determination of terms of R^2 is 0.985702, and the Adj R^2, as 0.972358 with a Pred R^2, was 0.949666. The contour and surface plots represent the interaction between variables.

Table 2. Design table of variable with responses

Run	X_1	X_2	X_3	X_4	Response	
		^oC	H	Rpm	Actual	Predicted
2	6	20	0	50	1500	1734.167
25	10	20	0	50	1770	1617.917
5	6	40	0	50	1750	1954.583
22	10	40	0	50	1470	1575.833
7	6	20	144	50	2920	2892.917
16	10	20	144	50	2270	2489.167
14	6	40	144	50	2560	2490.833
18	10	40	144	50	1790	1824.583
11	6	20	0	150	1880	1969.583
23	10	20	0	150	1750	1900.833
21	6	40	0	150	2570	2432.5
17	10	40	0	150	1950	2101.25
27	6	20	144	150	2610	2585.833
8	10	20	144	150	2310	2229.583
9	6	40	144	150	2150	2426.25
28	10	40	144	150	1960	1807.5
24	4	30	72	100	4020	3849.583
6	12	30	72	100	3150	3114.583
29	8	10	72	100	2980	2877.917
1	8	50	72	100	2780	2676.25
15	8	30	-72	100	1020	799.5833
10	8	30	216	100	1650	1664.583
12	8	30	72	0	1080	907.9167
26	8	30	72	200	1160	1126.25
19	8	30	72	100	5360	5646.667
3	8	30	72	100	5920	5646.667
30	8	30	72	100	5980	5646.667
20	8	30	72	100	5240	5646.667
13	8	30	72	100	5910	5646.667
4	8	30	72	100	5470	5646.667

X_1- pH; X_2- Temperature; X_3- Incubation time; X_4- Agitation

The equation Response = $5646.667 - 183.75\ X_1 - 50.4167\ X_2 + 216.25\ X_3 + 54.58333 X_4 - 65.625\ X_1X_2 - 71.875\ X_1X_3 + 11.875\ X_1X_4 - 155.625\ X_2X_3 + 60.625\ X_2X_4 - 135.625\ X_3X_4 - 541.146\ X_1^2 - 717.396\ X_2^2 - 1103.65\ X_3^2 - 1157.4\ X_4^2$

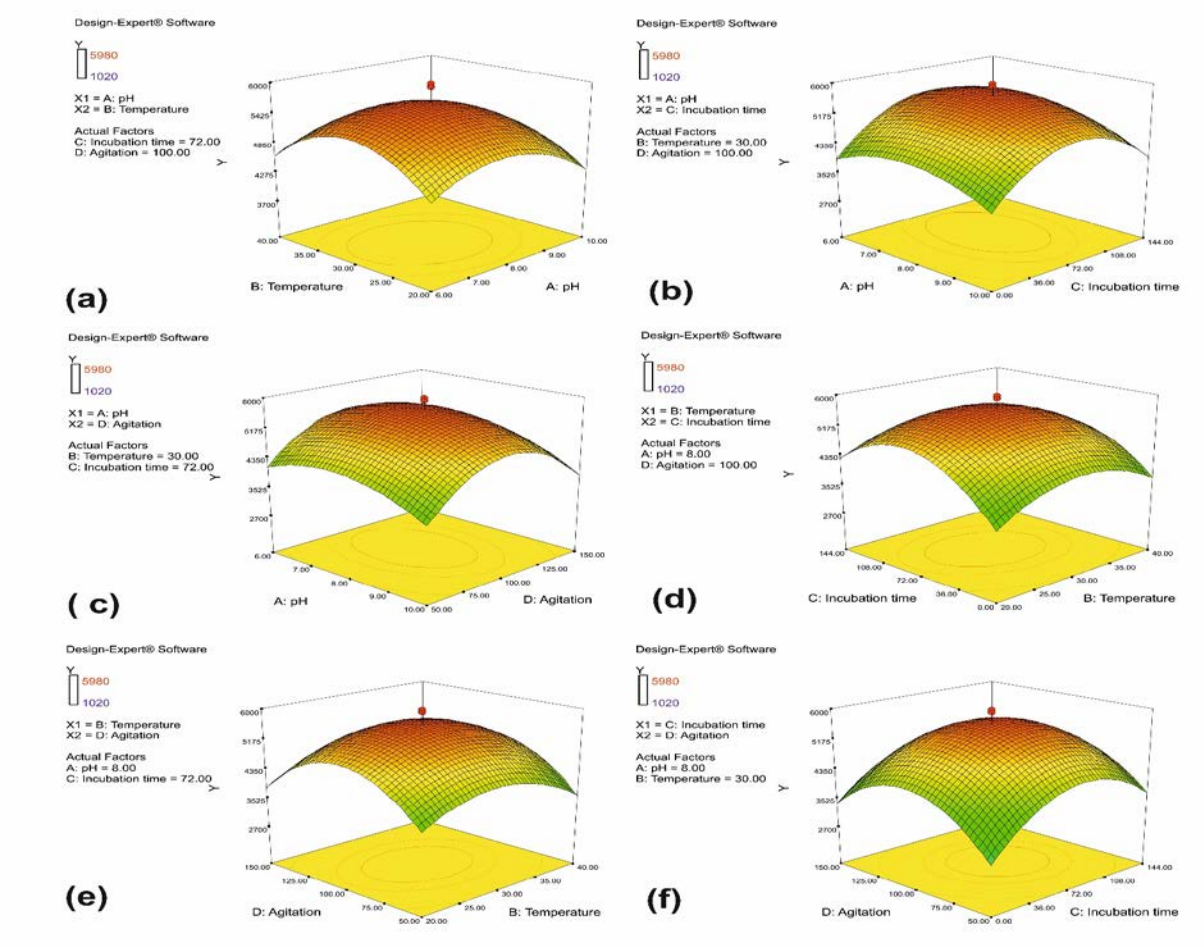

Figure 2. Three dimensional graphs showing the effect of pH (X_1), Temperature (X_2), Agitation (X_3) and Incubation time(X_4) on bacteriocin production

Table 3. ANOVA Table and regression analysis for selected model

Source	Sum of Squares	Df	Mean Square	F Value	p-valueProb > F
Model	71002762	14	5071626	73.8652	< 0.0001
X_1	810337.5	1	810337.5	11.80208	0.0037
X_2	61004.17	1	61004.17	0.888489	0.3608
X_3	1122338	1	1122338	16.34618	0.0011
X_4	71504.17	1	71504.17	1.041415	0.3237
X_1X_2	68906.25	1	68906.25	1.003578	0.3323
X_1X_3	82656.25	1	82656.25	1.203839	0.2899
X_1X_4	2256.25	1	2256.25	0.032861	0.8586
X_2X_3	387506.3	1	387506.3	5.643797	0.0313
X_2X_4	58806.25	1	58806.25	0.856478	0.3694
X_3X_4	294306.3	1	294306.3	4.286395	0.0561
X_1^2	8032150	1	8032150	116.9835	< 0.0001
X_2^2	14116300	1	14116300	205.5955	< 0.0001
X_3^2	33408936	1	33408936	486.5812	< 0.0001
X_4^2	36742357	1	36742357	535.1305	< 0.0001
Residual	1029908	15	68660.56		
Lack of Fit	495975	10	49597.5	0.464454	0.8586
Pure Error	533933.3	5	106786.7		
Cor Total	72032670	29			
Std. Dev.	262.0316		R-Squared		0.985702
Mean	2831		Adj R-Squared		0.972358
C.V. %	9.255796		Pred R-Squared		0.949666
PRESS	3625680		Adeq Precision		26.16025

Significant *$P \leq 0.05$

3.6. Purification and Characterization of Bacteriocin

After optimizing the cultural condition for enhanced production of bacteriocin, the concentration and purification were performed using ammonium sulphate precipitation, dialysis and column chromatography that was confirmed by the method of Lowry *et al*. (1951). The partially purified protein was analyzed for the molecular weight using SDS – PAGE that reveal 42 kDa when compared to that of standard protein marker.

3.7. FT-IR

The FTIR spectrum of bacteriocin from *Lactobacillus delbrueckii* subsp *lactis* is shown below. In bacteriocin treated cells shift in absorbance in low frequency at 3227.4, 2232.1 and 1871.8 cm^{-1} was observed. The shift in absorbance band in the region of 4000-3200 cm^{-1} indicated adsorption of water molecule. In addition, deformation in 2300 -2290 shows -C≡N→O changes take place. There was a shift from 1580 – 1490 to 2250-2670 that indicates the change of NH^{+}. At 1410 – 1260 the peaks were changed, indicating the disappearance of OH group. Similar results were reported by many researchers (Ravi Sankar *et al*., 2012)

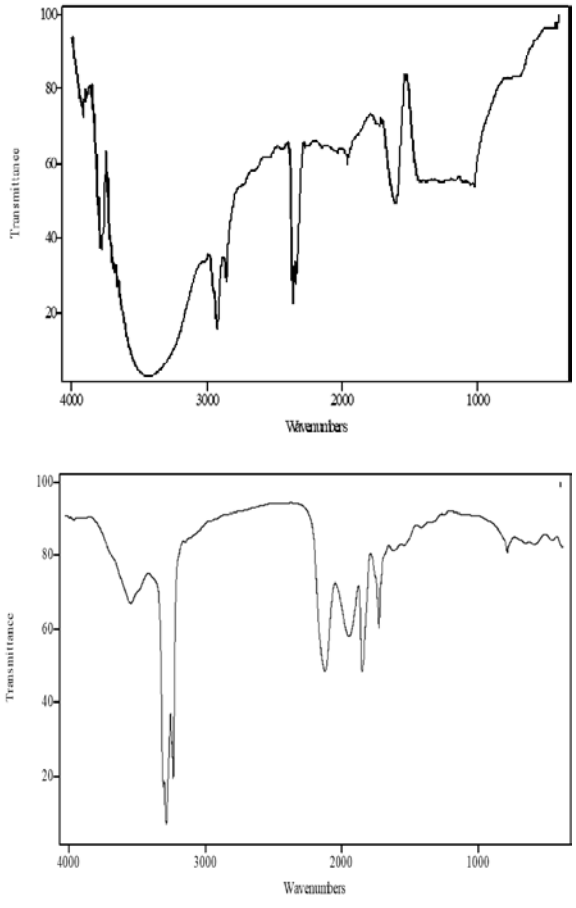

Figure 3. FTIR spectrum

4. Conclusion

Statistical designs (RSM using central composite design) were useful in the identification and optimization of important cultural conditions for bacteriocin production by the isolate from yogurt. Further characterization of the identified bacteriocin and technological evaluation of the isolate for the preparation of antibacterial compound are explored. The prospective application of these antimicrobial substances as bio-preservatives either as protective culture or as additives will be an alternative solution for the carcinogenic preservatives.

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