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Statistical optimization of bacteriocin produced from *Lactobacillus delbrueckii* subsp *bulgaricus* isolated from yoghurt

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Abstract

The Lactic Acid Bacteria (LAB) was isolated from yoghurt and identified as *Lactobacillus delbrueckii* subsp *bulgaricus* by biochemical methods and by 16s rDNA sequencing. The bacteria was screened for bacteriocin production by Agar well diffusion assay against four test organisms: *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis and Staphylococcus aureus*, it was found to inhibit the growth of three test organisms except *Klebsiella pneumoniae*. The production of bacteriocin was optimized using Response Surface Methodology (RSM) based on the results obtained from single factor method. The optimized parameters were statistically validated using the software MATLAB version 7.5.0.342 (R 2007b) from Math lab works Inc. and assumed that Starch (3%), Casein (3%), $FeSO_4$ (0.3%) and Tween 20 (0.24%) w/v supplemented to MRS media were optimum. Bacteriocin concentration before and after optimization was estimated by Lowry's method and was found to be 178.8µg/ml and 310µg/ml respectively.

Introduction

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of similar or closely related bacterial strains. They are antibiotic like agents encoded in the plasmids, chromosomes or transposons and are ribosomally synthesized extracellular bioactive peptides or peptide complexes that are bactericidal due to combined action of the bacteriocin and host autolysin or bacteristatic against other species. Over 150 Gram positive and 25 Gram negative bacteria produce bacteriocins: BACTIBASE (Bacteriocin data base) (Nes *et al*., 2007). Bacteriocins were discovered by Andre Gratia in the year 1925, but the term bacteriocin was coined by Jacob *et al*., (Konisky, 1982).

Lactic Acid Bacteria (LAB) includes the genera *Leuconostoc, Lactococcus, Lactobacillus, Enterococcus, Streptococcus, Aerococcus, Oenococcus, Vagococcus, Carnobacterium, Leuconostoc, Tetragonococcus,* and *Weisella.* (Iyapparaj *et al*., 2013). They are rod or coccus shaped, non-spore forming, Gram positive organisms that can ferment carbohydrates to form chiefly lactic acid (Parada *et al*., 2007), their DNA have a low G+C content (<55%). LAB show interesting physiological properties and technological applications such as resistance to proteolytic activity, bacteriophages, resistance to freezing, lyophilisation, production of polysaccharides and antimicrobial substances, lactose and citrate fermentation and can adhere and colonize digestive mucosa. In general, LAB has Generally regarded as safe status (GRAS) and play an important role in food fermentation given that a wide variety of strains are employed as starter cultures (or protective cultures) in the manufacture of meat, vegetable and dairy products. The most important contribution of these microbes is the preservation of the nutritional qualities of the raw material through extended shelf life, and the inhibition of spoilage and spoilage bacteria (Ananou *et al*., 2007).

Bacteriocins are generally cationic, hydrophilic or amphifilic molecules composed of 20-60 amino acid residues. They are stable in wide range of pH and temperatures. Their molecular weight ranges from <10 KD in case of small proteins to >30 KD in large proteins including one group of complex molecules with a lipid or polysaccharide moiety essential for its activity (Schillinger *et al.*, 1996; Nes and Holo, 2003).

Most bacteriocins have narrow inhibitory spectrum, i.e. they inhibit species which are closely related and some bacteriocins have broad inhibitory spectrum such as Nisin. Bacteriocins have a protein or peptide component which is essential for their bactericidal function, some consist of combinations of different proteins or are composites of proteins together with lipid or carbohydrate moieties.

Two major classes of killing actions are displayed by bacteriocins, they may form ion channels in the

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cytoplasmic membrane or exhibit nuclease activity upon gaining entry to a sensitive cell. Gram positive bacteria are membrane active whereas the lantibiotics show voltage dependence for membrane insertion, pores are formed in sensitive cell membranes leading to ion leakage and loss of PMF (proton motive force) hence, cell death. Bacteriocins adsorb to specific outer membrane receptor on Gram negative bacterial cell wall as first stage of interaction with a sensitive bacterium. Whereas, bacteriocins derived from Gram positive bacteria show less adsorption specificity. The Gram positive cell wall allows passage of relatively large molecule and hence there is no requirement for bacteriocin receptors as in Gram negative cells. The theichoic acid and lipotheichoic acid are important for initial interaction of cationic bacteriocins produced by Gram negative bacteria. Bacteriocins like Colicin are plasmid mediated but, many are not encoded by plasmid borne genes such as Nisin which is transposon associated.

The bacteriocin genes are arranged in multigene operon like structures, the structural protein is encoded by the first gene and additional gene products may be required for transcriptional regulation, modifications, processing, translocation to the exterior of the cell and self-protection (immunity). The self-immunity gene is present in association with the Bacteriocin gene. The immunity is brought about by immunity proteins encoded by "imm" genes which are in close proximity with the Bacteriocin structural and processing genes. In case of class II Bacteriocins, the immunity proteins are made up of 50-150 amino acid residues, resistance is brought about by production of antimicrobial substances in resistant mutants (Jack *et al*., 1995; Siegers and Entian, 1995; Nes *et al.*, 1996; Gravesen *et al*., 2002).

Bacteriocins are protein in nature, non-toxic to lab animals tested and generally non-immunogenic. They are inactive against eukaryotic cells, thermo resistant therefore can maintain antimicrobial activity after pasteurisation and sterilisation. They show broad bactericidal activity affecting most of the Gram positive bacteria and some damaged Gram negative bacteria including *Bacillus cereus, Salmonella, Pseudomonas aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus* (Ananou *et al*., 2007).

Considering the above characteristic and nature of bacteriocins an attempt has been made in the present work for its production optimization by Response surface methodology (RSM) which can be employed in studies related to bio preservation. RSM is an empirical statistical modelling technique employed for multiple regression analysis by means of quantitative data obtained from suitably designed experiments to simultaneously solved multivariable equations. This methodology can be used to evaluate the relative significance of several affecting factors (Rashmi and Padmavathi, 2014).

In the present study, RSM was used to optimize the bacteriocin production from *Lactobacillus delbrueckii* subsp *bulgaricus* in broth culture, the optimum conditions of different parameters obtained from single factor method was considered. The effects of Starch, Casein, $FeSO_4$ and Tween 20 on bacteriocin production were studied and sequential optimization based on statistical design was employed to enhance the production of bacteriocin.

Materials and Methods

The experiments were conducted in the Department of Microbiology, Centre for Post Graduate Studies, Jain University, Bangalore, to explore *Lactobacillus delbrueckii* subsp *bulgaricus* for the production of bacteriocin and its optimization.

Sample collection and identification

The *Lactobacillus* culture was isolated from locally available yoghurt and cultured in DeMan Rogosa and Sharpe's media (MRS). Gram stain was carried out to identify the cell morphology and checked for bacteriocin production by Agar well diffusion assay (Neha and Nivedita, 2009). The isolate was grown in 50 ml of MRS broth (pH 6) inoculated with 1% overnight inoculum and incubated at 30°C for 48hours. After incubation, the cells were separated by centrifuging for 10 min at 4°C at an rpm of 7,000. The cell free supernatant obtained was neutralised to pH 6.0 using 1N NaOH and was used as crude bacteriocin (Von *et al*., 2009). Nutrient agar plates preinoculated with test organisms, *Pseudomonas aeruginosa* (Gram negative), *Staphylococcus aureus* (Gram positive), *Klebsiella pneumoniae* (Gram negative) and *Proteus mirabilis* (Gram negative) were used onto which wells were cut and about 300 µl of the crude bacteriocin was added and incubated for 24hours at 30°C. The zones of inhibition were observed and the zone diameter were used to calculate the activity of bacteriocin which was measured in terms of Arbitrary Units per mille litre (AU/ml). One arbitrary unit is defined as the reciprocal of the highest serial two fold dilution showing a clear zone of growth inhibition of the indicator strain (Ivanova *et al*., 2000; Maria *et al.*, 2006; Vera *et al.*, 2007). The isolate was identified by standard biochemical tests and 16s rDNA technique (Yamamoto *et al*., 2003; Narayanapillai *et al*., 2012).

Table1. Experimental Range and Levels of Independent Variables

Variable with designate	Actual factor level at coded factor level					
	Code	-2	-1	0	1	2
Starch (g/100ml)	X1	1	1.5	2	2.5	3
Casein (g/100ml)	X2	1	1.5	2	2.5	3
FeSO4 (w/v)	X3	0.15	0.2	0.25	0.3	0.35
Tween 20 (ml/100ml)	X4	0.16	0.18	0.2	0.22	0.24

Experimental design

The optimum conditions of different parameters by single factor method were considered for optimization by RSM (Maria *et al*., 2006; Lim *et al.*, 2007; Meng *et al.*, 2012). Bacteriocin production by *Lactobacillus* sp using Starch, Casein, $FeSO_4$ and Tween 20 were studied and sequential optimization strategy based on statistical design was employed to enhance the production of bacteriocin. A fractional factorial design (2^4) was applied to elucidate the process parameters that significantly affect bacteriocin production (Table 1). The central composite design (CCD) was used to estimate the quadratic response surface from the factor levels for maximum production (Table 2).

Statistical analysis

The statistical software package MATLAB software version 7.5.0.342 (R 2007b) from the Math works Inc. was used to analyse the experimental data, ANOVA (analysis of variance) data and plotting surface. All the variables were taken at a central coded value of zero.

Protein estimation

The concentration of proteins in crude bacteriocin sample before and after optimization was estimated by Lowry's method. The results were recorded (Lowry *et al*., 1951).

Results and Discussions

Sample collection and identification

The morphology of the isolated culture based on Gram stain was found to be Gram positive bacilli. Agar well diffusion assay revealed that the bacteriocin produced was inhibitory against *Pseudomonas aeruginosa* (20.1mm), *Proteus mirabilis* (14.7mm) and *Staphylococcus aureus* (21.0mm) but not against *Klebsiella pneumoniae*. The culture was identified to genus level based on biochemical characterization and carbohydrate fermentation profile and was negative to arginine test, catalase test, gelatine liquefaction, indole production, Vogous proskeur test, nitrate reduction and cellulose degradation and positive to Methyl red test, citrate test and starch hydrolysis. It was also able to ferment carbohydrates such as sucrose, fructose, lactose, maltose, xylose, and dextrose with no gas production which matches with the typical biochemical and carbohydrate fermentation results of *Lactobacillus* sp.

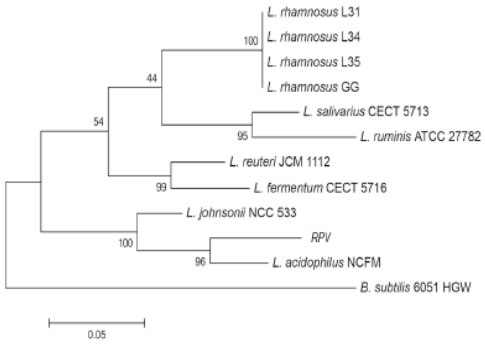

Figure 1. Phylogenetic tree of *Lactobacillus* isolate. The position of the isolate is shown as RPV

The molecular characterization of the isolate was done to identify up to species level using 16s rDNA sequencing and was analysed with BLAST which inferred 100% similarity with the existing strain of *L.delbdueckii* subsp *bulgaricus* ATCC 11842 (Figure 1).

Validation of optimized parameters by response surface methodology (RSM)

For optimum growth and bacteriocin production, the experimental and the predicted values obtained from Central Composite Design (CCD) in each run were as shown in Table 2. The equation showing relationship between the four variables Starch, Casein, Tween 20 and $FeSO_4$ for optimum growth and bacteriocin production is at OD 660 nm=c1+c2X Starch+c3XCasein+c4X$FeSO_4$+c5XTween 20+c6X StarchXCasein+c7XStarchX$FeSO_4$+c8XStarchXTween 20+c9XCaseinX$FeSO_4$+c10XCaseinXTween20 +c11X$FeSO_4$XTween20+c12XStarch^2+c13XCasein^2 +c14X$FeSO_4^2$+c15XTween 20^2

The experimental values were almost equal to the predicted values obtained from the central composite design (CCD) for validation of the four optimized parameters (Table 2).

The effects of interaction between the variables on growth and bacteriocin production were studied by plotting three-dimensional (3D) surface curves against two independent variables keeping other variables at their central (0) level. The calculated responses are as shown in Figure 2a, b, c, d, e and f.

Figure 2a shows the dependency of O.D at 660nm

Table 2. Full factorial central composite design (CCD) of four variables in coded and natural units along with the observed response

Run number	Starch (g/100ml)	Casein (g/100ml)	$FeSO_4$ % (w/v)	Tween 20 (g/100ml)	O.D at 660nm Expected value	O.D at 660nm Predicted value
1	0.15	0.15	0.2	0.18	0.2300	0.1992
2	0.25	0.15	0.2	0.18	0.1800	0.2017
3	0.15	0.25	0.2	0.18	0.1400	0.1842
4	0.25	0.25	0.2	0.18	0.1900	0.1692
5	0.15	0.15	0.3	0.18	0.1800	0.2085
6	0.25	0.15	0.3	0.18	0.2700	0.2735
7	0.15	0.25	0.3	0.18	0.2300	0.2110
8	0.25	0.25	0.3	0.18	0.2400	0.2585
9	0.15	0.15	0.2	0.22	0.1600	0.1503
10	0.25	0.15	0.2	0.22	0.2300	0.2365
11	0.15	0.25	0.2	0.22	0.1500	0.1240
12	0.25	0.25	0.2	0.22	0.2300	0.1928
13	0.15	0.15	0.3	0.22	0.1400	0.1422
14	0.25	0.15	0.3	0.22	0.3400	0.2909
15	0.15	0.25	0.3	0.22	0.1700	0.1334
16	0.25	0.25	0.3	0.22	0.2700	0.2647
17	0.1	0.2	0.25	0.2	0.1100	0.1199
18	0.3	0.2	0.25	0.2	0.3200	0.3374
19	0.2	0.1	0.25	0.2	0.1800	0.1799
20	0.2	0.3	0.25	0.2	0.1000	0.1274
21	0.2	0.2	0.15	0.2	0.1400	0.1574
22	0.2	0.2	0.3	0.2	0.1600	0.1998
23	0.2	0.2	0.25	0.16	0.3000	0.2743
24	0.2	0.2	0.25	0.24	0.2000	0.2315
25	0.2	0.2	0.25	0.2	0.1700	0.1753
26	0.2	0.2	0.25	0.2	0.1500	0.1753
27	0.2	0.2	0.25	0.2	0.1700	0.1753
28	0.2	0.2	0.25	0.2	0.1900	0.1753
29	0.2	0.2	0.25	0.2	0.2000	0.1753
30	0.2	0.2	0.25	0.2	0.1800	0.1753

on casein and starch. The variation of O.D was not so predominant with change in Casein though it was found to be higher with higher Starch. The highest value of the O.D was about 0.28 with starch value of 3 and casein value of 1. Figure 2b shows the dependency of O.D at 660 nm on $FeSO_4$ and Starch. The variation of O.D was not so predominant with lower values of either $FeSO_4$ or Starch. The value increased rapidly with simultaneous increase in the value of $FeSO_4$ or Starch. The highest value of the O.D was about 0.344 with starch value of 3 and $FeSO_4$ value of 0.30. Figure 2c shows the dependency of O.D at 660 nm on Tween 20 and Starch, the value of O.D decreased with decrease in the value of Tween 20 at lower starch value. However the trend was reverse with higher starch values. The peaks were found either with both the variables at their minima or the maxima. The highest value of the O.D was about 0.416 with starch value of 3 and Tween 20 value of 0.24. Figure 2d shows the dependency of O.D at 660 nm on $FeSO_4$ and Casein, the variation in the O.D value was almost constant with increase

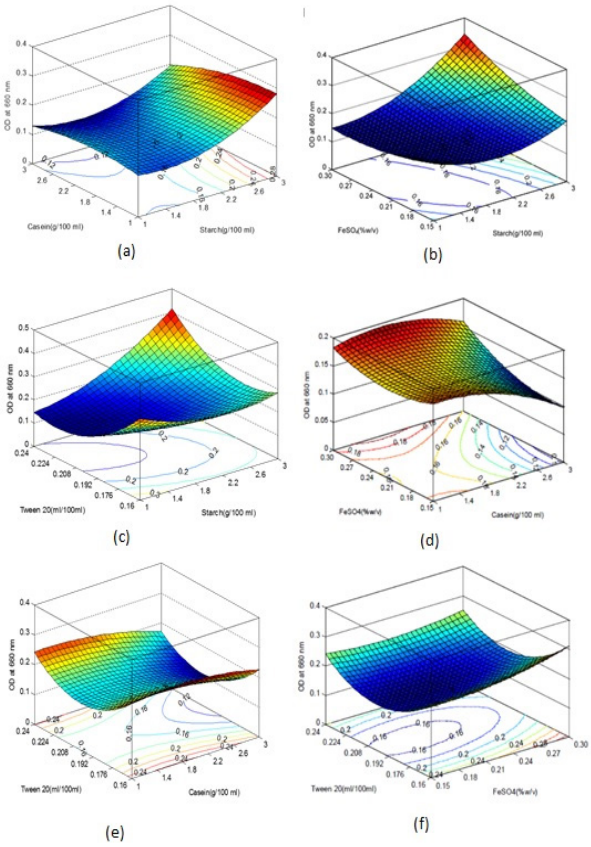

Figure 2. Dependency of O.D at 660nm by the four variables Casein, Starch, Tween 20 and $FeSO_4$
(a) Dependency of O.D at 660 nm on Casein and Starch.
(b) Dependency of O.D at 660 nm on FeSO4 and Starch.
(c) Dependency of O.D at 660 nm on Tween 20 and Starch.
(d) Dependency of O.D at 660 nm on FeSO4 and Casein.
(e) Dependency of O.D at 660 nm onTween 20 and Casein.
(f) Dependency of O.D at 660 nm on Tween 20 and $FeSO_4$.

in $FeSO_4$ values at lower Casein values whereas it decreased with increase in the value of Casein. The highest value of the O.D was about 0.194 with Casein value of 1.69 and $FeSO_4$ value of 0.3. Figure 2e shows the dependency of OD at 660 nm on Tween 20 and Casein, the variation in the value of O.D was almost constant with increase in Casein values either at lower or higher Tween 20 values. There was a parabolic dip seen in the mid-range of the Tween 20 values. The highest value of the O.D was about 0.264 with Casein value of 1.69 and Tween 20 value of 0.16. Figure 2f shows the dependency of O.D at 660 nm on Tween 20 and $FeSO_4$, the variation in the value of O.D was almost constant and on the lower side with increase in Tween 20 values either at lower or higher $FeSO_4$ values. There was a parabolic dip seen in the mid-range of the Tween 20 values. The highest value of the O.D was about 0.312 with $FeSO_4$ value of 0.3 and Tween 20 value of 0.16.

Through central composite design using RSM, the optimized O.D at 660 nm statistically comes out to be 0.50613 from the equation (1) with variables set as Starch (3g/100ml), Casein (3g/100ml), Tween 20(0.24 ml/100ml) and $FeSO_4$ 0.3% w/v. Therefore, to confirm the statistical results of the obtained O.D a single flask experiment was carried out where in MRS media was supplemented with the optimized concentration of the above four variables. The flask was incubated at 30°C for a period of 24 hours and checked for OD. The experimental result of the optimized O.D was found to be 0.4821 which is nearer to the statistical data obtained i.e., 0.50613 OD at 660nm. Hence, the optimized parameters were statistically validated. Through analysis of variance (ANOVA) the values of p was calculated to be 0.00177 which is less than 5 and value of R^2 was 0.8251 which is less than 1. Hence we can conclude that CCD was significant.

The growth of *L.salivarius* i24 was optimized by Lim *et al.* (2007) using RSM. It was estimated that 3.32% w/v of glucose, 4.31% w/v yeast extract, and initial pH of 6.10 were optimum for efficient cultivation of *L.salivarius* i24 using polynomial regression model. Magdalena *et al.* (2010) optimized the growth conditions of *Lactobacillus rhamnosus* PEN using Response surface methodology. The optimum conditions were deduced by Plackett-Burman design, and the conditions were found to be: glucose 13.4g/l, sodium pyruvate 3.4g/l, meat extract 7.2g/l, ammonium citrate 2.0g/l, sodium acetate 5.0g/l, potassium phosphate 2.0g/l. Meng *et al.* (2012) applied response surface methodology for the optimum production of lacticin LLC518 produced by *L.lactis* subsp *lactis* LLC518. The Plackett-Burman design showed that tryptone, KH_2PO_4 and $MgSO_4$ had prominent effects on the biosynthesis of lacticin LLC518. The central composite design revealed that the optimum conditions for bacteriocin production were sucrose 20g/l, yeast extract 5g/l, tryptone 8.18g/l, beef extract 10g/l, $MgSO_4$ 0.82g/l, K_2HPO_4 2.8g/l and Tween 80 5ml/l.

Protein estimation

The protein concentration before optimization was 178.8 µg/ml and its activity was 600 AU/ml. After optimization it was observed that there was an increase in concentration as well as its activity to 310 µg/ml and 800 AU/ml of crude bacteriocin. Through optimization we can observe that there was a two fold increase in concentration of protein and one fold increase in its activity.

Conclusion

Lactobacillus delbrueckii subsp *bulgaricus* and other *Lactobacillus* species are known to produce

bacteriocins but limited work has been carried out on optimization of bacteriocin production. An attempt has been made to statistically optimize the combined effect of different growth parameters and results obtained were satisfactory. Bacteriocins have preservative properties and can be used as a bio preservative which can replace chemical preservatives which have side effect on health of the consumer and well as the food to be preserved. From the results obtained, *Lactobacillus delbrueckii* subsp *bulgaricus* is a good producer of bacteriocin and can be used as a potent bio preservative in future.

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