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Optimization of Fermentation Process Parameter for Nisin Production from *Lactococcus lactis* (MTCC-440) Using a Statistical Experimental Design Technique

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Abstract: MRS broth, nisin and milk concentrations were optimized using a full factorial design to identify the significant effect of medium components towards nisin production using *Lactococcus lactis* (MTCC-440). Statistical optimization is preferable because it is helpful in evaluating the interactions among the possible influencing parameters with limited number of experiments whereas the conventional method of medium optimization involves changing one independent variable at a time while fixing all the others at a certain level which does not include the determination of interactive effects among the variables. Full factorial design was carried out in a different set sequentially till the optimum was reached. Nisin quantification was done by agar diffusion assay using *Streptococcus agalactiae* (NCIM-2401). Statistical analysis of results have shown that maximum production of nisin was found to be at MRS broth concentration 5.5 %, milk 0.7 % and nisin 0.25 μg/ml. By implementing the full factorial design, nisin production was increased from 206.1 μg/ml to 277.59 μg/ml, which was approximately 1.34-fold.

Key words: *Lactococcus lactis* · *Streptococcus agalactiae* · Nisin · Optimization · Factorial Design

INTRODUCTION

Nisin is a 34-amino acid, antimicrobial peptide produced by *Lactococcus lactis* during its fermentation. Nisin is unique as it is the only antibiotic presently used as a food preservative and is a protein. The application of nisin as a food preservative has been studied. It was used in foods for the first time in 1951 to prevent "blowing" of Swiss-type cheese caused by *Clostridium butyricum* [1]. *Bacillus* and *Clostridium* spores are sensitive to nisin [2] and the sensitivity appears to be more pronounced if the spores are heated [3-5]. Nisin is used extensively in European and other countries as a preservative in dairy products, vegetables, soups and sauces [6-8]. The presence of food components such as lipids and protein influence nisin activity [9]. Nisin is an effective antibotulinal agent at 12.5-250 μg/g. The higher nisin levels allow for the safe formulation of cheese spreads with higher moisture content and lower salt concentration. Nisin levels of 6 to 12.5 μg/g control non-*C. botulinum* spoilage in processed cheese [10]. Nisin can decrease *L. monocytogenes* cells to undetectable levels in 3 and 10 % fat ice cream stored at-18 °C [11]. Heating canned lobster in brine at 60 °C or 65 °C for 5 or 2 min, respectively, in combination with 25 μg/g nisin reduced *L. monocytogenes* by 3 to 5 logs [12]. Nisin has shown some potential for use in selected meat products. For example, it is found that 2 % lactate combined with 12.5 μg/g nisin was superior to nisin alone at controlling growth of total aerobes, *S. aureus* and *S. Kentucky* in fresh pork sausage stored at 4 °C for 10 days [13]. Nisin has also been suggested as an adjunct to nitrite in cured meats for the purpose of preventing the growth of *clostridia* [14, 15]. Nisin was used at 100 IU/ml to inhibit *lactobacilli* responsible for spoilage of kimchi, traditional Korean fermented vegetables [16]. Nisin has been evaluated for use as a component of antimicrobial packaging [17, 18]. In combination with chelating agents, nisin may reduce *E. coli* O157:H7 [19]. Nisin is an accepted food additive in various countries [20].

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The traditional method of optimization of parameters involves optimizing one parameter at a time. This is not only a time-consuming process, but often misses the interactive effects between components [21]. It also involves several experiments to determine the optimal levels, which may not often give the right picture. These draw-backs may be avoided by using response surface methodologies of experimental designs such as Plackett-Burman [22, 23] and Box-Behnken [24, 25] designs.

In this study, full factorial design was used for screening process to identify the critical, crucial and significant nutrient and also the interaction between two or more nutrients in relatively few experiments as compared to one-factor at a time techniques. Best conditions from previous set were selected for the zero level for the next set and experiments were repeated till optimum was reached. The experimental design was carried out using Design Expert 7.0.2 software [26].

MATERIALS AND METHODS

Bacterial Strains: *Lactococcus lactis* (MTCC-440) obtained from MTCC, Chandigarh, India is used for nisin production studies. Nisin sensitive organism (*Streptococcus agalactiae*-NCIM-2401) was obtained from NCIM, Pune, India. The cultures were revived in MRS broth and preserved in 20 % glycerol stocks at-80 °C for regular use.

Chemicals: All the chemicals, except skimmed milk and Tween-80 were obtained from Fluka, Switzerland. Skimmed milk (Amul Lite) was obtained from Amul, India. Tween-80 was procured from Sigma, USA.

Quantification of Nisin: An agar diffusion assay was used to quantify nisin using a sensitive indicator strain *Streptococcus agalactiae* (NCIM-2401) (27). *Streptococcus agalactiae* was grown in sterile media containing MRS broth (2 %, w/v) along with 0.1% (v/v) and Tween-80 at 37 °C and 180 rpm till the optical density reached to 0.8±0.2. Agar diffusion assay was carried out in Petri plates containing MRS broth (2 %, w/v), 0.1 % (v/v) Tween-80 and 1 % agar powder. Media for the assay was prepared and autoclaved at 121 °C for 15 minutes and then culture of *S. agalactae* was added to the autoclaved media when its temperature reached 45 ± 5 °C in such a way that the final optical density became 0.001 and then poured on to the plate. The media along with the culture was allowed to solidify and then 5 mm wells were created using a gel puncture. A stock solution of standard nisin was prepared and from this a range of solutions containing different concentration of nisin was prepared and loaded on to the wells. Plates were incubated at 37 °C for 20 h and then diameters were measured. Standard curve was prepared by taking the concentrations of nisin used and inhibition zone achieved [27].

Optimization of Fermentation Process

Inoculum Preparation: MRS broth (2 %, w/v) with 0.1 % (v/v) Tween-80 was autoclaved at 121 °C for 15 minutes. Frozen culture of *L. lactis* (500 µl) was inoculated on to the sterilized medium (cooled) and incubated at 37 °C and 180 rpm in an incubator shaker till the optical density reached 1.0 ± 0.2 [27]. In this process nisin yield was 206.1 µg/ml.

Fermentation Process: Experiments were carried out in 250-ml Erlenmeyer flask containing 50 ml of nutrient medium. Flasks were autoclaved at 121 °C for 15 minutes. After bringing it down to room temperature, inoculum was added to the flasks in such a way that the initial optical density became 0.01. The flasks were incubated in an incubator shaker for 20 h and then the culture was harvested by centrifugation at 10,800 g for 30 minutes [27]. Nisin quantification was done by agar diffusion assay.

Optimization of Media Concentration by Factorial Design: In single factor optimization, it was found that 6 % MRS broth and 0.5 % milk supplemented with 0.15 µg/ml of nisin had given better result in comparison with other combinations at 100 rpm and 30 °C which yielded 206.1 µg/ml of nisin [27]. These concentrations of MRS broth, milk and nisin were selected as zero level concentration for the factorial design. All variables (MRS broth, milk and nisin) were investigated at two levels designated as -1 (low level) and + 1 (high level). All the experimental runs were divided in to 2 blocks representing experiments performed at a time. The Prob>F value is used as a tool to check the significance of each variable. A Prob>F lower than 0.0500 indicated that the effect of parameter in question could be considered as significant at 95 % confidence level [28].

Full Factorial Experimental Design (Set-I): Full factorial design was carried out at two levels for three factors (MRS broth, milk and nisin) in replicates having six center points (three in each block) to have a significant model. Experimental variables at different levels used for the production of nisin in set-I are given in Table 1.

Table 1: Experimental variables at different levels used for the production of nisin (set-I)

Variables Block	Factors	Level -1	Level 0	Level +1
MRS (%)	1	5.5	6	6.5
Milk (%)	2	0.4	0.5	0.6
Nisin (μg/ml)	3	10	15	20

Full factorial experimental design (set-II)

Table 2: Experimental variables at different levels used for the production of nisin (set-II)

Variables Block	Factors	Level -1	Level 0	Level +1
MRS (%)	1	5.0	5.5	6.0
Milk (%)	2	0.5	0.6	0.7
Nisin (μg/ml)	3	15	20	25

Full factorial experimental design (set-III)

Table 3: Experimental variables at different levels used for the production of nisin (set-III)

Variables Block	Factors	Level -1	Level 0	Level +1
MRS (%)	1	4.5	5.0	5.5
Milk (%)	2	0.6	0.7	0.8
Nisin (μg/ml)	3	20	25	30

The total number of runs and the media ingredient concentrations are shown in Table 4. All the experiments were carried out in replicates to fulfill the required condition for lack of fit test i.e minimum of 3 degree of freedom for lack of fit and 4 degree of freedom for pure error (Stat Ease, USA) software [26].

Full Factorial Experimental Design (Set-II): From the set-I experiments, it was found that MRS broth concentration needed to be reduced whereas the concentration of milk and nisin needed to be increased to enhance nisin production. Experimental variables at different levels used for the production of nisin in set-II are given in Table 2.

Keeping all other fermentation operating parameters constant as per set-I, set-II experiments were carried out. The details of each run are shown in Table 7.

Full Factorial Experimental Design (Set-III): From the set-II experiments it was observed that MRS broth and milk have negative effect on nisin production where nisin has positive effect. Experimental variables at different levels used for the production of nisin in set-III are given in Table 3. Keeping all other fermentation operating parameters constant as of set-I and set-II except the concentration of media components, set-III experiments were carried out. The details of each run are shown in Table 1.

RESULTS AND DISCUSSION

Analysis of Effect of Different Factors of Set-I (Single and Combined) on Nisin Production: Responses obtained from the different run of set-I are given in Table 4. The Prob>F value was used as a tool to check the significance of each variable. A Prob>F lower than 0.0500 indicates that the effect of the parameter in question can be considered as significant at 95 % confidence level. In set-I experiment, very low probability value (Pmodel > F = 0.0001) demonstrated a very high significance for the regression model. F-value of this model is 65.68 and is significant as its P value Prob is <0.0001. The "Curvature F-value" of 208.22 implies that there is significant curvature (as measured by difference between the average of the center and factorial points) in the design space. There is only a 0.01 % chance that a "Curvature F-value" this large could occur due to noise. The "Lack of Fit F-value" of 0.60 implies the Lack of Fit is not significant relative to the pure error. There is a 75.16 % chance that this large "Lack of Fit F-value" could occur due to noise. Non-significant lack of fit is good. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this design (set-I) ratio of 28.796 indicated an adequate signal that could be used to navigate the design space [26].

Table 4: Design matrixes along with responses (nisin production) of set-I experiments

Run	Block	MRS (%)	Milk (%)	Nisin (μg/ml)	Nisin production (μg/ml)
1	1	6.0	0.5	0.15	197.42
2	1	6.0	0.5	0.15	209.86
3	1	5.5	0.4	0.10	144.49
4	1	6.0	0.5	0.15	207.81
5	1	6.5	0.6	0.10	166.36
6	1	5.5	0.4	0.20	159.37
7	1	5.5	0.6	0.20	241.11
8	1	6.5	0.4	0.10	162.83
9	1	5.5	0.6	0.10	175.96
10	1	6.5	0.4	0.20	168.86
11	1	6.5	0.6	0.20	177.31
12	2	6.0	0.5	0.15	204.26
13	2	6.5	0.6	0.20	166.71
14	2	6.5	0.6	0.10	154.43
15	2	6.0	0.5	0.15	203.16
16	2	6.5	0.4	0.20	164.21
17	2	5.5	0.6	0.10	171.25
18	2	6.0	0.5	0.15	211.11
19	2	5.5	0.4	0.10	149.31
20	2	6.5	0.4	0.10	161.53
21	2	5.5	0.4	0.20	161.27
22	2	5.5	0.6	0.20	233.69

Table 5: Analysis of variance for selected factorial model (set-I)

Source	Sum of squares	Mean square	F value	P value Prob > F
Block	42.15	42.15	-	-
Model	10611.49	1515.93	65.68	<0.0001
A	815.25	815.25	35.32	<0.0001
B	2887.72	2887.72	125.12	<0.0001
C	2170.86	2170.86	94.06	<0.0001
AB	2504.75	2504.75	108.53	<0.0001
AC	937.74	937.74	40.63	<0.0001
BC	830.45	830.45	35.98	<0.0001
ABC	464.73	464.73	20.14	0.0007
Curvature	4805.49	4805.49	208.22	<0.0001
Residual	276.95	23.08	-	-
Lack of fit	150.87	18.86	0.60	0.7516
Pure Error	126.08	31.52	-	-
Cor Total	15736.07	-	-	-

A-MRS B-MILK C-NISIN

R-Squared: 0.9746; Adj R-Squared: 0.9597; Pred R-Squared: 0.9211; Adeq Precision: 28.796; C.V. %:2.65

The goodness of fit of the model (for set-I experiments) was checked by the determination coefficient (R^2). In this model, R^2 was 0.9746 for nisin production which indicates 97.46 % of experimental data of the nisin production was compatible with the data predicted by the model (Table 4) whereas only 2.54 % of the total variations were not explained by this model. The R^2 value is always between 0 and 1 [29]. For a good statistical model, R^2 value should be close to 1.0. The adjusted R^2 value corrects the R^2 value for the sample size and for the number of terms in the model. The value of the adjusted determination coefficient (Adj R^2 = 0.9597) is also high which indicates significance of the model. Here in this case, the adjusted R^2 value is 0.9597, which was lesser than the R^2 value of 0.9746. The Pred R^2 of 0.9211 is in reasonable agreement with the Adj R^2 of 0.9597. The coefficient of variation (CV), indicative of the degree of precision with which the treatments are compared, was 2.65 %, showing greater reliability.

Pareto chart (Fig. 1) shows the effect of different media components (different factors) on nisin production. In this Pareto chart, it is found that t-value of effect of all the factors and their mutual interaction effect was higher than Bonferroni limit, which indicates that their concentrations are critical for nisin production. It further implicates that optimum concentrations of media were not achieved.

The predicted vs actual plot (Fig. 2) showed a satisfactory correlation between the experimental and predicted values of nisin production, where the points (experimental value) cluster around the diagonal line (predictd) indicated a good fit of the model.

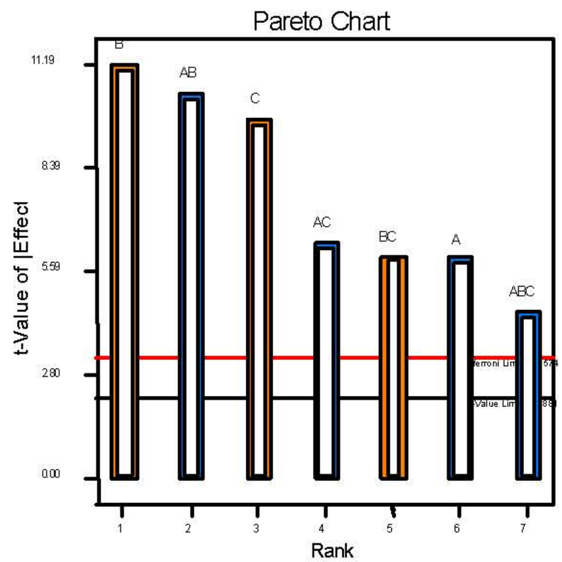

Fig. 1: Pareto chart showing the effect of single variable and interaction of effects on nisin production of set-I

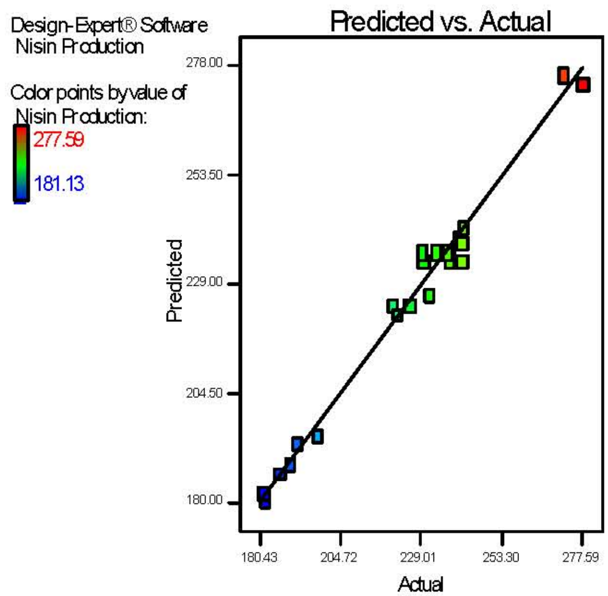

Fig. 2: Predicted versus actual response of experimental run of set-I

Total Contribution (Set-I): Effect of single factor and mutual interaction of different factors on nisin production were evaluated.

Table 6 shows that effect of MRS was negative, where the effects of milk and nisin were positive. Similarly, interaction effect of MRS with milk and with nisin was negative but effect of milk and nisin was positive. Effect of combination of all three factors on nisin production was also negative.

Table 6: Total contribution from different factors of set-I experiments

Serial Number	Factors	Effect
1	A	-14.27
2	B	26.86
3	C	23.29
4	AB	-25.02
5	AC	-15.31
6	BC	14.40
7	ABC	-10.77

A-MRS B-MILK C-NISIN

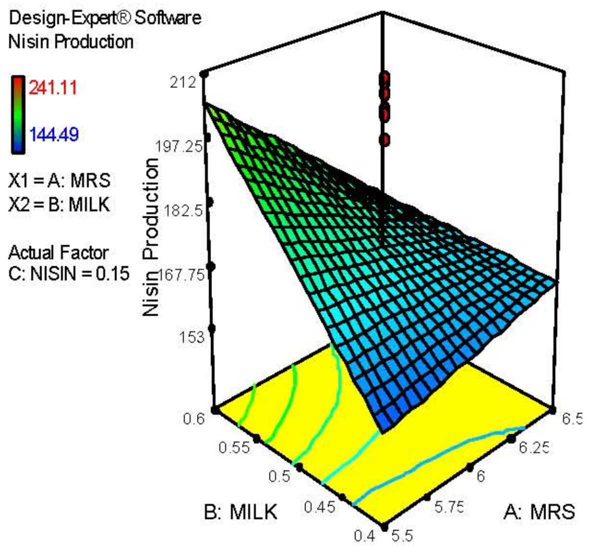

Fig. 3: Contour plot showing the effect of milk and MRS broth on nisin production at zero level concentration of nisin

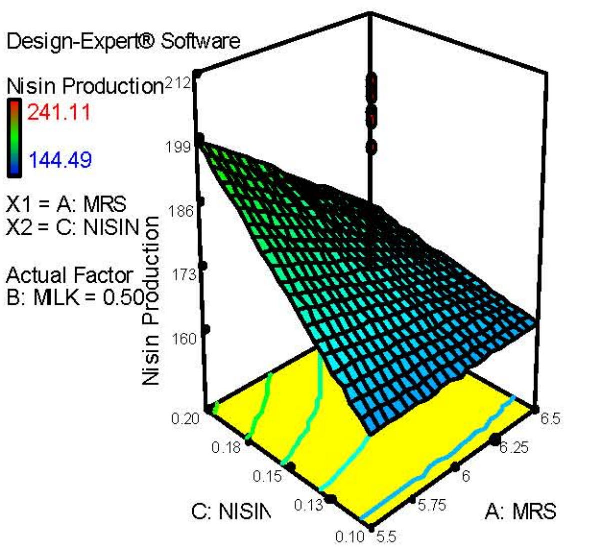

Fig. 4: Contour plot showing the interaction effect of nisin and MRS broth on nisin production at zero level concentration of milk

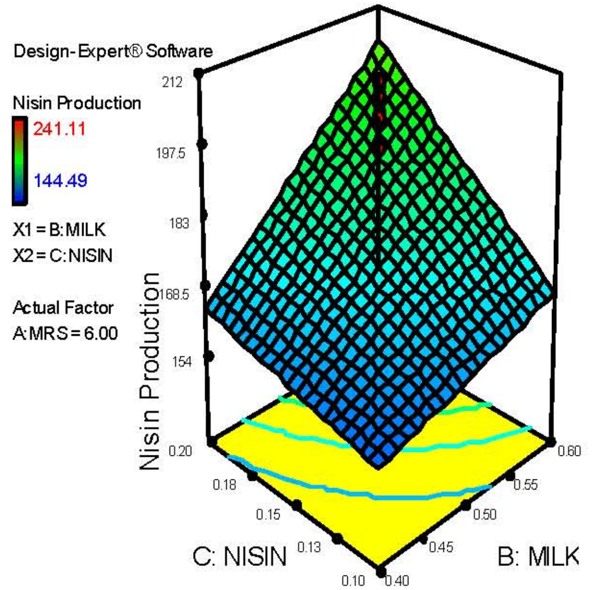

Fig. 5: Contour plot showing the effect of milk and nisin on nisin production at zero level concentration of MRS broth

Interaction Effects (set-I): Contour plot (Fig. 3) of Set-I experiments shows effect of MRS broth, milk and their mutual interaction in fermentation media on nisin production where nisin concentration is at zero level (0.15 μg/ml). Lower concentration of MRS broth and higher concentration of milk in fermentation media increased nisin production. Fig. 4 (contour plot) shows effect of MRS broth, nisin and their mutual interaction effect on *Lactococcus lactis* for nisin production at zero level concentration of milk (0.5 %). Lower concentration of MRS broth and higher concentration of nisin in fermentation media favored nisin production indicating that MRS broth concentration was needed to be reduced further and nisin concentration should be increased to reach the optimum concentration in the media. Effect of milk, nisin and their interaction effect on nisin production are shown in Fig. 5. This contour plot shows higher concentration of milk and higher concentration of nisin in the fermentation media favored nisin production which suggests that concentration of milk and nisin should be increased further to increase nisin production. From the results of Set-I factorial design, it is concluded that a new set of experiments needs to be designed where MRS broth concentration needs to be reduced and the concentration of milk and nisin needs to be increased in the fermentation media to reach the optimal concentration of media components. The best condition of set-I experiment was selected as the zero level for the set-II matrix design.

Table 7: Analysis of effect of different factors of set-II (single and combined) on nisin production

Run	Block	MRS (%)	Milk (%)	Nisin (μg/ml)	Nisin production (μg/ml)
1	1	5.5	0.60	0.20	237.46
2	1	6.0	0.50	0.15	219.99
3	1	5.0	0.70	0.25	277.59
4	1	5.0	0.50	0.25	239.98
5	1	6.0	0.70	0.15	221.39
6	1	5.5	0.60	0.20	241.15
7	1	6.0	0.70	0.25	191.06
8	1	6.0	0.50	0.25	233.29
9	1	5.0	0.70	0.15	181.76
10	1	5.0	0.50	0.15	186.23
11	1	5.5	0.60	0.20	229.65
12	2	5.0	0.50	0.15	189.41
13	2	5.5	0.60	0.20	229.31
14	2	6.0	0.50	0.15	231.13
15	2	6.0	0.50	0.25	241.11
16	2	5.5	0.60	0.20	236.61
17	2	5.5	0.60	0.20	237.23
18	2	5.0	0.50	0.25	241.33
19	2	6.0	0.70	0.15	225.29
20	2	6.0	0.70	0.25	197.37
21	2	5.0	0.70	0.25	271.91
22	2	5.0	0.70	0.15	181.13

Table 8: Analysis of variance for selected factorial model (set-II)

Source	Sum of squares	Mean square	F value	P value Prob > F
Block	22.56	22.56	-	-
Model	13891.80	1984.54	102.66	< 0.0001
A	4.74	4.74	0.25	0.6294
B	76.43	76.43	3.95	0.0701
C	4138.03	4138.03	214.06	< 0.0001
AB	1329.51	1329.51	68.77	< 0.0001
AC	6693.29	6693.29	346.24	< 0.0001
BC	0.022	0.022	0.001125	0.9738
ABC	1649.78	1649.78	85.34	< 0.0001
Curvature	931.67	931.67	48.19	< 0.0001
Residual	231.98	19.33	-	-
Lack of fit	124.22	15.53	0.58	0.7650
Pure Error	107.75	26.94	-	-
Cor Total	15078.01	-	-	-

A-MRS B-MILK C-NISIN

R-Squared: 0.9836; Adj R-Squared: 0.9740; Pred R-Squared: 0.9499; Adeq Precision: 32.160; C.V.%:1.96.

Analysis of Effect of Different Factors of Set-II (Single and Combined) on Nisin Production: Results of all the experiments of set II of full factorial design are given in Table 7. It was found that experiment having MRS broth concentration 5 % (w/v) along with nisin 0.7 % (v/v) and nisin 0.25 μg/ml yielded 277.59 μg/ml.

The Prob>F value was used as a tool to check the significance of each variable. In the second set, the Model F-value of 102.66 implies that the model was significant as its F vale is <0.0001, which is significant at 95% confidence limit. The "Curvature F-value" of 48.19 implies that there was significant curvature (as measured by difference between the average of the center and factorial points) in the design space. There was only a 0.01 % chance that a "Curvature F-value" this large could occur due to noise. The "Lack of Fit F-value" of 0.58 implies the Lack of Fit is not significant relative to the pure error. There is a 76.50 % chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit was good. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In set II experiment, ratio of 32.160 indicated an adequate signal. This model could be used to navigate the design space.

As stated earlier Fisher F-test with a very low probability value (Pmodel > F = 0.0001) demonstrates a very high significance for the regression model. R^2 was calculated to be 0.9836 for nisin production. This implies that 98.36 % of experimental data of the nisin production was compatible with the data predicted by the model (Table 8) and only 1.64 % of the total variations were not explained by the model. The R^2 value is always between 0 and 1 [29] and a value >0.75 indicates aptness of the model. For a good statistical model, R^2 value should be close to 1.0. The value of the adjusted determination coefficient (Adj R^2 = 0.9740) is also high to advocate for a high significance of the model. Here in this case, the adjusted R^2 value was 0.9740, which is lesser than the R^2 value of 0.9836. The Pred R^2 of 0.9499 was in reasonable agreement with the Adj R^2 of 0.9740. The value of CV was also low as 1.96 indicates that the deviations between experimental and predicted values are low.

Pareto chart (Fig. 6) shows the effect of different media components (different factors) on nisin production. In Pareto chart (Fig. 6), it was found that t-value of effect of nisin (factor C) and interaction effect of AC, AB and ABC were critical as their t-value of effect is higher than Bonferroni limit.

The preditced vs actual plot (Fig. 7) showed a satisfactory correlation between the experimental and predicted values of nisin production, where in the point (experimental value) cluster around the diagonal line (predictd) indicated a good fit of the model.

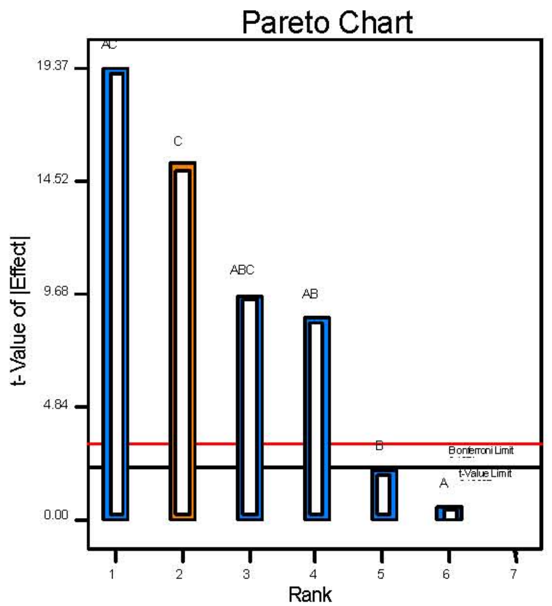

Fig. 6: Pareto chart showing the effect of single variable and interaction of effects on nisin production of set-II

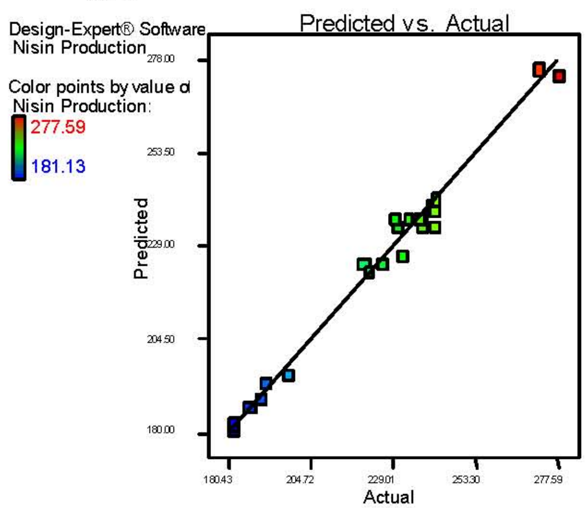

Fig. 7: Predicted versus actual response of experimental run of set-II

Total Contribution (Set-II): Effect of single factor or interaction of different factors on nisin production in set II is evaluated. Table 9 shows that effect of MRS broth and milk were negative but effect of nisin in media on nisin production was positive. Among the interaction of media components on nisin yield, it was found that all the interactions like effect of MRS broth and milk, milk and nisin, MRS and nisin and MRS, milk and nisin were negative.

Table 9: Effect of different paramters on contribution to nisin yield

Serial Number	Factors	Effects
1	A	-1.08875
2	B	-4.37125
3	C	32.1638
4	AB	-18.2313
5	AC	-40.9063
6	BC	-0.07375
7	ABC	-20.3087

A-MRS B-MILK C-NISIN

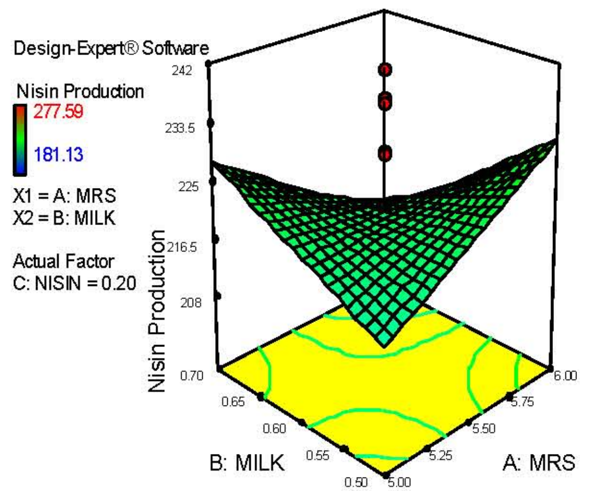

Fig. 8: Contour plot showing the effect of milk and MRS broth on nisin production at zero level concentration of nisin

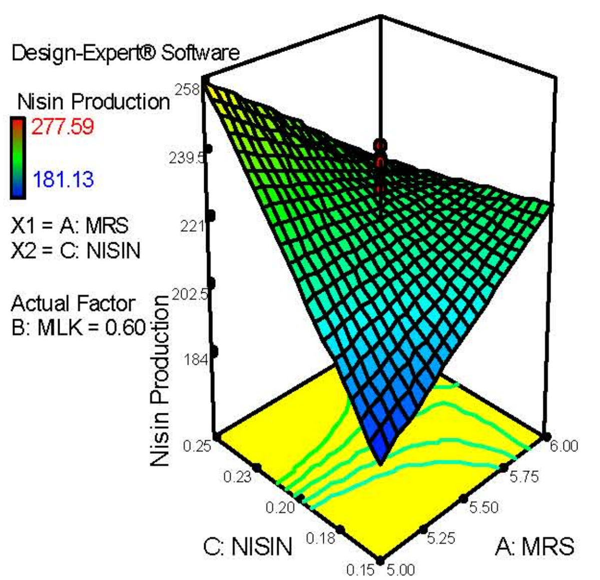

Fig. 9: Contour plot showing the effect of nisin and MRS broth on nisin production at zero level concentration of milk

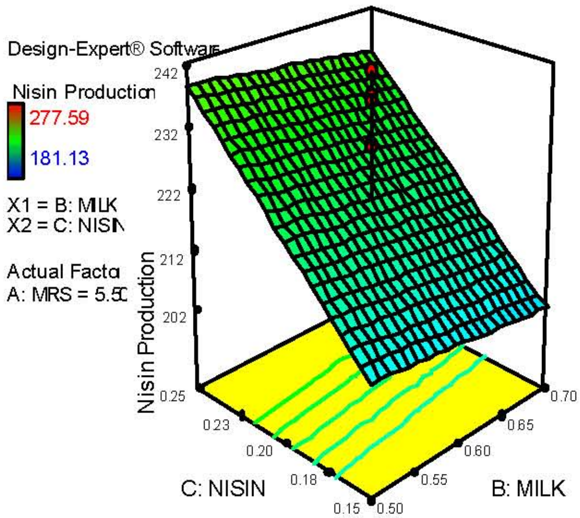

Fig. 10: Contour plot showing the effect of milk and nisin on nisin production at zero level concentration of MRS broth

Interaction Effects (set-II): Contour plot (Fig. 8) shows effect of MRS broth, milk and their mutual interaction effect on nisin production at zero level concentration of nisin (0.2 µg/mL) of set II experiment. This figure shows that higher concentration of milk and lower concentration of MRS broth had a positive effect on nisin production. On the other hand, it also shows that lower concentration of milk and higher concentration of MRS broth had positive response on nisin production. Interaction effect of nisin and MRS broth is also evaluated at zero level concentration of milk (0.6 %) in fermentation media (Fig. 9). This contour plot shows lower concentration of MRS broth and higher concentration of nisin had a positive response on nisin production. Interaction effect of nisin and milk at zero level concentration of MRS broth (5.5 %) is given in contour plot 10 which indicates that higher or lower concentrations of milk didn't not have much effect on nisin production whereas higher nisin had positive response on nisin production (Fig. 10).

From the set II factorial design experiments, it is found that MRS broth and nisin had overall negative effect on nisin production, whereas nisin contributed significantly to enhance nisin production. Hence, a third set experiment is carried out taking the best experimental condition from the set-II.

Analysis of Effect of Different Factors of Set-III (Single and Combined) on Nisin Production: Results of all the experiments of set III of full factorial design is given in Table 10. It was found that nisin yield between the experiments with media concentration having concentration at center points and other experiment is not significant.

Table 10: Experimental conditions and their response of set-III

Run	Block	MRS (%)	Milk (%)	Nisin (µg/ml)	Nisin Production (µg/ml)
1	1	5.5	0.80	0.30	284.35
2	1	4.5	0.80	0.20	247.19
3	1	5.0	0.70	0.25	274.69
4	1	4.5	0.80	0.30	260.69
5	1	5.5	0.60	0.30	256.09
6	1	4.5	0.60	0.20	262.39
7	1	5.0	0.70	0.25	276.73
8	1	5.5	0.60	0.20	261.83
9	1	5.5	0.80	0.20	272.39
10	1	5.0	0.70	0.25	276.89
11	1	4.5	0.60	0.30	261.11
12	2	4.5	0.80	0.20	254.19
13	2	5.0	0.70	0.25	273.73
14	2	4.5	0.80	0.30	263.64
15	2	4.5	0.60	0.30	256.32
16	2	5.5	0.80	0.20	261.17
17	2	5.0	0.70	0.25	275.38
18	2	5.5	0.80	0.30	249.73
19	2	4.5	0.60	0.20	272.89
20	2	5.0	0.70	0.25	273.23
21	2	5.5	0.60	0.30	252.63
22	2	5.5	0.60	0.20	256.26

Table 11: Anova For Selected Factorial Model (Set-III)

Source	Sum of squares	Mean square	F value	P value Prob > F
Block	92.78	92.78	-	-
Model	288.38	41.20	0.28	0.9496
A	20.73	20.73	0.14	0.7137
B	10.74	10.74	0.073	0.7914
C	45.19	45.19	0.31	0.5892
AB	0.15	0.15	0.001	0.9750
AC	112.10	112.10	0.76	0.3993
BC	52.38	52.38	0.36	0.5614
ABC	47.09	47.09	0.32	0.5816
Curvature	89.69	89.69	0.61	0.4496
Residual	1761.53	146.79	-	-
Lack of Fit	968.74	121.09	0.61	0.7439
Pure Error	5.54	1.39	-	-
Cor Total	2232.38	-	-	-

A-MRS B-MILK C-NISIN

R-Squared: 0.1407; Adj R-Squared:-0.3606; Pred R-Squared:-1.5744; Adeq Precision: 2.425; C.V. %:4.58

In set III experiments "Model F-value" of 0.28 implies the model was not significant relative to the noise. There was a 94.96 % chance that a "Model F-value" this large could occur due to noise. The "Curvature F-value" of 0.61 implies the curvature in the design space is not significant relative to the noise. There was a 44.96 % chance that a "Curvature F-value" this large could occur due to noise.

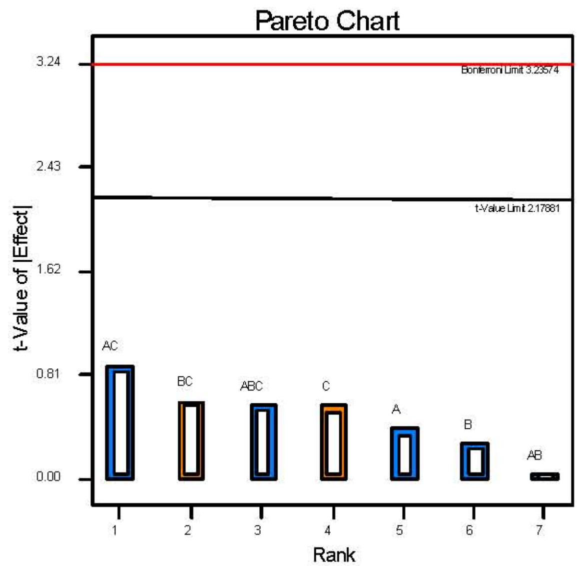

Fig. 11: Pareto chart showing the effect of single variable and interaction of effects on nisin production (Set-III)

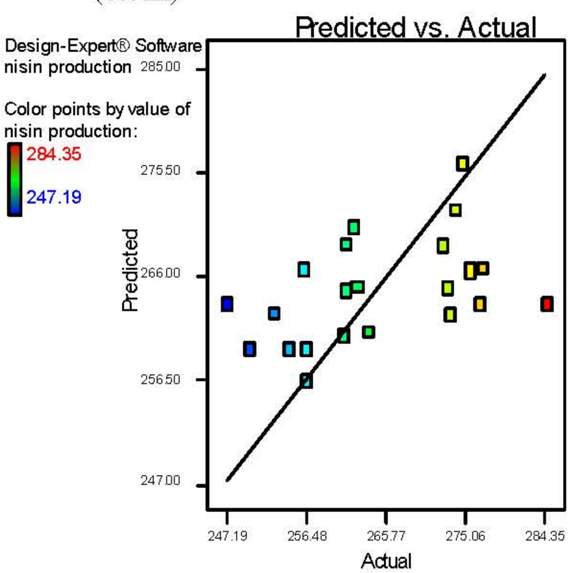

Fig. 12: Predicted versus actual response of experimental run of set-III

The "Lack of Fit F-value" of 0.61 implies the Lack of Fit is not significant relative to the pure error. There was a 74.39 % chance that a "Lack of Fit F-value" this large could occur due to noise. A ratio of 2.43 indicates an inadequate signal and we should not use this model to navigate the design space.

Fisher F-test with a very low probability value (Pmodel > F = 0.0001) demonstrates a very high significance for the regression model but in set-III Pmodel value was 0.9496 which is not significant at 95 % confidence level. R^2 was calculated to be 0.1407 and is very low. This implies that 14.07 % of experimental data of the nisin production was compatible with the data predicted by the model (Table 11) and 85.93 % of the total variations were not explained by the model. The value of (Adj R^2 =-0.3606) is too low to advocate for a high significance of the model. Here in this case, the adjusted R^2 value was-0.3606 (negative), which is lesser than the R^2 value of 0.1407. The Pred R^2 was also negative (-1.5744).

TheCo-efficient of variation (CV) was high (4.58) indicating the deviation experimental and predicted values were higher in comparison to set I and set-II.

Pareto chart (Fig. 11) shows the effect of different media components (different factors) on nisin production. It was found that t-value effect of all factors and their mutual interaction effect is less than the Bonferroni limit.

Predicted value was calculated by the software and it was plotted against the actual response obtained from set-III experiment (Fig. 12). The actual values are scattered very much which indicated the actual value did not correlate with predicted values.

The predicted vs actual plot (Fig. 12) didn't not show a satisfactory correlation between the experimental and predicted values of nisin production. The point (experimental value) scattered very much, thereby, indicating the absence of good fit of the model.

Table 12: Effect of different paramters on contributin to nisin yield

Serial Number	Factors	Effects
1	A	-2.276
2	B	-1.638
3	C	3.361
4	AB	-0.193
5	AC	-5.293
6	BC	3.618
7	ABC	-3.431

A-MRS B-MILK C-NISIN

Total Contribution (Set-III): From the results of set III experiments of full factorial design, it was found that effect of MRS and milk were negative whereas effect of nisin was positive, but less. Interaction effect of AB, AC and ABC were negative where BC was positive (Table 12).

Interaction Effects (Set-III): Contour plot (Fig. 13) represented the effect of MRS, milk and their mutual interaction effect on nisin production when nisin is at zero level (0.25 μg/ml). This plot indicates that there was no interaction between MRS broth and milk.

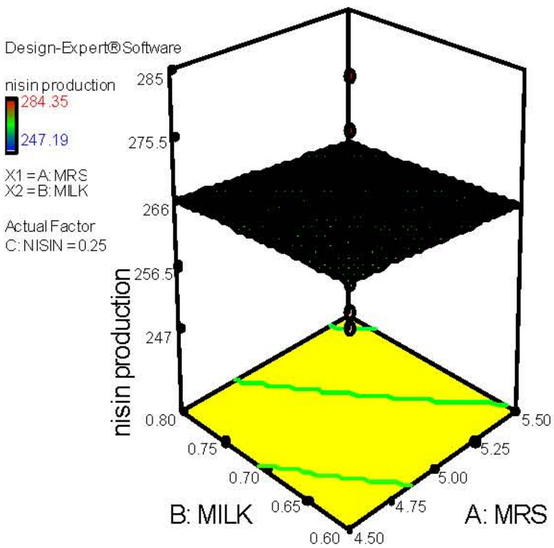

Fig. 13: Contour plot showing the effect of milk and MRS broth concentration on nisin production at zero level concentration of nisin

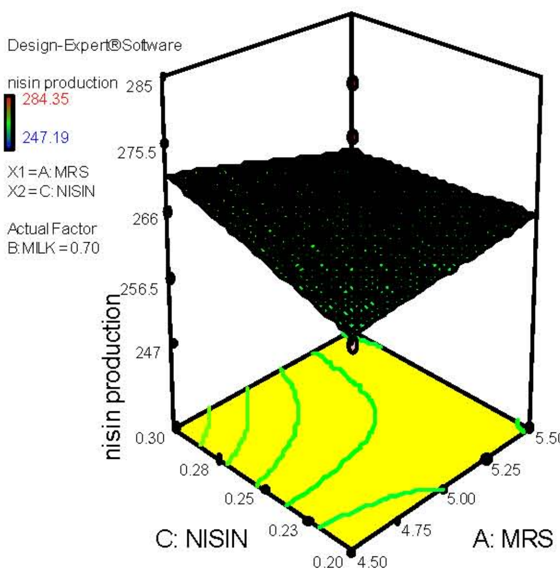

Fig. 14: Contour plot showing the effect of nisin and MRS broth on nisin production at zero level concentration of milk

Similarly, effect of MRS, nisin and their mutual interaction effect are shown in contour plot (Fig. 14) where concentration of milk is at zero level (0.7 %). This contour plot also shows that there wasn't much interaction between MRS broth and nisin to increase nisin yield. Fig. 15 shows contour plot of interaction effect of milk, nisin and their mutual interaction effect on nisin production where MRS broth is at its zero level (5.0 %).

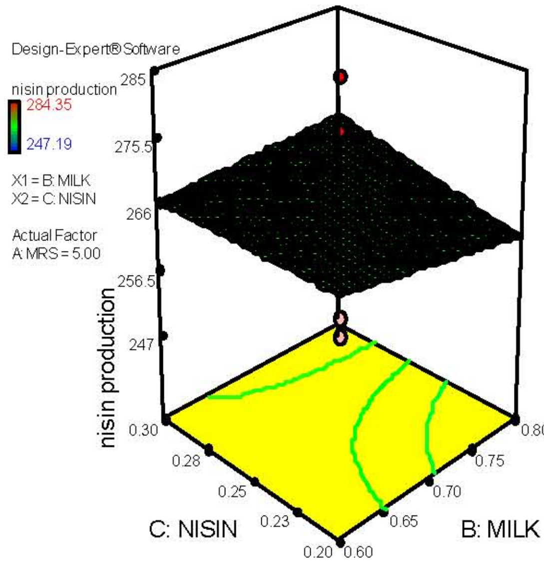

Fig. 15: Contour plot showing the effect of nisin and milk on nisin production at zero level concentration of MRS broth

It shows that there was almost no interaction between the factors.

From the Pareto chart (Fig. 11), it is found that none of the factors or their mutual interaction effect of set-III were higher than Bonferroni limit. This was also confirmed from the contribution table (Table 12). To have the significant model, the higher (+1) and lower (-1) concentration of factors can be changed. With changing the higher (+1) and lower (-1) value of the factors from set-I to Set-III as the optimum has been reached and the model becomes gradually insignificant at set-III. From the responses obtained from the different experimental sets, it can be concluded that the concentration of different factors became optimum in set-II as in set-III, the response did not increase as compared to the responses obtained from the set-II experiments.

CONCLUSION

The optimized concentrations of MRS broth, nisin and milk by full factorial design were found to be 5 %, 0.7 % and 0.25 μg/ml, respectively to obtain nisin yield of 277.59 μg/ml. When compared with one factor optimization experiments, nisin production yield increased from 206 to 277 μg/ml, which is approximately 1.34-fold.

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