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# APPLICATION OF EVOP FACTORIAL DESIGN TECHNIQUE FOR OPTIMIZATION OF NISIN PRODUCTION FROM *LACTOCOCCUS LACTIS* (MTCC-440)

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## ABSTRACT

Evolutionary Operation (EVOP) factorial design technique was sequentially used to optimize concentrations of various media ingredient such as MRS broth, nisin and milk in order to maximize nisin production using *Lactococcus lactis* (MTCC-440). Traditional method for optimization of media ingredient involves changing one variable at a time and keeping all other parameters constant. However, this method does not determine the interactive effects of different factors. EVOP factorial designing technique was applied consecutively and the results were analyzed statistically and next set of experiments were carried by getting the clue from the previous set of experiments till the effect of individual media components are less than the error limits. *Streptococcus agalactiae* (NCIM-2401) was used as an indicator strain for quantification of nisin by agar diffusion assay. Nisin yield increased when fermentation was carried out at 30°C at 100 rpm with final formulated media having media-ingredient concentrations MRS broth, milk and nisin 5.5 %, 0.7 % and 0.25 µg/mL respectively. Consequently, with successful implementation of EVOP factorial design technique Nisin production was increased from 206.1 µg/mL to 271.69 µg/mL, which was approximately 1.31 fold.

## KEYWORDS

Nisin, EVOP, Factorial, agar diffusion

## INTRODUCTION

Nisin is the only bacteriocin approved by the Food and Drug Administration (FDA) and commercially produced by the fermentation of the lactic acid bacterium- *Lactococcus lactis* sub sp. *lactis* in milk-based media under well-controlled culture conditions[1, 2, 3]. It is composed of 34 amino acids and has a molecular weight of 3500 Da. The antimicrobial activities of the industrially important lactic acid bacteria have been known for long and been traditionally applied, and these play an important role in food fermentations, food preservation and intestinal ecology[4, 5]. Among the antimicrobial proteins produced by lactic acid bacteria, nisin is the most studied[6, 7, 8]. The bioactive peptide nisin is synthesized by certain strains of *Lactococcus lactis* sub sp. *Lactis*[9] and is active against Gram-positive bacteria[6]. It is also effective against a wide variety of Gram-negative bacteria in the presence of ethylene di-amine tetra acetic acid (EDTA)[10,11, 12, 13]. The nisin precursor is synthesized in the early active growth phase, however the production rate is maximal towards the end of the exponential growth phase, and cells completely stop nisin biosynthesis while entering the stationary growth phase. This is indicative of the fact that nisin production is greatly influenced by the growth of bacteria and the fermentation media[14, 15, 16, 17].

During process development optimization experiments are generally conducted by trial-and-error or by changing one-factor-at-a-time (OFAT) technique. This method is inefficient in finding the true optimum conditions, especially due to the probable interaction among the factors[18]. The EVOP technique can be considered to be a multivariable sequential search technique, in which the effects of two or three factors are studied together and the responses are analyzed statistically to arrive at a decision. In this method sequentially search is made because the next phase experiment is based on the results of the earlier phase[19]. This program was first proposed for optimization of different operational parameters in a functioning chemical processing plant[18, 20, 21, 22, 23]. The EVOP factorial design technique has been reported as a useful technique to study bioprocesses[24, 25]. The main advantage of EVOP methodology is its clear cut decision procedure which directs the change of variables towards the objective maximum or minimum values.

The present study aims to find out the combined effect of media components such as MRS broth, milk and nisin concentration on nisin production using EVOP factorial design technique.

## MATERIALS AND METHODS

### *Bacterial strains*

*Lactococcus lactis* (MTCC-440), obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India, was used for nisin production studies. Nisin sensitive organism (*Streptococcus agalactiae* - NCIM2401) was obtained from National Collection of Industrial Microorganisms (NCIM). The cultures were revived in MRS broth and preserved in 20 % glycerol stocks at -80 $^{o}$C for further uses.

### *Chemicals*

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

***Quantification of nisin by agar diffusion assay***

An agar diffusion assay was used to quantify nisin using a sensitive indicator strain *Streptococcus agalactiae* (NCIM-2401). *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, 180rpm 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. agalactiae* was added to the autoclaved media when its temperature reached to 45±5°C in such a way that the final optical density becomes 0.001 and then poured on to the plate. Each plate contains approximately 30±1 ml media. 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 were 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.

***Optical density measurement***

Optical density of the culture was measured at 600 nm in which 1-ml sample was taken from the flask and centrifuged at 10,800 g for 15 minutes. Supernatant was discarded and cell pellet was washed twice with WFI (water for injection) [26]. Finally cell pellets were resuspended in WFI and OD was taken at 600 nm against WFI as a blank.

***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 to the sterilized medium after its temperature reached the room temperature. It was then incubated at 37 °C and 180 rpm in an incubator shaker till the optical density reached 1.0 ± 0.2.

***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 at 30 °C and 100 rpm for 20 h. The culture was then harvested by centrifuging at 10,800 g for 30 minutes. Supernatant was collected and nisin quantification was done by agar diffusion assay. In this process, the yield was found to be 206.1μg/mL [26].

***Fermentation process parameters optimization by EVOP factorial design***

EVOP factorial design technique was applied to find out the optimum concentrations of MRS broth, milk and nisin so as to achieve an optimum level of nisin production. To study the effect of these three media components on nisin production 8 ($2^3$) no. of experiments and two control experiments were necessary i.e. total 10 no. of experiments were necessary, where 2 stands for level and three stands for no. of factors. Two level, one at higher (+) and one at lower (-) level compared to search (control) level experiments were maintained for the EVOP factorial design. Experiments were divided in two blocks. Each block contained five experiments with one control level (search level) experiment. All the experiments were carried out in duplicates i.e. in two cycles (Cycle-I and Cycle-II) with a view to minimize the standard deviation and error limits. The results of Cycles I and II were recorded separately to determine their difference and average value. The effects of individual as well as interaction parameters were evaluated based on the average values of two cycle responses. The standard deviation and error limits were estimated at 95 % confidence level after obtaining the differences from Cycles I and II according to the relationship given in the published literature [27, 28].

Calculation of the effects of all individual parameters and that of all interactions can be made on the basis of theory of fractional factorials[29, 30]. The generalized form for the calculation of any effect is shown by the following expression.

Effect= l/e (summation of all higher level average responses of Block I and Block II)-l/e (summation of all lower level average responses of Block I and Block II).

Similarly, the change in mean effect of all the experimental conditions can be calculated from the below mentioned equation.

Change in mean effect=1/2(e+1) (summation of all average responses except control responses of Block I and Block II) -e/2(e+ 1) (summation of two control responses).

***Decision making procedure***

After the calculation of changes in the main effects and error limits, it was necessary to examine whether any change in the control (search) level condition would help to improve the response, and if so, then which was the desired direction of change [24]. For the above, magnitudes of the effects were compared with those of the error limits. If all or any of the effects are larger than the error limits, the change in the experimental conditions may yield better results. The decision on the desired direction of change of a variable has been described in the literature [25].

***Experimental conditions (set-I) for EVOP factorial design***

To perform first set of EVOP factorial design technique, the control level (zero level) experimental conditions ($E_{10}$ and $E_{20}$) were selected based on the results obtained in the earlier investigation[26]. The earlier results were derived by OFAT change technique i.e. changing one independent variable at a time while fixing all the others at a certain level. This method does not include the determination of interactive effects among the variables. The optimum media ingredient concentrations for maximum nisin production was MRS broth 6 % (w/v) supplemented with 0.5 % (v/v) milk and 0.15 µg/mL nisin through single factor optimization technique where the nisin yield was 206.1 µg/mL, when fermentation was carried out at 30°C and 100 rpm in an incubator shaker. These media ingredient concentrations were taken as the search level (zero level) to initiate EVOP factorial design. Thereafter, new experimental conditions were selected with lower and higher levels of media ingredients (MRS broth, milk and nisin) compared to the search level. The concentration of lower (-) and higher (+) values of media ingredients for set-I is given in Table 1. EVOP factorial design experimental conditions of set-I is given in Table 2.

Fermentation was carried out as described earlier for 20 h with higher (+) and lower (-) level of respective media concentrations and all experiments were repeated for two cycles. Nisin yield was estimated by agar diffusion assay. Difference in nisin yield between Cycles I and II, and average nisin yield between Cycle I and Cycle II was calculated to estimate the effects and error limits. The magnitude of effects, error limits and change in mean effect were examined as per the decision making procedure [25] to arrive at the optimum concentration.

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

| Variables Block | Level | | | |
|---|---|---|---|---|
| | Factors | -1 | Control (search) | +1 |
| MRS (%) | 1 | 5.50 | 6.00 | 6.50 |
| Milk (%) | 2 | 0.40 | 0.50 | 0.60 |
| Nisin (µg/mL) | 3 | 10.0 | 15.0 | 20.0 |

**Table 2**
***Experimental conditions of set-I***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 6.00 | 5.50 | 6.50 | 6.50 | 5.50 | 6.00 | 5.50 | 5.50 | 6.50 | 6.50 |
| Milk (%) (v/v) | 0.50 | 0.60 | 0.40 | 0.60 | 0.40 | 0.50 | 0.40 | 0.60 | 0.40 | 0.60 |
| Nisin (µg/mL) (w/v) | 0.15 | 0.20 | 0.20 | 0.10 | 0.10 | 0.15 | 0.20 | 0.10 | 0.10 | 0.20 |

***EVOP factorial design experimental conditions of (set-II) for EVOP factorial design***

From the results (Table 8) of set-I experiments, (magnitude of effects and error limits) it was found that optimum concentration of MRS broth, milk and nisin were not reached as the effects of many factors were positive and more than the error limits. So the best experimental condition giving the maximum nisin yield from the completed set of experiment (Set-I) i.e. ($E_{11}$) was chosen as the search level ($E_{10}$ and $E_{20}$) for next set of the experiments (set-II). The Higher (+) or lower (-) concentrations of media concentrations (MRS broth, milk and nisin) used for set-II experiments are given in Table 3. Experimental conditions of each run of set-II are given in Table 4.

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

| Variables Block | Level | | | |
|---|---|---|---|---|
| | Factors | -1 | Control (Search) | +1 |
| MRS (%) | 1 | 5.00 | 5.50 | 6.00 |
| Milk (%) | 2 | 0.50 | 0.60 | 0.70 |
| Nisin (µg/mL) | 3 | 15.00 | 20.00 | 25.00 |

**Table 4**
***Experimental conditions of set-II***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 5.50 | 5.00 | 6.00 | 6.00 | 5.00 | 5.50 | 5.00 | 5.00 | 6.00 | 6.00 |
| Milk (%) (v/v) | 0.60 | 0.70 | 0.50 | 0.70 | 0.50 | 0.60 | 0.50 | 0.70 | 0.50 | 0.70 |
| Nisin (µg/mL) (w/v) | 0.20 | 0.25 | 0.25 | 0.15 | 0.15 | 0.20 | 0.25 | 0.15 | 0.15 | 0.25 |

***EVOP factorial design experimental conditions of (set-III) for EVOP factorial design***

The results of set-II experiment were similar as those obtained from set-I. From the magnitude of effects and error limits of set II (Table 10), it is considered that the optimum concentration of nisin was not yet reached as effect of all the parameters were not equal or less than the error limits. So the best experimental condition giving the maximum nisin yield from the completed set of experiment (Set-II) i.e. ($E_{11}$) was chosen as the search level ($E_{10}$ and $E_{20}$) for next set of the experiments (set-III). The higher (+) or lower (-) concentrations of media concentrations (MRS broth, milk and nisin) used for set-III experiments are given in Table 5. Experimental conditions of each run of set-II are given in Table 6.

**Table 5**
***Experimental variables at different levels used for the production of nisin (set-III)***

| Variables Block | Level | | | |
|---|---|---|---|---|
| | Factors | -1 | Control (Search) | +1 |
| MRS (%) | 1 | 4.50 | 5.00 | 5.50 |
| Milk (%) | 2 | 0.60 | 0.70 | 0.80 |
| Nisin (µg/mL) | 3 | 20.00 | 25.00 | 30.00 |

**Table 6**
***Experimental conditions of set-III***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 5.00 | 4.50 | 5.50 | 5.50 | 4.50 | 5.00 | 4.50 | 4.50 | 5.50 | 5.50 |
| Milk (%) (v/v) | 0.70 | 0.80 | 0.60 | 0.80 | 0.60 | 0.70 | 0.60 | 0.80 | 0.60 | 0.80 |
| Nisin (µg/mL) (w/v) | 0.25 | 0.30 | 0.30 | 0.20 | 0.20 | 0.25 | 0.30 | 0.20 | 0.20 | 0.30 |

# RESULTS AND DISCUSSION

***Nisin yield of Set-I EVOP factorial design***

The experimental conditions for the first set of experiments, the corresponding nisin production of Cycles I and II, and their difference and average values are presented in Table 7. The calculation of error limits, effects and change in mean effect is done for set-I calculated by published procedure[25] and is shown in Table 8.

**Table 7**
***Experimental conditions and results of set-I***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 6.00 | 5.50 | 6.50 | 6.50 | 5.50 | 6.00 | 5.50 | 5.50 | 6.50 | 6.50 |
| Milk (%) (v/v) | 0.50 | 0.60 | 0.40 | 0.60 | 0.40 | 0.50 | 0.40 | 0.60 | 0.40 | 0.60 |
| Nisin (µg/mL) (w/v) | 0.15 | 0.20 | 0.20 | 0.10 | 0.10 | 0.15 | 0.20 | 0.10 | 0.10 | 0.20 |
| Nisin yield (µg/mL) (cycle I) | 198.33 | 238.63 | 170.99 | 163.33 | 146.56 | 207.00 | 156.23 | 173.09 | 165.99 | 173.65 |
| Nisin yield (µg/mL) (cycle II) | 208.13 | 230.00 | 166.25 | 157.50 | 153.13 | 199.88 | 157.31 | 167.06 | 157.50 | 164.69 |
| Difference (cycle I-cycle-II) | -9.80 | 8.63 | 4.74 | 5.83 | -6.57 | 7.12 | -1.08 | 6.03 | 8.49 | 8.96 |
| Average (µg/mL) | 203.23 | 234.31 | 168.62 | 160.41 | 149.84 | 203.44 | 156.77 | 170.07 | 161.74 | 169.17 |

**Table 8**
***Magnitude of effects and error limit of set-I***

| Factors | Effect |
|---|---|
| Effect of MRS broth | -12.763 |
| Effect of Milk | 24.248 |
| Effect of Nisin | 21.698 |
| Effect of MRS x Milk | -24.638 |
| Effect of MRS x Nisin | -13.883 |
| Effect of Milk x Nisin | 14.798 |
| Effect of MRS x Milk x Nisin | -13.858 |
| Change in mean effect | -25.572 |
| Standard deviation | 4.270 |
| Error limits: For average | ±6.038 |
| For Effects | ±4.287 |
| For change in mean | ±3.805 |

Table 8 shows that the change in mean effect is negative and is large compared to error limits, which also implies that the effect of MRS broth is negative where as the effect of milk and nisin is positive and are larger compared to the error limits. From the results obtained from set-I, it can be concluded that the concentration of milk and nisin needs to be increased and the concentration of MRS broth must be decreased to increase nisin production. The table also shows the effect of many factors individually and mutual interactive effects of many components are higher than the error limit which ensures that optimum level has not yet been achieved and hence further optimization needs to be done. Hence the parameters of the experiment which yielded more nisin in set-I i.e. $E_{11}$ where the concentrations of MRS broth, milk and nisin were 5.5 %, 0.6 % and 0.20 µg/mL respectively were selected as the search (zero level) for set-II experiments.

***Nisin yield of Set-II of EVOP factorial design***

The search level was fixed at the best conditions of Set-I for Set-II and that was at $E_{11}$ of Set–I .The experimental conditions and the results of Set-II experiments are presented in Table 9. Effects and error limits are shown in Table 10.

**Table 9**

***Experimental conditions and results of set-II***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 5.50 | 5.00 | 6.00 | 6.00 | 5.00 | 5.50 | 5.00 | 5.00 | 6.00 | 6.00 |
| Milk (%) (v/v) | 0.60 | 0.70 | 0.50 | 0.70 | 0.50 | 0.60 | 0.50 | 0.70 | 0.50 | 0.70 |
| Nisin (µg/mL) (w/v) | 0.2 | 0.25 | 0.25 | 0.15 | 0.15 | 0.20 | 0.25 | 0.15 | 0.15 | 0.25 |
| Nisin yield (µg/mL) (cycle I) | 235.36 | 274.52 | 236.25 | 217.41 | 184.15 | 236.86 | 242.47 | 179.12 | 223.19 | 186.82 |
| Nisin yield (µg/mL) (cycle II) | 232.24 | 268.86 | 243.99 | 223.5 | 186.39 | 231.71 | 237.63 | 184.03 | 227.91 | 194.13 |
| Difference (cycle I- cycle-II) | 3.12 | 5.66 | -7.74 | 6.09 | -2.24 | 5.15 | 4.84 | -4.91 | -4.72 | -7.31 |
| Average (µg/mL) | 233.80 | 271.69 | 240.12 | 220.45 | 185.27 | 234.28 | 240.05 | 181.57 | 225.55 | 190.47 |

**Table 10**
***Magnitude of effects and error limit of set-II***

| Factors | Effect |
|---|---|
| Effect of MRS broth | -0.496 |
| Effect of Milk | -6.698 |
| Effect of Nisin | 32.371 |
| Effect of MRS x Milk | -20.671 |
| Effect of MRS x Nisin | -40.076 |
| Effect of Milk x Nisin | -2.303 |
| Effect of MRS x Milk x Nisin | -19.971 |
| Change in mean effect | -11.715 |
| Standard deviation | 3.879 |
| Error limits: For average | ±5.484 |
| For Effects | ±3.894 |
| For change in mean | ±3.456 |

Similar observation was made in the set-II of the EVOP factorial design. The change in mean effect was negative and was higher compared to the error limits. Effect of MRS broth was lower than the error limit but effect of nisin was positive and higher than the error limits. Effect of milk was negative but was not in the range of error limits. This indicates that nisin concentration needs to be increased and concentrations of MRS broth and milk need to be reduced.

Therefore, the condition at search level of Set-II did not give the real optimum condition, though some of the effects were smaller than the error limit. Hence a third set of experiment was carried out taking the best condition of set-II, i.e. concentrations of MRS broth, milk and nisin 5.0 %, 0.7 % and 0.25 % respectively as the search level for Set-III.

***Nisin yield of Set-III EVOP factorial design***

The experimental conditions and results of the Set-III experiments are shown in Table 11 and the calculated effects and error limits are presented in Table 12.

**Table 11**
***Experimental conditions and results of set-III***

| Parameters | Experiments | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Block -I | | | | | Block-II | | | | |
| | $E_{10}$ | $E_{11}$ | $E_{12}$ | $E_{13}$ | $E_{14}$ | $E_{20}$ | $E_{21}$ | $E_{22}$ | $E_{23}$ | $E_{24}$ |
| MRS (%) (w/v) | 5.00 | 4.50 | 5.50 | 5.50 | 4.50 | 5.00 | 4.50 | 4.50 | 5.50 | 5.50 |
| Milk (%) (v/v) | 0.70 | 0.80 | 0.60 | 0.80 | 0.60 | 0.70 | 0.60 | 0.80 | 0.60 | 0.80 |
| Nisin (µg/mL) (w/v) | 0.25 | 0.30 | 0.30 | 0.20 | 0.20 | 0.25 | 0.30 | 0.20 | 0.20 | 0.30 |
| Nisin yield (µg/mL) (cycle I) | 274.13 | 258.75 | 255.12 | 270.14 | 264.56 | 272.98 | 260.23 | 245.10 | 256.99 | 258.19 |
| Nisin yield (µg/mL) (cycle II) | 270.37 | 264.63 | 253.22 | 260.45 | 273.06 | 275.88 | 255.31 | 255.05 | 262.33 | 250.69 |
| Difference (cycle I- cycle-II) | 3.76 | -5.88 | 1.90 | 9.69 | -8.50 | -2.90 | 4.92 | -9.95 | -5.34 | 7.50 |
| Average (µg/mL) | 272.25 | 261.69 | 254.17 | 265.30 | 268.81 | 274.43 | 257.77 | 250.08 | 259.66 | 254.44 |

**Table 12**
***Magnitude of effects and error limit of set-III***

| Factors | Effect |
|---|---|
| Effect of MRS broth | -1.195 |
| Effect of Milk | -2.227 |
| Effect of Nisin | -3.942 |
| Effect of MRS x Milk | 5.18 |
| Effect of MRS x Nisin | -4.23 |
| Effect of Milk x Nisin | 4.322 |
| Effect of MRS x Milk x Nisin | -7.005 |
| Change in mean effect | -11.481 |
| Standard deviation | 5.346 |
| Error limits: For average | ±7.559 |
| For effects | ±5.367 |
| For change in mean | ±4.763 |

A third set (set-III) of experiments was designed in which the best condition of set-II i.e.$E_{11}$ of set-II was selected as the search level ($E_{10}$ and $E_{20}$) for set-III. With the third set of experiments, it was found that the nisin yield did not increase and that the response (nisin yield) of other experiments was less than the search level (control) experiments. To arrive at real optimal condition, a few more sets of experiments are required in which the concentration of different media can be varied to have a real optimum level.

## CONCLUSION

Optimization of nisin production was done by implementing EVOP factorial technique which provides effect of individual and interaction effect on product formation. By employing EVOP factorial design technique, the nisin production increased from 206.1 µg/mL to 271.96 µg/mL, i.e. 1.31-fold increase. The final formulate media for nisin production is 5 % MRS broth supplemented with 0.7 % milk and 0.25 µg/mL nisin which yielded 271.69 µg/mL of nisin when fermentation was carried out at 100 rpm at 30 °C for 20 h.

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