Chapter 5

Optimization of ethanol production using immobilized stressed *Saccharomyces* cells

Introduction:

The multifaceted application and utility of ethanol is increasing gradually. To meet this acing demands, fermentation technology for the production of ethanol is gaining sharp momentum globally. Though researchers are going on to check the suitability of different microorganisms for ethanol industry, but still yeast is the primary choice for ethanol fermentation [1]. Due to its high production rate, high ethanol tolerance, adaptive nature and ability of fermenting wide range of sugars, yeasts especially *Saccharomyces cerevisiae* is the most common microorganism used in ethanol fermentation industry [2]. Hence, different strains of *S. cerevisiae* were extensively studied to make it more suitable in terms of stress tolerance, ability to adapt, viability etc. for industrial ethanol production [3]. To make it suitable, different engineered strains of *S. cerevisiae* as well as other organisms have been developed [4]. Such metabolic or genetic engineering have some major disadvantages like complexity in developmental methods, high mutation rate, risk of contamination, human safety etc. Moreover, these processes are prohibitively expensive. Hence, there is a need to develop cost-effective, eco-friendly and easy processes for the industrial production of ethanol. On the other hand, immobilization of yeast cells is also gaining interest in ethanol production industry. This technique offers higher yield in less time and also the chance of contamination as well as mutation is very low [3]. Hence this work is mainly focused on developing a cost-effective, eco-friendly approach to improve the ethanol production by exposing *S. cerevisiae* cells to nitrosative stress. These yeast cells can adapt under the stress conditions as per the requirement. Therefore, not only the ability of stress tolerance but also the metabolism may be modified [6, 7] to counteract the stress condition.

Hence, the primary objective of the work was set to develop a cost-effective, non-hazardous, easy approach to improve the ethanol production by using nitrosative stress exposed immobilized *S. cerevisiae* cells.

Results:

To assess the applicability of the approach, 0.5 mM acidified sodium nitrite treated yeast cells were immobilized using calcium chloride and sodium alginate. Immobilized cells were transferred to the minimal medium containing different concentrations of molasses and ammonium sulphate. CCRD-based RSM was applied to find out the optimal condition of ethanol production under the specified experimental set up.

Optimization of ethanol production by central composite rotatable design based (CCRD) response surface methodology (RSM):

Here, concentration of molasses (A), concentration of ammonium sulfate (B), and incubation time (C) were selected as the independent variables and the influence of these independent variables were tested for ethanol production using CCRD based RSM technique. The optimal level for each of the independent variables was determined. 19 experimental runs were performed to optimize the ethanol production and the results are represented in **Table 8** containing both the actual and predicted responses. Analysis of variance (ANOVA) was performed for the above mentioned experimental set up and represented in **Table 9**. *p* value of the model is 0.003, suggesting, the model is highly significant and it can efficiently predict ethanol production as the actual response. The significant terms of the model are concentration of molasses (A) [$p = 0.0094$], incubation time (C) [$p = 0.0043$], molasses concentration^2 (A^2) [$p = 0.0010$] and incubation time^2 (C^2) [$p = 0.0045$]. By subjecting these results of the experimental set up, a second-order polynomial regression equation was generated by the respective software to estimate the concentration of ethanol that is represented in actual terms.

Table 8: Experimental design along with model predicted and actual ethanol yield response

Run	Factor 1A: C-source (%)	Factor 2B: N- source (%)	Factor 3C: Incubation time (h)	Ethanol Actual (g/L)	Ethanol Predicted (g/L)
1	12.50	1.02	15.00	21.73	21.66
2	5.00	0.05	24.00	7.24	8.79
3	20.00	2.00	6.00	20.26	17.22
4	20.00	2.00	24.00	34.74	34.24
5	12.50	1.02	30.14	28.24	25.11
6	20.00	0.05	6.00	11.52	11.36
7	12.50	2.66	15.00	14.02	18.27
8	5.00	2.00	24.00	11.52	10.19
9	5.00	2.00	30.14	8.68	4.03
10	25.11	1.02	6.00	27.50	27.79
11	12.50	1.02	15.00	21.70	21.66
12	5.00	0.05	6.00	3.15	2.17
13	-0.11	1.02	15.00	0	5.23
14	12.50	1.02	15.00	21.69	21.66
15	12.50	1.02	15.00	21.75	21.66
16	12.50	1.02	-0.14	0	1.80
17	12.50	1.02	15.00	21.75	21.66
18	20.00	0.05	24.00	23.34	26.51
19	12.50	-0.61	15.00	12.35	10.20

Table 9: CCRD based RSM model

Source	Sum of squares	df	Mean square	*F* value	P value prob> F
Model	890.41	9	98.93	7.52	0.0030
A: C-source	142.10	1	142.10	10.80	0.0094
B: N-source	9.99	1	9.99	0.76	0.4061
C: Incubation time	188.79	1	188.79	14.35	0.0043
AB	4.44	1	4.44	0.34	0.5756
AC	26.35	1	26.35	2.00	0.1907
BC	51.01	1	51.01	3.88	0.0805
A2	299.72	1	299.72	22.78	0.0010
B2	113.57	1	113.57	8.63	0.0165
C2	180.27	1	180.27	13.70	0.0045

R1 (Ethanol concentration), Actual = –10.30525+ 1.38894 x A + 5.61195 x B + 1.03751 x C + 0.17658 x AB + 0.035870 x AC + 0.020085 x BC – 0.04312 x A^2 – 2.76112 x B^2 – 0.028324 x C^2

The R^2 value (coefficient of determination) of 0.9377 signifies that the model could predict and explain 93% of the variability. The predicted and adjusted R^2 value were 0.5256 and 0.8817 respectively, presence in a reasonable agreement with each other. Adequate precision ratio of the model is 13.864, showing, high signal to noise ratio. Generally adequate precision ratio of 4 is desirable to judge the significance level of the model. Overall, R^2, adjusted R^2, predicted R^2, and adequate precision ratio were significantly higher which makes the model fit for the prediction of the optimized level of each of the variables used for the actual response i.e. ethanol production.

Comparison of model actual and predicted values for ethanol response (g/L) is presented in **Fig. 25**. The observed and actual values were spread by a line of 45^o (angle) in the plot, suggesting a reasonable aliment of predicted with the actual responses. The response surface plots and their contour plots showed the degree of interactions among three independent variables for ethanol production **[Fig. 26–28]**. The optimal levels of the independent variables were also determined from the second-order polynomial

regression equation, generated from the system. It was found that the ethanol production was significantly increased from 11.88 to 27.54 g/L with the enhanced concentration of molasses (A) ranging from 5 to 20% W/V **[Fig. 26]**, at the fixed concentration of nitrogen source (1.22% W/V). The significance of this factor for ethanol production under the specified experimental condition was also validated by ANOVA (*p* value of 0.0094).

The interaction between the concentration of molasses (A) and incubation time (C) showed a positive effect on ethanol production under the specified experimental condition with a *p* value of 0.0043 **[Fig. 27]**. When the concentration of carbon source was fixed at 20% W/V, ethanol production was significantly enhanced from 16.37 to 32.9 g/L with the gradual increase in incubation time ranging from 6 to 24 h.

In addition to it, The interaction between concentration of ammonium sulfate as the nitrogen source (B) and incubation time (C) didn't show a strong effect on ethanol production **[Fig. 28]** , suggesting, a non-significant (p value of 0.0805) interaction between these two independent variables for ethanol production under the specified experimental set up.

After the rigorous analysis of the interaction among these three independent variables, finally the model was employed to extract the optimized levels of the independent variables for ethanol production under the specified experimental set up. Model predicted that 34.24 g/L ethanol can be produced after 24 h of incubation using medium containing 20% W/V molasses and 1.74% W/V ammonium sulphate. This data mostly corroborated with the wet lab data, where 35.24 g/L ethanol was produced under the same condition.

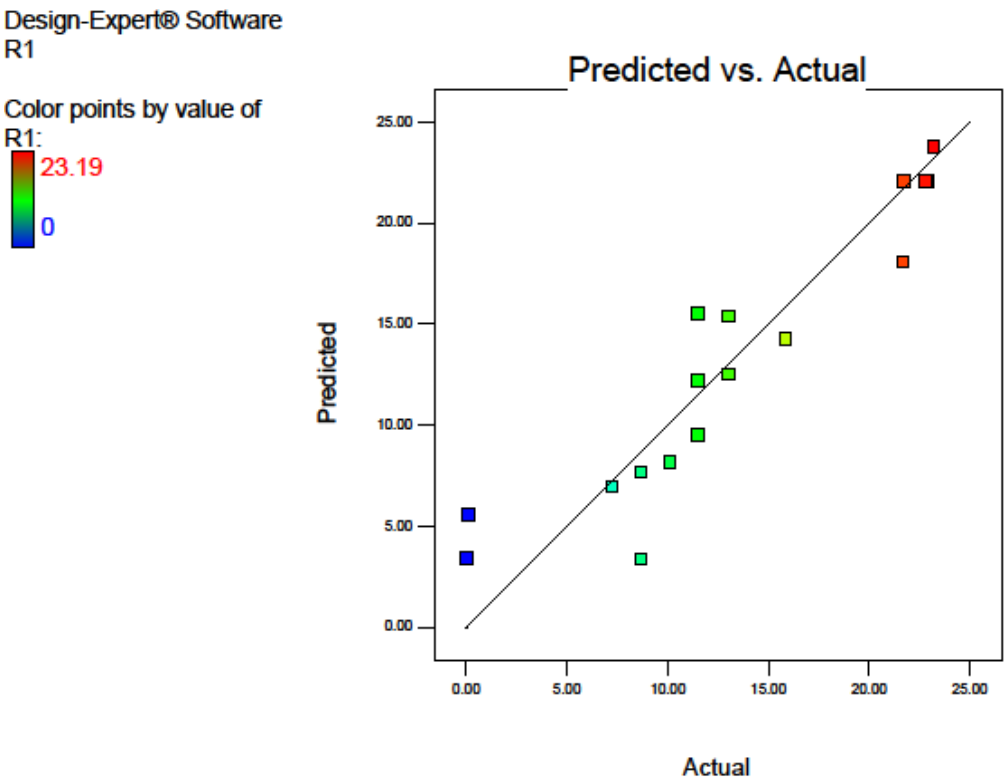

Fig. 25 Plot of actual values versus predicted values

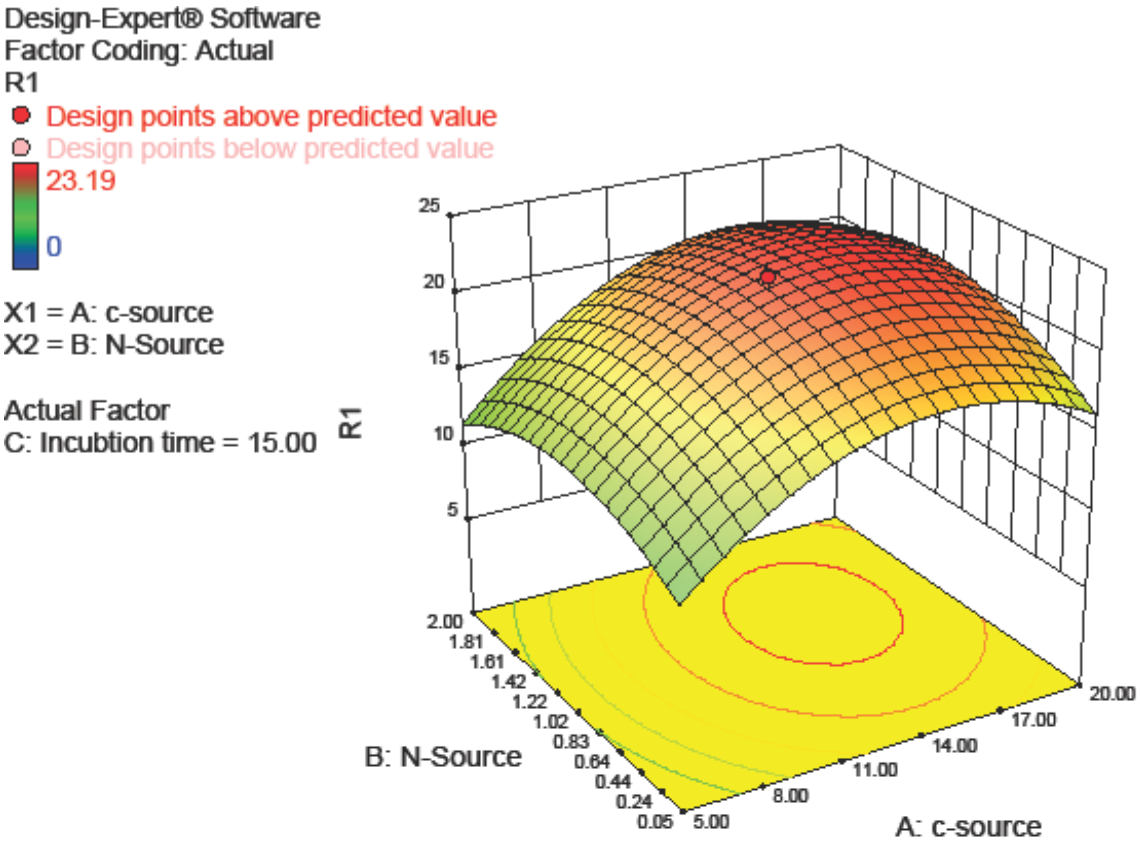

Fig. 26 Surface plot showing the effect of interaction between carbon source (Molasses) and nitrogen source (Ammonium sulfate)

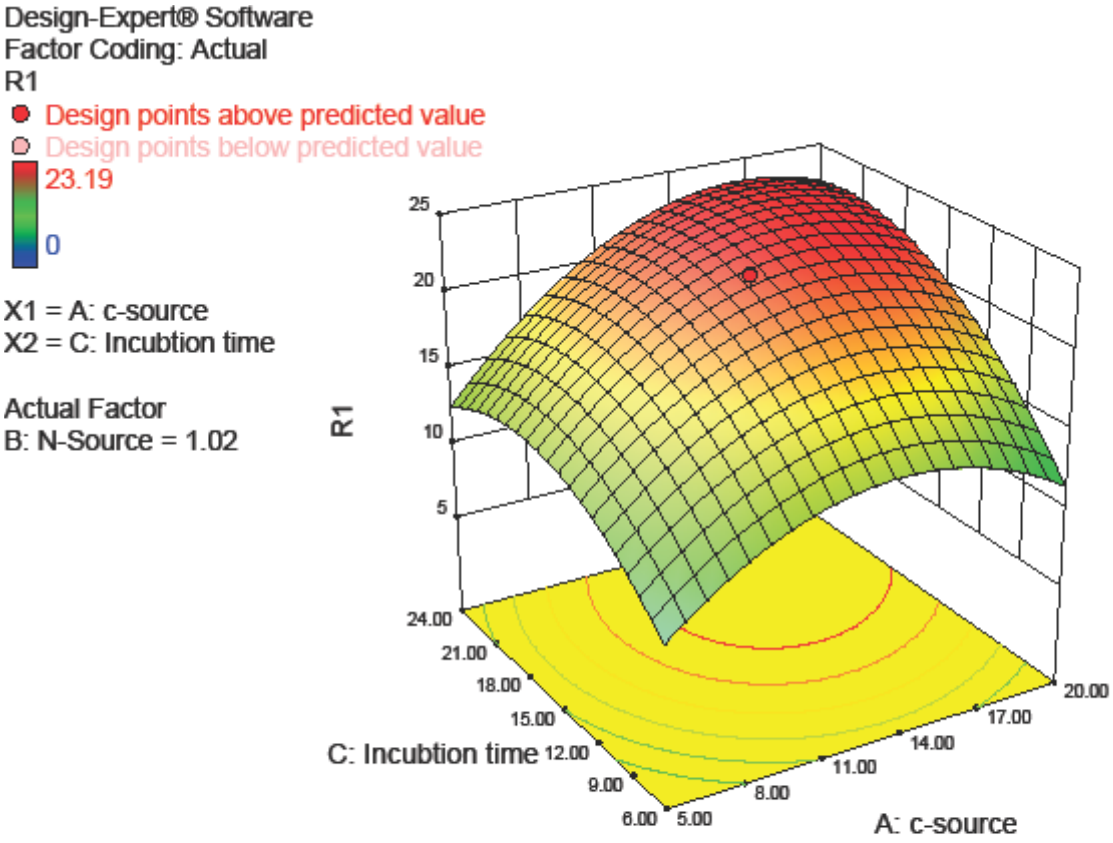

Fig. 27 Surface plot showing the effect of interaction between carbon source (Molasses) and incubation time

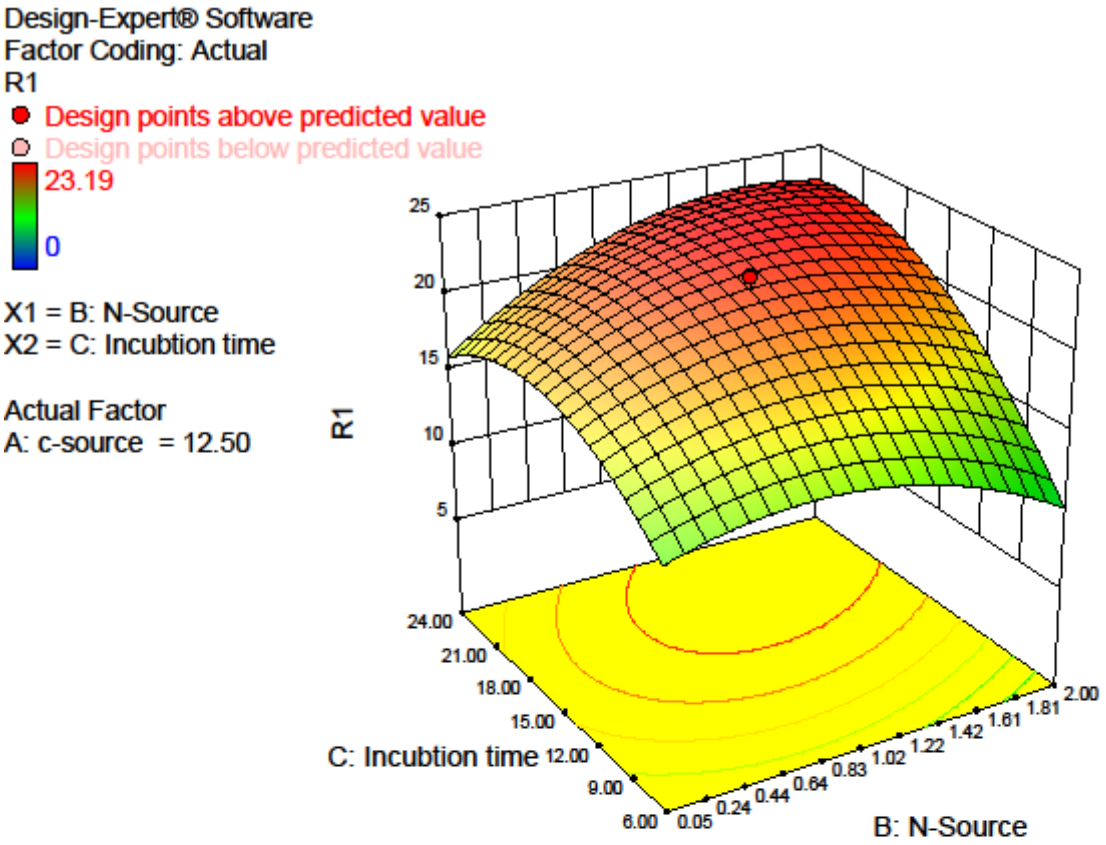

Fig. 28 Surface plot showing the effect of interaction between nitrogen source (Ammonium sulfate) and incubation time

Estimation of ethanol production by nitrosative stress exposed yeast cells grown in YPG and YPD Medium:

Yeast cells were first inoculated in YPD and YPG medium and after three hours 0.5 mM ac. Sodium nitrite was applied. Following an overnight incubation, nitrosative stress exposed cells were immobilized in calcium alginate beads and inoculated in RSM-optimized minimal medium to assess the ability of ethanol production of the nitrosative stress exposed yeast cells. It was found that nitrosative stress exposed

YPD grown yeast cells produced ethanol upto 2nd cycle without significant alteration in the production whereas the production was declined at the 3rd cycle **[Table 10]**. Interestingly, nitrosative stress exposed YPG grown yeast cells produced high concentration of ethanol upto 4th cycle and after that the production was declined significantly **[Table 11]**.

Table 10: Ethanol production by immobilized yeast cells grown in YPD medium

	Immobilization		
No. of cycle	1st	2nd	3rd
Ethanol production (g/L)	33±1	35±2	27±1

Table 11: Ethanol production by immobilized yeast cells grown in YPG medium

	Immobilization					
No. of cycle	1st	2nd	3rd	4th	5th	6th
Ethanol production (g/L)	31±1	36±2	39±1	35±1	24±1	19±1

Discussion:

In this study, to assess the applicability of the major finding of this work i.e. nitrosative stress induced yeast cells produce higher concentration of ethanol, was tried to assess. Hence, the experiments were designed with a view for future industrial application. Thus, minimal medium (containing ammonium sulphate and molasses) and immobilized yeast cells were used. CCRD-RSM software was also used in this work to find out the optimum condition under the specified experimental set up. From the obtained results, it was clear that factors i.e. concentration of molasses as the carbon source (A), concentration of ammonium sulfate as the nitrogen source (B) and incubation time (C) influenced ethanol production independently but their interaction had no significant effect on ethanol production. Moreover, R^2 value of the model was 0.9377 that indicates the excellent fitness of the model (93%). In addition to it, it was observed that nitrosative stress exposed YPG grown immobilized yeast cells produced ethanol more steadily as compared to nitrosative stress exposed YPD grown immobilized yeast cells. This was probably due to the production of higher concentration of ROS and subsequently high production of RNS in YPG medium [8]. Thus, it can be assumed that the altered physiology remained for a longer period of time in nitrosative stress exposed YPG grown immobilized yeast cells as compared to the nitrosative stress exposed YPD grown immobilized yeast cells. This resulted in enhanced ethanol production upto 4^{th} cycle by using nitrosative stress exposed YPG grown immobilized yeast cells.

References:

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