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Statistical Optimization of Batch Ethanol Fermentation of Sugarcane Molasses by *Candida tropicalis* Strain HSC-24

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Abstract: In this study response surface methodology RSM based on central composite face centered design CCFD was employed to optimize a sugarcane molasses batch fermentation process by the Egyptian yeast isolate *Candida tropicalis* strain HSC-24. A statistical quadratic model was developed to describe the interactive effect of five critical parameters; initial pH, molasses concentration wt.%, incubation temperature ^{o}C, mixing rate RPM and incubation period h on the bioethanol yield. The maximum bioethanol production of 36 g/L, with good utilization of different types of sugars was achieved in a batch fermentation process of initial pH5, 20% initial molasses concentration, 35^{o}C,100 RPM and 66 h, which recorded high conversion yield 0.6 g ethanol / g total sugars.
Keywords: Sugarcane molasses, batch fermentation, optimization, *Candida tropicalis*, bioethanol.

1. Introduction

The worldwide expansion of bioethanol production would come in parallel with the depletion of the reserves of non-renewable fossil fuels. The sucrose based substrate sugarcane molasses SCM, from the view point of industrial bioethanol production would be considered as a good candidate as it is renewable, abundant, and inexpensive with readily available source of fermentable sugars [1,2]. The fermentative yeast; *Saccharomyces cerevisiae* is the most widely employed yeast in bioethanol production from molasses [2-8]. To our knowledge, there is no reports has been published on the bioethanol fermentation from molasses by *Candida tropicalis*.

The aim of this study is to investigate and optimize a batch fermentation process of sugarcane molasses using a previously isolated yeast strain *Candida tropicalis* strain HSC-24 (accession No. KJ577477) [9].The response surface methodology was employed in this study as it is an empirical useful modeling system that would study the interactive effect of several independent parameters influencing different responses by varying the independent variables simultaneously by carrying out a limited number of experiments.

2. Materials and methods

2.1. Feedstock

Sugarcane molasses was purchased from Sugars and Integrated Industries Egyptian Distillation Plants in Hawamdeia City, Giza, Egypt and stored at 4^{o}C until use.

2.2. Media

Wickerham WH medium prepared according to Wickerham[10] was used for maintenance and inoculum preparation.

Medium for fermentation experiments was prepared as follows; 2 g KH_2PO_4, 10 g $(NH_4)_2SO_4$, 1 g $MgSO_4.7H_2O$ and 2 g yeast extract were dissolved in 1 L distilled water, molasses and pH were then adjusted according to the experimental conditions before sterilization, at 121°C for 20 min to avoid contamination.

2.3. Microorganism and inoculum preparation

The yeast strain *Candida tropicalis* strain HSC-24(accession No. KJ577477), used in this study was obtained from Petroleum Biotechnology lab., Egyptian Petroleum Research Institute EPRI[9]. Active cultures for fermentation experiments were prepared by growing HSC-24in WH medium for 48 h at 30°C in shaking incubator 150 rpm. Harvested cells were washed twice with sterile saline (8.5 g NaCl per 1 L distilled water), and then re-suspended in sterile saline to be used as a fresh and pure stock for inoculation.

2.4. Analytical Methods

The types and concentration of sugars in molasses were determined using high performance liquid chromatography HPLC (1200 Series Agilent HPLC, Santa Clara, CA, USA) equipped with a refractive index RI detector (model Agilent 1260 infinity, Santa Clara, CA,USA), and Spherisorb Amino (NH_2) Cartridge column (pore size 80Å, inner diameter 4.6 mm, length 250 mm, particle size 5 µm, Waters, Dublin, Ireland). The mobile phase was Acetonitrile: Water (80:20 v/v), flow rate was 1.5 mL/min, and injection volume was 10 µL and the column temperature was 35°C. Estimation of total reducing sugars was carried out by 3, 5-dinitro salicylic acid (DNS), and glucose was used as a standard sugar**.** Ethanol concentration (g/L) was measured by Gas chromatography (model 6890 (G1530A), Agilent, Santa Clara, CA,USA), equipped with flame ionization detector and nominal capillary column (HP-5, 5% phenyl- 95% methylsiloxane 30 m x 250 µm I.D., 5.00 µm film, Santa Clara, CA,USA). Nitrogen was the carrier gas, flow rate was 25 mL/min. Oven and detector temperature was 300°C, and the bioethanol yield was calculated according to the following equation:

$$Bioethanol\ yield\ (\%) = \frac{Produced\ bioethanol\ concentration\ \frac{g}{L}}{Amount\ of\ total\ sugars\ in\ the\ substrate\ \frac{g}{L}} \times 100 \quad (1)$$

The fermentation efficiency was calculated according to El-Refai et al. [4]:

$$\frac{Actual\ ethanol\ content}{Theoretical\ ethanol\ content} \times 100 \quad (2)$$

where, the theoretical ethanol content = total fermentable sugar x0.64 [6].

All experiments were carried out in triplicates, and the listed results are the average.

2.5. Fermentation experiments

Batch anaerobic fermentations were done in 100 mL Erlenmeyer flasks fitted with rubber stoppers, containing 50 mL of culture media with different molasses concentrations (wt.%) and pH values, adjusted according to the required experimental conditions and inoculated with 10% (v/v) yeast suspension (≈ 10^5 cells/mL). Incubation was performed in shaking incubator, set at different temperatures and shaking speeds according to the required experimental conditions. Samples for analyses were taken at the beginning and end of fermentation at different prescribed incubation periods.

2.6. Experimental Design

Response surface methodology (RSM) was used to optimize bioethanol production process from sugarcane molasses (SCM) and investigate the influence of different fermentation process variables on the bioethanol yield. The CCFD was applied to study process variables. The experimental runs were carried out according to a 2^5 full factorial design for the five identified design independent variables, namely; initial pH(A),

molasses concentration wt.% (B), incubation temperature ^{o}C (C), mixing rate RPM (D) and incubation period h (E), with low (-1) and high (+1) levels. The total number of experiments (runs) was given by the simple formula $[50 = 2^k + 2k + 8]$. Where; k is the number of independent variables ($k = 5$), this includes; 32 factorial points from 42 full factorial CCFD were augmented with 8 replicates at the center point to assess the pure error. Response selected was bioethanol yield. The levels were selected based on preliminary study results. The design factors (variables) with low -1 and high +1 levels, are namely A [4 and 6], B [15 and 25 wt%], C [25 and 35^{o}C], D [50 and 150 RPM] and E [24 and 72 h]. The central values (zero levels) chosen for experimental design were; pH5, 20%, 30^{o}C, 100 RPM and 48 h for A, B, C, D and E, respectively (Table.1.).

Table.1. Parameters and levels of the experimental design

Parameters	Levels		
	-1	**0**	**+1**
pH	4	5	6
Molasses concentration wt.% (w:v)	15	20	25
Temp ^{o}C	25	30	35
Mixing rate RPM	50	100	150
Incubation period h	24	48	72

2.7. Statistical analysis

Once the experiments were performed, the next step was to perform a response surface experiment to produce a prediction model to determine curvature, detect interactions among the design factors (independent variables) and optimize the process, i.e. determine the local optimum independent variables with maximum yield of bioethanol. The model used in this study, to estimate the response surface is the quadratic polynomial represented by the following equation:

$$Y = \beta_o + \sum_{i=1}^{5}\beta_i x_i + \sum_{i=1}^{4}\sum_{j=i+1}^{5}\beta_{ij} x_i x_j + \sum_{i=1}^{5}\beta_{ii} x_i^2 \qquad (3)$$

where, Y is the bioethanol yield (g/L), β_o is the value of the fixed response at the center point of the design, β_i, β_{ij} and β_{ii} are the linear, interactive and quadratic coefficients, respectively. x_i and x_j are the independent variables (factors) under study.

The statistical software Design Expert® 6.0.7., (State-Ease Inc., Minneapolis, USA) was used for design of experiments, regression and graphical analysis of the data obtained and for statistical analysis of the model to evaluate the analysis of variance (ANOVA) and it was used also for optimization of the bioethanol fermentation process.

2.8. Batch fermentation under optimum conditions

A batch fermentation of SCM was performed under the selected optimum conditions, in a self-sterilizer 10 L bioreactor (BiotronLiflus SL, Korian Republic) with a working capacity of 5 L, after the sterilization step, the broth was cooled then inoculated with 10% (v/v) yeast suspension (≈ 10^5 cells/mL). The batch fermentation was conducted for 72 h, under anaerobic conditions, and the produced ethanol and residual sugars' concentrations were determined, during prescribed time intervals.

3. Results and discussion

3.1. Molasses sugars

The collected SCM used in this study had total sugars of 292.82 g/L, where sucrose recoded the largest percentage 72.51%, followed by glucose 15.68%, fructose 8.94%, xylose 2.05% and 0.81% maltose. The SCM contained total reducing sugars TRS (69.6 g/L). The SCM was rich in fermentable sugars ≈ 55% (wt%) and the non-fermentable sugars recorded ≈ 5% (wt%).

3.2. Regression model and its validation

The main concern in this study is the actual amount of produced bioethanol, i.e. the actual yield of bioethanol relative to the amount of total sugars in the initial substrate (molasses) concentration.

The complete design matrix with experimental and predicted values of the produced bioethanol yield (%) is presented in Table 2. Based on CCFD and experimental data, the following second order quadratic model equation describing the influence of different considered variables on process yield were obtained:

$$Y = 33.1 - 3.91A - 1.56B + 2C + 6.24D + 3.82E - 16A^2 - 13B^2 + 8.48C^2 - 2.52D^2 + 12.5E^2 + 1.66AB - 4.03AC + 0.0938AD - 1.34AE + 3.47BC - 1.16BD - 5.22BE + 1.03CD - 4.53CE + 1.47DE \quad (4)$$

where Y is the bioethanol yield %, and positive sign in front of the terms indicate synergetic effect, whereas negative sign indicates antagonistic effect.

Table.2. Experimental Design matrix with experimental and predicted bioethanol yield by *Candida tropicalis* strain HSC-24

Run number	Initial pH A	Molasses concentration B	Incubation temperature C	Mixing rate D	Incubation period E	Bioethanol concentration g/L	Bioethanol yield %	
							Actual	Predicted
1	0	-1	0	0	0	10	23.0	21.6
2	-1	-1	-1	-1	+1	15.99	36.0	34.2
3	1	-1	-1	-1	+1	14.50	33.0	28.3
4	0	0	0	-1	0	14.02	24.0	24.3
5	0	0	0	0	+1	27.18	46.0	49.4
6	-1	0	0	0	0	13.87	24.0	21.0
7	0	0	-1	0	0	23.02	39.0	39.6
8	+1	-1	-1	+1	-1	8.01	18.0	16.7
9	+1	+1	+1	+1	+1	15.40	21.0	20.5
10	-1	+1	+1	-1	+1	14.91	20.0	20.6
11	-1	-1	+1	-1	-1	10.22	23.0	19.4
12	-1	-1	+1	-1	+1	11.32	25.0	28.2
13	+1	-1	+1	-1	-1	1.94	4.00	2.74
14	0	+1	0	0	0	14	19.0	18.5
15	+1	-1	+1	+1	+1	11.99	27.0	26.1
16	0	0	0	0	0	20.20	34.0	33.1
17	0	0	0	+1	0	22.75	39.0	36.8
18	-1	-1	-1	+1	-1	6.58	15.0	16.9
19	0	0	0	0	0	18.00	30.0	33.1
20	-1	+1	+1	+1	-1	27.30	37.0	41.8
21	0	0	+1	0	0	26.93	46.0	43.6
22	+1	+1	+1	+1	-1	23.35	32.0	32.1
23	+1	-1	+1	-1	+1	1.12	2.00	6.14
24	0	0	0	0	0	17.40	30.0	33.1
25	+1	0	0	0	0	6.86	12.0	13.1
26	0	0	0	0	0	18.30	31.0	33.1
27	0	0	0	0	0	20.00	34.0	33.1
28	+1	+1	-1	+1	-1	13.74	19.0	18.1
29	-1	+1	+1	+1	+1	27.22	37.0	35.6
30	0	0	0	0	0	19.04	32.0	33.1
31	-1	+1	-1	-1	-1	5.01	6.00	6.70
32	+1	+1	+1	-1	+1	6.00	8.00	5.15
33	+1	+1	-1	-1	-1	8.04	11.0	12.7
34	-1	-1	-1	-1	-1	2.15	5.00	7.31
35	+1	+1	-1	+1	+1	16.79	23.0	24.6
36	0	0	0	0	0	20.26	35.0	33.1
37	+1	-1	-1	-1	-1	2.23	5.00	6.74
38	+1	-1	-1	+1	+1	19.50	44.0	44.1
39	0	0	0	0	-1	27.49	47.0	41.7

40	0	0	0	0	0	18.10	31.0	33.1
41	+1	+1	+1	-1	-1	16.48	23.0	22.6
42	-1	-1	+1	+1	+1	22.37	51.0	47.8
43	+1	-1	+1	+1	-1	6.43	15.0	16.8
44	+1	+1	-1	-1	+1	9.30	13.0	13.4
45	0	-1	+1	+1	-1	14.13	32.0	33.2
46	0	+1	-1	-1	+1	10.41	14.0	12.7
47	0	+1	-1	+1	-1	11.41	16.0	11.7
48	0	+1	-1	+1	+1	16.34	22.0	23.6
49	0	+1	+1	-1	-1	24.06	32.0	32.7
50	0	-1	-1	+1	+1	20.98	48.0	49.7

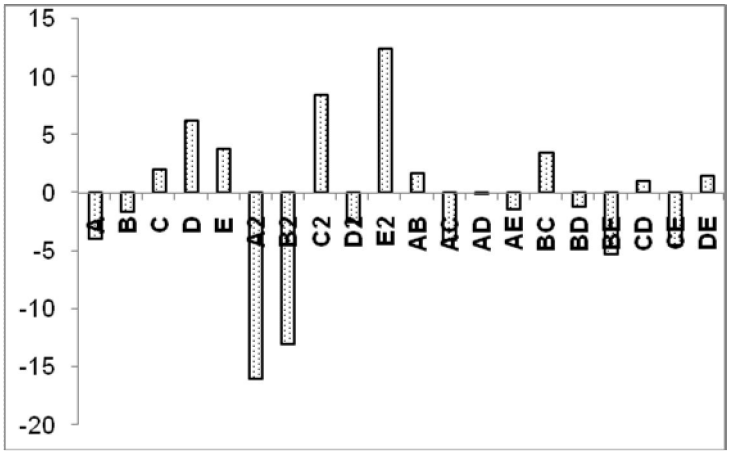
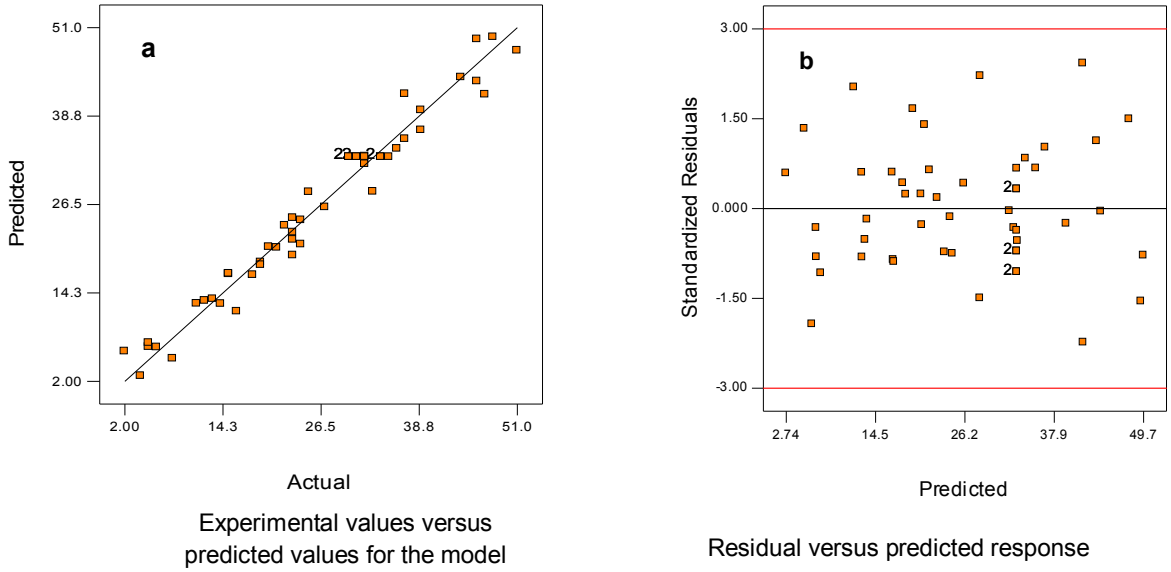


Figure.1. Pareto chart showing the effect of different independent variables on bioethanol production by *Candida tropicalis* strain HSC-24

Pareto charts, which are very useful in design of experiments, were used in this work; to make it much easier to visualize the main and interaction effects of all factors to the response variable i.e. bioethanol yield (Figure 1). The model identified that within the studied range of experiments, the mixing has the highest positive impact on the fermentation process followed by the incubation period and temperature, in a decreasing order. While, the initial pH has a higher negative impact on the bioethanol yield (%) than that of the initial molasses concentration. However, the quadratic effects of initial pH and molasses concentration have the highest negative impact on the fermentation process, followed by the negative quadratic effect of mixing rate. While, the quadratic effect of the incubation period and temperature have a positive impact on the fermentation process in a decreasing order. The positive interactive effect of the studied parameters can be ranked in the following decreasing order; initial molasses concentration and incubation temperature >initial pH and molasses concentration≈ mixing rate and incubation period > incubation temperature and mixing rate > initial pH and mixing rate. But, the negative interactive effect of the studied parameters can be ranked in the following decreasing order; initial molasses concentration and incubation period>incubation temperature and period > initial pH and incubation temperature >initial pH and incubation period > initial molasses concentration and mixing rate.

The validity of the fitted model was evaluated and their statistical significance was controlled by F-test. The analysis of variance (ANOVA) for the response surface full quadratic model is given in Table 3. It can be indicated that the models (eq. 4) is very highly statistically significant at 95% confidence level, with F-value of 42.2and very low probability p-value of < 0.0001, i.e., there is less than 0.01% chance that this error is caused by noise. The values of the determination coefficients, R^2andR^2_{adj}which measure the model fitting reliability, were calculated and found to be 0.967 and 0.944, respectively. This suggests that, approximately 96.7% of the variance is attributed to the variables, which indicated the high significance of the model, where, only 3.3 % of the total variations cannot be explained by the model equation 4, which ensures the good adjustment of the above predicted model to the experimental data. Confirmation of the adequacy of the regression model was reflected also by the good agreement between the experimental and the predicted values of the response variables as shown in Table.2. Where, the experimental bioethanol yield ranged from 2 to 51% and their corresponding predicted values were 6.14 and 47.8%, respectively. The “Adeq Precision” measures the signal

to noise ratio. A ratio greater than 4 is desirable. The ratio of 24.1 for model equation 4, indicated the adequate signal. This model is reliable and can be used to navigate the design space. The standard deviation SD and the coefficient of variance were low, recording; 3 and 11.6 for model eq.4, respectively.



Experimental values versus predicted values for the model

Residual versus predicted response

Figure.2. Validity of model Eq.4

The performance of the model can be observed by the plots of the predicted versus experimental results of bioethanol yield (Figure 2a), which showed high correlation coefficients (R^2 = 0.95), indicating that the predicted and experimental values were in reasonable agreement. This means that the data fit well with the model and gives a convincingly good estimate of response for the system in the studied experimental range. Figure 2b, presents a plot of the residual distribution, defined as the difference between calculated and observed values of the response variable studied, versus predicted response. The quality of the fit is good because the residual distribution does not follow a trend with respect to the predicted values of response variable, which indicate that the quadratic model adequately represent the biodiesel % yield over the studied experimental range.

Table.3. Analysis of variance of the fitted quadratic regression model Eq4:

Source	SS*	df*	MS*	F-value	p-value	Remarks
Model	7.60E+003	20	380	42.2	< 0.0001	Very highly significant
A	520	1	520	57.8	< 0.0001	Very highly significant
B	82.6	1	82.6	9.17	0.00512	Significant
C	136	1	136	15.1	0.000546	Highly significant
D	1.32E+003	1	1.32E+003	147.	< 0.0001	Very highly significant
E	497	1	497	55.2	< 0.0001	Very highly significant
A^2	635	1	635	70.5	< 0.0001	Very highly significant
B^2	419	1	419	46.6	< 0.0001	Very highly significant
C^2	178	1	178	19.7	0.000119	Highly significant
D^2	15.7	1	15.7	1.75	0.197	Non significant
E^2	385	1	385	42.8	< 0.0001	Very highly significant
AB	87.8	1	87.8	9.74	0.00405	Significant
AC	520	1	520.	57.7	< 0.0001	Very highly significant
AD	0.281	1	0.281	0.0312	0.861	Non significant
AE	57.8	1	57.8	6.41	0.0170	Possibly significant
BC	385	1	385	42.7	< 0.0001	Very highly significant
BD	42.8	1	42.8	4.75	0.0376	Possibly significant
BE	872	1	872	96.7	< 0.0001	Very highly significant
CD	34.0	1	34.0	3.78	0.0617	Possibly significant
CE	657	1	657	72.9	< 0.0001	Very highly significant

DE	69.0	1	69.0	7.66	0.00972	Significant
Residual	261	29	9.01			
Pure Error	26.9	7	3.84			
Corrected total	7.86E+003	49				

*SS: sum of squares df: degree of freedom MS: mean square

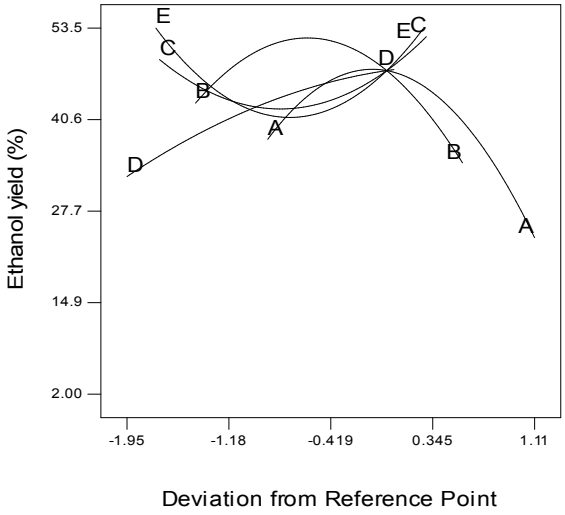


Figure.3. Perturbation plot for bioethanol yield from SCM by *Candida tropicalis* strain HSC-24

The perturbation plots (Figure 3) show the comparative effects of all the studied independent variables on the bioethanol yield %. The curvatures of the five studied factors from the center point confirm the statistical data obtained from analysis of variance (ANOVA, Table 3), that is, the significance of each parameter (coefficient). It is obvious from the sharp curvature of the independent variables initial pH (A) and molasses concentration incubation period (B, wt% w:v)), that the bioethanol yield increased with the increase of pH and molasses concentration, recording its maximum near the center point, i.e., pH5 and 20% molasses concentration and decreased with further increase of these two parameters. While the contrary occurred within the sharp curvatures of the incubation temperature (C, ^{o}C) and period (E, h), where the bioethanol yield recorded a decrease near the center point, i.e., 30^{o}C and 24 h but increased at longer incubation period and higher temperature. But the bioethanol yield increased with the increase of the mixing rate (D, RPM). The sensitivity of the fermentation process of SCM by *Candida tropicalis* strain HSC-24 and its bioethanol yield, towards the five studied parameters can be ranked in the following decreasing order; mixing rate > initial pH ≈ incubation period > incubation temperature > initial molasses concentration. The curvatures also confirm the data illustrated in Pareto chart (Figure 1). This was also confirmed by the analysis of variance (ANOVA) of the regression model, where the statistical significance of the main and interacting effects of different studied parameters on the bioethanol yield at 95% confidence level, were studied and illustrated in Table 3. The significance of each coefficient was determined by F-values and p-values. The larger the magnitude of the F-value and the smaller the p-values, the more significant is the corresponding coefficient. This implies that the main effects of initial pH and molasses concentration have very highly statistically ($p < 0.0001$) and statistically ($p = 0.00512$) significant negative impact on the bioethanol yield, respectively, and their quadratic effects i.e., their doubling, have a very highly statistically negative impact on the bioethanol yield ($p < 0.0001$). But, the incubation temperature ($p = 0.000546$), mixing rate ($p < 0.0001$) and incubation period ($p < 0.0001$) have highly statistically significant positive impact on the bioethanol yield, i.e., increase in the bioethanol yield with the increment of these parameters. Where, the quadratic effects of the incubation temperature ($p= 0.0001$) and incubation period ($p < 0.0001$) expressed highly statistically positive impact on the bioethanol yield, but the quadratic effect of mixing rate expressed a non-statistically significant negative impact on the bioethanol yield ($p = 0.197$). The interactive effect of the initial molasses concentration and incubation temperature has a very highly statistically significant positive impact on the bioethanol yield ($p < 0.0001$), while the interactive effects

of the initial pH and incubation temperature, the initial molasses concentration and incubation period and that of the incubation temperature and period have a very highly statistically negative impact on the bioethanol yield ($p <0.0001$).

3.3. Optimization of fermentation process

Three-dimensional response surfaces were plotted on the basis of the predicted model equation to investigate the interaction among the variables and to determine the optimum condition of each factor for maximum bioethanol yield %.

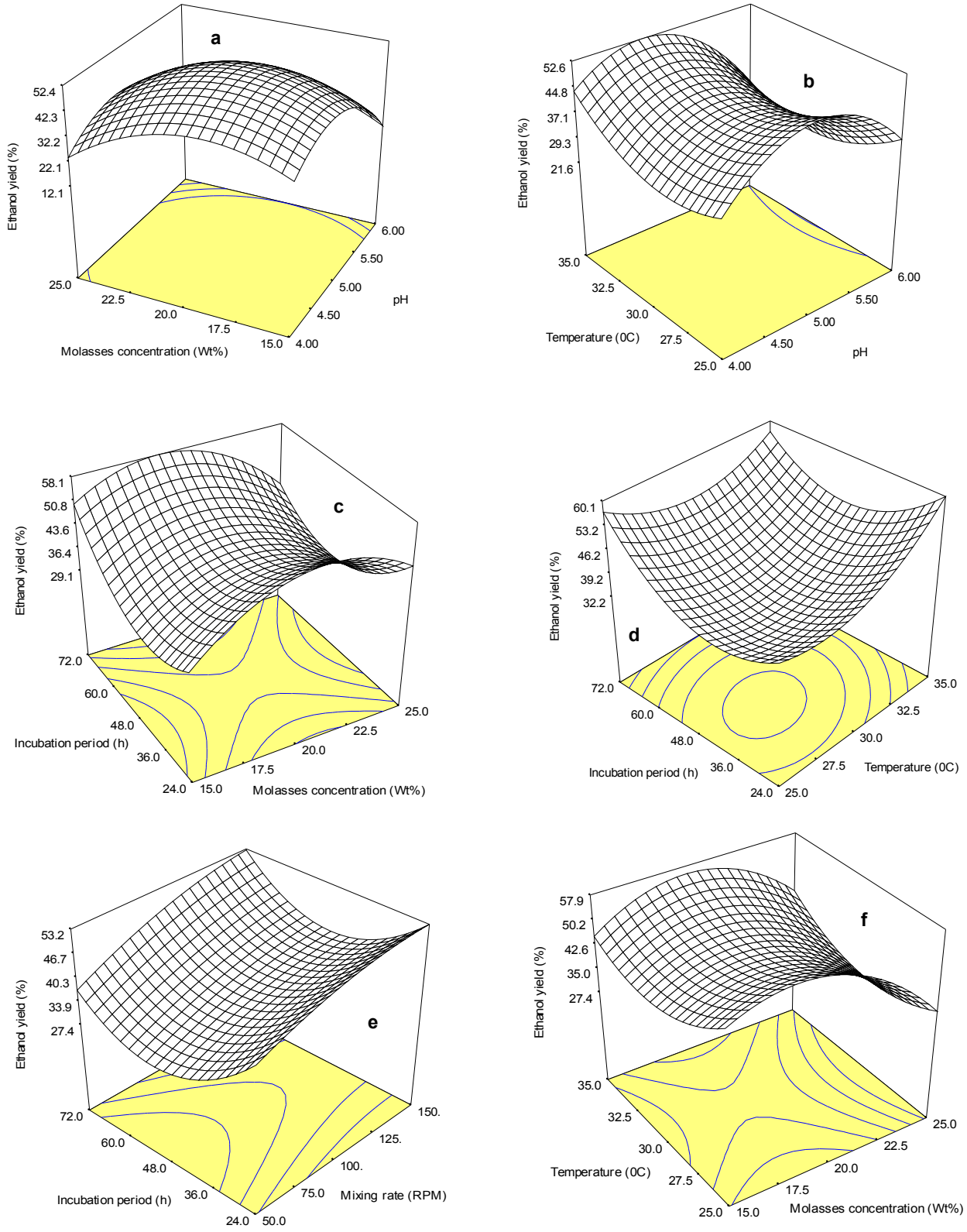


Figure.4. RSM and contour plots for bioethanol production from SCM *Candida tropicalis* strain HSC-24

The statistically significant positive interactive effect of initial pH and molasses concentration on the bioethanol yield($p = 0.004$)is obvious in the elliptical shape of the RSM plot (Figure 4a), where the production of ethanol increased with the increment of initial pH and molasses concentration recorded its maximum yield ≈ 52% at pH5 and 20% SCM, but it decreased with further increment of these two parameters. The very highly

statistically negative impact of the interactive effect of the initial pH and incubation temperature is illustrated within the RSM and contour plots (Figure 4b), where the bioethanol yield increased with incubation period and increment of initial pH but to a certain limit recording ≈ 53% bioethanol yield at pH5 within 72 h incubation period but sharply decreased at higher initial pH values. Also, the very highly statistically negative impact of the interactive effect of the initial molasses concentration and incubation period is obvious in RSM and contour plots (Figure 4c) where the bioethanol yield increased with incubation period and increment of initial molasses concentration but to a certain limit recording ≈ 58% bioethanol yield at 20% SCM concentration within 72 h incubation period but sharply decreased at higher initial SCM concentrations. The very highly statistically significant negative interactive effect of incubation period and temperature on the bioethanol yield ($p < 0.0001$) is obvious in the elliptical shape of the RSM plot (Figure 4d), where the production of ethanol increased with the increment of incubation period and temperature recorded its maximum yield ≈ 58% at 35^{o}C within 72 h. The statistically significant positive interactive effect of mixing rate and incubation period on the bioethanol yield ($p = 0.0097$) is obvious in the RSM and contour plots (Figure 4e), where the production of ethanol increased with the increment of incubation period and mixing rate recorded its maximum yield ≈ 53% within 72 h and mixing rate 150 RPM. while the RSM and contour plots (Figure 4f) showed the positive interactive effect of initial molasses concentration and incubation temperature on the bioethanol yield, where the bioethanol yield increased with the increment of temperature and initial molasses concentration recording its maximum ≈ 58%at 35^{o}C and 20% SCM concentration, but decreased with higher SCM concentrations.

Maiorella et al. [3], Cazetta et al. [1] and Shafaghat et al. [11] reported that the pH of fermentation medium as an important parameter affecting the microbial growth and product formation. Misono and Yamaguchi [12], reported that the optimum pH for the alcoholic fermentation of molasses at pH5 and decreased with the increase of pH to pH6. El-Refai et al. [4] reported the optimum pH value for the bioconversion of beet molasses to ethanol by *Saccharomyces cerevisiae* Y-7 to be pH5. Cazetta et al. [1] reported maximum bioethanol fermentation of molasses *by Zymomonas mobilis* at 35^{o}C and higher temperature has negative impact on fermentation process. El-Refai et al. [4] reported that, although the production of ethanol increased with the increment of molasses concentration up to 200 g/L, but the fermentation efficiency decreased with higher molasses concentrations, within the range of 250-300 g/L, due to osmotic disruptions, since osmotic pressure is one of the essential factors for by-products formation such as sorbitol and levan [1]. Ergun and Mutlu[5] reported that, the inorganic salts in molasses feedstock would exert some inhibitory effects on ethanolic fermentation and this effect increases with the increase of molasses concentration. Sedha et al. [6] reported the decrease of fermentation efficiency by increasing molasses concentration above 20% (w:v) and attributed this to the substrate inhibition and the increased accumulation of residual sugar. Morimura et al. [13] reported that the temperature tolerance has been found to depend upon sugar concentrations of the medium as observed that fermentation of molasses at 35°C was possible when sugar concentration was 20%(w:v) with no fermentation when sugar concentration was 22%(w:v).

The optimization process was carried out to determine the optimum values of the studied five parameters affecting the fermentation process of sugarcane molasses by *Candida tropicalis* strain HSC-24 to maximize the bioethanol production (g/L) and its yield%. This was done using Design Expert 6.0.7 software (State-Ease Inc., Minneapolis, USA). According to the software optimization step, the desired goal for each fermentation parameter (A initial pH, B initial molasses concentration wt%, C incubation temperature ^{o}C, D mixing rate RPM and E incubation period h) was defined within the studied levels range to achieve the highest performance. The program combines the individual desirability into a single number and then searches to optimize this function based on the response goal. Accordingly, the optimum conditions giving the maximum calculated bioethanol production of 35 g/L with bioethanol yield of 58% were; pH5, 20% initial molasses concentration, 35^{o}C,100 RPM and 66 h, with desirability function value of 1. The experimental result of these conditions was found to be 34 g/L with bioethanol yield of 56.3%. That indicates the process optimization based on CCFD of experiments was capable and reliable to optimize the bioethanol fermentation process of SCM by *Candida tropicalis* strain HSC-24.

3.4. Batch fermentation under optimum conditions

It is obvious from data listed in Table 4 and illustrated in Figure 5 that ethanol production increased with the depletion of sucrose, maltose, xylose, and total sugars TS. But the total reducing sugars TRS, glucose and fructose concentrations did not follow a certain trend, where the results of this typical fermentation showed that *Candida tropicalis* strain HSC-24, was able to hydrolyze sucrose to glucose and fructose, and then to selectively convert glucose to ethanol and biomass. In the beginning of the process, glucose started to accumulate (Figure 5a), since its production rate might have been faster than its consumption rate, and attained

a peak (within 16 h). After that, the glucose concentration started its continuous decrease, recording ≈ 82% consumption by the end of the process.

Table.4.Time course of sugars concentrations during fermentation of SCM by *Candida tropicalis* strain HSC-24 and bioethanol production

Parameters / Time h	Sucrose g/L	Glucose g/L	Fructose g/L	Maltose g/L	Xylose g/L	TRS g/L	TS g/L	Ethanol g/L	Yield %
0	43.79	9.47	5.4	0.49	1.24	13.56	60.39	0	0
8	16.28	11.56	6.87	0. 37	1.19	8.40	36.27	0.27	0.45
16	15.87	11.89	3.79	0.30	1.17	8.02	33.02	1.99	3.30
24	14.64	8.87	6.30	0.29	1.17	7.15	31.27	7.10	11.76
32	14.13	7.98	7.18	0.27	1.16	12.31	30.72	17.10	28.32
40	12.88	7.64	8.78	0.20	1.14	14.42	30.64	27.58	45.67
48	9.95	8.4	6.83	0.12	1.12	14.80	27.42	32.03	53.04
56	9.08	6.6	6.90	0.10	1.02	15.43	23.7	35.18	58.25
64	8.48	4.52	6.15	0.08	1.00	7.82	20.23	36.43	60.32
72	8.85	2.16	4.05	Nil	1.00	7.06	16.06	36.22	59.98

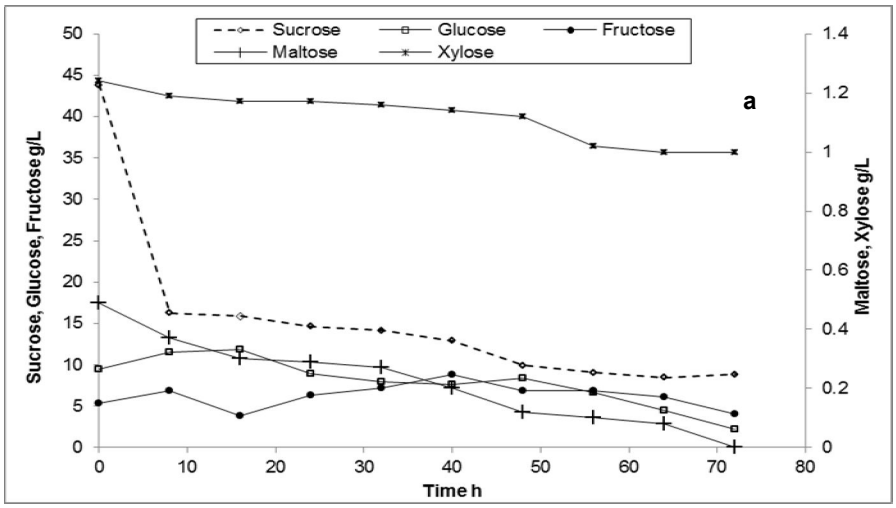


Figure. 5 a. Time profile of sugars consumption from SCM by *Candida tropicalis* Strain HSC-24

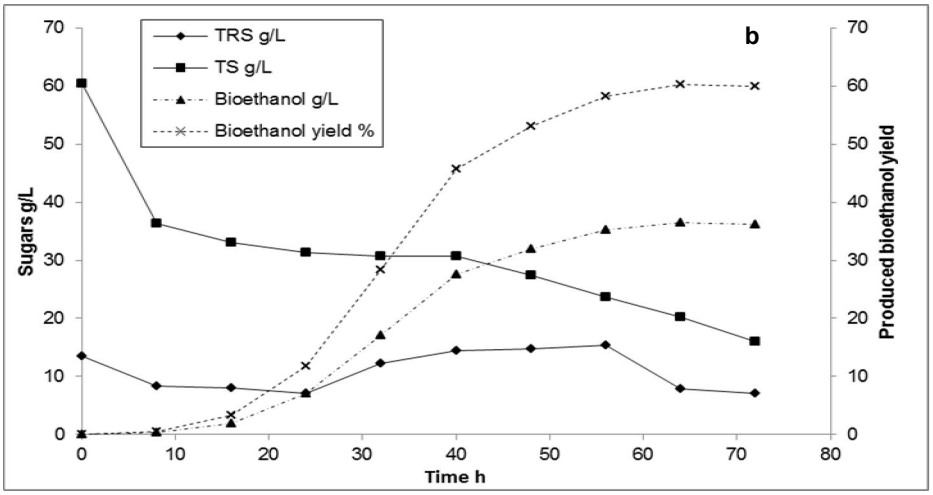


Figure.5 b. Time profile of sugars consumption and bioethanol production from SCM *Candida tropicalis* strain HSC-24

It has been reported that; yeast can ferment sucrose throughout its assimilation, which can be degraded by invertase enzymes to be taken up as glucose and fructose [8]. This might explain the overall decrease and consumption of sucrose throughout the time span of the ongoing fermentation process (Figure 5a) recording ≈ 80% consumption by the end of the process.

There was an overall decrease in different types of sugars in molasses feed stock, recording TS consumption of ≈73.41% (Figure 5b) with good ethanol yield, good fermentation efficiency and conversion yield of ≈ 60.32%,94.26% and$Y_{ethanol/TS}$ ≈ 0.6 g/g, respectively.

El-Refai et al. [4] reported 96.1% fermentation efficiency of 200 g/L beet molasses by *Saccharomyces cerevisiae* Y-7 at pH5, 30^{o}C within 72 h.Roukas[14] reported ethanol yield $Y_{ethanol/TS}$ = 0.3 g/g from beet molasses by *Saccharomyces cerevisiae*. Kopsahelis et al. [7] reported ethanol yield $Y_{ethanol/TS}$ = 0.47 g/g from waste molasses by *Saccharomyces cerevisiae*.

4. Conclusion

The response surface optimization of sugarcane molasses SCM fermentation process based on central composite face centered design CCFD of experiments was capable and reliable to maximize the bioethanol production by the Egyptian yeast isolate *Candida tropicalis* strainHSC-24 (accession No. KJ577477).The process ethanol yield reached 60.32%, with good fermentation efficiency of different types of sugars in the SCM and high conversion yield, which recorded 94.26% and $Y_{ethanol/TS}$ ≈ 0.6 g/g, respectively.

5. References

1. Cazetta, M. L., Celligoi, M. A. P. C., Buzato, J. B. and Scarmino, I. S. 2007. Fermentation of molasses by *Zymomonas mobilis*: Effects of temperature and sugar concentration on ethanol production. Bioresour. Technol. 98: 2824–2828.
2. El-Gendy, N. Sh., Madian, H. R. and Abu Amr, S. S. 2013. Design and optimization of a process for sugarcane molasses fermentation by *Saccharomyces cerevisiae* using response surface methodology.Int. J. Microbiol.Volume 2013, Article ID 815631, 9 pages, doi:10.1155/2013/815631
3. Maiorella, B. L., Blanch, H. W. and Wilke, C. R. 1984. Feed component inhibition in ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol.Bioeng. 24: 1155-1166.
4. El-Refai, A. H., El-Abyad, M. S., El-Diwany, A. I., Sallam, L. A. and Allam, R. F. 1992. Some physiological parameters for ethanol production from beet molasses by *Saccharomyces cerevisiae* Y-7.Bioresour. Technol. 42: 183-189.
5. Ergun, M. and Mutlu, S. F. 2000. Application of a statistical technique to the production of ethanol from sugar beet molasses by *Saccharomyces cerevisiae*.Bioresour. Technol. 73: 251-255.
6. Sedha, R. K., Verma, G., Gupta, R. P. and Tewari, H. K. 1984. Ethanol production from molasses using cell recycling of *Saccharomyces cerevisiae*. J. Ferment. Technol. 62, 471-476.

7. Kopsahelis, N., Agouridis, N., Bekatorou, A., Kanellaki, M. 2007. Comparative study of spent grains and delighted spent grains as yeast supports for alcoholic production from molasses. Bioresour. Technol. 98: 1440-1447.
8. Nakata, H., Tamura, M., Shintani, T. and Gomi, K. 2014. Evaluation of baker's yeast strains exhibiting significant growth on Japanese beet molasses and compound analysis of the molasses types. J. Biosci. Bioeng. 117(6): 715-719.
9. Hamouda, H. I., Nassar, H. N., Madian, H. R., El-Sayed, M. H., El-Ghamry, A. A., and El-Gendy, N. Sh. 2015. Isolation of fermentative microbial isolates from sugar- cane and beet molasses and evaluation for enhanced production of bioethanol.Energ.Source. Part A (Accepted in Press).
10. Wickerham, L. J. 1951. Taxonomy of yeasts. U.S. Dep. Agric. Tech. Bull., 1029: 1–56.
11. Shafaghat, H., Najafpour, G. D., Rezaei, P. S. and Sharifzadeh, M. 2010. Optimal growth of *Saccharomyces cerevisiae* (PTCC24860) on pretreated molasses for ethanol production: the application of response surface methodology. Chem. Ind. Chem. Eng. Q. 16(2): 199-206.
12. Misono, H. M. and Yamaguchi, Y. 1990. The alcoholic fermentation of molasses. J. Ferment. Technol. 8: 210-218.
13. Morimura, S., Ling, Z. Y. and Kida, K. 1997. Ethanol production by repeated batch fermentation at high temperature in a molasses medium containing a high concentration of total sugar by thermotolerant flocculating yeast with improved salt tolerance. J Ferment. Bioeng. 83: 271-74.
14. Roukas, T. 1996. Ethanol production from non-sterilized beet molasses by free and immobilized cells using fed-batch culture.Biotechnol.Bioeng. 43: 189-194.

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