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Optimization of Glycerol Production by a New Osmotolerant *Wickerhamomyces anomalus* AUMC 11687 Yeast Strain Using Response Surface Methodology

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GLYCEROL is considered as a great tool for industrial applications. Mechanism of glycerol production by yeast cell has become highly attractive and interesting. Many factors affect glycerol yield throughout fermentation process. For this reason, an osmotolerant glycerol producer yeast strain *Wickerhamomyces anomalus* AUMC 11687 was selected for optimization of glycerol production conditions by using response surface methodology. Four independent variables (temperature, pH, sugar and glycerol concentration as osmoregulatory) were studied using the Box-Behnken Design methodology with 4-factors and 3-levels. The best conditions were calculated by statistical regression analysis and surface plots. The optimal production parameters for maximum glycerol production were at 33.34ºC, 6.9 pH, 22.454% sugar concentration and 6.307% initial glycerol as osmoregulator. The concentration of glycerol and residual sugars under these optimal conditions were 129.65 and 7.404gL^{-1}, respectively. Results recorded during this study showed that the osmotolerant *W. anomalus* AUMC 11687 is a promising yeast with great potentials for application in glycerol production on large scale for industrial purposes.

Keyword: Glycerol, Optimization, Production, Response surface methodology, Yeast.

Introduction

Glycerol is an economically important alcohol with a little sweet taste and with applications in the food, beverage, pharmaceutical and chemical industries, it is also reported to enhance the flavor intensity (Omori et al., 1995; Jones et al., 2008), suppress the perceived roughness (Peleg et al., 1999; Jones et al., 2008), increase the worty off-flavor retention and the effect on the aroma volatility (Perpete & Collin, 1999, 2000; Jones et al., 2008). Glycerol is known as non-toxic tri-ol which is soluble in water and other polar solvents, but insoluble in non-polar organic solvents (Agarwal, 1990; Scanes et al., 1998; Kirk & Othmer, 1999).

Inside yeast cell, glycerol is mostly synthesized *via* the two-step reduction of dihydroxyacetone phosphate by glycerol-3phosphate dehydrogenase (GPD, EC 1.1.1.8) and glycerol-3-phosphatase (GPP, EC 3.1.3.21) (Påhlman et al., 2001). The most attractive ways of inhibiting ethanol and increasing glycerol production by using yeasts are (i) using of sulfites or bisulfites, which bind with acetaldehyde and shift the metabolism towards glycerol, (ii) using osmotolerant yeast strains, that accumulate glycerol as a compatible osmolyte to counterbalance high extracellular osmotic pressure (Brown, 1978; Rehm, 1988; Agarwal, 1990).

The effect of sugar concentration and osmotic stress on yield of glycerol produced by yeasts was recorded before. Raises in sugar concentration usually produce extremely increases in the yield

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of glycerol by yeasts. The increase in glycerol production is due to the greater osmotic stress obligatory on the organism (Vijakishore & Karanth, 1986; André et al., 1991; Albertyn et al., 1994).

This work was established to analyze the influence of temperature, pH, sugar concentration and addition of glycerol as osmoregulator on glycerol production by a new osmotolerant yeast strain named *Wickerhamomyces anomalus* AUMC 11687 and applying the response surface methodology.

Materials and Methods

Microorganisms and preparation of inoculum

Wickerhamomyces anomalus AUMC 11687 was isolated from sugar cane molasses and identified during previous study in our laboratory (Abdel-Latif, 2018). This strain was recorded as osmotolerant yeast and high glycerol producer. It had been genetically identified and deposed in Assiut University Mycological Center with AUMC 11687 and its sequence was recorded in gene Bank as *Wickerhamomyces anomalus* MF418013. The inoculum was prepared by preculturing aerobically in 100 ml conical flask containing 50mL seed medium (SM), which was composed of (g/L): glucose, 100; urea, 3; corn steep liquor, 3 and 1000 ml distilled water, at 30 ± 2ºC in shaking incubator at 150 rpm for 24hrs (Guo et al., 2006).

Fermentation process

Fermentation process was carried out at 250mL conical flask using fermentation medium composed of (glucose, 200g/L; urea, 2.5g/L; corn steep liquor, 3mL; KH_2PO_4, 3.5g/L). The medium was inoculated at 5% (v/v) with pre-cultured seed inoculum as 2×10^7cells/mL; fermentation period was 120hrs with shaking at 150rpm. The improvement of glycerol production conditions were performed by using response surface methodology for studying the influence of different tempratures (30, 35 & 40°C), glucose concentrations (200, 250 & 300g/L), pH (6, 7 & 8) and initial glycerol as osmoregulator (60, 80, 100g/L).

Analytical methods

Glucose concentration was determined by the dinitro-salicylic acid (DNS) method (Miller, 1959). Glycerol was determined in the filtrate by the method of Chitlaru & Pick (1989) One ml of periodate reagent ($NaIO_4$, 65mg in 90mL distilled water, acetic acid 10mL, ammonium acetate 7.7g) was added to 200µL of fungal or yeast filtrate, then 2.5mL acetylacetone reagent (acetylacetone, 2.5mL and isopropanol, 247.5mL) were added and mixed. Then, the mixture was incubated in water bath at 45ºC for 20min. After that, the optical density was measured by spectrophotometer at 410nm and compared to calibration standard.

Experimental design for optimizing glycerol production

A Box-Behnken design (BBD) (Box & Behnken, 1960) was used for analyzing the effect of independent variables and their interaction to get the optimum conditions of glycerol production. Four factors were chosen based on preliminary tests for this work, The factors were temperature (A), sugar concentration (B), pH (C), initial added glycerol as osmoregulator (D). Three levels of each factor were included as following in Table 1.

TABLE 1. Values of independent variables in in the Box-Behnken design

Parameters	Units	Coded levels		
		-1	0	+1
Temperature (A)	ºC	30	35	40
Sugar concentration (B)	g/L	200 (minimum)	250 (medium)	300 (maximum)
pH (C)	-	6	7	8
Glycerol concentration (D)	g/L	60	80	100

Statistical analysis

The design expert 11 software detected the Analysis of Variance (ANOVA), response surface plots and diagnostic checks. The mathematical model for the experimental design was selected on the basis of P value with high statistical significance, lack of fit test and high value of R, F-test and T-test were used to check the significance of the selected model and individual coefficients. The validity of the model was detected by the normal probability plot and the plots of the predicted versus experimental. Response surface plots were used to determine the combination between levels of the variables for the best glycerol production conditions.

Results and Discusstion

The suggested design matrix with experimental values of glycerol yield shown in Table 2 based on BBD and experimental data, the following second-order quadratic model equation illustrating the effect of different variables on glycerol yield was obtained:

$$Y= b_0 + b_1A + b_2B + b_3C + b_4D + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 \quad \text{(Eq. 1)}$$

where Y is dependent variable, A, B, C, D are independent variables. The complete optimization study comprised 29 combinations, which included three replicates for the center point (Table 2).

Analysis of variance for response surface quadratic model

The fitting of mode was determined with the coefficient of determination R^2 which was calculated to be 0.98 indicating that 98% of response variability could be explained by the previous model whereas the remaining 2% variation may have been due to other uncontrollable variables. The model considered as statically significant according to the F- test with 95% of confidence, as the F value of 152.0 is much higher than F (14, 14) = 2.48, the p-value of the model is (0.0001) which is less than 0.05 indicating model terms are significant. Sum of squares used as a mathematical way to find the function that best fits (varies least) from the data. According to Table 3, temperature (A), sugar concentration (B), pH (C) and initial glycerol added (D) are significant for glycerol production. Also, AB interaction has a significant effect on glycerol production at the confidence interval considered (95%). The lack of Fit F-value of 3.98 implies the Lack of Fit is not significant relative to the pure error. There is a 9.77% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit. The experimental results of Box-Behnken design were fitted to quadratic Equation (1). The dependent variables $Y_{\text{glycerol produced}}$ can be present by quadratic equation and interactive terms as below :

$$Y_{\text{glycerol produced}} = +130.35 - 8.79A - 5.78B - 3.07C - 1.41D - 5.57AB + 2.85AC + 2.12AD + 0.9950BC + 0.3300BD + 0.1625\ CD - 5.97A2 - 20.85B2 - 0.9455C2 - 2.65D2 \quad \text{(Eq. 2)}$$

Y_{glycerol} is glycerol production concentration g/L, $Y_{\text{residual sugars}}$ is residual sugar concentration g/L, (A) temperature C°, (B) sugar concentration g/L, (C) pH, (D) glycerol added g/L which acts as osmoregulator.

The performance of the model can be noticed by the plots of the predicted versus experimental results of glycerol concentration which showed high correlation coefficients (R2= 0.98) indicating that the predicted and experimental values were in reasonable agreement. This means that the data fit well with the models as shown in Fig. 1. The normality plot was also a very important parameter that proved the significance of the selected quadratic values. Figure 2 showed that all the points lie on the straight line which means that the residuals had a normal distribution.

Response surface plots

It was observed from the RSM plots that the interaction between temperature and sugar concentration was significant (Fig. 2), which means with raising temperature of glycerol, the production process reaches to its maximum concentration 129.65g/L at 33.34°C and decreased by further increasing of temperature. Also the production of glycerol increased by increasing sugar concentration and the best sugar concentration was 224.54g/L at 33.34°C. This result was compatible with the result of Zhuge et al. (2001) who recorded that the maximum glycerol concentration was 137g/L from 260g/L

glucose at the optimum temperature range (29–33°C) by *Candida glycerinogenes* and this is due to the high level of GPD1 gene expression and encoding GPDH activity which are participates in glycerol synthesis at high temperature. Within the tolerance range of osmotic stress, more glycerol was produced with the increasing of sugar concentration **(**Arroyo-López et al., 2010; Marti et al., 2011). Where the Hog1 kinase appears to stimulate the glycolytic flux and increase the transcription of the genes involved in glycerol synthesis (GPD1 and GPP2) and glycerol import (STL1); it may also be involved in the channel of aquaglyceroporin (FPS1) closure to prevent glycerol efflux (Albertyn et al., 1994; Ansell et al., 1997; Cordier et al., 2007).

TABLE 2. A Box-Behnken experimental design and the results of dependent variables response 1 (glycerol produced) by osmotolerant yeast strain (*Wickerhamomyces anomalus* AUMC 11687)

Ru	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
	A:temperature C	B:sugar concentration g/L	C:pH	D:glycerol added g/L	Glycerol produced
1	35	250	6	60	132.22
2	35	250	7	80	130.51
3	35	300	7	100	100.32
4	35	200	7	100	110.22
5	30	200	7	80	114.21
6	35	200	6	80	116.33
7	40	250	8	80	110.43
8	35	300	6	80	105.23
9	35	250	7	80	128.64
10	30	250	7	60	133.11
11	40	200	7	80	108.66
12	35	250	7	80	130.44
13	30	250	7	100	125.22
14	35	250	7	80	129.82
15	40	250	7	100	115.33
16	35	250	8	100	122.42
17	30	250	8	80	126.66
18	35	300	7	60	101.22
19	35	300	8	80	103.52
20	30	250	6	80	140.53
21	40	250	7	60	114.75
22	35	200	8	80	110.64
23	40	300	7	80	82.51
24	35	250	8	60	125.33
25	40	250	6	80	112.92
26	35	250	6	100	128.66
27	35	200	7	60	112.44
28	30	300	7	80	110.33
29	35	250	7	80	132.32

TABLE 3. ANOVA for the entire quadratic model of response (1) glycerol production concentration

Source	Sum of squares	d.f	Mean square	F value	P value
Mode	4577.83	14	326.99	58.76	< 0.0001
A-temperature	926.82	1	926.82	166.56	< 0.0001
B-Sugar conc	401.02	1	401.02	72.07	< 0.0001
C-pH	113.41	1	113.41	20.38	0.0005
D-Glycerol conc	23.80	1	23.80	4.28	0.0576
AB	123.99	1	123.99	22.28	0.0003
AC	32.38	1	32.38	5.82	0.0302
AD	17.94	1	17.94	3.22	0.0942
BC	3.96	1	3.96	0.7117	0.4131
BD	0.4356	1	0.4356	0.0783	0.7837
CD	0.1056	1	0.1056	0.0190	0.8924
A^2	231.51	1	231.51	41.61	< 0.0001
B^2	2820.63	1	2820.63	506.91	< 0.0001
C^2	5.80	1	5.80	1.04	0.3246
D^2	45.61	1	45.61	8.20	0.0125
Residual	77.90	14	5.56		
Lack of Fit	70.78	10	7.08	3.98	0.0977

df: degree of freedom

R^2= 0.98

F (0, 95, 14, 14) = 2.48

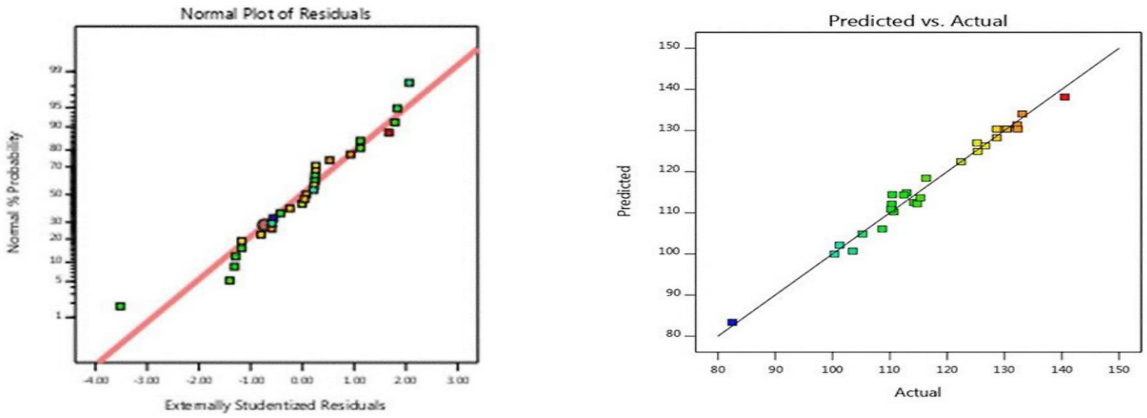

Fig. 1. Normal probability and predicted vs actual values

Glycerol production showed the maximum concentration at pH 6.9, it's clear that glycerol concentration increases by raising pH and the best glycerol concentration was near to pH 7. The effect of pH on glycerol production was reported by Arroyo-López et al. (2010) and indicated that the high pH-value slightly increases glycerol yield because aldehyde dehydrogenase enzyme becomes highly active at high pH-value (Wang et al., 2001).

The addition of glycerol to the fermentation medium decreases the water activity of the medium causing osmotic stress, Hohmann (1997) recorded that when yeast cells are exposed to changes in external osmolarity, they shrink as a consequence of the rapid loss of intracellular water. To compensate for the loss of turgor pressure, cells accumulate glycerol as osmolyte.

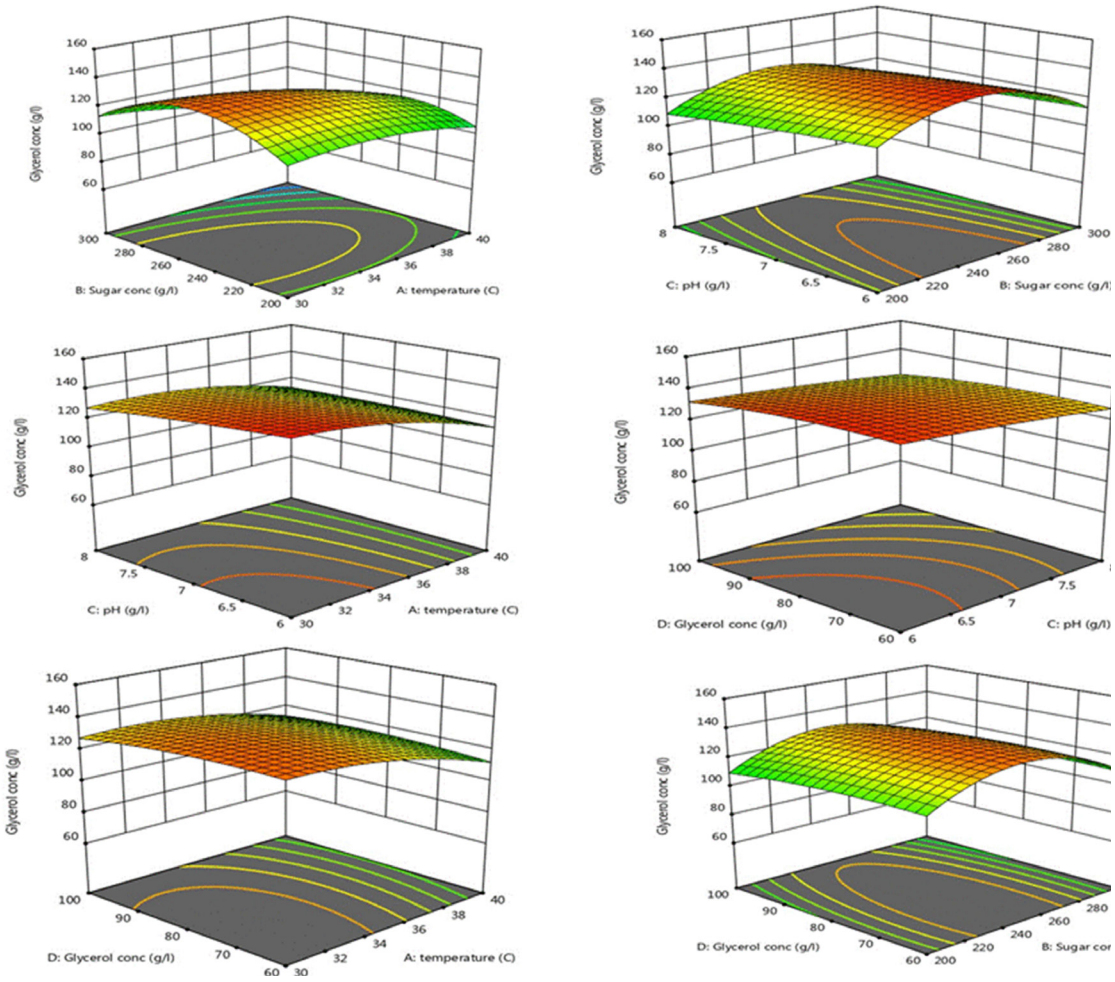

Fig. 2. Response surface plots indicated the influence of temperature and sugar concentration interaction on glycerol production at different pH and different concentration of initial added glycerol

Conclusion

The using of Box-Behnken design (BBD) was found to be applicable for modeling the production of glycerol depending on the changes in the process variables, temperature, sugar concentration, pH and initial addition of glycerol as osmoregulator. This study has indicated that the new osmotolerant *Wickerhamomyces anomalus* AUMC 11687 yeast strain is suitable for producing large amounts of glycerol on a laboratory scale. The optimal production parameters for maximum glycerol production were temperature 33.34ºC, pH 6.9, sugar concentration 224.45g/L and initial added glycerol concentration as osmoregulator of 63.07g/L the concentration of glycerol and residual sugars under these optimal conditions were 129.65 and 7.404g/L, respectively.

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تحسين أنتاج الجليسرول بواسطة عزلة الخميرة *Wickerhamomyces anomalus* AUMC 11687 باستخدام Response Surface Methodology

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يلعب الجليسرول دورا هاما فى الكثير من الصناعات، ولقد استحوز مؤخرا انتاج الجليسرول بواسطة الخمائر على الاهتمام الكبير. وهناك العديد من العوامل التى تلعب دورا مهما فى انتاج الجليسرول بواسطة الخمائر. تم انتخاب هذه العزلة *Wickerhamomyces anomalus* AUMC 11687 من الخمائر والتى تتميز بمقدرتها على انتاج الجليسرول بكميات كبيرة لدراسة الظروف المثلى لانتاج الجليسرول باستخدام البرنامج الاحصائى methodology response surface و لقد شملت الدراسة على اختبار اربعة من العوامل وهما درجة الحرارة وتركيز ايون الهيدوجين وتركيز السكر وأستخدام الجليسرول كمادة مقاومة للاسموزية. وكانت نتيجة هذه الدراسة أن الظروف المثلى لانتاج الجليسول هى درجة حرارة 33 درجة سيليزية وتركيز ايون الهيدروجين 6.9 وتركيز السكر هو 22.454% وتركيز الجليسرول كعامل مضاد للاسموزية هو 6.307% وهذه الظروف انتجت كمية من الجليسرول وقدرها 129.65 جرام/ لتر.