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Supplementation of sugarcane molasses for maximization of ethanol production by *Saccharomyces cerevisiae* using response surface method

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ABSTRACT: Dilute sugarcane molasses containing 19.2% fermentable sugars was supplemented with $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ to maximize the ethanol production by *Saccharomyces cerevisiae* TISTR 5596 using a central composite design of response surface method. The maximum ethanol (87.28 g/l) was produced when the dilute molasses was supplemented with 417 mg/l NH_4^+, 1.88 g/l PO_4^{3-} and 5 mg/l Mg^{2+}. The Ca^{2+} contaminated in the molasses was proposed as an indicative index for the requirement of phosphate and Mg^{2+} supplementation to maximize the ethanol production.

KEYWORDS: Calcium ion, phosphate, magnesium

INTRODUCTION

Thailand is ranked as the second largest cane sugar exporter in the world, and results in the annual generation of 3.9 million tons of molasses, a viscous, black-brown liquid, as a by-product of the sugar industry[1,2]. The molasses has sucrose as the major component (30–40%, w/w) and is rich in other nutrients, including minerals and vitamins, which are required for the growth and ethanol fermentation of microorganisms[3]. Molasses is a major raw material for fuel ethanol production in Thailand. *Saccharomyces cerevisiae*, the most popular yeast used for commercial ethanol production, has an invertase enzyme that can hydrolyse sucrose into glucose and fructose[4]. Hence *S. cerevisiae* can directly ferment molasses to ethanol. Molasses contains some amount of Ca^{2+} because CaO is used for clarification of the sugarcane juice during the sugar production process. These Ca^{2+} ions inhibit the invertase activity in a concentration-dependent manner with the toxic level reported to be 2.16% (w/v)[5] and entailed in reduction of the ethanol production level. Although molasses contains all the nutrients required for yeast growth and ethanol fermentation, their concentration might not be optimal, and so supplementation of molasses with specific nutrients may be necessary. Molasses supplemented with urea gave higher ethanol yield. As compared to the supplementation with urea alone, combination of urea and yeast autolysate improved the ethanol yield significantly[6]. In this study, the supplementation of molasses with four different nutrients was optimized via a three-factor-five-level central composite design (CCD) of response surface method (RSM).

MATERIALS AND METHODS

Molasses sample

The molasses sample was collected from the Angvien Industry sugar factory at Nakhon Ratchasima province, Thailand, and kept at 4 °C until use. The molasses was diluted to give 19.2% fermentable sugars and analysed for its chemical composition at the Food Research and Testing Laboratory (FRTL), Faculty of Science, Chulalongkorn University.

Microorganism

S. cerevisiae TISTR 5596 was obtained from the Thailand Institute of Science and Technology Research.

Molasses medium preparation

The molasses was diluted to 19.2% fermentable sugars with distilled water, and then clarified by centrifugation at 5000 rpm for 5 min. The resultant supernatant (dilute molasses) was analysed for the initial total sugar content by the phenol H_2SO_4 method[7] and then used for medium preparation.

Molasses medium was prepared by adding 2 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 0.75 g/l $MgSO_4 \cdot 7H_2O$, and 10 g/l yeast extract into the dilute molasses, mixing and adjusting the pH to 4.5 prior to autoclaving at 120 °C, 15 lb/in^2 (1.055 kg/cm^2) for 3 min. The initial reducing sugar concentration of the molasses medium, 192 g/l fermentable sugars, was analysed after autoclaving.

Inoculum preparation

A single colony of *S. cerevisiae* TISTR 5596, grown on modified yeast-peptone-dextrose (YPD) agar (100 g/l sucrose, 3 g/l yeast extract, 3 g/l Bactopeptone, 2 g/l agar, pH 5) at 30 °C for 48 h, was inoculated into 50 ml of modified YPD broth in a 250-ml flask and incubated at 30 °C, 200 rpm for 24 h. The obtained culture was transferred at 1% (v/v) to fresh modified YPD broth, incubated at the same condition as above and used as the inoculums.

Ethanol production

The inoculum was centrifuged at 4 °C, 8000 rpm for 10 min to precipitate the *S. cerevisiae* cells. The *S. cerevisiae* cells were then resuspended in the molasses medium (50 ml in a 250-ml flask) to a final cell number of 9.6×10^8 cells/ml. The inoculated medium was incubated at 30 °C under an oxygen limited condition (the fermenting flask was capped with a rubber stopper covered with parafilm, the stopper was connected to U-shape glass filled with $CuSO_4$ solution) with 130 rpm agitation. Samples were taken every 24 h and centrifuged (8000 rpm, 10 min) to remove the yeast cells, with the obtained supernatants being analysed for their ethanol content by gas chromatography[8]. Fermentable sugars was determined according to Lane and Eynon[9].

Table 1 Variables and levels screened by full-factorial design.

Variable	Parameter	Unit	Low level	High level
X_1	$(NH_4)_2SO_4$	g/l	0.0	2
X_2	KH_2PO_4	g/l	0.0	2
X_3	$MgSO_4 \cdot 7H_2O$	g/l	0.0	0.75
X_4	Yeast extract	g/l	0.0	10

Table 2 Experimental variable, parameter, range and level of independent variables in the central composite design.

Variable	Parameter	Range and level				
	(g/l)	−1.68	−1	0	1	−1.68
X_1	$(NH_4)_2SO_4$	0.32	1.0	2.0	3.0	3.68
X_2	KH_2PO_4	0.32	1.0	2.0	3.0	3.68
X_3	$MgSO_4 \cdot 7H_2O$	0.09	0.25	0.75	3.0	1.59

Full-factorial design based evaluation of the effect of nutrient supplementation of the molasses medium on the ethanol production level

A full-factorial design was used to screen for the effect of the concentration of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, and yeast extract in the molasses medium upon the ethanol production level by *S. cerevisiae* TISTR 5596. These four independent variables were examined in a factorial design of 16 experimental runs (combination), (Table 1). Each variable was examined at two levels: −1 for a low concentration and +1 for a high concentration. All experiments were performed in triplicate and the average value is reported.

The limits to which effects of independent variables were assigned from literature[10]. The significant level (p-value) of each variable was determined by F-test.

Central composite design (CCD)

A CCD with the three factors that significantly affected the ethanol production level ($(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$) were optimized by RSM using a three-factor-five-level CCD with three replicates at the centre point, which was fitted to the secondary-order response surface[11]. The CCD always contained twice as many star points as factors in the design, being the low and high values for each factor in this design. To maintain the rotatability, the α value was determined from the number of ex-

perimental runs in the factorial portion of the CCD, as $\alpha = [2^3]^{1/4} = 1.68$ for $k = 3$ factors $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$.

The variables, their values and the experimental design are shown in Table 2. A second-degree quadratic model with $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ as the variables and ethanol production as the response was established by the method of least squares;

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2,$$

where Y is the predicted response (ethanol production, g/l); X_1, X_2, and X_3 are the coded forms of the input variables $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$, respectively; a_0 is a constant; a_1, a_2, and a_3 are linear coefficients; a_{12}, a_{13}, and a_{23} are cross-product coefficients; a_{11}, a_{22}, and a_{33} are quadratic coefficients. The relationship between the coded forms of each input variable and the actual value of the ethanol production is described by $X_i = (x_i - X_0)/\Delta X$, where X_i is the dimensionless coded value of variable x_i, X_0 is value of x_i at the centre point and ΔX is the step change. The data from the experimental design were subjected to second-order multiple regression analysis using the least square regression method to obtain the parameter estimator of the mathematical model. SPSS Statistic 20.0 and STATISTICA 5.0 software (Statsoft, USA) were used for the regression analysis and graphical analysis of the data, respectively.

Validation of the experimental model

Ethanol production by *S. cerevisiae* TISTR 5596 in the optimized medium was performed to validate the obtained experimental model. The obtained result was then used to confirm the result derived from the RSM analysis.

RESULTS AND DISCUSSION

Chemical composition of the dilute molasses

The chemical composition of the dilute molasses, 192 g/l fermentable sugars, used for preparation of the fermentation medium is shown in Table 3.

Ethanol production from the molasses medium

Fermentation of the molasses medium by *S. cerevisiae* TISTR 5596 (9.6×10^8 cells/ml) yielded a maximum ethanol level (85.35 g/l) at 72 h of incubation (Fig. 1).

Table 3 Chemical composition of the dilute molasses[†]

Composition	Concentration	Method
Ca	2.8 g/l	Inhouse method based on AOAC(2010),984.27,975.03
Cu	nd*	
Zn	2.1 mg/l	
Mn	19 mg/l	
K	9.8 g/l	
P	210 mg/l	
N	2.8 g/l	Inhouse method based on AOAC(2012),991.20
Mg	1.2 g/l	Inhouse method based on AOAC(2010),984.27,975.03
Volatile acid (Acetic acid)	5.5 g/l	AOAC(2010),935.57,942.15
Non-volatile (Lactic acid)	8.2 g/l	
Sucrose	112.8 g/l	Asean Manual of Food Analysis (2011) pp 27–32
Glucose	nd**	
Fructose	96.5 g/l	
HMF	0.4 g/l	HPLC

[†] nd = not detectable; limit of detection: * 3.6×10^{-4} g/l, ** 1 g/l; HMF = hydroxymethylfurfural.

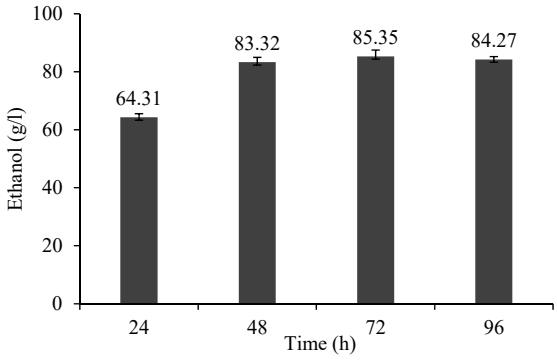

Fig. 1 Ethanol production in molasses medium by *S. cerevisiae* TISTR 5596.

Full-factorial design screening for nutrient supplements to the molasses medium that affect the ethanol production level

To evaluate the nutrient supplements in the molasses medium that had a significant improvement effect on the ethanol production level, the ethanol fermentation of the molasses medium was optimized using a full-factorial design of four variables ($(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and yeast extract) with 16 runs under two levels of each variable (Table 4). From the obtained data the effect of each variable was calculated and the significance of each variable was determined by F-test (Table 5). The F-values for $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ were highly significant with a

Table 4 Full-factorial design for screening of molasses medium-ingredient significantly affected on ethanol production.

Run no.	X_1	X_2	X_3	X_4	Ethanol (g/l)
1	−	−	−	−	76.92 ± 0.93
2	+	+	+	+	85.03 ± 0.36
3	+	−	−	−	80.25 ± 0.48
4	−	+	−	−	80.25 ± 1.16
5	−	−	+	−	79.18 ± 0.62
6	−	−	−	+	79.31 ± 0.25
7	+	+	−	−	80.03 ± 0.44
8	−	+	+	−	80.03 ± 0.06
9	−	−	+	+	81.05 ± 0.42
10	+	−	−	+	79.16 ± 0.32
11	+	−	+	−	81.18 ± 0.07
12	−	+	−	+	80.27 ± 0.30
13	+	+	+	−	85.21 ± 0.38
14	+	+	−	+	82.12 ± 0.57
15	+	−	+	+	82.48 ± 0.15
16	−	+	+	+	84.15 ± 1.22

$X_1 = (NH_4)_2SO_4$; $X_2 = KH_2PO_4$; $X_3 = MgSO_4 \cdot 7H_2O$; X_4 = yeast extract.

Table 5 Effect estimated for ethanol production from the results of full-factorial design.

Variable	Parameter	Mean square	F-value	p-value
X_1	$(NH_4)_2SO_4$	12.781	10.721	0.007
X_2	KH_2PO_4	19.272	16.166	0.002
X_3	$MgSO_4 \cdot 7H_2O$	25.000	20.970	0.001
X_4	yeast extract	6.917	5.802	0.035

confidence level of $\geqslant 99\%$. Thus the $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ level significantly affected the ethanol production level but yeast extract (at the tested levels) did not improve the ethanol production. Indeed, the maximum ethanol level (85.2 1 g/l) was produced in medium with no added yeast extract, and was almost the same as that of molasses medium (85.03 g/l) (Table 4).

Optimization of the modified molasses medium (without yeast extract) by RSM

The concentrations of $(NH_4)_2SO_4$ (X_1), KH_2PO_4 (X_2), and $MgSO_4 \cdot 7H_2O$ (X_3), as the three independent variables that had a significant effect on the ethanol production level according to the full-factorial result, were varied to maximize the ethanol production by a CCD of RSM. The correspondence between the coded levels and the real levels of the variables are shown in Table 2. The structure of the experiment, and the observed and predicted

Table 6 Experimental design used in response surface method of 3 independent variables; $(NH_4)_2SO_4$ (X_1), KH_2PO_4 (X_2), $MgSO_4 \cdot 7H_2O$ (X_3); observed and predicted ethanol production.

Run	Code-setting level			Observed	Predicted
no.	X_1	X_2	X_3	(g/l)	(g/l)
1	−1	−1	−1	83.09 ± 0.85	82.62
2	−1	−1	1	78.59 ± 1.25	80.14
3	−1	1	−1	87.17 ± 0.98	85.77
4	−1	1	1	84.50 ± 0.92	83.22
5	1	−1	−1	79.30 ± 1.57	77.97
6	1	−1	1	74.31 ± 1.92	76.10
7	1	1	−1	82.83 ± 1.04	81.67
8	1	1	1	78.86 ± 1.47	79.72
9	−1.68	0	0	83.05 ± 0.70	84.20
10	1.68	0	0	79.05 ± 2.37	77.36
11	0	−1.68	0	81.58 ± 0.91	79.07
12	0	1.68	0	82.81 ± 1.27	84.76
13	0	0	−1.68	82.39 ± 0.71	83.82
14	0	0	1.68	80.30 ± 2.22	78.87
15	0	0	0	85.12 ± 0.80	84.97
16	0	0	0	85.11 ± 0.79	84.97
17	0	0	0	85.11 ± 0.79	84.97

Table 7 ANOVA for the regression model representing ethanol production[†]

Model	SS	df	MS	F-value	p-value
Model	471.881	9	52.431	13.702	0.000
Residual	156.883	41	3.826		

[†] $R^2 = 0.750$; $R = 0.866$; SS, sum of squares; df, degree of freedom; MS, mean square; significance level at 99%.

Table 8 Regression coefficients and their significances for ethanol production from results of CCD experimental design.

Term	Coefficient	t-statistic	p-value[†]
(Constant)	74.618	23.106	0.000
X_1	3.405	2.018	0.050**
X_2	5.773	3.421	0.001*
X_3	6.249	1.842	0.073
X_1^2	−1.485	−4.480	0.000*
X_2^2	−1.081	−3.260	0.002*
X_3^2	−6.001	−3.876	0.000*
X_1X_2	0.136	0.340	0.735
X_1X_3	0.303	0.380	0.706
X_2X_3	−0.035	−0.044	0.965

[†] **Significant at 5% level ($p < 0.05$); *significant at 1% level ($p < 0.01$).

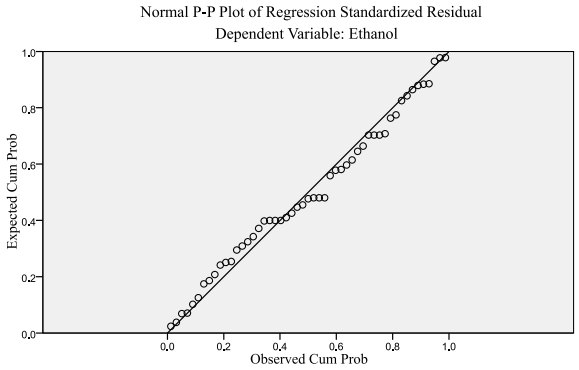

Fig. 2 Observed ethanol production versus predicted ethanol production under the optimized condition.

(from the second-order model) results are shown in Table 6. Seventeen experiments were performed in triplicate and the central point was repeated three times to allow the determination of the standard error. The CCD experimental results and regression analysis followed a second-order polynomial equation, with the ethanol production level as an empirical function of the test variables in coded units as

$$Y = 74.618 + 3.405X_1 + 5.773X_2 + 6.249X_3 + 0.136X_1X_2 + 0.303X_1X_3 - 0.035X_2X_3 - 1.485X_1^2 - 1.081X_2^2 - 6.0013X_3^2, \quad (1)$$

where Y is the predicted response (ethanol production) and X_1, X_2, and X_3 are the coded values of $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7\,H_2O$, respectively. The statistical significance of Eq. (1), as evaluated by the Fisher's F-test ANOVA for the response surface quadratic model, is shown in Table 7. It is evident that the model was significant ($p < 0.001$) at 99% confidence level and that the model was suitable to use in the experiment [10]. The model did not show a lack of fit and presented a determination coefficient (R) of 0.866 [12]. From the R^2 value (0.750), the model could explain about 75.7% of the actual variance. The student t-distribution and the corresponding p-value, along with the parameter estimated, are shown in Table 8, where X_2 and X_1 clearly had significant effect at 99% and 95% confidence levels, respectively, for a linear effect on the response (and thus on ethanol production). The quadric term of these three variables (X_1^2, X_2^2, and X_3^2) also had a significant effect at 99% confidence level and so on ethanol production.

The normal P–P plot of the regression standardized residual (Fig. 2) demonstrated a good agreement between the experimental and predicted ethanol production levels, the latter obtained from the empirical model using Eq. (1), over the tested range of each variable.

Localization of the optimal concentration of important nutritional variables

The optimal value of each of the three important nutritional variables was determined from the contour and response surface plots. The effect of the $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7\,H_2O$ concentration on ethanol production when that for KH_2PO_4 was fixed at its middle value (2 g/l) revealed the optimal $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7\,H_2O$ concentration range was 0.9–1.2 g/l and 0.4–0.6 g/l, respectively, (Fig. 3a). Likewise, the effect of the $(NH_4)_2SO_4$ and KH_2PO_4 concentration on the ethanol production level when $MgSO_4 \cdot 7\,H_2O$ was fixed at its middle level (0.75 g/l) revealed that the optimal $(NH_4)_2SO_4$ and KH_2PO_4 concentration range was 1.0–1.3 g/l and 2.8–3.3 g/l, respectively, (Fig. 3b). Finally, for the effect of the KH_2PO_4 and $MgSO_4 \cdot 7\,H_2O$ concentration on the ethanol production level with $(NH_4)_2SO_4$ fixed at its middle level (2 g/l), the optimal KH_2PO_4 and $MgSO_4 \cdot 7\,H_2O$ concentration range was 3.0–3.5 g/l and 0.4–0.6 g/l, respectively, (Fig. 3c). From these 3D response surface plots and their corresponding contour plots, the optimal values of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4 \cdot 7\,H_2O$ were deemed to be 1.3, 2.7, and 0.5 g/l, respectively, with a predicted maximum ethanol production of 86.49 g/l.

Validation of the experimental model

The theoretically determined optimal value of the key three nutritional variables, $(NH_4)_2SO_4$ at 1.3 g/l, KH_2PO_4 at 2.7 g/l, and $MgSO_4 \cdot 7\,H_2O$ at 0.5 g/l to yield a predicted ethanol production of 86.49 g/l, was tested by performing the fermentation of the dilute molasses supplemented with these concentrations of $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7\,H_2O$. This yielded an ethanol level of 87.28 g/l, which is close to the predicted ethanol production level. The non-optimized molasses medium contained nutrients, $(NH_4)_2SO_4$, KH_2PO_4, and $MgSO_4 \cdot 7\,H_2O$, which significantly affected the ethanol production and their concentrations were close to those of the optimized molasses medium. This might be a reason of slightly increase of ethanol production level after the optimization. In fact the dilute molasses is supplemented with only $(NH_4)_2SO_4$ in industrial fuel ethanol production. Based on this study, it yielded ethanol 80.25 g/l.

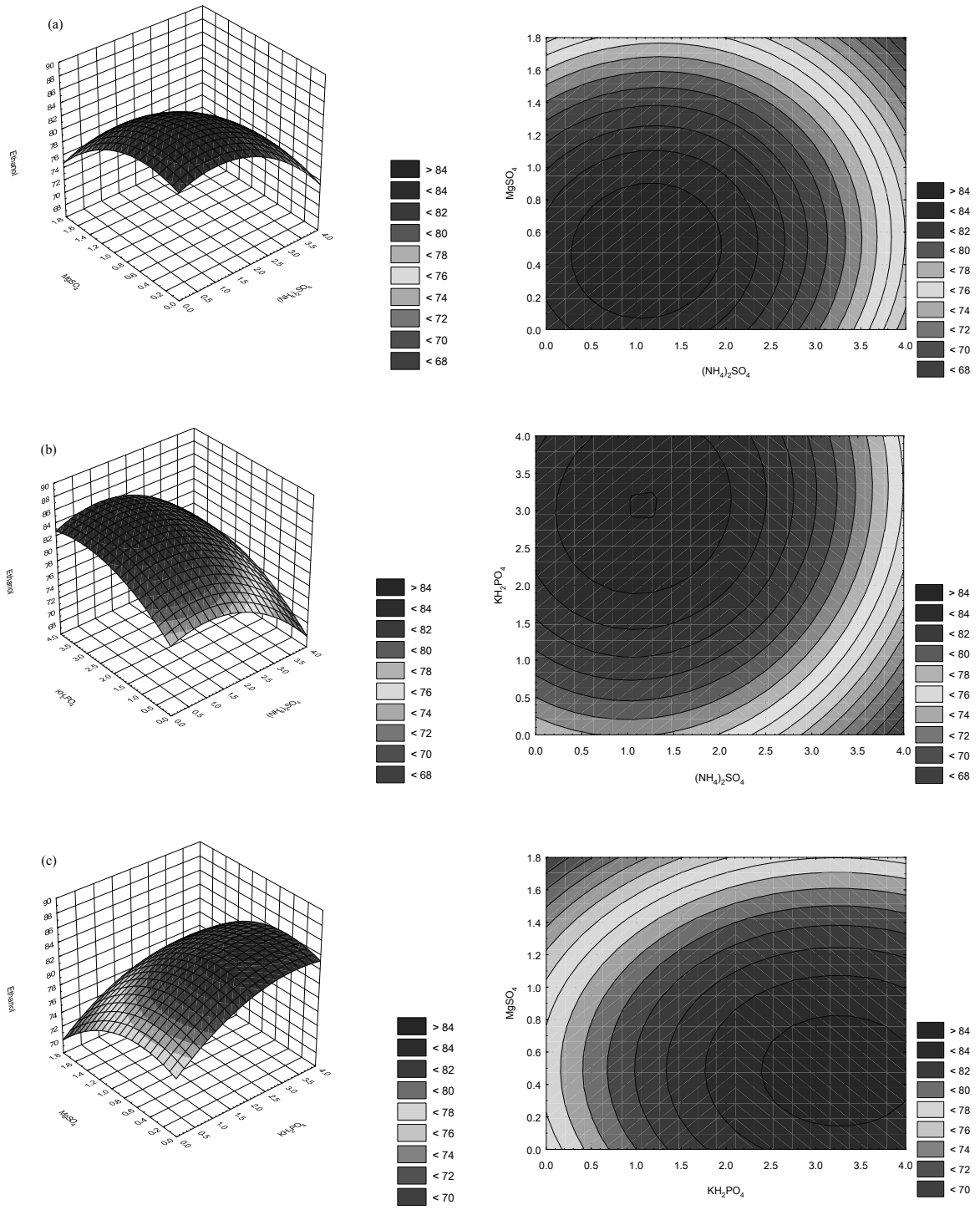

Fig. 3 Response surface and contour plots described by model, representing ethanol production (g/l) as the combined effects of (a) $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$, (b) $(NH_4)_2SO_4$ and KH_2PO_4, and (c) KH_2PO_4 and $MgSO_4 \cdot 7H_2O$.

Further addition of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ or the non-optimized molasses medium increased the ethanol level to 85.21 g/l (Table 4). Hence the RSM optimization further increased the ethanol level to 87.28 g/l equating to 8.76% increase of ethanol above the fermentation of molasses supplemented with only $(NH_4)_2SO_4$, indicating the importance of K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ supplementation.

Molasses contains some amount of Ca^{2+} and this Ca^{2+} precipitates the phosphate and Mg^{2+} present in the molasses. Although this phenomenon removes Ca^{2+} which inhibits *S. cerevisiae* invertase activity, it also removes phosphate and Mg^{2+} which are important for the growth and vitality of the yeast, being involved in metabolic and bioenergetic pathways. Indeed, Mg^{2+} is known to activate over 300 different enzymes. Furthermore, the ethanol produced during the fermentation causes an alteration in the yeast cellular membrane lipids and dysfunction of the H^+-ATPase activity, leading to the leakage of cellular components, including phosphate, and inhibition of the membrane transport systems, including the uptake of nutrients like Mg^{2+} [13]. Hence it was necessary to supplement the phosphate and Mg^{2+} levels in the molasses to obtain the optimal ethanol production by the yeast.

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