Optimization of fermentation conditions for the production of the mezcal from *Agave salmiana* using response surface methodology

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Abstract

Response surface methodology was applied to optimize the fermentative phase for the mezcal production from *Agave salmiana*, a Mexican alcoholic beverage. A second order and complete factorial design was used to obtain models describing the relationship between the ethanol production, process productivity, and product yield with respect to the fermentation temperature and the initial sugar concentration. The results shown that the fermentative conditions affected the composition of higher alcohols (referred as quality indicator) in the mezcal as well as the amount of ethanol. The highest ethanol production was attained by employing the following predicted optimum operational conditions: temperature of 28°C and an initial sugar concentration of 105 g/l. However, the maximum productivity process was attained with 34.6 °C and 90 g/l, whereas the maximum product yield and the best mezcal at 28 °C and 77 g/l. Results shows that the simultaneous optimization for high alcohol production and fast production rate are not compatible, since high alcohol production requires a high substrate concentration, which inhibited the growth rate.

KEYWORDS: alcoholic fermentation, optimization, response surface methodology, spirits, substrate inhibition.

INTRODUCTION

Mezcal is an alcoholic beverage obtained through the fermentation and distillation of the *Agave* syrup. Species of *Agave* plants such as *Agave salmiana, A. angustifolia* Haw, *A. potatorum, A. durangensis* and others are used as raw materials [1]. However, only wild-type plants of *A. salmiana* are used in the Mexican altiplano. The mezcal production process includes five phases: cooking, milling, fermenting, distilling and aging. During the cooking phase, the raw material is softened to make easy the milling phase, the inuline and other fructo-oligosacharides are hydrolyzed to single sugars (mainly fructose), and some other organic compounds are generated by the Maillard reaction [2]. Events during each one of the mezcal production phases have the potential to affect the final quality and yield, thus the need to evaluate them. However, special attention must be given to the fermentative phase, which produces the ethanol and other compounds that directly define the main characteristics of mezcal. Factors such as initial sugar concentration and temperature are important variables on the fermentation and they could modify the ethanol production (referencias).

The goal of this work was the optimization of the fermentative phase for improving the mezcal production. Response surface methodology and a 3k full factorial design were used to determine the influence of temperature and sugar concentration on the mezcal production from *A. salmiana*.

EXPERIMENTAL

2.1 Culture medium and fermentation conditions

The *Agave* syrup from *A. salmiana* and inocula were kindly provided by Juan Zarur. *Agave* syrup was centrifuged at 7000 *g* for 10 min and pasteurized at 65°C. Batch cultures were carried out in Erlenmeyer

flasks containing 1000 ml of *Agave* syrup with 1 g/l of ammonium sulfate. The syrup was inoculated at an initial optical density (OD_{620nm}) of 0.1 and incubated in a water bath at constant temperature according the experimental design (described below). The initial sugars concentration and temperature were fixed according to the experimental design described below. Potential redox was monitored with an autoclaveable redox electrode (Applikon, Schiedam, The Netherlands) and data were registered in a PC interfaced with potentiometer (B&C Electronics, Italy) using a RS232 port. Broth samples were harvested each hour and centrifuged at 5,000 *g* for 5 min; supernatants were collected and stored at 4 ºC for further analysis.

2.2 Kinetic studies and determination of the fermentation parameters

The fermentations were carried out using the same procedure described in the secc. 2.1 using different initial concentration of sugar (between 0 and 200 g/l) and temeprature of 32.5°C. The ethanol production (*EP*) was defined as the amount of ethanol produced by litter of culture media at the end of the exponential phase. The specific growth rate was determined by linear regression of the plot Ln *X* versus time, at the exponential growth phase. The productivity process (*PP*) was defined as the amount of ethanol produced by liter and per hour and the process yield ($Y_{P/S}$) was deffined as the amount of ethanol produced by amount of sugar consumed. The process parameters were obtained as follow:

$$PP = \frac{EP}{t} \tag{1}$$

$$Y_{P/S} = \frac{P_f - P_i}{S_i - S_f} \tag{2}$$

Where PP is the productivy process ($g_{ethanol}$/l-h), EP the production of ethanol ($g_{ethanol}$/l), t the time (h), $Y_{P/S}$ is the process yield ($g_{ethanol}/g_{susbstrate}$), P_f the final concentration of ethanol ($g_{ethanol}$/l) and P_i is the initial concentration of ethanol ($g_{ethanol}$/l), S_f the final sugar concentration (g/l), S_i the initial sugar concentration (g/l).

The data of specific growht rate were fitting to the inhibition by substrate model descrived by the eq. (3) and the terms were obtained using Solver algoritm from Excel^{TM} (Microsoft Co.).

$$\mu = \frac{\mu_{max} S}{k_s + S + \frac{S^2}{k_i}} \quad (3)$$

Where μ is the specific growth rate (h^{-1}), S is the initial concentration of sugar (g/l), k_s is the saturation constant (g/l), k_i is the inhibition constant (g/l) and μ_{max} is the maximum specific growth rate (h^{-1}).

2.3 Experimental design

A two-factor factorial experimental design was used to determine the influence of initial sugar concentration (factor A) and temperature (factor B) as independent variables on the fermentation process for mezcal. The treatments were arranged according to a factorial 3^2 designs; and they were carried out in duplicates as independent experiments in order to account for non-adjustable data and allow the calculation of the analysis of variance (ANOVA). The treatments were applied randomly in a complete blocks experimental design (Table 1). A 3k full factorial design was selected, since the expected model has curvature, due to quadratic termin from subtrate inhibition model. Furthermore, having a wide interval of interest in initial sugar concentration (35-105 g/l) justifies the use of the third level in the experimental design (3k instead of 2k), because it is known that the wider the interval of the

factor to study, the greater the variability of the results.,then the a model with curvature in the response surface due to the addition of a third level in the factors (Montgomery 2004). Statistical analysis was performed with Statgraphics v 5 (Manugistics Inc. Rockville) software according to Montgomery [3]. The quadratic model for predicting the optimal value was expressed according to following equation 4 [Montgomery]:

to fit a polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (4)$$

Where Y is the response variable, X_1, X_2 and X_3 are independent variables for temperature, substrate concentration induction, respectively. β_0 is the intercept term, β_1 and β_2 are linear coefficients, β_{12} is the interactive coefficients, and β_{11} and β_{22} are quadratic coefficients. The model was evaluated with significance, good fit and R^2 values. The Eq. 4 was used to build surfaces graphs for the model. The interaction of one factor with the others was studied using the three-dimensional plots. The analysis of RSM, analysis of variance (ANOVA) and the optimal conditions were identified using with Statgraphics v 5 (Manugistics Inc. Rockville) software according to Montgomery [3]. The optimal values were obtained solving the regression equation (4) by the Newton-Raphson method and analyzing the response surface contour (De León et al. 2004 and 2007). The adjusted models for ethanol production (EP), productivity process (PP) and product yield ($Y_{p/s}$) were evaluated by the F-test from ANOVA. The significant effects on dependent variables were determined by T-test with a probability value (P-value) smaller than 0.05.

2.3 Distillation procedure

The fermented *Agave* syrup was distilled using an eight-plats Vygrux column (SEV, Puebla, Mexico) and a heating mantle (Electrothermal, UK). The distillation temperature was monitored with a thermo-par (Hanna Inst. Italy). Two fractions were collected, the first one (rich in methanol) collected below 68°C was discarded and the second fraction (rich in ethanol) was collected in the range of 68 to 85°.

2.4 Analytical methods

Biomass concentration was determined from OD_{620nm} using a spectrophotometer Cary Bio-50 (Varian Inc., Australia) and converted to dry cell weight (DCW) with a standard curve. Reducing sugar concentration was determined by the dinitro-salicilic acid (DNS) method using fructose as standard [4]. The concentration of ethanol and other major compounds of mezcal (substances with concentration larger than 10 mg/l) were measured in a gas chromatograph 6890N (Agilent technologies, Wilmington, DW) equipped with a FID detector, an auto-sampler 7863 (Agilent technologies, Wilmington, DW) and a capillary column HP-Innowax (30 m x 0.25 mm i.d., 0.25 µm film thickness; Agilent technologies, Wilmington, DW). The analytical conditions have been described elsewhere [5]. All samples were analyzed in duplicates, and average of each compound concentration was used for comparing the different fermentative conditions.

RESULTS AND DISCUSSION

Kinetic studies

Kinetic behavior of the batch culture for the treatment 6 at an initial sugar concentration of 70 g/l and temperature of 37°C is shown in Fig. 1. Cultures at other culture conditions showed similar trends as

1 those in Fig. 1, although rates of the various parameters measured, their maximum concentrations, and
2 times to reach them were different in each case. For the culture of the treatment 6, the cell concentration
3 increased exponentially at a specific growth rate of 0.32 h^{-1}. Since the culture is non-axenic, the
4 observed specific growth rate is the average of growth rates of all type of microorganisms. Biomass
5 reached a maximum of 1.04 g/l, and thereafter it remained constant (Fig. 1A). In all cultures, ethanol
6 concentration showed a growth-associated behavior and the maximum ethanol concentration attained
7 was 23 g/l for this culture (Fig. 1B). The redox potential decreased from +135 to -163 mV and followed
8 an inverse relationship with respect to ethanol production (Fig. 1B). These results shown that
9 measurement redox potential could be used for a rapid and on-line surrogate determination of ethanol
10 during mezcal fermentation and other alcoholic beverages produced from different *Agave* plants.
11 Similar to the results obtained here, Berovic et al., reported that during the fermentation of cabernet
12 sauvignon must, the redox potential decreased from +190 to -240 mV a culture temperature of 26°C,
13 while at 18°C it decreased from +190 to -90 mV. They concluded that the must fermented at 26°C was
14 converted into a more stable and reductive environment [6].
15
16 The influence of initial sugar concentration on specific growth rate (μ) is shown in Fig 2. As sugar
17 concentration increased, μ followed a substrate inhibition-type fashion. The maximum specific growth
18 rate (μ_{max}), saturation constant (K_s) and inhibition constant (K_i) were 0.6 h^{-1}, 16.82 g/l and 47.78 g/l,
19 respectively. Catabolite inhibition of enzymes in the fermentative pathway becomes important at higher
20 substrate concentrations, indicating the onset of substrate inhibition as a result of high osmotic pressure
21 and low water activity [7]. Thatipamala et al. reported a substrate inhibition above 150 g/l for yeast
22 cultures during ethanol batch fermentation at 30°C using a minimum medium with yeast extract [8]. In
23 our case, the substrate inhibition was observed above 40 g/l. Since we used a complex medium, perhaps

other compounds present in the *Agave* syrup, such as furfural, Maillard products and saponins may exert an additional inhibitory effect on the cell growth [9-11].

Optimization of fermentation conditions

Table 1 shows the summary of results for *EP*, *PP* and $Y_{p/s}$. The *EP* values varied in the range of 12.36±0.17 to 37.68±0.11 g/l for the treatments 2 and 7 respectively. The analysis of variance for the adjusted model showed that *EP* was significantly affected only by initial sugar concentration (Table 2). The second-order equation with *EP* as a function of temperature and initial sugar concentration is descrived by the eq (5):

$$EP = -48.779 + 0.641529\,A + 2.76363\,B - 0.00113331\,A^2 - 0.00523595\,AB - 0.0401\,B^2 \quad (5)$$

The standard error was 2.1536 and the correlation coefficient (R^2) was 96.6%. These values indicate a good fit between the model and the experimental data and can explain the majority of variance in the EP. Applying the Newton-Raphson method to Eq. (5), the highest predicted EP of 36.63 g/l is attained when temperature and initial sugar concentration were 28°C and 105 g/l, respectively. Fig. 3 shows the predicted dependence of EP on the temperature and initial sugar concentration, based on equation (5). A maximum or minimum response could not be observed within the range of study. Thus, increasing sugar concentration in the culture medium yield an increment of ethanol production.

The PP values varied in the range of 0.95±0.09 to 2.21±0.08 g/l-h for the treatments 1 and 5, respectively. The analysis of variance for the adjusted model showed that PP was significantly affected

by A, B, A^2 and B^2 (Table 3). The mathematical model representing PP as a function of A and B in the experimental region studied is expressed by Eq. (6).

$$PP = -15.9837 + 0.0437542\ A + 0.938191\ B - 0.000261204\ A^2 + 0.0000951587\ AB - 0.0136975\ B^2 \quad (6)$$

The standard error of the model was 0.14823 and according to R^2 value, the predictors included in the model explain 92.5% of the variance in PP. Applying the Newton-Raphson method to Eq. (6) the maximum predicted PP of 2.2 g/l-h is attained when temperature and initial sugar concentration were 34.6°C and 90 g/l, respectively. Fig. 4 shows the predicted dependence of PP on the temperature and initial sugar concentration, based on equation (6). This figure shows that, both linear and quadratic coefficients of temperature and sugar concentration affected the PP. Also, a maximum response can be observed within the range of study. Chen, observed that during the alcoholic fermentation from glucose syrup using *Saccharomyces cerevisiae*, the highest alcohol productivity was 21 g/l-h at substrate concentration of 12°Brix (approx. 120 g/l). However, the ethanol production was only 6 % by weigh [12]. In our case we observed a maximum PP of 2.2 g/l-h, perhaps the low productivity attained here is the result of the inhibitory effect of other compounds in the *Agave* syrup.

The $Y_{P/S}$ values varied in the range of 0.27±0.01 to 0.46±0.02 for the treatments 2 and 5 respectively. The analysis of variance for the adjusted model showed that $Y_{P/S}$ was significantly affected by both the linear (A) and quadratic (A^2) terms of the initial sugar concentration (Table 4). The mathematical model representing $Y_{P/S}$ in the range of study is expressed by Eq. (7):

$$Y_{P/S} = 0.0729124 + 0.0134648A - 0.0078323B - 0.000083217A^2 - 0.000023730AB + 0.0001226B^2 \quad (7)$$

The standard error of the model was 0.02492 and according to R^2 value, the predictors included in the model explain 89.5% of the variance in $Y_{P/S}$. In this case, the maximum predicted $Y_{P/S}$ of 0.44 was attained when temperature and initial sugar concentration were 28°C and 77 g/l, respectively. Fig. 5 shows the predicted dependence of $Y_{P/S}$ on the temperature and initial sugar concentration, based on eq (7). It can be observed that, lineal and quadratic coefficients of sugar concentration affected the $Y_{P/S}$ and a maximum response was observed within the range of study. Since, the importance of ethanol as liquid fuel, several reports about the optimization of fermentation conditions for the ethanol production have been published [12-15]. Criteria such as yields, productivity and ethanol production can be used to evaluate alcohol fermentation. However, multiple optimization is not easy. For instance, high alcohol production and fast production rate are not compatible because the first one requires high substrate concentration, which in turn inhibits the growth rate. The Response surface methodology (RSM) has been successfully applied to the optimization of medium composition [He, 2004], gene expression [Teresita, 2007, De Leon, 2003], and parameters of food preservation and fermentation process [Ratnam, Bandaru, 2006].

Effect of temperature and sugar concentration on mezcal composition

Table 5 shows a summary of the concentration of volatile compounds present in mezcal obtained by distilling the *Agave* syrup fermented under the treatments described in the experimental design. In all cases the ethanol concentration was set at 36 % (v/v) according to an Official Mexican Norm [1]. It can be observed that the composition of volatile compounds in mezcal depends on the fermentative

conditions. Methanol is produced from pectin and lignin present in the vegetal-cell wall [16], whereas higher alcohols such as propanol, n-butanol, 2-methyl-propanol, 2/3 methyl-1-butanol are produced by the catabolism of amino acids [17]. The culture conditions may affect the microbial dynamic and metabolic pathways resulting in mixtures of alcohols with different composition. Higher alcohols concentration is a quality indicator used during tequila production [18], grape wine [6] and spirits obtained from Jerusalem artichoke [19] because they contribute to organoleptic properties and the bouquet of alcoholic beverages, then we used the same criteria as indicator of quality for mezcal. The amount of higher alcohols obtained in mezcals produced under the different fermentative conditions varied in the range of 201 to 313 mg/l, for the treatments 3 and 4 respectively. Thus mezcal obtained at 28°C and 70 g/l was the best mezcal. Pinal et al., reported that the type of yeast strain, temperature and C/N ratio had a significant influence in the level of higher alcohols produced for tequila production [18]. It has been reported that propanol is produced by *Lactobacillus* genus [20], whereas other higher alcohols are produced by yeasts such as *S. cerevisiae, Pichia fermentans* and others [21]. The origin of ethyl 2-hydroxypropanoate and ethyl acetate is not clear; they can be produced by *Lactobacillus* or by extra-cellular esterification reaction [22]. The major compounds are the main responsible in conferring aroma and organoleptic properties to the mezcal. Therefore, differences on the composition of alcoholic beverages result from differences in the microbial community and their metabolism during the fermentation phase. Further identification of microorganisms involved on the fermentative phase, and the subsequent selection of main strains may provide a better understanding of the process and a better production of the mezcal as well.

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Figure captions

Figure 1. Behavior of a typical batch culture for mezcal production at an initial sugar concentration of 70 g/l and temperature of 32.5°C. A) Biomass concentration (●), reducing sugar conc. (○). B) Ethanol conc. (■), redox potential (Δ).

Figure 2. Effect of initial sugar concentration on specific growth rate at 32.5°C. Line draws the substrate inhibition-type fashion with a maximum specific rate (μ_{max}), saturation constant (K_s) and inhibition constant (K_i) of 0.6 h^{-1}, 16.82 g/l and 47.78 g/l, respectively.

Figure 3. Dependence of ethanol production on the temperature and initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

Figure 4. Dependence of productivity process on the temperature and initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

Figure 5. Dependence of product yield on the temperature and initial sugar concentration in the alcoholic fermentation of syrup from *A. salmiana*.

Table 1. Experimental design and summary of results for dependent variables.

	Independent variable		Dependent variable		
Treatment No.	**Factor A (g/l)**	**Factor B (°C)**	***EP* (g/l)**	***PP* (g/l-h)**	$Y_{P/S}$ **(g/g)**
1	35	28.0	14.26 ± 1.28	0.95 ± 0.09	0.30 ± 0.01
2	35	32.5	12.36 ± 0.17	1.24 ± 0.02	0.27 ± 0.01
3	35	37.0	13.14 ± 0.64	1.34 ± 0.07	0.30 ± 0.01
4	70	28.0	24.05 ± 0.57	1.35 ± 0.03	0.42 ± 0.00
5	70	32.5	28.76 ± 1.03	2.21 ± 0.08	0.46 ± 0.02
6	70	37.0	23.94 ± 1.02	1.99 ± 0.08	0.40 ± 0.00
7	105	28.0	37.68 ± 0.11	1.60 ± 0.01	0.38 ± 0.01
8	105	32.5	34.48 ± 1.34	2.03 ± 0.08	0.35 ± 0.03
9	105	37.0	33.26 ± 3.11	2.05 ± 0.19	0.36 ± 0.01

Treatments were conducted in a random order. Experimental results are averages of two independent experiments and their respective standard deviation. A: Initial sugar concentration, B: Temperature, *EP*: Ethanol production, PP: Productivity process, $Y_{P/S}$: Product yield.

Table 2. Analysis of variance for the adjusted model for ethanol production

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	*F*-Ratio	*P*-Value
constant	-48.779					
A	0.641529	81.607	1	81.607	17.59	0.0015
B	2.76363	0.493174	1	0.493174	0.11	0.7505
A^2	-0.00113331	7.70951	1	7.70951	1.66	0.2238
AB	-0.00523595	5.44055	1	5.44055	1.17	0.3020
B^2	-0.0401	2.63754	1	2.63754	0.57	0.4666
Total error		51.0201	12	4.63819		
Total (corr.)		1515.79	17			

A: Initial sugar concentration, B: Temperature, DF: Degrees of freedom, *F*: Fisher test, *P*-value: probability distribution value. The correlation coefficient (R^2) was 0.966 and the standard error was 2.1536.

Table 3. Analysis of variance for the adjusted model for process productivity

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	F-Ratio	P-Value
constant	-15.9837					
A	0.0437542	1.55297	1	1.55297	70.65	0.0000
B	0.938191	0.722114	1	0.722114	32.85	0.0001
A^2	0.000261204	0.409536	1	0.409536	18.63	0.0012
AB	0.0000951587	0.001797	1	0.001797	0.08	0.7802
B^2	0.0136975	0.307748	1	0.307748	14.00	0.0033
Total error		0.241788	12	0.0219807		
Total (corr.)		3.2404	17			

For abbreviations, see Table 2. The correlation coefficient (R^2) was 0.925 and the standard error was 0.14823.

Table 4. Analysis of variance for the adjusted model for product yield

Source	Polynomial coefficients	Sum of Squares	DF	Mean Square	*F*-Ratio	*P*-Value
constant	0.0729124					
A	0.0134648	0.0493078	1	0.0493078	79.42	0.0000
B	-0.0078323	4.3517E-7	1	4.3517E-7	0.00	0.9794
A^2	-0.000083217	0.0415684	1	0.0415684	66.96	0.0000
AB	-0.000023730	0.000111751	1	0.000111751	0.18	0.6796
B^2	0.0001226	0.0000246678	1	0.0000246678	0.04	0.8456
Total error		0.00682916	12	0.000620833		
Total (corr.)		0.0652157	17			

For abbreviations, see Table 2. The correlation coefficient (R^2) was 0.895 and the standard error was 0.02492.

Table 5. Concentration (mg/l) of volatile compounds present in mezcal produced under different fermentative conditions.

Rt[a] (min)	Compound	Treatment No.								
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
4.46	Ethyl acetate	269±1	146±0	50±1	103±2	400±7	104±0	115±8	780±1	158±2
4.63	Methanol	1795±2	1782±3	1537±37	1583±13	1648±22	1682±3	1554±105	1671±2	1640±2
6.91	n-Propanol	218±0	195±1	191±4	272±3	234±3	216±0	270±17	276±0	244±1
7.87	2-Methyl-propanol	ND[c]	ND	ND	ND	ND	ND	5±0	ND	ND
8.64	n-Butanol	ND	10±1	ND	29±5	14±6	12±2	8±3	7±0	10±2
9.62	2/3-Metyl-1-Butanol	ND	8±0	ND	12±0	9±0	9±0	13±1	14±0	12±0
11.72	Ethyl 2-hydroxypropanoate	104±1	121±1	124±3	235±3	182±1	140±1	124±8	158±1	70±0
13.26	Acetic acid	67±0	58±1	90±4	29±3	72±4	63±1	23±1	68±2	77±4
13.524	Furfuraldehyde	15±0	33±0	ND	11±0	ND	ND	ND	ND	ND
	Higher Alcohols[b]	212±0	213±2	201±17	313±3	257±3	237±3	296±15	297±0	266±1

[a]Rt: Retention time in the HP-Innowax column. [b]Sum of alcohol with three or more carbons. [c]ND: Not detectable. Ethanol concentration was fixed to 36 % v/v. Data are the average ± standard deviation of two independent experiments as described in materials and methods.

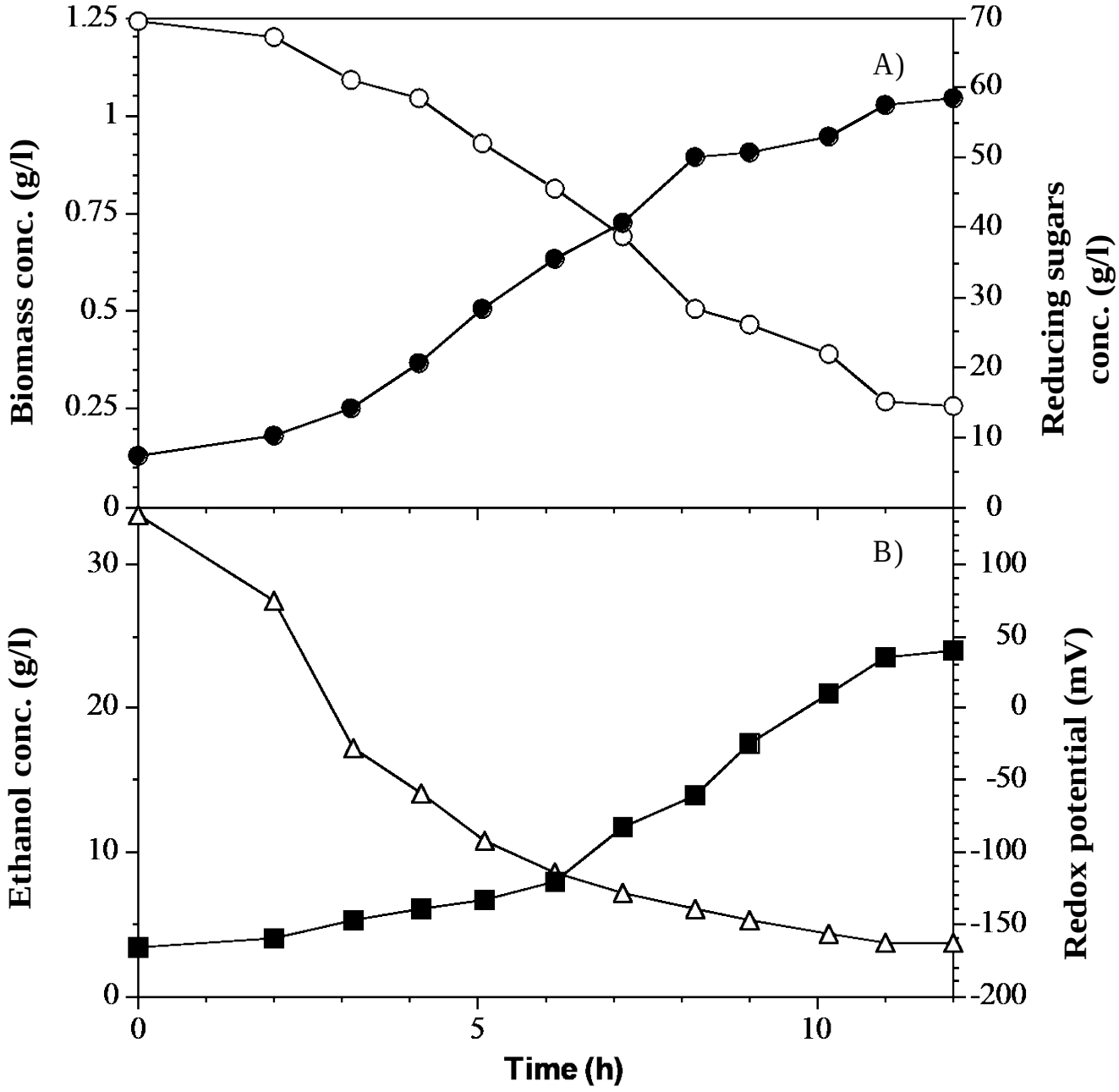
A)
B)
Biomass conc. (g/l)
Reducing sugars conc. (g/l)
Ethanol conc. (g/l)
Redox potential (mV)
Time (h)
1.25
1
0.75
0.5
0.25
0
70
60
50
40
30
20
10
0
30
20
10
0
100
50
0
-50
-100
-150
-200
0
5
10

Fig. 1

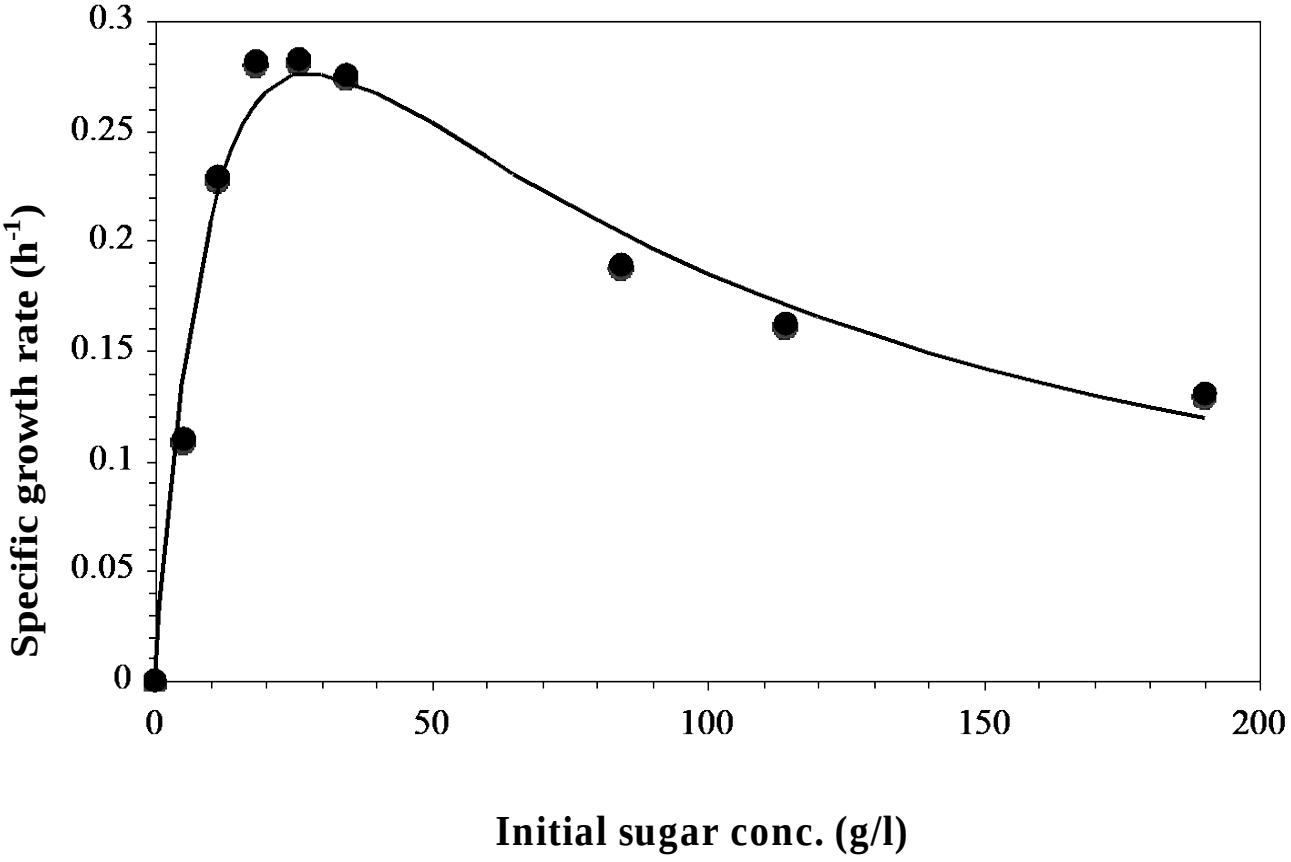
Specific growth rate (h-1)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
200
Initial sugar conc. (g/l)

Fig. 2.

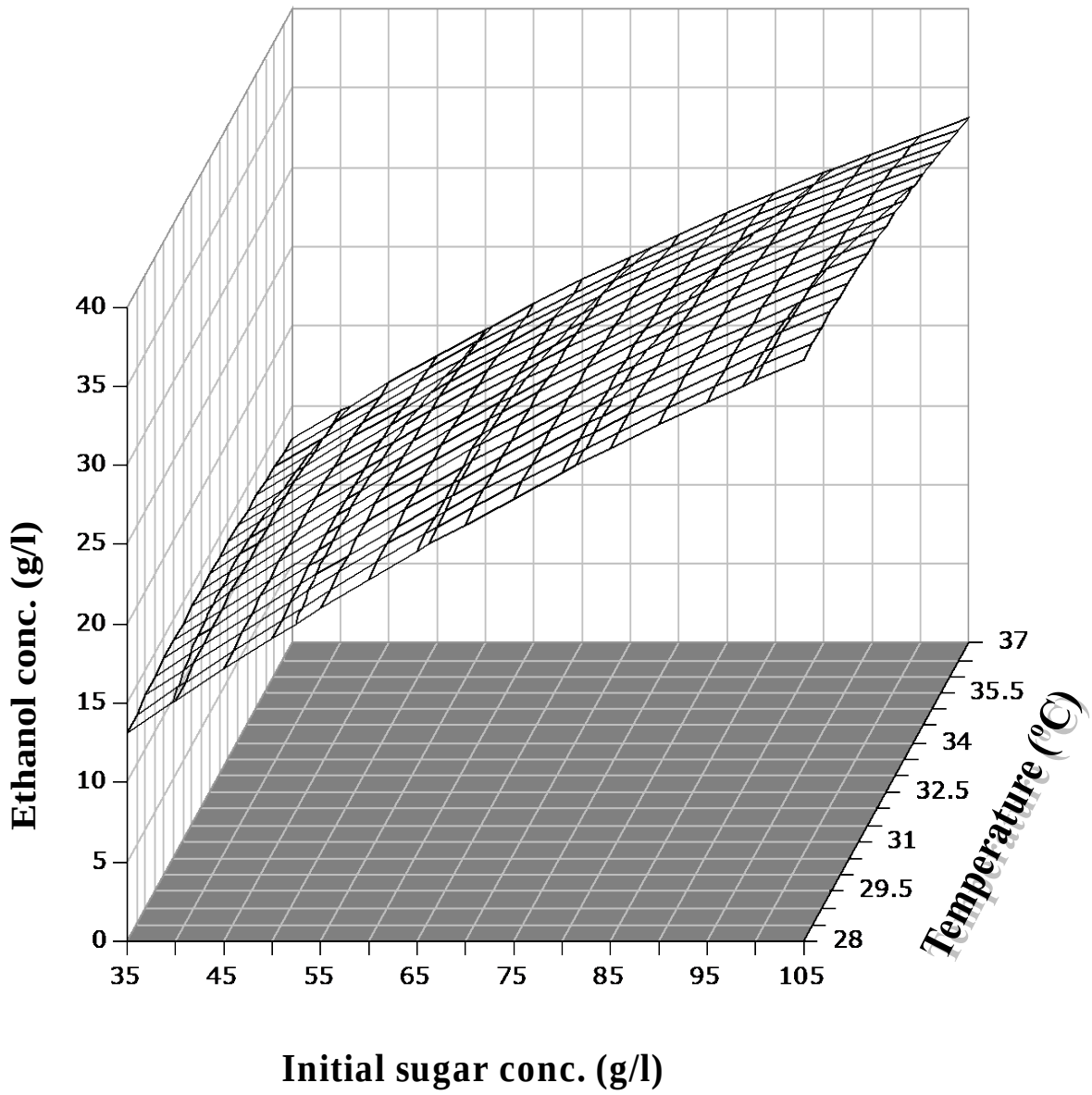
Ethanol conc. (g/l)
0
5
10
15
20
25
30
35
40
35
45
55
65
75
85
95
105
Initial sugar conc. (g/l)
28
29.5
31
32.5
34
35.5
37
Temperature (°C)

Fig. 3

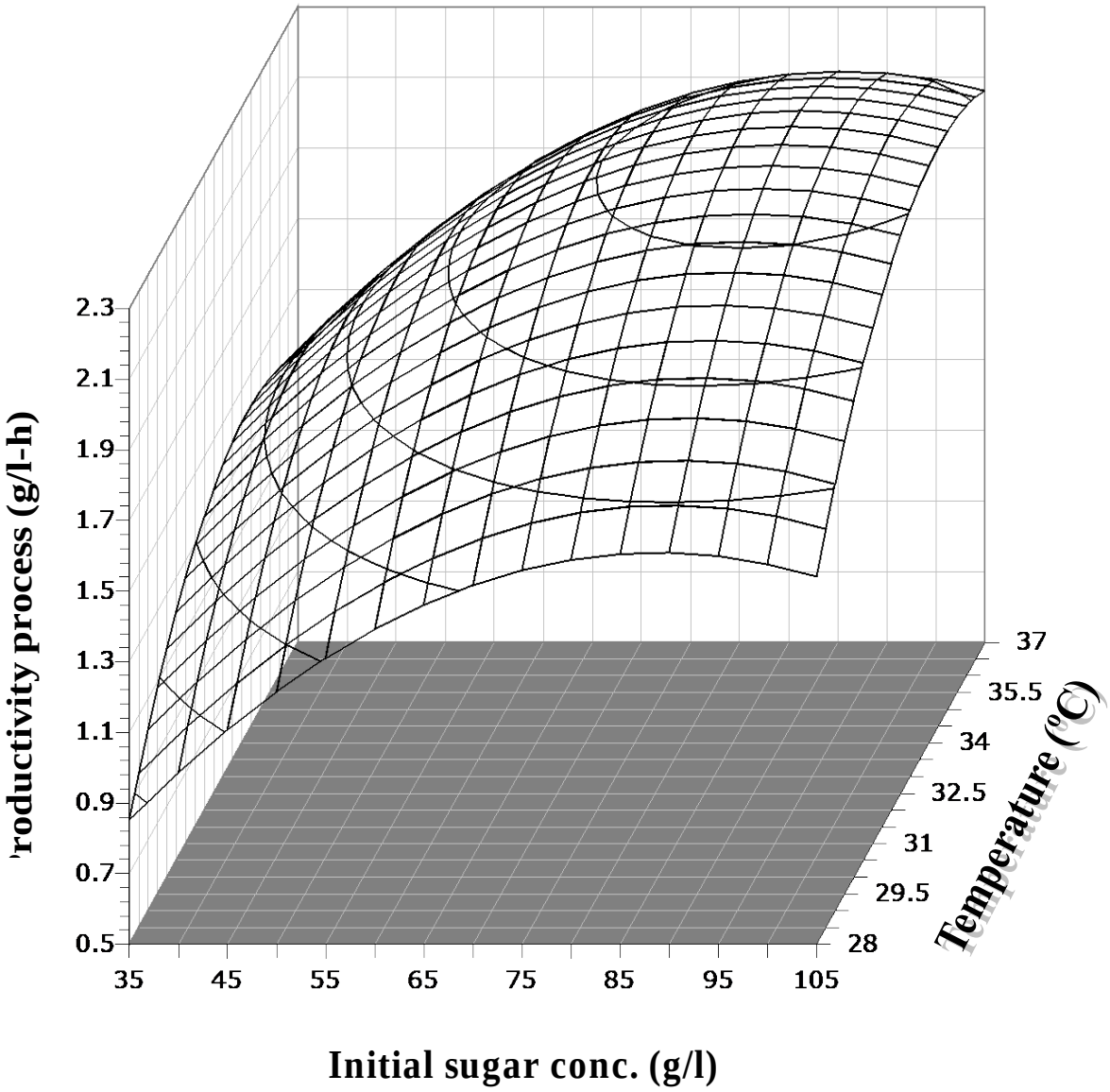
roductivity process (g/l-h)
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
35
45
55
65
75
85
95
105
Initial sugar conc. (g/l)
37
35.5
34
32.5
31
29.5
28
Temperature (°C)

Fig. 4

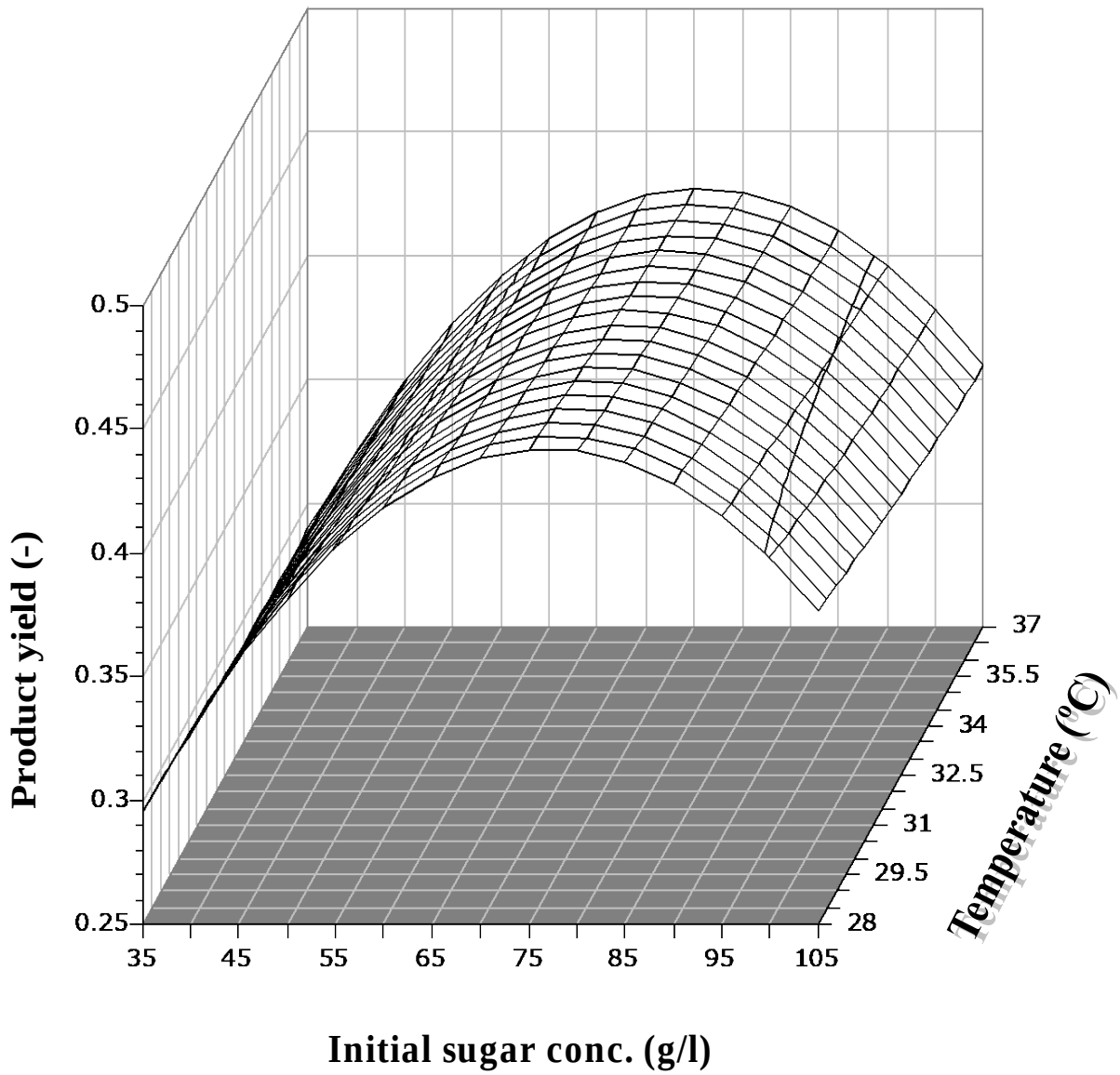
Product yield (-)
0.5
0.45
0.4
0.35
0.3
0.25
35
45
55
65
75
85
95
105
Initial sugar conc. (g/l)
37
35.5
34
32.5
31
29.5
28
Temperature (ºC)

Fig. 5