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Optimization of ethanol production from apple pomace through solid-state fermentation using enzymes and yeasts combination through response surface methodology

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Apple pomace (AP) which accounts for 25% of original fruit mass is a by-product from the apple processing industry. Solid-state fermentation of AP was conducted on laboratory scale in 250 ml flask at 30°C, agitation speed of 55 rpm at different pH levels of 4, 4.5 and 5.0 using the Y_{51} strain, *Saccharomyces cerevisiae* (SC) ATCC 9673 and their combination. The sample was treated with both α-amylase and cellulase enzyme collectively for higher reducing sugar content. Ethanol yield of 5.23% (v/v) was obtained in the case wherein strain Y_{51} was inoculum at pH of 4.5 and the fermentation period was 72 h. Response surface methodology was used to design the experiments as well as for the data analysis. Optimization of various process conditions was done using software Design-Expert 7.1.6.

Key words: Apple pomace, *saccharomyces cerevisiae*, Y_{51}, cellulase, optimization.

INTRODUCTION

Sustainable food production and waste valorisation have become important issues in modern life and are becoming important issues in the food industry. Food producers generate high amounts of biological by-products and waste that could be used for other purposes as well. The use of agro-industrial wastes in solid state fermentation is economically important and can minimize various environmental problems. The direct disposal of agro-industrial residues as a waste on the environment represents an important loss of biomass, which could be bioconverted into different metabolites, with a higher commercial value (Vendruscolo et al., 2007).

AP is the solid residue that remains after the extraction of juice from apple and its disposal as such causes considerable economic (Miller et al., 1982) and environmental (Hang and Woodams, 1986) problems. Conventional process of juice recovery removes 75% of fresh weight as juice and 25% as pomace (Vendruscolo et al., 2008). More than 500 food processing plants in India produces about 1.3 million tones of AP annually which involves annual disposal expenditure of 0.5 million US dollars (Jewell and Cummings, 1984). Hence, there is a strong need to have an integrated approach for AP waste utilization and its treatment.

Globally, several million tones of AP are generated. Owing to the high carbohydrate content, it is used as a substrate in a number of microbial processes for the production of organic acids, enzymes, single cell protein, ethanol, low alcoholic drinks and pigments (Bhushan et al., 2008). AP also serves as the potential source of

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Table 1. Selected process variables and their assigned levels.

Independent variable		Coded levels		
Name	Code	-1	0	1
		Actual levels		
Yeast strains	X_1	Y_{51}	SC	Combination
pH	X_2	4.0	4.5	5.0
Time	X_3	24	48	72

ethanol. Jain and Singh (2006) has reported the ethanol yield of 4.074% (v/v) in inoculated fermentation using Y_{51} strain at pH of 4.5 and the sample was kept for 72 h of incubation at 30°C. Kumar and Sahgal (2008) reported the ethanol yield of 5.02% (v/v) when Y_{51} was inoculated to the substrate combination of 75% AP plus 25% molasses at 72 h of fermentation.

Enzymatic hydrolysis of various feedstocks for ethanol production has been widely attempted with considerable amount of success (Aswathy et al., 2010). From the preliminary experiments it was found that AP sample when treated with the enzyme combination of α- amylase and cellulase having higher amount of reducing sugar, that is, 10.85% as compared to the samples which are treated separately. The α-amylase and cellulase concentration were calculated as 25 and 2 mg/g of dry matter in AP sample, respectively (Kumar and Wyman, 2009).

Optimisation of different parameters, by the traditional 'one-factor-at-a-time' method requires a considerable amount of time and effort. An alternative potential approach is a statistical approach, such as response surface methodology (RSM), one of the most widely used statistical techniques for bioprocess optimisation (Liu and Tzeng, 1998). Optimization includes finding "best available" values of some objective function given a defined domain including a variety of different types of objective functions and different types of domains. RSM can be used to evaluate the relationship between a set of controllable experimental factors and outcomes. The interactions among the possible influencing factors can be evaluated with a restricted number of experiments.

This study comprised of improvement of ethanol yield from apple pomace by using the various combinations of enzymes and yeast isolates. The combined effect of independent variables on the responses is also being investigated using second order model. The study also reveals the optimum condition for the maximum ethanol yield by using RSM.

MATERIALS AND METHODS

Substrate procurement and pretreatment

Apples of *Red delicious* variety were procured from the local market of Pantnagar as per requirement and stored in the refrigerator at 4°C until needed for experiments. AP was prepared using hydraulic press in the bioconversion laboratory of the Department of Post Harvest Process and Food Engineering (PHPFE), GBPUAT Pantnagar. Sterilization of AP was done by using autoclave unit at 15 psi (121°C) for 15 to 20 min. The initial moisture content of AP was calculated by using hot-air oven method and was observed to be 80% (wb).

Characteristics of apple pomace

Initially apple pomace has 5.80 to 7.20% of reducing sugar, after the treatment of α- amylase and cellulase collectively it increased to10.65 to 13.10%. AP is a poor source of nitrogen. Therefore, from the micronutrient analysis salt of the essential nutrient Nitrogen, that is ammonium sulphate 0.02 g/150 ml of sample was added before fermentation.

Microorganisms and enzymes used

Saccharomyces cerevisiae ATCC 9673 was procured from IMTC Chandigarh while Y_{51} strain was procured by natural fermentation of AP in the bioconversion laboratory of the department. Both strains (*SC* 9673 and Y_{51}) were tested for the ethanol producing quality and were found to have independent growth which ensures that the growth of one does not affect the growth of another. Hence, combination of both yeast strains was considered as an independent variable in the experimental design. Yeast isolates (Y_{51}, *SC* 9673) were grown on Yeast- Peptone- Dextrose medium for 48 h at 30°C kept at 120 rpm in incubator shaker.

Experimental design

Design Expert 7.1.6 (Stat-Ease Inc., Minneapolis, USA) was used for experimental design and also for statistical and regression analysis of the data. A Box- Behnken design with three independent variables was used: Type of yeast (X1), pH (X2) and fermentation time (X3). The coded and actual range of the selected variables is given in Table 1, which resulted in 17 experimental runs, including five central points.

The variables which were kept constant during the experimental run are fermentation temperature (30°C), agitation speed (55 rpm), dilution level (1:10), sample size (150 ml), amount of amylase (0.068 g/ 150 ml), amount of cellulase (0.0055 g/ 150 ml), enzyme treatment (α-amylase + cellulase) with the incubation period (1 h) and inoculum rate 10% (v/v) of the sample size as standardized by Jain and Singh (2006).

Fermentation for production of ethanol

Fermentation experiments were carried out at the process

Table 2. Response surface design and corresponding response values for ethanol production.

Exp. No.	Variable			Responses			
	Yeast strains	pH	Time (h)	Utilized sugar conc. (%)	Change in pH	Cell count 10^6 cfu/ml	Ethanol conc. (%v/v)
1	SC	5.0	24	1.24*	1.32**	0.09*	0.65
2	SC	4.0	72	7.65	0.53	150	0.94
3	Y_{51}	4.0	48	4.29	0.36*	57	3.95
4	SC	4.0	24	1.57	1.04	0.109	0.75
5	COM	4.5	48	3.94	0.64	110	1.26
6	SC	4.5	48	4.78	0.55	63	0.98
7	COM	4.0	48	4.26	0.58	8.9	1.09
8	SC	4.5	48	4.94	0.52	69	1.05
9	SC	4.5	48	5.22	0.48	73	1.12
10	COM	4.5	24	1.35	0.66	9.5	0.58*
11	COM	4.5	72	8.86**	1.05	158**	1.43
12	Y_{51}	4.5	72	8.61	0.76	156	5.23**
13	Y_{51}	5.0	48	4.70	1.13	82	4.86
14	SC	4.5	48	5.08	0.54	59	1.17
15	Y_{51}	4.5	24	1.52	1.31	0.103	2.42
16	SC	4.5	48	4.64	0.45	67	0.89
17	SC	5.0	72	7.85	1.14	146	1.28

*Minimum value; ** Maximum value.

conditions as mentioned in Table 1. Samples were withdrawn at the specified intervals and were tested for their sugar, ethanol, pH and viable count individually. The change of pH was monitored by digital pH meter. Colony forming unit were determined by serial dilution pour plating method (Seeley et al., 1991). The amount of sugar was estimated by Dinitrosalicylic acid method of Miller (1972). The fermentation worth was distilled and then amount of ethanol was estimated using GC according to the method of Lancas and de Moreas (2007). A calibration curve was constructed using ethanol standards in water. *n* Propanol at a concentration of 5% (v/v) was used as an internal standard to correct for unequal injection volumes in gas chromatography. The distilled samples and the standards all contain the same concentration of the internal standard. The calibration curve is constructed by dividing the peak area of the ethanol by the peak area of the internal standard and plotting the ratio against the concentration of the ethanol. The peak area ratio is independent of injection volume. The amount of ethanol was determined by the formula:

$$\frac{\text{X \% Ethanol}}{\text{Peak area ratio for sample}} = \frac{\text{5\% Ethanol}}{\text{Peak area ratio for sample}}$$

Data analysis

A full second order mathematical model was fitted into each response. The adequacy of the model was tested using coefficient of determination (R^2) and Fisher's F-test. The effects of variables on responses were then interpreted. If the model was found adequate, the best fit equations were developed in order to draw contour plots for showing the effect of independent variables on those responses and to select the optimum range of variables for an acceptable product.

RESULTS AND DISCUSSION

The experimental data given in Table 2 was analysed employing multiple regression technique to develop response functions and variable parameters optimized for best outputs.

Sugar utilization

The maximum sugar (8.86%) was utilized in the case of combination of yeast strains with the initial fermentation pH of 4.5 and the fermentation period of 72 h, it was due to the fact that CFU's of yeast cells was maximum in this case. Sugar was metabolized by yeast cells for its growth and was subsequently converted into ethanol. Yeast favours an optimum value of pH (4.3-4.7) for their growth and hence utilize maximum amount of sugar during this range (Neuberg, 1958).

The coefficient of determination (R^2) for the regression model for utilized sugar was 99.20%. Model was highly significant ($p<0.05$) with F as 95.92. Effect of independent variables was highly significant ($p< 0.01$) at linear level, while the level of significance at quadratic level was 5%.

Change in pH

Coefficient of determination (R^2) was 97.43%, model was

highly significant ($p<0.05$) with F value of 29.48. It was observed that all the 3 parameters viz. yeast strain, pH and fermentation time affected the change in pH at 1% level of significance.

Cell count

During the fermentation process throughout, an followed by a decrease. Yeast, during the log phase of increasing pattern was observed for the cell count their growth cycle utilized the nutrients and hence leads to ethanol production. Higher value indicates the conditions where sample of pomace was treated with combination of yeast strain at pH level of 4.5 and fermentation time was 72 h. Fermentation time affected the cell concentration at 1% level of significance.

Ethanol yield

Maximum conversion of sugar into ethanol 5.23% (v/v) was observed when the sample was treated with Y_{51} strain at the pH level of 4.5 and for 72 h of fermentation. The reason behind this is that strain Y_{51} converts the sugar into ethanol under the optimum initial pH of 4.5 which is favourable for yeast growth. Similar results for Y_{51} strain were obtained by Jain and Singh (2006) and Kumar and Sahgal (2008). Effect of independent variables on ethanol yield was highly significant ($p< 0.01$) at linear and quadratic level. Yeast strain affected the ethanol yield at 1% level of significance and fermentation time affected at 5% level of significance.

Effect of yeast strains, pH and fermentation time on utilized sugar

$$\text{Utilizedsugar} = 4.93 - 0.089X1 - 0.005X2 + 3.41X3 - 0.18X1X2 + 0.11X1X3 + 0.13X2X3 - 0.064X12 - 0.57X22 + 0.22X32 \quad (1)$$

Full second order model, Equation (1) was fitted into utilized sugar and experimental conditions using multiple regression analysis.

Effect of yeast strains, pH and fermentation time on change in pH

Model was highly significant ($p<0.05$) with F value of 29.48 and hence found to be satisfactory in describing change in pH content.

$$\text{Change in pH} = 0.51 - 0.079X_1 + 0.21X_2 - 0.11X_3 - 0.18X_1X_2 + 0.23X_1X_3 + 0.085X_2X_3 + 0.055X_1^2 + 0.11X_2^2 + 0.38X_3^2 \quad (2)$$

Effect of yeast strains, pH and fermentation time on cell concentration

The coefficient of determination (R^2) for the regression model for this parameter was 95.40%. Model was highly significant ($p<0.05$) with F value of 16.13 and therefore was sufficient in describing cell concentration.

$$CFU = (6.620E+007) - (1.088E + 006X_1) + (1.526E + 007X_2) + (7.502E + 007X_3) + (1.902E + 007X_1X_2) - (1.849E + 006X_1X_3) (9.952E + 005X_2X_3) + (2.563E + 006X_1^2) + (4.288E + 006X_2^2) + (1.214E + 007X_3^2) \quad (3)$$

Effect of yeast strains, pH and fermentation time on ethanol yield

The coefficient of determination (R^2) for the regression model for ethanol yield was 96.47%, which implies that the model could account for 96.47% data. Lack of fit was significant but model can be considered adequate as it had a high R^2 value.

$$\text{Ethanol yield} = 1.04 - 1.51X_1 + 0.17X_2 + 0.56X_3 - 0.19X_1X_2 - 0.49X_1X_3 + 0.11X_2X_3 + 1.63X_1^2 + 0.12X_2^2 - 0.26X_3^2 \quad (4)$$

The result of regression analysis of all the dependent parameters is given in Table 3.

Process optimization

The objective of the study was to get the optimized condition where the best product can be obtained among the experiments performed. The optimized condition could be a single point or a range of points in which all the possible combinations would yield good results.

Optimization of independent variables

Optimization is a process of making compromises between responses, to achieve a common target. The responses namely ethanol yield, utilized sugar, change in pH and cell count were considered for optimization. The goal setup for optimization is given in the Table 4.

The validity of the model was proved by fitting different values of the variables into the model equation and by carrying out the experiment at those values of the variables. During optimization 10 solutions were obtained, out of which the one that suited the criteria most was selected. The most suitable optimum point is given in the Table 5. The model F- value was found to be highly significant at 1% level of significance in case of all the responses observed. Hence second order model was fitted to predict all the dependent parameters. The contours were drawn using the best fit model equations

Table 3. Result of regression analysis for dependent parameters.

Source	Ethanol conc.		Utilized sugar		Change in pH		CFU	
	Coeff.	P value	Coeff.	P value	Coeff.	P value	Coeff.	P value
Cons	1.04	0.03	4.93	0.01	0.51	0.01	6.620E+007	0.07
X_1	-1.51	0.01***	-0.089	47.38	-0.079	2.40**	-1.088E+006	87.18
X_2	0.17	30.23	-5E-003	96.72	0.21	0.01***	1.526E+007	5.13*
X_3	0.56	0.69***	3.41	0.01***	-0.11	0.65***	7.502E+007	0.01***
X_1X_2	-0.19	40.67	-0.18	30.75	-0.18	0.26***	1.903E+007	7.73*
X_1X_3	-0.49	5.20*	0.11	54.67	0.23	0.05***	-1.849E+006	84.63
X_2X_3	0.11	61.59	0.13	45.05	0.085	6.47**	-9.952E+005	91.68
X_1^2	1.63	0.01***	-0.064	70.61	0.055	19.12	2.563E+006	78.31
X_2^2	0.12	57.85	-0.57	0.96*	0.11	1.90**	-4.288E+006	64.68
X_3^2	-0.26	25.04	0.22	22.23	0.38	0.01***	1.214E+007	21.76
R^2%	96.47		99.20		97.43		95.40	
F value	21.27		95.92		29.48		16.13	
LOF	S		NS		S		S	

*** , ** , * Significant at 1, 5 and 10% level of significance respectively; ns = Non significant, s = significant, cons = constant; X_1 = yeast strains, X_2 = pH , X_3 = Fermentation time (min).

Table 4. Constraints for optimization.

Variable	Goal	Lower limit	Upper limit
Yeast strains	None	-1	+1
pH	Is in range	-1	+1
Fermentation time	Maximum	-1	+1
Ethanol yield	Maximum	0.58	5.23
Utilized sugar	Maximum	1.24	8.86
Change in pH	Maximum	0.36	1.32
Cell count	Maximum	90000	1.58E+008

Table 5. Optimum levels of variables.

Independent variable	Coded levels	Actual levels
Yeast strains (X_1)	-1	Y_{51}
pH (X_2)	1	5
Fermentation time (X_3)	1	72 h

for the centre point as well as for optimum point as shown in the Figures 1 to 4.

Conclusion

The present study has shown a promising potential for utilising apple pomace as a novel substrate for the production of ethanol. However, the yield 5.23% (v/v) was low, but it can be further improved with different possible combinations of sugar reducing enzymes and also by blending AP with other potential sources for production of ethanol, that is, sugar cane, sugar beet, molasses, corn, grains (wheat, maize, and barley), tubers, biomass etc. The statistically based optimisation procedure, using response surface methodology was proved to be an effective technique in optimising fermentation conditions.

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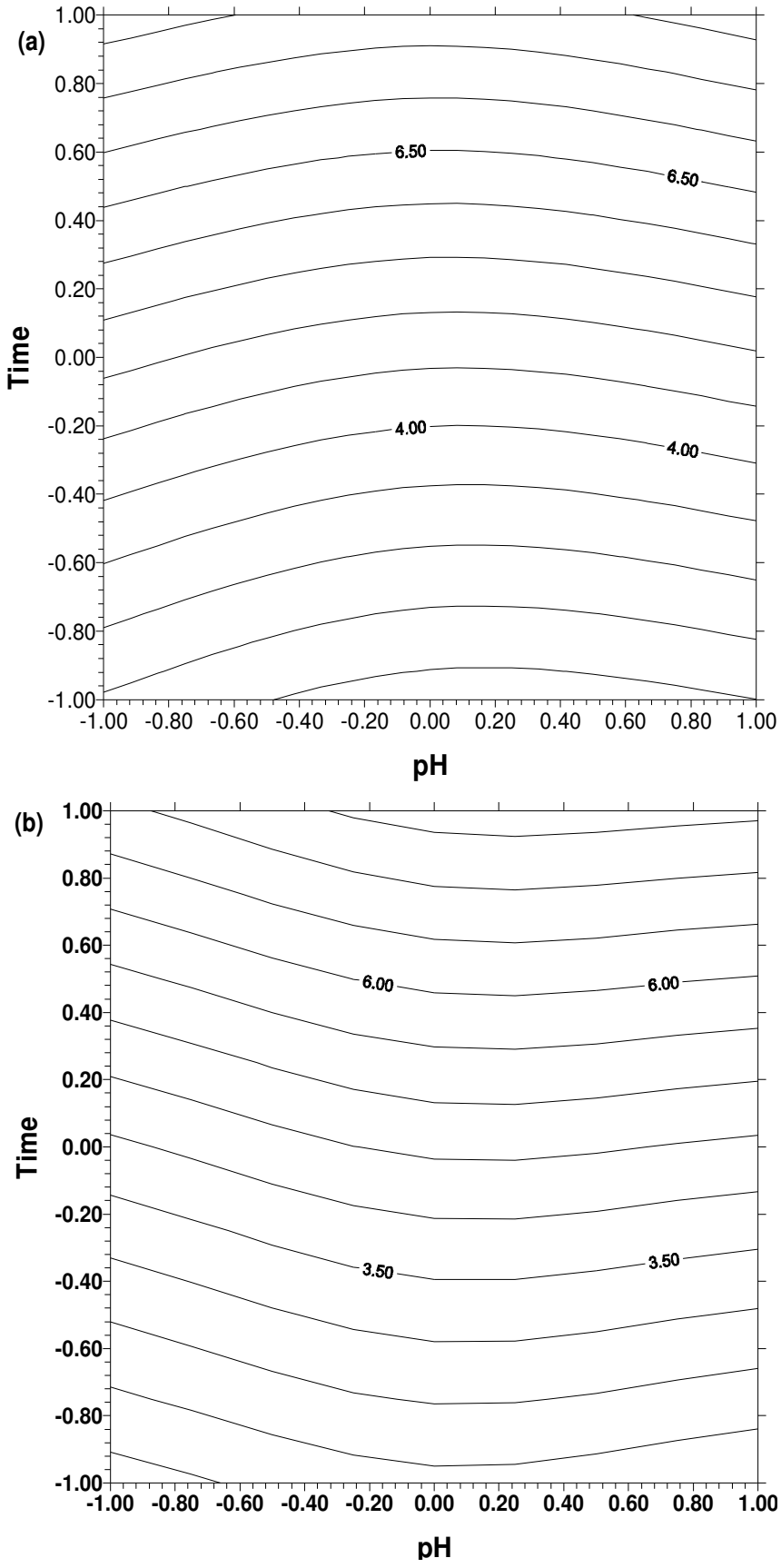

Figure 1. Contour plots for utilized sugar. (a) At centre point; (b) At optimum point.

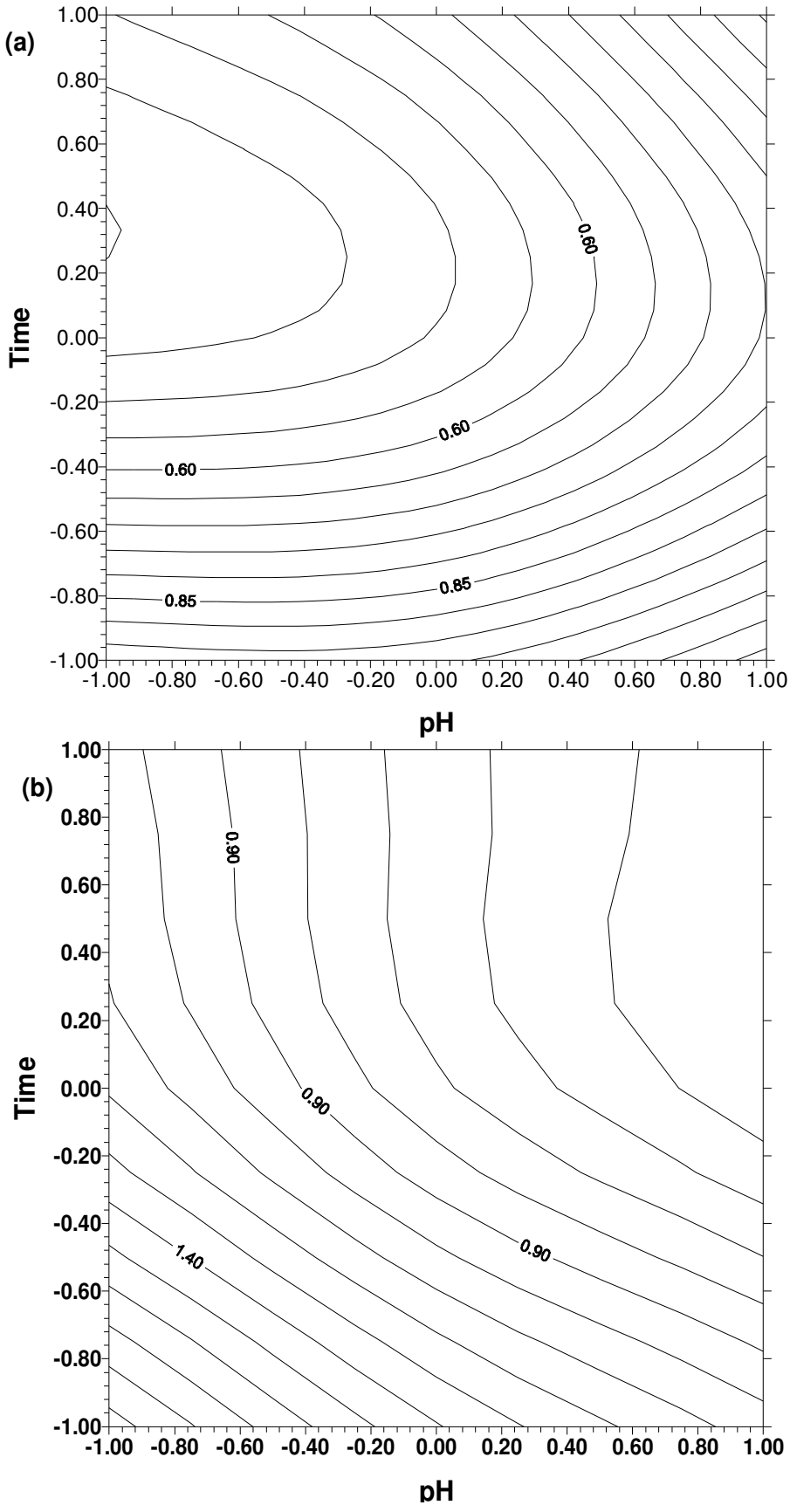

Figure 2. Contour plots for change in pH. (a) At centre point; (b) At optimum point.

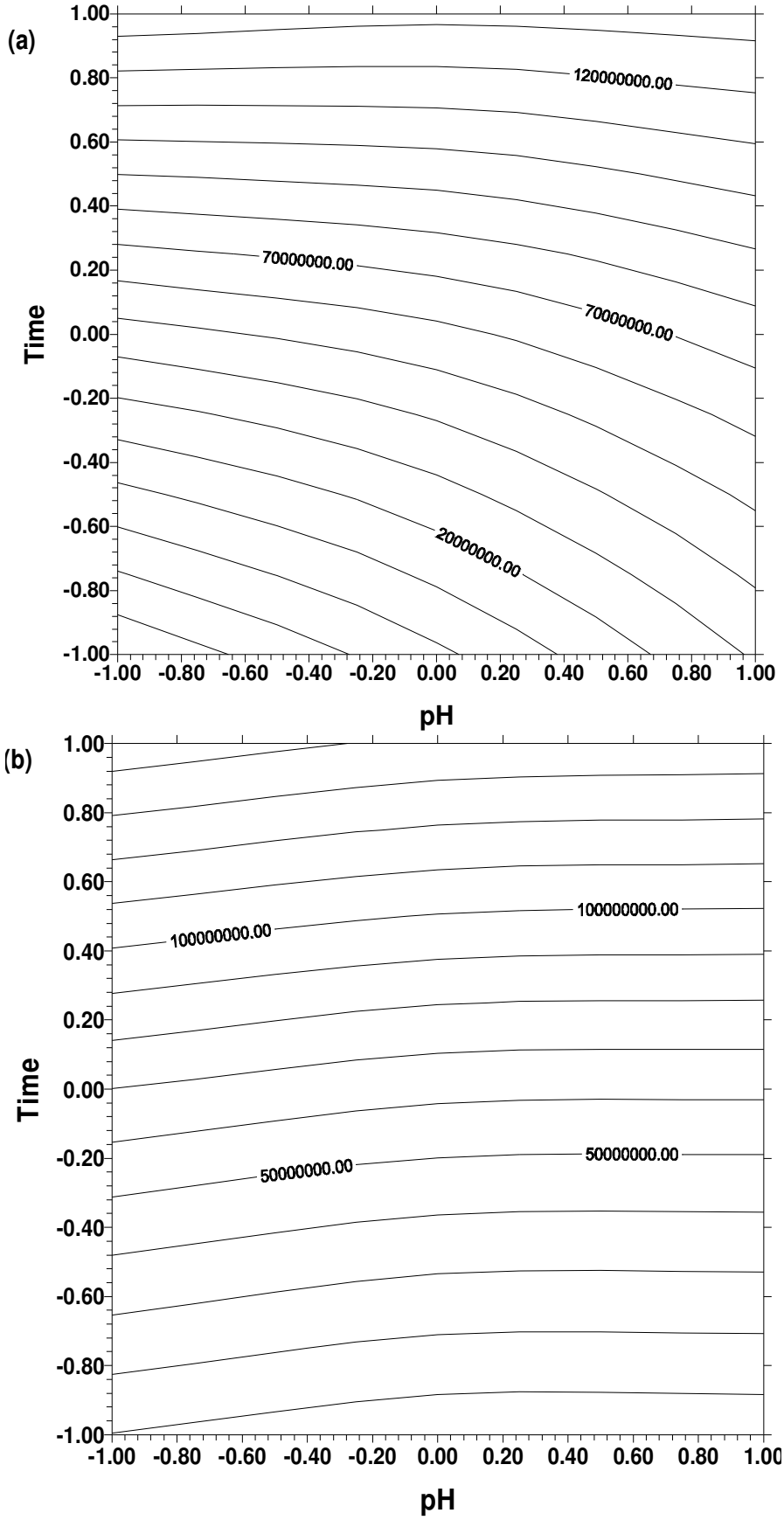

Figure 3. Contour plots for cell count. (a) At centre point; (b) At optimum point.

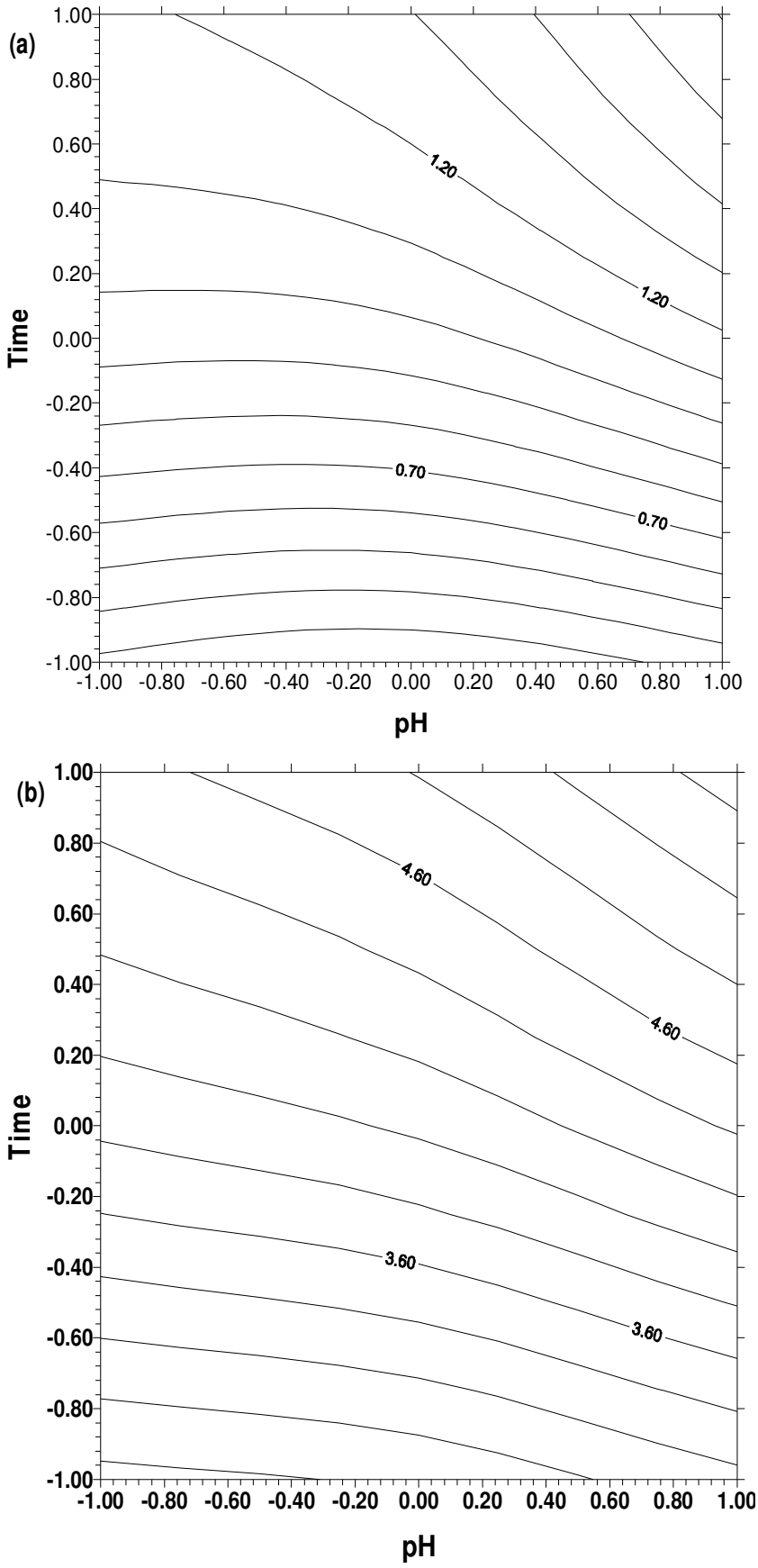

Figure 4. Contour plots for ethanol yield. (a) At centre point; (b) At optimum point.

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