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Full Length Research Paper

Optimization of fermentation process for preparation of mulberry fruit wine by response surface methodology

Chu-Yan Wang[1,3], Yan-Wei Liu[1], Jun-Qiang Jia[1,2], Thasma Raman Sivakumar[1], Tao Fan[3], and Zhong-Zheng Gui[1,2*]

[1]Jiangsu University of Science and Technology, Zhenjiang 212018, China.
[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China.
[3]Anhui Academy of Agricultural Sciences, Hefei 230061, China.

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Response surface methodology (RSM) has been the most widely used optimization method in recent years. Lately, many studies successfully employed this methodology to improve enzyme production processes of industrial interest. In this context, the mulberry (*Morus alba* L.) fruit was used as raw material to brew fruit wine. The effects of fermentation parameters (temperature, pH value, inoculum size and fermentation time) on the alcoholicity of mulberry wine were investigated. Based on single factor and three factor influence level tests by following the Plackett-Burman design, the optimum extraction yield was analyzed by response surface methodology (RSM). The results of RSM showed that the optimal condition for mulberry fermentation was defined as pH, 3.2; inoculum size, 0.53%; fermentation temperature, 31.4°C and fermentation time of 6 days. Under this optimal condition, alcoholicity of the wine reached 12.46%.

Key words: Mulberry wine, fermentation condition, response surface analysis, optimization.

INTRODUCTION

Phytochemical rich plants played a significant role in diet based therapies to cure various maladies (Butt et al., 2009). Genus *Morus* (mulberry) is one of such example that consists of over 150 species, among these *Morus alba* L. is the dominant one (Srivastava et al., 2006). Mulberry fruit, a kind of mature fruit of mulberry tree, contains many kinds of nutrient compounds, such as vitamin, amino acid and mineral matters. Ercisli and Orhan (2007) reported total phenolics, total flavonoids and ascorbic acid in fresh *M. alba* fruit as 181 mg/100 g (gallic acid equivalent), 29 mg/100 g (quercetin equivalent) and 100-300 mg/100 g on dry weight basis, respectively (Bae and Suh, 2007; Zadernowski et al., 2005). It is also of significant importance and presence of essential fatty acids which has already proved its worth as nutraceuticals. Its medicinal worth was attributed to the presence of active ingredients and it is also of significant importance functions (Doi et al., 2001; Ercisli and Orhan, 2007).

Mulberry fruit is a traditional Chinese edible fruit that is used effectively in folk medicines to treat fever, protect liver from damage, strengthen the joints, facilitate discharge of urine and lower blood pressure. Recently, it has gained an important position in the local soft drink market, although it has biological and pharmacological effects that are still poorly defined. Studies showed that mulberry fruit has significant effects in anti oxidation, reducing LDL (low density lipoprotein) level, delaying ageing and beautifying skin (Halliwell, 1992; Tomoyuki et al., 2006; Wang et al., 2011). In some European countries, *M. alba* and other mulberries are grown for fruit productions that have certain application in some traditional foodstuffs (Ercisli, 2004). At present, various food-grade mulberry fruit products (that is, juice, jam, and dried fruit) have been developed commercially as func-

*Corresponding author. E-mail: srizzgui@hotmail.com. Tel: 86-511-8561-6716.

tional foods in some traditional sericulture countries, such as China, Japan and Korea. However, *M. alba* fresh fruit is hardly commercialized. Due to its fragile structure and low stability in storage, it is usually processed as jelly or juice. Another possibility is to commercialize its fermented product. It has been widely reported that cardio protective effect can be achieved by moderate consumption of some alcoholic fermented beverages (such as wine or beer) as the consequence of their content of phenolic compounds (Gorinstein et al., 2000) and ethanol.

Response surface methodology (RSM) is a statistical method that use quantitative data based on adequate experimental planning to determine and simultaneously resolve multivariate equations. This method has been extensively used to optimize chemical and biochemical processes, such as production of enzymes (Hajji et al., 2008; Jiang et al., 2010), composition of cultivation media (Kunamneni and Singh, 2005), conditions of enzymatic hydrolysis (Shieh and Lai, 2000), parameters for polymer synthesis and parameters for food processing (Ozer et al., 2004).

In this study, mulberry fruit was used to develop mulberry fruit wine by fermentation. We defined four factors namely inoculum size, temperature, fermentation time and pH value by response surface methodology (RSM) to gain the optimum fermentation condition. The optimal fermentation conditions for mulberry wine were investigated. This study will provide a good reference for future industrial production of mulberry fruit wine.

MATERIALS AND METHODS

Mulberry fruit and microbial strain

Fresh mulberry fruit was harvested from the plantation of mulberry (the Sericultural Research Institute, Anhui Academy of Agricultural Sciences, Hefei, China). The fruits were manually selected, while those that were green or damaged were discarded. Completely ripe (uniform black color) berries were washed with tap water and immediately frozen and stored at -70°C until the time for analysis.

The yeast, *Saccharomyces cerevisiae* CFTRI 101, was obtained from Hubei Angela yeast Co., Ltd, China. The dry yeast was activated according to the procedure described by Zhang et al. (2009). Briefly, the dried yeast was added to 2% granulated sugar solution (1:10, w/v) in a warm bath for 20 min at 37°C, and shaken every 10 min until small amount of tiny bubbles appeared.

Mulberry fruit juice preparation and alcoholic fermentation

Frozen fruit samples were thawed at room temperature and crushed in a blender with a fruit blender at the speed of 100 ×*g* for 10 min. The crushed fruit was treated with pectinase, filtered with an industrial commercial sieve, and squeezed to extract the juice. The juice was ameliorated to 22°Brix with sucrose and then subjected to analysis of fermentation.

There are three steps for alcoholic fermentation. (1) Main-fermentation: A micro scale fermentation method described by Sampaio et al. (2007) was used for fermenting the fruit. The fruit juice was placed into the fermentor with 50 mg/L sulfur dioxide, then inoculated with a cell suspension of *S. cerevisiae* CFTRI 101, and finally kept at 20°C for seven to 10 days. The cultures were collected every two days from the center of the fermentation vessels and then subjected to analytical determinations (reducing sugars, acidity and ethanol). (2) Post-fermentation: After the fermentation, the upper wine juice was transferred by siphon to another clean containers without any top gap, and placed still for 10 to 20 days. (3) Aging: The clarified alcoholic broth was moved to another clean container added with gelatin solution as clarify agent, fermented at 0 - 5°C for one to two months. After the separation and fine filtration, the original wine was obtained

Ethanol determination

Ethanol was determined by using a gas chromatography (GC-6890, Agilent Inc., USA) equipped with a flame ionization detector (FID) and a 20 m × 0.25 mm × 0.25 μm fused-silica capillary column (DB-FFAP). The mass spectral ionization temperature was set at 230°C. The mass spectrometer was operated in the electron impact ionization mode at a voltage of 70 eV. The column was held at 40°C for 3 min, and then increased from 40 to 160°C at 3°C/min, held at 160°C for 2 min, and finally increased to 220°C at a rate of 8°C/min, then held for 3 min. Nitrogen was used as the carrier gas with a flow rate of 2.6 ml/min; and n-propanol was used as internal standard. Reducing sugars were determined by the 3,5-dinitrosalicylic acid reaction with glucose as standard (Pérez-Gregorio, 2011).

Single factor testing

Effect of fermentation temperature on the alcoholicity

Adjustment of sugar content 22°Bx and pH 3.6, the mulberry fruit juice was inoculated with 0.2% of *S. cerevisiae* and fermented for five days with different temperature of 20, 25, 30, 35 and 40°C, respectively. The alcoholicity was determined with the above method described.

Effect of pH value on the alcoholicity

Fixed the sugar content 22°Bx, temperature 30°C and inoculum size 0.2%, the mulberry fruit juice was fermented for five days at different pH level of 3.0, 3.3, 3.6, 3.9 and 4.2, respectively. The alcoholicity was determined with the above method described.

Effect of inoculum size on the alcoholicity

Setup the sugar content 22°Bx, temperature 30°C and pH 3.6, the mulberry fruit juice was fermented for five days with the inoculation size 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35% of *S. cerevisiae* respectively. The alcoholicity was determined with the above method described.

Effect of fermentation time on the alcoholicity

Setup the sugar content 22°Bx, temperature 30°C and pH 3.6, the mulberry fruit juice was inoculated with 0.2% of *S. cerevisiae* and fermented with different time 2, 3, 4, 5, 6, 7 and 8 d, respectively. The alcoholicity was determined with the above method described.

Experimental design

The effect parameters were optimized using response surface

Table 1. Levels of factors used for optimization of mulberry fruit wine production.

Level	Factor			
	x_1 Temperature (°C)	x_2 pH level	x_3 Inoculum size (%)	x_4 Time (d)
-1	25	3.0	0.35	3
0	30	3.3	0.50	4
+1	35	3.6	0.65	5

Table 2. Plackett-Burman central composite design.

Order	x_1	x_2	x_3	x_4	Alcoholicity (y, %)
1	0.00	0.00	-1.00	1.00	11.82
2	0.00	0.00	1.00	1.00	11.70
3	0.00	0.00	1.00	-1.00	11.36
4	0.00	0.00	-1.00	-1.00	11.60
5	0.00	-1.00	0.00	1.00	11.71
6	0.00	-1.00	0.00	-1.00	11.02
7	0.00	1.00	0.00	1.00	10.59
8	0.00	1.00	0.00	-1.00	10.39
9	-1.00	-1.00	0.00	0.00	9.31
10	-1.00	1.00	0.00	0.00	9.29
11	1.00	-1.00	0.00	0.00	11.07
12	1.00	1.00	0.00	0.00	10.06
13	1.00	0.00	0.00	-1.00	11.21
14	1.00	0.00	0.00	1.00	11.67
15	-1.00	0.00	0.00	1.00	10.51
16	-1.00	0.00	0.00	-1.00	9.97
17	1.00	0.00	1.00	0.00	10.97
18	1.00	0.00	-1.00	0.00	11.27
19	-1.00	0.00	1.00	0.00	9.87
20	-1.00	0.00	-1.00	0.00	9.78
21	0.00	-1.00	-1.00	0.00	11.08
22	0.00	-1.00	1.00	0.00	10.89
23	0.00	1.00	-1.00	0.00	10.11
24	0.00	1.00	1.00	0.00	9.86
25	0.00	0.00	0.00	0.00	11.91
26	0.00	0.00	0.00	0.00	11.87
27	0.00	0.00	0.00	0.00	11.95

methodology (RSM) (Hajji et al., 2008). A three-level-four-factor central composite design (CCD) obtained by using the software SAS 8.1 (SAS Institute Inc., USA) was employed to find out the interactive effects of four independent variables, viz. temperature (x_1), pH level (x_2), inoculum size (x_3), and fermentation time (x_4). The range and centre point values of three independent variables are presented in Table 1.

Plackett-Burman (PB) design was employed to optimize conditions for fermentation with response surface methodology (RSM). PB design involved a set of 12 experiments in -1 and +1 levels. The following parameters were investigated: A, temperature (20, 25, 30, 35 and 40°C); B, pH level (3.0, 3.3, 3.6, 3.9 and 4.2); C,inoculum size (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35%); and D, fermentation time (2, 3, 4, 5, 6, 7 and 8 days). Based on the results of PB design, effective factors were selected to cover a wide range to ascertain the tendency of alcoholicity variation (Table 2).

Data analysis

The model and formula were performed by RSM. The values were calculated as the mean of individual experiments in triplicate. The statistical significance was analyzed by Student's *t* test and regression analysis and the data were fitted by using the Expert

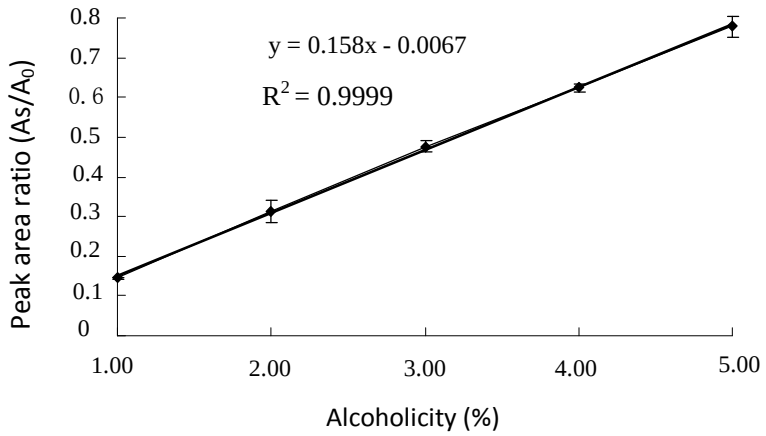

Figure 1. The standard curve of alcoholicity.

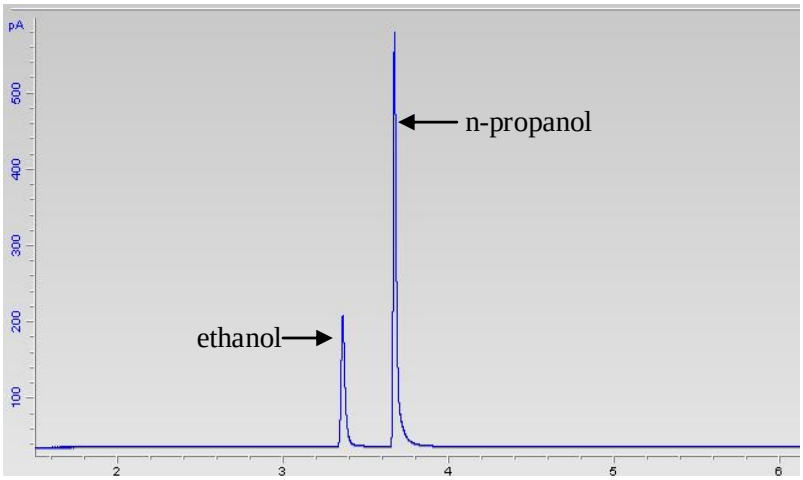

Figure 2. Typical gas chromatogram of alcoholicity.

Design 8.0.6 for Windows software (SPSS Inc., USA).

RESULTS

Determination of alcoholicity using gas chromatography (GC)

Typical gas chromatograms of alcoholicity fermented from mulberry fruit were conducted following the Chinese National Standard Method of Port Wine. The n-propanol was used as the standard. The correlation between alcoholicity and response was best described by the following equation: ratio of peak area of ethanol to peakarea of n-propanol = alcoholicity $\times$ 0.158 - 0.0067, r^2 = 0.9999. The result shows that there existed a good linear relationship between alcoholicity and the area response (Figure 1). Alcoholicity content was detected by GC in Figure 2. Peak 1 represents ethanol and Peak 2 presents to n-propanol.

Selection of potential factors

Based on previous study (Jiang et al., 2010), certain extents of functional factors were performed to confirm the influencing internal tendency. From Table 1, we first conducted, single factor tests of the temperature as x_1 (20, 25, 30, 35 and 40°C), pH level as x_2 (3.0, 3.3, 3.6, 3.9 and 4.2), inoculum size as x_3 (0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35%), and time as x_4 (2, 3, 4, 5, 6, 7 and 8 d) to alcoholicity y (%) (Table 2).

Fermentation temperature

Fermentation temperature is the key factor that influences taste of fruit wine. Generally, the optimum

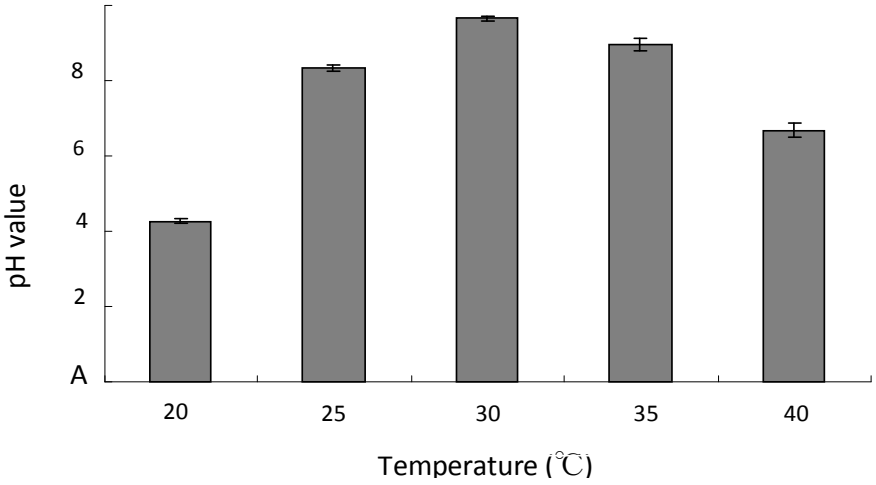

Figure 3. Effect of fermentation temperature on alcoholicity. Values are represented as mean±SD (n=3).

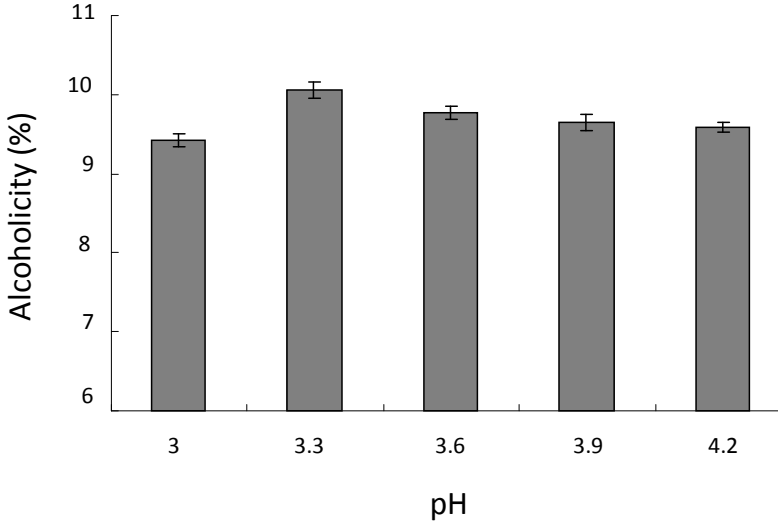

Figure 4. Effect of pH value on alcoholicity. Values are represented as mean ± SD (n = 3).

temperature range is 25 to 35°C. Higher temperature shortens fermentation period. But, too high temperature leads to low quality of fruit wine with bad taste.

Base on the fermentation condition of sugar content, 22°Bx, pH 3.6, and *S. cerevisiae* inoculum size 0.2%, temperature was set as 20, 25, 30, 35 and 40°C respectively. The alcoholicity determination was conducted after five days of fermentation. Figure 3 shows that the alcoholicity was reached at the highest when temperature was set as 30°C.

pH value

With respect to pH, the other factors were set as sugar content 22°Bx, *S. cerevisiae* inoculum size 0.2%, and temperature at 30°C. The alcoholicity was programmed at different pH levels as 3.0, 3.3, 3.6, 3.9 and 4.2 after fermentation for four days. The optimal pH for the alcoholicity was 3.3 (Figure 4).

Inoculum size

As shown in Figure 5, inoculum size was selected as 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35% while the other factors were set as sugar content 22°Bx, temperature 30°C, pH 3.3 and fermentation time 4 d. The highest alcoholicity was observed at inoculum size of 0.2 g/100 mL (that is, 0.2%).

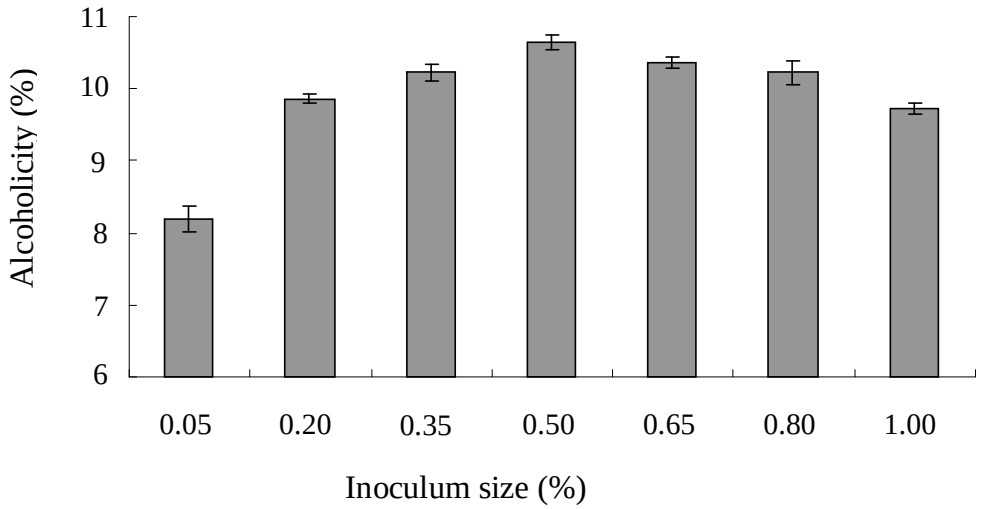

Figure 5. Effect of yeast inoculum size on alcoholicity in fruit wine. Values are represented as mean ± SD (n=3).

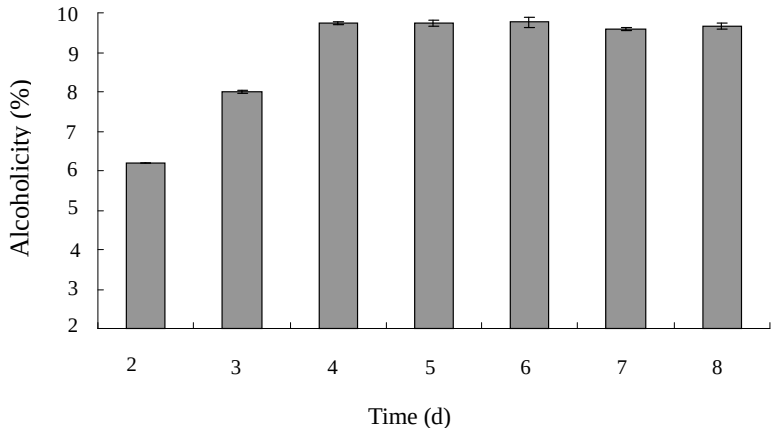

Figure 6. Effect of fermentation time on alcoholicity in fruit wine. Values are represented as mean ± SD (n=3).

Fermentation time

After fixing the factors as sugar content 22°Bx, *S. cerevisiae* inoculum size 0.2%, temperature 30°C and pH 3.3, different fermentation periods from two to eight days were selected for checking the alcoholicity. The result in Figure 6 shows that the alcoholicity was increasing rapidly from two to four days and remaining stable at later days. Therefore, the appropriate fermentation time was selected as four days.

Optimization of fermentation conditions

The response selection is a critical stage in optimization. The responses were directly related to the parameters that define the quality of the fruit wine. Based on the single factor experiments and the principle of Plackett-Burman design, temperature (x_1), pH level (x_2), inoculum size (x_3), and fermentation time (x_4) were chosen to be as independent variables. Meanwhile, alcoholicity volume fraction was regarded as response value. The response surface analysis was adapted to optimize the process conditions of mulberry fruit wine fermentation (Figure 7).

According to the software of SAS8.2, the result of regression analysis is shown in Table 3. By applying multiple regression analysis, the results were fitted to a second-order polynomial equation. Thus the alcoholicity regression model for SMP fitted in terms of encoded factors was obtained as follows:

$$Y = 11.91 + 0.625x_1 - 0.4092x_2 - 0.0833x_3 + 0.1725x_4 - 0.02275x_1x_2 - 0.115x_1x_3 - 0.0175x_1x_4 - 0.0175x_2x_3 - 0.1125x_2x_4 + 0.1025x_3x_4 - 1.03875x_1^2 - 1.01x_2^2 - 0.36375x_3^2 + 0.03x_4^2$$

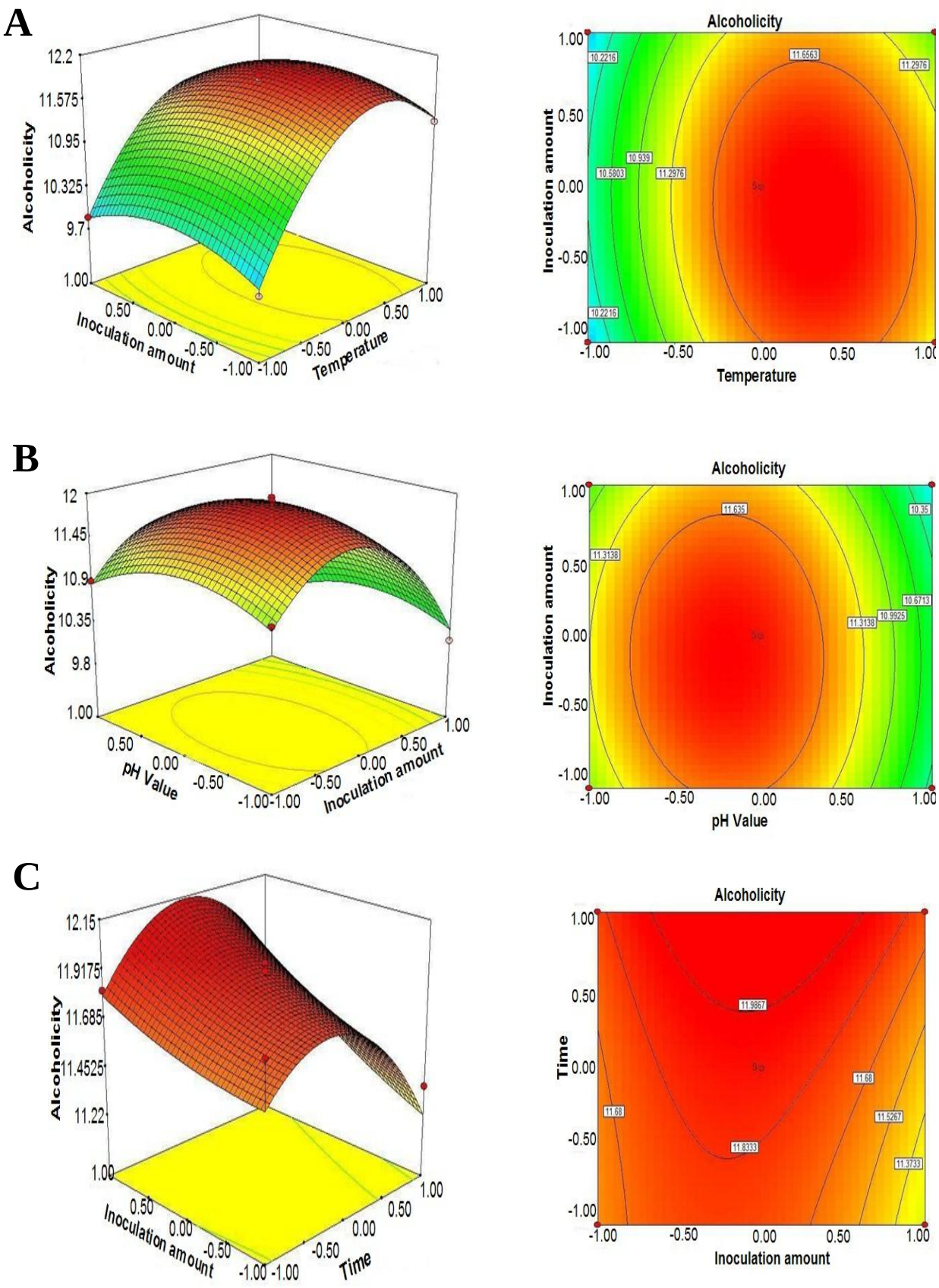

Figure 7. Response surface and contour plots for alcoholicity. **A.** Effect of temperature (x_1) and inoculum size (x_3). **B.** Effect of pH value (x_2) and the inoculum size (x_3). **C.** Effect of inoculum size (x_3) and fermentation time (x_4).

Table 3. ANOVA for the regression response surface model.

Source	Sum of Square	df	Mean Square	F Value	P-value Prob>F	Significant
Model	20.07	14	1.43	64.81	<0.0001	**
x_1	4.69	1	4.69	211.89	<0.0001	**
x_2	2.01	1	2.01	90.81	<0.0001	**
x_3	0.01	1	0.01	4.73	0.0474	*
x_4	0.40	1	0.40	18.07	0.0008	**
x_1x_2	0.21	1	0.21	9.36	0.0085	**
x_1x_3	0.053	1	0.053	2.39	0.1443	
x_2x_4	0.051	1	0.051	2.29	0.1526	
x_3x_4	0.021	1	0.021	0.95	0.3462	
x_{12}	7.07	1	7.07	319.43	<0.0001	**
x_{22}	6.68	1	6.68	302.08	<0.0001	**
x_{32}	0.81	1	0.81	36.69	<0.0001	**
x_{42}	0.010	1	0.010	0.47	0.5046	
Residual	0.31	14	0.022			
Lack of fit	0.29	10	0.029	4.94	0.0687	Not significant
Pure Error	0.023	4	5.8×10^{-3}			
Cor Total	20.18	28				
R-Squared	0.9848					
Adj-Squared	0.9696					

**Extremely significant difference at P<0.01; *significant difference at P<0.05.

Table 3 shows the significance of the linear relationship between dependent variable and independent variables. The model could account for 96.6% of the response value, showing a good fit of the model. The non-significance of lack of fit showed that the quadratic regression equation could forecast the response value well. The significant terms of x_1, x_2, x_4, x_1x_2, x_1^2, x_2^2 and x_3^2 declared that they could have a big influence on the response value.

The actual response values (12.46%) was close to the predicted one (12.56%), suggesting that the regression model for the design was available. The model “Prob > F” value was less than 0.001 (Table 3), showing that the model test is remarkable. Lack of fit was not significant. Model calibration coefficient R^2_{AJR} = 0.9696 means that the model can explain the change in 96.96% response value, and only about 3.04% of the total variance does not explain with this model. Results suggesting that this model navigates the design space. X_1^2, X_2^2, X_3^2 and X_4^2 are significant model terms in Table 3. The results suggest that the most important independent indices were the range of temperature (x_1), pH value (x_2), inoculum size (x_3), and fermentation time (x_4) in order.

In order to estimate the fermentation factors, plotted graphs were made among the influencing parameters. The response surface graph depicted by the two out of three factors formed a series of approximate circles with one center regardless of diverse gradients; simultaneously vaults hanging down took shape at the three-dimensional chart and reached the maximum. The alcoholicity (12.46%) from the fermentation broth by *S. cerevisiae* would be calculated by first order local deviation with equivalence to zero, when x_1, x_2, x_3 and x_4 were set to 31.4°C, 3.2, 0.53% and 6 d, respectively.

DISCUSSION

Morus is a genus of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, North America, and Southern Europe. Mulberry leaves have historically been used for its foliage, to feed silkworm (*Bombyx mori* L.). In traditional Chinese medicine, dried mulberry fruits have been used as a tonic and sedative, as have the leaves and root bark of the mulberry, suggesting that the mulberry fruits have compounds with physiological functions that offer protection to humans. In some European countries, the berries of *Morus alba* are consumed as a fresh fruit or as various confectionary products such as jam, marmalade, frozen desserts, pulp, juice, paste, and ice cream. Phytochemical studies on the fruits of *Morus* species showed the presence of fats and fatty acids, vitamin C, minerals, phenolics, and flavonoids (Ercisli and Orhan, 2007). Based on the fact that nearly all the sugar content in mulberry fruit consists of monosaccharide (example fructose), the sugar of the concentrated fruit juices is easily absorbed by the digestive system (Ustun and Tosun, 1997).

A new approach in wine production involves the use of selected yeast strains, as well as controlled temperature

during fermentation. The alcoholic fermentation of mulberry fruit juice into wine is carried out by a complex and temporally dynamic interplay between the changing communities of microorganisms involving bacteria and principally yeasts. Wine properties such as wine flavor, quality, consistency and economic value depend in part on the species and strains of yeasts that develop during the fermentation (Fleet, 2003). The yeast, *S. cerevisiae* has been widely used for wine making by fermentation of tropical fruit juice (Epifanio et al., 1999; Reddy and Reddy, 2009). The yeast strains, *S. cerevisiae* CFTRI 101 was selected for the mulberry wine fermentation in this study.

Fruit maturity also influences the synthesis of higher alcohols during fermentation; higher alcohols concentration was low in Semillon wines made from late harvested grapes (Ribereau-Gayon and Sudarud, 1991). Both the spontaneous and enzymatic clarification of apple and grape juice increases the higher alcohol concentration (Mangas et al., 1994; Aragon et al., 1998). To get the higher alcohol concentration, we selected the maturity mulberry fruit for the wine fermentation.

Additionally, it has been known that the preservation of health beneficial components in wine depends to a great extent on the main fermentation conditions (such as temperature and pH). Temperature has influence on fermentation - to some extent temperature increases the yeast growth, speed of enzyme action, cell sensitivity to the toxic effect of alcohol increases with temperature due to increased membrane fluidity. This may partially explain the rapid decline in yeast viability at temperatures above 20°C during wine fermentation (Torija et al., 2002). The fermentation at low temperatures such as <15°C leads to more aromatic and paler wines (Walker, 1998).

Ethanol production varied with pH change of fruit juice. The mango juices fermentation studies show that the lowest concentration (5% w/v) of ethanol was produced at pH 3.0 and highest (7.8% w/v) was at pH 5.0 (Reddy and Reddy, 2011). Low or high pH values are known to cause chemical stress on yeast cell. The enzyme aldehyde dehydrogenate activity is increased at high pH values and acetic acid is produced. This oxidation generates a molecule of NADH, which requires re-oxidation to maintain the redox balance of the cell (Walker, 1998).

Optimum temperature and pH value are necessary for yeast growth and ethanol production. In the present study, RSM was used to determine the optimum process parameters that yield high alcoholicity contents. Based on the single factor experiments and PBD, the whole experiment acquired a satisfying result. The optimal fermentation conditions and fermentation temperature = 31.4°C, pH level = 3.2, inoculum size = 0.53%, and fermentation time = 6d. Under these conditions, the alcoholicity volume fraction was up to 12.46%. The second regression model established in the experiment was accuracy and validity. Ezeronye (2004) showed that the wines produced from the fruit must had percentage alcohol levels ranging from 10.6 to 12.6. Our result has a good consistency to this range. Meanwhile, the parameters of predicted conditions and mulberry fruit wine fermentation is process fitted for the model. The predicted and experimental values were not significantly different. It is suggested that the models obtained can be used to optimize the fermentation process of fruit wine from *Morus alba*.

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