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*Full Length Research Paper*

# Optimization of the alcoholic fermentation of blueberry juice by AS 2.316 *Saccharomyces cerevisiae* wine yeast

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**Central composite experimental design together with response surface methodology (RSM) was employed to optimize the fermentation temperature, pH and inoculums size for maximum production of ethanol and minimum production of volatile acidity during alcoholic fermentation of blueberry wine. A second-order polynomial model was fitted to the content of ethanol and volatile acidity of runs as the responses. Analysis of variance revealed that the quadratic models were well adjusted to predict the experimental data. Lack-of-fit tests were not significant and determination coefficients ($R^2$) were higher than 92.33%. Through the statistically designed optimization, the optimal condition of alcoholic fermentation including temperature of 22.65°C, pH of 3.53 and inoculums size of 7.37% were found. Under this optimal condition, the production of ethanol and volatile acid of blueberry wine could be achieved reaching up to 7.63% and 0.34 g $l^{-1}$, respectively. The wine obtained using optimum fermentation conditions was the favorite choice of consumers.**

**Key words:** Blueberry wine, ethanol, fermentation, response surface methodology, yeast.

## INTRODUCTION

Blueberry (*Vaccinium corymbosum* L.) is characterized by antioxidant power, as determined with the oxygen radical absorbing capacity assay and other methods (Prior et al., 1998; Cho et al., 2004; Wang et al., 2000), and great potential health benefit, mostly attributable to their high concentration of bioactive compounds, including anthocyanins, flavonoid, procyanidins, phenolic acids (Prior et al., 1998; Cho et al., 2004; Ehlenfeldt et al., 2001; Wang et al., 2008), which play an important role in enhancing brain function activity, anti-cancer activities, and reducing the number of cardiovascular disease events, inhibiting oxidation of human low-density lipoproteins, fighting against human pathogens and preventing urinary tract infections (Zafra-Stone et al., 2007; Kraft et al., 2005; Joshipura et al., 2001; Galli et al., 2002; Prior et al., 2007; Molan et al., 2009). Immature and other ‘‘cull” blueberries are discarded because blueberries were mainly utilized in “fresh” market, “export” market and processing. In fact, these discarded blueberries contain high amounts of various bioactive compounds with antioxidant activity and most of these compounds can pass from the fruits to wines and remain active (Satora et al., 2009). There are intensive studies on finding new potential uses for blueberries to be value-added products with no agricultural residue such as fruit wine (Martin et al., 2005; Su and Silva, 2006, Su and Chien, 2007; Vasantha Rupasinghe et al., 2007).

Alcoholic fermentation is the important step in providing best quality wine. Several researchers have reported that many factors, including fermentation temperature, pH, inoculums size, sugar concentration, type of fermentation can significantly influence the ethanol content of fruit wine. Many physico-chemical conditions play an important role in ethanol content of wine (Kumar et al., 2009). The selected strains of *Saccharomyces cerevisiae*

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show different characteristics of alcoholic fermentation and influence on wine quality (Erten et al., 2006). To our knowledge, there is no report on the alcoholic fermentation of blueberry wine using *S. cerevisiae* and response surface methodology experiment design.

It is important to note that higher volatile acidity content in wine is not accepted by consumers. Different countries established limits for volatile acid content in fruit wine by law, which may not be higher than 1 to 1.5 g $l^{-1}$ (Lonvaud-Funel, 1995). When alcoholic fermentation is too slow or when it stops, higher content of acidity will encourage the activity of spoilage microbes, oxidation of wine, and result in poor color and wine stability. *S. cerevisiae* can produce higher acetic acid when compared with non *S. cerevisiae* species, especially *Saccharomyces bayanus/uvarum* (Antonelli et al., 1999; Eglinton and Henschke, 1999). Tempe-ratures during alcoholic fermentation induce the development of undesired microorganisms; therefore, undesirable volatile acids could be produced (Torija et al., 2003).

Response surface methodology (RSM) is a statistical technique that has been successfully applied to estimate the relationship between experimental factors and results in alcoholic fermentation and other fermentation media (Kumar et al., 2009).

*S. cerevisiae* AS2.316 commercial wine yeast has been commonly used in Chinese fruit wine industry.

The objective of this study was to model and evaluate the combined effects of fermentation temperature, pH and inoculums size level on volatile acidity and ethanol content of blueberry wine, applying the response surface methodology.

## MATERIALS AND METHODS

About one hundred kilogram of mature Premier Rabbiteye Blueberry (*Vaccinium ashei* Reade) was kindly supplied by Anhui Academy of Agricultural Sciences in July, 2010. *S. cerevisiae* AS2.316 supplied by CICC (Beijing, China Center of Industrial Culture Collection) was used in this study. The Pectinex BE XXL used in the juice processing was from Novo Nordisk A/S (Bagsvaerd, Denmark). Deionized water was produced using a Milli-Q unit (Millipore, Bedford, MA). Other reagents were of analytical grade.

### Blueberry juice preparation

Blueberry fruit were homogenized in a crusher XBLL-21B (Shuaijia Electronic Science and Technology Co. Ltd. Shanghai, China) for 2 min and Pectinex BE XXL (0.2 ml $l^{-1}$) was then added, as it was most active at 30°C for 2 h. The juice was extracted by passing through four layers of fine cheese cloth and mixed together. The juice was then clarified at 2°C for 8 h, and separated from the sediments with siphon by careful pouring into another vessel. The juice had 12°Brix, a pH value of 3.02, a total acid content of 0.87%, a Brix/acid ratio of 13.8. Blueberry juice was then sterilized with 50 mg $l^{-1}$ sodium metabisulphite. The finished juice had a Brix value of 15° by adding 48 g $l^{-1}$ sucrose and 0.5 g $l^{-1}$ ammonia sulfate. The pH was set to the desired value by means of 1N sodium hydroxide addition before inoculation.

### Inoculums preparation

*S. cerevisiae* AS2.316 was grown in YM (malt extract 0.3%, peptone 0.5%, yeast extract 0.3%, glucose 1% and agar 2%) slants at 25°C for 48 h. The inoculums were prepared by inoculating the slant culture into 25 ml of the sterile YM liquid medium taken in 100 ml Erlenmeyer flask at 25°C for 24 h. The inoculums were then transferred to 500 ml conical flasks having 400 ml of the finished blueberry juice for pre-culture.

### Production media and fermentation condition

Laboratory fermentations using 2 L vessels were carried out. The finished juice was divided into different batches and 1.6 L aliquots were placed in each vessel. When the yeast concentration of *S. cerevisiae* AS2.316 (CICC) of inoculums preparation was $10^6$ cells $ml^{-1}$, mosts were used for inoculation at varied ratios. Yeast concentration was carried out in a Neubauer improved bright-lined counting chamber (1 mm depth) and the result was expressed as total cell counts (Tian et al., 2009). After inoculation, blueberry juice was fermented at stationary conditions and different temperatures for 8 days.

Alcoholic fermentation was considered complete when the °Brix value remained constant for three consecutive days. After the alcoholic fermentation, the young wines were separated from the sediments with siphon by careful pouring into another vessel. Samples of the young wines were taken for analysis immediately.

### Analyses

Blueberry wine was analysed to determine the following parameters (AOAC 2003): °Brix (AOAC procedure number 31.009), pH (AOAC procedure number 31.203), ethanol (AOAC procedure number 11.003), volatile acidity (AOAC procedure number 11.039).

### Experimental design

Based on prior trials in the laboratory, fermentation temperature, pH and inoculums size were found to be the more critical variables in the production of ethanol and volatile acid of blueberry wine by *S. cerevisiae* AS2.316. The impact of fermentation temperature, pH and inoculums size level on ethanol content and volatile acidity of blueberry wine fermented with *S. cerevisiae* AS2.316 was studied through a central composite experimental design (CCD). A quadratic model was employed to study the combined influence of three independent variables namely temperature ($X_1$, °C), pH ($X_2$) and inoculums size ($X_3$, %). The measured dependent variables (Y) were ethanol ($Y_1$, %) and volatile acidity ($Y_2$, g $l^{-1}$) of the wine. Response surface methodology (RSM) proposed by Montgomery (1997) was used to analyze the results by a commercial statistical package, Design-Expert version 7.01 (Stat-ease Inc., Minneapolis, MN, USA). The second order polynomial function was used to represent the variance for each factor assessed as follows:

$$Y=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{12}X_1X_2+b_{23}X_2X_3+b_{13}X_1X_3 \quad (1)$$

Where, Y is the predicted response, $X_1$, $X_2$, $X_3$ are independent variables; $b_0$ is the offset term; $b_1$, $b_2$ and $b_3$ are linear effects; $b_{11}$, $b_{22}$ and $b_{33}$ are squared effects and $b_{12}$, $b_{23}$ and $b_{13}$ are interaction terms. Fermentation conditions were carried out changing pH from 3.16 to 3.84 and temperatures from 18.61 to 30.39°C and inoculums size from 0.95 to 11.05% (Table 1). Evaluation of optimal conditions was performed for ethanol content and volatile acidity of blueberry wine. Each type of fermentation was done in triplicate,

**Table 1.** Coded and actual values of the factors in central composite design.

| Factor | Variable | Low actual | High actual | Low coded | High coded |
|---|---|---|---|---|---|
| $X_1$ | Temperature (°C) | 21 | 28 | -1 | 1 |
| $X_2$ | pH | 3.3 | 3.7 | -1 | 1 |
| $X_3$ | Inoculums size (%) | 3 | 9 | -1 | 1 |
| **Response** | **Variables** | **Obs.*** | **Min.** | **Max.** | **Mean** |
| $Y_1$ | Ethanol (%) | 20 | 3 | 7.4 | 5.7 |
| $Y_2$ | Volatile acidity (g $l^{-1}$) | 20 | 0.32 | 0.95 | 0.58 |

*Observed run values. Min, Minimum; Max, maximum.

**Table 2.** Central composite design matrix.

| Run number | Temperature (°C) ($X_1$) | pH ($X_2$) | Inoculum size (%) ($X_3$) | Ethanol (%) ($Y_1$) | Volatile acidity (g $l^{-1}$) ($Y_2$) |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 7.4 | 0.34 |
| 2 | 0 | 0 | 0 | 7.2 | 0.42 |
| 3 | 0 | -1.682 | 0 | 5.4 | 0.59 |
| 4 | 0 | 0 | 0 | 7.2 | 0.39 |
| 5 | 0 | 0 | 0 | 7.4 | 0.44 |
| 6 | 0 | 0 | 1.682 | 6.4 | 0.58 |
| 7 | 0 | 1.682 | 0 | 5 | 0.74 |
| 8 | -1.682 | 0 | 0 | 6.2 | 0.38 |
| 9 | 1 | 1 | 1 | 5.1 | 0.71 |
| 10 | 0 | 0 | 0 | 7.3 | 0.32 |
| 11 | 1 | -1 | -1 | 4.4 | 0.6 |
| 12 | 0 | 0 | -1.682 | 3.5 | 0.89 |
| 13 | 1 | 1 | -1 | 3 | 0.95 |
| 14 | -1 | -1 | -1 | 4.7 | 0.79 |
| 15 | -1 | 1 | -1 | 4.5 | 0.62 |
| 16 | 1.682 | 0 | 0 | 4.8 | 0.85 |
| 17 | -1 | 1 | 1 | 6.9 | 0.47 |
| 18 | -1 | -1 | 1 | 5.9 | 0.58 |
| 19 | 1 | -1 | 1 | 5 | 0.53 |
| 20 | 0 | 0 | 0 | 7.4 | 0.36 |

and data were expressed as the mean ± standard error of the mean.

### Sensory evaluation

Sensory analysis (Millgaard et al., 1999) of the blueberry wine samples produced under the optimized and original conditions were carried out by 10 panelists (7 females and 3 males) from the College of Tea and Food Science, Anhui Agricultural University. They were asked to rank the wines according to their nine-point Hedonic scale, viz aroma, taste, appearance and overall acceptability. The analysis was carried out in triplicate.

## RESULTS AND DISCUSSION

### Checking of the fitted models

Based on the pre-experiment, three variables which significantly influenced ethanol and volatile acid production of blueberry wine including temperature, pH and inoculums size were investigated. Tables 1 and 2 show the design matrix and the corresponding experimental data. The following second order polynomial equation yielded by applying multiple regression analysis on the experimental

**Table 3.** Analysis of variance for the experimental results of the central composite design.

| Source | df | F-value | | P-value | |
|---|---|---|---|---|---|
| | | $Y_1$ | $Y_2$ | $Y_1$ | $Y_2$ |
| Model | 9 | 172.53 | 13.38 | 0.0001 | 0.0002 |
| $X_1$ | 1 | 147.60 | 17.03 | 0.0001*** | 0.0021* |
| $X_2$ | 1 | 4.32 | 3.42 | 0.0644**** | 0.0941**** |
| $X_3$ | 1 | 392.46 | 19.25 | 0.0001*** | 0.0014* |
| $X_1X_2$ | 1 | 23.65 | 15.19 | 0.0007** | 0.0030* |
| $X_1X_3$ | 1 | 4.34 | 0.06 | 0.0637**** | 0.8147**** |
| $X_2X_3$ | 1 | 39.09 | 0.28 | 0.0001*** | 0.6081**** |
| $X_1^2$ | 1 | 281.13 | 15.69 | 0.0001*** | 0.0027* |
| $X_2^2$ | 1 | 376.53 | 23.76 | 0.0001*** | 0.0006** |
| $X_3^2$ | 1 | 466.65 | 37.86 | 0.0001*** | 0.0001*** |
| Residual | 10 | | | | |
| Lack of fit | 5 | 3.82 | 3.96 | 0.0837 | 0.0786 |
| Pure error | 5 | | | | |
| Total | 19 | | | | |
| $R^2$ | | 0.9936 | 0.9233 | | |
| $R^2_{Vad}$ | | 0.9878 | 0.8543 | | |

$Y_1$- Ethanol, $Y_2$- volatile acidity; *P < 0.05- significant at 5% level, **P < 0.001- significant at 1% level, ***P < 0.0001- significant at 0.1% level, **** not significant.

data was found to represent the ethanol and volatile acidity production, respectively:

$$Y_1=7.32-0.5X_1-0.086X_2+0.82X_3-0.67X_1^2-0.78X_2^2-0.87X_3^2-0.26X_1X_2+0.34X_2X_3-0.11X_1X_3 \quad (2)$$

$$Y_2=0.38+0.082X_1+0.037X_2-0.087X_3+0.077X_1^2+0.094X_2^2+0.12X_3^2+6.25\times10^{-3}\times X_1X_2-0.014X_2X_3+b_{13}X_1X_3 \quad (3)$$

$X_1$, $X_2$ and $X_3$ are the coded values of the variables temperature, pH and inoculums size, respectively. The comprehensive model based on the experimental observations has been development for ethanol production by using yeast strains. Table 3 shows the predicted levels of ethanol and volatile acidity production from the blueberry wine using Equations 2 and 3. The second-order regressions were statistically significant (P<0.01) by ANOVA analysis and the quadratic polynomial models were well fitted to the experimental data (Table 2). The lack of fit (Table 3), which measures the fitness of the model, did not result in a significant F value for ethanol production and volatile acid production, indicating that these models are sufficiently accurate for predicting those responses.

## Ethanol concentration

Ethanol content is the important factor to improve wine quality. From the regression model ($Y_1$) of ethanol concentration, the value of the determination coefficient ($R^2$= 0.9936) indicates that 99.36% of the variance could be explained by this model. The value of the adjusted determination coefficient ($R^2_{Vad}$ = 0.9878) shows high significance of the model. Among the model terms, $X_1$, $X_3$, $X_2X_3$, $X_1^2$, $X_2^2$ and $X_3^2$ were significant with a probability of 99% and $X_1X_2$ was significant with a probability of 95% (Table 3). The ethanol production was not significantly affected by the interactions between $X_1$ and $X_3$. The pH ($X_2$) model term is not significantly involved in ethanol production during alcoholic fermentation.

## Volatile acidity concentration

Volatile acids of the wine should be as lowest as possible. From the regression model ($Y_1$) of volatile acidity with $R^2$ = 0.9233 indicates that 92.33% of the variance could be explained by this model. The adjusted $R^2$= 0.8543 was insignificant with the model. Among the model terms, $X_3^2$ was significant with a probability of 99% and $X_1$, $X_3$, $X_1X_2$ $X_1^2$ and $X_2^2$ were significant with a probability of 95% (Table 3). The volatile acids production was not significantly influenced by the interactions between $X_1$ and $X_3$ or $X_2$ and $X_3$. The pH ($X_2$) model term is not significantly involved in volatile acid production during alcoholic fermentation.

## Optimization

The 3-D graphs obtained from the calculated RSM were useful, explaining the interaction of variables and the

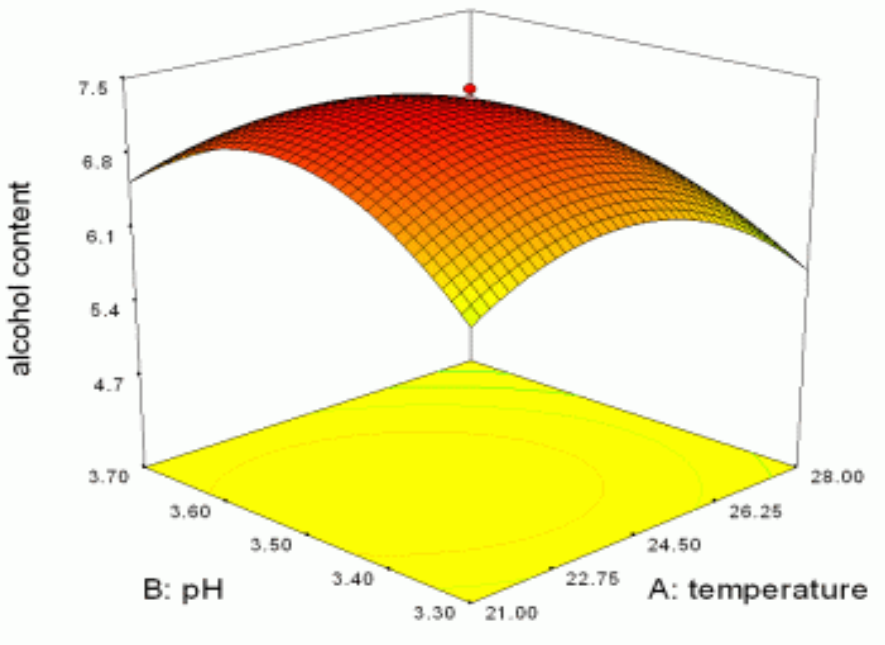


**Figure 1.** Response surface plots of ethanol (%) showing the interactive effect of temperature and pH.

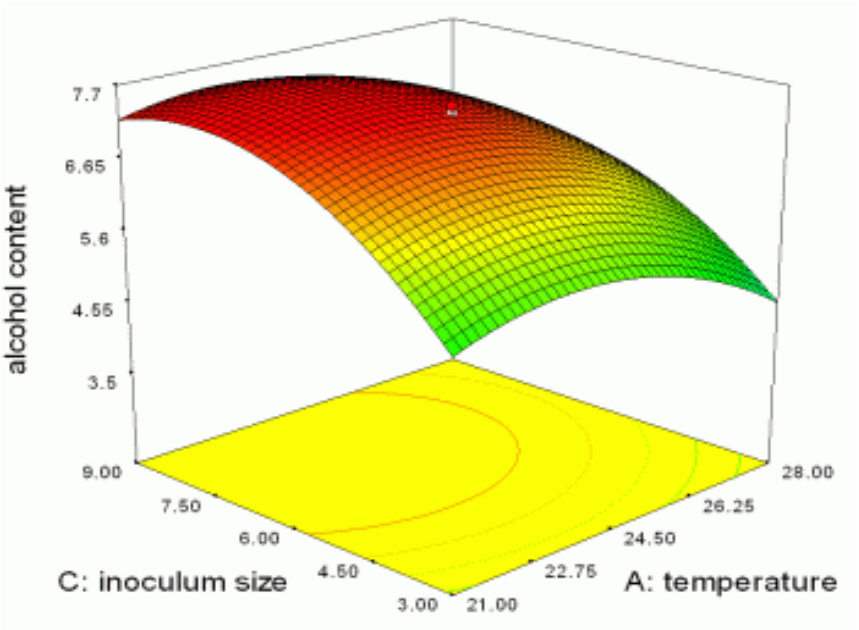


**Figure 2.** Response surface plots of ethanol (%) showing the interactive effect of temperature and inoculum size.

optimum levels of each variable required for the maximum production of ethanol (Figures 1 to 3) and minimum production of volatile acid (Figures 4 to 6). The greatest response of ethanol yield to pH occurred at the intermediate level of temperature (Figure 1). Kumar et al. (2009) reported a similar result. At higher inoculums size, the greatest response of ethanol yield was found when temperature is optimum (Figure 2). Maximum ethanol content was produced under condition of higher inoculums size along with optimum pH (Figure 3). The minimum yield of volatile acid production was obtained at lower temperature and minimum pH (Figure 4). The

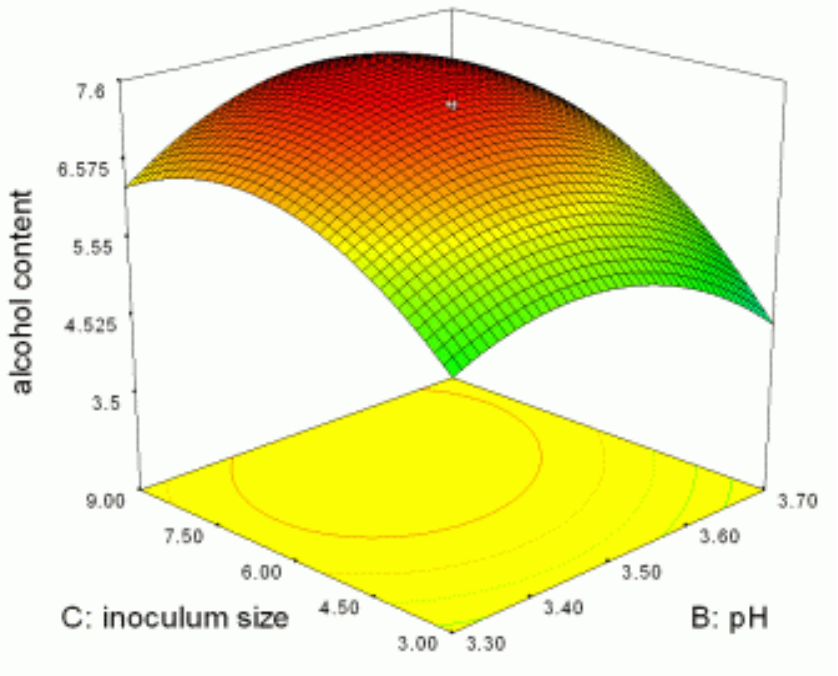


**Figure 3.** Response surface plots of ethanol (%) showing the interactive effect of pH and inoculum size.

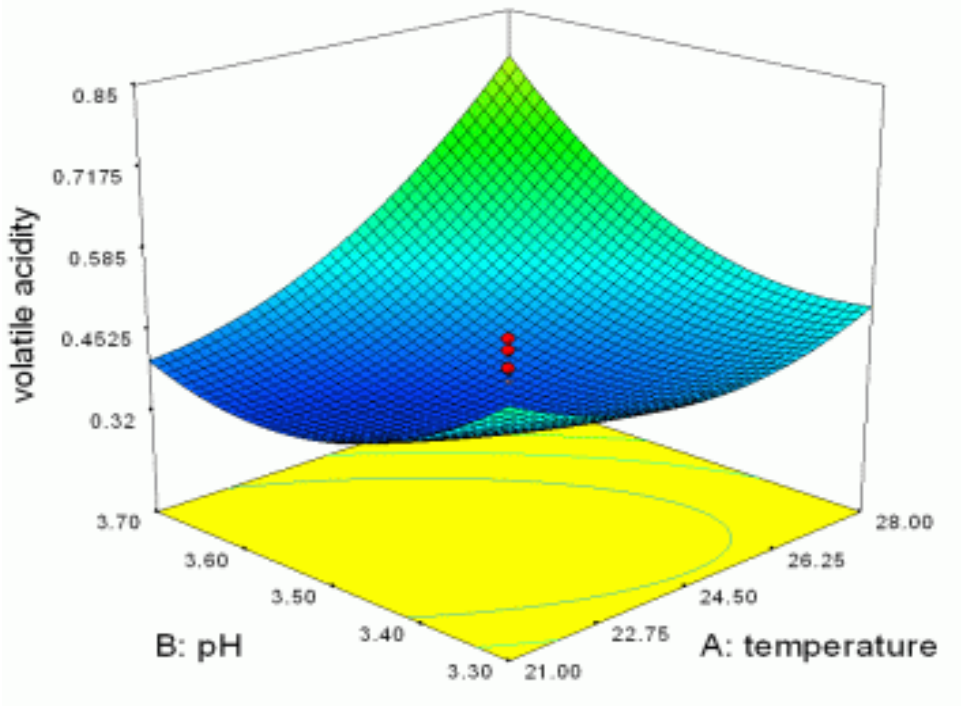


**Figure 4.** Response surface plots of volatile acidity (g $l^{-1}$) showing the interactive effect of temperature and pH.

minimum yield of volatile acid was observed at high inoculums size and the optimum temperature (Figure 5). The minimum yield of volatile acid was obtained at intermediate inoculums size and pH (Figure 6). The

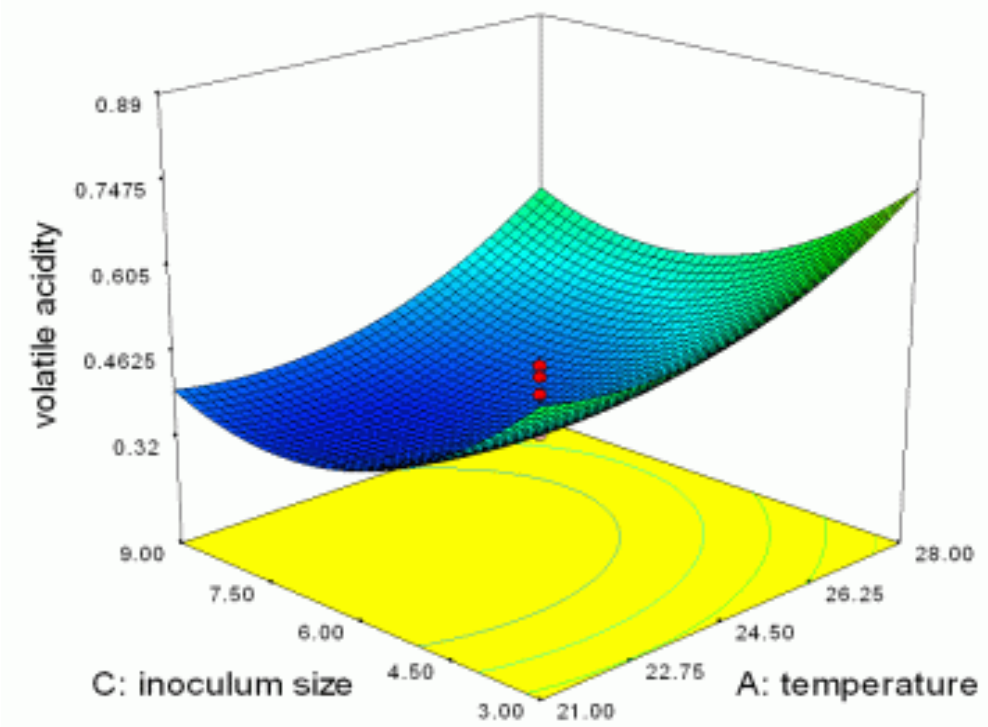


**Figure 5.** Response surface plots of volatile acidity (g $l^{-1}$) showing the interactive effect of temperature and inoculum size.

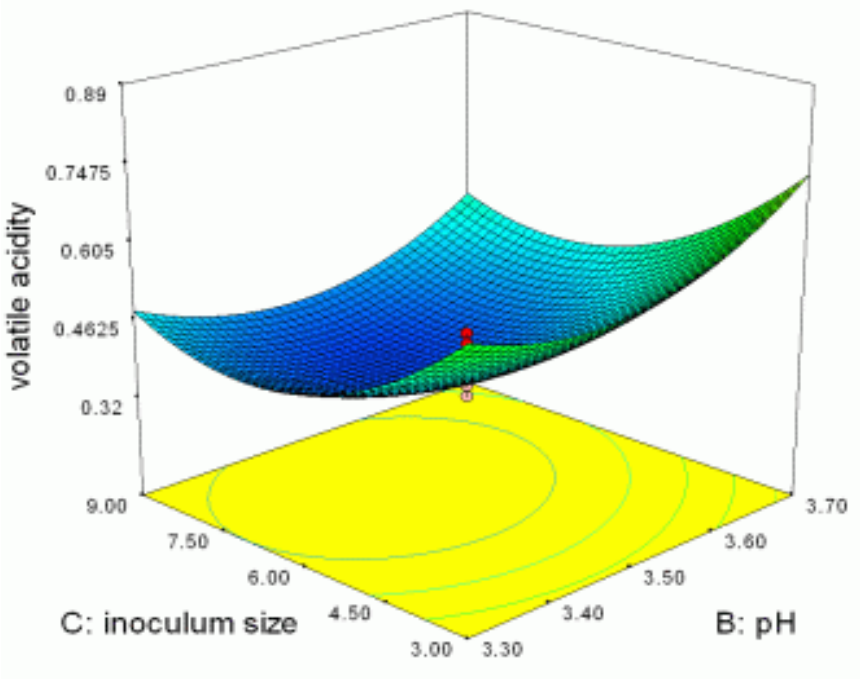


**Figure 6.** Response surface plots of volatile acidity (g $l^{-1}$) showing the interactive effect of pH and inoculum size.

finished optimized fermentation conditions achieved with RSM were 22.65°C (temperature), 3.53 (pH) and 7.37% (inoculums size). Under these conditions, the predicted response values of ethanol content and volatile acid

production were 7.63% and 0.34 g $l^{-1}$, respectively. Three replications were coincident with the predicted value and it is evident that the use of model has helped to get higher content of ethanol content and lower volatile acid content. The sensory analysis was used to evaluate the overall quality of wine samples. The wine produced under the optimized conditions was found to be maximum score of overall acceptance (7.82 ± 0.65) in term of aroma (7.45 ± 0.25), taste (6.82 ± 0.85) and appearance (8.24 ± 0.75), whereas, the sensory analysis of the original wine has the overall acceptance (6.22 ± 0.35) with respect to aroma (5.8 ± 0.2), taste (5.85 ± 0.25) and appearance (6.6 ± 0.46). The wine obtained using optimum fermentation conditions was the best choice of consumers.

## Conclusion

Many factors can affect the ethanol content and volatile acid content of blueberry wine; RSM together with central composite design (CCD) were used to optimize alcoholic fermentation conditions. The results suggested that the maximum ethanol content and minimum volatile acid production of blueberry wine fermentation with *S. cerevisiae* AS2.316 commercial wine yeast could reach 7.63% and 0.34 g $l^{-1}$ under the optimal condition: temperature, 22.65°C; pH, 3.53; inoculums size, 7.37%. The wine obtained using optimal fermentation conditions was the best choice of consumers.

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