The Effect of Temperature, pH and SO_2 on Ethanol Concentration and Sugar Consumption Rate (SCR) in Apple Wine Process

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Summary: The goal of this study was to examine the effects of operating parameters on ethanol concentration (ethanol) in apple wine production process. Examined parameters were temperature (T), pH and sulphurdioxide concentration (SO_2). Experiments were planned and executed according to a full two-level factorial experimental design method. The studied levels were 18°C and 25°C for temperature, 3 and 4 for pH and 50 and 150 ppm for SO_2. Ethanol concentration of apple wine for each set of experiments was determined by GC/MS. Experimental data were analyzed by using both graphical and quantitative Exploratory Data Analysis (EDA) Techniques. The main effect of each factor on sugar consumption rate (SCR) was also examined. The results show that the effect of examined operating parameters on ethanol was negative. High temperature level caused faster fermentation rate than the one caused by low temperature. Low level of pH and high level of SO_2 inhibited the activities of both harmful microorganisms and wine yeast.

Keywords: Apple wine, Cider, Design of experiment, Statistical analysis, Ethanol.

Introduction

Wine production is one of the most attractive fermentation processes with respect to scientific studies and researches, since chemical and microbiological events have a combined complex effect on many qualitative and quantitative properties of wines. Although wine is known to be produced by the fermentation of grape, it is also produced by the fermentation of plant and fruits such as sake from rice in Japan and cider from apple especially in England, Canada, USA and Australia. Wine product on from plant and fruits is especially important with respect to the agricultural economy all over the world. According to 2008 report of The National Agricultural Statistics Service (NASS) of America, about 43% of the processed apples were used in juice and cider production [1]. As reported by Rowles [2], the companies producing hard cider and apple wine have mostly been established in America and England and have been expanding their investment all over the world.

Apple wine, which is also called *cider*, can be defined as an alcoholic beverage produced by the fermentation of apple cider or apple juice. However in the US and some parts of Canada, the alcoholic beverage is commonly known as "hard cider" while "cider" usually refers to a non-alcoholic unfiltered apple juice with a distinct sweet-tart taste. The fermentation process consists of two different stages. The first stage is the yeast fermentation in which sugar is converted to ethanol and the higher alcohols by the yeasts which are added or naturally found on the apple peel. The second one is called the malolactic fermentation in which L(-)-malic acid is converted to L(+)-lactic acid and carbon dioxide by the lactic acid bacteria being in the apple juice or in the natural fermentation area [3]. Ethanol is the most important alcohol formed in wine and volumetrically constitutes 10-13%. It is crucial or the stability, aging, and sensory properties of wine.

In apple wine production, there is a risk of contamination during the growing, harvesting, and juice preparation stages. To provide the necessary microbial stability, addition of sulfur dioxide (SO_2) or metabisulphite into apple juice is the most suitable and traditional method [3]. On the other hand, use of it at even very low concentration causes some health problems such as allergic reaction. Because of the health issues; International Organizations (Joint FAO/WHO Expert Committee on Food Additives) recommended its total elimination or at least reductions in its amount. Nevertheless, the legal permitted limit is 200 ppm (U.S. Food and Drug Administration and European Commission) [4].

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SO_2 effectiveness depends on the pH of the juice. The suggested necessary concentration of SO_2 is 50 ppm when the pH ranged from 3 to 3.3, 100 ppm for pH 3.3-3.5, and 150 ppm for pH 3.5 - 3.8. A desirable juice pH range for cider making is 3.2-3.8. At higher pH values, the fermentation will be subject to contamination and to serious flavor problems. At lower pH infection is safeguarded but the final cider will be unacceptably sharp to the palate and may never be pleasant to drink [5].

The influence of low level of pH and high level of SO_2 on the retardation of the onset of yeast and malolactic fermentation stages, which is the result of the inhibited metabolic activity of yeast cell, could be seen in literature [6-8]. Also, ethanol formation could be prohibited at high level of SO2 resulting from the binding of SO_2 with acetaldehyde which is the product of the decarboxylation reaction of pyruvate in anaerobic fermentation [9].

The medium temperature strongly affects the characteristic properties of both alcoholic and malolactic stages [6]. For example, the rate of acetic acid formation, which negatively affect the organoleptic properties of wine, increase with increasing temperature. The decrease in the concentrations of higher alcohols and ethanol with increasing temperature, mainly formed during yeast metabolism, was observed and reported by Herrero *et al.* [10]. Moreover, the bounded and free forms of SO_2 depend on the cider temperature [11].

Many valuable studies on apple wine production processes related with kinetic properties, modeling and qualitative properties are available in literature [5, 12, 13]. However, it was observed that the studies systematically examining the main and interaction effects of operating parameters on the ethanol content of cider by using quantitative and qualitative methods are limited. This experimental study was realized to simultaneously examine the effect of the operating parameters on ethanol and SCR in apple wine production process which was the first stage of our optimization and control studies. The main, two and three factor interaction effects of temperature, pH, and SO_2 concentration were examined by applying both statistical and graphical methods called two-tailed student's *t* test, Exploratory Data Analysis (EDA) and Normal Plot of Effects [14].

Experimental

Yeast

The yeast used for the fermentation was Saccharomyces cerevisiae Narince III was brewery yeast isolated from an Anatolian white grape type of Narince grapes. It was supplied as plate culture from Ankara University, Faculty of Engineering, and Department of Food Engineering. In order to prepare the pre culture of yeast for wine production inoculation was made from fresh plate culture to liquid medium.

10 ml apple juice containing 1 % yeast extract, 2 % malt extract, 1 % glucose, 0.1 % $(NH4)_2SO_4$ and 0.05 % K_2HPO_4 was used as liquid medium and it was sterilized for 20 min at 121^{o}C in an autoclave before inoculation of yeast. After yeast inoculation to liquid medium at sterilized conditions in a laminar flow cabinet then it was cultured at 30°C for 12 hours in an incubator. This inoculation was transferred to fermentation medium of 1 L consisting the same components and, at the same volumetric ratio with the pre culture at the sterilized conditions.

Fermentation Medium

Golden apples obtained from The Haymana Research and Application Farm (Ankara University, Faculty of Agriculture, Turkey) were processed in order to obtain the apple juice. Possible air contamination was blocked. Apple juice was divided into two parts for 50 ppm and 150 ppm SO_2 application according to the experimental design. Potassium metabisulphite was used for the SO_2 application. These juices were clarified by precipitating at 10°C for 24 hours. Apple juices were transferred to 1 L batch reactors in which the juice pH was regulated from the initial pH of 3.8 to 4 or 3 by adding NaOH or HCl according to the experimental design.

Analytical Assay

Ethanol in apple wine was determined by gas chromatography-mass spectrometry (GC/MS). The GC/MS system was a Shimadzu GC/MS-QP 5000 with a GL-Science TC-5 column (30 m x 0.32 mm internal diameter, 0.25 µm film thickness). Temperature programming was as follows; held at 35°C for 2 min., ramped to 85°C at 2°C/min. and held for 2 min., ramped to 150 ^{0}C at 2 ^{0}C/min and held for 2 min., and finally ramped to 185°C at 6 rate of 4°C/min. The injection volume was 1 µL, the port temperature was 150°C, and the detector transfer line was 300°C. Helium was used as a carrier gas at a flow rate of 1.8 mL/min.

Statistical Analysis

The standard deviation and standard error of the factor effects were calculated as 1.1 and 0.63 by

using Eq. (2) and (3) respectively. Calculated t values from Eq. (l) were compared with the tabulated t_{cr} value as given in Table-1. As can be seen at 0.2 significance level pH-SO_2 two factor interaction and T-pH- SO_2 three factor interaction had greater t values than the t_{cr} value, indicating that these factor effects were different from zero specified by the null hypothesis.

Table-1: Calculated *t* values of factor effects.

Factor	Estimated Absolute Effect	Calculated *t* values	Tabulated $t_{cr/2,3-1}$ α =0.2
T	0.73	1.16	
pH	0.62	0.99	
SO_2	0.42	0.67	
T-pH	0.32	0.51	1.886
T- SO_2	0.03	0.05	
pH- SO_2	1.62	2.57*	
T-pH- SO_2	1.48	2.35*	

*Factor having effect different from zero at 0.2 significance level

Experimental Design

Full two-level non replicated factorial design was used to systematically analyze the experimental data. Factors were studied at two levels called low and high and coded as "+1" and "-1" respectively as given in Table-2. The number of experimental run was eight for three factors. Three replicated experiment was done at center points of factors to determine the process variability. The experimental design matrix was given in Table-3. Factor levels were determined according to the standard order of runs [15]. Experiments were executed randomly in order to remove the effects of possible extraneous factors on experimental results. Throughout the study, it was assumed that experimental data were normally distributed with mean zero and fixed variance. Factor effects and sum of squares were estimated according to the Yates Algorithm [16] given in Table-4.

Exploratory Data Analysis (EDA)

Main Effect Plot was applied to determine the most important factor defined as the one causing significant shift in location of ethanol when factor level was changed from low to high settings. Mean ethanol values were put on the axis y. Examined factors with levels were put on the axis x. Related numerical values and graphical illustration were given in Table-5 and Fig. 1 respectively.

Table-2: Factor levels.

Factor	Symbol	Real Levels			Codified Values		
		maximum	minimum	center	maximum	minimum	center
Temperature (X_1)	T	25°C	18°C	21.5°C	+1	-1	0
pH (X_2)	pH	4	3	3.5	+1	-1	0
SO_2 concentration (X_3)	SO_2	150 ppm	50 ppm	100 ppm	+1	-1	0

Table-3: Experimental design matrix.

Number of experiment	T	pH	SO_2	Ethanol (% v/v)
1	-1	-1	-1	6.9
2	+1	-1	-1	8.0
3	-1	+1	-1	9.7
4	+1	+1	-1	7.2
5	-1	-1	+1	9.6
6	+1	-1	+1	7.7
7	-1	+1	+1	6.2
8	+1	+1	+1	6.6
9	0	0	0	6.2
10	0	0	0	8.1
11	0	0	0	6.2

Table-4: Yates Algorithm.

Run Number	Factors				Algorithm			Factor identification	Estimate of Effect (3)+2^{k-1}
	T	pH	SO_2	Ethanol	(1)	(2)	(3)		
1	-1	-1	-1	6.9	14.90	31.80	61.90	average	--
2	+1	-1	-1	8.0	16.90	30.10	-2.90	T	-0.73
3	-1	+1	-1	9.7	17.30	-1.40	-2.50	pH	-0.62
4	+1	+1	-1	7.2	12.80	-1.50	-1.30	T-pH	-0.32
5	-1	-1	+1	9.6	1.10	2.00	-1.70	SO_2	-0.42
6	+1	-1	+1	7.7	-2.50	-4.50	-0.10	T- SO_2	-0.03
7	-1	+1	+1	6.2	-1.90	-3.60	-6.50	pH- SO_2	-1.62
8	+1	+1	+1	6.6	0.40	2.30	5.90	T-pH- SO_2	1.48

Table-5: Average ethanol concentration (% v/v) for main effect plot.

Factor	at high level	at low level
T	7.38	8.10
pH	7.42	8.05
SO_2	7.53	7.95

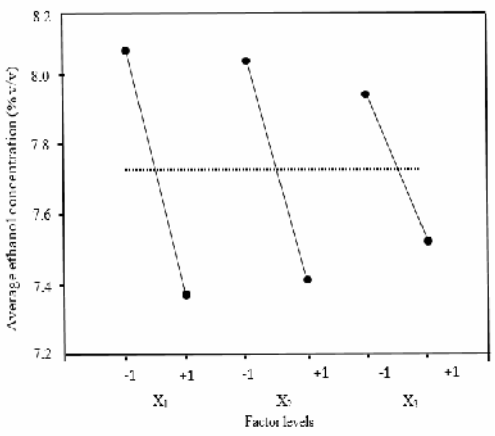

Fig. 1: Main effect plot.

The two-factor interaction is defined as the average change in response caused not only by one input factor level but also by the other input factor level. In case of three-factor interaction, the two-factor interaction depends on the remaining third factor level. In this study, T-pH, T-SO_2 and pH-SO_2 were; the two-factor interaction terms. The interaction between T-pH-SO_2 was the three-factor interaction term.

Generally, factor interaction is defined by a nonparallel line in a line graph. Crossed or eventually crossed lines illustrate the interaction, too [17]. The two-factor Interaction plots were given in Fig. 2(a), 2(b) and 2(c) and related numerical values were given in Table-6. The Figures and Tables of three-factor interaction were given in Fig. 3(a), 3(b), Fig. 4(a), 4(b) and Table-7, respectively.

The statistical hypothesis test is a method of making decisions about a hypothesis by using data and suitable statistical methods. A hypothesis test includes two statements called null and alternative hypotheses. The null hypothesis illustrates the possibilities which are not to be expected. The hypothesis testing is based on the acceptance of the trueness of the null hypothesis. The alternative hypothesis is the supported claim by the researches. When a statistical hypothesis is done, two types of error can arise. The probability of Type I error or the probability of the wrongly rejected true null hypothesis is called significance level and symbolized with α. The value of α is selected such that the probability of Type I error must be as possible as low [18].

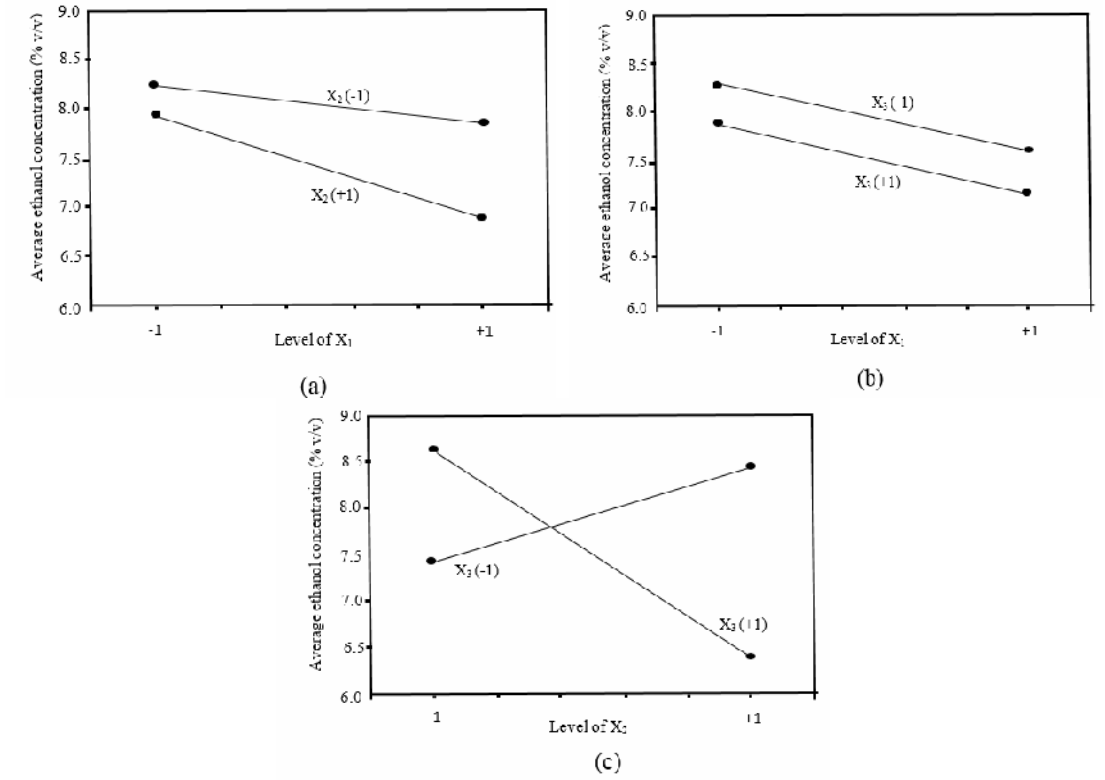

Fig. 2: Two factor interaction plots (a) Temperature (X_1) and pH (X_2) (b) Temperature (X_1) and SO_2 concentration (X_3) (c) pH (X_2) and SO_2 concentration (X_3).

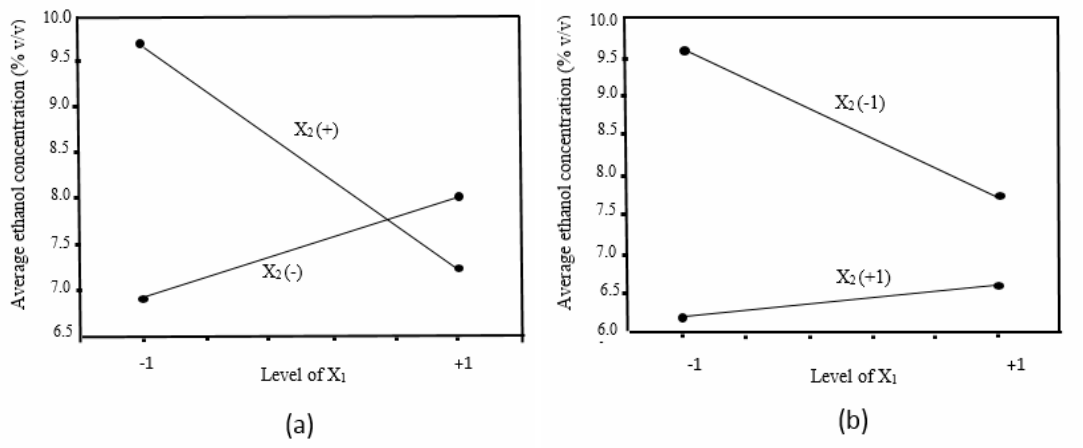

Fig. 3: Three factor interaction plots (a) SO_2 concentration at 50 ppm (X_3: -1) (b) SO_2 concentration at 150 ppm (X_3: +1).

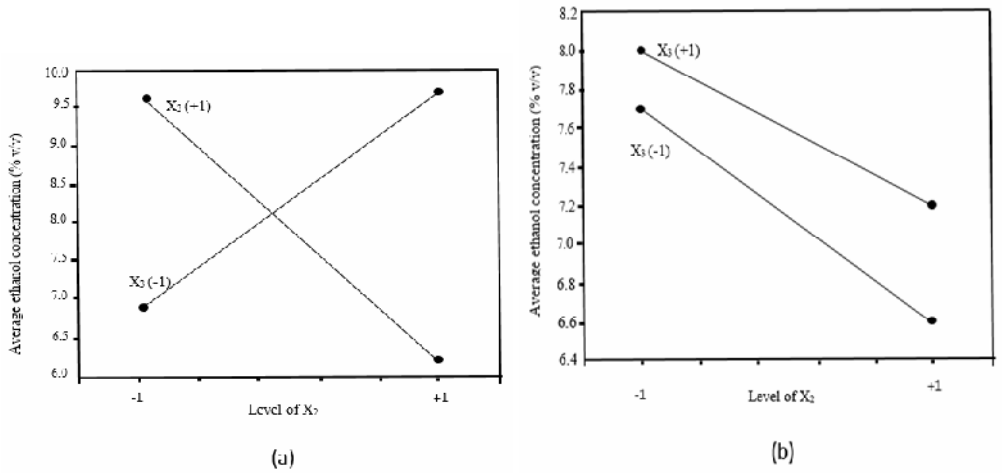

Fig. 4: Three factor interaction plots (a) Temperature at 18°C (X_1: -1) (b) Temperature at 25°C (X_1: +1).

In this study, following null (H_0) and alternative hypothesis (H_a) were set up and tested in the case of Type I error at 0.2 significance level.

H_0: All factor effects = 0
H_a: All factor effects ≠ 0.

The t statistics of factors were calculated from the following equation and given in Table-8.

$$t = \frac{|X_i|}{seX_i} \qquad i = 1\ldots.12\ldots.123 \qquad (1)$$

seX_i is the standard error of factor effects and defined;

$$seX_i = \frac{s}{\sqrt{n}} \qquad (2)$$

$$s = \sqrt{\frac{center\ point}{n-1}} \qquad (3)$$

Table-6: Average ethanol concentration (% v/v) for two factor interaction plots.

		pH		SO_2	
		at high level	at low level	at high level	at low level
T	at high level	6.90	7.85	7.15	7.60
	at low level	7.95	8.25	7.90	8.30

		SO_2	
		at high level	at low level
pH	at high level	6.40	8.45
	at low level	8.65	7.45

Table-7: Average ethanol concentration (% v/v) for three-factor interaction plots.

		T (SO_2 at high level)		T (SO_2 at low level)	
		at high level	at low level	at high level	at low level
pH	at high level	6.6	6.2	7.2	9.7
	at low level	7.7	9.6	8.0	6.9

Table-8: Steps of normal probability plot.

Factor	Estimated Effect	Rank	Probability (R-0.5)/n	Theoretical quintile "z"
pH- SO_2	-1.62	1	0.071	-1.46838
T	-0.73	2	0.21	-0.80642
pH	-0.62	3	0.36	-0.35846
SO_2	-0.42	4	0.5	-1.4E-16
T-pH	-0.32	5	0.64	0.358459
T- SO_2	-0.03	6	0.78	0.772193
T-pH- SO_2	1.48	7	0.93	1.475791

"s" is the standard deviation of the process determined by using the data obtained from three replicated center point experiment given in Table-3. The null hypothesis was rejected at significance level of 0.2 when the calculated t values, given in Table-1 were greater than the two-tailed tabulated t statistic [19].

Normal probability plot was suggested by Daniel in 1959 especially as a non-replicated factorial design analysis and is used to define significant and non-significant factor effects [20]. According to this method, experimental data follow a normal distribution with mean zero and fixed variance. Data deviating from normality assumption are defined to be significant because they have different mean and variance from normality assumption.

The steps for constructing Normal Probability plot are given below;

1. Effects were ordered from smallest to largest
2. Ordered values were ranked from 1 to 7
3. Cumulative probabilities (R-0.5)/n were calculated for each rank
4. "z" values corresponding for each probability were obtained by using standard normal distribution table
5. XY scatter graph was plotted by placing ordered effects on horizontal axis and z-score on vertical axis

Data placed ahead of straight line was called significant factor effect. On the other hand, data placed on straight line was called non-significant factor effect. In this study data for normal probability plot was given in Table 8. In Fig. 5 normal probability plot of factor effects was given.

Results and Discussion

In Fig. 1, the main effects of temperature, pH and SO_2 concentration on ethanol were examined. As can be seen, all the main factors have negative effects since the variation in factor levels caused a decrease in ethanol. The greatest amount of ethanol on average (8.1% v/v) was obtained at 18°C. The least value (7.38 % v/v) was obtained at 25°C. Temperature difference of 7°C between low and high levels caused 0.7% v/v decrease in ethanol. The variation in ethanol was 0.42% v/v decrease with the change of level in SO_2. The pH had a greater decreasing effect than SO_2 by causing 0.63% v/v decrease. The most important factor was the temperature since it caused the largest variation in response.

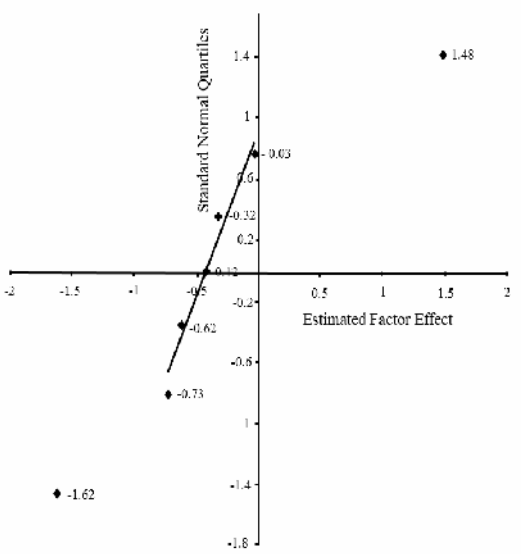

Fig. 5: Normal probability plot of factor effects.

The two-factor interaction of T-pH was examined in Fig. 2(a). If the lines extended, lines could be crossed by indicating an interaction. The highest average amount of ethanol (8.25% v/v) was reached at 18°C and pH 3. When pH value was at 4 and 3, the changes in ethanol with the variation in T were 1.05% v/v and 0.4% v/v decrease, respectively. At 18°C and at different pH levels the variation in response was 0.3% v/v decrease. At 25°C and at different pH levels the variation in ethanol was 0.95% v/v decrease.

The two-factor interaction of T-SO_2 was examined in Fig. 2(b). Maximum average ethanol (18.3% v/v) was reached at 18°C and at 50 ppm of SO_2. Minimum ethanol (7.15% v/v) was obtained at 25°C and 150 ppm SO_2. At both low and high T, the variation in ethanol with the change of SO_2 level was 0.4% v/v decrease. The variation in ethanol with the change of T was about 0.7% v/v decrease at both high and low SO_2 levels. There was no direct interaction between T and SO_2.

In Fig. 2(c), a significant interaction was observed between pH and SO_2. Minimum ethanol (6.4% v/v) was reached at pH 4 and at 150 ppm of SO_2. Maximum ethanol (8.65% v/v) was obtained at

pH 3, and at 150 ppm of SO_2. It is clear that a significant decrease (2.25% v/v) in ethanol occurred when the pH was increased from 3 to 4 at 150 ppm of SO_2. The pH effect was smaller at 50 ppm than at 150 ppm of SO_2. When pH was changed from 3 to 4 at 50 ppm level of SO_2, the variation was 1% v/v increase in ethanol. The variations in ethanol with the change of SO_2 level at pH 3 and 4 were 1.2% v/v increase and 2.0% v/v decrease respectively.

In Fig. 3(a), SO_2 level was set at 50 ppm and the variation between T and pH was examined. A significant shift in location of ethanol was clear. The shift was greater at pH 4 than at pH 3 when T was changed from 18°C to 25°C. The variation was 2.5% v/v decrease and 1.1% v/v increase at pH 4 and 3 respectively. At 18°C the variation in ethanol with the change of pH level was 2.8% v/v increase. However, at 25°C the variation in ethanol with the change of pH level from low to high was 0.9% v/v increase.

In Fig. 3(b), the shift in ethanol with the change of T and pH at 150 ppm of SO_2 was given. The variations in ethanol at 18°C and 25°C with the change of pH were 3.4% v/v and 1 % v/v decrease respectively. The amount of ethanol dropped 1.6% v/v when the T was increased from 18°C to 25°C at pH 3. The variation in ethanol at pH 4 with the change of T was 1.2% v/v increase.

In Fig. 4(a), the crossed lines showed an important interaction between pH and SO_2 at 18°C. On the contrary, as shown by Fig. 4(b), at 25°C, pH-SO_2 interaction was smaller than at 18°C.

From Fig. 5, it was observed that two-factor interaction of pH- SO_2 and the three-factor interaction of T-pH- SO_2 placed ahead off straight line with the magnitude of -1.62 and 1.48, respectively. These factors deviated from the normality assumption.

The related Fig was given in Fig. 6. Maximum SCR (8.2 g sugar/L. day) was obtained with faster fermentation at 25°C than 18°C at which minimum SCR (5.9 g sugar/L. day) was observed. It was also observed that the SCR values were progressed faster to the shorter stationary phase and, ended sharply and quickly at 25°C than at 18°C.

The data, obtained from two-level factorial experimental design, were systematically analyzed by using graphical and statistical methods to explore the combined and individual effects of the temperature, pH and SO_2 on ethanol and SCR in apple wine. The operating temperature was found to be the most important main effect according to the Fig. 1 and Table-1. Nevertheless, the two-factor interaction of pH- SO_2 and the three-factor interaction of T-pH-SO_2 effects were concluded to be effective on ethanol at 20% significance level with -1.62 and 1.48 magnitudes, respectively.

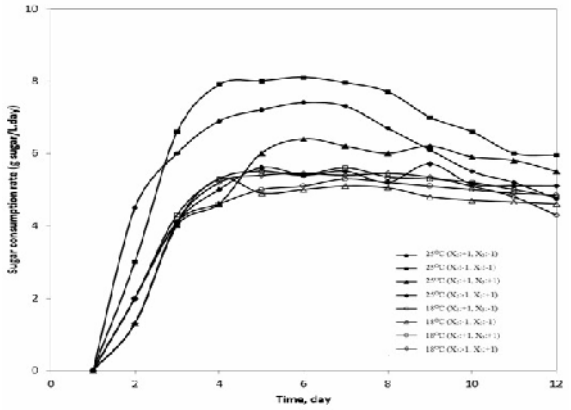

Fig. 6: Sugar consumption rates at high (25°C) and low (18°C) temperatures.

To overcome the disadvantage of nonreplicated experimental design at low and high levels of factors, the center point experiment was replicated three times and the normal probability plot of effects was examined. The low probability of statistical significance level α=0.2, (or the probability of rejecting true null hypothesis) was the function of small sample size. It could be increased by replication and the more significant results could be obtained [21, 22].

From Fig. 3 and 4, it was concluded that at 18°C, pH-SO_2 interaction was more dominant than at 25°C because of the slow fermentation rate at 18°C. Also, the selected low level of SO_2 could not ensure enough hygienic condition despite of acidic properties of low level of pH, since acidic pH level could cause an improved hygienic condition [8]. At 150ppm SO_2 contamination was prevented and high ethanol obtained at low level of pH.

The lowest ethanol content was observed at 25°C than at 18°C confirming the results observed by Sener *et.al*. [19] in which decreasing, ethanol yield (g ethanol/g biomass) was reported by increasing temperature from 18°C to 25°C. From Table-7 and Fig. 4, the negative effects of high levels of SO_2 and pH together with the low level temperature on the ethanol could be seen.

As can be seen from Fig. 6, high temperature (25°C) caused faster fermentation rate Roza *et.al*. [13] reported similar process dynamic properties such as slow and low yeast activity, ethanol evaluation and SCR values at controlled low temperature (15°C) in laboratory, plot and semiplot scales. In addition, a sharp and quick decrease in *S.cerevisiae* activity at the end of the fast fermentation, obtained from the industrial scale fermentation in which temperature changed freely from 15°C to 27°C, reported by them confirming our observation carried out at 25°C [23]. At 18°C, slowly progressing fermentation with lag phase and prolonged stationary phase were observed similar to the findings at 10°C recorded by Bilbao *et al.* [24].

The slowest SCR (5.9 g sugar/L. day) was observed at 18°C under pH 3 and 150 ppm SO_2. But under these conditions ethanol was 9.6(% v/v) as can be seen from Table-7. This could be attributed to slow fermentation rate at low temperature (18°C). On the other hand, the influence of high level of SO_2 (150 ppm) on ethanol could be seen at 25°C from Table-7. The high level of SO_2 inhibited the yeast growth and metabolic activities at high level of pH as reported in literature [6, 7, 9]. Ton *et.al*. stated that when the initial sulfur dioxide content in must augmented from 12 ppm to 312 ppm, the fermentation time of the free yeast was 8.9h- 29.7h higher than that of the immobilized yeast [8]. Also, the selected low level of SO_2 could not ensure enough hygienic condition despite of low level of pH. Since acidic pH level could improve hygienic condition. At 150 ppm SO_2 with low pH, contamination was prevented and high ethanol obtained.

Conclusion

Temperature, pH and SO_2 in the apple wine production process were important operating parameters and they should be pre-determined and controlled. According to statistical analysis, main effects of these parameters were found to be important between low and high levels. As it was expected, the two factor interaction of pH-SO_2 and the three-factor interaction of T- pH- SO_2 effects were concluded to be effective on ethanol process at 20% significance level with -1.62 and 1.48 magnitudes respectively. These factor effects didn't occur by chance and were found to be different from experimental error. 150 ppm of SO_2 was determined very high to provide suitable hygienic stability since at this level of SO_2 the activities of both harmful microorganisms and wine yeast were inhibited. By regarding both ethanol production and sugar uptake rate dynamic properties, the level of operating temperature was concluded to be 18°C. Three factor interaction was meaningful when we consider the dependence SO_2 solubility in water on temperature for example at 10°C 162.1 g/L, at 20°C 112.9 g/L, 30°C 78.1 g/L and the dependence of SO_2 dissociates equilibrium on pH.

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