

The Influence of Inoculum Level on Fermentation and Flavour Compounds of White Wines Made from cv. Emir

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ABSTRACT

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In this study, the influence of the addition of a commercial wine yeast (*Saccharomyces cerevisiae*) at inocula of 1×10^4 to 1×10^7 cells/ml in Emir must was investigated with a focus on yeast growth, fermentation rate, ethyl alcohol and flavour compound formation. Spontaneous fermentation without inoculation was also performed. Higher peak counts were observed with higher amounts of *S. cerevisiae* yeast. Addition of various amounts of yeast led to the earlier disappearance of non-*Saccharomyces* yeasts. The fermentation rate was improved with higher amounts of yeast, but ethanol production was not affected. Concentrations of higher alcohols increased with increasing inoculum levels, especially inoculum sizes of 1×10^6 cells/ml and 1×10^7 cells/ml. The amount of ethyl acetate was reduced with increased inoculum levels.

Key words: cv. Emir, fermentation, inoculum level, *Saccharomyces cerevisiae*, volatile compounds, wine.

INTRODUCTION

Yeasts are primarily responsible for the alcoholic fermentation of grape juice into wine¹⁰. They originate from the flora of grapes and winery equipment and from added starter cultures, if used¹⁶. Wine fermentation is either performed conventionally without inoculation or by the addition of selected wine yeast into grape juice^{11,17,18,34}.

In conventional wine making, natural (spontaneous) alcoholic fermentation of grape juice is conducted by a sequence of different yeast species. At the beginning, non-*Saccharomyces* yeasts, *Kloeckera* (teleomorph *Hanseniaspora*) and *Candida* in particular, initiate the alcoholic fermentation of grape juice. These yeasts progressively die off with an increase in ethanol concentration, and leave more ethanol tolerant *Saccharomyces* (*S. cerevisiae*) to complete the fermentation^{7–9,21,28,35}. The inoculation of grape must with selected and/or commercial strains of *S. cerevisiae* in wine making is used in newer and also in traditional wine producer countries at the present time. The process offers better control of alcoholic fermentation

by establishing a high population of wine yeast and affects wine quality^{7,17,18,34}.

The major products of yeast metabolism are ethanol and CO₂. In addition to these products, yeast produces many flavour compounds as secondary products during alcoholic fermentation. The main compounds that form the fermentation bouquet are higher alcohols, esters, acids and carbonyl compounds. Small amounts of these volatile compounds, depending on odour threshold, contribute positively to wine quality^{19,29,33}.

Although there are various studies on the effects of pitching levels of brewer's yeasts on beer fermentation^{12,30,32}, information concerning the effects of the pitching level of the wine yeast *S. cerevisiae* on wine fermentation is scarce. Mateo et al.²⁰ found that inoculum levels affected wine fermentation and volatiles.

Vitis vinifera L. cv. Emir is a native grape variety grown in the Nevsehir-Ürgüp region (ancient Cappadocia) of Turkey. The Emir variety has important potential in terms of white grape production and yields one of the good-quality wines in Turkey^{4,23,24}.

The objective of the present work was to study the influence of the inoculum level at inocula of 1×10^4 to 1×10^7 cells/mL, at intervals of a log unit, on wine fermentation and flavour formation of white wines made from Emir grapes.

MATERIALS AND METHODS

Yeast strain

The commercial active dried culture of *S. cerevisiae* (Fermiblanc N°SM 102-Gist Brocades) was obtained from INRA, France.

Fermentation conditions

About 300 kg of healthy grapes of cv. Emir were purchased from the Nevsehir-Urgup region of Turkey. The grapes were transported to the Pilot Winery of the Department of Food Engineering, Faculty of Agriculture, Cukurova University. They were crushed and pressed in a horizontal press (Sarksan, Ankara). Free and pressed grape juices were combined and 40 mg/L of sulphur dioxide (i.e. 0.8 mL/L from a 5% solution of sulphur dioxide) was added. The grape juice was allowed to settle at 15°C for 24 h and then racked.

Fermentations were performed, in duplicate, in 20 L glass vessels. Commercial wine yeast was suspended in

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sterile warm water at 35°C for 30 min according to the producer's instructions. Cells were counted using a counting chamber and then added to the fermentation vessels at the level of 1×10^4 , 1×10^5 , 1×10^6 and 1×10^7 viable cells/mL. Simultaneously, spontaneous fermentation, without inoculation, was also performed. All vessels were closed with fermentation locks and were maintained at 18°C throughout the fermentation. Fermentation was monitored by measuring specific gravity. At the end of alcoholic fermentation, wines were racked and 40 mg/L of sulphur dioxide was added. The wines were then bottled for general wine analysis. For flavour analysis, the samples were stored at -18°C until analysed.

Enumeration of yeast population

During fermentation, samples were taken aseptically for yeast counts. One mL of sample was serially diluted as required in 0.25% saline and 0.1 mL of diluted sample was spread on malt extract agar and lysine agar (Difco). Total yeast were enumerated on malt extract agar. Non-*Saccharomyces* yeasts were counted on L-lysine agar (a synthetic medium of glucose, vitamins, inorganic salts, and L-lysine as sole nitrogen source on which *Saccharomyces* spp. are unable to grow). Plates were incubated at 25°C for 3–5 days and yeast colonies were counted^{5,8,17}. The total *Saccharomyces* spp. count was calculated from the total yeast and non-*Saccharomyces* yeast counts.

Analytical determinations

Density was determined by the pycnometric method at 20°C and ethanol was determined by pycnometer at 20°C after distillation. Reducing sugar was analysed according to the Luff-School method. Volatile acidity was determined by steam distillation and expressed as g of acetic acid/L. Total acidity was analysed by titration with 0.1 N NaOH solution, phenolphthalein was used as indicator and expressed as g of tartaric acid/L. The above methods are described in the standard methods for analysis of musts and wines^{1,25}.

Volatile higher alcohols, esters and carbonyl compounds were analysed with a 5890 gas chromatograph (Hewlett-Packard, Stockport, UK). Samples were centrifuged in capped tubes at 4°C to remove the yeast cells. The cell-free samples were diluted to 4% ethanol and 5 mL of diluted sample, 2 g of NaCl and 50 µL of internal standard (a mixture of 200 mg/L of 3-heptanone and 18.2 mg/L of 2,3-hexanedione) were added into vials, sealed and a 1 mL sample was injected into the column using a headspace autosampler (Perkin Elmer). The column was 60 m long, 0.25 mm ID and 0.4 µm thick from Chrompack CP-Wax-57-CB (Middleburg, Netherlands). The column temperature was kept at 43°C for 2 min, and increased to 92°C at 1.8°C/min. It is then increased from 92°C to 180°C at 30°C/min and held at that temperature for 4 min. The stream from the column was split 1:1 to a flame ionisation detector. The carrier gas was helium at the flow rate of 2.2 mL/min. The flavour compounds were tentatively calculated by comparing the retention times with those from calibration standard curves on a data handling system (Hewlett-Packard)¹³.

Replicates for all analytical determinations were carried out in triplicate.

Statistical analysis

Data of wine composition were analysed for statistical significance by one-way analysis of variance (ANOVA). Means were compared by Duncan's test. The statistical analysis was carried out using the software SPSS 9.0 for windows²⁶.

RESULTS AND DISCUSSION

Effect of inoculum level on the course of fermentation

The decrease in specific gravity is given in Fig. 1. Inoculating the grape must at different rates of total yeast *S. cerevisiae* influenced the fermentation time. There were significant differences in fermentation performance with different inoculum levels. Faster fermentations were observed with an increase at the addition of pitching rates. Therefore, the fermentation time needed ranged from 7 to 13 days, depending on inoculum level. The fastest fermentation performance occurred with an inoculum level of 1×10^7 cells/mL, followed by inoculum levels of 1×10^6 , 1×10^5 , 1×10^4 cells/mL. The slowest fermentation rate was with spontaneous fermentation without inoculation. Several workers^{12,17,20,32} have stated that increasing the inoculum level resulted in a faster fermentation rate with brewer's and wine yeast strains.

Effect of inoculum level on yeast growth

The development of total *Saccharomyces* spp. during the fermentations is given in Fig. 2. At the beginning of fermentation, the fresh grape juice exhibited a total *Saccharomyces* yeast count of 3.92 log cfu/mL. As the inoculum level rose, the maximum yeast count in the fermenting grape must increased and the maximum yeast number was obtained as 8.05 log cfu/mL with inoculum level of 1×10^7 cells/mL on day 3. With the experiment with the highest yeast amount, total *Saccharomyces* spp. showed a long stationary growth and at the end of fermentation, viable counts varied from 7.26 log cfu/mL to 7.46 log cfu/mL.

The growth of non-*Saccharomyces* yeasts is given in Fig. 3. At the beginning of fermentation, the number of

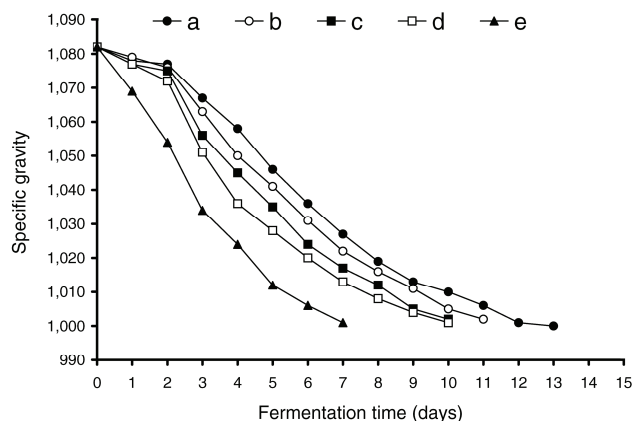


Fig. 1. The decrease in specific gravity during fermentations. a: Spontaneous, b: 1×10^4 cells/mL yeast, c: 1×10^5 cells/mL yeast, d: 1×10^6 cells/mL yeast and e: 1×10^7 cells/mL yeast.

these yeasts was 3.54 log cfu/mL. The maximum amount of total non-*Saccharomyces* yeasts was obtained on day 2 and was 4.76 log cfu/mL with spontaneous fermentation (without inoculation) and with an inoculum level of 1×10^4 cells/mL. The maximum amounts were 4.66, 4.15 and 3.81 log cfu/mL by day 1–2 with inoculum levels of 1×10^5 , 1×10^6 , 1×10^7 cells/mL, respectively. The non-*Saccharomyces* yeasts did not exhibit a stationary phase. After maximum growth, the decline phase occurred during fermentation. As the inoculum level increased, the disappearance time decreased and these yeasts were not counted from day 6 to day 10, depending on inoculum levels.

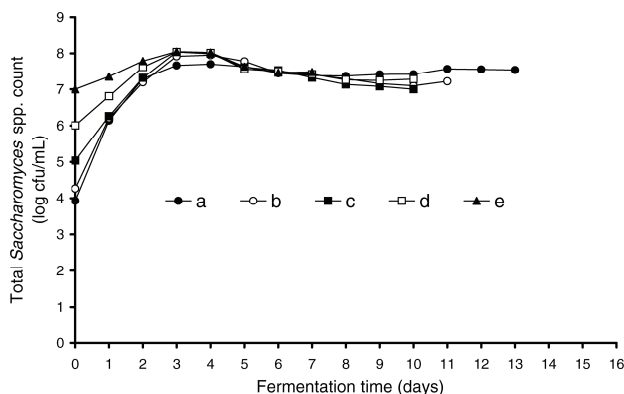


Fig. 2. The development of total *Saccharomyces* spp. during fermentation. a: Spontaneous, b: 1×10^4 cells/mL yeast, c: 1×10^5 cells/mL yeast, d: 1×10^6 cells/mL yeast and e: 1×10^7 cells/mL yeast.

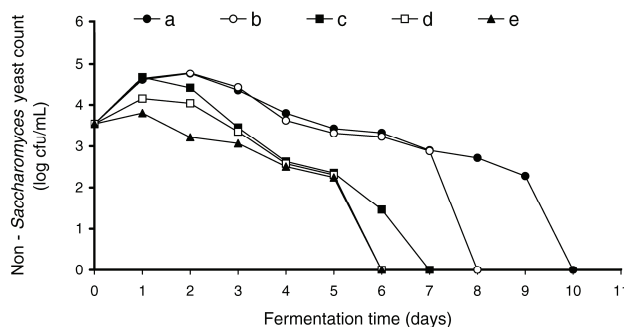


Fig. 3. The growth of non-*Saccharomyces* yeasts during fermentation. a: Spontaneous, b: 1×10^4 cells/mL yeast, c: 1×10^5 cells/mL yeast, d: 1×10^6 cells/mL yeast and e: 1×10^7 cells/mL yeast.

It is thought that during the alcoholic fermentation, the non-*Saccharomyces* yeasts disappear after 3 or 4 days of fermentation, with an increase in ethanol concentration, and the main wine yeast *S. cerevisiae* completes the fermentation^{3,6}. Disappearance of the non-*Saccharomyces* yeasts is recently attributed to the quorum sensing phenomenon, where microbial cells communicate with each other and thus regulate their population growth^{16,22}. However, it is reported that in conventional and selected yeast added fermentations, the non-*Saccharomyces* yeasts grew and survived longer during the fermentation^{8,9,14,17,21,23,36}.

General wine composition

Table I gives the general wine composition. The ethyl alcohol content of the wines was between 10.15% (v/v) and 10.30% (v/v). All wines were fermented to dryness with less than 1.17 g/L of reducing sugar remaining. Total acidity of the wines ranged from 6.07 to 6.84 g/L as tartaric acid. The amount of volatile acidity was found to be between 0.22 to 0.47 g/L as acetic acid in wines inoculated with different levels, but spontaneous fermentation without inoculation produced 0.54 g/L. Fleet and Heard¹⁷ have stated that the volatile acidity of wines produced from spontaneous fermentation was found to be higher than that of the wine produced from the addition of selected wine yeast.

Effect of inoculum level on flavour compounds

The main producer of flavour compounds during alcoholic fermentation is the yeast. A number of these compounds make an important contribution in the typical odour and taste of alcoholic beverages².

The effect of inoculum size on the flavour composition of the wines is given in Table II. When inoculum size increased, the total amount of higher alcohols rose from 232.79 mg/L to 386.39 mg/L. A total concentration of higher alcohols below 300 mg/L contributes positively to wine quality, but amounts higher than 400 mg/L may detract from the quality^{19,21,27}. The relative amounts of isobutanol (2-methyl-1-propanol), 2-methyl-1-butanol (active amyl alcohol) and 3-methyl-1-butanol (isoamyl alcohol) were greater for the higher inoculum levels. The lowest concentration was obtained with spontaneous fermentation, without inoculation, and the highest with an inoculum level of 1×10^7 cells/mL. The amount of n-propanol (1-propanol), however, decreased with increasing inoculum size compared to non-inoculated wine. Mateo et al.²⁰ reported that increasing the inoculum level increased the relative amount of 3-methyl-1-butanol and n-propanol, but the relative concentration of isobutanol decreased.

Table I. General wine composition.

	Spontaneous	1×10^4 cells/mL yeast	1×10^5 cells/mL yeast	1×10^6 cells/mL yeast	1×10^7 cells/mL yeast	Sig. ^a
Density (20°C)	0.9945 ^a	0.9935 ^{bc}	0.9922 ^d	0.9939 ^{ab}	0.9929 ^c	*
Ethanol (v/v)	10.28	10.16	10.30	10.15	10.23	ns
Residual sugar (g/L)	1.17	1.08	1.15	1.13	1.13	ns
Total acidity (g/L as tartaric acid)	6.24 ^b	6.12 ^{bc}	6.07 ^c	6.84 ^a	6.23 ^b	*
Volatile acidity (g/L as acetic acid)	0.54 ^a	0.22 ^d	0.25 ^d	0.47 ^b	0.31 ^c	*

^a Sig.: Significance, *, displays the significance at 5% by LSD. Values not sharing the same superscript letter within the horizontal line are different according to the Duncan test, ns: not significant.

Table II. The effect of inoculum size on flavour compounds of wines.

Flavour compounds (mg/L)	Spontaneous	1×10^4	1×10^5	1×10^6	1×10^7	Sig. ^a
		cells/mL yeast	cells/mL yeast	cells/mL yeast	cells/mL yeast	
Higher alcohols						
Iso butanol	36.93 ^b	40.97 ^b	33.44 ^b	44.10 ^b	60.65 ^a	*
2-Methyl-1-butanol	34.24 ^b	44.83 ^{ab}	35.33 ^b	50.86 ^a	55.16 ^a	*
3-Methyl-1-butanol	142.83 ^d	178.96 ^c	163.92 ^c	213.83 ^b	255.70 ^a	**
<i>n</i> -Propanol	18.79 ^a	15.25 ^{ab}	12.34 ^b	12.89 ^b	14.88 ^b	*
Total	232.79	280.01	245.03	321.68	386.39	
Esters						
Ethyl acetate	136.53 ^a	79.23 ^c	62.26 ^c	87.04 ^b	40.01 ^d	**
Isobutyl acetate	0.03	0.02	0.02	0.04	0.03	ns
Ethyl butyrate	0.07 ^b	0.07 ^b	0.13 ^a	0.08 ^b	0.08 ^b	**
Iso amyl acetate	0.61 ^c	0.77 ^c	1.59 ^a	1.05 ^b	0.78 ^c	**
Ethyl hexanoate	0.11 ^b	0.10 ^b	0.27 ^a	0.14 ^b	0.11 ^b	**
Total	137.35	80.19	64.27	88.35	41.01	
Carbonyl compounds						
Diacetyl	0.15	0.05	0.17	0.04	0.05	ns
2,3 Pentanedione	0.05	0.02	0.01	0.01	0.01	ns
Acetaldehyde	13.99	12.60	11.08	14.88	16.22	ns
Total	14.19	12.67	11.26	14.93	16.28	
Main total	398.52	372.87	320.56	424.96	443.68	

Sig.: Significance, *, ** display the significance at 5%, and 1%, respectively, by LSD. Values not sharing the same superscript letter within the horizontal line are different according to the Duncan test, ns: not significant.

Esters are important contributors to the fruity or floral flavours of wines^{15,31}. Increasing the inoculum level led to a marked decrease in the amount of ethyl acetate. Spontaneous fermentation without inoculation produced 136.53 mg/L of ethyl acetate. The lowest level was obtained in the wine inoculated with 1×10^7 cells/mL. The highest level of ethyl acetate in non-inoculated must could be due to the higher persistence of non-*Saccharomyces* yeasts during the alcoholic fermentation in agreement with Nurgel et al.²³, Xu et al.³⁵, Ciani et al.⁸ and Gadre-Cerdán and Ancín-Azpilicueta.¹⁸ Mateo et al.²⁰ reported similar results with ethyl acetate. It is reported that concentrations of ethyl acetate below 50 mg/L do not contribute to wine flavour, while amounts higher than 200 mg/L result in defects in wine quality¹⁵.

The concentration of isoamyl acetate (3-methyl butyl acetate) increased with increasing inoculum size. Wine produced with an inoculum size of 1×10^5 cells/mL had the highest level at 1.59 mg/L. The lowest content, 0.61 mg/L isoamyl acetate, was found in the spontaneous fermentation without inoculation. The concentration varied from 0.77 mg/L to 1.05 mg/L in other wines inoculated at different levels. Simpson²⁹ and Etiévant¹⁵ stated that among the esters, isoamyl acetate with a banana and fruity odour and a 1 mg/L flavour threshold, was the most abundant contributor to wine aroma. Mateo et al.²⁰ stated that isoamyl acetate concentrations, depending on yeast strain inoculated, were higher or lower than the ones present in non-inoculated samples. In the present study, the influence of inoculum size on isobutyl acetate, ethyl butyrate and ethyl hexanoate (ethyl caproate) was less remarkable and the highest amount of ethyl hexanoate was formed with an inoculum level of 1×10^5 cells/mL. Ethyl hexanoate has a 0.1 mg/L of flavour threshold in the wine and gives an apple-like and fruity flavour to wine¹⁵. Concentrations of isobutyl acetate and ethyl hexanoate, with different inocu-

lum size and yeast strain, varied in the wines²⁰. Inoculum size had no effect on the carbonyl compounds of acetaldehyde, diacetyl and 2,3-pentanedione in this study.

CONCLUSIONS

Yeast inoculum level significantly affected wine fermentation. It shortened the fermentation time. The non-*Saccharomyces* yeasts disappeared quickly with increasing inoculum size. Formation of isobutanol, 2- and 3-methyl-1-butanol and therefore the total amount of higher alcohols increased with an increase in the inoculum level. The concentration of ethyl acetate decreased, but the amount of isoamyl acetate and ethyl hexanoate increased compared to the non-inoculated must sample. Studies on the influence of inoculum size on wine quality are limited and therefore further study is still required.

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