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Fermentation Activity of Yeast in Pinot Noir Must

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Abstract

This study presents the effect of some B vitamins and mineral nitrogen on yeast growth rate and the rate of the alcoholic fermentation of wine must, as well as on the amount of some wine components produced as by-products of the alcoholic fermentation of sugar. Thiamine, pantothenic acid and pyridoxine were added to the pasteurised sulphured must both as a sterile solution, each at a concentration of 1mg/l, and as a mixture. Secondary ammonium phosphate was added at a concentration of 0.8gl^{-1}of must. The experiment showed that the addition of thiamine, the vitamin mixture and ammonium phosphate stimulated yeast growth and the course of the alcoholic fermentation of must, leading to an increased production of glycerine and volatile acids in the wine produced, whereas the supplementation with pantothenic acid and pyridoxine did not substantially enhance wine yeast growth, the fermentation process and the amount of must by-products.

Key words: *must, wine yeast, B vitamins, wine, glycerine, volatile and total acids, alcohol*

1. Introduction

Wine as the product of the alcoholic fermentation of grape juice contains a number of B-complex vitamins that contribute to some degree to its nutritional i.e. biological value. These vitamins partly originate from grape i.e. must, and are partly synthetised by wine yeast during the alcoholic fermentation of must. Moreover, substantial amounts of vitamins can enter the wine harvesting, throughout the alcoholic fermentation, clarification and after bottling. Many of these reactions are left to nature and microorganisms present on the grapes (M. J. TORIJA & al. [1]). The surface of the grapes contains a large variety of moulds, bacteria and yeasts. In particular, a great variety of yeasts are present on grapes, but only a minor portion can participate in alcoholic fermentation. Apart from the principal wine yeast, *Saccharomyces cerevisiae,* spontaneous alcoholic fermentation of grape must is a complex process carried out by the sequential action of different yeast genera and species (G.M. HEARD & G.H. FLEET [2]) found on the grapes, in the must and in the wine, contributing to the flavour of wines (M.G. LAMBRECHTS & I.S. PRETORIUS [3]). The early stages of the alcoholic fermentation are dominated by the growth of *non-Saccharomyces* yeasts, characterized by a low fermentative power. Of these, *Hanse- niaspora (Kloeckera)* and *Candida* (e.g. *Candida stellata, C. pulcherrima)* (G.M. HEARD & G.H. FLEET [4]) are more frequently the

principal yeasts both in spontaneous and inoculated fermentation (G.H. FLEET& al. [5], G.M. HEARD & al. [6], I. PARDO & al.[7]). The fermentation activity of wine yeast i.e. its growth rate and rate of fermentation are mostly affected by B-complex vitamins: thiamine (B-1), pantothenic acid (B-3), pyridoxine (B-6), biotin (B-7) and mesoinositol (B-8), with biotin and thiamine exhibiting the strongest stimulating effect (A. ANTONELLI & al. [8]). Must always contains B vitamins at levels dependent on a number of factors, including vine cultivar, grape ripeness, and the composition and properties of the soil used for vine cultivation . Wine must contains 0.2-0.5 mgl^{-1} thiamine, 0.8-1.5 mgl^{-1} pantothenic acid, 0.6-2.0 mgl^{-1} pyridoxine, a very small amount of biotin – below 0.01 mg/l, in contrast to the substantial content of mesoinosite in both the grape and must ranging from 250 to 1000 mgl^{-1} (I. BENDA & al. [9]). The fermentation activity of wine yeast can also be affected by nitrogen added to must in the form of mineral salts to provide nutrition for yeast and ensure the synthesis of protoplasmic proteins during yeast cell growth. The rate of fermentation is dependent on fermentation conditions, particularly fermentation temperature, influence on yeast growth rate. Higher temperatures generally lead to an increase in fermentation rate. The effect of temperatures studied (C. CHAROENCHAI & al. [10]) on the fermentation rate for 22 different strains of yeast. They found that higher temperatures increased growth rates. However, some different species of yeast have different temperature limitations. Sacchromyces cerevisiae and S. bayanus tend to be more ethanol tolerant at higher temperatures than non-Saccharomyces yeast, such as Klockera apiculata. S. bayanus used (J. M. EGLINTON & al. [11]) to modify the chemical and sensory profile of wine, and determined that S. bayanus produced higher concentrations of some higher alcohols, particularly 2-phenylethanol, compared to S. cerevisiae. S. bayanus yeasts typically produce more 2-phenylethyl acetate than S. cerevisiae (A. ANTONELLI & al. [8]). The non-*Saccharomyces* yeasts contribute to the fermentation since they can reach populations of about up 10^6-10^7 cells/ml, affecting both the kinetics of growth and metabolism of *Saccharomyces* (C. LEMA & al. [12]). These yeasts are capable of anaerobic as well as aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients, and may produce secondary compounds affecting the bouquet of the final wine. The characterization of wine yeasts of different species for by-product formation has underlined that the yeast species itself is a prominent factor in determining the wine composition (A.C. HOUTMAN & al. [13], I. BENDA & al. [9], T. HERRAIZ & al. [14], P. ROMANO & al. [15], V. BRANDOLINI & al. [16]). In particular, the characterization of *S. cerevisiae,* the principal actor of the winemaking, has revealed that, in addition to the production of ethanol, this yeast generates many secondary metabolites that are key determinants of wine quality (G.H. FLEET & al. [17], C. LEMA & al. [18], I.S. PRETORIUS & al. [3], G.H. FLEET & al. [19]). *Saccharomyces cerevisiae* is a yeast that have been known to humans for thousands of years as it has been used in fermentation processes like the dough leavening, winemaking and brewing. In nature, yeast cells are found primarily on the skin of ripe fruits such as grapes FELDMANN [20]. The choice of yeast strain offers great potential to modulate wine aroma profiles to definable styles and predetermined consumer market specifications. The influence of yeasts on the flavour of wine depends on several variables as grape variety, viticultural practices, soil composition, which will influence the sensory descriptive analyses. In addition, oenological practices, including yeast and fermentation conditions, have a prominent effect on the primary flavours wines (Lilly & al. [21], Jose & al. [22], Callejon & al. [23], Carrascosa & al. [24]). The wide use of commercial starter cultures, mainly applied to reduce the risk of

spoilage and unpredictable changes of wine flavour, can ensure a balanced wine flavour, but it may also cause a loss of characteristic aroma and flavour determinants (Molina & al. [25]). Yeast strains that produce the highest levels of volatile thiols were responsible for wines with the highest perceived intensity of fruitiness, and these wines are preferred by tasting panels. While the 'green' characters wines can be manipulated through vineyard management, the 'tropical fruity' characters appear to be largely dependent on the wine yeast strain used during fermentation (Swiegers & al. [26]). Varietal character has been successfully attributed to some polyfunctional mercaptans which are released by the yeast during fermentation (Campo & al. [27]).

2. Material and methods

The material used in the experiment included Pinot Noir must, pH 3.48, containing 22.6% sugars and 8.2gl^{-1} total acids. The experiment was conducted in 0.5l glass bottles filled with 400ml must which was pasteurised and, then, sulphured with 150$mgl^{-1}SO_2$. The vitamins thiamine (B-1), pantothenic acid (B-3) and pyridoxine (B-6) were added to the must as a sterile solution under aseptic conditions, each at a concentration of 1mgl^{-1}. Both the individual effects of the vitamins and the effect of the vitamin mixture on the fermentation activity of yeast were assessed. The experiment included another concurrent treatment involving the supplementation of the must with 0.8gl^{-1} of secondary ammonium phosphate $(NH_4)_2HPO_4$ as a nutrient nitrogen source for wine yeast. The must was inoculated with a pure culture of *Sacch. cerevisiae var ellipsoideus* yeast at about 2 million cells per ml. The alcoholic fermentation of the must was carried out in the thermostat at 28°C, an optimal temperature for wine yeast growth and activity. The must alcoholic fermentation dynamics was monitored by weighing the test bottles once a day at the same time to obtain the amount of carbon dioxide released as an indicator of fermentation dynamics. The effect of the abovementioned vitamins on yeast growth was observed using the yeast biomass produced i.e. the number of yeast cells found in the fermented must after fermentation. Yeast cell numbers were determined by direct plate counting using a microscope and a Dürker-Türk counting chamber, and expressed as millions per millilitre. Upon the alcoholic fermentation of the must, the wine produced was tested not only for alcohol and total acids as its main ingredients, but also for glycerine and volatile acids as by-products of the alcoholic fermentation of sugar. These wine components were determined by standard methods commonly used in enological laboratories. The analysis of the experimental data was performed by analytical statistics with the help of statistical package IBM SPSS Statistics 20 [34]. The significance of differences between the treatments studied traits was conducted using analysis of variance for the risk level of 5% and 1%.

3. Results and discussion

Data on the dynamics of the alcoholic fermentation of the test must, expressed through the amount of carbon dioxide released, are presented in Table 1. The data suggest that the thiamine supplementation of the must prior to fermentation considerably enhanced its alcoholic fermentation, compared to the must that received no vitamin supplementation (control). In this case, the highest rate of must fermentation was observed on days 2 and 3, with most of the sugar being decomposed within this time.

Table 1. Dynamics of the alcoholic fermentation of must, g CO_2/400 ml must

Treatment	Amount of CO_2 released across days									$\bar{x} \pm S\bar{x}$
	1	2	3	4	5	6	7	8	9	
Control	0.4	9.0	12.3	9.3	4.8	2.6	1.8	0.9	0.8	4.7^{a}±0.7
Thiamine	0.4	14.7	12.7	7.7	2.7	1.4	1.1	0.7	0.8	4.7^{a}±0.9
Pantothenic acid	0.4	8.6	11.8	9.2	4.2	2.7	2.0	1.2	0.9	4.6^{a}±0.7
Pyridoxine	0.4	8.5	11.8	9.4	4.2	2.5	2.0	1.2	1.1	4.6^{a}±0.7
Mixture	0.4	14.4	12.8	7.6	2.4	1.5	1.2	0.8	0.9	4.7^{a}±0.9
$(NH_4)_2HPO_4$	0.8	9.4	14.6	11.9	2.9	1.1	0.7	0.4	0.4	$4,7^{a}$±0.9
$\bar{x} \pm S\bar{x}$	0.5^{h}±0.03	10.8^{b}±0.6	12.7^{a}±0.2	9.2^{c}±0.3	3.5^{d}±0.2	2.0^{e}±0.1	1.5^{f}±0.1	**0.9^{g}±0.1**	**0.8^{g}±0.04**	
	Treatment			**Day**			**Treatment x Day**			
F-test	1.43^{NS}			1695.60**			24.76**			

Means in columns followed by the same letter are not significantly different according to Fisher's protected *LSD* values ($P = 0.01$); ^{NS} Non significant; **significant at 0.01;

The analysis of variance showed that the amount of CO_2 released was not significantly different between the tested treatments ($p>0.05$), but not statistically significantly different in the days of observation ($p>0.01$). Only was not observed the difference between the eighth and ninth days (tab. 1). The interaction of investigated factors (Treatment x Day) also showed statistical significance. This suggests that these factors are interdependent and that the interaction must focused especially attention. It can be seen that the days when a larger amount of CO_2 liberated (from the third to sixth) were significantly interacting with all treatments, while the liberation of in the first and last days for all treatments not recorded statistically significant interactions. The addition of pantothenic acid and pyridoxine practically did not increase the rate of the alcoholic fermentation of the must as the fermentation dynamics was quite similar to that of the must non-supplemented with vitamins (control). The supplementation of the vitamin mixture led to the fermentation dynamics highly similar to that obtained by thiamine supplementation, indicating that thiamine was the only stimulating agent. Ammonium phosphate supplementation was found to enhance fermentation similarly to thiamine supplementation, with the highest rate of fermentation reached later, on days 3 and 4. Data on the amount of yeast biomass expressed as the number of yeast cells per unit volume of fermented must are given in Table 2. Results in Table 2 show that the content the number of yeast cells significantly different among all the tested treatments ($p<0.01$).The results suggest that thiamine addition prior to fermentation led to a considerable increase in yeast growth rate, resulting in the number of yeast cells almost double the number found in the must that received no vitamin supplementation (control). The addition of pantothenic acid and pyridoxine did not enhance yeast growth, as indicated by the number of yeast cells being almost identical in these treatments to that in the control.

Table 2. Number of yeast cells in the must fermented, millions/ml

Number	Treatment	Number of yeast cells	F-test
1.	Control	143^{d}	89420**
2.	Thiamine	293^{b}	
3.	Pantothenic acid	140^{e}	
4.	Pyridoxine	137^{f}	
5.	Mixture	309^{a}	
6.	$(NH_4)_2HPO_4$	197^{c}	

Means in columns followed by the same letter are not significantly different according to Fisher's protected *LSD* values ($P = 0.01$); ** 1% significance

The amount of yeast produced after the addition of the vitamin mixture was similar to that obtained by thiamine supplementation, suggesting that yeast cell growth was stimulated only by thiamine. Yeast growth was also enhanced by ammonium phosphate supplementation, but to a lesser extent than by thiamine addition.

Data on the yeast biomass produced and the fermentation dynamics show the same pattern, and quite logically so, as increasing wine yeast biomass enhances the alcoholic fermentation of must. The amounts of major wine components are presented in Table 3. The main products of the alcoholic fermentation of sugar include ethyl alcohol and carbon dioxide. In addition, small amounts of by-products are produced, the most noteworthy among them being glycerine and acetic acid (volatile acids).

Table 3. Major wine components

Number	Treatment	Wine components			
		Alcohol vol %	Total acids gl^{-1}	Volatile acids gl^{-1}	Glycerine gl^{-1}
1.	Control	13.2^{a}	7.2^{a}	0.60^{d}	7.3^{d}
2.	Thiamine	13.3^{a}	7.4^{a}	0.77^{a}	7.8^{b}
3.	Pantothenic acid	13.3^{a}	7.4^{a}	0.72^{b}	7.1^{f}
4.	Pyridoxine	13.5^{a}	7.3^{a}	0.71^{b}	7.2^{e}
5.	Mixture	13.3^{a}	7.6^{a}	0.78^{a}	8.1^{a}
6.	$(NH_4)_2HPO_4$	13.4^{a}	7.5^{a}	0.68^{c}	7.4^{c}
F-test		0.182^{NS}	0.178^{NS}	86.40**	424.25**

Means in columns followed by the same letter are not significantly different according to Fisher's protected *LSD* values ($P = 0.01$); ^{NS} Non Significant; ** 1% significance

Test ANOVA (tab. 3) shows that among the tested treatments were no statistically significant differences in alcohol content and total acids ($p>0.05$). Volatile acids and glycerin demonstrate statistical significance between treatments ($p<0.01$). Thus, control material recorded a significantly lower value of volatile acids compared to all other treatments. The difference between the volatile acid treatment Thiamine and Mixture, as well as differences between treatment Pantothenic acid and Pyridoxine were not significantly different. Among all other treatments defined differences volatile acids were statistically significant ($p<0.01$). The content of glycerin is significantly different among all treatments ($p<0.01$). The present data show no major differences in the amount of alcohol produced across treatments. Likewise, no substantial differences were observed in the amount of total acids. Importance is given to the data on the amount of volatile acids (acetic acid) and glycerine as by-products of sugar decomposition. The data reveal that thiamine supplementation of must prior to fermentation leads to an increased production of volatile acids and glycerine. Most of the glycerine and acetic acid are produced at the first stage of the alcoholic fermentation of sugar, and all the more so if must fermentation at this stage is intensified. Thiamine supplementation of wine must increases the rate of fermentation and yeast growth rate, resulting in an increased production of glycerine and volatile acids in the wine. The beneficial contribution of yeast becomes more significant when starter cultures for winemaking are able to optimise grape quality. During wine fermentation, yeast metabolic activity operates at two different levels: by producing new aromatic compounds and by transforming aromatic precursors present in the grape must. In grape must, there are certain compounds which are transformed to aromatic compounds only by yeast metabolic pathways. In fact, it is demonstrated that by fermentation of grape must of different origin, the same yeast produces different wines in

consequence of the qualitative and quantitative differences in the grape must composition (C. DELFINI & L. BARDI [28]). Over the years, much work has been devoted to investigating biochemical mechanisms, which today enables us to describe routes by which the flavour compounds in alcoholic beverages are formed. The successful use of sophisticated instrumental methods has shown that the flavour of alcoholic beverages is composed of a very large number of compounds. More than 1000 volatile compounds have been identified and of these, more than 400 are produced by yeasts during fermentation (L. NYKANEN [29]). The nature and concentrations of these end-products are determined by the yeast species that participate in the fermentation. Wine flavour is composed by a wide variety of compounds with different aromatic properties. It includes flavour compounds originating from the grapes (varietal flavour), compounds formed during operations of extraction and conditioning of must (pre- fermentative flavour), other compounds produced by yeasts and bacteria during alcoholic and malolactic fermentation (fermentative flavour) and compounds that appear during the ageing process (post-fermentative flavour), as reviewed by (P. SCHREIER [30], R. B. BOULTON & al. [31], A. RAPP [32]). Volatiles identified in wines are usually dominated by fermentation products, since these compounds are present in the highest concentrations. Therefore, conversion of grape sugars to alcohol and other end-products by specific yeast populations may yield wines with distinct organoleptic quality. The various yeast species and strains that develop during the overall fermentative process metabolise grape juice constituents, principally the sugars, to a wide range of volatile and non-volatile end-products, which influence and determine the types and concentrations of many products that contribute to the aroma and flavour characteristics of the wine. The major volatile products of yeast metabolism, ethanol and carbon dioxide, make a relatively small contribution to wine flavour. Conversely, organic acids, higher alcohols and esters and to a lesser extent acetaldehyde constitute the main group of compounds that form the "fermentation bouquet" (A. RAPP & G. VERSINI [33]). When present in excess concentrations, these compounds may also be regarded as undesirable.

4. Conclusions

The results obtained in this study suggest that the pre-fermentation thiamine supplementation of wine must enhances both yeast cell growth and alcoholic fermentation of must, leading to an increased production of glycerine and volatile acids as sugar by-products. The addition of pantothenic acid and pyridoxine results in no substantial increase in yeast growth rate and the rate of the alcoholic fermentation of wine must. The increase in the rate of yeast growth and alcoholic fermentation of must by the addition of the vitamin mixture is almost identical to that resulting from thiamine supplementation, indicating that the stimulation is mainly due to thiamine. The addition of ammonium phosphate as a nitrogen nutrient for the vitamin yeast leads to an increase in both yeast growth rate and the rate of the alcoholic fermentation of must similarly to that induced by thiamine supplementation.

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