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High-Temperature Wine Making Using the Thermotolerant Yeast Strain *Kluyveromyces marxianus* IMB3

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Abstract

Kluyveromyces marxianus IMB3 yeast cells were immobilized on delignified cellulosic material, apple, and quince separately. Both immobilized and free cells were used in high-temperature wine making, and their fermented grape must contained 3 to 4% alcohol. Semisweet wines were produced by the addition of potable alcohol to the fermented must. Preliminary sensory evaluation of the produced semisweet wines showed good flavor and aroma. The final product contained extremely low levels of higher and amyl alcohols while ethyl acetate was at levels usually present in wines. The ferment produced may be blended with other products to improve their quality.

Index Entries: Wine making; high temperature; immobilized cells; volatile byproducts; fermentation.

Introduction

Kluyveromyces marxianus IMB3 is a thermotolerant fermentative ethanol-producing yeast strain, which is capable of producing ethanol at high temperatures using various sugar carbon substrates *(1,2)*. A number of

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specific applications for this yeast in industrial use have recently been reviewed *(3)*.

Many researchers in the fermentation industry have also recently focused their interest on cell immobilization for fuel-grade and potable alcohol production as well as wine making. Many reports have proposed various supports for cell immobilization in the wine-making process *(4–10)*. The main criteria for the industrialization of immobilized cell technology in wine making are cost-effectiveness, availability, food-grade purity, and positive contribution to taste and aroma.

Delignified cellulosic materials and apple have been proposed as immobilization supports for yeast used in wine making at room and low temperature *(11,12)*. Using these supports, it was concluded that there were indications of aroma and taste improvement of the wines produced, which were mainly attributed to a decrease in the percentages of higher alcohols and an increase in ethyl acetate relative to the total volatiles produced. Fermentation of grape must using the thermotolerant yeast *K. marxianus* IMB3, which is known to ferment sugars *(13)* and to produce compounds that contribute to the aroma of alcoholic beverages *(14)*, has not been reported before. High-temperature fermentation using this organism may therefore give a distinctive aromatic character to the wines produced, which may lead to a product with an overall improved quality.

The aim of the present study was to investigate the possibility of using the thermotolerant yeast strain *K. marxianus* IMB3 for high-temperature wine making and, specifically, for the production of semisweet wines.

Materials and Methods

Yeast Strain

The thermotolerant *K. marxianus* IMB3 yeast strain isolated from an Indian distillery environment was used *(15)*. It was grown and maintained at 45°C using a medium containing (w/v): 4% glucose; 0.3% yeast extract; 0.2% peptone; 0.2% KH_2PO_4; 0.2% $(NH_4)_2SO_4$; 0.1% $MgSO_4 \cdot 7H_2O$, and 0.01% $MnSO_4 \cdot H_2O$ in distilled water. The medium was sterilized at 130°C for 15 min. Yeast cells were harvested by centrifugation. Pressed wet wt cells (15–20 g) were used in the fermentation process.

Grape Must

Achaia Clauss, one of the largest wineries in Greece, supplied the concentrated grape juice (32.5 °Be≈55%). Grape juice, ≈11.5 °Be from Roditis grape variety, was concentrated by evaporation at 50°C under vacuum. Afterward, the concentrated grape juice was sulfurized by the addition of sulfur dioxide from cylinders up to a level of 1.5 g of total SO_2/L and more. All fermentation musts were prepared from concentrates by the addition of distilled water to obtain the desirable °Be density. The must was sterilized at 130°C for 15 min.

Effect of Initial °Be Density and Cell Concentration

To determine the optimum initial °Be density, fermentations were carried out at 45°C using free cells at 7, 9, 11, and 12 initial °Be densities. The initial cell concentration used was 10 g of wet wt cells/L.

Fermentations were carried out at 45°C using different initial cell biomass concentrations of 10, 30, and 40 g of wet wt cells/L. The initial °Be density of the must was 7 (≈12% w/v sugar concentration).

Immobilization on Delignified Cellulose

The preparation of delignified cellulose was carried out as previously described *(11)*. Cell immobilization was carried out using 93 g of wet delignified cellulose placed in a 1-L conical flask containing 500 mL of culture medium with 6% glucose and nutrients. Ten grams (wet wt) of yeast cell biomass was added to the flask and allowed to ferment for 8 h at 45°C. The fermented liquid was decanted, the support was washed twice with 500 mL of must, and the resulting biocatalyst was used for wine production.

Immobilization on Apple and Quince Pieces

Apple and quince were cut into ≈2-cm cubes. For the immobilization of cells, 425 g of apple (or quince) pieces was placed in a 1-L glass cylinder to which 500 mL of culture medium was added, and immobilization was carried out to produce the biocatalyst as just described. Once immobilization was achieved on the apple and quince pieces, it was not possible for us to determine the biomass concentration.

Fermentation of Grape Must

The different biocatalysts were introduced into 400 mL of must with an initial °Be density of 7 (12%), and fermentations carried out separately to determine the most appropriate support for wine production using *K. marxianus* IMB3 yeast. All fermentations were carried out without any agitation. Samples were collected and analyzed for volatile compounds (methanol, acetaldehyde, ethyl acetate, propanol-1, isobutanol, and amyl alcohols).

Semisweet Wine Making

Fermentations using immobilized *K. marxianus* IMB3 on apple and quince pieces at an initial must °Be density of 7 and until a final °Be density of 3 were conducted. Semisweet wines were produced by adding potable alcohol to the fermentation broth to achieve a 16% (v/v) final concentration and taking into account the ethanol that was produced during fermentation. Samples were collected and analyzed for ethanol, residual sugar, and volatile byproducts, and the wines were evaluated by a sensory evaluation test.

Analyses

Alcoholic degrees were measured after distillation of samples using a Gay-Lussac alcohol-meter. The determination of ethanol enabled us to calculate the ethanol productivity, defined as the grams of ethanol per liter of liquid volume produced per day.

Residual sugars were determined by high-performance liquid chromatography using a Shimadzu chromatograph consisting of an SCR-101N stainless steel column, an LC-9A pump, a CTO-10A oven at 60°C, and an RID-6A refractive index detector. Triple distilled water was used as a mobile phase with a flow rate of 0.8 mL/min using butanol-1 as an internal standard. Samples of 0.5 mL of wine and 2.5 mL of a 1% solution of butanol-1 were diluted to 50 mL, and 40 μL of the latter solution was injected directly into the column. Residual sugar concentrations were calculated using standard curves and expressed as grams per liter. Conversion was calculated using the following equation:

(Initial sugar concn. – residual sugar concn.)/Initial sugar concn. × 100

Determination of Volatile Byproducts

Acetaldehyde, ethyl acetate, propanol-1, isobutanol, and amyl alcohols were determined by gas chromatography using a stainless steel column packed with Escarto-5905 containing 5% Squalene, 90% Carbowax-300, and 5% (v/v) diethylhexyl sebacate *(16)*. Nitrogen was used as carrier gas at a 20 mL/min flow rate. Injection port and detector temperatures were 210 and 220°C, respectively, and the column temperature was programmed at 60–70°C. Butanol-1 (0.5% [v/v]) was used as an internal standard. Four-microliter samples of wine were injected into the column and the concentrations determined from standard curves. Methanol was also determined by gas chromatography using Porapack S as the column material with nitrogen as the carrier gas at a 40 mL/min flow rate. The column temperature was programmed at 120–170°C, rising at a rate of 10°C/min, and the temperature of the injector and detector was 210 and 220°C, respectively.

Preliminary Sensory Evaluation

Wines produced by fermentation of must using free and immobilized cells of *K. marxianus* IMB3 on apple and quince were tested for their aroma characteristics and compared with a commercial semisweet wine produced locally using similar must types. Nine tasters familiar with wine tastes were asked to give scores on a scale of 0–10 using locally approved protocols in our laboratories for taste and aroma quality. The sensory evaluation was a blind test, and samples were provided in dark glasses.

Results and Discussion

Effect of Initial °Be Density and Free-Cell Concentration

The test to determine the effect of the initial °Be density for *K. marxianus* IMB3 yeast strain fermentation clearly showed that the highest ethanol

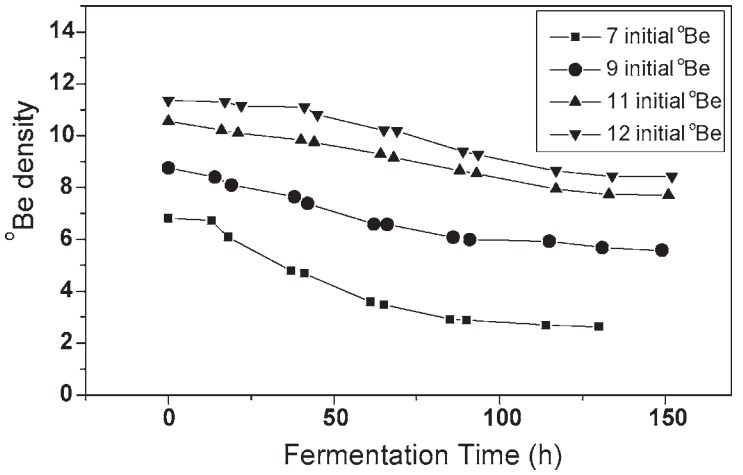

Fig. 1. Fermentation kinetics of grape must using *K. marxianus* free cells at 45°C and various initial °Be densities.

Table 1
Effect of Cell Concentration and Initial °Be Density on Fermentation of Grape Must Using Free Cells of *K. marxianus* IMB3 at 45°C

Free-cell concentration (g/L)	Initial °Be density	Fermentation time (h)	Ethanol concentration (% [v/v])	Residual sugar (g/L)
10	12	152.0	2.6	146.2
10	11	151.0	2.4	134.3
10	9	149.0	2.7	96.9
10	7	130.0	3.3	48.5
30	7	70.0	3.3	53.6
40	7	96.5	2.6	70.1

concentration was obtained at an initial density of 7 °Be, as shown in Fig. 1 and Table 1. The effect of initial free-cell concentration on fermentation at an initial density of 7 °Be is also presented in Table 1 and showed an initial cell concentration of 30 g/L of free cells to be the most suitable for wine production. An initial cell concentration of 40 g/L had no positive or negative effect on ethanol production. The free-cell experiments were carried out to provide some initial indications of suitable inoculum and must concentrations; however, they do not exactly reflect the most suitable conditions for use in the immobilization process.

Figure 1 shows the fermentation kinetics of grape must at various initial °Be densities. At an initial °Be density of 7 the fermentation rate was highest. Higher initial °Be densities resulted in a reduction in fermentation rate.

Fermentation Using Immobilized Yeast Cells

Cell immobilization usually leads to an increase in fermentation rate. Our aims were (1) to produce the maximum amount of ethanol within the shortest fermentation time while leaving an unfermented 2 to 3 °Be density, and (2) to produce a wine with a distinctive aromatic potential and a good overall quality. The supports we used were considered to be suitable for wine making because they all were of food-grade purity, available naturally, and cost-effective. To determine the best support material for immobilization, *K. marxianus* cells were immobilized on delignified cellulose, apple, and quince. The supported biocatalysts were introduced into 400 mL of must having a 7 °Be initial density and allowed to ferment. The results showed that immobilized cell fermentation carried out at 45°C improved conversion of sugars. Apple- and quince-supported biocatalyst had lower fermentation times (46 and 57 h, respectively), and higher ethanol concentration of 3.2 and 3.6 (% [v/v]), respectively; both were therefore selected for semisweet wine making.

Volatile byproducts formed during fermentation of grape must using free and immobilized cells of *K. marxianus* on delignified cellulose, apple, and quince pieces are shown in Table 2. The concentrations of all analyzed volatile compounds except for methanol were at extremely low levels. The very low levels of amyl and higher alcohols may contribute to the overall value of the product, because it is well known that high concentrations of these compounds worsen the organoleptic character of wines *(17)*. The methanol content of the produced wines ranged from 96 to 134 ppm. In traditional fermentations, the methanol content is usually in the range of 100–200 mg/L *(18,19)*.

Semisweet Wine Making

Six semisweet wines were produced using cells immobilized on apple and quince pieces. Table 3 shows the kinetic parameters of fermentation of grape must using immobilized cells of *K. marxianus* on apple and quince pieces. These wines were produced using must with an initial density of 7 °Be. The fermentation was stopped when a density of ≈3 °Be was obtained, and enhancement of the alcohol content to 16% (v/v) was carried out using potable alcohol.

The concentrations of volatile byproducts contained in these semisweet wines are shown in Table 4. The concentrations of amyl (2-methyl-butanol-1 and 3-methyl-butanol-1) and higher alcohols were significantly low. The improved quality of flavor of the produced wines may be owing to the low concentrations of both higher alcohols and amyl alcohols, which are considered to be the main contributors to the off flavor and are also toxic.

The concentrations of ethyl acetate were mainly low and only once detected at 90 ppm. However, there was no indication of any vinegar odor in the final product; on the contrary, a fruity aroma was predominant.

Table 2
Volatile Byproducts Formed During Wine Production at 45°C
Using *K. marxianus* Yeast Cells Immobilized on Delignified Cellulose, Apple, and Quince Pieces Compared With Free Cells[a]

Support	Initial °Be density	Methanol (ppm)	Acetaldehyde (ppm)	Ethyl acetate (ppm)	Propanol-1 (ppm)	Isobutanol (ppm)	Amyl alcohols (ppm)	Total volatiles minus methanol (ppm)
Delignified cellulose	7	96	3	Traces	Traces	Traces	Traces	3
Apple	7	123	Traces	Traces	Traces	Traces	Traces	0
Quince	7	134	Traces	Traces	Traces	Traces	13	13
Free cells	7	123	1	Traces	Traces	Traces	14	15

[a]No potable alcohol was added in these wines.

Table 3
Kinetic Parameters in Fermentation of Grape Must Using Apple- and Quince-Supported *K. marxianus* IMB3 During Semisweet Wine Production at 45°C Before Addition of Potable Alcohol

Support	Sample	Fermentation time (h)	Ethanol concentration (% [v/v])	Residual sugar (g/L)	Ethanol productivity (g/[L·d])	Conversion (%)
Apple	1	45.0	3.4	50.3	14.3	64
	2	31.0	4.2	26.2	25.7	81
	3	24.5	3.3	53.0	25.5	62
	4	42.0	3.0	60.8	13.5	57
Quince	1	56.0	4.3	44.5	14.6	68
	2	50.0	3.8	29.7	14.4	79

Table 4
Volatile Byproducts in Final Wines (After Addition of Potable Alcohol up to 16%) Formed During Semisweet Wine Production at 45°C Using *K. marxianus* Yeast Cells Immobilized on Apple and Quince Pieces

Support	Wine	Methanol (ppm)	Acetaldehyde (ppm)	Ethyl acetate (ppm)	Propanol-1 (ppm)	Isobutanol (ppm)	Amyl alcohols (ppm)	Total volatiles minus methanol (ppm)
Apple	1	82	Traces	74	Traces	Traces	Traces	74
	2	63	Traces	48	1	Traces	7	56
	3	68	8	90	8	Traces	10	116
	4	31	Traces	Traces	14	Traces	Traces	14
Quince	1	101	Traces	41	Traces	Traces	11	52
	2	70	1	23	2	Traces	11	37

Methanol concentrations in the produced wines were similar to levels usually detected in products of natural fermentation. The addition of potable alcohol to the fermented must did not affect the volatile byproduct compositions (Table 4), because potable alcohol is rectified in the alcohol distilleries.

Preliminary Sensory Evaluation Test

Nine evaluators were asked to judge the aroma and taste of three wines and compare them with commercially available semisweet ones. These included one wine produced using apple-supported biocatalyst, one wine produced using quince-supported biocatalyst, and one using free cells. All three wines were semisweet wines adjusted to 16% (v/v) alcohol content. *K. marxianus* IMB3 wines achieved significantly better results for test and aroma compared with the commercial wine (data not shown). Aroma in particular was much better for the *K. marxianus* IMB3 wines. The commercial wine results demonstrated not only a lower overall score for both taste and aroma, but also much more variability among the tasters.

Conclusion

Although fermentation of grape must using *K. marxianus* IMB3 yeast cells resulted in low levels of alcohol and relatively high residual sugars, the final alcohol-adjusted products were of relatively good quality. This may be owing to the extremely low levels of higher and amyl alcohols produced at higher fermentation temperatures. Preliminary sensory tests ascertained the fine taste and the special character of the wines produced. These results suggest the potential for using this fermented must in the production of semisweet wines or for blending with other musts to improve their quality.

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