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New Alcohol Resistant Strains of *Saccharomyces cerevisiae* Species for Potable Alcohol Production Using Molasse

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ABSTRACT

Two alcohol resistant strains of *Saccharomyces cerevisiae* species were isolated from a Greek vineyard plantation. The strain AXAZ-1 gave a concentration of 17.6% v/v alcohol and AXAZ-2 16.5%, when musts from raisin and sultana grapes, respectively, were employed in alcoholic fermentations. They were found to be more alcohol tolerant and fermentative in the fermentation of molasse than the traditional baker's yeast. Specifically, using an initial °Be density of 16°Be at the repeated batch fermentation process, in the first as well as fourth batch, the better AXAZ-1 gave final °Be densities of 6.0 and 10.5 respectively, and the baker's yeast 11.6 and 14.5.

Index Entries: Alcohol; molasse; *Saccharomyces cerevisiae.*

INTRODUCTION

An interesting research field in alcoholic fermentation is the study of new microorganisms able to ferment faster and sugar solutions more concentrated than those are employed in industrial practice by using the

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traditional baker's yeast *Saccharomyces cerevisiae*. Specifically, a newly isolated *S. cerevisiae* strain (*1*) and *Z. mobilis* for faster fermentation (*2*), as well as *Z. mobilis* (*2*), *S. oviformis* (*3*), and *S. bayanus* (*4*) that are relatively alcohol resistant have been reported. The use of an alcohol resistant microorganism is needed to reduce energy demand and cost.

From the aforementioned microorganisms the alcohol resistant *Z. mobilis* was widely studied as a promising bacteria in grade-fuel alcohol production (*5–15*). For this bacteria, however, there is no industrial process, practice, or experience for its culture preparation as there is for *S. cerevisiae*. The latter advantage makes it clear that finding an alcohol resistant and fermentative yeast strain of *S. cerevisiae* species suitable for molasse fermentation will be of technological importance.

The attempt to isolate alcohol resistant yeast strains suitable for alcohol production from the Greek argicultural area was based on the thought that occasionally in Greece concentrated musts of 14–18°Be are produced, completing the fermentation needed for the production of dry wines. In this work, two new particular alcohol resistant and fermentative *S. cerevisiae* strains isolated from raisin grapes are described. They were examined for their suitability for reducing the energy demand and cost in the potable and grade-fuel alcohol production using molasse.

MATERIALS AND METHODS

Isolation of the Strains and Culture Conditions

The new strains AXAZ-1 and AXAZ-2 were isolated by the authors in the Food Technology Laboratory, Department of Chemistry, University of Patras, Patras, Greece on October 15, and November 5, 1988, respectively. Musts with high initial °Be densities (17.5 and 17.7°Be), obtained from raisin grapes were employed. Grapes were collected from a vineyard plantation of Ano Ziria-North Peloponnisos, located at an altitude of 500 m. Musts of 500 mL vol were incubated at 30°C and allowed to ferment in the absence of grape skins. Just before the end of the fermentation, 1 mL of fermenting mass, obtained by a sterilized pipete from the bottom, was added to 5 mL liquid medium and incubated at 30°C. The culture was purified by poured plate technic. Identifications were made by using an API 20C AUX yeast identification system. For microscopie studies, a LEITZ orthoplan microscope was used.

The culture media was sterilized at 130°C for 15 min and contained 2% glucose, 0.1% $(NH_4)SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4$, and 0.4% yeast extract in distilled water. For petri dish cultures, 2% agar was added. This synthetic culture medium was used for the preparation of the inocula employed in all fermentations carried out in this work.

Batch Fermentation of Grape Must

For each of the strains AXAZ-1 and AXAZ-2, inocula were prepared in 1 L at late log phase. Cells were separated by centrifugation and transferred to 250 mL must prepared from sultana or raisin grape (having a desirable high initial °Be density: 16.1, 16.5, 17.2, 17.5) so that the wet weight of initial cell was 20 g/L. Inoculated musts were placed in 250 mL glass cylinders and incubated at 26°C. In one half of the runs, the strain AXAZ-1 was used, and the strain AXAZ-2 in the other half. All runs were made without stirring, and the kinetics of fermentation was followed by measuring the °Be density at various time intervals. After the end of the fermentation the wines obtained were analyzed for alcohol, residual sugar, and volatile by-products.

Repeated Batch Fermentations of Molasses

The strains AXAZ-1 and AXAZ-2, as well as baker's yeast *Saccharomyces cerevisiae,* were grown separately in a synthetic liquid culture media with a composition as described above. The cell mass of each culture was transferred after centrifugation in rarefied molasses of 5°Be density, pH 4.7, and contains 0.5 g/L KH_2PO_4. They were incubated at 30°C for fermentation. Two repeated batch fermentations were carried out for each culture in the same conditions and media, obtained after centrifugation of cells. These three batch fermentations of the strains were made in order to obtain the cells in the molasses media. After that the cells of each culture were transferred separately in glass cylinders each containing 500 mL rarefied molasse of 13°Be, to obtain a cell concentration of 30 g/L (wet weight). The pH was adjusted to 4.7 by adding sulfuric acid, and the molasse media contained 0.5 g/L KH_2PO_4. The three glass cylinders obtained were incubated at 30°C. Repeated batch fermentations for each culture in the same conditions and molasses media at 13°Be were obtained after centrifugation of cells just before each fermentation. In the same way, repeated batch fermentations as described above were organized and carried out for each strain using molasse, first at 15 and then 16°Be density. The cell concentration of the strains and the conditions of the fermentations were similar to those of 13°Be. Finally, kinetics of the fermentations were performed at initial °Be densities of 13 and 16°Be by measuring the °Be density at various time intervals.

Alcohol and Residual Sugar Determination

Alcoholic degrees were obtained after distillation of samples employing a Gay-Lusac alcohol meter. Residual sugar was determined in all samples using the anthrone test (*16*).

RESULTS

The morphology of cells of the isolated strains are shown in Figure 1. The cells of the strains AXAZ-1 and AXAZ-2 are larger and more oval than those of baker's yeast. Identification by the API test indicated than the strains are of the *S. cerevisiae* species with a credibility of 99% for AXAZ-1, and 95% for AXAZ-2.

The fermentation rates of the strains are relatively fast. AXAZ-1 renders the kinetics of must fermentation faster than AXAZ-2. Increasing the initial °Be density of must from 16.1 to 17.5 increased the fermentation time for strain AXAZ-2 over AXAZ-1 two-fold (Fig. 2). In all cases studied AXAZ-1 gave a higher ethanol concentration and lower residual sugar than AXAZ-2. At the highest initial °Be density employed, the final ethanol concentration obtained by AXAZ-1 was equal to 17.6 in contrast to 14.8 of AXAZ-2, which indicated that AXAZ-1 was more resistant to alcohol concentrations and osmotic pressure (Table 1).

In order to compare the isolated strains with baker's yeast in the alcohol production using molasse, repeated batch fermentations were made, and fermentations kinetics were performed at various initial °Be densities. The results are presented in Tables 2 and 3 and in Figure 3.

However, in the case of molasses fermentation the proposed strains gave about similar ethanol production rates even at the relatively high initial °Be density of 16°Be. These rates of fermentation were clearly higher than those obtained by baker's yeast. Similarly, the strains indicated the same alcohol tolerance and are more alcohol resistant than baker's yeast. The ethanol yield factors were also higher than baker's yeast, and repeated batch fermentations studied also show that the strains were more resistant than baker's yeast.

DISCUSSION

This research effort was recently undertaken in Greece by the authors. The strains were investigated especially in the Ano Ziria area of Achaia province, because this argicultural area usually produces wines with relatively high ethanol concentrations. The reasons for this are that in plantation of vines of this area, raisin vineyards are cultivated and musts with high initial °Be densities can be obtained, and the presence of alcohol resistant yeast strains.

The isolated strains of the *S. cerevisiae* species and these strains are not resistant to high alcohol contents in order to produce wines with alcohol concentrations as high as 16–17.6 v/v as are the isolated strains AXAZ-1 and AXAZ-2. These alcohol concentrations are a little higher than those obtained by the fermentative and alcohol resistant *Zymononas mobilis* used in the fermentation of raisin extracts (*17*). Furthermore, the

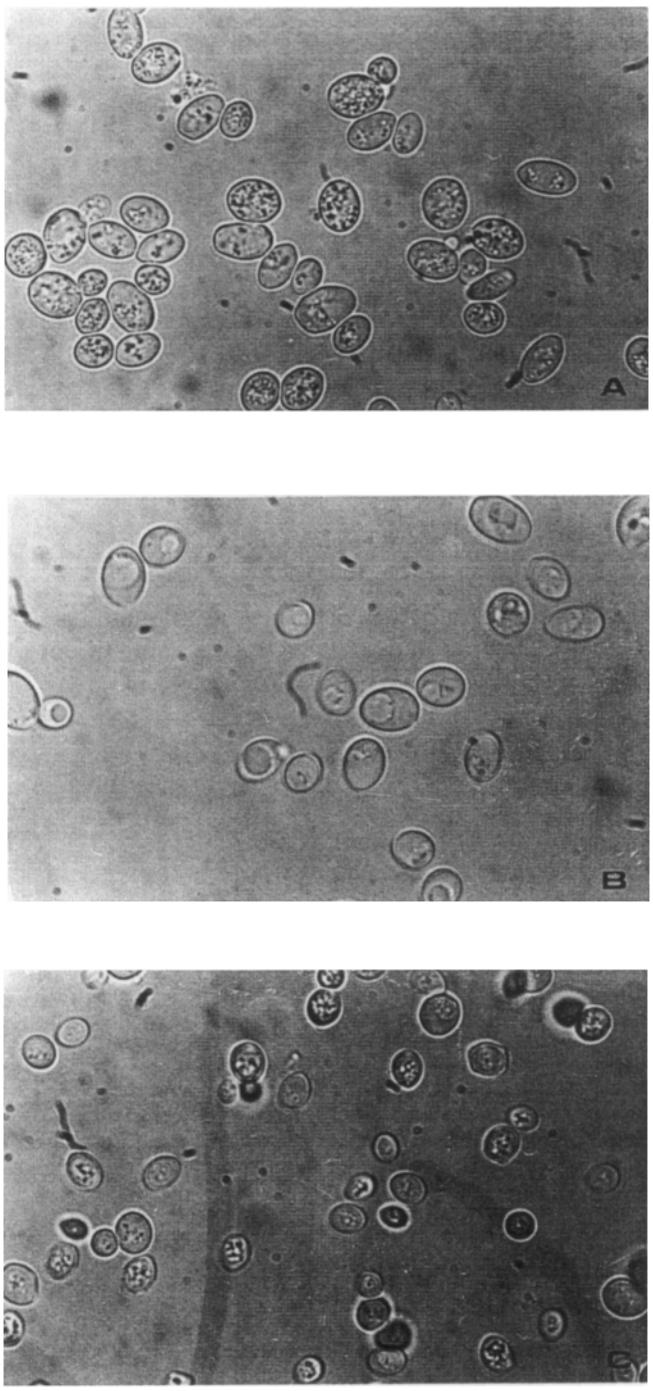

Fig. 1. Photographs of cells at ×1500 of the alcohol resistant *S. cerevisiae* strains AXAZ-1 (A) and AXAZ-2 (B) as compared with those of baker's yeast (C).

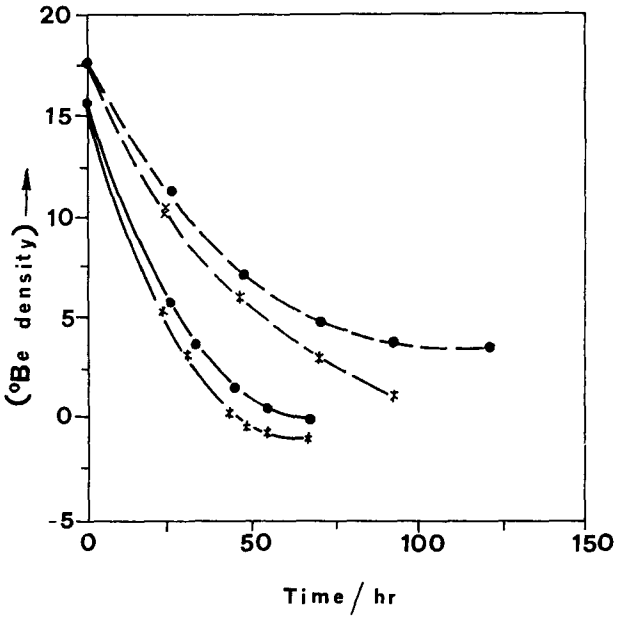

Fig. 2. Fermentation kinetics observed in the fermentation of grape must at different relatively high initial °Be densities using the proposed alcohol resistant *S. cerevisiae* strains. AXAZ-1 (*); AXAZ-2 (●).

Table 1
Final Ethanol Concentration in the Fermentation of Grape Must by *S. cerevisiae* Strains

Experiments	Strains	Grape must	Initial °Be density	Final ethanol concentration, % v/v	Residual sugar, g/L
1	AXAZ-1	Sultana	16.1	17.0	4.9
	AXAZ-2	Sultana	16.1	16.5	15.8
2	AXAZ-1	Sultana*	16.5	16.1	25.5
	AXAZ-2	Sultana*	16.5	14.5	43.5
3	AXAZ-1	Raisin*	17.2	16.8	7.2
	AXAZ-2	Raisin*	17.2	16.0	21.5
4	AXAZ-1	Raisin**	17.5	17.6	35.3
	AXAZ-2	Raisin**	17.5	14.8	67.5

*Must concentrated by freeze drying.
**Must prepared from partly dried grapes.

Table 2
Kinetic Alcoholic Fermentation Parameters Obtained in the Repeated Batch Fermentations by the Strains as Compared with Those of Baker's Yeast, Using Molasse

Initial °Be density	Repeated batch fermentation	AXAZ-1				AXAZ-2				Baker's yeast			
		F.T., h	E.C., g/L	E.Y.F., g/g	E.P., g/L·d	F.T., h	E.C., g/L	E.Y.F., g/g	E.P., g/L·d	F.T., h	E.C., g/L	E.Y.F., g/g	E.P., g/L·d
13	1	35	62	0.50	43	30	63	0.51	50	50	59	0.48	28
	10	25	62	0.50	60	40	64	0.51	38	40	59	0.48	35
15	1	55	77	0.50	34	55	78	0.50	34	65	73	0.47	30
	6	55	79	0.51	35	60	79	0.51	32	65	76	0.49	28
16	1	95	83	0.48	21	130	82	0.48	15	220	55	0.32	6
	4	265	82	0.48	7	270	79	0.46	7	334	50	0.32	4

Abbreviations
F.T. = Fermentation time
E.C. = Ethanol concentration
E.Y.F. = Ethanol yield factor
E.P. = Ethanol productivity

Table 3
°Be Density Obtained at the Same Fermentation Time
by the Use of Strains and Baker's Yeast in the Repeated Batch Fermentations
of Molasse at Various Initial °Be Densities

Initial °Be density	Repeated batch fermentations	Fermentation time, h	°Be density		
			AXAZ-1	AXAZ-2	Baker's yeast
13	1	20	7.1	7.2	10.0
13	3	20	6.6	5.8	7.0
13	5	20	5.6	5.7	6.7
13	7	20	5.5	5.4	6.2
13	9	20	5.4	5.4	6.5
13	10	20	5.4	5.5	6.7
15	1	30	8.1	6.9	7.9
15	2	30	7.7	7.2	8.8
15	3	30	8.6	8.9	9.8
15	4	30	8.5	8.7	9.8
15	5	30	9.1	8.9	10.2
15	6	30	9.2	9.3	10.2
16	1	59	6.0	6.3	11.6
16	2	59	8.0	7.6	12.4
16	3	59	11.6	—	15.6*
16	4	59	10.5	10.6	14.5*

*These °Be densities were obtained at 106 h fermentation time.

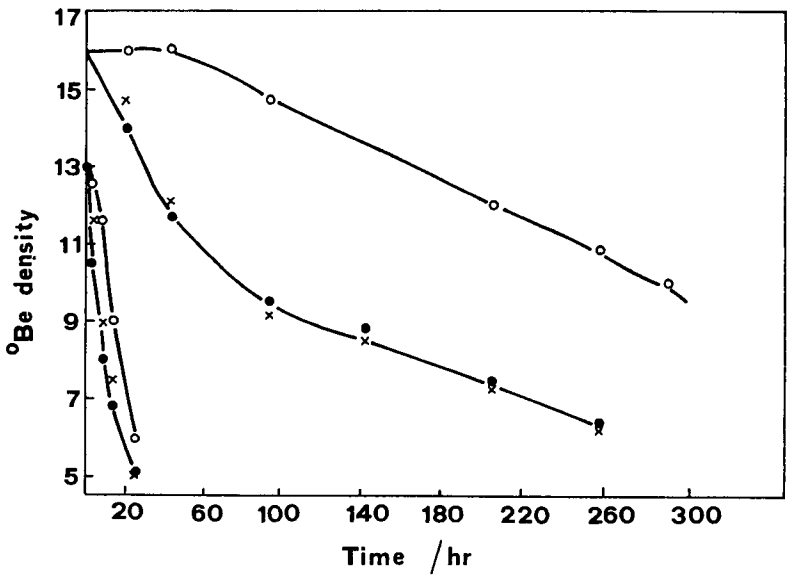

Fig. 3. Fermentation kinetics obtained in the alcoholic fermentation of molasse using the proposed strains as compared with baker's yeast. AXAZ-1 (●); AXAZ-2 (X); Baker's yeast (○).

experiments using molasse showed that the isolated strains are suitable for its fermentation. They gave higher alcohol productivity and yield, and the alcohol concentrations obtained were higher than those obtained through the use of traditional baker's yeast. The latter contributes to a reduction of the energy demand in the alcohol production plant, a fact that is a prerequisite for grade-fuel alcohol production, and interesting for potable alcohol production. In the repeated batch fermentations the strains were more resistant than baker's yeast presently used in the alcohol production plant. This accommodates obtaining more repeated batch fermentations with the same yeast culture that leads to higher alcohol production yield and higher ethanol concentrations.

An observation made during the experimental part of this work was the reduction of the fermentability of the strains when they were cultured for a long period in synthetic media containing glucose. It was found that the strains became fermentative in molasses fermentation broth after an acclimation obtained with three repeated batch fermentations in diluted molasse of 5°Be.

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