Kissiris-Supported Yeast Cells: High Biocatalytic Stability and Productivity Improvement by Successive Preservations at 0 °C

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Successive preservations at 0 °C of cells of the strain Visanto-1 immobilized on mineral kissiris increased the biocatalytic stability of the cells and the ethanol and wine productivities. Specifically, immobilized yeast cells were viable for 2.5 years, producing wines by both batch and continuous processes. In addition, the ethanol and wine productivities were at least fivefold higher compared with those obtained from immobilized cells on the same support without successive preservations at low temperature. The results with preservation showed higher conversion and ethanol concentration than those in fermentations with kissiris-supported biocatalyst without successive preservations at 0 °C.

Keywords: *Wine; ethanol; productivity; immobilized yeast cells; psychrophile; stain; preservation; mineral; kissiris; fermentation technology*

INTRODUCTION

Immobilization of cells has received considerable attention in recent years because fuel-grade alcohol production needs higher alcohol productivity to reduce cost. In addition, researchers have focused their interest on cell immobilization for use in potable alcohol production (Koutinas and Kanellaki, 1990; Koutinas et al., 1991) and wine making (Otsuka, 1980; Nakanishi and Yokotsuka, 1987; Mori, 1987; Fumi et al., 1987; Hamdy, 1990; Lommi and Ahvenainen, 1990; Bakoyanis et al., 1992). In light of these efforts and the advantages of volcanic mineral kissiris for cell immobilization, kissiris was proposed as a useful cell support (Kana et al., 1989). The low cost and natural abundance of kissiris prompted further research with kissiris-supported biocatalysts for the study of continuous potable alcohol production (Koutinas et al., 1991) and low-temperature wine making (Bakoyianis et al., 1992). The low-temperature wine making at 0−7 °C, performed after immobilization of the phychrophilic yeast strain Visanto 1, indicated that the prepared kissiris-supported biocatalyst reduced the activation energy (E_a). Likewise, this mineral promotes molasse ethanol fermentation with free cells (Tsoutsas et al., 1990; Koutinas et al., 1993). On the basis of this promotion, two pilot plants, with bioreactors of 7000 and 100 000 L total working volume, have been installed and financed by the European Union as part of the STRIDE program. Experience acquired from this project leads to the conclusion that to employ immobilization of cells in industry, solid-supported biocatalysts must have a very large biocatalytic stability in addition to abundance in nature and low cost. Furthermore, the biocatalyst must be preserved even during periods when alcohol production and wine making are halted. Therefore, the aims of this investigation were to study the biocatalytic stability of kissiris-supported biocatalyst by preservation at 0 °C after every repeated batch fermentation series, and to examine the effect of this preservation process on the ethanol productivity.

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MATERIALS AND METHODS

Visanto-1, a psychrophilic and alcohol-resistant yeast strain, was employed in the present investigation and in a recent study of low-temperature wine making after its immobilization on mineral kissiris (Bakoyianis et al., 1992). Pressed wet weight cells (20 g) of the strain were grown at a late log phase in a liquid culture medium containing 2% glucose, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4$, and 0.4% yeast extract. For cell growth, pH was adjusted to 5.6. Reactor temperatures were controlled at 30 °C for batch and 28 °C for continuous fermentations by placing the reactor in a constant-temperature water bath.

Raisin extracts were prepared from the variety Trechumena as recently described (Koutinas and Kanellaki, 1990). Grape must was prepared from the variety Rosaki. The °Be density was adjusted to 12 after the addition of concentrated must. All media and musts were sterilized at 130 °C for 15−20 min. All grape must was used without nutrient addition. In the case of raisin extracts, 0.1% $(NH_4)_2SO_4$ and 0.1% KH_2PO_4 were added as nutrients, and pH was adjusted to 5.6 by the addition of sodium hydroxide solution. Mineral kissiris was used as support for immobilization of cells (Kana et al., 1989).

Ethanol was determined by alcoholic degrees (milliliters of ethanol/100 mL of wine), after distillation of samples, with a Gay Lussac alcohol meter. Residual sugar was analyzed according to the Lane-Eynon procedure (Egan et al., 1981).

Preservation of Immobilized Cells. Immobilization of cells on mineral kissiris was achieved by a method described in previous study (Kana et al. 1989). Three sterilized glass cylinders were used; each cylinder contained 150 g of kissiris and 150 mL of fermenting liquid. At the beginning, six repeated fermentations of synthetic media containing 11.3% glucose were carried out for biomass attachment. Then, the 7th to 12th batches were performed at 30 °C with the raisin extract. Then, glass cylinders were filled with a 7 °Be density raisin extract and stored at 0 °C in a refrigerator. When the °Be densities were dropped into the range 1.7−1.2, new amounts of raisin extracts were added to replace the fermented liquid. The procedure just described was repeated twice for a preservation time of 18 days. In sequence, immobilized cells were employed for repeated batch fermentations at 30 °C and then were preserved at 0 °C. This method of preservation was repeated many times after every series of repeated batch fermentations at 30 °C, and immobilized cells continued to have significant activity at 900 days from the beginning of the fermentation.

Repeated Fermentation Batches. The fermentations were studied systematically after 8, 60, 300, and 900 days, and three to six repeated batches were performed each time

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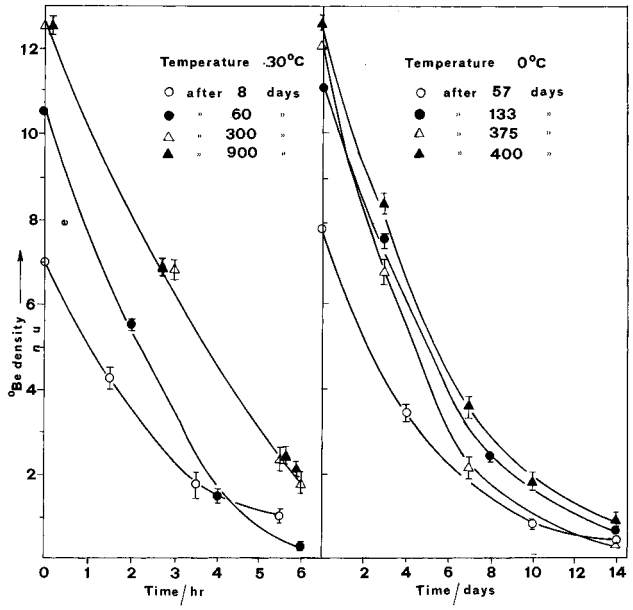

Figure 1. Alcoholic fermentation kinetics of raisin extracts, observed in repeated batch fermentations carried out after different times from the beginning of immobilization and successively at 30 and 0 °C. Every curve corresponds at the 5th repeated batch fermentation in the case of 30 °C and at the 3rd of 0 °C.

with initial °Be densities of 8, 10.5, 11.5, and 12.5, respectively. In addition, during preservation at 0 °C, slow fermentations were carried out every time after substitution of must, and these fermentations were examined as repeated batches. The fermentations were also studied in a series of three to seven batches each time and successively during the period of 900 days. In this case, the initial °Be density was also successively increased from 7.7 to 13, as indicated in Figure 1. Kinetics of the fermentations were followed by measuring the °Be density at various time intervals. In every three samples, three replicate fermentations were performed. All values were the mean of three repetitions. The standard deviation for ethanol concentration was $<\pm 3$, for ethanol productivity was $<\pm 20$ at 30 °C and $<\pm 1$ at 0 °C, and for conversion was $<\pm 6$.

Continuous Wine Making. After 300 days of batch fermentations and successive preservations of immobilized cells, kissiris-supported biocatalyst from two glass cylinders was transferred into two reactors for continuous wine making. The two reactors were linked together so that the outlet of the first reactor was joined with the inlet of the second one. The reactors were glass towers (each of 250 mL total working volume and 150 mL liquid volume), and 150 g of kissiris-supported biocatalyst was placed in each of them. All operations of the continuous process were performed as described in a recent study (Bakoyianis et al., 1992). The system was supplied with must, and the flow rate was increased and reduced from 600 to 2500 mL/day as indicated in Figure 3. The reactor was operated for 64 days, and the biocatalyst was

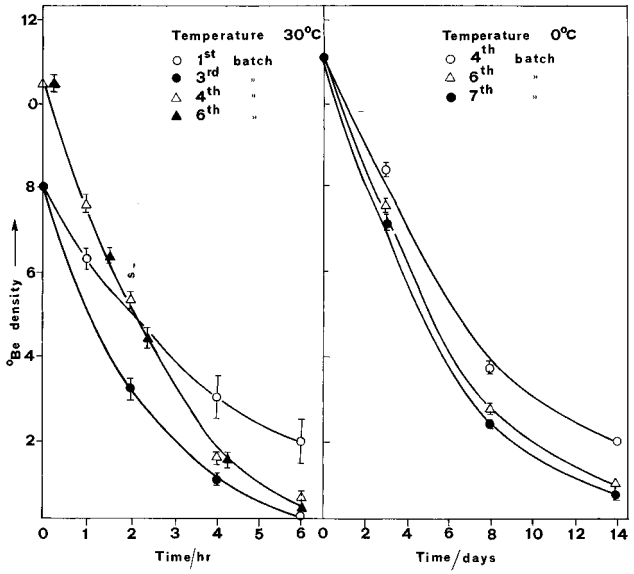

Figure 2. Repeated batch alcoholic fermentation kinetics of raisin extracts in a series of experiments.

then transferred into glass cylinders for further preservation and repeated batch fermentations at 30 °C until 900 days.

The effects of dilution rate on wine and ethanol productivity as well as on residual sugar, conversion, and yield were examined. These parameters were calculated as described in previous studies (Koutinas et al., 1991; Bakoyianis et al., 1992). Wine productivity was calculated on the basis of total working volume.

RESULTS AND DISCUSSION

Raisin extracts and grape must are used as raw materials for wine making and potable alcohol production and were therefore chosen for this study. Kissiris-supported yeast cells reduce the activation energy (E_a) and were found to be suitable for low-temperature wine making (Bakoyianis et al., 1992). These results suggested that kissiris would be suitable for preservation at 0 °C. To examine the biocatalytic stability of kissiris-supported biocatalyst, experiments of repeated batch fermentations were carried out at 30 °C, every time after successive preservations at 0 °C, for 900 days. In the first series of fermentation batches, an improvement of the ethanol production rate was observed from batch to batch. This improvement allowed us to organize the repeated batch fermentation series after every preservation according to increased initial °Be density. Such organization was performed to examine the improvement of productivity at higher alcohol concentrations. The results are summarized in Figures 1 and 2, as well as in Table 1.

Table 1. Fermentation Kinetic Parameters Observed in Batch Fermentations of Raisin Extracts Carried Out in the Presence of the Same Immobilized Yeast Cells on Mineral Kissiris As Related with the Progress of Preservation of Immobilized Cells

temp (°C)	time from the beginning (days)	repeated fermentation batches after every preservation at 0 °C	time at 0 °C of every preservation (days)	initial °Be density	final °Be density	wine productivity (g/L·d)	fermentation time (h)	ethanol concn. (g/L)	ethanol productivity[a] (g/L·d)	conversion (%)
30	8	5	0	7	1	2618	5.5	46	200.7	85.7
30	60	3	18	7.7	1.2	4114	3.5	51	350	84.4
30	300	6	29	10.5	0.3	2400	6	84	336	97.1
30	900	3	580	12.5	0.2	2352	10	98	235.6	98.4
0	14	2	–	7	2.9	51	284	31	2.6	58.5
0	75	3	–	8	0.2	120	120	62	12.4	97.5
0	135	4	–	11	0.3	40	360	88	5.9	97.3
0	375	3	–	12	0.2	43	336	96	6.9	98.3
0	900	3	–	12.5	0.5	47.7	488	97	4.8	96

[a] Calculated on liquid volume.

Table 2. Results of Continuous Wine Making (at 28 °C) by Immobilized Yeast Cells on Mineral Kissiris after Their Successive Preservation at 0 °C and after Total Preservation Time of 300 Days

dilution rate								ethanol conversion yield factor	
calcd on liquid volume (h^{-1})	calcd on total working volume (h^{-1})	test duration (h)	feed concn. (g/L)	residual sugar (g/L)	wine productivity (g/L·h)	ethanol conc. (g/L)	ethanol productivity[a] (g/L·h)	(%)	(g/g)
0.11	0.07	48	218	22.0	66.7	86.4	9.5	89.9	0.40
0.08	0.05	64	218	0.6	50.0	101.6	8.1	99.8	0.47
0.14	0.08	48	218	0.7	83.0	101.6	14.2	99.8	0.47
0.17	0.10	48	218	0.1	100.0	104.0	17.7	100.0	0.48
0.29	0.18	48	218	5.0	175.0	95.2	27.6	97.7	0.44
0.35	0.21	48	218	29.0	208.3	84.0	29.4	86.7	0.39
0.25	0.15	24	218	7.3	150.0	93.6	23.4	96.7	0.43
0.21	0.13	96	218	0.6	125.0	104.0	21.8	99.8	0.48
0.18	0.11	840	218	1.5	108.3	101.6	18.3	99.5	0.47

[a] Calculated on liquid volume.

The results clearly show that the immobilized cells preserved successively at 0 °C have biocatalytic stability for at least 2.5 years. Ethanol production rate and ethanol and wine productivity were improved as the time period using the same biocatalyst was increased and even when the initial °Be density was increased. This improvement was also observed from batch to batch, in a series of repeated batch fermentation. The ethanol productivity with preservation, obtained at 30 °C and final ethanol concentration of 84 g/L was at least threefold higher than the productivity at final ethanol concentration of 54 g/L and without successive preservation at 0 °C of kissiris-supported biocatalyst (Kana et al., 1989). This improvement of the productivity with preservation compared to without preservation is in agreement with results recently reported by Bakoyiannis et al. (1992). Improvement in productivity was also obtained at 0 °C, and the improvement was higher than that obtained by Bakoyianis et al. (1992) at low temperatures without preservation. Conversion was also high, even at relatively high final ethanol concentrations.

To examine the ethanol and wine productivity of immobilized cells in a continuous process, experiments were carried out after a long period of successive preservations. The flow rate of must was changed for a period of 20 days to 600−2500 mL/day, and afterwards was kept constant at 1300 mL/day. The initial $^{o}B_e$ density of must was relatively constant, and all must was prepared from the same cultivar. The results are presented in Table 2 and Figure 3.

The results indicate that the reactor was operable for >2 months. The °Be density of the effluent was at the level of dry wines when the flow rate of must was <1500 mL/d. Even in the case of higher flow rates and in the range 2500−1600 mL/day, the alcoholic degrees were >10.8. With relatively high ethanol productivity (21.8 g/L·h, calculated on the basis of liquid volume and 13.5 g/L·h calculated on the basis of total working volume), the wine produced had an alcoholic degree of 12.7% (v/v), which remained constant for 44 days. The highest ethanol productivity obtained was 29.4 g/L·h (86.7%) and the lowest was 9.1 g/L·h 99.8%).

Preservation of immobilized cells resulted in the highest ethanol productivities obtained by continuous fermentation with kissiris-supported yeast cells. Specifically, this preservation of cells increased ethanol and wine productivity at least fivefold compared with continuous fermentations carried out with the same biocatalyst and without preservation of immobilized cells (Koutinas et al., 1991; Bakoyianis et al., 1992). Likewise, the ethanol productivity and conversion achieved in this study was higher than continuous fermentation

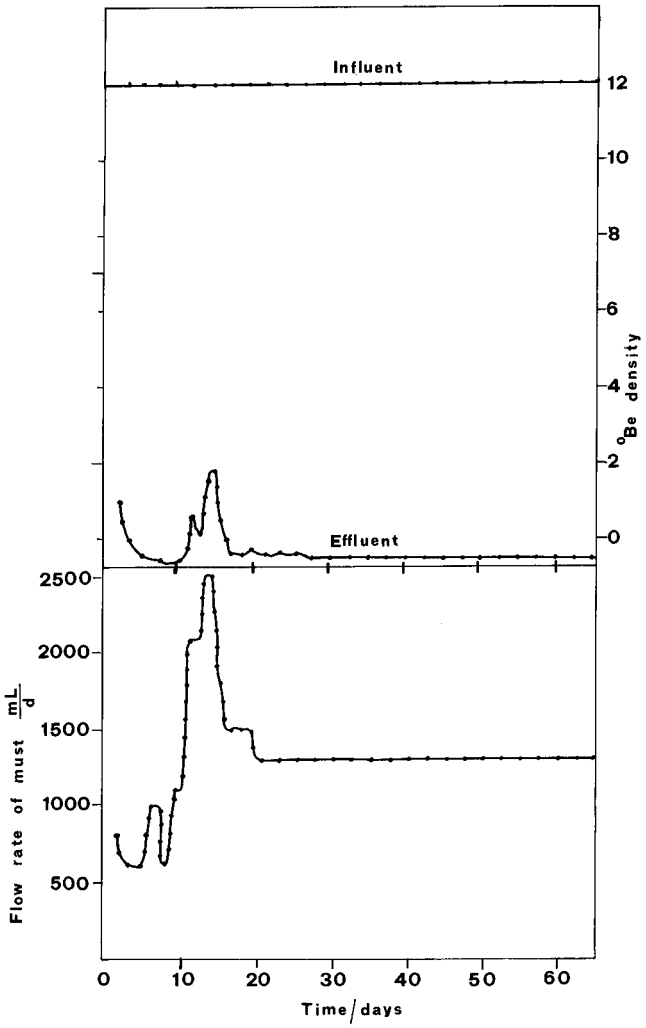

Figure 3. The °Be density converted as related to flow rate of must in the continuous wine making.

with immobilized Zymomonas mobilis on γ-alumina pellets without preservation at 0 °C (Koutinas and Kanellaki, 1990).

The continuous bioreactor filled with kissiris-supported biocatalyst was viable for alcohol production and wine making with operational stability for 2.5 months (Koutinas et al., 1991; Bakoyianis et al., 1992). However, the results obtained in this investigation show that the biocatalytic stability can be >2.5 years. The long-term stability is prerequisite for a solid-supported biocatalyst to be employed in an industrial process. Moreover, kissiris-supported or any other biocatalyst will have to be preserved throughout the year for days during which the installation needs maintainance (e.g.,

vacation periods). Therefore, the possibility of preservation at a low temperature of kissiris-supported biocatalyst during these periods is of technological importance and makes this abundant and low-cost support able to fulfill all the prerequisites for cost-effective immobilization and industrial application. Furthermore, the large improvement of ethanol and wine productivity, after preservation of immobilized cells at 0 °C, is also of technological importance. According to the results of this work, one can predict that a bioreactor with 10 000 L of total working volume, continuously operating, produces 30 000−50 000 L of wine or 5000 L of alcohol per day.

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