Low-Temperature Wine Making by Immobilized Cells on Mineral Kissiris

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Kissiris-supported biocatalyst, prepared by immobilization of the alcohol resistant and psychrophile strain Visanto 1 on mineral kissiris, was found to be suitable for low-temperature (5–16 °C) continuous wine making. It is also reported that the ethanol productivity at 5 °C by continuous fermentation was equal to what should be observed at 22–25 °C by natural fermentation. The important productivity obtained at low temperatures can be attributed to the reduction of the activation engery E_a, caused by the support mineral kissiris. The continuous reactor was operated for 75 days without any diminution of the ethanol productivity. Finally, the wine was produced with lower total and volatile acidity as compared with wines obtained by natural fermentation at 22–25 °C.

INTRODUCTION

Continuous wine making by a solid supported biocatalyst has as prerequisites the use of an abundant and cheap support material as well as the use for immobilization of an alcohol resistant yeast strain. The advantages of this process are as follows: (1) The fermentation performs at higher than 10% v/v alcohol, at which musts during fermentation are usually not infected. (2) Musts are protected from the air during fermentation, and the aerobic acetic bacteria do not grow easily in the fermentation broth. (3) Higher wine productivities are obtained (Iconomou et al., 1992). (4) No sulfur dioxide must be added before the fermentation to accomodate growth of the alcohol resistant *Saccharomyces elipsoideous*.

Although the immobilization of yeast is widely studied for alcohol production and the above advantages as well as the possibility to study the quality of the wine produced by continuous fermentation are important for manufacturers and researchers, publications concerning wine making by continuous fermentation are not abundant in the literature. However, grape wine was continuously produced by fermentation of grape juice with immobilized *Saccharomyces cerevisiae* IAM-4175 cells on sodium alginate granules of 3-mm diameter (Keniichi, 1980). Also, batch fermentation of white wine from Koshu grape using immobilized yeast was reported (Kotoyoshi and Koki, 1987). Furthermore, continuous wine making at 26 °C, by the alcohol resistant kissiris-supported biocatalyst prepared by the immobilization of alcohol resistant strain AXAZ-1 of *S. cerevisiae* species, is also reported (Iconomou et al., 1992). Kissiris is a porous volcanic mineral, the composition of which was given in detail in a previous study (Tsoutsas et al., 1990), and contains mainly 70% SiO_2. Tsoutsas et al. (1990) reported that this mineral promotes molasses fermentation and enables it to be carried out at even higher ethanol concentrations. Likewise, bakers' yeast *S. cerevisiae* was immobilized on kissiris for potable and grade-fuel alcohol production in batch (Kana et al., 1989) and continuous processes (Koutinas et al., 1991).

Manufacturers have the experience that the wine produced at low temperatures has important odor and taste. However, publications concerning wine making at temperatures lower than 10 °C, to our knowledge, are absent in the literature. Fermentations using free cells and performed in the temperature range 10–15 °C with relatively high fermentation time are reported (Nadesda, 1978; Giolfi et al., 1985; Mauricio et al., 1986). Finally, wine production by continuous fermentation at low temperatures has not been reported yet in the literature. Wine making at temperatures lower than 15 °C is not usual on an industrial scale. In this case the wine productivity of the bioreactor is very low.

On the basis of the above presentation, one may conclude that to obtain an increase in the ethanol and wine productivities and to study wine making at low temperaturs, the study of continuous fermentation of must, by the use of an alcohol resistant and psychrophile yeast strain immobilized on mineral kissiris, is also required. Therefore, this is the subject of the present work.

MATERIALS AND METHODS

The psychrophile and alcohol resistant yeast strain Visanto 1 was recently isolated from grapes of a vineyard plantation of the Aegean island Santorini (T. Argiriou, M. Kanellaki, and A. Koutinas, 1991, unpublished results). Pressed wet weight cells (20 g) of the strain, grown in a liquid culture media, and the preparation of continuous culture media in the case of glucose solutions were as described in a previous study (Iconomou et al., 1992). For the cell growth the pH was adjusted to 5.6. All media were sterilized at 130 °C for 15–20 min. Reactor temperatures were controlled periodically at 27, 16, 13, 10, 7, and 5 °C by placing the reactor in a constant-temperature water bath. Low temperatures were obtained using a Haake EK12 freezing apparatus.

Grape must was prepared from cultivar Sideritis. It was sterilized at 130 °C for 15–20 min. The initial °Be density was 11.2–11.4. In the case in which the °Be density of must was prepared by crushing the grapes, it was lower than 11.2, and it was adjusted by dilution with glucose. All must was used without nutrient addition. For immobilization mineral kissiris was used (Kana et al., 1989).

Ethanol was determined as alcoholic degrees (milliliters of ethanol per 100 mL of wine) which were obtained after distillation of samples using a Gay Lusac alcohol meter. Residual sugar was analyzed according to the Lane–Eynon procedure (Egan et al., 1981). Total acidity of the wine was titrated with 0.1 N NaOH; volatile acidity was also estimated after steam distillation of samples and titration of the distillate with 0.1 N NaOH (Zoeklein et al., 1990).

Determination of Immobilized Cells. Wet weight cells of the strain Visanto 1, equal to 20 g/L of liquid synthetic media

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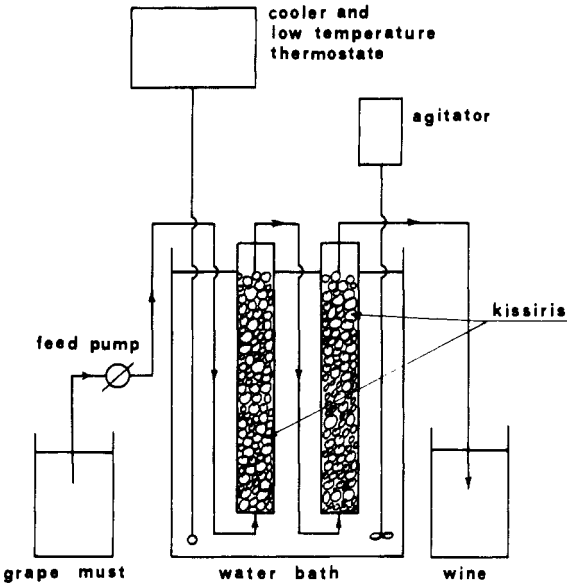

Figure 1. Pilot plant operation for continuous low-temperature wine making using the psychrophile kissiris-supported biocatalyst.

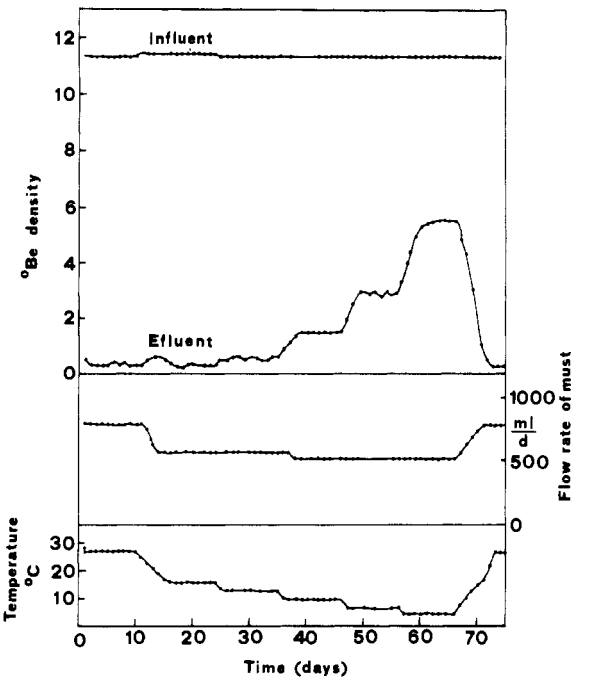

Figure 2. Flow rate of must and °Be density converted as related to temperature in the range 5–27 °C.

containing 12% glucose, were used for immobilization on mineral kissiris. It was made according to a method previously reported (Kana et al., 1989). Simultaneously, a fermentation by the same strain and cell concentration at similar liquid media and conditions, in the absence of kissiris, was also performed. After 4 h of fermentation time, kissiris was separated from the liquid media and washed with 200 mL of liquid media containing no cells and the concentration of cells was determined by centrifugation in the liquid media as grams of wet weight per liter. Likewise, to determine the cell concentration in the media without kissiris, measurements by centrifugation were made. The difference in the cell concentration, between the liquid media fermented in the presence of kissiris and those in absence of it, was the grams of wet weight cells immobilized on kissiris. It was found to be 6 g of wet weight cells/150 g of this porous mineral material.

Batch Fermentations of Must. Must obtained from grape Sideritis was fermented simultaneously in batch systems by free cells and separately with those of immobilized cells at temperatures of 7, 13, and 27 °C. Fermentations, using free cells and separately cells immobilized on mineral kissiris, were performed in two glass cylinders of 500 mL. For those of free cells the liquid contained 6 g of wet weight cells and had an initial °Be density of 11.5. For the fermentation using immobilized cells on mineral kissiris, the immobilization was made as described in a recent study (Kana et al., 1989). The prepared biocatalyst was transferred to the glass cylinder, and after the addition of grape must, the fermentation took place in a parallel way with those of free cells and the continuous process. The concentration of free cells on a wet weight basis was equal to the concentration of the immobilized ones. In every pair of samples (immobilized and free) three replicate fermentations were carried out at every temperature studied.

Pilot Plant. Continuous wine making was obtained with two reactors linked together so that the outlet of the first reactor was joined with the inlet of the second, as shown in Figure 1. They were glass tower reactors (each of 1500 mL total working volume and 1000 mL liquid volume) and in each of them was placed 750 g of kissiris as the immobilization support in the continuous process. Must was pumped in an up flow stream with the aid of a high accuracy peristaltic pump (Cole Parmer Instruments Co., Chicago, IL).

Experimental Procedure. The first reactor of the two-reactor system was charged with synthetic media containing glucose and cells of the strain and operated for biomass attachment as described in a previous study (Iconomou et al., 1992). After that, the second reactor was joined with the outlet of the first and it was pumped continuously for 2 days with the synthetic media containing glucose. Subsequently, the system was supplied with must having an initial °Be density rather constant, within the range 11.2–11.4 °Be. The flow rate was reduced as the temperature was lowered, from an initial 800 to 500 mL/day. The reactor was operated for 75 days. The starting temperature was 27 °C, and it was lowered periodically from 27 to 5 °C, as indicated in Figure 2. The lowering of the temperature, when necessary, was carried out at the rate of 2–3 °C/day so as to avoid shock of the biocatalyst. At every temperature samples were taken, after at least 4 days of pumping, so as to obtain a steady state of the reactor. When steady state was obtained, four or five samples were collected, one every 24 h, and analyzed for °Be density, residual sugar, alcohol concentration, and total and volatile acidity.

The effect of temperature on alcohol productivity as well as on total and volatile acidity was examined. Ethanol productivity, given on Table I, was expressed as grams of ethanol per liter produced in 1 day and calculated on the basis of total working volume, by multiplying the dilution rate by ethanol concentration. Dilution rates were calculated by dividing the flow rate of liquid by the total volume of the fermentors. Ethanol productivity, given in Table II, was calculated on the basis of liquid volume.

RESULTS AND DISCUSSION

To obtain operational stability of the reactor in the case of wine making, an alcohol resistant strain recently isolated from the vineyard plantation of the Aegean island Sandorini was used. This strain was found to be psychrophile, and it was deemed necessary to be employed for low-temperature wine making. Likewise, it was shown that kissiris-supported biocatalyst, prepared by the immobilization of a yeast strain on the porous mineral kissiris, is alcohol resistant and convenient for continuous wine making (Iconomou et al., 1992), since wines have relatively high alcohol concentration. The system of the reactors filled with the biocatalyst was operated with must for 10 days at 27 °C. This was done for biomass attachment and for stability of the system. Then the temperature was subsequently diminished from 27 to 5 °C. The initial °Be density of must was relatively constant, and all must was prepared from the same cultivar (grape Siderilis) to obtain the comparison of the results. These are summarized in Figure 2 and Table I.

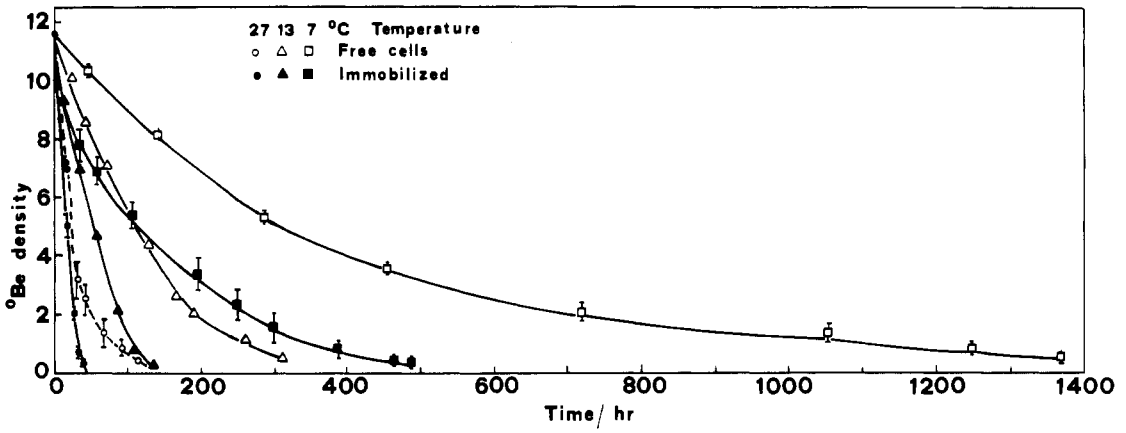

Figure 3. Alcoholic fermentation kinetics of must, observed at temperatures of 7, 13, and 27 °C and carried out in batch reactors separately by free and immobilized cells on mineral kissiris.

Table I. Characteristics of the Wine Produced by Continuous Fermentation at Low Temperatures

temp, °C	wine produced, mL/day	ethanol concn, % (v/v)	residual sugar, g/L	ethanol productivity, g L^{-1} day^{-1}	total acidity of the wine, g of tartaric acid/L	volatile acidity, g of acetic acid/L
16	550	11.1	7.9	17.0	4.3	0.73
16	550	11.2	4.7	16.4	4.1	0.73
16	550	11.2	5.7	16.4	4.2	0.63
16	550	11.1	6.8	16.3	4.1	0.63
13	550	10.1	12.3	14.8		
13	550	10.3	10.1	15.1	4.0	0.55
13	550	10.0	12.4	14.7	3.9	0.60
13	550	10.0	12.2	14.7	4.0	0.62
13	550	10.5	10.1	15.1	4.0	0.60
10	500	9.4	33.5	12.5		
10	500	9.3	34.8	12.4	3.9	
10	500	9.5	33.2	12.6	3.8	0.53
10	500	9.2	37.4	12.3	3.5	0.61
10	500	9.5	33.7	12.6	3.4	0.52
7	500	7.5	49.1	10.0	3.4	0.66
7	500	8.0	51.1	10.7	3.6	0.66
7	500	8.1	47.2	10.8	3.4	0.66
7	500	8.0	48.5	10.7	3.4	0.60
5	500	7.2	65.3	9.6	3.2	0.58
5	500	6.6	61.3	8.8	2.9	
5	500	6.0	83.6	8.0	3.4	0.56
5	500	6.0	86.5	8.0	3.3	0.53

Table II. Ethanol Productivities in Fermentations of Must by Free and Immobilized Cells at 7, 13, and 27 °C

	ethanol productivity, g L^{-1} day^{-1}		
		immobilized cells	
temp, °C	free cells	batch	continuous
27	16.0	38.5	72.8
13	4.8	12.9	22.5
7	1.2	3.5	15.8

The results clearly show that the reactor was operated over 2 months, except that the temperature was low for a long period. The °Be density of the effluent was relatively constant up to 13 °C. When the temperature dropped to lower values, the °Be density of the effluent was subsequently increased. The flow rate of must was also relatively constant, except at the temperature of 27 °C, due to higher activity of the system. The ethanol productivity of the system obtained at 5 °C was reduced to about 60% at 27 °C. This productivity at 5 °C is equal to that obtained by the natural fermentation at temperatures of 22–25 °C. Total acidity of the wine prepared at 5 °C was 47% of that of must, which was found to be equal to 6.75 g of tartaric acid/L. It is estimated that this reduction is higher than that obtained in wines produced by natural fermentation. This is an advantage in the case of must with high total acidity which must be reduced to obtain better organoleptic character. The reduction of the total acidity can be attributed to the increase of crystallization of monopotassium tartrate as the temperature drops to lower values. Total acidity becomes lower as the temperature drops. Volatile acidity also dropped as the temperature was reduced from 27 (0.83 g of tartaric acid/L) to 5 °C. The latter is due to the reduction of the activity of acetic bacteria as well as to the reduction of the rate of reaction which oxidizes acetaldehyde to acetic acid, as the temperature is lowered. When measurements at 5 °C were finished, the temperature was increased to 27 °C, as indicated in Figure 2, and the °Be density of the effluent attained the level at which it was at the start of the experiment. The latter indicates that the operational stability of the system for wine making at low temperatures was at least of 2.5-months duration. This operational stability of the system can be attributed to the alcohol resistance of kissiris-supported biocatalyst (Iconomou et

al., 1992) as well as to the psychrophilation of the yeast strain used.

Batch fermentations performed at 7 °C by free cells (four replicates) gave an average ethanol productivity 1.9 $g L^{-1} day^{-1}$, a value at least 5-fold lower than those obtained by immobilized cells in continuous fermentation. This fact was the reason for undertaking more systematic experiments to prove that kissiris-supported biocatalyst is at low tempertures much more active than free cells. Therefore, batch fermentations by free cells and separately by kissiris-supported biocatalyst were attempted at 27, 13, and 7 °C. The cell concentrations in the case of free cells and those of immobilized were equal, and the wet weight cells immobilized was determined as described under Materials and Methods. Fermenttion kinetics were also obtained by measuring the °Be density at various time intervals. The results are presented in Figure 3.

Kissiris-supported biocatalyst used in the batch system increases the ethanol production rate at all temperatures studied, as compared with that of free cells. Inspection of the figure shows that the reduction of the fermentation time was significantly increased as the temperature was diminished. This increased activity is agreement with the promotion effect obtained in molasses fermentation and in disagreement with no promotion of raisin extract fermentation, both carried out by free cells and in the presence of kissiris (Tsoutsas et al., 1990). The high reduction in the fermentation time at low temperatures of 7 and 13 °C is an advantage for the use of this biocatalyst in wine making at low temperatures. Likewise, continuous wine making increases further the rate of the fermentation. The latter is concluded from the increased values of ethanol productivity obtained by the continuous process as presented in Table II. The results, therefore, clearly show that kissiris-supported biocatalyst increases the productivity as compared with free cells and the continuous process is more efficient. At 7 °C the ethanol productivity in continuous wine making was 13-fold higher than that obtained by free cells. This result and others, presented above, support the use of kissiris-supported biocatalyst in wine making at low temperatures by continuous fermentation.

Figure 3 illustrates that kissiris-supported biocatalyst causes an increase in the fermentation time smaller than those of free cells as the temperature of the fermentation drops. For example, one can observe at 3 °Be density, in kinetic curves of the figure, that kissiris-supported biocatalyst gives increases of the fermentation time of 317% and free cells 494% as the temperature drops from 27 to 13 °C. This means that the proportion of reaction speed constants at 27 and 13 °C, K_{27}/K_{13}, becomes lower in the case of immobilized cells on kissiris as compared with that of free cells. Using the Arrhenius equation

$$\log \frac{K_2}{K_1} = \frac{E_a}{2303R}\left[\frac{T_2 - T_1}{T_2 T_1}\right]$$

the activation energy E_a is reduced in the case of kissiris-supported biocatalyst.

The latter supports the idea that the solid support mineral kissiris behaves as a catalyst or as a promoter (Giannakoudakis, 1964) of the catalytic activity of the enzymes involved in the process. The reduction of the activation energy E_a explains the relatively high ethanol productivity obtained at low temperatures. The above explanation further supports the use of kissiris-supported biocatalyst for low-temperature wine making.

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Received for review December 3, 1991. Accepted March 16, 1992.