Low-Temperature Continuous Wine Making by Kissiris-Supported Biocatalyst: Volatile Byproducts

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The formation of methanol, ethanol, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols (mixture of 2-methylbutanol-1 and 3-methylbutanol-1) in continuous wine making by kissiris-supported biocatalyst, in comparison with free cells, was studied. The effect of temperature was also examined. Methanol content was not affected by the process and temperature, but the concentrations of ethyl acetate, propanol-1, isobutyl alcohol, amyl alcohols (total content of 2-methylbutanol-1 and 3-methylbutanol-1), and total volatiles determined plus methanol were reduced by the reduction of the temperature. Although the concentration of the main aromatic compound, ethyl acetate, was slightly reduced in the wine, its percentage content in the total volatiles determined plus methanol was increased. Continuous wine making by the aforementioned biocatalyst gave higher ethyl acetate concentrations compared to that with free cells.

INTRODUCTION

Research efforts in past years have been undertaken to employ the cheap and abundant mineral kissiris as cell immobilization support for industrial continuous alcoholic fermentation processes. In this effort, the immobilization of *Saccharomyces cerevisiae* cells on it was obtained, and fermentation of glucose and raisin extract was performed in a batch bioreactor (Kana et al., 1989). Continuous potable alcohol production by immobilized *S. cerevisiae* using glucose, raisin extract, and molasses was recently reported (Koutinas et al., 1991). Continuous wine making for a long period was performed by alcohol-resistant, kissiris-supported biocatalyst, prepared by immobilizing the alcohol-resistant *S. cerevisiae* strain AXAZ-1 on mineral kissiris (Iconomou et al., 1992). Kissiris-supported biocatalyst, prepared by immobilization of the alcohol-resistant and psychrophile strain Visanto 1, has proved that it reduces the activation energy E_a and increased significantly the fermentation rate of must at low temperatures of 10, 7, and 5 °C (Bakoyianis et al., 1992). The last contribution makes clear that low-temperature wine making, using the aforementioned biocatalyst, is simplified in industrial practice.

Volatile byproducts of alcoholic fermentation, such as methanol, ethanal, and amyl alcohols (2-methylbutanol-1 and 3-methylbutanol-1), are mainly responsible for the flavor of alcoholic beverages (Greenshields, 1974). Although the formatioin of methanol by hydrolyzation of poly(galacturonate) (pectins) methoxyl groups by pectolytic enzymes has been reported by Reinhard (1969) and Lee et al. (1975) and the formation of other volatile compounds in wines and spirits by naturally occurring yeast cells has been investigated by several authors in the past (Guymon et al., 1961; Crowel and Guymon, 1963; Hieke and Volbrecht, 1974; Greenshields, 1974; Cabezundo et al., 1974, 1983; Reinhard, 1976; Lisle et al., 1978; Bertrand, 1978; Postel and Adam, 1979, 1980; Suarez et al., 1981; De Corostiza et al., 1982; Giolfi and Distefano, 1983; Fahrasmane, 1985; Kana et al., 1988), literature on the formation of volatiles by immobilized yeast cells is scarce. The formation of volatiles in products obtained by repeated batch fermentations of raisin extracts, using immobilized *S. cerevisiae* on mineral kissiris, was recently reported (Kana et al., 1992).

Volatiles formed by continuous wine making had not been studied. Likewise, many more volatiles in low-temperature continuous wine making were not reported in the literature.

Our experience with low-temperature continuous wine making, using kissiris-supported biocatalyst, has shown (1) the higher potential of immobilized cells to ferment some substitutes at low temperatures in comparison with free ones and (2) the production of wine with a fine aroma.

Therefore, the aims of this study were (1) the formation of volatiles in continuous wine making by the use of kissiris-supported biocatalyst as compared to those formed by fermentation using free cells and (2) the formation of volatiles at low temperatures in continuous wine making by kissiris-supported biocatalyst.

MATERIALS AND METHODS

Strains and Fermentations. The yeast strain Visanto 1, psychrophilic and alcohol resistant, was used. It was recently isolated from grapes of a vineyard on the Aegean island Santorini (Argiriou et al., unpublished results). The preparation of Visanto 1 inoculum, its immobilization on mineral kissiris and biomass attachment, and the continuous fermentation of must were conducted as previously described (Bakoyianis et al., 1992).

Preparation of Must and Immobilization Support. 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. If the °Be density of must prepared by crushing the grapes was lower than 11.2, it was adjusted by the addition of glucose. All must was used without nutrient addition. For immobilization, mineral kissiris was used (Kana et al., 1989). This mineral, which is well-known in Greece as elafropetra or Thiraiki gi, is a volcanic rock, comprised of volcanic glasses and with a petrification similar to that of granite. Kissiris is usually formed by the foam thicknening of volcanic lava and is characterized as a natural glass foam. The gas volume released during its formation is almost equal to the glass mass. This is the reason for its porosity and specific surface area. Its composition is similar to that of the igneous rock type of alkali granite and it contains more than 70% SiO_2, 13% Al_2O_3, and other inorganic oxides. Kissiris is a cheap mineral material whose sale price does not exceed $50/ton.

Ethanol and Byproduct Determination. Ethanol was determined as alcoholic degrees (milliliters of ethanol/100 mL of

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Table I. Effect of Temperature on Formation of Volatiles in the Continuous Wine Making by Kissiris-Supported Biocatalyst

temp, °C	ethanol concn, % v/v	methanol		ethanal		ethyl acetate		propanol-1		isobutyl alcohol		amyl alcohol		total volatiles determined plus methanol	
		mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH
27	11.1	210	2.4	28	0.32	70	0.79	28	0.32	26	0.29	152	1.71	304	3.4
27	11.2	180	2.0	30	0.33	72	0.80	30	0.33	24	0.27	142	1.58	298	3.3
27	11.2	193	2.1	26	0.29	77	0.86	32	0.36	28	0.31	138	1.54	301	3.4
16	11.1	151	1.7	18	0.20	48	0.54	43	0.48	19	0.21	134	1.51	262	3.0
16	11.2	137	1.5	32	0.36	57	0.64	45	0.51	19	0.21	133	1.48	286	3.2
16	11.2	160	1.7	26	0.29	72	0.81	45	0.50	23	0.26	131	1.48	298	3.3
16	11.1	143	1.6	23	0.26	71	0.80	41	0.46	23	0.26				
13	10.1	130	1.6	19	0.23	54	0.67	27	0.33	16	0.20	115	1.40	231	2.9
13	10.3	165	2.1	33	0.41	68	0.83	tr	tr	17	0.21	126	1.53	244	3.0
13	10.0	160	2.0	28	0.35	70	0.88	tr	tr	21	0.26	120	1.50	239	3.0
13	10.0	153	1.9	22	0.28	82	1.03	tr	tr	19	0.24	107	1.34	230	2.9
13	10.5	163	1.9	25	0.30	65	0.77	tr	tr	19	0.23				
10	9.4	150	2.0	31	0.41	57	0.76	tr	tr	12	0.16	121	1.61	221	2.9
10	9.3	178	2.4	35	0.47	54	0.73	tr	tr	10	0.13	105	1.41	204	2.7
10	9.5	161	2.1	37	0.49	58	0.76	tr	tr	10	0.13	115	1.51	220	2.9
10	9.2	190	2.6	30	0.41	55	0.75	tr	tr	13	0.18				
10	9.5	117	1.5	37	0.49	58	0.76	tr	tr	9	0.12	92	1.21	196	2.6
7	7.5	150	2.5	48	0.80	57	0.95	tr	tr	10	0.17	101	1.68	216	3.6
7	8.0	176	2.8	33	0.52	54	0.84	tr	tr	9	0.14	96	1.50	192	3.0
7	8.1	183	2.9	47	0.73	42	0.65	tr	tr	9	0.14	109	1.68	207	3.2
5	7.2	178	3.1	44	0.76	34	0.59	tr	tr	5	0.09	46	0.80	129	2.2
5	6.6	195	3.7	32	0.61	36	0.68	tr	tr	5	0.10	49	0.93	122	2.3
5	6.0	205	4.3	38	0.79	38	0.79	tr	tr	5	0.10	52	1.08	133	2.8

Table II. Volatile Byproducts Formed in Batch Wine Making Using Free Cells

temp, °C	ethanol concn, % v/v	methanol		ethanal		ethyl acetate		propanol		isobutyl alcohol		amyl alcohols		total volatiles determined plus methanol	
		mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH	mg/L	mg/g of EtOH
27	11.5	188	2.0	15	0.16	42	0.46	40	0.43	18	0.20	154	1.67	269	2.9
27	11.3	204	2.3	14	0.15	46	0.51	48	0.53	22	0.24	110	1.22	240	2.7
27	11.4	190	2.1	16	0.18	43	0.47	54	0.59	23	0.26	171	1.88	307	3.4
7	11.3	170	1.9	36	0.40	7	0.08	43	0.48	14	0.15	108	1.19	208	2.3
7	11.4	191	2.1	38	0.42	9	0.10	47	0.52	17	0.19	113	1.24	224	2.5
7	11.2	172	1.9	34	0.38	8	0.09	49	0.55	18	0.20	117	1.31	226	2.5

wine) which were obtained by distillation of samples using a Gay-Lussac alcoholometer. Quantitative determinations of volatile byproducts were made with a Shimadzu gas chromatograph GC-8A connected with the integrator C-R6A Chromatopac. For methanol, Porapak S as column material and N_2 as the carrier gas (40 mL/min) were employed. The injector port and detector temperatures was 180 °C. The column temperature was programmed between 140 and 180 °C. 3-Pentanol was used as the internal standard, and samples of 1 μL of the wine were injected directly into the column.

Ethanal, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols (total amount of 2-methylbutanol-1 and 3-methylbutanol-1) were determined using a stainless steel column, packed with Escarto 5905 [consisting of squalene 5%, Carbowax 300 90%, and bis(2-ethylhexyl)sebacate 5% v/v], with N_2 as the carrier gas (20 mL/min). The injection port and detector temperatures were 210 °C, and the column temperature was 58 °C. The internal standard was 3-pentanol, at a concentration of 0.5% v/v. Samples of 2 μL of the wine were injected directly in the column (e.g., without extraction).

RESULTS AND DISCUSSION

The effect of temperature on the formation of the volatile compounds at continuous wine making by kissiris-supported biocatalyst was studied by placing the reactor in a constant-temperature water bath and adjusting the temperature periodically to 27, 16, 13, 10, 7, or 5 °C. At every temperature, samples were taken after at least 4 days of pumping to obtain steady state of the reactor. When steady state was obtained, samples were collected every 24 h and analyzed for ethanol, methanol, ethanal, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols (total amount of 2-methylbutanol-1 and 3-methylbutanol-1). To compare volatile formation by continuous wine making using kissiris-supported biocatalyst with those formed by free cells, batch fermentations were carried out at 27 and 7 °C, using the same must and identical initial °Be density and yeast strains. The results are summarized in Tables I and II.

Methanol concentration was not significantly affected in the continuous fermentations using kissiris-supported biocatalyst as the temperature was reduced from 27 to 5 °C. The same effect was also obtained in batch fermentations using free cells. Furthermore, continuous fermentation, employing the aforementioned biocatalyst, results to about the same methanol concentrations as those of free cells. The increased values of methanol per gram of ethanol, observed in the continuous fermentation at 7 and 5 °C, are due to their lower ethanol concentrations. This increase does not mean that the completion of the fermentation will give larger methanol contents, because higher ethanol concentrations of the wines of 27, 16, and 13 °C do not give larger ethanol concentrations and methanol/ethanol ratios.

The ethanal content was greater in wines produced by continuous fermentation at temperatures lower than 10 °C. This increase was larger in the case of wines prepared with free cells by batch fermentation. Ethanal content in wines obtained by free cells at 27 °C was about half that obtained by continuous fermentation at the same temperature. At the low temperature of 7 °C this difference was significantly reduced.

The ethyl acetate content of the continuous wine making was reduced as the temperaturer became lower than 10 °C. At the temperature of 7 °C the reduction was 30% and at 5 °C 50%, as compared with those of 27 °C. In the case of free cells, from 27 to 7 °C the concentration of this volatile was reduced 80%. In continuous wine making at temperatures higher than 7 °C, the concentration of ethyl acetate was greater than that obtained in the fermentation at 27 °C carried out with free cells. At the temperature of 7 °C, continuous wine making produces wines with at least a 6-fold higher ethyl acetate content as compared to those produced by free cells.

In the continuous wine making process the volatiles propanol-1 and isobutyl alcohol are reduced at low temperatures as the temperature is diminished. Propanol-1 drops significantly after 13 °C. At 5 °C the concentration of isobutyl alcohol is about 25% that at 27 °C. The reduction of these compounds is also shown when they are expressed as milligrams per gram of EtOH. The concentration of propanol-1 did not become lower as the temperature dropped from 27 to 7 °C in the case of free cells, and it is higher than that obtained by continuous fermentation of must. Likewise, isobutyl alcohol was not significantly reduced in the fermentation using free cells as it was by continuous fermentation.

The concentration of amyl alcohols (total content of 2-methylbutanol-1 and 3-methylbutanol-1) in the continuous process was reduced as the temperature dropped, although the diminution was not so great. Large reduction was observed at the temperature of 5 °C, where it is also shown when the concentration is expressed as milligrams per gram of EtOH. No significant difference of their content was observed in the wines prepared by the use of free cells as compared with those of continuous fermentation.

Finally, the content of total volatiles determined plus methanol in the case of wines prepared by continuous fermentation was decreased as the temperature was reduced. This is also shown in the expression of volatiles as milligrams per gram of EtOH. At 5 °C they were only 41% of the volatiles at 27 °C. The reduction of volatiles as the temperature dropped was lower in the case of free cells as compared with those of continuous wine making.

The increase of total volatiles determined plus methanol found in the continuous wine making at 27 °C, as compared with those of free cells, agrees well with that obtained by the same biocatalyst in batch fermentations (Kana et al., 1992). According to the last reference, batch fermentations, carried out by the aforementioned biocatalyst, increased more greatly the total volatiles than the continuous wine making, as is presented in this work.

Temperature and continuous wine making do not affect methanol. The formation of methanol in wines is attributed to hydrolyzation of methoxyl groups of poly(galacturonate)s (pectins) by pectolytic enzymes (Reinhard, 1969; Lee et al., 1975) and organic acids at the relatively high temperature effected at the sterilization of must. The above two factors explain why the fermentation of methanol was not affected by the immobilization, continuous fermentation, and low temperature.

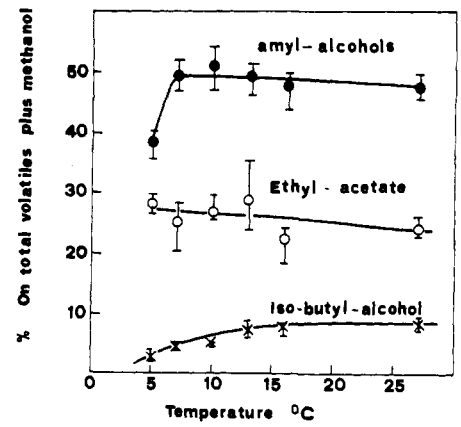

Figure 1. Contents of amyl alcohols (2-methylbutanol-1 and 3-methylbutanol-1), ethyl acetate, and isobutyl alcohol on total volatile determined plus methanol.

The increase of the ethanol content at low temperatures may be due to relatively lower alcoholic dehydrogenase activity (Koutinas and Pefanis, 1990) in the final step of alcoholic fermentation mechanism, by which acetaldehyde is converted to ethanol.

Wines, prepared by continuous fermentation using the proposed biocatalyst at low temperatures, had an improved aroma. The latter may be attributed to the diminution of propanol-1 and isobutyl alcohol at low temperatures and to the relatively high ethyl acetate concentrations. Also, it may be attributed to the increase of percent ethyl acetate and to the reductiion of isobutyl alcohol calculated on total volatiles determined plus methanol (Figure 1). Likewise, this figure shows that the percent amyl alcohols (2-methylbutanol-1 and 3-methylbutanol-1) on total volatiles determined is slightly increased until 7 °C and drops at 5 °C. The latter may improve the quality of the wine.

The improved aroma observed should be a good reason to undertake in the future systematic experiments for sensory studies of the prepared wines and their distillates.

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