Wine Production Using Yeast Immobilized on Apple Pieces at Low and Room Temperatures

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A biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae* strain AXAZ-1 on apple pieces. It was examined by electron microscope and studied during the fermentation of grape must for batch wine-making. The immobilized yeast showed an important operational stability without any decrease of its activity even at low temperatures (1−12 °C). Specifically, at 6 °C the biocatalyst favored wine production within 8 days, which is less time than is required for the natural fermentation of grape must. At 1 °C wine production was effected in 1 month. This speeding up of the fermentation could be accepted and adopted by the industry for scaling up the wine-making process. Total and volatile acidities were similar to those found in dry wines. The concentrations of higher alcohols (propanol-1 and isobutyl alcohol) were low. The presence of amyl alcohols proved to be temperature dependent and decreased with the temperature decrease. The values of ethyl acetate concentrations were relatively high, up to 154 mg/L. This probably contributes to the fruity aroma and excellent taste of the produced wines. There was no indication of vinegar odor in the product given that the volatile acidity was <0.47 g of acetic acid/L. From the GC-MS analysis it was concluded that cell immobilization did not create serious changes in the product (wine) with regard to the qualitative composition of the aroma compounds.

Keywords: *Wine-making; immobilized cells; apple; fermentation; volatile byproducts*

INTRODUCTION

Cell immobilization in alcoholic fermentation is an attractive and rapidly expanding research area because of its technical and economical advantages compared to the free cell system (*1*, *2*). However, for application in the food industry, further research is needed to obtain cells immobilized on a support that is more hygienic for food, cheap, abundant in nature, suitable for low-temperature fermentation, and will lead to an improvement of the aroma and taste of the final product. Wine manufacturers know that low-temperature fermentation leads to higher quality products. Many researchers have proposed various supports for cell immobilization in the wine-making process (*3*−*5*). However, these supports are considered to be inconvenient for wine-making because they are inorganic materials. The use of alginates proposed by some other researchers (*6*−*9*) hardly offered a good alternative and was abandoned. Use of delignified cellulosic materials proposed by Bardi and Koutinas (*10*) proved to be very effective for low-temperature alcoholic fermentation.

It is well-known that cider is produced from apple juice. This drink has a pleasant taste and aroma (*11*). Therefore, it was thought that it would be very interesting to use pieces of apple as a support of the immobilization of cells in wine-making. This support is of food-grade purity and abundant in nature. Given that large quantities of apples and apple pulp are destroyed every year, it was thought that the use of apple pieces as a support for cell immobilization would possibly lead to a product with fine taste and aroma because of the transfer of some of their constituents into the wine.

The aim of this investigation was to find a new support for cell immobilization suitable for wine production and good enough to use for low-temperature fermentation.

MATERIALS AND METHODS

Yeast Strain. AXAZ-1, an alcohol-resistant and psychrophile *Saccharomyces cerevisiae* strain isolated (*12*) from the Greek agricultural area, was used in the present study. The yeast strain AXAZ-1 was isolated from grapes collected from a vineyard plantation of Ano-Ziria North Peloponnisos, Greece. Musts with high initial °Be densities (17.5 and 17.7 °Be) were obtained from raisin grapes. They were fermented in the absence of grape skins. Just before the end of the fermentation, 1 mL of the fermenting mass was added to 5 mL of a liquid medium consisting of glucose, 2%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.1%; $MgSO_4$, 0.5%; and yeast extract, 0.4%; the mixture was incubated at 30 °C. This culture was purified by the poured plate technique. This yeast strain was grown on a medium consisting of glucose, 4%; $(NH_4)_2SO_4$, 0.1%; KH_2PO_4, 0.1%; and $MgSO_4$, 0.5%. Pressed wet weight cells (15−20 g) were prepared accordingly and employed directly in the fermentation process.

Must. All musts were prepared from concentrated ones by the addition of distilled water, so that the final °Be density ranged from 12 to 14 °Be. Achaia Clauss, one of the largest wineries and the oldest one in Greece, supplied us with concentrated grape juice (32.5 °Be). Grape juice, ∼11.5 °Be from Roditis grape variety, was concentrated by evaporation at 50 °C under vacuum. After that, the concentrated grape juice was sulfurized by the addition of sulfur dioxide from

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cylinders up to the level of 1.5 g of total SO_2/L and more. In all diluted musts no nutrient addition or any other adjustment took place. Musts were sterilized at 130 °C for 15 min.

Support and Immobilization of Cells. For the immobilization of cells, pieces of apple were used as support. Pieces of apple (425 g) were placed in a glass cylinder of 1 L, and 500 mL of culture medium were added. The culture medium contained 12% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.5% $MgSO_4$ in distilled water and had a pH of 5.6. There was no pH adjustment. Ten grams of AXAZ-1 strain was also added to the cylinder and allowed to ferment until an approximate 0.5 °Be density was reached. The fermented liquid was decanted, and the support was washed twice with 400 mL of must that was used for the next fermentation. After that, the biocatalyst was used for wine production. The first batch was carried out with 400 mL of liquid, whereas all of the others were done with 300 mL, because a decrease of the volume of apple in the cylinder was observed (see Results and Discussion). In all, 30 repeated fermentation batches were performed using a must of initial density of 12 °Be.

Low-Temperature Wine-Making. The amount of apple-supported biocatalyst, prepared as described above by the immobilization of cells on apple pieces, was introduced in 300 mL of must having an initial density of 12 °Be. A series of repeated batch fermentations were performed at each temperature, which was successively decreased as follows: 25, 20, 15, 12, 9, 6, and 1 °C. All fermentations were carried out without any agitation. When the fermentation was completed, the liquid was decanted and the support was washed twice, with 300 mL of must each time. After that treatment, the biocatalyst was used for the next fermentation batch. At the end of every batch, samples were collected and analyzed for ethanol, residual sugar, free cells, total and volatile acidity, and volatile composition.

Analyses. Alcoholic degrees were measured after distillation of samples and employment of 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. Wine productivity was calculated as grams of wine per liter of total volume produced per day.

Residual sugars were determined by high-performance liquid chromatography, using a Shimadzu HPLC 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. Three times distilled water was used as mobile phase with a flow rate of 0.8 mL/min, and butanol-1 was used 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. The residual sugar concentration was calculated using standard curves and expressed as grams of residual sugar per liter.

Total acidity was estimated by titration of samples by 0.1 N NaOH solution and volatile acidity by titration with 0.1 N NaOH of distillates obtained by steam distillation of wine samples (*13*).

To determine wet free cell concentrations, the experimental procedures of Klein and Kressdorf (*14*) and Bajpai and Margaritis (*15*) were used. The concentration was expressed in grams of wet weight per liter.

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 consisting of squalene, 5%; Carbowax-300, 90%; and diethylhexyl sebacate, 5% (v/v) (*16*). Nitrogen was used as carrier gas at 20 mL/min. Injection port and FID detector temperatures were 210 and 220 °C, respectively. The column temperature was programmed at 60−70 °C. In all cases, the internal standard was butanol-1 at a concentration of 0.5% (v/v). Samples of 4 μL of wine were directly injected into the column, and the concentrations of the aforementioned volatile compounds were calculated from standard curves. Methanol was also determined by gas chromatography using Porapack S as column material. Nitrogen was used as carrier gas at 40 (mL/min). The column temperature was programmed at 120−170 °C. The temperatures of the injector and FID detector were 210 and 220 °C, respectively.

Gas Chromatography—Mass Spectrometry (GC-MS). *Headspace Analysis.* Ten milliliters of the sample containing 2.2 g of Na_2SO_4 was left for 45 min at 60 °C before being subjected to headspace analysis. The sample volume injected was 1 mL. The injector was operated in split mode. Conditions used in the GC-MS are referred to below.

Solvent Extraction. The volatiles of the wines produced were isolated according to the method described by Maio et al. (*17*) and Priser et al. *(18)*. Wine (200 mL), 5 mL of CH_2Cl_2, and 30 g of sodium chloride were placed into a specific extraction device (*17*). The round-bottom flask was cooled in melting ice, and the wine−CH_2Cl_2 mixture was stirred at 500 rpm for 2 h. The emulsion formed was broken down by passing it through a specific filter (C_THP Acrodisc syringe filters GP 0.45 μm) with a gastight syringe. The organic extract was dried over anhydrous sodium sulfate in a small glass vial and stored at −5 °C until further analysis.

All compounds were identified on a GC-MS of Fisons Instruments (GC 8000 series, MS: MD 800). A Chrompack WCOT fused silica column was used (CP-Sil 8, 30 m, 0.32 mm i.d, 0.25 μm film thickness). Helium was used as carrier gas (linear velocity of 1 mL/min). Oven temperature was programmed from 35 °C for 2 min and then raised to 50 °C at a rate of 4 °C/min. After a period of 5 min at 50 °C, the temperature was raised to 230 °C with a rate of 4 °C/min. The injector temperature was 230 °C. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV. The identification was effected by comparison with standard compounds and data obtained from NIST and Wiley libraries.

Electron Microscope Study. For the electron microscope studies, a piece of apple-supported biocatalyst was taken and coated with gold in a Balzers SCD 004 sputter coater for 2 min, so as to obtain an increase in the electron conductivity. The prepared sample was studied in a Cambridge Stereoscan 120 scanning electron microscope.

RESULTS AND DISCUSSION

Immobilization and Fermentation. For the immobilization of cells pieces of apple were mixed with a liquid culture of an alcohol-resistant and psychrophile yeast strain and left for ~8 h. The prepared biocatalyst was washed and used for repeated batch fermentations of grape must for wine-making at room and low temperatures starting with an initial density of 12 °Be.

Figure 1 shows the clarity of the fermented liquid and the fermentation obtained by immobilized cells. Their existence was proved by the electron micrographs (Figure 2). The stability of productivity in the repeated batch fermentations and the low free cell concentrations are shown in Table 1.

To determine the volume of the apple pieces that remained in the cylinder after a long period of fermentative activity, the percentage of the volume of apple that remained in the cylinder after each batch for the first 12 repeated batch fermentations was measured. The must used had an initial density of 12 °Be. A decrease in the volume of the apple biocatalyst was observed as the number of batches increased. More specifically, after the immobilization, the volume of the apple pieces that remained in the cylinder was 94.4% of the initial volume. The main decrease was observed after the first fermentation batches. This was probably due to the conversion of the apple sugar to ethanol and/or cells. After that period, the apple volume remained

Figure 1. Apple-supported biocatalyst in the fermentation of grape must.

stable until the end of the experiment (duration of 7 months) due rather to the residual cellulose, another nonfermentable constituent of apple. The results are summarized in Table 1 and Figures 1 and 3.

Table 1 shows the results of 30 repeated batch fermentations of grape must performed at temperatures ranging from 1 to 25 °C. The whole process was carried out by apple-supported biocatalyst. The duration was ~7 months without any loss of the biocatalyst activity. The productivity is considered to be an important factor even at the very low temperatures (1−9 °C) used. More specifically, at 6 °C the biocatalyst produced wine within 8 days, which is less time than is required for the natural fermentation of grape must, whereas at 1 °C production of wine was effected after a period of 1 month. This is a very useful observation for the wine industry as it may scale-up the whole wine-making process. Residual sugar concentrations show that this observation might be useful for dry wine production. Wine and ethanol productivities are at least 10-fold higher than those obtained by natural fermentation. Total and volatile acidities were similar to those found in dry wines. The total acidity of the product is a little increased due to the transfer of apple acids to the product (wine) or to the formation of acids during fermentation. This was previously observed by some other researchers during fermentation of cider-apple juices (*11*), but it is within the normal limits of the dry wines (4−6 g of tartaric acid/L). The total acidity is in the range 4.5−5.5 g of tartaric acid/L at 25−12 °C, but it becomes slightly lower as the temperature drops from 9 to 1 °C. This reduction can be attributed to the increase of crystallization of tartrate salts with the decrease in temperature.

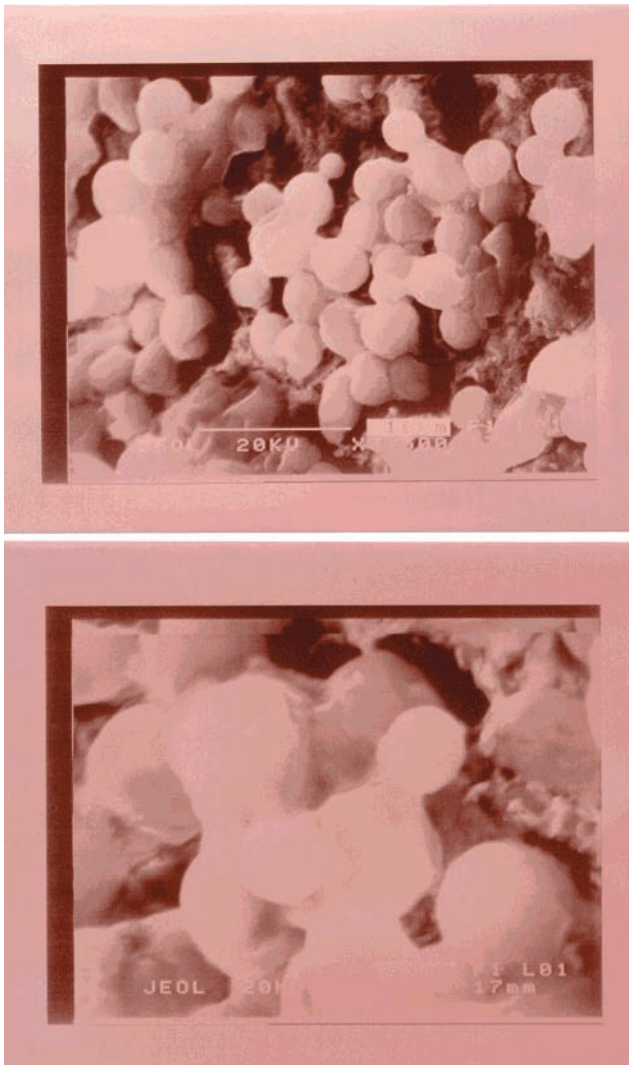

Figure 2. Electron micrographs showing immobilized cells on apple-supported biocatalyst.

Volatile Byproducts. *Higher Alcohols.* The concentrations of higher alcohols (propanol-1 and isobutyl acohol) were low. They ranged from traces up to 32 ppm (Table 2).

The concentration of amyl alcohols decreased with the temperature decrease. At 1 °C the amount of amyl alcohols was ~20% of that at 25 °C. Likewise, at this low temperature, extremely low concentrations were found for propanol-1 and isobutyl alcohol. In general, low temperature greatly reduced the amount of higher alcohols. These results show that the product is of improved quality because of low concentrations of higher alcohols. The reduction of the amount of amyl alcohols in wines at low temperatures by apple-supported biocatalyst is in agreement with results that were reported for biocatalyst supported on mineral kissiris, gluten, and delignified cellulosic materials. The reduction for propanol-1 and isobutyl alcohol is greater for apple-supported biocatalyst.

Ethyl Acetate. Due to the significant effect of this compound on organoleptic characteristics, the concentration of ethyl acetate was also examined.

The ethyl acetate concentration increased as the temperature decreased. Although the values of ethyl acetate concentrations were relatively high up to 154

Table 1. Fermentation Parameters Obtained at Low-Temperature Wine-Making by Repeated Batch Fermentations with Immobilized Cells of the Strain AXAZ-1 on Apple Using Must of 12 °Be Initial Density

temp (°C)	repeated fermentation batches	initial °Be density	fermentation time (h)	daily wine productivity (g/L)	ethanol concn (% vol)	daily ethanol productivity (g/L)	residual sugars (g/L)	final concn of free cells (g/L)	volatile acidity (g of acetic acid/L)	total acidity (g of tartaric acid/L)
25	1	12.1	80	116	10.8	26	30.8	0.4	0.22	4.6
25	2	12.0	58	141	11.8	38	35.9		0.17	4.7
25	3	11.9	49	167	11.9	47	29.5	0.6	0.26	5.1
25	4	12.4	46	201	12.3	51	31.7	0.7	0.26	5.3
25	5	12.2	52	178	11.5	47	35.2	0.8	0.26	5.3
20	6	12.5	50	184	12.5	50	28.3	0.7	0.26	5.3
20	7	12.4	45	206	13.1	56	36.2	0.5	0.26	5.0
20	8	12.2	47	198	13.3	54	33.6	1.3	0.31	5.4
20	9	12.4	42	218	12.2	55	34.4	1.4	0.31	5.3
20	10	12.3	39	236	12.5	60	32.6	1.6	0.31	5.3
15	11	12.3	51	186	12.7	47	35.9	1.6	0.22	5.5
15	12	12.2	49	194	11.3	44	31.6	2.2	0.29	4.8
15	13	12.1	49	192	12.2	50	14.2	1.9	0.28	4.8
15	14	12.0	44	215	13.0	56	8.0	2.1	0.29	4.5
15	15	12.1	45	212	11.6	49	14.0	2.1	0.38	5.5
12	16	12.2	50	189	13.0	49	8.9	2.1	0.38	4.5
12	17	12.1	50	189	12.7	48	11.8	1.8	0.30	5.1
12	18	12.0	49	195	11.9	46	25.6	2.4	0.41	5.5
12	19	12.0	49	193	12.3	47	25.4	0.6	0.38	5.1
12	20	12.0	49	192	12.3	48	12.7	1.8	0.30	5.1
9	21	12.0	81	118	13.1	31	14.5	0.5	0.24	4.6
9	22	12.1	72	132	11.8	31	52.1	0.5	0.25	4.7
9	23	12.0	87	109	12.2	27	23.6	0.6	0.47	4.9
9	24	12.0	88	108	12.3	27	9.2	0.5	0.41	4.2
6	25	12.0	180	53	11.5	12	30.4	0.5	0.34	4.4
6	26	12.0	175	54	11.2	12	29.4	0.5	0.34	4.4
6	27	12.0	170	56	12.6	14	37.6	0.5	0.34	4.3
1	28	12.0	528	18	12.5	4	22.0	0.7	0.25	4.4
1	29	12.0	1343	7	11.4	2	20.3	0.2	0.24	4.2
1	30	12.0	761	12	12.5	3	27.5	0.4	0.31	4.3

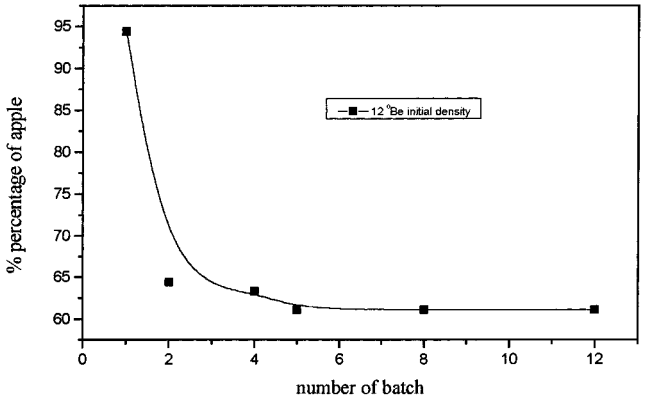

Figure 3. Decrease of apple volume as a function of batch number.

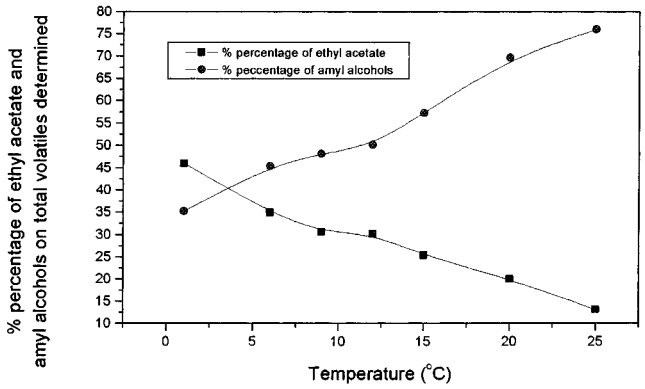

Figure 4. Percentage of ethyl acetate and amyl alcohols on total volatiles determined as a function of temperature.

mg/L, there was no indication of vinegar odor in the final product. On the contrary, it had a fruity aroma and a fine taste.

Other Volatiles. The acetaldehyde content in wines usually lies in the range 13−40 mg/L (*19*) but may reach 75 mg/L (*20*). In the present experiments, acetaldehyde concentrations up to 53 mg/L were observed. However, in most cases they were lower (Table 2).

Methanol is formed from methylated pectic substances (pectins) by the action of pectin esterase present in plants and microorganisms. The methanol content of the wines produced from batch fermentations of must by cells immobilized on apple was <100 mg/L. In traditional fermentation the usual range of methanol content is 0.1−0.2 g/L.

GC-MS Analysis. As the immobilized cells show differences in the specific metabolic activity in relation to free cells, the use of GC-MS became necessary. This analysis led to a comparison of the aroma composition of the wine produced by immobilized cells with that of the traditional product.

For the evaluation of the aromatic profile of the produced wine two techniques were employed, the extraction one and static headspace (Tables 3−6) (Figures 5−8). More peaks were observed when the extraction technique was employed. However, common compounds were identified in both the extraction and headspace techniques. These were ethanol, acetic acid ethyl ester, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, acetic acid butyl ester, 3-methylbutyl acetate, 2-methylbutyl acetate, butanoic acid ethyl ester, 2-methylbutyric acid ethyl ester, 1-hexanol, hexanoic acid ethyl ester, phenyl ethyl alcohol, octanoic acid ethyl ester, and acetic acid 2-phenylethyl ester. All of these compounds are considered to be important contributors to the wine aroma. The majority of these compounds are esters, and the positive contribution they

Table 2. Volatile Byproducts Formed by Repeated Batch Fermentations of Must by Cells Immobilized on Apple, Using Must of an Initial °Be of 12

temp (°C)	batch	acetaldehyde (ppm)	ethyl acetate (ppm)	propanol-1 (ppm)	isobutyl alcohol (ppm)	amyl alcohols (ppm)	total volatiles (ppm)
25	1	tr[a]	37	tr	16	188	241
25	2	26	43	2	27	308	406
25	3	tr	66	6	29	305	406
25	4	25	50	9	30	338	452
25	5	14	46	4	16	265	345
20	6	6	77	9	22	324	438
20	7	tr	68	9	20	309	406
20	8	26	93	11	23	301	454
20	9	22	90	7	20	284	423
20	10	17	108	13	21	297	456
15	11	45	119	14	23	276	477
15	12	28	87	11	18	221	365
15	13	36	104	15	22	232	409
15	14	36	123	20	29	263	471
15	15	32	110	15	26	236	419
12	16	47	133	18	28	233	459
12	17	19	145	25	31	247	467
12	18	40	150	25	27	204	446
12	19	53	117	28	32	226	456
12	20	13	128	25	31	208	405
9	21	50	139	23	28	208	448
9	22	50	154	21	29	259	513
9	23	39	118	25	27	185	394
9	24	30	124	22	29	188	393
6	25	33	97	10	18	141	299
6	26	52	118	5	18	136	329
6	27	6	91	11	21	121	250
1	28	40	77	8	tr	90	215
1	29	9	116	3	tr	59	187
1	30	49	77	2	tr	58	186

[a] Traces.

Table 3. Volatile Components Isolated from Wine with Extraction Technique[a]

time	compound	certainty of identification	time	compound	certainty of identification
0.976	acetaldehyde	*b*	6.460	3-methyl-1-butanol acetate	*b*
1.092	ethanol	*b*	6.544	2-methyl-1-butanol acetate	*b*
1.559	acetic acid ethyl ester	*b*	7.577	dihydro-(3*H*)-furanone	*c*
1.626	2-methyl-1-propanol	*b*	8.927	3-hydroxybutanoic acid ethyl ester	*c*
2.493	propanoic acid ethyl ester	*b*	11.428	heptanol	*c*
2.859	3-methyl-1-butanol	*b*	11.628	3-(methylthio)-1-propanol	*b*
2.959	2-methyl-1-butanol	*b*	11.861	dihydro-2-methyl-3(2*H*)-thiophenone	*c*
2.976	2,2-dimethyl-1-propanol	*c*	13.248	hexanoic acid	*b*
3.610	acetic acid isobutyl ester	*b*	13.298	hexanoic acid ethyl ester	*b*
3.726	2,3-butanediol	*b*	17.415	methyl-2-furoate	*d*
4.143	3-pentanol	*b*	19.015	phenylethyl alcohol	*b*
4.226	butanoic acid ethyl ester	*b*	20.432	*N*-(3-methylbutyl)acetamide	*c*
4.526	lactic acid ethyl ester	*b*	22.633	octanoic acid	*b*
5.210	4-methyl-1-pentanol	*b*	23.066	octanoic acid ethyl ester	*b*
5.277	3-ethoxy-1-propanol	*c*	24.050	2,3-dihydrobenzofuran	*d*
5.443	3-methyl-1-pentanol	*b*	25.167	isobutyric acid phenethyl ester	*c*
5.577	butyric acid, 2-methylethyl ester	*b*	25.200	acetic acid, 2-phenyl ethyl ester	*b*
5.743	2-furanmethanol	*c*	27.334	2-methyl-4-hydroxyacetophenone	*d*
5.843	2-methyl butanoic acid	*c*	29.634	decanoic acid	*b*
6.210	1-hexanol	*b*	30.168	ethyl dec-9-enoate	*c*

[a] Solvent used CH_2Cl_2. Fermentation was carried out by cells of AXAZ-1 immobilized on apple pieces at 15 °C. [b] Positive identification from mass spectra and retention time that agree with authentic compounds. [c] Positive identification from mass spectra data. [d] Tentative identification from mass spectra data.

have to the aroma of wines is well-known. The main acids identified by the extraction technique were hexanoic, heptanoic, octanoic, and decanoic acid. Although their flavor impact is considered to be negligible, it is also known that there is a positive correlation between the concentration of C_6, C_8, and C_{10} fatty acids and wine quality. More specifically, at or just below threshold levels fatty acids contribute to the complexity of the bouquet of a wine. These are in accordance with the findings of other researchers. No acids were identified by the headspace (HS) technique. This was attributed to the high boiling point they have. Therefore, it is not very easy to have enough vapor in the headspace under the specific conditions used in the analysis.

Alcohols identified in the extract include ethanol, hexanol, phenyl ethyl alcohol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,2-dimethyl-1-propanol, 2,3-butanediol, 3-pentanol, 4-methyl-1-pentanol, 3-ethoxypropanol, 3-methyl-1-pentanol, 2-furanmethanol, heptanol, and 3-methylthio-1-propanol. The dihydro-2(3*H*)-furanone (buterolactone) was detected only in the extract. This verifies the findings of other

Table 4. Headspace Analysis (HS) of Volatile Components[a]

time	compound	certainty of identification	time	compound	certainty of identification
1.126	ethanol	*b*	6.560	3-methylbutyl acetate	*b*
1.676	acetic acid ethyl ester	*b*	6.660	2-methylbutyl acetate	*b*
1.776	2-methyl-1-propanol	*b*	13.428	hexanoic acid ethyl ester	*b*
2.076	1-butanol	*b*	14.278	acetic acid hexyl ester	*c*
2.593	propionic acid ethyl ester	*b*	19.096	benzeneethanol (2-phenylethanol)	*b*
2.826	2,4,5-trimethyl-1,3-dioxalane	*d*	23.164	octanoic acid ethyl ester	*b*
2.893	1,1-diethoxyethane	*b*	25.298	acetic acid phenethyl ester	*c*
2.943	3-methyl-1-butanol	*b*	30.249	ethyl dec-9-enoate	*b*
3.059	2-methyl-1-butanol	*b*	30.549	decanoic acid ethyl ester	*b*
3.710	isobutyl acetate	*b*	32.233	octanoic acid, 3-methylbutyl ester	*d*
4.326	butyric acid ethyl ester	*b*	34.233	farnesene	*b*
4.676	*n*-butyl acetate	*c*	36.950	dodecanoic acid ethyl ester	*b*
5.677	2-methylbutyric acid ethyl ester	*c*	38.434	3-methylbutyl decanoate	*c*
6.343	1-hexanol	*b*	47.369	ethyl 9-hexadecanoate	*b*

[a] Fermentation was carried out by cells of AXAZ-1 immobilized on apple pieces at 15 °C. [b] Positive identification from mass spectra and retention time that agree with authentic compounds. [c] Positive identification from mass spectra data. [d] Tentative identification from mass spectra data.

Table 5. Volatile Components Isolated from Wine with Solvent Extraction Technique[a]

time	compound	certainty of identification	time	compound	certainty of identification
1.059	acetaldehyde	*b*	6.493	hexanol	*b*
1.092	ethanol	*b*	6.760	3-methyl-1-butanol acetate	*b*
1.180	dichloromethane (solvent)	*b*	6.860	2-methyl-1-butanol acetate	*b*
1.659	ethyl acetate	*b*	7.927	2(3*H*)-furanone, dihydro	*b*
1.743	2-methyl-1-propanol	*b*	9.161	α-pinene	*b*
2.276	2-pentanone	*d*	12.228	3-methylthio-1-propanol	*b*
2.543	3-hydroxy-2-butanone	*b*	13.862	ethyl hexanoate	*b*
2.626	ethyl propanoate	*b*	14.262	hexanoic acid	*b*
2.859	2,4,5-trimethyl-1,3-dioxolane	*b*	17.012	butanoic acid, 3-hydroxy-, ethyl ester	*d*
2.943	1,1-diethoxyethane	*b*	18.196	2,5-dimethyl-3(2*H*)-furanone	*d*
2.993	3-methyl-1-butanol	*b*	19.613	phenylethyl alcohol	*b*
3.043	2-methyl-1-butanol	*b*	20.813	2-ethylhexanoic acid	*b*
3.393	4-pentenol	*c*	23.230	butanedioic acid, diethyl ester	*b*
3.543	2-methylpropanoic acid	*c*	23.931	octanoic acid	*b*
3.776	acetic acid, 2-methylpropyl ester	*b*	24.064	ethyl octanoate	*b*
3.876	2,3-butanediol	*b*	25.147	benzofuran	*d*
4.160	butanoic acid	*d*	26.531	2-furanethanol, *β*-ethoxy	*d*
4.426	ethyl butanoate	*b*	27.048	phenylethyl acetate	*b*
4.726	propanoic acid, 2-hydroxy-, ethyl ester	*b*	36.834	undecenoic acid	*d*
5.743	butanoic acid, 3-methyl	*d*	37.968	decanoic acid	*b*
5.943	2-furanmethanol	*b*	38.768	ethyl dec-9-enoate	*b*
6.027	benzene, ethyl	*c*	39.818	ethyl decanoate	*b*
6.093	2-methylbutanoic acid	*d*			

[a] Solvent used was CH_2Cl_2. Fermentation was carried out by free cells at 15 °C. [b] Positive identification from mass spectra and retention time that agree with authentic compounds. [c] Positive identification from mass spectra data. [d] Tentative identification from mass spectra data.

Table 6. Headspace Analysis of Volatile Components[a]

time	compound	certainty of identification	time	compound	certainty of identification
1.209	ethanol	*b*	6.544	2-methyl-1-butyl acetate	*b*
1.392	propanol	*b*	13.262	ethyl hexanoate	*b*
1.609	ethyl acetate	*b*	14.112	hexyl acetate	*b*
1.743	2-methyl-1-propanol	*b*	18.530	α-linalool	*d*
2.043	butanol	*b*	18.963	phelylethyl alcohol	*b*
2.576	ethyl propanoate	*b*	23.031	ethyl octanoate	*b*
2.776	2,4,5-trimethyl-1,3-dioxolane	*d*	25.165	phenylethyl acetate	*b*
2.826	1,1-diethoxyethane (acetaldehyde, diethyl acetal)	*c*	30.133	ethyl dec-9-enoate	*b*
2.959	3-methyl-1-butanol	*b*	30.420	ethyl decanoate	*b*
3.009	2-methyl-1-butanol	*b*	32.104	octanoic acid, 3-methylbutyl ester	*d*
3.543	acetic acid, 2-methylpropyl ester	*b*	36.822	ethyl dodecanoate	*b*
4.260	ethyl butanoate	*b*	38.305	3-methylbutyl decanoate	*d*
6.460	3-methyl-1-butyl acetate	*b*			

[a] Fermentation was carried out by free cells at 15 °C. [b] Positive identification from mass spectra and retention time that agree with authentic compounds. [c] Positive identification from mass spectra data. [d] Tentative identification from mass spectra data.

workers of the same research group (*21*). Traces of some sulfurous compounds such as 3-(methylthio)-1-propanol and dihydro-2-methyl-3(2*H*)-thiophenone were detected only in the extract. Usually, these compounds have a negative result on the aroma of the wine, and special attention should be paid.

The major aldehyde detected in the extract was acetaldehyde. The hexanol was present only in small

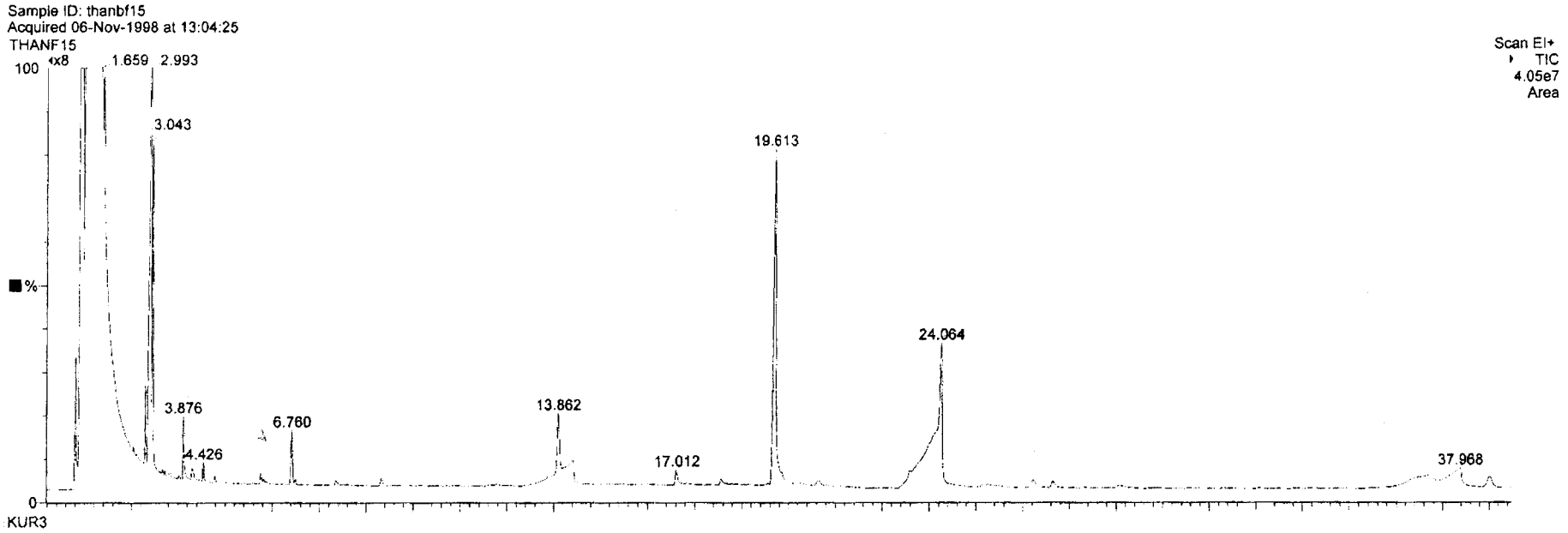

Figure 5. Typical chromatogram of aroma compounds identified by GC-MS. Technique used extraction with CH_2Cl_2. Fermentation was carried out by free cells at 15 °C.

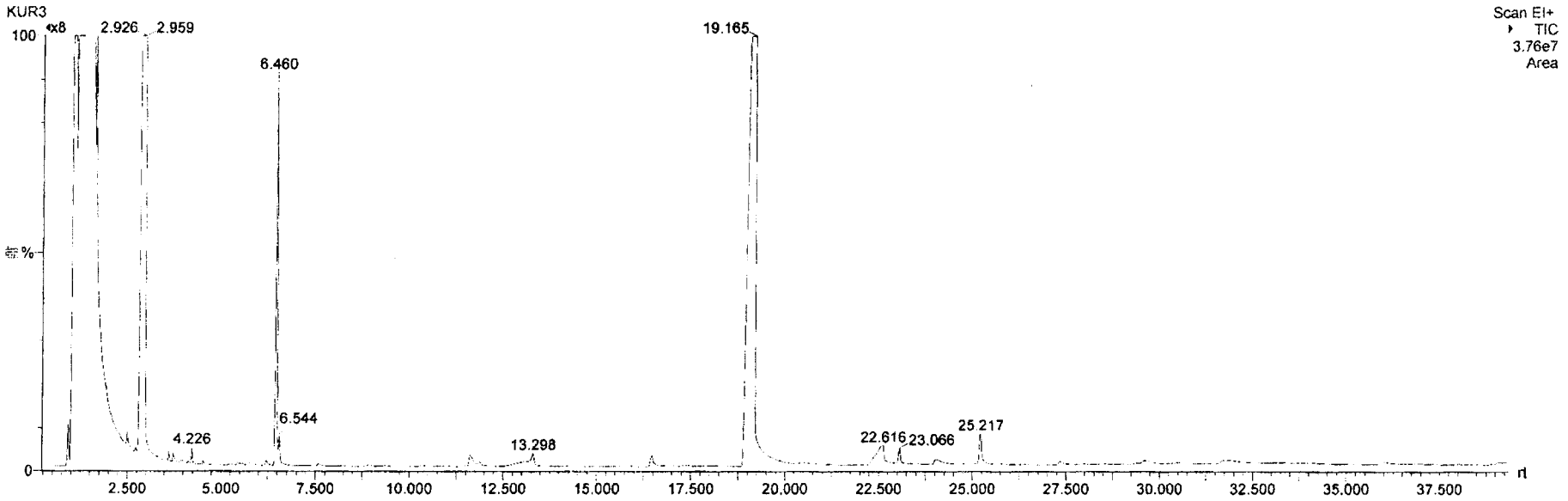

Figure 6. Typical chromatogram of aroma compounds identified by GC-MS. Technique used extraction with CH_2Cl_2. Fermentation was carried out by cells immobilized on pieces of apple at 15 °C.

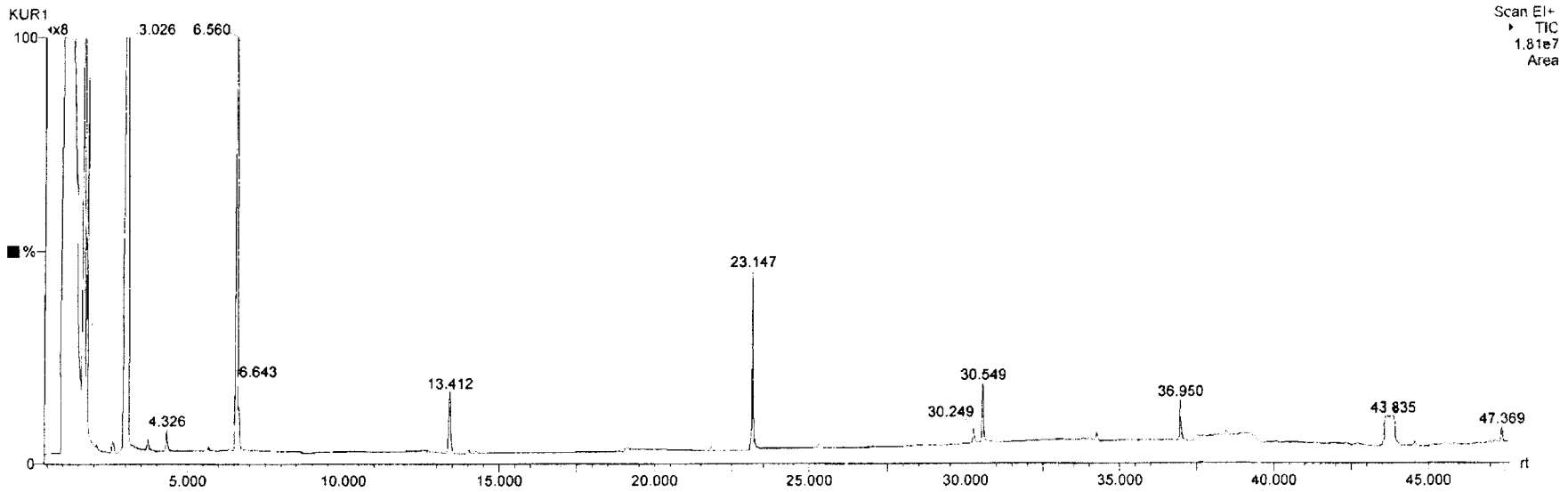

Figure 7. Typical chromatogram of aroma compounds identified by GC-MS. Technique used headspace. Fermentation was carried out by cells immobilized on pieces of apple at 15 °C.

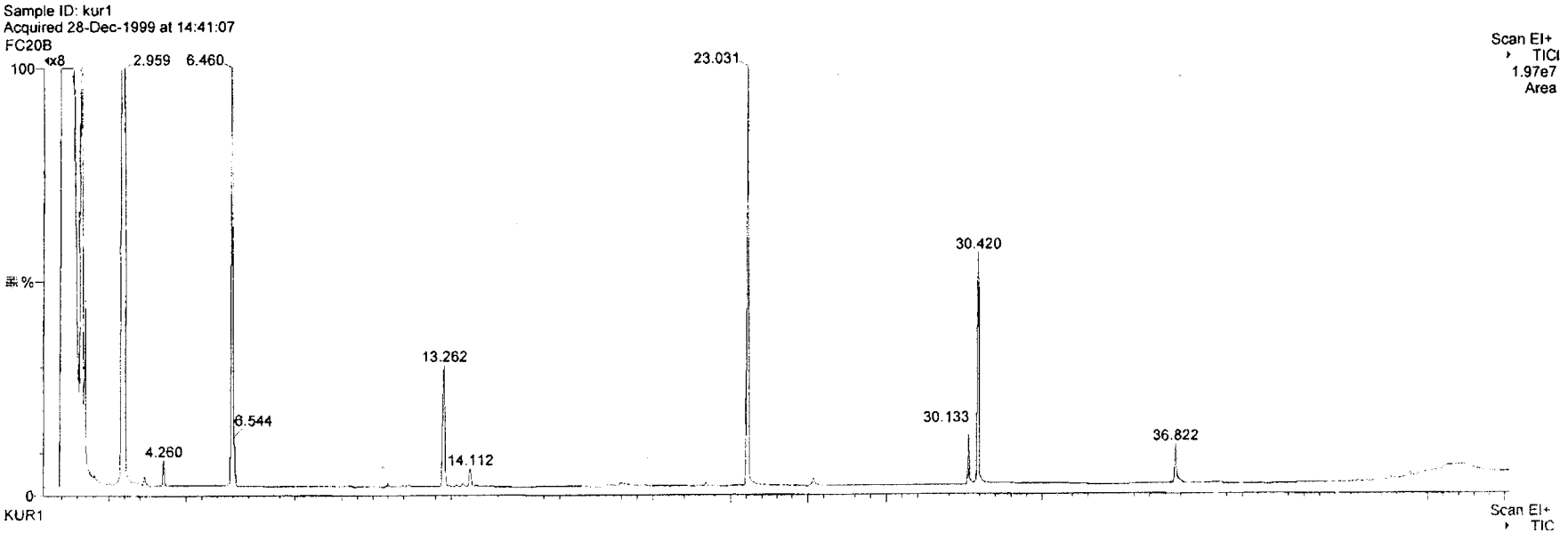

Figure 8. Typical chromatogram of aroma compounds identified by GC-MS. Technique used headspace. Fermentation was carried out by free cells at 15 °C.

amounts (traces). No other aliphatic compound with six carbon atoms was present. This is a positive indication as these compounds, which are responsible for the green flavor of leaves, may affect negatively the wine aroma.

After the GC-MS analysis, one can say that almost all of the volatiles found in the wine, which was produced by immobilized cells on apples, were present in wines produced by natural fermentation. These results lead to the conclusion that cell immobilization does not create serious changes in the product with regard to the qualitative composition of the aroma compounds.

With regard to the most abundant byproducts in the wine, a reduction of the percentage of higher alcohols and a relative increase of the percentage of ethyl acetate in the total volatiles were observed in the low-temperature fermentations carried out by cells immobilized on apple pieces (Figure 4). It is known that the relatively high concentration of ethyl acetate (>150 mg/mL) may donate a sour vinegar off-flavor to the wines. However, in this case, only in one stage of the fermentation were ethyl acetate values >150 mg/L observed. The recorded volatile acidity was <0.47 g/L, ranging mainly between 0.20 and 0.35 g/L (*13*). This was not enough to spoil the flavor of the final product and had no effect on the overall sensory characteristics. Preliminary sensory tests carried out in the laboratory have ascertained the fruity aroma, the fine taste, and the overall better quality of the wines produced by using cells immobilized on apple pieces at low temperatures.

From the aforementioned results one can conclude that pieces of apple offer a very good and effective support material for low-temperature alcoholic fermentation. Their use leads not only to higher productivity but also to a product with better sensory characteristics.

This preliminary but objective evaluation of the wine quality produced by use of cells immobilized on pieces of apple leads to some useful conclusions. First, the special types of wines produced were of very good quality with distinctive aromatic potential, and, second, large quantities of apples, which otherwise are destroyed and pollute the environment, can be beneficially used.

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