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Wine fermentations by immobilized and free cells at different temperatures. Effect of immobilization and temperature on volatile by-products

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Abstract

A biocatalyst was prepared by immobilization of *Saccharomyces cerevisiae*, strain AXAZ-1, on delignified cellulosic material (DCM) and gluten pellets (GP). Repeated batch fermentations were conducted using these biocatalysts and free cells, separately, at different temperatures. The volatile constituents were extracted with dichloromethane and the extracts were subsequently analyzed by HRGC/MS. Wines produced by DCM contained higher amounts of esters, at every temperature studied, whereas those produced by GP biocatalyst contained higher amounts of alcohols. Free cells and DCM biocatalyst gave wines with similar contents of alcohols. Wines produced by DCM biocatalyst gave better ratios of esters to alcohols and had dominating fruity aromas. GC/MS analysis proved that cell immobilization did not create serious changes in qualitative composition of the wine aroma. As regards the quantitative profile, the combined effect of temperature and immobilization resulted in the production of wines with more fruity character because of the higher ratio of esters to alcohols.

Keywords: Wine; Immobilization; Volatiles; GC/MS; Fermentation; Temperature; Esters; Alcohols; Acids

1. Introduction

Wine aroma is the result of a complex combination of components that gives each wine its distinctive character. It has been shown that the main fraction of the aroma compounds is produced during fermentation. These compounds are acetates and ethyl esters, higher alcohols, fatty acids, ketones and aldehydes (Herraiz, Reglero, Cabezudo, Martin-Alvarez, & Herraiz, 1991; Rapp & Mandery, 1986; Schreier, 1979).

Temperature is one of the most influential factors affecting fermentation. Several authors have reported the effect of temperature on yeast metabolism and the production of volatile compounds (Aragon, Atienza, & Climent, 1998; Castellari, Magrini, Passarelli, & Zambonelli, 1995; Etievant, 1991; Killian & Ough, 1979; Suomalainen & Lehtonen, 1979). The modern practice, in most winemaking regions, is to conduct white wine fermentations between 8 and 15 °C, because they give more fruity wines. In this respect, it is important to increase the synthesis of fruit esters, such as isoamyl acetate, isobutyl and hexyl acetates. The esters are both synthesized and withheld to a greater degree at low fermentation temperatures (Jackson, 1994).

Immobilized cell systems, not only increase productivity and improve the cost of bioprocesses (Colagrande, Silva & Fumi, 1994; Dervakos & Webb, 1991; Divies, Cachon, Cavin, & Prevost, 1994), but also influence yeast metabolism and, consequently, wine aroma (Melzoch, Rychtera, & Habova, 1994; Rosi, Costamagna, Bertuccioli, Clementi, & Cruciani, 1987; Russell & Stewart, 1992). There are some reports in the literature about the combined effects of temperature and immobilization on the formation of the major volatiles produced during wine fermentation (Bakoyianis, Kana,

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Kaliafas, & Koutinas, 1993; Bardi, Koutinas, Psarianos, & Kanellaki, 1997; Kana, Kanellaki, & Koutinas, 1992). However, publications concerning the analysis of the minor volatile components produced using immobilized yeast cells are scarce in the literature.

The aim of this study was to determine the influence of fermentation temperature and immobilization on the quantitative profile of minor volatile compounds produced during fermentation.

2. Materials and methods

2.1. Yeast strain

The psychrophilic and alcohol resistant yeast strain AXAZ-1 was used. It was isolated from the Greek agricultural area (Argiriou, Kaliafas, Psarianos, Kanellaki, Kana, & Koutinas, 1992) and was grown in a medium consisting of glucose (2%), yeast extract (0.4%), $(NH_4)_2SO_4$ (0.1%), KH_2PO_4 (0.1%) and $MgSO_4.7H_2O$ (0.5%) at 25 °C.

2.2. Must

Grape must was prepared from the cultivar Moshofilero. It was sterilized at 130 °C for 15 min. The initial °Be density was 11 and total acidity 5.9, expressed as g of tartaric acid/l. The must was used without any nutrient addition.

2.3. Immobilization supports

For the immobilization of yeast cells, delignified cellulosic material and gluten pellets were used as supports. Delignified cellulosic material (DCM) was prepared from sawdust after lignin removal with sodium hydroxide solution (Bardi & Koutinas, 1994).

Gluten pellets (GP) were prepared from wheat dough after starch removal. Gluten was shaped by hand into pellets of 1.5–2.0 cm diameter and dried at 105 °C for 5 h (Bardi, Bakoyianis, Koutinas, & Kanellaki, 1996).

2.4. Immobilization

In an 1l culture medium, containing 12% glucose, 20 g wet weight cells of the *Saccharomyces cerevisiae* strain AXAZ-1 were spread. The pH was adjusted to 4.8 by addition of dilute sulfuric acid. The resulting broth was mixed with 200 g wet-delignified cellulosic material and left to ferment for 6–8 h. After that time, the supernatant liquid was decanted and the solid was washed twice with 400 ml of culture medium containing glucose and then with 400 ml of must. The resulting biocatalyst was directly used for wine making by repeated batch fermentations.

In the case of immobilized biocatalyst on gluten pellets, the same process was followed. For yeast immobilization, 238 g of gluten pellets were used.

2.5. Fermentations

The prepared biocatalysts were separately used for the fermentation. One litre of must was transferred into a 2 l-glass cylinder. Cotton plugs were fitted to prevent contamination. The glass cylinder for each fermentation batch was incubated at temperatures of 20, 15, 10 °C. All fermentations were carried out without any agitation. When the fermentation was completed ($d = 0.992$ g/ml), the supernatant liquid was decanted and the support was washed twice with 400 ml of must each time. After that, the immobilized biocatalyst was used for the next fermentation batch.

Similar runs were carried out, simultaneously, with the same initial concentration (10 g/l) of free cells. Yeast cells immobilized on DCM weighed 4.9 g of wet weight cells/100 g of DCM (Bardi & Koutinas, 1994) and on gluten pellets 4.2 g of wet weight cells/100 g of GP (Bardi et al., 1996).

After the end of each fermentation, the produced wines were centrifuged at 3000 rpm for 10 min and stored at low temperature.

2.6. Isolation of volatile compounds

The volatile constituents of the wines produced were isolated with a combination and modification of the methods described by Moio, Chambellant, Lesschaeve, Issanchou, Schlich, and Etievant (1995) and Priser, Etievant, Nicklaus and Brun (1997). Wine (200 ml), internal standard (2-Octanol), dichloromethane (5 ml) and sodium chloride (30 g) were poured into a specially built conical flask. The conical flask was purged with nitrogen for 1 min in order to remove air, cooled in melting ice and the wine/CH_2Cl_2 mixture was stirred at 500 rpm for 2 h. The wine/CH_2Cl_2 emulsion formed during stirring was broken down by passing through a special filter (GHP Acrodisc Syringe Filters, GF 0.45μm) with the help of a gas-tight syringe. The organic extract was dried over anhydrous sodium sulfate and then filtered once more. The whole process was performed in the absence of air. The filtrate was transferred into a small glass-vial and stored at −5 °C for further analysis.

2.7. Gas chromatography-mass spectrometry (GC/MS)

Analyses of dichloromethane extracts were performed on a GC-MS of Fisons instruments (GC 8000series, Model: 8060 MS: MD800) with the injector in the splitless mode for 5 min. A Chrompack WCOT fused silica column was used (CP-Wax 52CB, 60 m × 0.32

mm, DF = 0.25 μm). Helium was used as carrier gas at a flow rate of 2.0 ml/min.

One μl of sample extract was injected. Oven temperature was programmed to 35 °C for 2 min and then raised to 50 °C at a rate of 4.0 °C/min. After a period of 5 min at 50 °C, the temperature was raised to 230 °C at a rate of 4 °C/min. The injector temperature was maintained at 230 °C, the source temperature at 200 °C and the interface temperature at 250 °C. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70eV and mass range 29–400 m/z. Identification of compounds was effected by comparing the retention times with those of authentic compounds and by spectral data obtained from Wiley and Nist libraries. The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the internal standard (IS) and multiplying this ratio by the initial concentration of the IS (expressed as μg/l). The peak areas were measured either from the full scan chromatogram, or by selecting fragments (Table 1). Each determination was carried out in triplicate.

Table 1
Composition of wines produced by free (FC)[a]or immobilized cells on DC[b] and GP[c] at temperatures of 20, 15 and 10 °C

Compound (μg/L)	DC20	GP20	FC20	DC15	GP15	FC15	DC10	GP10	FC10	m/z[d]
Esters										
Ethyl propanoate	18	92	12	47	98	31	67	106	34	29,57,102
Ethyl butanoate	130	87	130	123	79	97	190	85	118	43,60
Ethyl hexanoate	250	200	315	205	159	165	188	94	92	29–400
Ethyl lactate	38	21	40	32	27	33	17	22	19	45,75
Ethyl octanoate	8	3	143	22	6	50	28	5	11	88,101,127
Acetates										
Ethyl acetate	668	485	619	577	487	440	548	566	419	43,61
Propyl acetate	39	22	26	21	17	8	17	20	8	43,61,73
Isobutyl acetate	258	143	137	70	87	57	69	77	27	43,56,73
3-and 2-methylbutyl Acetate	6360	3347	4675	4549	3045	2348	3755	2809	608	29–400
Hexyl acetate	2	1	2	2	1	1	2	1	1	61,84
2-phenylethyl acetate	1956	913	1511	1576	855	845	1151	775	191	29–400
Sum of esters and acetates	9727	5315	7611	7221	4861	4076	6032	4560	1528	
Alcohols										
1-Propanol	210	358	256	285	392	259	323	543	241	31,59
Isobutanol	861	2651	627	515	2247	1260	457	2404	449	29–400
1-Butanol	5	6	7	5	6	7	4	7	3	31,41,56
3-and 2-methyl-1-butanol	22208	28822	21774	21749	26138	22755	17793	23060	16838	29–400
4-Penten-1-ol	2	3	3	3	3	8	9	4	3	53,67,68
4-Methyl-1-pentanol	3	2	2	2	2	2	1	2	1	56,69,84
3-Methyl-1-pentanol	6	7	4	5	7	7	4	7	3	56,69,84
3-Pentanol	4	8	4	7	9	9	8	10	4	31,55,59
1-Hexanol	15	100	22	48	81	61	48	94	69	56,69,84
2-Phenylethanol	12234	13468	10096	10610	11849	9534	8012	10090	6443	29–400
3-Methylthio-1-propanol	333	1821	423	625	1817	1160	432	1213	511	29–400
Sum of alcohols	35882	47246	33217	33855	42553	35062	27090	37435	24565	
Acids										
Acetic acid	73	30	873	7	19	20	11	61	81	45,60
Butanoic acid	20	14	17	8	9	10	24	10	10	60,73
3-Methyl butanoic acid	41	87	55	29	79	34	23	75	23	57,60,87
Hexanoic acid	2172	1546	2627	1536	1494	1398	1613	1361	1640	29–400
Octanoic acid	7070	2136	8204	5844	1982	4772	5113	1919	5023	29–400
Decanoic acid	756	346	1212	742	290	876	381	161	277	60,73,129
Sum of acids	10131	4160	12988	8166	3873	7111	7165	3586	7054	
Total volatiles	55740	56722	53816	49242	51287	46248	40287	45581	33147	

[a] FC, free cells
[b] DC, delignified cellulosic material
[c] GP, gluten pellets
[d] m/z, ions used in quantitation of the compounds (each determination was carried out in triplicate)

3. Results and discussion

According to Bardi and Koutinas (1994) and Bardi et al. (1996), DCM and GP act as promoters of alcoholic fermentation, even at very low temperatures. They are characterized by greater fermentation rates and productivities than free cells.

The qualitative results of this study (results not shown) have shown that the wines produced, either with free or immobilized cells on DCM and GP, have very similar profiles of volatile constituents, at every temperature studied. The differences between them are not significant. So, the next step was to proceed to the quantification of the volatile by-products. The main compounds produced during fermentation by yeasts are alcohols, esters and acids. This study was focused on these three classes of volatile compounds, which have a very strong influence on wine aroma, and subsequently, on its quality.

3.1. Esters

Important aroma compounds in wine are the ethyl esters of fatty acids, as well as acetates. They are formed enzymatically during fermentation and contribute to the fruity and floral sensory properties of the wine (Nordstrom, 1964). Acetates are more important than ethyl esters and among them, 3-methylbutyl acetate and hexyl acetate are considered as the most powerful contributors to wine aroma. Other significant positive contributors detected in all wines are 2-phenylethyl acetate and ethyl 2-hydroxypropanoate (Etievant, 1991). There are many factors influencing the production of esters. However, the most important are the conditions used during fermentation.

As shown in Table 1, the amount of total esters, formed either by free or immobilized cells, decreased as the fermentation temperature dropped. The rate of decrease was high for free cells and low in the case of GP biocatalyst. Wines produced by DCM biocatalyst contained greater amounts of esters, at every temperature studied. Then followed GP and free cells.

The production of alcohol acetates during fermentation is dependent on the activity of at least three acetyltransferases (Lilly, Lambrechts, & Pretorious, 2000). Immobilization of yeast cells might alter the activity of these enzymes positively, leading to increased synthesis of esters.

3.2. Higher alcohols

Higher alcohols are considered to have a negative impact on wine quality. Their formation is dependent on the fermentation temperature (Etievant, 1991). An increase in fermentation temperature resulted in increased concentration of total alcohols (Table 1). GP-produced wines contained the highest amount of alcohols at every temperature studied. This was expected, because of the enrichment of must with amino acids from the hydrolysis of the gluten (Bardi et al., 1997). Herraiz et al., (1989) have reported that higher alcohols increase with amino acid increase in the must. Lopez, Santamaria, Gutierrez, and Iniguez (1996) reported that the amino acids of the grape must were assimilated at a lower rate with temperature decrease. These findings may offer an explanation for the increased amounts of alcohols produced, by immobilized and free cells at higher temperatures. In wines produced by DCM and free cells, the concentrations of total alcohols were almost identical at every temperature studied.

3.3. Acids

The flavour impact of fatty acids is generally considered to be negligible. At or just below the threshold levels, fatty acids contribute to the complexity of the bouquet of a wine. Above the recognition threshold they have a negative influence on wine fragrance (Jackson, 1994).

The total amount of acids decreased with temperature decrease, in free and immobilized cells. GP-supported biocatalyst produced wines with the smaller amounts of acids (Table 1). Assimilable nitrogen of must is a factor that influences the production of fatty acids (Etievant, 1991). Thus, the increased amount of amino acids, due to the hydrolysis of the gluten, might inversely affect the production of acids from yeasts. DCM biocatalyst and

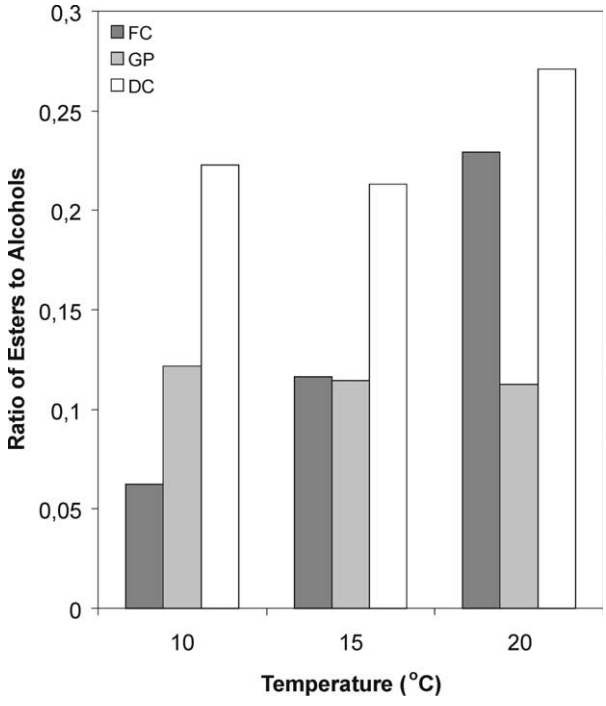

Fig. 1. Effect of temperature on the ratio of ester-to-alcohol.

free cells gave wines with similar contents of volatile acids.

3.4. Ratio of esters to alcohols

From the histogram (Fig. 1), it can be seen that DCM-supported biocatalyst showed better ratios of esters to alcohols at every temperature studied. As a consequence, the wines produced had a dominating fruity aroma. In wines produced by GP-supported biocatalyst, this ratio slightly increased with temperature decrease, showing that more esters were produced (in relation to alcohols) as the temperature decreased. In contrast, the ratio for free cells was drastically reduced with temperature decrease. This means that free cells produced more alcohols than esters, as the temperature decreased.

4. Conclusions

It can be concluded that the combined effects of temperature and immobilization produces wines, which possess a more fruity character because of the better ratio of esters to alcohols. DCM-supported biocatalyst may offer a good alternative for wine fermentations because it produces wines with more distinct fruity character, lower fusel alcohol content and intermediate acidity. Furthermore, DCM constitutes a cheap, and abundant support of food grade purity for yeast immobilization, which performs fast fermentations, leading to improvement of the economics of the whole process.

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