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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Storage of Immobilized Yeast Cells for Use in Wine-Making at Ambient Temperature

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A comparative study of the storage and reuse of immobilized yeast cells on apple pieces, kissiris, and γ-alumina was carried out. The immobilized biocatalysts were allowed to remain in the fermented alcoholic liquid after the end of each fermentation batch for extended periods at 30 °C before reactivation in batch fermentation for wine-making. The results showed that the biocatalysts were able to reactivate and ferment after successively increased periods of storage compared to free cell systems both on glucose medium and on grape must. In glucose medium, apple-, kissiris-, and γ-alumina-supported biocatalysts reactivated after 120, 80, and 83 days, respectively. Possible storage periods for grape must were lower but remained high. Immobilized yeast biocatalyst on apple pieces produced wines with an improved volatiles composition compared to kissiris- and γ-alumina-supported biocatalysts. There were no significant negative effects on the fermentation activity and volatile byproduct composition.

KEYWORDS: Preservation; immobilized cells; wine-making; yeast biocatalyst

INTRODUCTION

Use of immobilized cells in alcoholic fermentation has received increasing attention due to its technical and economic advantages when compared to free cell fermentation systems (*1*, *2*). Many researchers have proposed various supports for cell immobilization in the wine-making process such as glass beads (*3*), alginates (*4*−*7*), gluten pellets (*8*), and delignified cellulosic material (*9*). However, none has been used for wine-making on an industrial scale.

This is mainly due to the difficulty in finding a low-cost support material that is abundant in nature, is durable, is of food-grade purity, and has the ability to be preserved for long periods when alcohol production and wine-making are halted. Argiriou et al. (*10*) proposed preservation of immobilized cells by cooling at 0 °C. Even though the results were promising, this method requires capital investment. A cheaper and more effective preservation method has therefore been sought.

Volatile byproducts of alcoholic fermentation determine the quality and flavor of the wine (*11*), and therefore many authors have investigated their formation in wines (*11*−*14*). Due to their importance, preservation methods should not negatively affect them.

Apple pieces (*15*, *16*), kissiris (a cheap, porous volcanic mineral found in Greece, similar to granite, containing 70% SiO_2, 13% Al_2O_3, and other inorganic oxides) (*13*, *17*, *18*), and γ-alumina (porous cylindrical pellets) (*19*, *20*) have all been successfully used as supports for immobilization in wine-making. Yeast cells immobilized on kissiris have also been shown to increase biocatalytic stability, ethanol, and wine productivities during successive preservations at 0 °C (*10*).

The aim of this study therefore was to investigate possible storage duration and characteristics of immobilized yeast cells on apple pieces, kissiris, and γ-alumina at ambient temperature after fermentation of grape must in order to obtain a cost-effective preservation method for immobilized yeast cells suitable for wine-making.

MATERIALS AND METHODS

Yeast and Must. A locally available baker's yeast strain was used in the present study. Concentrated grape must was diluted with distilled water to a final °Be density of 11.5−12.0 [~19−24% (w/v) initial sugar concentration]. The must was used without any nutrient addition or adjustments. It was sterilized at 130 °C for 15 min before use.

Support and Immobilization of Cells. Immobilization of cells on apple pieces (*15*), kissiris (*17*), and γ-alumina pellets (length, 5 mm; diameter, 2.5 mm; pore volume, 0.4 cm^3/g; surface area, 1.40 m^2/g) (*19*) was carried out separately as described in previous studies.

Fermentations. Fermentations were carried out using 425 g of supported biocatalyst on apple pieces, 250 g of supported biocatalyst on kissiris, and 200 g of supported biocatalyst on γ-alumina, separately. The yeast-supported biocatalysts were introduced in 300 mL (500 mL for the first batch using apple-supported biocatalyst) of either (a)

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Table 1. Fermentation Parameters Obtained by Repeated Batch Fermentations of Glucose Using Immobilized Yeast Cells and Free Yeast Cells after Storage at 30 °C

support	repeated batch fermentation	storage time (days)	fermentation time (h)	ethanol concn (% v/v)	residual sugars (g/L)	daily ethanol productivity (g/L)
apple	1	0	27	10.2	24.6	72
	4	3	30	11.8	1.0	75
	5	5	64	11.7	0.6	35
	6	8	86	11.4	2.5	25
	7	12	99	12.2	7.5	23
	8	18	85	12.4	0.5	28
	9	24	101	11.6	0.2	22
	10	37	116	12.0	tr[a]	20
	11	50	122	11.8	tr	18
	12	60	143	11.4	21.3	15
	13	81	108	11.9	0.1	21
	15	120	140	10.8	0.1	15
	16	150	129	10.2	tr	15
kissiris	1	0	48	9.9	25.2	39
	4	2	49	11.7	tr	45
	5	3	56	12.1	0.3	41
	6	6	62	12.2	0.3	37
	7	10	80	12.2	0.7	29
	8	15	114	11.7	0.4	19
	9	18	93	12.2	0.2	25
	10	24	117	11.1	0.4	18
	11	30	144	11.4	Tr	15
	12	39	78	12.1	1.4	29
	13	49	NA[b]	12.0	0.3	NA
	14	64	148	12.0	tr	15
	15	80	246	7.8	24.9	6
γ-alumina	1	0	54	11.9	9.3	42
	4	3	75	11.9	tr	30
	5	7	65	10.9	0.5	32
	6	9	218	10.1	13.6	9
	7	13	254	11.7	tr	9
	8	15	155	11.6	0.4	14
	10	28	156	11.5	tr	14
	11	41	222	12.1	4.1	10
	12	52	253	10.3	37.8	8
	13	61	229	12.8	tr	11
	14	83	220	11.8	tr	10
free cells	1	3	58	12.1	7.1	40
	2	7	125	11.8	3.0	18
	3	10	161	12.0	16.6	14
	5	15	196	11.8	tr	11
	6	18	187	12.2	tr	12
	7	24	175	12.2	tr	13
	8	30	166	12.0	17.2	14
	9	40	180	11.4	14.9	12

[a] tr, traces. [b] NA, not available.

synthetic medium containing 22% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.5% $MgSO_4$ or (b) grape must and allowed to ferment. Fermentations were also carried out using free yeast cells. The free cell concentration in the first batch was 14 g (wet weight)/L. All fermentations were carried out stationary at 30 °C. At the end of every fermentation batch, the biocatalysts were allowed to remain in the fermented liquid at 30 °C for increasing time periods, after which the liquid was decanted and the biocatalysts were washed twice with 300 mL of synthetic medium or must and used in a subsequent batch fermentation. Samples were collected after the end of each preservation period and analyzed for ethanol, residual sugar, and volatile byproducts. In a second experiment, the biocatalysts on apple pieces, kissiris, and γ-alumina remained in the fermented liquid at 30 °C for 6 months and were then tested in a fermentation run.

Analyses. Alcohol concentration was measured after distillation of samples and use of a Gay-Lussac alcohol-meter. Ethanol productivity was defined as the grams of ethanol per liter of liquid volume produced per day.

Residual sugar was determined by high-performance liquid chromatography (HPLC), using a Shimadzu 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 the 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% (v/v) solution of butanol-1 were diluted to 50 mL, and 40 μL was injected directly to the column. The residual sugar concentration was calculated using standard curves and

Table 2. Fermentation Parameters Obtained by Repeated Batch Fermentations of Grape Must with Immobilized Yeast Cells and Free Yeast Cells after Storage at 30 °C

support	repeated batch fermentation	initial °Be density	storage time (days)	fermentation time (h)	ethanol concn (% v/v)	residual sugars (g/L)	daily ethanol productivity (g/L)
apple	1	11.7	0	32	11.1	25.1	66
	4	11.6	3	25	11.6	1.8	88
	5	11.9	6	58	11.8	0.6	39
	6	11.5	9	84	11.5	0.6	26
	7	11.6	11	103	11.5	0.6	21
	8	11.5	13	97	11.5	0.5	22
	10	11.2	24	108	11.0	1.9	19
	11	11.6	30	128	11.5	1.9	17
	12	11.6	40	87	11.4	1.2	25
kissiris	1	11.7	0	56	10.4	36.8	35
	4	11.8	2	54	11.7	3.0	41
	5	11.3	6	83	11.3	0.5	26
	6	11.7	9	93	11.5	0.5	23
	7	11.6	15	99	11.2	2.4	21
	8	11.5	18	105	11.5	5.6	21
	9	11.6	24	106	14.4	1.5	20
	10	11.6	30	131	11.6	0.6	17
	11	11.6	40	95	11.6	0.5	23
	12	11.6	50	83	11.6	2.4	26
γ-alumina	1	11.0	0	63	10.8	11.9	33
	4	11.7	3	78	11.4	7.7	28
	5	11.4	6	123	11.4	3.8	18
	6	11.3	9	128	11.1	4.8	16
	7	11.2	12	354	11.1	25.0	6
	8	11.2	16	230	10.8	18.1	9
	9	11.7	18	372	10.4	31.7	5
free cells	1	11.4	3	104	11.4	4.2	21
	2	11.5	7	103	11.4	2.5	21
	3	11.3	10	NA[a]	11.3	0.7	NA
	4	11.6	13	147	11.5	1.6	15

[a] NA, not available.

Table 3. Fermentation Parameters Obtained by Repeated Batch Fermentations of Grape Must with Immobilized and Free Yeast Cells after Storage for 6 Months at 30 °C

support	repeated batch fermentation	storage time (months)	initial °Be density	fermentation time (h)	ethanol concn (% v/v)	residual sugars (g/L)	daily ethanol productivity (g/L)
kissiris	1	0	12.4	74	12.3	tr[a]	32
	2	0	12.3	86	12.1	7.7	27
	3	6	11.9	82	11.7	2.8	27
	4	0	12.5	143	12.0	4.9	16
γ-alumina	1	0	11.8	96	11.5	11.9	23
	2	0	11.9	110	11.4	11.8	20
	3	6	11.5	128	11.2	4.5	17
	4	0	11.8	134	11.3	8.4	16
apple	1	0	11.7	34	11.0	tr	61
	2	0	12.0	30	11.7	tr	74
	3	6	12.0	30	11.8	tr	75
	4	0	12.4	103	11.5	5.5	21
free cells	1	0	12.5	127	11.7	11.8	17
	2	0	11.9	95	11.3	19.7	23
	3	6	12.2	136	12.0	3.9	17
	4	0	12.4	571	11.2	20.8	4

[a] tr, traces.

Table 4. Volatile Byproducts Formed in Wines Prepared by Repeated Batch Fermentations Using Immobilized Yeast Cells and Free Yeast Cells after Storage at 30 °C

support	repeated batch fermentation	storage time (days)	acetaldehyde (ppm)	ethyl acetate (ppm)	propanol (ppm)	isobutyl alcohol (ppm)	amyl alcohols (ppm)	methanol (ppm)
apple	1	0	68	75	tr[a]	7	266	37
	4	3	17	28	tr	13	191	59
	5	6	14	18	4	12	194	85
	6	9	16	26	7	14	217	77
	7	11	15	30	9	13	198	79
	8	13	7	14	6	16	184	53
	10	24	62	68	14	27	228	51
	11	30	50	53	9	24	206	98
	12	40	43	40	8	19	292	51
kissiris	1	0	tr	39	7	31	249	66
	4	2	24	28	3	21	246	85
	5	6	22	26	tr	16	255	81
	6	9	16	23	tr	17	260	62
	7	15	19	23	2	41	216	56
	8	18	15	28	3	46	188	50
	9	24	56	63	8	43	209	61
	10	30	96	83	9	44	222	96
	11	40	107	96	9	37	227	85
	12	50	118	62	27	57	176	90
γ-alumina	1	0	43	86	10	35	261	65
	4	3	27	35	4	23	268	71
	5	6	17	28	6	18	235	68
	6	9	18	34	9	20	246	67
	7	12	18	22	9	18	293	69
	8	16	30	15	7	19	284	74
	9	18	41	46	11	26	293	72
free cells	1	3	52	45	6	41	244	28
	2	7	43	44	14	26	173	31
	3	10	48	36	12	19	202	30
	4	13	49	tr	27	58	193	51

[a] tr, traces.

expressed as grams of residual sugar per liter. All values were the mean of three repetitions. The standard deviation for ethanol concentration was $<\pm 0.2$, and that for residual sugar was $<\pm 2$.

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) (*21*). Nitrogen was used as carrier gas (at a rate of 20 mL/min). Injection port and detector temperatures were 210 and 220 °C, respectively. The column temperature was programmed at 62−70 °C. Butanol-1 was used as an internal standard at a concentration of 0.5% (v/v). Four microliter samples were directly injected into the column, and the concentrations of the above volatile compounds were determined from standard curves. Methanol was also determined by gas chromatography using Porapack S as column material. Nitrogen was used as carrier gas (at a rate of 40 (mL/min). The column temperature was programmed at 120−170 °C. The temperatures of the injector and detector were 210 and 220 °C, respectively. Two microliter samples were directly injected into the column, and the concentration of methanol was determined from standard curves. All values were the mean of three repetitions. The standard deviations for acetaldehyde, ethyl acetate, propanol, isobutyl alcohol, amyl alcohols, and methanol were $<\pm 9$, $<\pm 7$, $<\pm 5$, $<\pm 10$, $<\pm 20$, and $<\pm 10$, respectively.

RESULTS AND DISCUSSION

Fermentations Using Glucose Synthetic Medium and Grape Must. The duration and effective storage of immobilized cell biocatalysts at 30 °C was selected to reflect ambient temperature in such industrial fermentations. Biocatalysts suitable for industrial use should be able to be stored for a maximum of 1 month while retaining activity for at least 1 year of storage with product consistency and stable quality. Materials such as apple pieces (*15*, *16*), kissiris (*17*, *18*), and γ-alumina (*19*, *20*) were used as supports for immobilization because they are abundant and cheap and have been successfully used for cell immobilization and wine-making.

The results of repeated batch fermentations carried out after increasing periods of incubation of immobilized and in free cells are summarized in **Table 1**. Immobilized cells were found to be more stable on storage than free cells. Immobilized cells on apple pieces, kissiris, and γ-alumina pellets were able to ferment after storage for 120, 80, and 83 days, respectively, and without any observed problems associated with contamination. Free cells, on the other hand, were unable to ferment after storage for 40 days. In summary, apple-, kissiris-, and γ-alumina-supported biocatalysts were stored and able to reactivate after a maximum of 518, 340, and 330 days, respectively (**Table 1**). In immobilized cells on kissiris and γ-alumina, storage for periods longer than 80 and 83 days, respectively, resulted in contamination of the fermented broth. However, they were able to ferment even when the fermented broth was contaminated (data not shown). Contamination of the fermented broth was determined visually and by sensory evaluation (taste and smell). Contamination was probably due to the ambient sanitation conditions and was unrelated to the fermentation systems themselves.

Immobilized cells on apple pieces were not able to ferment after 150 days of storage; a high quantity of unfermented glucose (stuck fermentation) remained, although no contamination was observed. Fermentation times increased as the time of storage increased. However, they fell within the range obtained by the alcohol production and wine-making industries. Ethanol pro-

ductivity was reduced when the duration of storage increased because there was an increase in the fermentation times. Specifically, fermentation time increased by 477, 510, 370, and 310% when immobilized yeast cells on apple pieces, kissiris, γ-alumina, and free cells were stored for 120, 64, 61, and 30 days, respectively. The corresponding reductions of ethanol productivity were 79, 85, 76, and 70%.

Fermentations with increasing times of storage using both immobilized and free cells were also carried out using grape must. The results are summarized in **Table 2**. When grape must was used, storage times were shorter compared to fermentations using glucose synthetic medium; however, they remained high and were longer in the immobilized biocatalysts compared to the free cell system. The reduced biocatalysts' stability when stored in grape must, was probably due to depletion of nutritional factors required by the yeast when the yeast was confined to grape must over many growth cycles, in contrast to being stored in an enriched synthetic medium. Apple- and kissiris-supported biocatalysts had longer survival times (40 and 50 days, respectively) compared to γ-alumina-supported biocatalyst and free cells. After 40 and 50 days for apple- and kissiris-supported biocatalysts, respectively, contamination of the fermentation broth occurred. Immobilized cells on γ-alumina pellets were unable to ferment after 18 days of storage, whereas free cells were contaminated. The period needed to achieve complete fermentation was longer as time of storage increased. Specifically, fermentation time increased by 272, 148, 475, and 141% when immobilized yeast cells on apple pieces, kissiris, γ-alumina, and free cells were stored for 30, 40, 16, and 10 days, respectively. However, we believe that these times would still be acceptable to the alcohol production and wine-making industry. The increase in fermentation time was lower when grape must was used in all cases except from γ-alumina-supported biocatalyst. The corresponding reductions of ethanol productivity were 62, 26, 85, and 29%. Ethanol and residual sugar concentrations were similar in concentration to those found in dry and semisweet wines.

To investigate the possibility of preservation of immobilized cells for extended periods, immobilized cells were allowed to stand for 180 days before resumption of fermentation. The results are summarized in **Table 3**. Contamination of the fermented broth was observed in the free cell fermentation, whereas the immobilized biocatalyst systems fermented, although requiring longer completion times compared to the fermentations in which there were no storage intervals. Ethanol and residual sugar concentrations in these fermentations were similar to those found in dry wines. Fermentation time rose by 177, 121, 332, and 480% for immobilized yeast cells on apple pieces, kissiris, γ-alumina, and free cells, respectively, when stored for 6 months. The increase in fermentation time was lower compared to the successive storage periods. The corresponding reductions of ethanol productivity were 58, 20, 70, and 79%.

Volatile Byproducts. To investigate the effect of the successive storage on the aroma of the wines, the pattern of the most abundant volatile byproducts was determined (**Table 4**). The results show that ethyl acetate concentrations remained relatively constant in all cases except when immobilized cells on kissiris were used, when it increased with increased storage time.

Acetaldehyde, methanol, and higher alcohols were not significantly affected. Wines produced using immobilized cells on apple pieces contained lower amounts of higher alcohols (propanol-1, isobutanol, and amyl alcohols), leading to an increase of ethyl acetate percentage on total volatiles, thus contributing positively to the aroma and overall quality of the wine.

Because the consistency of wines produced after storage of the biocatalysts was similar to the consistency before storage of the biocatalsts, we assume that the original yeast culture was present, as changes in the yeast microflora would result in changes in product consistency and quality.

Technological Consideration. A longer active life of biocatalysts is of great importance in industrial productions, particularly when the production is halted. Longer storage time reduces waste of the bioreactor contents, restarting time, preparations, and cost. The results indicate that the immobilized biocatalysts could be easily stored and reactivated during a minimum of 30 days. The immobilized biocatalysts retained their activity after storage for up to 6 months, showing that they could be preserved from the end of a wine-making season until the next year. Furthermore, with proper handling the transfer of the immobilized biocatalysts from the production facility to the wine-making industry could be carried out without the need for cooling or freeze-drying.

The use of apple-supported biocatalyst in wine-making appears to have an advantage over kissiris- and γ-alumina-supported biocatalysts, which is strengthened by its food-grade purity and improved volatile byproduct composition. Considering the higher productivity and time of preservation of the immobilized cell system compared to free cells, the possibility for industrial application of immobilized cells in wine-making has great potential.

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