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# Low-Temperature Alcoholic Fermentation by Delignified Cellulosic Material Supported Cells of Kefir Yeast

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A novel system for low-temperature alcoholic fermentation of glucose is described. This system consists of kefir yeast immobilized on delignified cellulosic materials. Batch fermentations were carried out at various pH values, and the effect of temperature on kinetic parameters, in the range of 5−30 °C, was examined. At pH 4.7 the shortest fermentation time was obtained. The formation of volatiles indicates that the concentration of amyl alcohols (total content of 2-methylbutanol-1 and 3-methylbutanol-1) is reduced as the temperature becomes lower. Propanol-1 and isobutyl alcohol formation drops significantly below 15 °C. The percentage of ethyl acetate increases as the temperature is diminished. At 5 °C the content of total volatiles in the product was only 38% of the volatiles formed during fermentation at 30 °C.

**Keywords:** *Kefir; glucose; fermentation; low temperature; immobilization*

## INTRODUCTION

Kefir yeast is a known culture employed to produce from milk the traditional Russian alcoholic drink "kefir". This yeast, which ferments lactose, seems to have a potential for alcohol production using milk whey. To produce potable or fuel grade alcohol from milk whey, the latter should be mixed with a raw material with a higher sugar concentration to avoid whey condensation and consequently a high energy demand. Potable alcohol production and the production of an alcoholic drink from milk whey should be accompanied by the formation of volatile byproducts that are constituents of alcoholic beverages and contribute to the typical organoleptic character.

A series of efforts have been made to obtain alcohol production employing lactose. Various strains of *Kluyveromyces fragilis* (O'Leary et al., 1977; Gawel and Kosikowski, 1978; Guillaume et al., 1980) were proposed. Marchant et al. (1996) reported the use of the thermotolerant yeast *Kluyveromyces marxianus* IMB3 for high-temperature fermentation of lactose. Co-immobilization of *Saccharomyces cerevisiae* and the enzyme β-galactosidase (Roukas and Lazarides, 1991) was also examined. Finally, recombinant DNA technique was used to provide strains of *S. cerevisiae* suitable to ferment lactose (Sreekrishna and Dickson, 1985; Poro et al., 1992). It is obvious that further research on kefir yeast needs to be done to prove its suitability for industrial fermentation of raw materials which contain or provide glucose in the fermented liquid.

Proper immobilization of cells is an important factor in alcoholic fermentation. Delignified cellulosic (DC) materials have been proposed as an immobilization support of yeast strains for wine-making (Bardi and Koutinas, 1994) and brewing (Bardi et al., 1996). The authors of these studies report that DC material supported biocatalyst reduces the activation energy and contributes to low-temperature fermentation. There is also an indication of aroma and taste improvement of wine and beer produced by low-temperature fermentation; this improvement was manifested by a reduction of amyl alcohols and other higher alcohols, as well as by an increase of the percentage of ethyl acetate on total volatiles (Bardi et al., 1996, 1997).

The aim of this paper is to produce alcohol by low-temperature fermentation using psychrophilic microorganisms and to detect the possibility for low-temperature wine-making and brewing. Kefir yeast cells were immobilized on a DC material, and fermentation of glucose was carried out at temperatures ranging from 5 to 30 °C. The fermentation process was monitored by measuring kinetic parameters and the formation of volatiles.

## MATERIALS AND METHODS

Kefir yeast, a commercial product that is employed to produce the kefir drink, was used in the present study. It was grown on complete medium containing 4% glucose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% $KH_2PO_4$, and 0.5% $MgSO_4 \cdot 7H_2O$ in distilled water. This complete medium was sterilized at 130 °C for 15 min. Pressed wet weight cells (15−20 g) were prepared at late log phase. Cells were separated by centrifugation and transferred in a synthetic medium containing glucose for fermentation. The batch culture medium in the case of glucose solutions had an initial glucose concentration of 120 g/L and nutrients as described above. The pH was adjusted to 4.7 by the addition of sulfuric acid. To obtain optimum pH, a series of tests were carried out to relate pH and fermentation time, as described below.

The preparation of wet DC material and the immobilization of cells of kefir yeast were performed as described in a previous study (Bardi and Koutinas, 1994).

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**Table 1. Effect of Temperature on Kinetic Parameters of Alcoholic Fermentation of Glucose by DC Material Supported Cells of Kefir Yeast[a]**

| temp (°C) | repeated batch fermentations | fermentation time (h) | ethanol concn (% v/v) | residual sugar (g/L) | yield (g/g) | ethanol productivity (g/L/h) | conversion (%) |
|---|---|---|---|---|---|---|---|
| 30 | 1−5 | 7.7 | 6.4 | 5.8 | 0.31 | 6.8 | 96.6 |
| 25 | 6−10 | 11.3 | 8.6 | 1.6 | 0.40 | 6.0 | 99.4 |
| 20 | 11−15 | 13.3 | 9.0 | 1.2 | 0.42 | 5.3 | 99.3 |
| 15 | 16−20 | 23.5 | 9.1 | 1.5 | 0.43 | 3.1 | 99.1 |
| 10 | 21−25 | 64.7 | 8.1 | 4.2 | 0.38 | 1.0 | 97.5 |
| 5 | 26−28 | 137.7 | 7.9 | 13.9 | 0.40 | 0.4 | 91.9 |

[a] Standard deviations for ethanol concentration were <±1.7, for residual sugar <±13.7, for yield <±0.05, for productivity <±2, and for conversion <±8.

**Table 2. Effect of Temperature on Formation of Volatiles in the Alcoholic Fermentation of Glucose by DC Material Supported Cells of Kefir Yeast**

| | | ethanol | ethanal | | ethyl acetate | | propanol-1 | | isobutanol | | amyl alcohols | | total volatiles | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| temp (°C) | repeated batch fermentations | concn (% v/v) | mg/L | mg/ alc deg[a] | mg/L | mg/ alc deg | mg/L | mg/ alc deg | mg/L | mg/ alc deg | mg/L | mg/ alc deg | mg/L | mg/ alc deg |
| 30 | 1−5 | 6.4 | 27.5[b] | 4.3 | 24.5 | 3.9 | 20.5 | 3.2 | 45.4 | 7.2 | 123.4 | 19.4 | 241.2 | 37.9 |
| 25 | 6−10 | 8.6 | 23.9 | 2.8 | 30.1 | 3.6 | 15.6 | 1.8 | 17.2 | 2.0 | 85.8 | 10.0 | 182.5 | 21.3 |
| 20 | 11−15 | 9.0 | 26.4 | 2.9 | 28.3 | 3.2 | 10.0 | 1.1 | 14.0 | 1.6 | 68.3 | 7.7 | 142.8 | 16.0 |
| 15 | 16−20 | 9.1 | 22.5 | 2.5 | 30.5 | 3.4 | 8.5 | 1.0 | 10.9 | 1.2 | 65.1 | 7.2 | 137.5 | 15.1 |
| 10 | 21−25 | 8.1 | 31.4 | 3.8 | 19.3 | 2.4 | 7.7 | 0.9 | 7.4 | 0.9 | 51.3 | 6.3 | 117.1 | 14.3 |
| 5 | 26−28 | 7.9 | 22.8 | 2.9 | 25.0 | 3.2 | 4.9 | 0.6 | 5.7 | 0.7 | 32.5 | 4.2 | 92.2 | 11.7 |

[a] mg/alc deg means milligrams of each volatile per 1% alcohol v/v. [b] Standard deviations for ethanal (concentration in mg/L) was <±37, for ethyl acetate <±10, for propanol-1 <±9, for isobutanol <±6, for amyl alcohols <±17, and for total volatiles <±43.

Residual sugar was determined in all samples by HPLC analysis as is described under Ethanol and Byproducts Determination.

Wet free cell concentrations were determined using the absorbance experimental procedure (Klein and Kressdorf, 1983; Bajpai and Margaritis, 1986) and are given in grams of wet weight per liter, using standard curves.

**Ethanol and Byproduct Determination.** Ethanol and residual sugar were determined by high-performance liquid chromatography (HPLC). A Shimadzu HPLC chromatograph, Model LC-9A, connected with an integrator, C-R6A Chromatopac, column SCR-101N (packed with a cation exchange resin—sulfonated polystyrene—divinylbenzene copolymer), CTO-10A column oven, and a refractive index detector RID-6A were employed. The elution was made using water distilled and filtered three times. The determination was performed using a pressure of 78−82 atm, and the flow rate of the mobile phase was 0.8 mL/min. An oven temperature of 60 °C was used. Samples of 0.5 and 2.5 mL of 1% butanol as internal standard were added in a 50 mL volumetric flask with distilled and filtered water. This solution was filtered using microfilters of 0.45 μm hole size and injected directly into the column.

Quantitative determinations of volatile byproducts were made with a Shimadzu gas chromatograph GC-8A, connected with the integrator Chromatopac C-R6A. 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 (4 m long, 1/8 in. i.d.), 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 butanol, at a concentration of 0.5% v/v. Samples of 2 μL of the fermented liquid were injected directly in the column.

**Effect of pH on Fermentation Time.** Fermentations were performed at 30 °C using synthetic media containing 170 g/L glucose and nutrients. Fermentations were made at pH 5.6, 4.7, 4.0, 3.6, and 3.2, and the pH was adjusted by the addition of sulfuric acid. Five repeated batch fermentations were performed at each pH value using the same kefir yeast culture from batch to batch, in all pH values, which every time were measured after centrifugation of samples. This was made to obtain the steady state of fermentation. The fermentation time of the fifth batch at each pH value was measured.

**Effect of Temperature on Repeated Batch Fermentation by DC Material Supported Cells of Kefir Yeast.** An amount of 120 g wet weight of DC material supported kefir yeast, prepared by immobilization of cells as described in the work of Bardi and Koutinas (1994), was introduced into 250 mL of liquid medium containing 170 g/L glucose (pH adjusted to 4.7) in a 1 L glass cylinder. The glass cylinder for each fermentation batch was incubated at 30, 25, 20, 15, 10, and 5 °C as indicated in Tables 1 and 2, and the fermentations were carried out without agitation. Just before the fermentation was completed, the liquid was filtered by a Büchner funnel and the support was washed three times, each time with 400 mL of the liquid medium containing glucose. The biocatalyst was pressed on the funnel to remove the liquid, and then it was used for the next fermentation batch. At the end of every batch a sample was collected and analyzed for ethanol, residual sugar, and volatile byproducts as previously described. When the weight of the support was reduced by >10%, the appropriate amount was added. All values were the mean of three runs.

## RESULTS AND DISCUSSION

**Fermentation Process.** The DC material is a solid of food grade purity that has proved its suitability as a support for cell immobilization of *S. cerevisiae*, because it reduces the activation energy and therefore is convenient for low-temperature fermentations (Bardi and Koutinas, 1994). These are the reasons this material was used as a support for immobilization of kefir yeast in the present study. The DC material obtained was mixed with a liquid culture of kefir yeast for immobilization. The biocatalyst prepared was studied at various pH values. It was used for repeated batch fermentations of a liquid culture containing glucose. A total of 30 batches at various temperatures ranging from 5 to 30 °C were performed to study the activity of the biocatalyst at low-temperature fermentations and its ability to carry out repeated batch fermentations. The results are summarized in Figure 1 and Table 1.

Figure 1 shows that the fermentation is carried out more rapidly at pH 4.7, which is the optimum point. After this optimization study, all experiments in this work were performed at pH 4.7. The fermentation time

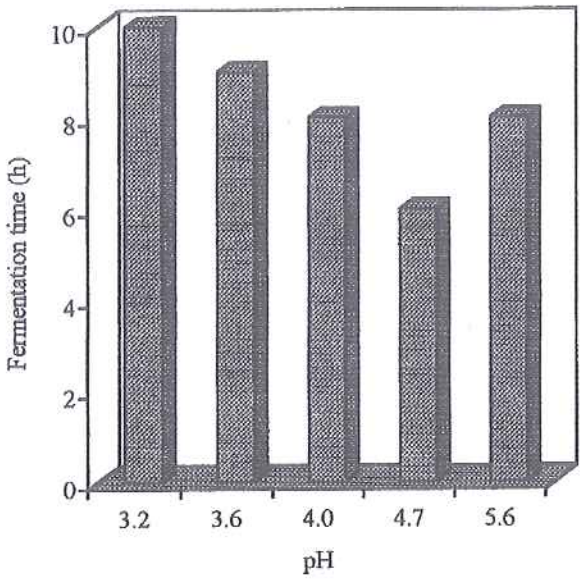


**Figure 1.** Effect of pH on fermentation time of alcoholic fermentation of glucose by DC material supported cells of kefir yeast.

at pH 3.2 is 70% longer compared to that obtained at a pH value of 4.7. This indicates that pH affects significantly the rate of fermentation of glucose by kefir yeast. These results are not in accordance with those reported by Kanellaki et al. (1989), who used *S. cerevisiae* to ferment glucose and found that shorter times of fermentation can be obtained at lower pH values.

Table 1 shows repeated batch fermentations of glucose at room and low temperatures in the range of 5−30 °C. Batches that were carried out at various temperatures show that the fermentation time up to 5 °C could be acceptable by the industry, and therefore DC material supported kefir yeast cells are active. This stability of the activity for 28 fermentation batches indicates that the immobilization of kefir yeast is successful. It is important to note that the fermentation rate is comparable to that obtained by DC material supported cells of *S. cerevisiae* (Bardi and Koutinas, 1994). Table 1 also shows that the biocatalyst, DC material supported cells of kefir yeast, has a carry-through property at low temperatures. Parameters such as fermentation time, ethanol concentration, residual sugar, yield, and conversion are acceptable in industrial fermentations for the production of alcoholic drinks. The fermentation time and ethanol productivity that are obtained at 30 and 25 °C indicate a promising biocatalyst even for fuel grade alcohol production. Likewise, the yield in the range 0.40−0.43 could be of practical importance. However, higher yield is expected with raw material because synthetic media containing glucose induce cell growth. The low yield obtained at 30 °C is due to the fact that this temperature facilitates cell growth. Fermentation time and ethanol production are important up to 15 °C and could be acceptable by the alcohol production industry. Also, this is a significant event for cold countries and the quality of the product. Furthermore, the values of these kinetic parameters below 10 °C may lead to the important industrialization of low-temperature wine-making and brewing. Ethanol concentration is considered to be satisfactory at all temperatures below 25 °C, whereas the low concentration obtained at 30 °C due to low yield attributed to cell growth. Conversion is very high at all temperatures studied.

**Volatiles.** At the end of each repeated batch fermentation, samples were collected and analyzed for ethanol, ethanal, ethyl acetate, propanol-1, isobutyl alcohol, and amyl alcohols (total amount of 2-methylbutanol-1 and 3-methylbutanol-1). The effect of temperature on the formation of the total volatile compounds at repeated batch fermentation of glucose is demonstrated in Table 2.

Ethanal content was not significantly affected by the temperature in the range of 5−30 °C. This result is not in agreement with values reported by Bakoyianis et al. (1993), who used the immobilized *Visanto* strain of *S. cerevisiae* species in continuous wine-making.

The ethyl acetate content remained in general constant in the fluid product, as the temperature was reduced. This stability is also shown even when it is expressed as milligrams per alcoholic degree (milligrams of volatile per 1% alcohol v/v). The concentrations of ethyl acetate obtained by immobilized kefir yeast for glucose fermentation were slightly less than half of those found in wines produced by continuous fermentation using immobilized cells of *S. cerevisiae* (strain *Visanto*) on mineral kissiris (Bakoyianis et al., 1993).

In the repeated batch fermentations of glucose using DC material supported cells of kefir yeast, the volatiles propanol-1 and isobutyl alcohol are reduced at low temperatures, as the temperature is decreased. Propanol-1 and isobutyl alcohol drop significantly after 15 °C. At 5 °C the concentration of isobutyl alcohol is <15% of that at 30 °C. The reduction of these compounds is also shown when they are calculated as milligrams per alcoholic degree. This reduction of these compounds is in agreement with that reported for immobilized cells of *S. cerevisiae* on mineral kissiris (Bakoyianis et al., 1993).

The concentration of amyl alcohols (total content of 2-methylbutanol-1 and 3-methylbutanol-1) is significantly reduced as the temperature drops. At 5 °C a large reduction was observed. In absolute numbers, the concentration of these alcohols is reduced by about one-fourth from 30 to 5 °C. These results are also observed when the concentration is expressed as milligrams per alcoholic degree.

Finally, the content of total volatiles determined in the product obtained after fermentation of glucose by immobilized cells of kefir yeast was decreased as the temperature was reduced. This is also shown in the expression of volatiles as milligrams per alcoholic degree. At 5 °C they were only 38% of the volatiles at 30 °C.

From the above presentation of the results, it is obvious that all higher alcohols are decreased as the temperature drops. That means the enzymes are capable for the formation and are successively inactivated by the reduction of temperature. This does not happen for ethyl acetate and ethanal.

The reduction of volatiles at low temperature found in this work for immobilized kefir yeast on DC material is in agreement with the results that were obtained by immobilized cells of *S. cerevisiae* on mineral kissiris in a previous study (Bakoyianis et al., 1993).

Figure 2 shows that the percentages of amyl alcohols (2-methylbutanol-1 and 3-methylbutanol-1) and isobutanol on total volatiles are reduced as the temperature

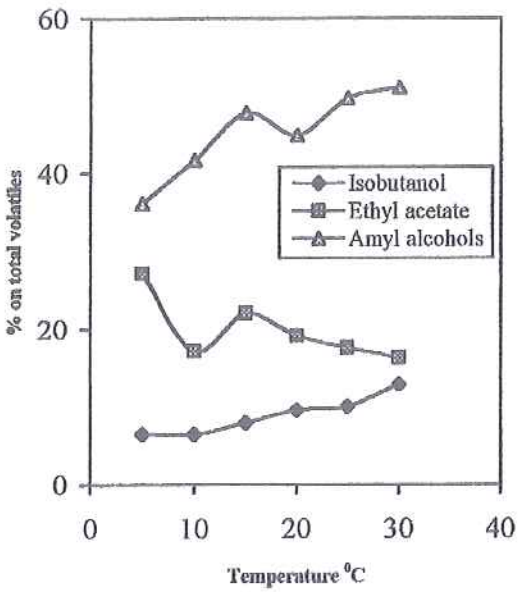


**Figure 2.** Contents of amyl alcohols (2-methylbutanol-1 and 3-methylbutanol-1), ethyl acetate, and isobutyl alcohol on total volatiles determined.

drops. In contrast, the percentage of ethyl acetate increases as the temperature is diminished. These results indicate an improvement of the aroma of the fermentation product due to the process.

Kefir yeast seems to be a suitable microorganism for the alcoholic fermentation of raw materials containing glucose. It would therefore be interesting to extend this study also to lactose and whey on an industrial scale.

It is interesting to note that kefir yeast supported on DC material is suitable for fermentations at low temperatures. This implies that drinks produced by using this technique may have an improved nutritional value, because their content in higher alcohols (toxic at high concentrations) is low. The results and conclusions that are obtained in this study for kefir yeast are in agreement with the results that were obtained for low-temperature fermentations in the case of strains of *S. cerevisiae* species (Bakoyianis et al., 1992, 1993; Bardi and Koutinas, 1994; Bardi et al., 1997).

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