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Continuous Whey Fermentation Using Kefir Yeast Immobilized on Delignified Cellulosic Material

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Delignified cellulosic-supported biocatalyst, prepared by immobilization of kefir yeast on delignified cellulosic material (DCM), was found to be suitable for continuous, modified whey fermentation. The modified whey contained 1% raisin extract and molasses. Ethanol productivities ranged from 3.6 to 8.3 g $L^{-1}day^{-1}$, whereas parameters such as ethanol concentration, residual sugars, and daily fermented whey productivity were acceptable for the production of potable alcohol and alcoholic drinks in industrial fermentations. The continuous fermentation bioreactor was operated for 39 days, stored for 18 days at 4 °C, and operated again for another 15 days without any diminution of the ethanol productivity. The concentrations of higher alcohols (propanol-1, isobutyl alcohol, and amyl alcohols) were low. The main volatile byproducts formed in the continuous process were similar to those observed in alcoholic beverages, and the fermented whey had a good aroma. The concentrations of higher alcohols were very low when compared to that of ethyl acetate, therefore resulting in a quality product. The possibility of using such a process for the production of potable alcohol or a novel, low-alcohol content drink is proposed.

KEYWORDS: Whey; continuous fermentation; delignified cellulosic material; immobilized cells; kefir; alcoholic drinks

INTRODUCTION

Immobilization of yeast cells on various supports for the preparation of a biocatalyst suitable for continuous production of fermented beverages or potable alcohol has received considerable attention in recent years. A series of efforts have been made to obtain such an alcohol production process using immobilized cells (*1*, *2*) and cheap substrates as carbon source such as lactose (*3*−*7*).

Proper immobilization of cells is an important factor in immobilized alcoholic fermentation processes. Deliginified cellulosic materials (DCM) have been proposed as a suitable immobilization support of yeast strains for wine-making (*8*), brewing (*9*), and potable alcohol production (*10*, *11*). There were indications of aroma and taste improvement in wine and beer produced using DCM-supported biocatalyst (*12*, *13*) compared with other supports.

Kefir yeast is a known culture of various species of the genera *Kluyveromyces*, *Candida*, *Saccharomyces*, and *Pichia* and some lactic acid bacteria of the genus *Lactobacillus* (*14*). This mixed culture is able to ferment lactose and therefore has the potential for potable alcohol production from whey as a substrate. The use of milk whey in mixtures with low-cost raw materials such as molasses or raisin extracts and the application of a continuous process may significantly decrease potable alcohol production costs and may also lead to the production of novel alcoholic drinks. Many efforts have been made in the past to use whey as a raw material for the production of such a novel drink (*15*−*19*). These efforts mainly concerned the addition of fruit juices such as mango, banana, pineapple, guava, and strawberries in whey and high-temperature fermentation.

The aims of this study were to investigate the potential of using kefir yeast immobilized on DCM in a continuous process and to evaluate the possibility of the production of a novel, low-alcohol-content drink using such an immobilization process. Such a process may have an additional benefit of utilizing the large quantities of whey that are produced by the dairy industry and which are difficult to dispose of or treat.

MATERIALS AND METHODS

Yeast Strain. Kefir yeast, a commercial product usually used to produce the kefir drink, was used in the present study. It was grown on milk whey in a bioreactor at 30 °C. Aerobic conditions were obtained by supplying air through a sterilizing filter. Pressed wet cells (15−20 g) were prepared at the late exponential phase. Cells were separated by centrifugation and used directly for immobilization.

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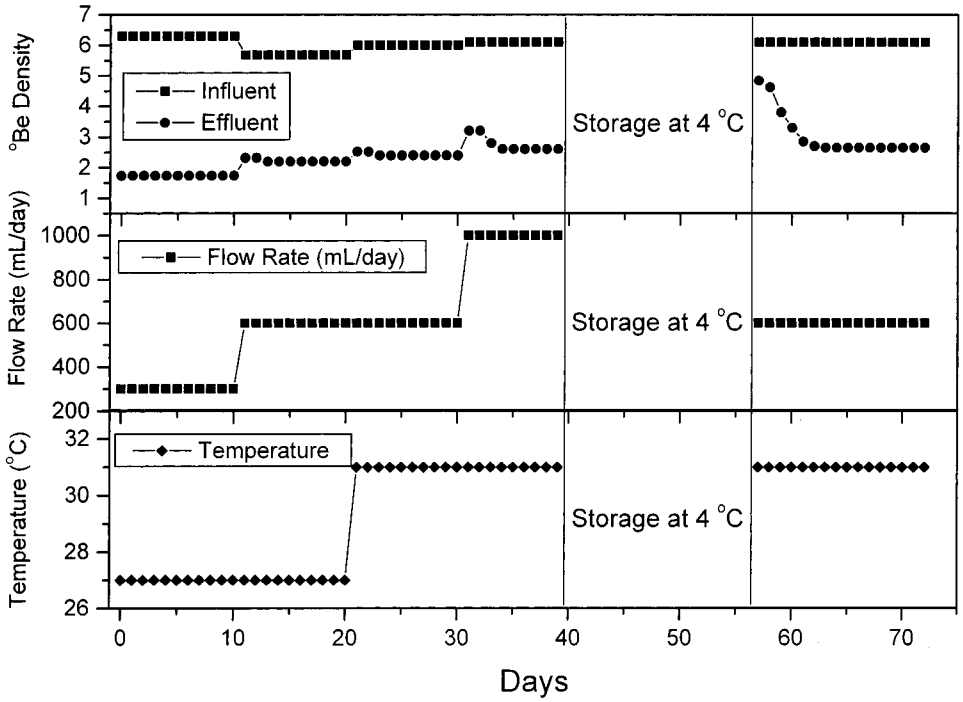

Figure 1. Operational stability of the bioreactor in relation to flow rate and temperature before and after interruption due to storage (in refrigeration) at 4 °C for 18 days.

Whey Source and Preparation of Fermentation Liquid. Whey was produced in the laboratory from commercial milk. Milk was placed in a water bath at 37 °C, 0.1 g/L of rennin was added, and the mixture was left for 1 h. After that, the curd was cut in squares (diameter ~ 1 cm) and left undisturbed for 10 min; whey was obtained by cloth filtration. The whey so produced contained 5% lactose and 0.8% proteins. The liquid used for continuous fermentation was obtained by the addition of 1% (w/v) of raisin extract to the whey and adjustment of the °Be density to ≈6.0 by addition of molasses. The pH was adjusted to 6.6 using a saturated and sterilized solution of Na_2CO_3, and the liquid (modified whey) was stored at 4 °C.

Support and Immobilization of Cells. Wet DCM was prepared as described previously (*8*). To prepare the DCM-supported biocatalyst, 55 g of DCM, 250 mL of whey, and 5 g of kefir yeast were placed in a 500 mL flask. The flask was incubated at 30 °C and allowed to ferment for 6 h. During this stage, adjustment of the pH to 5.5 took place, as described above. When the immobilization was completed, the fermented liquid was decanted and the supported biocatalyst was washed twice with 250 mL of whey; the biocatalyst was then used for continuous fermentation.

Pilot Fermentation. Continuous fermentation was carried out in a glass bioreactor. The reactor was placed in an incubator and had 2000 mL total volume to which 1000 mL of modified whey and 750 g of DCM-supported biocatalyst were added. Modified whey was pumped into the bioreactor in an up-flow stream with the aid of a high-accuracy peristaltic pump (Cole Parmer Instruments Co., Chicago, IL).

Experimental Procedure. Modified whey with an initial density of ≈6.0 °Be was continuously supplied to the bioreactor. The reactor was operated for 39 days continuously, stored at 4 °C for 18 days, and operated again for another 15 days (**Figure 1**). The fermentation temperatures studied were 27 and 31 °C. The pH in the bioreactor was continuously adjusted to 5.5 during operation, as described previously. Samples were collected at different flow rates and temperatures (**Table 1**) starting after at least 6 days of pumping to allow time to achieve steady conditions in the bioreactor. Samples were collected every 24 h and analyzed for °Be density, residual sugar, alcohol concentration, and volatile byproducts.

Analyses. Alcohol concentrations were determined using a Gay−Lussac alcoholmeter after distillation of samples. Ethanol productivity was defined as the grams of ethanol per volume produced per day, and it was calculated by multiplying the dilution rate by ethanol concentration. Dilution rates were calculated by dividing the flow rate of liquid by the total volume of the fermentor. Fermented whey productivity was calculated as grams of whey per liter of total volume produced per day and was calculated by multiplying the flow rate (mL/day) by the density of fermented whey (≈1 g/L) and dividing by the total volume (2 L).

Residual sugar was determined by high-performance liquid chromatography, using a Shimadzu chromatograph with an SCR-101N stainless steel column, an LC-9A pump, a CTO-10A oven at 60 °C, and an RID-6A refractive index detector. Triple-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 fermented product and 2.5 mL of a 1% solution of butanol-1 diluted to 50 mL and 40 μL were injected directly onto the column. Residual sugar concentration was calculated using a standard curve and 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, $<\pm 2$.

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 diethyl hexyl sebacate 5% (v/v) (*20*). 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 70 °C. In all cases, the internal standard was butanol-1 at a concentration of 0.5% (v/v). Samples of 4 μL of fermented product were directly injected into the column, and the concentrations of the volatile compounds were determined using standard curves. All values were the mean of three repetitions. The standard deviation for all volatile byproducts was $<\pm 5$.

RESULTS AND DISCUSSION

Continuous Fermentation. DCM is a solid of food grade purity that has been previously shown to be an effective support material for cell immobilization of *S. cerevisiae* (*8*), kefir yeast (*10*), and *Kluyveromyces marxianus* (*19*). The fermentation of whey in a continuous bioreactor was carried out to investigate the operational stability of the immobilized kefir yeast on DCM and suitability for continuous process.

Table 1. Kinetic Parameters during Continuous Fermentation of Whey Using DCM-Supported Kefir Yeast at Two Different Temperatures before and after Storage at Refrigeration Temperature (4 °C) for 18 Days

before/after storage	temp (°C)	initial °Be density	flow rate (mL/day)	daily product productivity (g/L)	ethanol concn (% vol)	residual sugar (g/L)	ethanol productivity (g L^{-1} day^{-1})
before	27	6.3	300	150	3.5	10.1	4.1
		6.3	300	150	3.0	18.1	3.6
		6.3	300	150	3.3	21.2	3.9
		6.3	300	150	4.3	1.2	5.1
		5.7	600	300	3.5	<1	8.3
		5.7	600	300	2.5	11.4	5.9
		5.7	600	300	3.4	7.4	8.1
		5.7	600	300	2.8	<1	6.6
before	31	6.0	600	300	2.6	20.9	6.2
		6.0	600	300	2.6	17.2	6.2
		6.0	600	300	3.0	10.0	7.1
		6.0	600	300	3.5	5.0	8.3
		6.1	1000	500	1.6	52.5	6.3
		6.1	1000	500	1.5	44.3	5.9
		6.1	1000	500	1.5	46.6	5.9
		6.1	1000	500	1.7	41.4	6.7
after	31	6.1	600	300	2.5	31.1	5.9
		6.1	600	300	2.7	26.2	6.4
		6.1	600	300	2.6	17.0	6.2
		6.1	600	300	2.2	17.0	5.2

Kefir yeast had the advantage of fermenting lactose and producing high amounts of ethanol, which was considered to be advantageous for whey fermentation. The use of DCM-supported biocatalyst, prepared by the immobilization of kefir yeast on DCM, was reported to be suitable for the production of alcohol under batch fermentation conditions (*10*). To investigate continuous whey fermentation with DCM-supported biocatalyst, the initial °Be density of the modified whey was kept relatively constant. The results of the continuous fermentation are summarized in **Figure 1** and **Table 1**. Raisin extract and molasses were added to the whey before fermentation. The first was added to promote the fermentation (*21*) and the second to increase the sugar concentration. The reactor was operated continuously for 39 days. The biocatalyst was then stored for 18 days at 4 °C and later operated again for another 15 days without any loss of biocatalyst activity. It should be noted, however, that it took 4 days to reach full pre-storage-period fermentative activity levels following the interruption. The trials on storage of the biocatalyst at low temperature for a certain period of time and the use of two different temperatures (27 and 31 °C) were carried out at the request of our industrial partner (MEVGAL). Different flow rates were tested during the experiments to establish the maximum ethanol productivity of the biocatalyst. The pH dropped during fermentation and was continuously adjusted to 5.5. This was carried out as it was usually observed that kefir cells became inactive at pH <4.0. The °Be density of the effluent was relatively constant during the experiment but never fell below 1.7 due to the effect of residual solids from the molasses and to the production of small amounts of lactic acid by the lactic acid bacteria found in the kefir culture. Ethanol productivities were comparable to those obtained by continuous fermentation of must at low temperatures using the inorganic support mineral kissiris (*22*). DCM-supported kefir yeast gave a productivity of ~10% of that obtained by DCM-supported psychrophilic *Saccharomyces cerevisiae* yeast strain AXAZ-1 (*23*). However, the productivity obtained in our case is considered to be industrially cost-effective, because it was able to produce 1 L of fermented liquid for drink and potable alcohol production in 2−3 days. The biocatalyst was able to produce alcohol even after a refrigeration storage period of 18 days at 4 °C without any decrease in ethanol productivity. This was also observed by other researchers during wine production using immobilized *S. cerevisiae* cells on mineral kissiris (*24*). Our bioreactor was operated for 72 days, which shows a relatively high operational stability. This is of industrial importance and contributes positively to the use of immobilization to reduce the need for repeated batch setup, which is costly and difficult for the industry. Parameters such as ethanol concentration, residual sugars, and daily fermented whey productivity were all comparable to industrial fermentations for the production of potable alcohol and alcoholic drinks (*25*).

As DCM proved to be a suitable support for continuous whey fermentation with the advantage of being abundantly available, cheap, and easy to use for immobilization, one further consideration evaluated was the quality of the fermented product as a beverage.

Volatile Byproducts. Because potable alcohol is employed as the raw material to produce various kinds of alcoholic drinks such as liqueurs, sweet wines, and distillates, it was necessary to analyze the formation of volatile byproducts and aromatic compounds of the fermentation product. The formation of volatile compounds during continuous whey fermentation using DCM-supported biocatalyst is shown in **Table 2**.

The maximum concentrations of higher alcohols (propanol-1 and isobutyl alcohol) detected were ≤16 and ≤28 ppm, respectively. The concentrations of amyl alcohols were also low (maximum value detected was 89 ppm) at both fermentation temperatures. The fusel alcohol concentrations were at levels that are usually present in beers and wines produced by *S. cerevisiae* conventional batch process or immobilized on DCM (*13*). Similar levels of higher alcohols were also recently reported in batch fermentations carried out using immobilized kefir on DCM (*11*).

Due to the significance of ethyl acetate in the quality of alcoholic beverages, the formation of this volatile compound was also examined. The concentrations of ethyl acetate in these experiments ranged from traces to 95 mg/L. Such concentrations are reasonably typical of fermented beverages (*13*, *25*, *26*). However, these concentrations are at the high end in the context

Table 2. Volatile Byproducts in the Continuous Fermentation of Whey Using DCM-Supported Kefir Yeast at Two Different Temperatures before and after Storage at Refrigeration Temperature (4 °C) for 18 Days

before/after storage	temp (°C)	acet-aldehyde (ppm)	ethyl acetate (ppm)	propanol-1 (ppm)	isobutyl alcohol (ppm)	amyl alcohols (ppm)
before	27	50	80	11	9	66
		Tr[a]	95	10	14	89
		55	84	10	7	77
		59	84	10	11	78
		Tr	85	8	11	73
		Tr	50	4	5	73
		Tr	41	3	7	83
		Tr	36	2	6	63
before	31	68	79	Tr	28	44
		50	24	Tr	18	Tr
		48	6	2	22	Tr
		34	23	7	23	42
		Tr	8	9	10	Tr
		Tr	8	12	9	Tr
		Tr	7	16	8	Tr
		Tr	Tr	12	Tr	Tr
after	31	Tr	Tr	12	3	Tr
		Tr	Tr	16	3	Tr
		Tr	Tr	13	Tr	Tr
		Tr	45	12	Tr	Tr

[a] Tr = traces.

of the fusel alcohol levels observed. Lower concentrations of fusel alcohol and higher concentrations of ethyl acetate often result in a significant improvement in the aroma of the final fermented product, which is consistent with our observation of an enhanced aroma of the fermented whey. Similar observations were also reported when immobilized cells of *K. marxianus* yeast immobilized DCM were used in the fermentation of whey at high temperature (*19*).

The acetaldehyde content in the fermented beverages usually ranges between 13 and 40 mg/L (*27*) and may reach up to 75 mg/L (*25*). In the experiments reported here acetaldehyde concentrations ranged from traces to 68 ppm (**Table 2**), which is well within the expected ranges in similar alcoholic drinks. It is believed that further development of the approach proposed in these experiments would allow acetaldehyde levels to be reduced significantly.

Finally, a fermented whey alcoholic product can be used as raw material to produce alcoholic drinks. A product similar to kefir drink has been already produced with accepted organoleptic quality (unpublished data). A low-alcohol-containing drink can also be produced through mixing fermented whey product with fruit juices.

Conclusions. A DCM-supported kefir culture proved to be suitable for continuous fermentation of whey. The biocatalyst showed a high operational stability and was able to produce alcohol without any decrease in productivity even after storage at low temperature. Ethanol content was similar to that expected in industrial fermentations. The concentrations of higher alcohols (propanol-1, isobutyl alcohol, and amyl alcohols) were low. The main volatile byproducts formed in the continuous process were also similar to those observed in alcoholic beverages, whereas the fermented whey had an advantage of improved aroma associated with increased ethyl acetate. It is concluded that DCM-supported kefir can be developed for continuous whey fermentation for the production of potable alcohol or low-alcohol-content drinks.

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