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Whey Liquid Waste of the Dairy Industry as Raw Material for Potable Alcohol Production by Kefir Granules

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Kefir granular biomass was used in the fermentation of sweet whey and proved to be more effective compared to single-cell biomass of kefir yeast. The operational stability of the biocatalyst was assessed by carrying out 20 repeated batch fermentations. Levels of ethanol productivity reached 2.57 g L^{-1} h^{-1}, whereas the yield was 0.45 g/g. The fermentation time was only 8 h. Mixtures of sweet whey with molasses were fermented at initial densities ranging from 4.2 to 10.2 °Be and resulted in ethanol yield factors between 0.36 and 0.48 g of ethanol/g of utilized sugar. Lower °Be values led to an increase of percentages of ethyl acetate on total volatiles determined and a reduction of amyl alcohols. The addition of 1% black raisin extract to whey appears to promote whey fermentation, whereas the same was not observed in the case of white sultana extract addition. It was finally established that it is preferable to ferment mixtures of whey−molasses by adding molasses in whey after the completion of whey fermentation.

KEYWORDS: Kefir; granules; fermentation; whey; molasses

INTRODUCTION

Cheese whey is the liquid remaining after the precipitation and removal of milk casein during cheese-making. This byproduct represents ∼85−90% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4.5−5.0% w/v), soluble proteins (0.6−0.8% w/v), lipids, and mineral salts (*1*).

Cheese whey represents an important environmental problem because of the high volumes produced and its high organic matter content, exhibiting a COD = 60000−80000 ppm (*2*). Worldwide production of whey is of the order of 130 million tonnes per year, with cheese production increasing at a yearly rate of ∼3%. The pressure of antipollution regulations demands that the dairy industry develop new technologies that can change whey from waste to a valuable product (*3*).

The treatment of whey by fermenting lactose to ethanol has received wide attention to date. Many researchers have reported alcohol production from whey using *Kluyveromyces fragilis* (*4*, *5*) and strains of *Kluyveromyces marxianus* (*6*, *7*) and *K. fragilis* (*8*, *9*) immobilized on alginates. *Candida kefir* and *Candida tropicalis* are two other strains employed in salted whey alcoholic fermentation (*10*).

Kefir yeast culture is employed to produce from milk the traditional Russian alcoholic drink "kefir". It is a mixed culture of various species of the genera *Kluyveromyces*, *Candida*, *Saccharomyces*, and *Pichia* and various lactic acid bacteria of the genus *Lactobacillus*, which form granules during cell growth under aerobic condition (*11*).

This mixed culture of kefir yeast, which ferments lactose, seems to have a potential for alcohol production using cheese whey. The production of ethanol, however, from nonconcentrated whey is not economically feasible because the levels of ethanol concentration obtained reach only 2%, making the distillation process too expensive (*12*). The fermentation of mixtures of whey with other raw materials, such as molasses, will avoid the condensation of whey and consequently the high energy demand. Recently, an attempt was made to use delignified cellulosic (DC) material supported kefir yeast in the low-temperature alcoholic fermentation of glucose (*13*) and to study the efficiency of this biocatalyst to ferment synthetic media containing various carbohydrates (*14*), as a precursor study for further research to ferment whey or mixtures of whey with raw materials to reinforce final alcohol concentration.

This work was conducted to evaluate potable alcohol production by alcoholic fermentation of whey and its mixtures with molasses that can be carried out by kefir granules, which are rapidly formed during aerobic fermentation.

MATERIALS AND METHODS

Cheese whey was obtained from a regional dairy industry (Mevgal Milk Industry, Koufalia, Greece) and contained 5% w/v lactose, 0.3% fat, 0.7% protein, and 0.5% ash. Molasses employed was a product of

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the sugar refinery (Thessaloniki, Plati, Greece). Kefir yeast commercially available (Meliton S.A.), which is used in Caucasus for a homemade kefir drink, was employed in this study. Cell growth was performed on cheese whey, supplemented with 0.16% w/v KH_2PO_4 and 0.75% w/v $(NH_4)_2SO_4$ (Riedel-de Häen, Seelze, Germany). This medium was pasteurized at 75 °C for 15 min. Pressed wet weight cells were prepared at late log phase.

Production of Kefir Granular Biomass. Wet weight cells of 10 g were separated by centrifugation and transferred to a plexiglass bioreactor of 1.5 L working volume. A fed batch aerobic fermentation was carried out at 30 °C, employing a high-accuracy peristaltic pump, using again as fermentation broth supplemented cheese whey; the air supply of 4 L/min passed through sterile microfilters. The process lasted 7 h, and kefir granules were gradually formed. Wet weight cell concentrations were determined after centrifugation of samples.

Comparison of Kefir Granules and Kefir Biomass Produced from Synthetic Media. A mixture of whey−molasses of 250 mL was introduced in a glass cylinder with 5 g wet weight of granular kefir biomass and incubated at 30 °C simultaneously with another similar glass cylinder containing also 250 mL of a mixture of whey−molasses and 5 g of kefir biomass produced from synthetic media containing 2.0% glucose, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4$, and 0.4% yeast extract. The two mixtures were allowed to ferment, and kinetics were performed by measuring the °Be density at various time intervals.

Fermentations. Twenty repeated batch fermentations of cheese whey were carried out with kefir granules. For each fermentation, an amount of 5 g of wet kefir granules was introduced into 250 mL of pasteurized cheese whey in a 0.5 L glass cylinder. The medium contained 1% raisin extract of 12 °Be density. The glass cylinder was incubated at 30 °C, and kinetics of fermentations were monitored by measuring the °Be density at various time intervals. The initial pH value was ~5.6. At the end of every batch the biomass was separated by centrifugation (4000 rpm, 10 min) and the fermented liquid removed.

Mixtures of cheese whey and molasses were also fermented by kefir granules, without the addition of raisin extracts. Molasses was added in whey before pasteurization so the final °Be density would be increased successively by 1, 2, 3, 4, 5, 6, and 7 units. Three batches of each concentration were performed. All batch fermentations were stopped at ~2 °Be density. At the end of each batch fermentation a sample was collected and analyzed for ethanol, residual sugar, and volatile byproducts. Whey−molasses fermentations were also performed by the addition of molasses after the completion of whey fermentation and contained 1% raisin extract.

Consideration of Raisin Extracts as Promoters. Three glass cylinders each contained 250 mL of whey and 5 g of kefir granular biomass and were incubated at 30 °C. In the second cylinder 2.5 mL of raisin extract from black raisin was added and in the third also 2.5 mL of raisin extract from white raisin (sultana).

Kinetics of fermentation were performed by measuring °Be density at various time intervals. Raisin extracts were prepared by hot extraction of 100 g of black raisin or sultana with 200 mL of tap water, contained in an Erlenmeyer flask and heated for at least 3 h in a constant-temperature water bath adjusted at 72 °C.

Ethanol and Residual Sugar Determination. Ethanol and residual sugar (lactose, galactose, and glucose in the case of whey fermentations, lactose, galactose, glucose, fructose, and sucrose in the case of whey−molasses mixtures) 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 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, whereas 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. Ethanol productivity was expressed as grams of ethanol produced per liter of bioreactor per hour and calculated by dividing
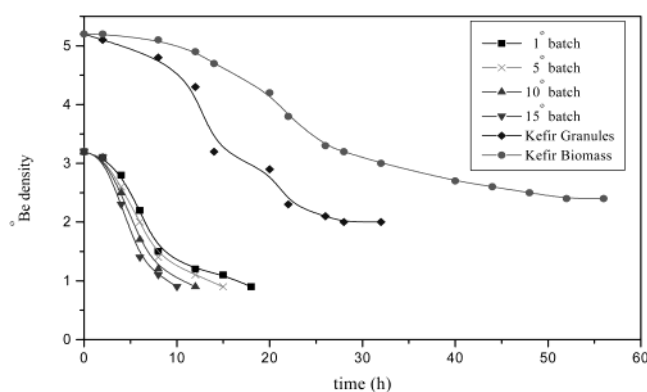

Figure 1. Kinetics of repeated batch fermentation of cheese-whey by kefir granules and comparison of kinetics of whey–molasses fermentation by kefir granules and by kefir biomass produced from synthetic media.

ethanol concentration, expressed as grams per liter by the fermentation time, whereas ethanol yield factor was calculated by dividing the ethanol concentration (grams per liter) by grams of sugars utilized. Conversion is the percentage of sugars fermented and calculated using the equation

$$\frac{\text{initial sugar concn} - \text{residual sugar}}{\text{initial sugar concn}} \times 100$$

Volatile Byproducts Determination. Quantitative determination of volatile byproducts was 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, i.d. = $^1/_8$ in.), packed with Escarto 5905 [consisting of squalene 5%, Carbowax 300 90%, and bis(2-ethylexyl)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 into the column.

RESULTS AND DISCUSSION

It has been observed that kefir granular biomass is formed rapidly by the aerobic fermentation of whey in a bioreactor, whereas single-cell biomass is formed using synthetic media containing glucose. The difference between kefir granules and kefir grains is that the latter are formed slowly during milk fermentation. **Figure 1** illustrates that granular biomass was more effective in comparison with single-cell product. For this reason all experiments were performed using kefir granules.

To assess the operational stability of the fermentation of whey and mixtures of whey−molasses, repeated batch fermentations were performed for at least 20 batches.

It is apparent from the results that the ethanol productivity was improved up to the 15th fermentation batch and remained constant during the following batches, in the case of whey fermentation. As far as fermentation of mixtures of whey−molasses is concerned, the ethanol productivity remained constant for 21 successive fermentation batches. This stability of the system was also proved by a similar stability of ethanol yield factor and conversion. Kinetic parameters were continuously improved as the number of batches increased. This behavior is due to the fact that kefir granules performed a gradual adaptation from batch to batch to anaerobic conditions.

The production of ethanol from nonconcentrated whey is not economically feasible because the levels of ethanol obtained reach only 2.5%, making the distillation process expensive. Thus, the fermentation of mixtures of whey with other raw materials, such as molasses, is necessary in order to avoid whey
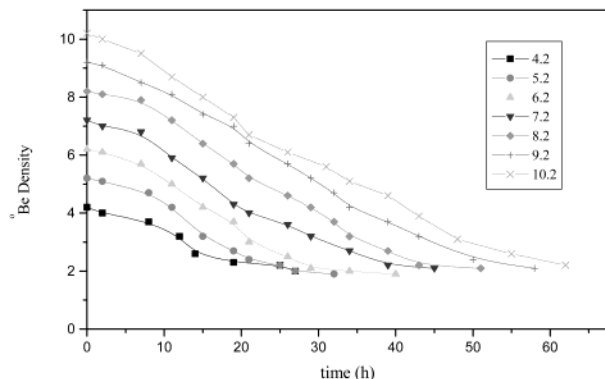
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Figure 2. Effect of initial °Be density on the kinetics of alcoholic fermentation of whey–molasses mixtures by kefir granules.

Figure 3. Effect of initial °Be density in whey–molasses mixtures fermented by kefir granules on percentage of the most important volatiles on total volatiles.

Figure 4. Effect of black raisin and sultana raisin extract on the kinetics of alcoholic fermentation of whey by kefir granules.

condensation and consequently a high energy demand. Some efforts have been made in the past to study the alcoholic fermentation of such mixtures (*15*, *16*).

In this study kefir granules were employed in the fermentation of mixtures of whey with molasses having the advantage of separation of biomass without using centrifugal separators. The mixtures fermented had initial °Be densities varying from 4.2 to 10.2. In each case three repeated batches were carried out. It is clear that the biocatalyst used has a carry-through property in the fermentation of whey–molasses mixtures. The ethanol concentration values obtained were high, whereas yields obtained were in the range of 0.36–0.48, which could be of practical importance as whey is a raw material of negligible cost. Parameters such as conversion, fermentation time, and ethanol productivity were acceptable in the industrial fermentations for the production of alcoholic drinks.

Figure 2 shows the effect of initial °Be density on the kinetics of the alcoholic fermentation of whey–molasses mixtures by kefir granules. The common characteristic of all these fermentations was the fact that they stopped at 2 °Be. This relatively high concentration can be attributed to nonfermentable diluted constituents of whey and molasses and not to residual sugar concentration, which was low.

Apart from being effective, a biocatalyst should produce alcohol of good quality, which means monitoring the volatile byproducts of alcoholic fermentation to ensure the produced ethanol is potable. Samples at the end of fermentation of mixtures were analyzed for the main volatile byproducts such as ethyl acetate, propanol-1, amyl alcohols, ethanal, and isobutanol. Results are presented in **Figure 3**, where percentages of ethyl acetate, amyl alcohols, and ethanal on total volatiles are plotted against initial °Be density of whey–molasses mixtures. It is reported that as the initial °Be density increases, the percentage of amyl alcohols is increased, whereas the percentages of ethanal and ethyl acetate are gradually decreased.

This is in agreement with the results reported by Athanasiadis et al. (*14*). The decrease of amyl alcohols and the increase of ethyl acetate as the °Be density is reduced lead to improvement of the aroma of this fermented liquid, which could be used as raw material for potable alcohol production. This improvement was observed during the laboratory work performance. Therefore, potable alcohol will be produced with improved quality as the initial °Be density is decreased.

To expand the study of the effect of raisin extract in the promotion of fermentation of whey by kefir yeast (*17*), kinetics of whey fermentation by the presence of extracts of black raisin and white sultana were performed. Results presented in **Table 1** and **Figure 4** show a significant drop of fermentation time
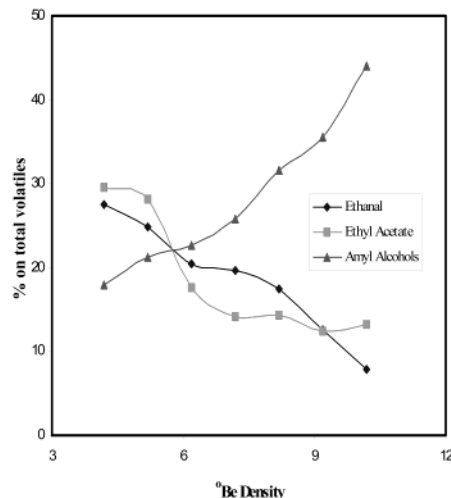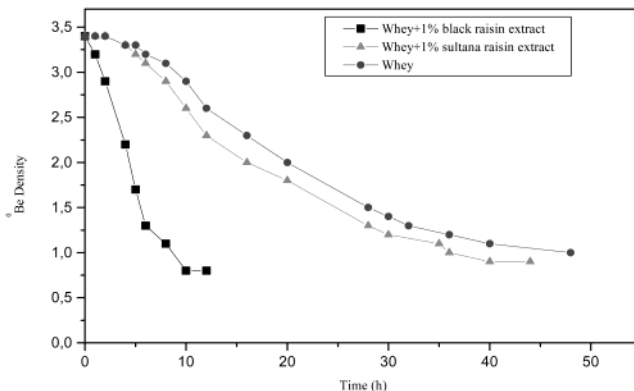
by the addition of 1% raisin extract, confirming the promising results of Athanasiadis et al.'s study (*17*). On the contrary, this positive effect was not observed in the case of white sultana extract, which means no promotion. This obvious difference will facilitate experimental work to determine the active component that catalyzes the fermentation of whey by kefir yeast. The effect of 1% black raisin extract can be also manifested by comparison of **Figures 1** and **4**, regarding repeated batch fermentation by the presence and absence of 1% raisin extracts.

For further improvement of fermentation of mixtures of whey–molasses, fermentations of whey were carried out first, to reduce the inhibition time caused by inhibitors existing in molasses. This was also performed to avoid inhibition due to higher osmotic pressure and competition of yeasts to ferment a mixture of lactose–sucrose. Then molasses was added, and the results of this second fermentation are illustrated in **Table 2**. These results indicate higher ethanol concentration and ethanol yield factor in comparison with the fermentation of mixtures of whey–molasses from the beginning.

The above results prove that kefir granular biomass is more effective than the single-cell biomass and that the promotion of whey fermentation can be performed by using black raisin extracts only. The active component might be a substance that is present in black raisin and absent in white sultana. It can be determined by comparison of the composition of the two prod-

Table 1. Effect of Black and Sultana Raisin Extract on Kinetic Parameters of Alcoholic Fermentation of Whey by Kefir Granules at 30 °C[a]

fermentation	fermentation time (h)	EtOH concn (% v/v)	residual sugar (g/L)	EtOH yield factor (g/g)	EtOH productivity (g L^{-1} h^{-1})	conversion (%)
whey	48	1.7	9.8	0.33	0.28	80
whey + 1% sultana raisin extract	40	1.9	8.5	0.36	0.38	83
whey + 1% black raisin extract	10	2.5	5.1	0.44	1.98	90

[a] All values are the mean of three repetitions. The standard deviation for the fermentation time was <±2, that for ethanol concentration <±0.5, that for residual sugar <±2.9, that for yield <±0.04, that for ethanol productivity <±0.06, and that for conversion <±7.

Table 2. Kinetic Parameters of Whey–Molasses Fermentation by Kefir Granules When Molasses Was Added in Fermented Whey at 30 °C[a]

°Be density after the addition of molasses	fermentation time[b] (h)	EtOH concn (% v/v)	residual sugar (g/L)	EtOH yield factor (g/g)	EtOH productivity (g L^{-1}h^{-1})	conversion (%)
3	28	4.4	8.2	0.46	1.24	90
4	37	5.3	12.4	0.47	1.13	88
5	48	6.1	14.2	0.46	1.01	88
6	51	6.8	10.1	0.43	1.06	93

[a] All values are the mean of three repetitions. The standard deviation for the fermentation time was <±4, that for ethanol concentration <±0.6, that for residual sugar <±3.1, that for yield <±0.05, that for ethanol productivity <±0.05, and that for conversion <±7. [b] Fermentation time refers to the duration of fermentation after the addition of molasses.

ucts. It is more preferable to ferment the whey first and add the molasses when the whey fermentation has been completed.

Fermentation of the resulting mixture will lead to higher ethanol yields and lower fermentation times compared to those obtained when whey and molasses are fermented as a mixture from the beginning. The best composition of volatiles is obtained at lower initial °Be densities.

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