



Study of whey fermentation by kefir immobilized on low cost supports using ^{14}C -labelled lactose



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HIGHLIGHTS

- ▶ ^{14}C -labelled lactose uptake rate by kefir immobilized on BSG and MSR was studied.
- ▶ Lactose uptake rate by immobilized cells was correlated to fermentation rate in whey.
- ▶ Biocatalysts with low cost BSG, MSR, GP or DCM increase the whey fermentation rate.
- ▶ BSG cellulosic biocatalyst can in 8 h ferment the lactose of whey and produce alcohol.
- ▶ Hydrophilic cellulose protects immobilized cells and increase biocatalytic activity.

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ABSTRACT

Brewer's Spent Grains (BSG) and Malt Spent Rootlets (MSR) were used as supports for kefir cells immobilization and the role of lactose uptake rate by kefir in the positive activity of produced biocatalysts during whey fermentation was investigated. Lactose uptake rate by the immobilized cells was recorded using ^{14}C -labelled lactose and the effect of various conditions (pH, temperature and kind of support) on it and consequently on fermentation time and ethanol production was examined. The results showed that lactose uptake rate was correlated to fermentation rate and increased as temperature was increased up to 30 °C at pH 5.5. The same results have been recently noticed by using biocatalysts with Delignified Cellulosic Materials (DCM) and Gluten Pellets (GP), but fermentation time of about 7 h by kefir immobilized on DCM and BSG resulted to two fold lower than that on GP and MSR. The highest alcohol concentration was observed by MSR.

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1. Introduction

It is well known that microorganisms cells take up carbohydrates through their cell wall and it is obvious that this diffusion of carbohydrate molecules could play an important role on the fermentation rate. Therefore, research to examine cell carbohydrate uptake rate will visualize this phenomenon and will create the practical and theoretical background in supported productivity in bioprocesses. As recently has been reported, ^{14}C -labelled lactose was used for monitoring lactose uptake rate by free kefir cells and by immobilized ones in an attempt to confirm directly the positive effect of immobilized kefir on fermentation rate (Golfinopoulos et al., 2009, 2011, 2012). So, it was found that lactose uptake rate is strongly correlated to fermentation rate and increased during whey fermentation by kefir cells immobilized

on Delignified Cellulosic Materials (DCM) and Gluten Pellets, (GP), in comparison with fermentation by free cells (FC).

Whey is the main liquid by-product of the dairy activities, in which a large quantity of the lactose of the milk is removed (~4.2–5%). But, it is usually discarded as a waste in the environment, representing a grave pollutant. So, except of protein and whey lactose recovery processes, the improvement of whey lactose fermentation was necessary for the production of various bioproducts, through biotechnological means. In that way the streams of whey could be used as an abundant and renewable potential raw material for microbial fermentations (Panesar et al., 2007). Over the last two decades, the natural mixed culture kefir was chosen for whey exploitation, due to its ability to ferment the lactose of whey mainly to ethanol and lactic acid (Athanasiadis et al., 2002; Panesar et al., 2010).

In the present work two new biocatalysts prepared by immobilization of kefir on Brewer's Spent Grains (BSG) or Malt Spent Rootlets (MSR) were used for whey fermentation. The BSG

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and MSR are important solid by-products of the brewing industry and have been used in animal feed production (Bekatorou et al., 2007) and as ingredients in the food industry due to their high nutritional properties (Loetterle et al., 2011). They are available at low or no cost throughout the year and are produced in high quantities not only by large, but also by small breweries.

Cells immobilized on BSG have been applied successfully as biocatalysts in various brewing processes (Kopsahelis et al., 2007) but their use as supports for kefir immobilization to develop biocatalysts for use in whey fermentation has not been reported before. The production of potable alcohol or whey alcoholic beverages using kefir immobilized on BSG and MSR is a very attractive perspective, because they are natural, easily available, abundant and have food grade purity and many dietary fibers.

In this study the lactose uptake rate by kefir cells immobilized on BSG and MSR during whey fermentation was examined and the results were compared with the previously reported ones (Golfinopoulos et al., 2009, 2011, 2012). For this reason the optimum physicochemical conditions like pH, temperature, and kind of support for lactose uptake rate by the new biocatalysts were investigated for fast whey fermentation. The results were compared with results by DCM and GP biocatalysts as well as by FC in an attempt to propose the best system for fast and easy removal of lactose from whey.

2. Methods

2.1. Microorganism and cell growth

The kefir yeast commercial product bought from the local market used in Caucasus for homemade kefir drink was employed in the present work. As the manufacturer and researchers report the kefir grains are mainly consisting of (% w/w) 5.0 ± 0.6 protein, 8.2 ± 1.6 sugars και 80.7 ± 1.3 H₂O (Rimada and Abraham, 2001). Cell growth and production of biomass were done according to previous work (Golfinopoulos et al., 2009).

2.2. Materials and media

Liquid cheese whey used for all fermentation experiments was obtained from the regional dairy industry "Agricultural Cooperative Union of Kalavryta" (Kalavryta, Greece). It remained after the production of feta cheese and after removal of whey proteins lactalbumins and lactoglobulins and contained about (% w/w) 5 of lactose, 0.8 of proteins, while its pH value was 6.5. The BSG and MSR were obtained from the Athenian Brewery S.A. and used as cells supports. The BSG contained about (% w/w on dry matter) 21.6 of crude protein, 6.8 of fats, 17 of cellulose, 22 of lignin, 28 of non-cellulosic polysaccharides and 4.7 of ash. The MSR consist of about (% w/w on dry matter) 36.2 protein contents, 12.1 cellulose, 18.4 fibers, 13.3 carbohydrates, 3.7 glucose, 1.0 polyphenols, 0.6 fats and 3.0 ash. The compositions of BSG, MSR and whey provided by the manufacturers.

2.3. Preparation of supports and cell immobilization

For cell immobilization fresh BSG were utilized, while the MSR were sieved and the particles with diameter less than 0.36 mm were selected and remained in water for two days before use. All media were sterilized by autoclaving at 120 °C for about 15 min prior to their use. Then, kefir immobilization on BSG or MSR was performed according to previous investigations, where DCM and GP were also used as supports for kefir cells immobilization (Kopsahelis et al., 2007; Golfinopoulos et al., 2012). Finally, the produced immobilized biocatalysts were washed twice with 12%

lactose culture medium for removal of free cells and used for whey fermentation.

2.4. Effect of pH and temperature on lactose fermentation rate and lactose uptake rate by kefir immobilized on BSG or MSR during whey fermentation

In order to study the effect of pH value and temperature on whey fermentation rate and lactose uptake rate by kefir cells immobilized on BSG or MSR, whey fermentations experiments were carried out in 500-mL Erlenmeyer flasks without agitation or air supply, as immobilized biocatalysts were distributed in all reacting volume and the lactose fermentation is an anaerobic process. Specifically, an amount of about 122.00 g BSG biocatalyst or 120.00 g of wet MSR biocatalyst was added in 250 mL of pasteurized whey and fermentations were carried out (i) at various pH values 4, 5, 5.5, 6 and 6.5 at 30 °C and (ii) at various temperatures of 10, 20, 25 and 30 °C under 5.5 pH value.

In all cases initial trial pH value was achieved by the addition of tartaric acid (7% w/v). During of each whey fermentation the pH value was monitored by pH meter (Hanna 9024C) at time intervals of about two hours and maintained stable to the selected trial pH by the addition of 6 M NaOH solution.

At the beginning of each run a small quantity of ¹⁴C-labelled lactose [¹⁴C-D-glucose-1-¹⁴C], (ARC 0466 lactose 0.1 mCi/mL) was added, in order to determine the lactose uptake rate by kefir during the processes by liquid scintillation.

Samples of the fermented liquids were collected on fixed time intervals and stored at –20 °C until further analysis. Also, whey samples at the beginning and at the end of each fermentation were used for determination of Ca²⁺ ions concentration.

Fermentation kinetics was monitored by total sugar measurements by HPLC and ethanol concentration by GC. For statistical reasons all fermentations were carried out in triplicate and the recorded results were the mean value of the three repetitions.

2.5. ¹⁴C-labelled lactose determinations

The determinations of ¹⁴C-labelled lactose and liquid scintillation measurements were performed according to a recent investigation (Golfinopoulos et al., 2011).

2.6. Determination of residual sugar, ethanol and Ca²⁺ ions concentration

Residual sugars in the fermented whey samples were determined in a Shimadzu LC-9A HPLC system comprising a Shim-pack SCR-101 N, an LC-9A, an RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit. Ultra pure water obtained by a Milli-Q water purifier system, (resistivity 18.2 MΩ cm⁻¹), was used as the mobile phase (0.8 mL/min), and 1-butanol (0.1%v/v), was used as an internal standard. Column temperature was 60 °C. Sample dilution was 1% v/v, and the injection volume was 40 μL.

Ethanol was determined according to a recent investigation (Golfinopoulos et al., 2012).

Finally, the Ca²⁺ ions concentration was measured by Flame Atomic Absorption Spectrometry (FAAS) using a Shimadzu (AA-6500) spectrophotometer equipped with SR hollow cathode lamps for good background correction and a corrosion resistant nebulizer. Analytical precision was better than 10% on the basis of replicate analyses.

2.7. Statistical methods and data analysis

For every whey fermentation, the standard deviation (SD) of lactose uptake rate, residual sugar and ethanol concentration was calculated with the PC program Origin 8. The data were analyzed using the analysis of variance technique. Significant differences between means were identified by multiple range tests (considered significant for $P < 0.05$). Statistical analyses were carried out using the Computer software, Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 11.0 for Windows.

3. Results and discussion

In the present work the use of ^{14}C -labelled lactose in order to determine lactose uptake rate by cells was a very convenient laboratory method as labelled and unlabelled lactose molecules follow the same metabolic pathways. So, the relation between kefir fermentation ability and carbohydrate uptake rate by kefir cells was really clarified by the ^{14}C -labelled lactose determination during whey fermentation.

The results showed that the highest fermentation rate and lactose uptake rate by BSG or MSR immobilized biocatalyst were recorded at temperature of 30 °C and at 5.5 pH value, as it was reported previously for kefir FC and GP or DCM biocatalysts (Golfnopoulos et al., 2009, 2011, 2012).

As in Fig. 1(a) and (b) illustrated both new biocatalysts were found suitable for lactose whey fermentation and impressively more effective in comparison with free cells. Moreover, Fig. 1(a) shows that the fermentation time for DCM and BSG dropped 50% in comparison with GP and MSR and about 90% compared with FC. Specifically, for BSG and DCM the whey fermentation lasted only about 8 and 7.2 h, respectively! Also, residual sugar was affected by the kind of support and was significantly low ($P < 0.05$) when DCM and BSG biocatalysts were used for whey fermentation, in comparison with fermentations by GP and MSR biocatalysts.

This result shows potential industrial fermentations using mainly BSG, because of the low cost of this material and the simplicity of processing. The BSG can be used for cell immobilization support without any chemical treatment (e.g. delignification) which means easier handling and lower costs. Therefore, biocatalyst made by kefir cell immobilization on BSG can be acceptable by even small cheese-dairies as a reactor with small volume is required to ferment the produced whey. Furthermore, due to the fact that BSG are industrial wastes produced in large quantities and negligible cost, their utilization is critical as a solution to their disposal problem. Eventually, using BSG biocatalyst could fast and easily reduce the whey polluting load and produce a more nutritious animal feed.

The increase of the promotional activity of porous cellulose (DCM) and spent grains toward protein gluten can be attributed: (i) to their higher surfaces in relation to Gluten Pellets surface for equal weights and so to the increase of lactose concentration on the surface of DCM, and (ii) to the increased lactase enzyme activity and lactose hydrolysis rate, because the immobilized cells are protected on these hydrophilic cellulosic supports (DCM, BSG) in contrary to GP support and FC.

As has been already reported for whey fermentation by free kefir cells the fermentation time was found about 3-fold higher of that of synthetic media containing lactose (Golfnopoulos et al., 2009). The whey contains much more chemical constituents than the synthetic media – e.g. Ca^{2+} ions – which adsorbed on cell walls, prevent the adsorption of lactose and reduce diffusion of lactose into the cell for the hydrolysis (Akrida-Demertzi and Demertzi, 1988; Akrida-Demertzi et al., 1990). Here is obvious that using biopolymers the fermentation rate was much higher in the case of whey as compared with lactose synthetic medium (Golfnopoulos

et al., 2009). The inhibition in whey fermentation using free cells (Golfnopoulos et al., 2011) by Ca^{2+} ions was eliminated, maybe due to cluster formation of Ca^{2+} ions with cellulose which leaves the liquid medium. Indeed, using the atomic adsorption spectroscopy (AAS) method, in the present work was found that the concentration of calcium in the whey was decreased about 34% during fermentation by BSG biocatalyst, whereas concentrations of magnesium, iron and zinc were remained almost constant.

Also, the formed lactic acid, not acting as yeasts inhibitor, generates hydrogen bonds with hydroxyl groups of cellulose and leaves the liquid medium (Rhee and Tanaka, 2000). This explains why whey is fermented in reduced fermentation time by porous cellulose supported kefir cells as compared with free cells (Golfnopoulos et al., 2011). Recently it was observed that lactose uptake rate by free kefir cells was strongly correlated to lactose fermentation rate in synthetic media containing lactose (Golfnopoulos et al., 2009), as well as in whey (Golfnopoulos et al., 2011). Also, new results indicated that the use of kefir immobilized on GP and DCM reduced sharply whey fermentation time and increased lactose uptake rate (Golfnopoulos et al., 2012). As Fig. 1 illustrates the aforementioned results were verified and also lactose uptake rate by kefir cells immobilized on BSG or MSR was correlated to lactose fermentation rate during whey fermentation too.

It is clear that lactose uptake rate by kefir immobilized on GP, DCM, BSG and MSR is related to whey fermentation rate and the increase of fermentation rate is accompanied by the increase of lactose uptake rate by immobilized biocatalysts. Also, comparing free cells with immobilized ones on above materials it is evident that lactose uptake rate by immobilized kefir cells during whey fermentation is higher than that with free cells. Furthermore, comparing BSG and DCM supports with GP it is clear that immobilization of kefir cells on cellulosic supports leads to higher fermentation rate and lactose uptake rate than those for GP support.

In Fig. 1(c), the effect of the kind of support on ethanol concentration during whey fermentation is illustrated. The highest ethanol concentration was observed by kefir cells immobilized on MSR compared with that produced by kefir FC and immobilized on GP, DCM or BSG. Specifically, ethanol concentration was significantly higher ($P < 0.05$) in the case of MSR biocatalyst compared to that from fermentations conducted by kefir FC or by the rest biocatalysts. But in Fig. 1(a) is shown that the residual sugar concentration was significantly higher ($P < 0.05$), while the sugar conversion was significantly lower ($P < 0.05$) in the whey fermentation by MSR biocatalyst compared with fermentation by FC and GP, DCM or BSG biocatalysts. It would be explained, assuming that lactic acid bacteria, contained in kefir microflora, could also metabolise, except lactose, the whey amino acids via non-transaminating reactions and endogenous transamination, as has been reported elsewhere (Liu et al., 2003).

However, the produced ethanol by kefir immobilized on DCM and BSG was also high compared with that of FC and GP biocatalyst. Ethanol concentration during whey fermentation by kefir cells immobilized on MSR reached near 3.24 mL/100 mL of whey, while by kefir immobilized on BSG and DCM about 2.13 and 2 mL/100 mL, respectively. The potential of dairy distillation of the fermentation broth in order to obtain such a low ethanol concentration is very expensive. However, it is clear that the ethanol production in presence of kefir cells immobilized on MSR and BSG or DCM was about 2.5-fold and 1.5-fold higher respectively compared with the ethanol obtained during whey fermentation by kefir FC. Therefore, further scientific research on cell immobilization is required in order to increase ethanol concentration during whey fermentation by kefir.

As shown in Fig. 1(c) the highest alcohol concentrations were obtained during whey fermentation by kefir cells immobilized on

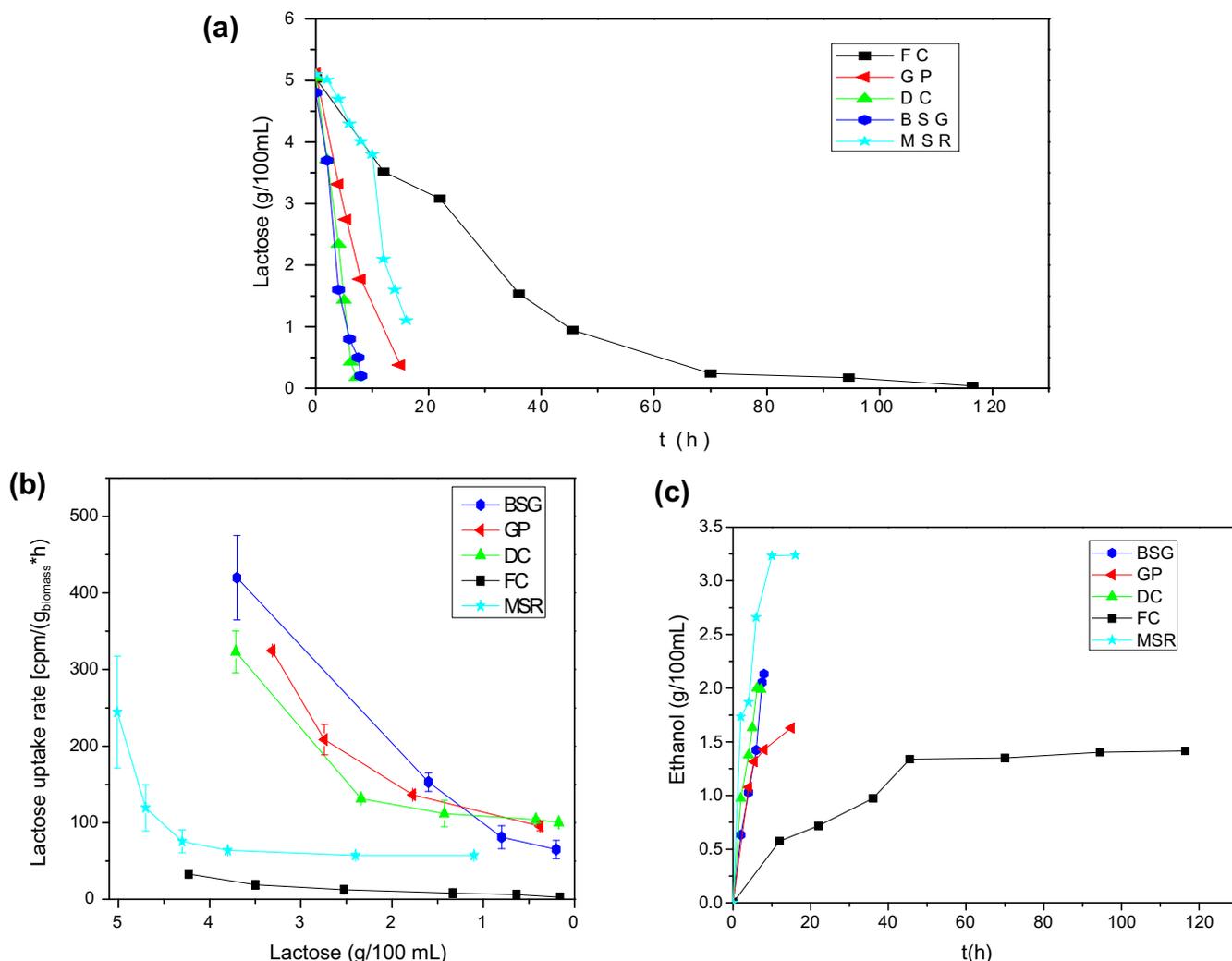


Fig. 1. (a) Fermentation kinetics of lactose by kefir free cells (FC) and immobilized on Gluten Pellets (GP), Delignified Cellulosic Material (DCM), Brewer's Spent Grains (BSG) and Malt Spent Rootlets, (MSR), (b) in relation with lactose uptake rate and (c) ethanol concentration during whey fermentation at 30 °C and 5.5 pH value (for DCM, pH = 5).

MSR, but the highest lactose uptake rates were obtained in fermentations by BSG and DCM biocatalysts Fig. 1(b). This implies that alcohol may be an inhibitor for lactic bacteria contained in kefir microflora, even at those concentrations – e.g. 3.24 g/100 mL – and therefore it plays an important role on the microorganisms fermentation activity and lactose uptake rate. Similar inhibition effect has been reported by other researchers (Brown et al., 1981; Maiorella et al., 1983). In addition other by-products such as acetaldehyde, lactic acid, acetic acid, 1-propanol and 2 methyl-1-butanol produced in whey fermentation by kefir may act as inhibitors for microorganisms contained in kefir microflora (Maiorella et al., 1983).

4. Conclusions

The ¹⁴C-labelled lactose uptake rate by kefir immobilized on BSG and MSR was correlated to fermentation rate during whey fermentation. Low cost biopolymers such as BSG, MSR, GP and DCM can be used as supports for kefir immobilization and the prepared biocatalysts strongly increase the fermentation rate. Especially cheap and food grade BSG cellulosic biocatalyst can in 8 h reduce the whey polluting load and produce potable alcohol, although ethanol at concentrations ~3.24 g/100 mL may act as an inhibitor during fermentation. The hydrophilic character of cellulose

contributes to the protection of immobilized kefir cells and therefore to their increased biocatalytic activity.

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