



Research perspectives and role of lactose uptake rate revealed by its study using ^{14}C -labelled lactose in whey fermentation

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ABSTRACT

The present investigation examines the effect of pH, temperature and cell concentration on lactose uptake rate, in relation with kinetics of whey fermentation using kefir and determines the optimum conditions of these parameters. Lactose uptake rate was measured by adding ^{14}C -labelled lactose in whey. The results reveal the role of lactose uptake rate, being the main factor that affects the rate of fermentation, in contrast to the activity of the enzymes involved in lactose bioconversion process. Lactose uptake rate results discussion showed that mainly Ca^{2+} is responsible for the reduced whey fermentation rate in comparison with fermentations using synthetic media containing lactose. Likewise, the results draw up perspectives on whey fermentation research to improve whey fermentation rate. Those perspectives are research to remove Ca^{2+} from whey, the use of nano and microtubular biopolymers and promoters such as γ -alumina pellets and volcan foaming rock kissiris in order to accelerate whey fermentation.

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1. Introduction

Whey is the liquid waste of dairy industry containing 4.8%–5.3% lactose. Owing to its high organic load of 40–70 g/L BOD_5 and 60–80 g/L COD (Athanasiadis et al., 2004), whey represents an important environmental problem. Last decade due to its ability to ferment lactose, kefir was thought to be an ideal industrial microflora of microorganisms for the exploitation of whey lactose (Athanasiadis et al., 2001, 2004, 2005; Kourkoutas et al., 2002; Koutinas et al., 2007, 2009). In the frame of this effort cell immobilization of kefir co-culture on delignified cellulosic material (Athanasiadis et al., 2001), the production of freeze-dried kefir as starter culture (Papavasiliou et al., 2008), and the fermentation efficiency of thermally dried kefir (Papapostolou et al., 2008) were reported.

Kefir is a mixed culture consisting of various yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces*, *Pichia*) and lactic acid bacteria of the genus *Lactobacillus* (Garrote et al., 1997; Pintado et al., 1996; Witthuhn et al., 2005). Yeasts and lactic acid bacteria co-exist in a symbiotic association and are responsible for lactose fermentation of milk and whey (Leroi and Pidoux, 1992). Recently, research efforts have been undertaken in order to study kefir co-culture fermentation ability in a model system containing kefir co-culture and lactose. In the frame of this attempt synthetic media containing lactose were used and the lactose uptake rate was recorded by using ^{14}C -labelled lactose (Goulinopoulos et al., 2009). Likewise, a study of

glucose uptake rate by *Saccharomyces cerevisiae* was previously reported (Soupioni et al., 1998).

In the present work, based on the results obtained on synthetic media containing glucose or lactose, ^{14}C -labelled lactose was used to study kefir co-culture fermentation ability in a raw material like the whey. Due to whey is a very complex medium of high production capacity, the differences between synthetic media containing lactose and the raw material whey were extensively discussed. Whey fermentation resulted to much lower fermentation ability as compared with synthetic media containing lactose (Athanasiadis, 2003). The results of this investigation can be attributed to the theoretical background and approach of intracellular enzyme activity as well as to fermentation rate increase by cell immobilization. Therefore, the aim of this investigation was to examine the effect of various conditions on lactose uptake rate in whey fermentation using kefir, in order to reveal the role of uptake rate. This aim concerns the production of foods, such as kefir drink stimulating product, or the production of fuel grade ethanol, both related with environmental purposes. Likewise, finding perspectives of research on whey fermentation helps to approach new ways for the fermentation rate increment.

2. Methods

2.1. Microorganism and cell growth

Kefir, a commercial product usually used to produce kefir drink, was employed in the present study. Kefir grains were preserved

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into 1 L fresh pasteurised full cream milk, which was renewed every week, at approximately 4 °C. Cell growth of kefir biomass took place in a sterilized synthetic medium (2% lactose, 0.4% yeast extract, 0.1% (NH₄)₂SO₄, 0.1% KH₂PO₄ and 0.5% MgSO₄ · 7H₂O) at 30 °C under anaerobic conditions. Pressed wet-weight cells were prepared and used directly in anaerobic fermentation. All treatments were carried out in triplicate and the mean values are presented.

2.2. ¹⁴C-labelled lactose determination

At the beginning of each run 1 mL of labelled lactose [D-glucose-1-¹⁴C], (ARC 0466 lactose, and 0.1 mCi/mL) was added as in previous investigations (Golfinopoulos et al., 2009; Soupioni et al., 1998). The labelled lactose was fermented in the same way as the non-active one. At various time intervals, samples of 2 mL were filtered using cellulose membrane filters (0.45 μm) and ¹⁴C within the cells was determined by a liquid scintillation counter. The amount of the labelled lactose consumed by a specific amount of kefir biomass during fermentation was determined and expressed as cpm of lactose per gram of kefir biomass per hour.

2.3. Liquid scintillation measurements

All cellulose filters with cells contained ¹⁴C were put one by one in appropriate vials and 5 mL of liquid scintillation cocktail Opti Fluor (Perkin Elmer) was added. The measurements were performed on a PACARD-3255 liquid scintillation counter, interfaced to an APPLE-2 personal computer for data evaluation.

2.4. Determination of residual sugar and ethanol

Residual sugar concentrations in the whey samples were determined on a Shimadzu LC-9A HPLC system consisting of a Shim-pack SCR-101N column, an LC-9A pump, an RID-6A refractive index detector, a CTO-10A column oven, and a DGU-2A degassing unit. Three times distilled water 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 injection volume was 40 μL.

Ethanol was determined by gas chromatography using Porapac S column. Nitrogen was used as carrier gas at 40 mL/min. The column temperature was programmed at 120–170 °C at a rate 10 °C/min. The temperatures of the injector and FID detector were 210 and 220 °C, respectively. For the ethanol determination, a total volume of 2 μL for each sample was injected directly into the column and the concentration of ethanol was determined using standard curves. 1-Butanol was used as internal standard at a concentration of 0.5% (v/v) (Kopsahelis et al., 2009). All analyses were carried out in triplicate and the mean data are presented (standard deviation for all values was about ± 5%). Ethanol yield is the ratio of g ethanol/g of utilised sugar during the fermentation. Conversion was calculated by the following equation:

$$\frac{(\text{Initial sugar conc.} - \text{Residual sugar conc.})/\text{Initial sugar conc.} \times 100}{100} \quad (1)$$

2.5. Effect of pH value, temperature and cell concentration on lactose uptake rate during whey fermentation

Cheese whey (rennet whey) was obtained from a regional dairy industry (Agricultural Cooperative Union of Kalavryta, Kalavryta, Greece) and after pasteurization was introduced into a 250 mL Erlenmeyer flask.

To study the effect of pH value, a series of whey fermentations were carried out at 30 °C at different pH values, as 4, 5, 5.5, 6 and 6.5, using liquid biomass of 2.4% w/v. Trial pH value was achieved by the addition of tartaric acid (7% w/v), as the original whey pH was 6.5. During whey fermentation the pH value was maintained stable to the selected trial pH, by the addition of 6 M NaOH solution.

The effect of temperature on lactose uptake rate by kefir biomass was studied at various temperatures of 10, 20, 25 and 30 °C. The experiments were conducted under 5.5 pH value, which was achieved by the addition of tartaric acid (7% w/v) into the original whey. pH remain constant during the fermentation to the aforementioned value, by the addition of a 6 M NaOH.

Furthermore, the effect of kefir biomass concentration was studied by a series of fermentations using 32, 40, 48 and 56 g/L. Fermentations were carried out at 30 °C and the pH value of the fermented medium was kept constantly at 5.5 by the addition of 6 M NaOH solution. Initial trial pH value (5.5) was achieved by adding tartaric acid (7% w/v).

The kinetics of fermentation were monitored by specific lactose measurements in g/100 mL with HPLC. The amount of the consumed labelled lactose during a fermentation time interval was measured by liquid scintillation and expressed as cpm lactose per gram of biomass per hour. The recorded results were the mean value of three repetitions. The produced ethanol concentration, was expressed as grams per 100 mL of whey.

2.6. Data analysis

The data were analysed 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

Lactose uptake rate has been recently studied in synthetic media containing lactose (Golfinopoulos et al., 2009). However, whey differs extremely in chemical composition as compared with synthetic media containing lactose. Due to whey has shown differences in fermentation rate (Athanasiadis et al., 2005) using kefir microflora, a study that relates lactose uptake rate with fermentation kinetic, could reveal ways to affect lactose uptake rate and factors affecting it and are contained in whey. Therefore, a study concerning whey lactose uptake rate versus kinetic of whey fermentation is necessary. In the frame of the above claim the effect of pH, temperature, cell concentration and the ethanol content, on kefir-lactose uptake rate in whey fermentation were examined.

3.1. Effect of pH and ethanol on kefir-lactose uptake rate in whey fermentation

Fig. 1a and b illustrate the effect of pH on fermentation kinetics and lactose uptake rate respectively. It is clear that the optimum pH value for whey fermentation by kefir was 5.5. This result was identified with results achieved in previous study where synthetic lactose medium was fermented by kefir (Golfinopoulos et al., 2009). Likewise, Fig. 1b shows that the pH 5.5 was the value at which the highest lactose uptake rate was recorded, while the minimum one was observed at pH 4 value. Furthermore, residual sugar and ethanol concentration were also significantly affected (P < 0.05) by the alteration of pH value. More specific, at pH 5.5 ethanol concentration was significantly higher (P < 0.05) and resid-

Table 1
Effect of pH, temperature and initial biomass on kinetic parameters of whey fermentations using kefir.

Temp (°C)	Initial biomass (g/L)	pH	Initial Sugar (g/L)	Fermentation Time (h)	Ethanol Concentration (%v/v)	Ethanol Yield (g/g)	Residual Sugar (g/L)	Conversion (%)
30	24	4	5.05 ± 0.04	113	1.13 ± 0.05	0.24 ± 0.01	1.39 ± 0.03	72.47
30	24	5	4.98 ± 0.00	92	1.49 ± 0.17	0.24 ± 0.02	0.16 ± 0.03	96.78
30	24	5.5	5.03 ± 0.01	116.5	1.79 ± 0.04	0.28 ± 0.00	0.03 ± 0.03	99.40
30	24	6	5.00 ± 0.00	88.5	1.55 ± 0.03	0.25 ± 0.00	0.18 ± 0.02	96.40
30	24	6.5	5.02 ± 0.00	90	1.24 ± 0.05	0.20 ± 0.00	0.30 ± 0.06	94.02
10	24	5.5	5.32 ± 0.02	350	1.42 ± 0.04	0.42 ± 0.01	2.67 ± 0.03	49.81
20	24	5.5	5.30 ± 0.01	286.5	1.90 ± 0.08	0.35 ± 0.02	0.95 ± 0.11	82.07
25	24	5.5	5.24 ± 0.00	126	1.98 ± 0.06	0.42 ± 0.02	1.53 ± 0.06	70.80
30	24	5.5	5.17 ± 0.00	86	1.97 ± 0.12	0.30 ± 0.01	0.07 ± 0.00	98.64
30	32	5.5	5.35 ± 0.49	96.5	2.18 ± 0.02	0.33 ± 0.03	0.16 ± 0.09	97.00
30	40	5.5	5.60 ± 0.06	95	2.33 ± 0.02	0.34 ± 0.00	0.16 ± 0.05	97.14
30	48	5.5	5.26 ± 0.02	45	1.77 ± 0.05	0.31 ± 0.00	0.68 ± 0.05	87.07
30	56	5.5	5.30 ± 0.02	68.5	1.70 ± 0.09	0.26 ± 0.01	0.16 ± 0.02	96.98

ual sugar significantly lower ($P < 0.05$), compared to the other tested pH values (Table 1). Therefore, the significant effect of pH value on kefir natural co-culture fermentation can be attributed to the fact that lactose hydrolysis is affected by pH either through pH effect on lactase enzyme activity or through low resistance of various lactic acid bacteria contained in kefir microflora, at low pH values. The fermentation time in the case of whey was about 3fold higher compared to that of synthetic media containing lactose (Goufopoulos et al., 2009). This difference can be attributed to the fact that much more ions are contained in milk whey, which are attracted by the cell wall (Akrida-Demertzi et al., 1990; Akrida-Demertzi, 1987) and therefore reduce diffusion of lactose into the cell. Those results at 5.5 pH value were obtained, even though significantly ($P < 0.05$) higher alcohol concentrations were contained (Fig. 1c) (Table 1). Taking into consideration the aforementioned results, it seemed that alcohol inhibition on lactic acid bacteria contained in kefir microflora, does not affect the fermentation activity and lactose uptake rate, as shown in Fig. 1b.

3.2. Effect of temperature and ethanol on lactose uptake rate by kefir, in whey fermentation

Fig. 2a and b show the effect of temperature on kinetics of whey fermentation related with lactose uptake rate. The optimum temperature for the rate of fermentation and lactose uptake rate was 30 °C. Residual sugar was significantly ($P < 0.05$) affected by temperature and was significantly lower ($P < 0.05$) at 30 °C (Table 1). Ethanol concentration was also significantly higher ($P < 0.05$) at 25 and 30 °C compared to fermentations conducted at 10 and 20 °C (Table 1). Fig. 2a indicates big differences between the temperatures that were studied. Those differences were much bigger than those obtained by using synthetic media containing lactose (Goufopoulos et al., 2009). Lactose uptake rate was extremely increased at higher temperatures. That result leads to conclusion that the increase of the fermentation rate is mainly attributed to the increase of lactose uptake rate and secondly to the increase of the activity of the enzymes that are involved in lactose bioconversion process. The experimental effect of lactose uptake rate, obtained in the frame of our research, versus enzyme activity, on the rate of fermentation, is firstly stressed. Another factor, is that a lot of lactic acid bacteria which comprise 83%–90% of the kefir grains microbial count composition are mesophilic (Simova et al., 2002). In addition, the dominant microorganism in total kefir microflora, *Lactococcus lactis*, is also mesophilic (Jianzhong et al., 2009). Moreover, yeast count comprising 10%–17% of total microflora composition, show maximum ability to ferment lactose at these temperatures. The aforementioned discussion shows that the increase of fermentation rate as the temperature is increased has a complexity as regards the affecting factors. That is an original con-

clusion taking into account that the up today knowledge was the increase of lactose bioconverting enzyme activity by the increase of temperature (Whitaker, 1996). When comparing fermentation using synthetic media containing lactose with whey fermentation, the bigger differences obtained by temperature to temperature in the case of whey can be attributed to much higher uptake of ions such as Ca^{++} by the cell wall (Akrida-Demertzi et al., 1990). That reduces lactose uptake as it was observed for the case of glucose by the presence of Ca^{++} (Akrida-Demertzi et al., 1988; 1990; 1991; Akrida-Demertzi, 1987). The stress of cells at low temperatures (Garbutt M.J., 1997) could be also considered as a factor that reduces lactose uptake. Stress obtained by the formation of alcohol does not seem to cause any effect to the uptake of lactose (Fig. 2c).

3.3. Effect of cell concentration, on lactose uptake rate by kefir in whey fermentation

The Fig. 3a and b show the effect of cell concentration on the kinetics of whey fermentation related with lactose uptake rate. The increase of initial cell concentration resulted to an increase of the fermentation rate and to faster lactose concentration drop. However, the increase of fermentation rate is relatively low in comparison with the effect of pH and temperature. This small effect was also identified with the small differences of whey lactose uptake rate. This smaller effect, even though cell concentration increase means also an increase of enzyme concentration, can be attributed to the chemical composition of whey. Whey contains more Ca^{++} and other ions than lactose containing synthetic media. That is complied with results reported by Goufopoulos et al., 2009. The uptake of ions (Akrida-Demertzi et al., 1988; 1990; Akrida-Demertzi, 1987) by the cell wall inhibits the diffusion of lactose. Furthermore, Fig. 3c indicates in correlation with Fig. 3a and b, that ethanol does not seem to play any role in the rate of fermentation and whey lactose uptake rate, even though ethanol is an inhibitor especially for the bacteria contained in kefir.

As illustrated, lactose uptake rate was higher in the case of 48 g/L initial cell concentration during the first hours of the fermentation. After that point, the highest lactose uptake rate was observed by using 40 g/L initial cell concentration. Ethanol concentration was also significantly ($P < 0.05$) higher when 40 g/L initial cell concentration was used, compared to all other cases (Table 1). It is worth mentioning, that lactose uptake rate was decreased at high initial cell concentration (56 g/L). This could be explained as lactose uptake rate, which is expressed as cpm of lactose per gram of kefir biomass, per litre of whey and per hour, shows the highest dispersion of lactose substrate on larger amount of kefir cells and therefore reduction of lactose uptake rate of an individual kefir cell is observed. But when lactose synthetic med-

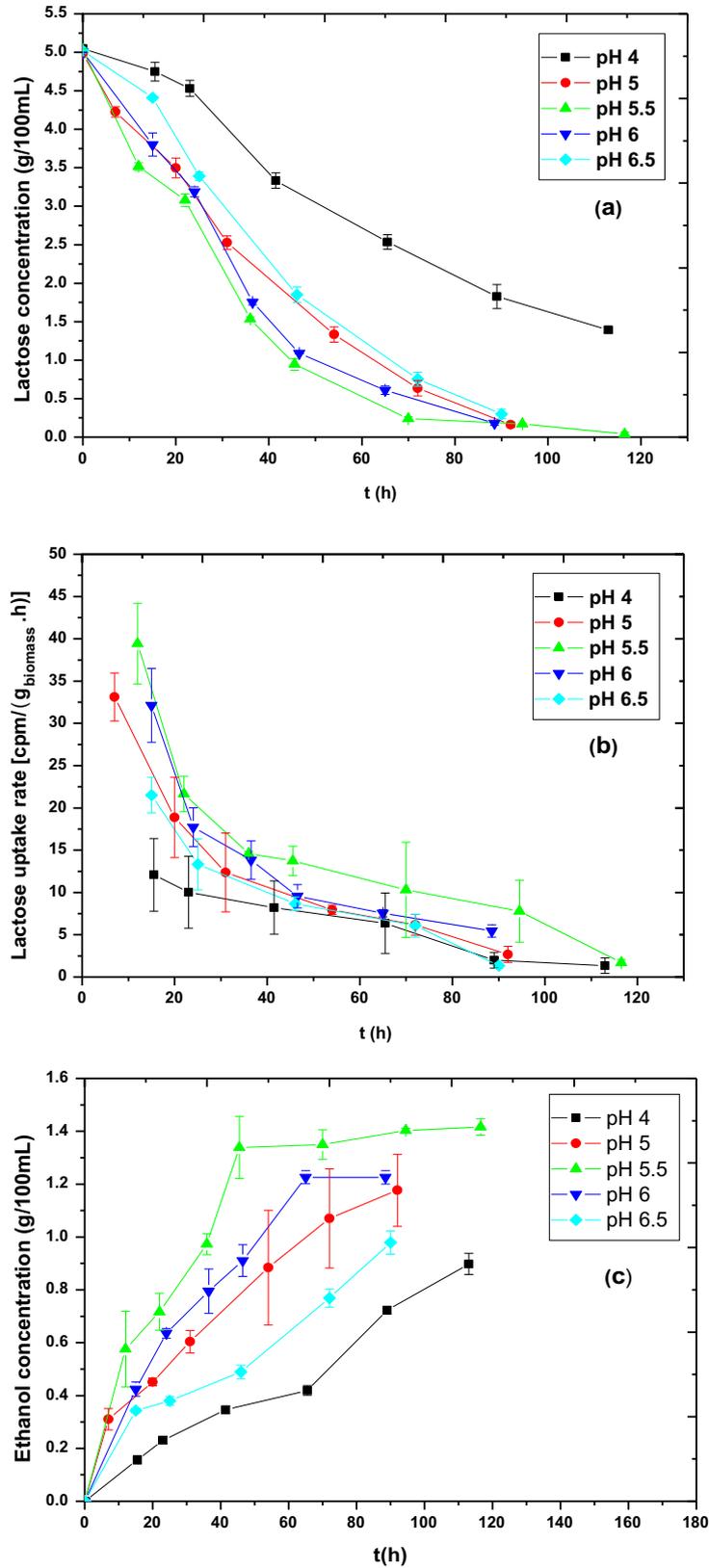


Fig. 1. (a) Fermentation kinetics of whey, (b) in relation with lactose uptake rate and (c) ethanol concentration during whey fermentation, observed at various initial pH values.

ium was fermented by kefir the optimum cell concentration was 16 g/L (Goulinopoulos et al., 2009). This difference between fermentation media, might be attributed to the complexity of whey medium in comparison to a synthetic one.

3.4. Perspectives of the investigation

Taking into account the results of this investigation, showing lower lactose uptake rate in whey fermentation as compared with

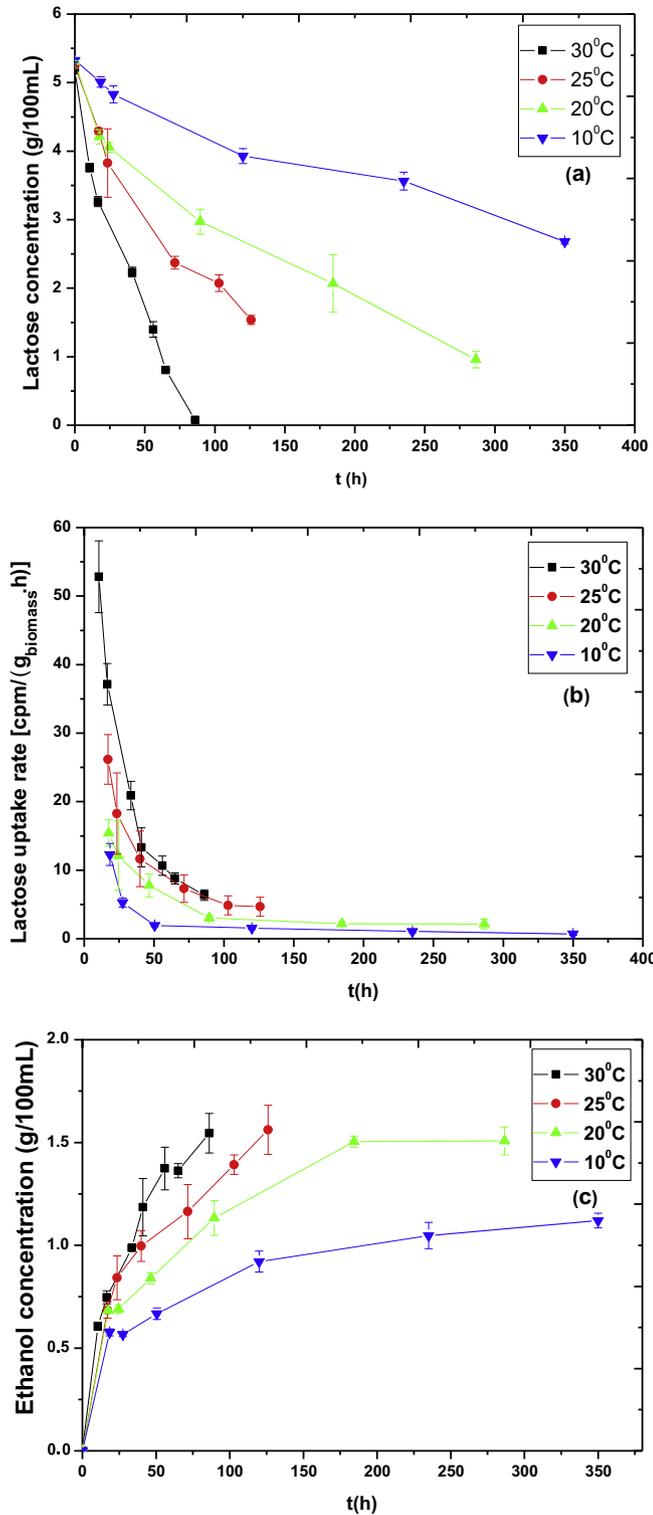


Fig. 2. (a) Fermentation kinetics of whey, (b) in relation with lactose uptake rate and (c) ethanol concentration during whey fermentation, observed for various temperatures.

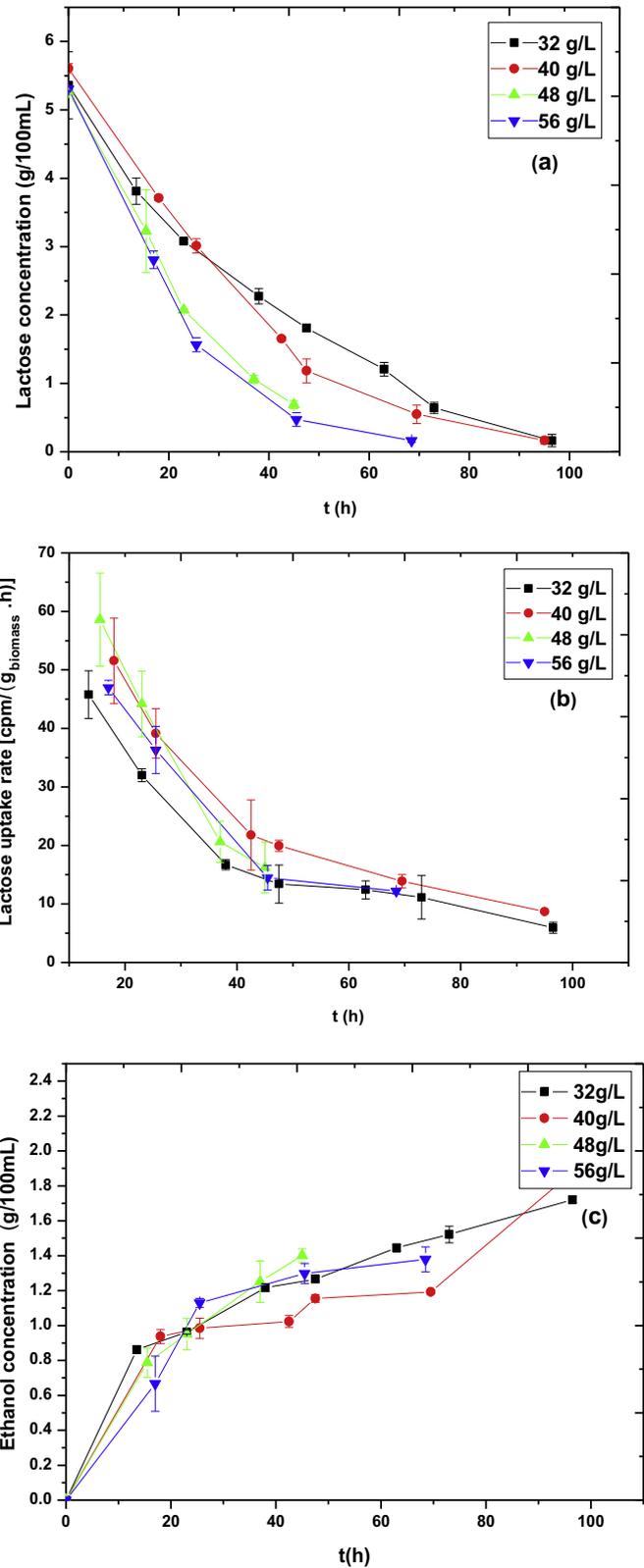


Fig. 3. (a) Fermentation kinetics of whey, (b) in relation with lactose uptake rate and (c) ethanol concentration during whey fermentation, observed at various kefir biomass concentrations.

synthetic media containing lactose, a research in order to increase whey fermentation could be planned. In the frame of this possibility, projects to examine the effect of the removal of ions in whey fermentation can be organized. Furthermore, a research can be conducted aiming to investigate the reasons of the much higher whey fermentation rate obtained by using nanotubular cellulose as immobilization support (Mitik-Dineva et al., 2008; Koutinas,

2008), compared with the fermentation rates obtained by using other immobilization supports. Likewise, a research project could be organized in order to examine promoters and their effect in glucose and lactose uptake rate. A known promoter of the

fermentation is γ -alumina pellets (Kanellaki et al., 1989) and the volcanic foaming rock kissiris (Tsoutsas et al., 1990).

4. Conclusions

Lactose uptake rate was strongly correlated to fermentation rate and increased during whey fermentation by kefir cells, as temperature was increased up to 30 °C. Moreover the highest lactose uptake rate was recorded at 5.5 pH value. High cell concentration didn't play any role in the fermentation of whey. The complex chemical composition of whey reduced fermentation rates and lactose uptake in comparison with synthetic media containing lactose. Likewise, ethanol didn't affect whey lactose uptake. The main reason for lactose uptake rate reduction in whey fermentation as compared with synthetic medium containing lactose is the presence of Ca^{++} in whey. The results of our investigation show the need and perspective to conduct studies in order to find alternative and more effective processes for whey fermentation. These processes could involve cell immobilization on nano and microtubular biopolymers, the use of promoters and the removal of ions and mainly Ca^{++} from whey.

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