Journal of Dairy Research (2001) **68** 653–661. © *Proprietors of Journal of Dairy Research 2001*
DOI: 10.1017/S0022029901005131 *Printed in the United Kingdom*

Polysaccharide production by kefir grains during whey fermentation

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(*Received 1 November 2000 and accepted for publication 2 July 2001*)

Summary. Fermentation of deproteinised whey with kefir grains CIDCA AGK1 was studied focusing on polysaccharide production from lactose. Kefir grains were able to acidify whey at different rates depending on the grain/whey ratio. During fermentation, kefir grains increased their weight and a water-soluble polysaccharide was released to the media. Exopolysaccharide concentration increased with fermentation time, reaching values of 57·2 and 103·4 mg/l after 5 days of fermentation in cultures with 10 and 100 g kefir grains/l, respectively. The polysaccharide fraction quantified after fermentation corresponded to the soluble fraction, because part of the polysaccharide became a component of the grain. Weight of kefir grains varied depending on the time of fermentation. Polysaccharide production was affected by temperature. Although the highest concentration of polysaccharide in the media was observed at 43 °C at both grain/whey ratios, the weight of the grains decreased in these conditions. In conclusion, kefir grains were able to acidify deproteinised whey, reducing lactose concentration, increasing their weight and producing a soluble polysaccharide.

Keywords: Kefir, lactic acid bacteria, exopolysaccharide, fermented whey.

Kefir is a fermented milk product of ancient origin. It has a low alcohol content and can be distinguished from other fermented milks by its peculiar starter culture: the kefir grains. Kefir grains are clusters of microorganisms held together by a matrix of protein and polysaccharide (Bottazzi *et al.* 1994; Abraham & De Antoni, 1999). They are gelatinous and irregular particles that resemble cauliflower florets with a diameter ranging from 0·1 to 4 cm. Kefir microflora depends on the origin of the grain and consists mainly of lactic acid bacteria (LAB), yeast and acetic acid bacteria. LAB are represented by homofermentative and heterofermentative lactobacilli, lactococci and leuconostoc. Lactose fermenting and non-fermenting yeasts are also present in a symbiotic association with the bacteria (Garrote *et al.* 1997).

LAB are generally recognised as safe (GRAS), and are known to produce extracellular polysaccharides (EPS), which contribute to the texture of the resulting fermented milk. Microbial polysaccharides can be used as thickeners, viscosifying, emulsifying, or gelling agents in foodstuffs (Cerning, 1995; Marshall & Rawson, 1999).

The polysaccharide produced by kefir microorganisms is commonly known as kefiran. It is a water-soluble branched glucogalactan containing equal amounts of D-glucose and D-galactose (Mukai *et al.* 1988; Micheli *et al.* 1999). Antitumour activity

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of kefiran has been reported (Murofushi *et al.* 1983). Several efforts have been directed to the isolation of polysaccharide-producing LAB from kefir grains. Polysaccharide biosynthesis is generally ascribed to *Lactobacillus kefir* (Toba *et al.* 1987) or *Lactobacillus kefiranofaciens* (Fujisawa *et al.* 1988; Yokoi *et al.* 1990). Recently, a *Lactobacillus* strain producing an exopolysaccharide (EPS) with similar properties to kefiran was isolated from the grains (Micheli *et al.* 1999).

Whey is produced in large amounts by dairy industries and thus is readily available to use as substrate for the production of ethanol, lactic acid, food yeasts, and protein (Boyaval, 1988; Krischke *et al.* 1991; Chiarini *et al.* 1992). Whey has been used to grow polysaccharide-producing microorganisms that consume lactose, decreasing the whey biological oxygen demand (BOD) (Gassem *et al.* 1997; Fialho *et al.* 1999). Little is known about polysaccharide production in milk by kefir grains and to our knowledge, no results have been published about polysaccharide production in whey.

The purpose of this work was to evaluate the ability of kefir grain to ferment deproteinised (DP-)whey, focusing on polysaccharide production from lactose. Variation of the grain/milk ratio produced changes in acidity and viscosity of fermented milk. Taking into account the different properties of the kefir beverages obtained with different grain/milk ratios (Garrote *et al.* 1998), two grain/whey ratios were studied.

Materials and methods

Starter culture

Kefir grains CIDCA AGK1 were originally obtained from a household in Argentina. The grains were maintained at –20 °C and were reactivated by successive subcultures in UHT low fat milk.

Chemicals and culture media

Yeast extract, tryptone, MRS-agar and MRS broth (De Man *et al.* 1960) were obtained from Difco (Detroit, MI 48232, USA). Agar was obtained from Britania (Buenos Aires, Argentina). Anthrone, urea and thin layer chromatography reagents were obtained from Sigma (St. Louis, MO 63178, USA). Silica gel G type 60 plates were purchased from Merck (D-64271 Darmstadt, Germany). Yeast extract-Glucose-Chloramphenicol-agar (YGC-agar) and bromcresol purple (BCP) were obtained from Merck. Commercial UHT skim milk, obtained from Sancor (Santa Fe 2322, Argentina) was used to propagate the grains and prepare the DP-whey.

Preparation of deproteinised whey

DP-whey was prepared by adjusting skim milk to pH 4·6 with 2 M-HCl and then heating for 30 min at 100 °C and filtering (Whatman No. 1 paper). The resulting supernatant was adjusted to pH 6·8 with 2 M-NaOH, heated 30 min at 100 °C and filtered again to obtain DP-whey. The resulting DP-whey was sterilised at 121 °C for 15 min. DP-whey composition obtained by this procedure was as follows: $53{\cdot}4 \pm 3{\cdot}1$ g lactose/l (determined by the anthrone method; Southgate, 1976), $3{\cdot}19 \pm 0{\cdot}25$ g/l of total nitrogen expressed as protein and $2{\cdot}74 \pm 0{\cdot}06$ g/l of non-protein-nitrogen expressed as protein equivalent (conversion factor: 6·38) determined by the Kjeldahl method according to IDF standard 20 B (International Dairy Federation, 1993).

DP-whey fermentation

Kefir grains were washed with sterile water and inoculated into 100 ml DP-whey at 10 and 100 g/l. After incubation at the controlled temperature, grains were

separated from the fermented whey by filtration through a plastic sieve (sanitised by immersion in 700 ml ethanol/l and then washed with sterile water) and washed prior to the next culture passage (subculture).

Growth and acidification kinetics were performed at 20 °C (unless stated otherwise) in DP-whey. At regular time intervals, samples were taken and pH was determined. Numbers of viable bacteria and yeasts, lactose concentration, grain weight increment and polysaccharide production were also determined in each sample.

To evaluate the effect of temperature, whey aliquots were inoculated with kefir grains and incubated at temperatures of 20, 30, 37 and 43 °C until pH 4 was reached. Polysaccharide concentrations in DP-whey and grain weight increments were determined in each sample.

Enumeration of viable microorganisms

Both viable bacteria and yeasts concentrations were determined in kefir grain suspensions and in fermented whey as previously published (Garrote *et al.* 1997, 1998) by plating serial dilutions prepared in tryptone (1 g/l) on Lee's agar, MRS-agar and YGC-agar plates. The results were expressed as colony-forming units (cfu)/ml of fermented DP-whey and cfu/g of kefir grain.

Weight increment of kefir grains

Samples (1 g) were weighted in a Precisa 205A analytical scale (precision $\pm 0{\cdot}1$ mg) and added to 10 and 100 ml of DP-whey. After different times of incubation, grains were separated from the fermented whey by filtration, dried between tissue papers and weighed again. In each case, kefir grains were dried until constant weight. Weight increment was expressed as the difference between final and initial grain weight.

Kefir grain composition

Water content of grains was determined by drying at 100 °C until a constant weight was reached. Sugar content was determined by the anthrone method. Under the assay conditions, all the carbohydrates containing hexoses were determined (Southgate, 1976). Protein content was determined by the Kjeldahl method according to IDF standard 20 B (International Dairy Federation, 1993).

Polysaccharide isolation and quantification

After removal of kefir grains by filtration, fermented DP-whey was heated in a boiling water bath for 15 min in order to dissolve the polysaccharide attached to cells and to inactivate the enzymes that could hydrolyse the polymer. Cells were removed by centrifugation at 10000 ***g*** and 20 °C for 15 min in a Sorvall RC-5B plus centrifuge (Sorvall Products, L.P. Newtown, CN, U.S.A). Polysaccharide from the fermented DP-whey was precipitated by the addition of two volumes of cold ethanol and maintained at 4 °C for 24 h. Then, samples were centrifuged at 10000 ***g*** and 4 °C for 15 min. Pellets were resuspended in hot water and samples were dialysed against distilled water for 24 h at 4 °C (cut-off: 1000 Da). Total sugars from the dialysed solution were determined by the anthrone method. All the samples were tested for the absence of lactose.

Lactose determination

Lactose from fermented whey samples and polysaccharide pellets was determined by thin layer chromatography (TLC) on Silica gel G type 60 plates using *n*-propanol-

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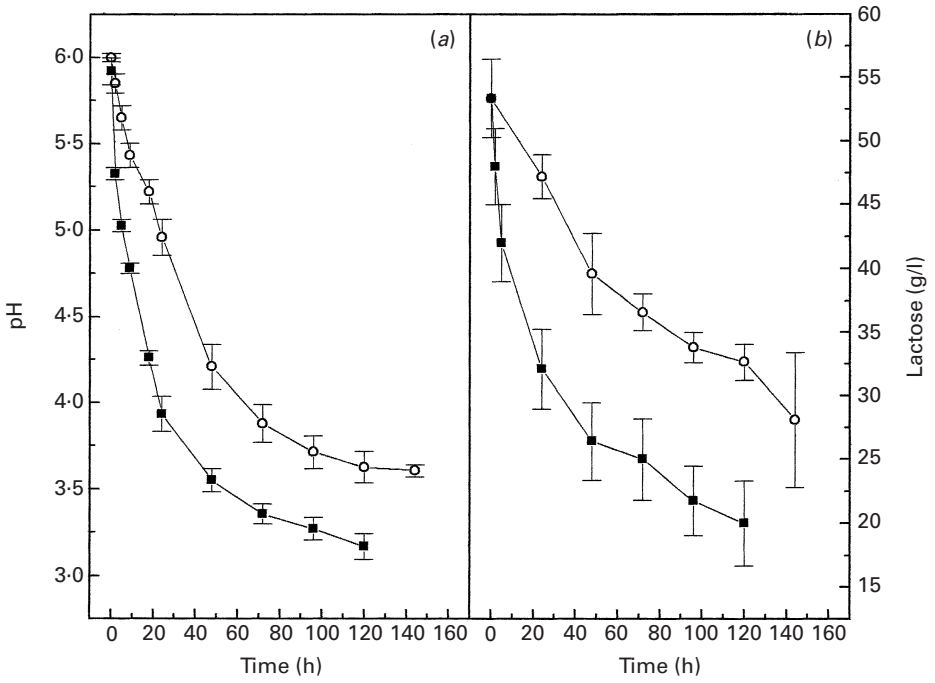

Fig. 1. Kinetics of acidification (*a*) and lactose consumption (*b*) in whey. Key: (○) 10 g of kefir grains per litre of whey, (■) 100 g of kefir grains per litre of whey.

acetic acid-water (70:20:10) as running solvent. TLC plates were developed with *p*-amino benzoic acid 7 g/l and *o*-phosphoric acid 30 g/l in methanol (Zweig & Sherma, 1978). Sugars were identified by comparison with retention factor of standard sugars. TLC plates were scanned and the intensity of the bands was determined with Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA 94547, USA). Lactose was used to construct a calibration curve.

Statistical analysis

Differences in grain composition, lactose consumption, polysaccharide production and grain weight increment were tested for significance by analysis of variance (ANOVA). Differences were considered at $P \leqslant 0{\cdot}05$.

RESULTS

Chemical and microbiological composition of kefir grains grown in DP-whey

Chemical composition of grains after 20 subcultures in DP-whey was 50 ± 6 g protein/kg, 82 ± 16 g polysaccharide/kg and 807 ± 13 g water/kg. Grain microflora was composed of $1{\cdot}8 \times 10^8 \pm 1{\cdot}4 \times 10^8$ cfu LAB/g and $2{\cdot}1 \times 10^7 \pm 1{\cdot}7 \times 10^7$ cfu yeasts/g. These values did not differ ($P \leqslant 0{\cdot}05$) from those obtained with the same grains grown in milk (Abraham & De Antoni, 1999).

Fermentation of DP-whey by kefir grains

Different grain/whey ratios produced different acidification rates (Fig. 1*a*). A higher rate of acid production was observed in cultures with 100 g inoculum/l, and pH $3{\cdot}93 \pm 0{\cdot}10$ was reached after 24 h incubation. On the other hand, for cultures using 10 g inoculum/l, 60 h incubation was needed to reach pH 4. Lactose

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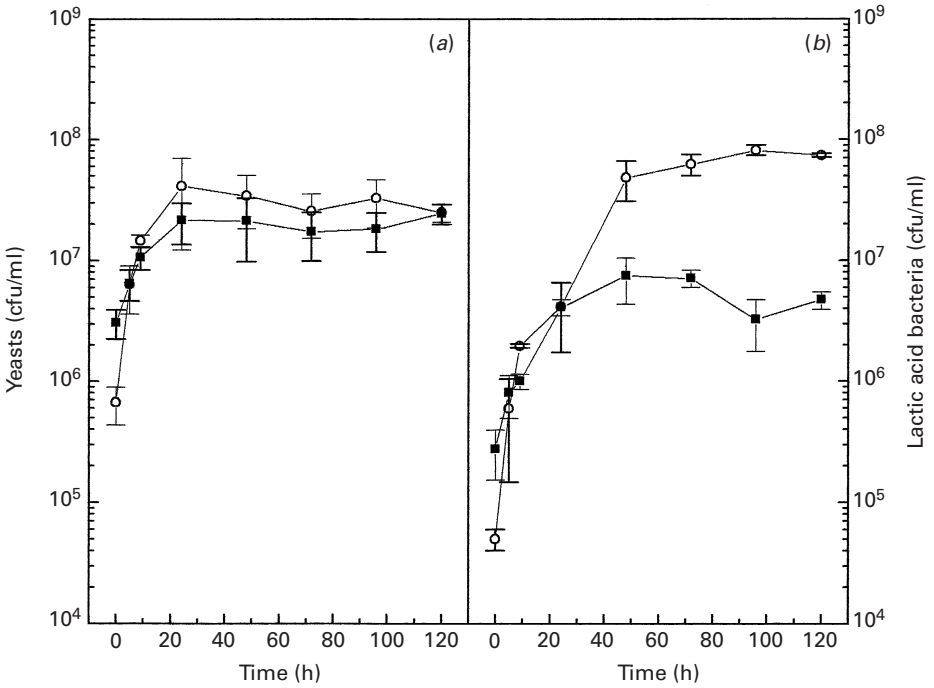

Fig. 2. Growth kinetics of yeasts (*a*) and lactic acid bacteria (*b*) in whey. Key: (○) 10 g of kefir grains per litre of whey, (■) 100 g of kefir grains per litre of whey.

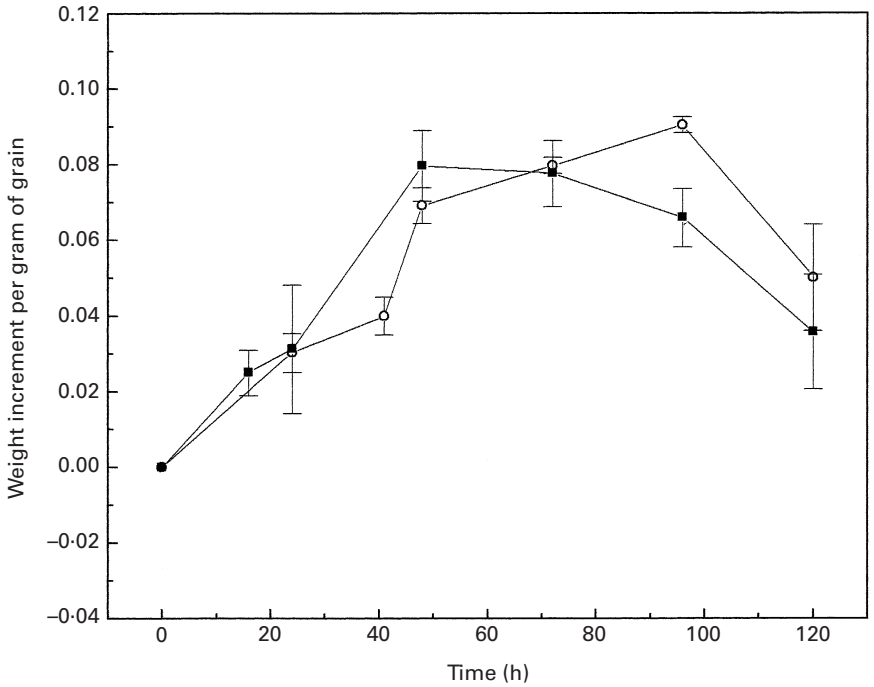

Fig. 3. Weight increment of kefir grains in whey versus time of fermentation at 20 °C. Key: (○) 10 g of kefir grains per litre of whey, (■) 100 g of kefir grains per litre of whey.

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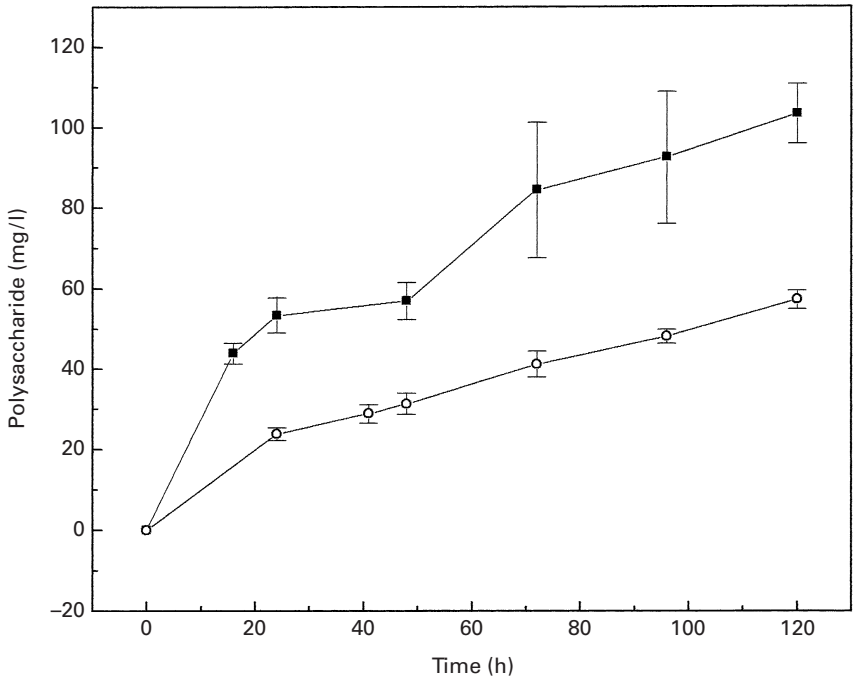

Fig. 4. Kinetics of polysaccharide production in whey at 20 °C. Key: (○) 10 g of kefir grains per litre of whey, (■) 100 g of kefir grains per litre of whey.

consumption corresponded to the drop in pH (Fig. 1*b*). After 24 h incubation, lactose concentration had decreased significantly ($P \leqslant 0{\cdot}05$) from $53{\cdot}4 \pm 3{\cdot}1$ g/l to $47{\cdot}2 \pm 1{\cdot}7$ g/l and $32{\cdot}1 \pm 3{\cdot}1$ g/l, where levels of inoculum were 10 and 100 g/l, respectively. After 120 h, lactose concentration was reduced to 32·6 and 20·0 g/l, respectively.

A liberation of microorganisms from the kefir grain was observed soon after mixing the grains with DP-whey (Fig. 2, $t = 0$). The number of yeasts and LAB released was one log unit higher when 100 g grain/l was inoculated. The growth kinetics of LAB and yeasts are shown in Fig. 2. The yeasts reached stationary phase after 24 h incubation ($2{\cdot}1$–$4{\cdot}1 \times 10^7$ cfu/ml) with both grain/whey ratios, suggesting that the growth kinetics were similar regardless of the initial number of yeasts (Fig. 2*a*). The number of viable bacteria in cultures with 10 g inoculum/l increased 3 log units after 48 h incubation, whereas in cultures with 100 g/l, the number of viable LAB increased 1·5 log units. LAB reached stationary phase when the pH of cultures was approximately 4, indicating that pH may affect the growth of LAB.

After 48 h fermentation with the higher inoculum, grain weights increased $0{\cdot}08 \pm 0{\cdot}01$ g /g of grain. This value was constant up to 96 h (means obtained at 48, 72 and 96 h were not significantly different, $P \leqslant 0{\cdot}05$). The weight fell significantly ($P = 0{\cdot}02$) by 120 h, (Fig. 3). When the inoculum was 10 g/l, grain weights increased until 96 h before finally decreasing. This result indicates that after a certain time of incubation or under certain environmental conditions, the grains might dissolve, liberating their components to the media.

Polysaccharide production by kefir grains in whey

As shown in Fig. 4, a water-soluble polysaccharide was released to the media during whey fermentation. When 100 g inoculum/l was used, polysaccharide

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Table 1. *Effect of temperature on polysaccharide production in whey fermented with different grain/whey ratios*

(Values are means ± SD for $n = 4$† or $n = 2$‡; independent experiments)

	Grain/whey ratio: 10 g/l		
Temperature (°C)	Hours to reach pH 4·00	Weight increment (g/g grain)†	Polysaccharide concentration (mg/l)‡
20	41	$0{\cdot}040 \pm 0{\cdot}005^{a}$	$28{\cdot}9 \pm 2{\cdot}3^{a}$
30	27	$0{\cdot}050 \pm 0{\cdot}020^{a}$	$30{\cdot}0 \pm 4{\cdot}9^{a}$
37	21	$-0{\cdot}010 \pm 0{\cdot}004^{b,c}$	$40{\cdot}1 \pm 5{\cdot}4^{a}$
43	19	$-0{\cdot}055 \pm 0{\cdot}012^{c}$	$60{\cdot}8 \pm 3{\cdot}4^{c,d}$
	Grain/whey ratio: 100 g/l		
	Hours to reach pH 4·00	Weight increment (g/g grain)†	Polysaccharide concentration (mg/l)‡
20	16	$0{\cdot}025 \pm 0{\cdot}006^{a}$	$43{\cdot}9 \pm 2{\cdot}7^{a,b}$
30	6	$0{\cdot}030 \pm 0{\cdot}014^{a}$	$51{\cdot}1 \pm 1{\cdot}6^{b,c}$
37	5·5	$-0{\cdot}003 \pm 0{\cdot}006^{b}$	$66{\cdot}6 \pm 5{\cdot}4^{d}$
43	4·5	$-0{\cdot}027 \pm 0{\cdot}009^{c}$	$78{\cdot}7 \pm 5{\cdot}1^{e}$

[a,b,c,d,e] Means for each parameter without a common superscript are significantly different ($P \leqslant 0{\cdot}05$)

concentration was $50{\cdot}4 \pm 4{\cdot}4$ mg/l after 24 h fermentation reaching a value of $103{\cdot}4 \pm 7{\cdot}4$ mg/l after 120 h incubation. When 10 g inoculum/l was used, concentration of polysaccharide was $57{\cdot}2 \pm 2{\cdot}3$ mg/l after 120 h incubation. Although higher values of polysaccharide concentration were obtained in cultures with the higher grain/whey ratio, polysaccharide concentration per gram of grain was higher with the lower level of inoculum.

Polysaccharide production at different temperatures

Table 1 shows the effect of incubation temperature on fermentation time to reach pH 4, grain weight increment and polysaccharide production. The time to reach pH 4 decreased with increase of incubation temperature, as expected. When fermentation was performed at 20 and 30 °C grain weight increased, whereas incubation at higher temperatures resulted in a decrease in weight. The highest amount of polysaccharide and the highest decrease in biomass was obtained at 43 °C, indicating that the polysaccharide quantified under these conditions would be in part produced by the microflora and also from dissolution of the grain. These results were obtained at both grain/whey ratios.

DISCUSSION

The disposal of whey represents an environmental problem in Argentina and proper disposal of this by-product has been a concern for the dairy industry. Milk whey is a source of protein with a high nutritional value and consequently it is used to obtain whey protein isolates (WPI) or whey protein concentrates (WPC). A suitable way to take advantage of the residual DP-whey is as a substrate for the production of useful products such as bacterial exopolysaccharide. Kefir grains CIDCA AGK1 grew and produced exopolysaccharide in DP-whey from lactose, suggesting that whey proteins were not required for this process; lactose would

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be the only carbon source. Additionally, the decrease in the amount of fermentable sugar contributed to a decrease in the BOD of the whey.

Two kinetics were observed during whey fermentation with kefir grains: (a) grains increased their weight as a consequence of the growth of microorganisms and the biosynthesis of grain components (Fig. 3), and (b) each kind of microorganism grew free in the whey (Fig. 2). Kefir grains varied in weight depending on time of fermentation. However, the drop in the grain weight after 96 h incubation (Fig. 3) was not reflected in an increment of viable microorganisms (Fig. 2), suggesting a balance between the microorganisms originating from the dissolution of the grains and those that would lose viability because of the low pH.

Dairy LAB are weak EPS producers. EPS production in milk depends strongly on the fermenting species even at strain level and range in concentration between 50 and 600 mg/l (Cerning, 1995). The amount of polysaccharide produced by kefir grains (100 mg/l) in DP-whey was similar to that obtained with other LAB grown in synthetic media (Kimmel *et al*. 1998; Petry *et al*. 2000). However, the amount of polysaccharide quantified in this system represented the soluble fraction, ignoring that part of the polysaccharide incorporated into the grains, observed as grain weight increase. When grains decreased in weight, the total amount of polysaccharide quantified included soluble EPS and polysaccharide released from grains. Consequently, care should be taken when maximum concentration of polysaccharide without reduction of grain weight is to be obtained. The decrease in weight was not reflected in a higher production of soluble polysaccharide. This observation could be ascribed to a degradation of the polysaccharide. Several authors reported that LAB liberate cytoplasmic hydrolases after certain incubation times, which could degrade the polysaccharide (Gancel & Novel, 1994; Pham *et al*. 2000). Glucose, galactose and one unidentified oligosaccharide where detected by TLC in whey samples after 96 h fermentation. The unidentified oligosaccharide may be a product of polysaccharide hydrolysis at long fermentation times, since it was not detectable at short times of incubation (data not shown).

Temperature of fermentation affected both the increment of grain biomass and the amount of polysaccharide. The highest level of polysaccharide was determined at the temperature where the highest decrease in biomass was obtained. On the other hand, the highest concentrations of polysaccharide were obtained at 20 and 30 °C, considering soluble polysaccharide as well as that contained in the grain.

Because polysaccharide can be obtained from fermented whey and from the kefir grain (Toba *et al*. 1987; Mukai *et al*. 1988, 1991; Pintado *et al*. 1996; Micheli *et al*. 1999), time and temperature of fermentation have to be considered in order to obtain optimum polysaccharide production.

Whey fermentation by kefir grains could be a sensible solution for the use of this by-product from the dairy industry. A new application to whey would increase its market value. The procedure described in this work not only decreases the level of lactose, reducing waste disposal problems, but also highlights a source of potentially useful polysaccharide produced by a GRAS starter.

P. S. Rimada is a research fellow of UNLP. A. G. Abraham is a member of the Researcher Career of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The authors are grateful to L. A. Brandi and M. S. Zuluaga for technical assistance. Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), CONICET, and Universidad Nacional de La Plata (UNLP) supported this work.

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