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Production of freeze-dried kefir culture using whey

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Abstract

A freeze-dried kefir culture produced using whey as raw material was studied in three steps. The first step examined the effect of air flow rate and agitation in a bioreactor for the production of biomass. The highest daily biomass productivity of 14.1 g dry weight L^{-1} was obtained when 8.3 vvm air flow rate was used, where vvm is volume of air per unit volume of medium min^{-1} and is defined as the ratio of air volume fed to the volume of ungassed broth in the fermenter min^{-1}. In the second step, production of freeze-dried cultures was studied testing various cryoprotective agents and cooling rates. Fermented whey proved to be a suitable cryoprotective medium, providing 86% survival of the kefir culture and showing satisfactory metabolic activity of freeze-dried cultures. The third step involved evaluation of the freeze-dried product in carbohydrate fermentations, showing a high operational stability during repeated batch fermentations. The use of freeze-dried kefir culture in food production as a value-added starter culture thus appears possible.

Keywords: Kefir; Whey; Freeze-drying; Fermentation

1. Introduction

Kefir culture is used in fermenting milk to produce the traditional drink ''kefir'' with low alcohol content. Kefir grains are gelatinous white or cream-coloured, water-insoluble, irregular granules with diameter ranging 0.3–3.5 cm, which resemble small cauliflower florets. They are composed mostly of proteins and polysaccharides in which the complex microflora is enclosed (Garrote, Abraham, & De Antoni, 1997; Zourari & Anifantakis, 1988). This mixed culture consists of various yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia*), various lactic acid bacteria of the genus *Lactobacillus, Lactococcus*, *Leuconostoc* and acetic acid bacteria (Garrote et al., 1997; Luis, Lopez, & Lema, 1993; Pintado, Lopes Da Silva, Fernades, Malcata, & Hogg, 1996; Witthuhn, Schoeman, & Britz, 2005). Yeast and lactic acid bacteria co-exist in a symbiotic association and are responsible for an acid–alcoholic fermentation. This mixed culture is able to utilize lactose and therefore whey could be used as a raw material for kefir production (Iconomopoulou et al., 2001; Koutinas, Athanasiadis, Bekatorou, Iconomopoulou, & Blekas, 2005). Immobilization of kefir yeast on delignified cellulosic (DC) material was previously reported (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001). The immobilized biocatalyst was found to be suitable for continuous alcohol production using whey (Kourkoutas, Psarianos, et al., 2002) and for whey beverage production (Athanasiadis, Paraskevopoulou, Blekas, & Kiosseoglou, 2004). Likewise, kefir culture has been proposed as baker's yeast in baking (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005) while, recently, freeze-dried kefir culture was evaluated as a starter in cheese production (Kourkoutas et al., 2006).

Freeze-drying is considered to be a valuable preservation method for foods and, to a greater extent, for microorganisms. The major advantages of freeze-drying are: (i) the avoidance of special requirements during storage; (ii) the avoidance of cooling facilities during dispatch of the product; and (iii) the convenience in handling freeze-dried powders compared with frozen products. However, the freeze-drying process exposes the cells to a stressful processing step. Since freeze-drying results in loss of cell

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viability, factors such as cooling rate and cryoprotective agents are critical for the viability of cells (Benry & Hennebert, 1991; Gehrke, Pralle, & Decker, 1992). A number of publications concerning freeze-drying of a variety of microorganisms are available in the literature (Bekatorou, Koutinas, Psarianos, & Kanellaki, 2001; Boss, Filho, & Vasco de Toledo, 2004; Iconomopoulou, Kanellaki, Psarianos, & Koutinas, 2000; Palmfeldt, & Hahn-Hägerdal, 2000; Palmfeldt, Rådström, & Hahn-Hägerdal, 2003; Tal, Rijin, & Nussinovitch, 1997). Recently, the effects of sugars on survival of microorganisms present in kefir during freeze-drying were studied (Chen, Lin, & Chen, 2006). Although whey has been extensively used as a fermentation medium for production of a number of products, such as oligonucleotides, single cell protein, potable alcohol, and alcoholic beverages (Athanasiadis, Boskou, Kanellaki, Kiosseoglou, & Koutinas, 2002; Athanasiadis et al., 2004; Belem & Lee, 1999; Hosseini, Shojaosadati, & Towfighi, 2003; Kourkoutas, Dimitropoulou, et al., 2002; Kourkoutas, Psarianos, et al., 2002; Parrondo, Garcia, & Diaz, 2000), to the authors' knowledge, it has never been used as a cryoprotective medium.

Since production of wet cultures is incompatible with commercial operations due to the physical statue of the culture, the objectives of the present research were: (i) to investigate efficient production of freeze-dried kefir culture using whey as raw material; and (ii) to study the effect of freeze-drying on the metabolic and fermentative activity of the freeze-dried product, in order to produce a marketable starter culture for dairy fermentations.

2. Materials and methods

2.1. Whey preparation

Whey was produced in the laboratory from commercial bovine milk (MEVGAL, S.A., Thessaloniki, Greece), which was heated to 37 °C, whereupon 0.01% commercial rennet (Vlacha, Athens, Greece) was added and left undisturbed for 2 h for curd formation. Subsequently, the curd was cut in squares (diameter ~1 cm), left undisturbed for 10 min and was cloth filtered at room temperature (18–22 °C). Whey contained ~5% lactose (lactose was determined by HPLC as described in Section 2.5) and ~0.8% protein (protein was determined using the Kjeldahl procedure as described in Section 2.5).

2.2. Kefir culture

Kefir culture, isolated from commercially available kefir grains (MELITON S.A., Thessaloniki, Greece) usually used to produce kefir drink, was employed in the present study, and grown on a synthetic medium consisting of 4% lactose (Sigma-Aldrich, Buchs, Switzerland), 0.4% yeast extract (Fluka, Buchs, Switzerland), 0.1% $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany), 0.1% KH_2PO_4 (Fluka) and 0.5% $MgSO_4 \cdot 7H_2O$ (Merck) at 30 °C. The pH of the synthetic medium prior to inoculation was 5.6. The synthetic medium was sterilized at 130 °C for 15 min prior to use. Pressed wet weight cells (≈0.5–1.0 g dry weight) were prepared by growing in the above medium and employed directly in the aerobic fermentations.

Kefir culture, 1 g (dry weight) and 500 mL of whey supplemented with 4 g L^{-1} $(NH_4)_2SO_4$ (Merck), 2 g L^{-1} KH_2PO_4 (Fluka), and 1% (v/v) Tween 80 (Fluka) were added to a glass bioreactor (University of Patras glassworks, Patras, Greece; 42.5 cm height and 7.4 cm internal diameter, total volume 1800 mL) and allowed to ferment at 30 °C, until constant optical density (maximum biomass concentration). Sterilized air was continuously supplied to the reactor using an air compressor (Compressori D' Aria LT 50 HP 1.5, Fini, Bologna, Italy). Foam was controlled by adding adequate amounts of antifoam (Silicon antifoaming agent, Merck) when necessary. The pH was continuously adjusted to 5.5–6.0 using 2 M NaOH. The effect of size of pores in the perforated air distribution tube, air flow rate and agitation was studied. Therefore, pore sizes of 0.5, 1.0, 3.0 and 8.0 mm diameter in the perforated air distribution tube, air flow rates of 1.0, 2.3, 5.0 and 8.3 vvm, where vvm is volume of air per unit volume of medium min^{-1} and is defined as the ratio of air volume fed to the volume of ungassed broth in the fermenter min^{-1}, and agitation at 300, 450 and 600 rpm were tested. In order to overcome errors arising from concentration of whey components due to water loss through exhaust air, sterile distilled water was added manually to the bioreactor when necessary to counterbalance water loss. Aerobic fermentations were carried out in triplicate and the results presented are the mean values (standard deviation for all values was about ±5% in most cases). Samples were collected at various intervals (every ~3–5 h) and analysed for biomass concentration, and level of lactic acid, residual sugar, and ethanol, as described in Section 2.5.

2.3. Freezing, freeze-drying and storage

Kefir culture produced from the above aerobic fermentation process using 0.5 mm diameter pores in the perforated air distribution tube, 8.3 vvm air supply, and no agitation, were freeze-dried. Kefir culture, corresponding to 0.5 g dry weight, was suspended in 4 mL of a cryoprotective agent and the mixture was then frozen at −45 °C in a controlled-rate freezer (BioCool, FTS Systems, NY, USA), as similar temperatures have been previously proposed for freezing microbial cultures (Bekatorou, Koutinas, Kaliafas, & Kanellaki, 2001; Blanquet et al., 2005; Iconomopoulou, Psarianos, Kanellaki, & Koutinas, 2002). The effect of cryoprotective agents and cooling rate on viability and fermentation activity of kefir cells was studied. The cryoprotective agents used were $\frac{1}{4}$ ringer solution, unfermented and fermented whey, glucose, fructose, sucrose, trehalose (all from Merck), and lactose

(Sigma-Aldrich) at 5% and 10%, 1% honey solution (Attiki, Athens, Greece) in distilled water, 1% glycerol, and skim milk (both from Merck), while the cooling rates tested were 1, 3, 4, and 5 °C min^{-1}. The frozen samples were freeze-dried overnight at 5×10^{-3} bar and at −45 °C in a Freeze Dry System, Freezone 4.5 (Labconco, Kansas City, Missouri, USA) and then employed in batch fermentations. The effect of storage at 4 °C of freeze-dried kefir culture on kefir cells viability and fermentation activity was also examined. The freeze-dried cultures were packaged aerobically in a sterilized vial sealed with hydrophobic cotton and stored in a refrigerator (RH 77–79%) for 12, 30, 90 and 180 d. Water content of the freeze-dried cultures before and after storage was in the range 0.95–0.98%.

2.4. Batch alcoholic fermentations using freeze-dried kefir culture

In order to study the effect of cryoprotective agent and cooling rate on fermentative activity of freeze-dried kefir culture, batch fermentations of synthetic medium consisting of 5% lactose and nutrients [0.4% yeast extract (Fluka), 0.1% $(NH_4)_2SO_4$ (Merck), 0.1% KH_2PO_4 (Fluka), and 0.5% $MgSO_4 \cdot 7H_2O$ (Merck)], as described above, were carried out at 30 °C, using 2.5 g L^{-1} freeze-dried kefir culture.

In addition, the effects of carbohydrate substrate, initial sugar concentration and initial freeze-dried kefir culture concentration on alcoholic batch fermentations at 30 °C using freeze-dried kefir culture were studied. Freeze-drying of kefir culture was carried out using fermented whey as cryoprotective agent at a cooling rate of 3 °C min^{-1}.

The effect of fermentation temperature (30, 15, and 5 °C) was studied in a series of repeated batch fermentations of a synthetic medium containing 5% glucose, 5% fructose, 5% sucrose and 5% lactose, separately, and nutrients as described above. The initial freeze-dried kefir culture concentration was 2.5 g L^{-1}. When the fermentation was completed, the kefir culture was centrifuged and the fermented liquid was collected. Then, kefir culture was washed with synthetic medium that was used for the next fermentation run, centrifuged again and used in the next repeated batch fermentation.

All fermentations were carried out in a stationary mode and the pH was continuously adjusted to 5.0–5.5 in fermentations of glucose, fructose and lactose and to 4.7 (optimum pH for the enzyme invertase) (Koutinas & Kanellaki, 2006) in sucrose fermentations using a 2 M NaOH solution. At the end of each fermentation run, samples were collected and analysed for lactic acid, residual sugar and ethanol concentration.

2.5. Chemical analyses

Moisture content of the freeze-dried cultures was calculated by determining the weight before and after heating at 120 °C overnight (Association of Official Analytical Chemists, 1995). Biomass concentrations (dry weight L^{-1}) in whey during aerobic fermentations were determined according to method of Klein and Kressdorf (1983) using standard curves by relating optical density (at 700 nm) to cell dry weight. At the end of each fermentation run, biomass concentration was also determined after centrifugation at $2700 \times g$ for 20 min. Dry biomass was obtained after centrifugation and drying at 105 °C for 24 h. Total nitrogen in whey, expressed as crude protein, was determined using the Kjeldahl procedure (Kirk & Sawyer, 1991).

Ethanol and residual sugar levels were determined by size-exclusion high-performance liquid chromatography (SE-HPLC), using a Shimadzu (Kyoto, Japan) chromatograph with a SCR-101N stainless steel column, a LC-9A pump, a CTO-10A oven at 60 °C and a RID-6A refractive index detector. Triple-distilled water was used as mobile phase with a flow rate of 0.8 mL min^{-1} and 1-butanol (Merck) was used as an internal standard. Samples of 0.5 mL and 2.5 mL of a 1% (v/v) solution of 1-butanol were diluted to 50 mL and 40 μL were injected directly to the column. Concentrations of ethanol and residual sugar were calculated using standard curves and expressed in terms of percentage (v/v) and g of residual sugar L^{-1}, respectively.

Lactic acid was determined by ion-exchange HPLC, using a Shimadzu chromatograph with a Shim-pack IC-A1 stainless steel column, a LC-10A pump, a CTO-10A oven at 40 °C and a CDD-6A conductivity detector. A solution of 2.5 mM phthalic acid (Merck) and 2.4 mM Tris (hydroxymethyl) aminomethane (Merck) (pH 4.0) in triple-distilled water was used as mobile phase with a flow rate of 1.5 mL min^{-1}. Samples (0.25 mL) were diluted to 25 mL with triple-distilled water and 60 μL were injected directly to the column. Lactic acid concentrations were calculated using standard curves.

Maximum growth rate μ_{max} was determined by linear regression (indicated by the correlation coefficient, r^2) from the plots of optical density against time. Biomass productivity was expressed as g (dry weight) biomass produced L^{-1} liquid volume of the bioreactor per day. Biomass yield was expressed as g (dry weight) of biomass produced g^{-1} of utilized sugar. Ethanol productivity was expressed as g of ethanol produced per day per litre liquid volume of bioreactor.

Conversion was calculated by the following equation:

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

2.6. Microbiological analyses

Counts of yeasts, lactobacilli and lactococci in pressed wet weight kefir culture were determined as colony-forming units (cfu g^{-1}). Representative duplicate portions (10 g) of pressed wet culture samples, before and after aerobic whey fermentations, were blended with 90 mL of sterilized 2%

tri-sodium citrate (Merck) solution, subjected to serial dilutions in sterilized $\frac{1}{4}$ ringer solution, and subsequently plated on agar plates.

The following tests on microbiological analysis were performed: (i) yeasts on malt agar (Fluka) (pH was adjusted to 4.5 by sterile solution of 10% lactic acid) incubated at 30 °C for 72 h; (ii) lactococci on M-17 agar (Fluka) incubated at 30 °C for 72 h; and (iii) lactobacilli on acidified MRS agar (Fluka) incubated at 37 °C for 72 h anaerobically (Anaerobic jar, Anerocult C, Merck). All incubations were further extended up to 120 h, but no extra colonies were observed. Gram staining was performed for lactic acid bacteria confirmation. Results are presented as mean colony-forming units on solid media culture plates containing between 30 and 300 colonies g^{-1} of pressed wet weight culture.

2.7. Determination of kefir culture viability

The number of viable cells before and after freeze-drying was determined as colony-forming units (cfu). Decimal dilutions were prepared from the kefir culture suspension before freezing and plated on agar plates containing lactose synthetic medium and nutrients (2% lactose, 0.4% yeast extract, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.5% $MgSO_4 \cdot 7H_2O$, and 2% agar), as described above. The freeze-dried samples were resuspended in distilled and sterilized water by shaking for 3 min at 200 rpm, incubated for 15 min at room temperature, decimally diluted and subsequently plated. The agar plates were incubated aerobically at 30 °C for 72 h. The survival rate was calculated as cfu after freeze-drying divided by cfu before freezing.

2.8. Statistical analysis

All treatments were carried out in triplicate and the mean values are presented (standard deviation for all values was about $\pm 5\%$ in most cases). The experiments were designed and analysed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results; coefficients, ANOVA tables and significance ($p < 0.05$) were computed using Statistica v.5.0 (StatSoft, Inc, Tulsa, USA).

3. Results and discussion

3.1. Production of Kefir culture using whey

Batch aerobic fermentations were carried out until maximum biomass concentrations were obtained (Fig. 1). Smaller pore diameter in the air distribution tube and higher air flow rates resulted in increased maximum growth rates. Agitation did not increase biomass concentrations, but increased maximum growth rates and biomass productivity, as higher biomass concentrations were obtained in shorter fermentation times (Table 1). The highest biomass concentration, 14.0 g dry weight L^{-1}, was produced when pores of size 0.5 mm were used without agitation. However, the highest daily biomass productivity, 14.1 g dry weight L^{-1} was obtained when the same pores and air flow rate, at 600 rpm were used. Ethanol and lactic acid were measured, and were the main by-products (up to 2.9%, v/v, and 9.7 g L^{-1}, respectively). Similar results reporting ethanol production in aerobic fermentations in excess of sugars, known as the Crabtree effect (caused by over-saturation of the respiratory pathways), have been previously reported (González-Siso et al., 2000; González-Siso, Ramil, Cerdán, & Freire-Picos, 1996; Van Dijken & Scheffers, 1986). Residual sugar concentration was low, leading to high values of conversion (89.8–99.0%).

In all cases, the microbial balance in kefir culture remained unaltered after the aerobic whey fermentations. Counts of yeasts, lactobacilli and lactococci before and after whey fermentations were $\approx 10^7$, 10^{10} and 10^8 cfu g^{-1}, respectively.

3.2. Effect of cryoprotective medium and cooling rate on freeze-drying of kefir culture

To investigate the optimum conditions for freeze-drying, 16 cryoprotective agents and four cooling rates using the optimum cryoprotective agent were tested (Table 2). After freeze-drying, kefir was used in alcoholic fermentation of a 5% lactose synthetic medium, to study its activity. Freeze-dried kefir culture was directly introduced into the fermentation medium and fermentation was almost immediately initiated.

Fresh whey and the liquid remaining after aerobic kefir production (i.e., the fermented whey) were tested as cryoprotective media during freeze-drying of kefir culture, as whey which contains lactose and fermented whey would probably act as cryoprotective agents. Whey and fermented whey were compared to usually used cryoprotective agents such as glycerol, carbohydrates, 1% honey solution and skim milk (Blanquet et al., 2005; Saarela, Virkajärvi, Alakomi, Sigvart-Mattila, & Mättö, 2006; Tan et al., 1995). The type of cryoprotective agent significantly affected percentage survival rate of freeze-dried kefir culture ($p < 0.01$) and all fermentation parameters studied ($p < 0.01$). The highest survival rate (86%) was observed when fermented whey was used as the cryoprotective agent, probably due to adaptation of cells, or perhaps due to the presence of trace nutrients in the fermented whey. Similar percentage survival rates were previously reported during freeze-drying of yeasts (Berni & Hennebert, 1991). The high survival rates obtained in almost all cases could be attributed to the fact that kefir culture cells were harvested at the early stationary phase, as no cell growth was observed at higher fermentation times (Fig. 1). Survival of freeze-drying is commonly found to be higher for stationary-phase cells than for exponentially growing cells (Potts, 1994). Stationary phase, induced by carbon starvation, triggers a general stress response, which involves induction of a wide range of stress proteins (Hecker &

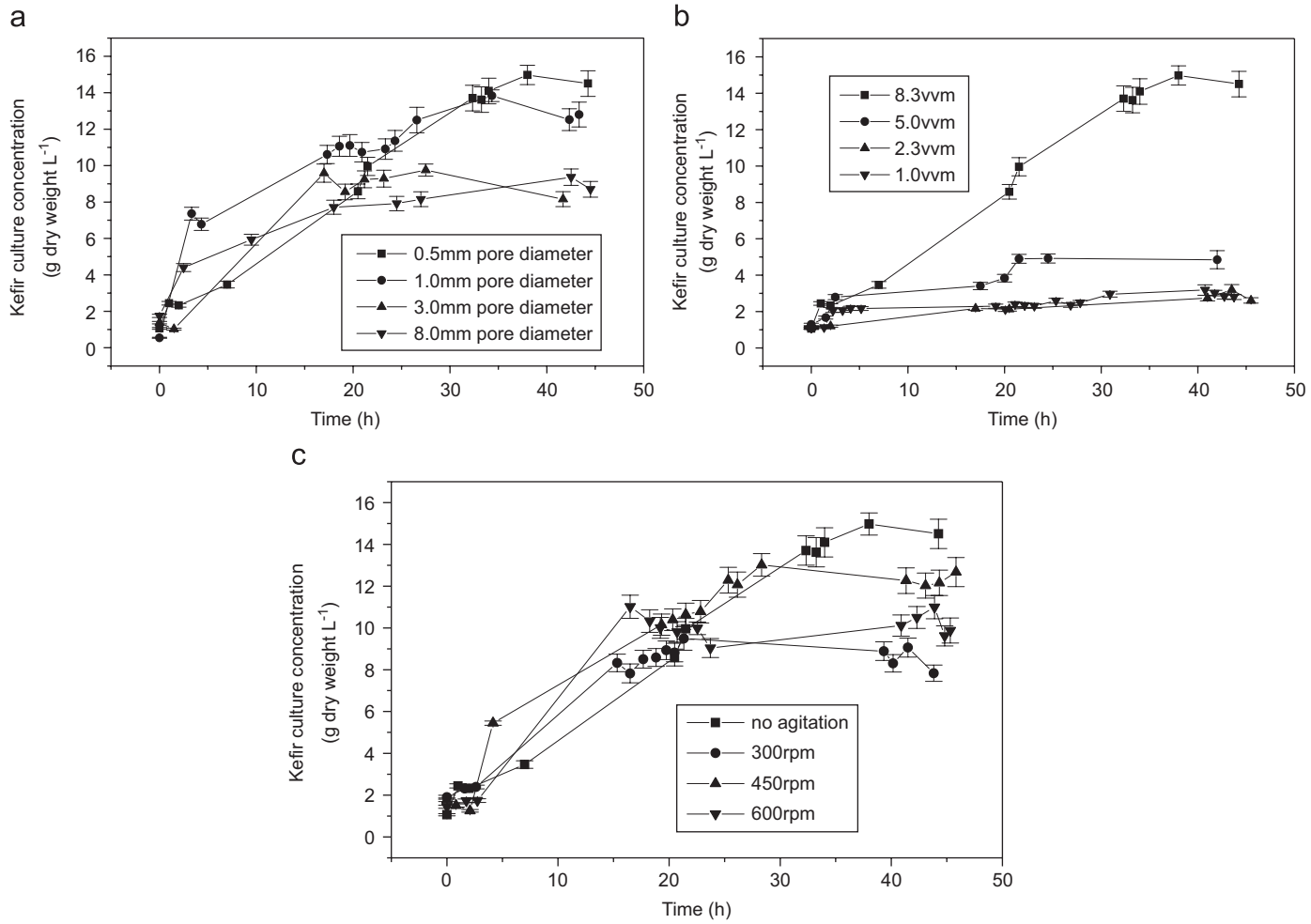

Fig. 1. Effect of: (a) pore diameter of perforated air distribution tube (air flow rate 8.3 vvm, no agitation); (b) air flow rate (pore diameter of perforated air distribution tube 0.5 mm, no agitation); and (c) agitation (air flow rate 8.3 vvm, pore diameter of perforated air distribution tube 0.5 mm) on aerobic fermentation of whey during kefir culture production. Standard deviations are shown by error bars.

Table 1
Effect of diameter of perforated air distribution tube pore, air flow rate and agitation on aerobic production of kefir culture using whey as raw material

Perforated tube pore diameter (mm)	Air flow rate (vvm)	Agitation (rpm)	Fermentation time (h)	μ_{max} (h^{-1})	Biomass produced (g dry weight L^{-1})	Ethanol concentration (% vol)[a]	Residual sugar (g L^{-1})	Lactic acid concentration (g L^{-1})	Daily biomass productivity (g dry weight L^{-1})	Biomass yield (g dry weight g^{-1} utilized sugar)	Conversion (%)
0.5	8.3	No	$38\pm2^{a,b,d,e}$	$0.43\pm0.03^{a,e}$	14.0 ± 0.5^{a}	$0.1\pm0.01^{a,e}$	$0.8\pm0.1^{a,c,d,f}$	$6.0\pm0.4^{a,f}$	$8.8\pm0.3^{a,f}$	$0.28\pm0.01^{a,d}$	$98.4\pm0.2^{a,c,d,f}$
1.0	8.3	No	$34\pm1^{a,b,c}$	0.47 ± 0.02^{a}	12.8 ± 0.3^{a}	Tr*,[a]	1.7 ± 0.2^{a}	7.7 ± 0.5^{b}	9.0 ± 0.2^{a}	0.27 ± 0.01^{a}	96.6 ± 0.4^{a}
3.0	8.3	No	28 ± 1^{c}	0.47 ± 0.01^{a}	8.8 ± 0.3^{b}	Tr[a]	4.3 ± 0.3^{b}	5.6 ± 0.2^{a}	7.5 ± 0.2^{b}	0.19 ± 0.01^{b}	91.4 ± 0.6^{b}
8.0	8.3	No	42 ± 3^{d}	0.30 ± 0.01^{b}	8.4 ± 0.4^{b}	0.9 ± 0.1^{b}	4.0 ± 0.5^{b}	1.0 ± 0.2^{c}	4.8 ± 0.2^{c}	0.18 ± 0.01^{b}	92.0 ± 1.0^{b}
0.5	1.0	No	41 ± 2^{e}	0.03 ± 0.01^{c}	2.2 ± 0.2^{c}	2.5 ± 0.2^{c}	2.5 ± 0.3^{e}	2.7 ± 0.3^{d}	1.3 ± 0.1^{d}	0.05 ± 0.01^{c}	95.0 ± 0.6^{e}
0.5	2.3	No	44 ± 2^{e}	0.04 ± 0.01^{c}	2.2 ± 0.2^{c}	1.0 ± 0.1^{d}	1.4 ± 0.2^{c}	4.1 ± 0.3^{e}	1.2 ± 0.1^{d}	0.05 ± 0.01^{c}	97.2 ± 0.4^{c}
0.5	5.0	No	25 ± 1^{f}	0.15 ± 0.01^{d}	3.9 ± 0.4^{d}	0.9 ± 0.1^{d}	0.5 ± 0.01^{d}	4.3 ± 0.1^{e}	3.7 ± 0.3^{e}	0.08 ± 0.01^{c}	99.0 ± 0.2^{d}
0.5	8.3	300	21 ± 1^{g}	0.45 ± 0.04^{e}	8.5 ± 0.5^{e}	0.7 ± 0.1^{f}	0.8 ± 0.1^{f}	9.7 ± 0.5^{g}	9.7 ± 0.6^{f}	0.17 ± 0.01^{e}	98.4 ± 0.2^{f}
0.5	8.3	450	28 ± 2^{h}	0.45 ± 0.04^{e}	12.0 ± 0.5^{f}	0.2 ± 0.01^{e}	1.4 ± 0.2^{f}	7.6 ± 0.4^{h}	10.3 ± 0.4^{f}	$0.25\pm0.01^{d,f}$	97.2 ± 0.4^{f}
0.5	8.3	600	17 ± 1^{g}	0.51 ± 0.05^{e}	10.0 ± 0.5^{e}	0.3 ± 0.01^{e}	5.1 ± 0.6^{g}	$6.2\pm0.2^{h,f}$	14.1 ± 0.7^{g}	0.22 ± 0.01^{f}	89.8 ± 1.2^{g}

[a]Tr: Traces. Statistical differences ($p<0.05$ for fermentation time and $p<0.01$ for the rest of parameters) for various treatments within a column are indicated by different letters in superscript.

Volker, 2001). Short fermentation times and high ethanol concentrations were obtained in fermentations by freeze-dried kefir culture using 5% or 10% sucrose, 10% fructose, 10% glucose, 10% trehalose, $\frac{1}{4}$ ringer solution and fermented whey as cryoprotective agents separately. Likewise, the highest lactic acid concentration (9.0 g L^{-1}) was

Table 2
Effect of cryoprotective agents and cooling rate on percentage of survival rate and metabolic activity of freeze-dried kefir culture during fermentation of synthetic medium containing 5% lactose at 30 °C[a]

Cryoprotective agent	Cooling rate (°C min^{-1})	Percentage survival rate	Fermentation time (h)	Ethanol concentration (% vol)	Daily ethanol productivity (g L^{-1})	Lactic acid concentration (g L^{-1})	Residual sugar (g L^{-1})	Conversion (%)
Wet kefir culture		100^{a}	40 ± 3^{a}	2.4 ± 0.2^{a}	11.4 ± 1.0^{a}	$5.2\pm0.2^{a,f,s,t}$	3.5 ± 0.5^{a}	93.0 ± 1.0^{a}
$\frac{1}{4}$ Ringer solution	3	68 ± 3^{b}	$68\pm9^{a,b}$	$1.4\pm0.2^{b,e,l}$	$3.9\pm0.6^{b,e}$	$8.3\pm0.5^{b,e,k,q,v,w}$	$2.7\pm0.2^{a,b}$	$94.6\pm0.4^{a,b}$
Unfermented whey	3	$78\pm3^{b,c}$	$117\pm23^{c,e}$	$0.5\pm0.1^{c,g}$	0.8 ± 0.2^{c}	3.0 ± 0.2^{c}	$2.8\pm0.3^{a,b}$	$94.4\pm0.6^{a,c}$
Fermented whey	3	$86\pm2^{d,c,k}$	$115\pm6^{c,e,s}$	$1.5\pm0.1^{d,e,m,n}$	$2.5\pm0.1^{d,e,f,g,i,l,m,n,o}$	$7.2\pm0.5^{d,e,h,j,m,o,x}$	$5.7\pm0.2^{c,m}$	$88.6\pm0.3^{d,n}$
5% Glucose	3	$76\pm2^{b,d}$	$96\pm12^{b,c,d,i,o,l}$	$0.8\pm0.1^{c,f,h}$	$1.6\pm0.1^{c,f}$	$5.0\pm0.5^{f,g}$	5.0 ± 0.5^{c}	90.0 ± 1.0^{d}
5% Fructose	3	$77\pm2^{b,d}$	$117\pm17^{c,f}$	$0.7\pm0.1^{g,h}$	$1.1\pm0.1^{c,g}$	$5.5\pm0.5^{a,g,h,i,u}$	7.7 ± 0.2^{d}	84.6 ± 1.0^{e}
5% Sucrose	3	$77\pm4^{b,d}$	$75\pm7^{a,d,g}$	$2.1\pm0.2^{a,i}$	$5.3\pm0.5^{b,h}$	$8.9\pm0.4^{k,j,l}$	3.5 ± 0.5^{a}	93.0 ± 1.0^{a}
5% Lactose	3	$80\pm2^{c,d,e}$	$118\pm10^{c,h}$	$0.7\pm0.1^{c,h}$	$1.1\pm0.1^{c,i}$	$8.2\pm0.5^{b,l,m,n,r,u}$	$2.3\pm0.2^{b,e,g}$	$95.4\pm0.4^{b,c,f}$
10% Glucose	3	$80\pm2^{c,d,f,i}$	$65\pm5^{a,i,j}$	$2.2\pm0.2^{a,j}$	$6.4\pm0.6^{h,j}$	$7.7\pm0.2^{b,d,l,p}$	$2.6\pm0.2^{a,e,f}$	$94.8\pm0.4^{a,f,g}$
10% Fructose	3	$80\pm4^{c,d,g,i}$	$55\pm5^{a,k}$	$2.2\pm0.2^{a,k}$	7.6 ± 0.7^{j}	$6.0\pm0.5^{a,g,o,p}$	$1.6\pm0.2^{g,i,j,l}$	$96.8\pm0.4^{f,h,j,k}$
10% Sucrose	3	$80\pm3^{c,d,h}$	$69\pm8^{a,l,m}$	$1.7\pm0.2^{i,l,m}$	$4.7\pm0.6^{b,k}$	$8.5\pm1.0^{b,d,l}$	0.4 ± 0.1^{h}	99.2 ± 0.2^{i}
10% Lactose	3	$73\pm3^{b,e,f,g,h,i}$	$119\pm20^{c,n,o}$	$0.6\pm0.1^{c,h}$	$1.0\pm0.1^{c,l}$	$7.0\pm0.5^{d,i,p,q,r}$	$1.3\pm0.2^{h,i}$	$97.7\pm0.1^{h,i}$
1% Honey solution	3	68 ± 3^{b}	$144\pm15^{e,f,h,n,p}$	$1.0\pm0.1^{b,c,h}$	$1.3\pm0.1^{c,m}$	$4.8\pm0.4^{g,s}$	$0.8\pm0.1^{h,j}$	$98.4\pm0.2^{i,j}$
Skim milk	3	$71\pm2^{b,e,f,g,h}$	$114\pm10^{c,p}$	$1.1\pm0.1^{b,f,h,n}$	$1.8\pm0.2^{c,n}$	$7.1\pm0.3^{b,d,i,p}$	0.5 ± 0.1^{h}	99.0 ± 0.2^{i}
1% Glycerol	3	$78\pm3^{b,d}$	$58\pm6^{a,d,q}$	$1.0\pm0.1^{b,c,h}$	$3.3\pm0.4^{b,o}$	$6.9\pm0.4^{d,t,u,v}$	$1.9\pm0.4^{b,f,i,k}$	$96.2\pm0.8^{b,c,g,k,l,m}$
5% Trehalose	3	$83\pm4^{c,d,i,j}$	$85\pm14^{b,c,g,j,k,m,q,r}$	$0.6\pm0.1^{c,h}$	$1.3\pm0.2^{c,d}$	$4.7\pm0.2^{a,g}$	$2.8\pm0.4^{a,e,k}$	$94.4\pm0.8^{a,f,m}$
10% Trehalose	3	$73\pm5^{b,e,f,g,h,j}$	$65\pm7^{a,d,r}$	$1.9\pm0.2^{d,i,j,k}$	$5.5\pm0.6^{h,k}$	$9.0\pm1.2^{l,w}$	$0.8\pm0.3^{h,l}$	$98.4\pm0.6^{h,i,l}$
Fermented whey	1	81 ± 5^{k}	164 ± 12^{t}	$0.6\pm0.1^{\circ}$	0.7 ± 0.1^{p}	7.3 ± 0.3^{x}	3.9 ± 0.3^{n}	$92.2\pm0.6^{\circ}$
Fermented whey	4	85 ± 2^{k}	$139\pm10^{s,t}$	$0.9\pm0.1^{\circ}$	1.2 ± 0.1^{q}	7.7 ± 0.5^{x}	6.4 ± 0.3^{m}	87.2 ± 0.6^{n}
Fermented whey	5	84 ± 2^{k}	74 ± 4^{u}	$0.8\pm0.1^{\circ}$	2.0 ± 0.1^{r}	7.1 ± 0.4^{x}	4.5 ± 0.2^{n}	$91.0\pm0.4^{\circ}$

[a]Statistical differences ($p<0.01$) for various treatments within a column are indicated by different letters in superscript.

observed when 10% trehalose was used as cryoprotective agent, showing that it may constitute a suitable cryoprotecting agent for survival of lactic acid bacteria. However, use of fermented whey resulted in relatively high lactic acid content (7.2 g L^{-1}). Residual sugar was low in almost all cases, while up to 99.2% conversion was found when 10% sucrose was used as cryoprotective agent.

As fermented whey proved to be a suitable cryoprotective agent for freeze-drying of kefir culture, with the advantage of negligible cost, it was used as the cryoprotective medium when the effect of cooling rate was studied. It is well-known that the optimal freezing rate should be slow enough to prevent formation of intracellular ice crystals and fast enough to avoid serious cell damage due to concentration effects (Gehrke et al., 1992). Therefore, it was decided to study cooling rates in the range 1–5 °C min^{-1}, as it has been previously reported that optimum cooling rates for yeast and bacterium cells are between 1 and 10 °C min^{-1} (Gehrke et al., 1992; Mazur, 1970) and especially 3 °C min^{-1} for *S. cerevisiae* yeast (Berni & Hennebert, 1991). Cooling rate did not significantly affect the viability of the freeze-dried kefir culture and the lactic acid content ($p>0.05$). However, it had a significant effect on the remaining fermentation parameters ($p<0.01$ for fermentation time, daily ethanol productivity, residual sugar and conversion and $p<0.05$ for ethanol concentration). Although the cooling rates tested did not result in significant differences in viability of kefir culture during freeze-drying, it seemed that cooling rate of 3 °C min^{-1} resulted in improved metabolic activity of kefir culture cells, as the highest ($p<0.05$) ethanol concentration (1.5% v/v) and daily ethanol productivity (2.5 g L^{-1}) ($p<0.01$) were found in fermentation using freeze-dried kefir culture cooled at 3 °C min^{-1}. Although shorter ($p<0.01$) fermentation times (74 h) were observed in fermentations using freeze-dried kefir culture cooled at 5 °C min^{-1}, significantly ($p<0.05$) lower ethanol concentrations were obtained. The results are in accordance with findings presented in previous studies of freeze-drying of *S. cerevisiae* (Bekatorou, Koutinas, Kaliafas, et al., 2001; Berni & Hennebert, 1991).

3.3. Use of freeze-dried kefir culture in batch fermentations

The freeze-dried kefir culture was used for fermentation of various carbohydrates in order to evaluate its metabolic activity. The influence of initial sugar concentration, initial biomass concentration and storage time of freeze-dried kefir culture were examined (data not shown).

Type of carbohydrate and initial sugar content affected significantly fermentation time, ethanol and lactic acid concentration, daily ethanol productivity, residual sugar, and extent of conversion ($p<0.01$) and a strong interaction between them was also observed ($p<0.01$). In general, shorter fermentation times were observed on fermentations of glucose for 30–92 h, and times increased when fructose and the disaccharides sucrose and lactose were used in fermentations, the ranges being 69–94, 51–117 and 115–307 h, respectively. The highest ethanol concentration (6.7%, v/v) was recorded for fermentation with 12% initial

sucrose level, while the highest ($p<0.01$) daily ethanol productivities (13.9 and 13.4 g L^{-1}, respectively) were obtained in fermentations with 5% and 12% glucose. The highest ($p<0.01$) lactic acid contents (9.2 and 9.7 g L^{-1}) were detected in fermentations with 8% and 12% glucose, respectively.

The effect of initial biomass concentration on fermentation of carbohydrates was also investigated. The type of carbohydrate and initial biomass concentration affected significantly all parameters studied ($p<0.01$), with a strong interactive effect ($p<0.01$). Higher initial biomass concentrations resulted in shorter ($p<0.01$) fermentation times only in fermentations using fructose and lactose. Duncan's multiple range test showed that the highest ($p<0.01$) ethanol productivity (13.9 g L^{-1}) was recorded when 2.5 g L^{-1} freeze-dried kefir culture was used in fermentation of glucose. Lactic acid content was reduced with increasing initial biomass in fermentations of monosaccharides, while the opposite effect was observed in fermentations of disaccharides. Residual sugar level was low in all cases, especially in glucose fermentations, and high levels of conversion, up to 100%, were thus measured.

To investigate the effect of storage on survival and metabolic activity, freeze-dried kefir culture was stored at 4 °C for various periods up to 6 months (data not shown), as higher survival rates have been reported at low storage temperatures (Champagne, Mondou, Raymond, & Roy, 1996; Ndoye, Weekers, Diawara, Guiro, & Thonart, 2007; Wang, Yu, & Chou, 2004). Survival rates of freeze-dried kefir culture stored for 12, 30, 90 or 180 d were 67%, 73%, 71% and 62%, respectively. Storage had a significant effect on all kinetic parameters during lactose fermentation ($p<0.01$). In all cases, storage significantly decreased ethanol concentration and, consequently, daily ethanol productivity. The highest ($p<0.01$) lactic acid content (9.2 g L^{-1}) was detected in fermentation using freeze-dried kefir culture stored for 12 d.

3.4. Suitability for repeated batch fermentations

In order to study the operational stability of the freeze-dried kefir culture, it was used in 12 repeated batch fermentations of glucose, fructose, sucrose or lactose. To investigate the possibility of fermentation at low temperatures (≤15 °C), the effect of temperature was also studied (data not shown). The high number of repeated batch fermentations showed high operational stability of freeze-dried kefir culture; fermentations were continued for up to 2 months without any significant loss of metabolic activity.

Carbohydrate substrate and fermentation temperature significantly ($p<0.01$) affected fermentation time and there was also a strong interactive effect ($p<0.01$). Ethanol concentration was only affected by the type of carbohydrate ($p<0.01$), while daily ethanol productivity and residual sugar depended on both the type of carbohydrate ($p<0.05$ and $p<0.01$, respectively) and the fermentation temperature ($p<0.01$). Lactic acid content was affected by carbohydrate substrate ($p<0.01$) and there was a strong interaction between carbohydrate substrate and fermentation temperature ($p<0.01$).

An improvement of fermentation time and productivity was observed from batch to subsequent batch fermentations at each temperature, except in lactose fermentations at temperatures ≤15 °C, which could be attributed to the adaptation of freeze-dried kefir culture. There were no significant differences in fermentation times during fermentation of glucose, fructose and sucrose, but higher fermentation times (up to 528 h) were observed in lactose fermentations, especially at low temperatures (data not shown). Generally, higher ethanol concentrations and daily ethanol productivities were observed in glucose fermentations (2.2–2.5% v/v and 4.5–75.8 g L^{-1}, respectively), while the highest ($p<0.01$) lactic acid content (14.4 g L^{-1}) was found in fermentation of sucrose at 5 °C. Residual sugar level was low in all cases, except in lactose fermentations at low temperatures, where levels of residual lactose up to 2.15% were detected.

4. Conclusions

Whey may be a practical base for kefir culture production, and fermented whey was a suitable cryoprotective medium during freeze-drying. The freeze-dried culture retained high survival rate and showed good metabolic activity and fermentation efficiency, indicating good potential as a value-added starter culture in dairy technology.

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References

Association of Official Analytical Chemists. (1995). *Official methods of analysis. Moisture in cheese*. 33.7.04. Method 948.12.

Athanasiadis, I., Boskou, D., Kanellaki, M., Kiosseoglou, V., & Koutinas, A. A. (2002). Whey liquid waste of the dairy industry as raw material for potable alcohol production by kefir granules. *Journal of Agricultural and Food Chemistry*, *50*, 7231–7234.

Athanasiadis, I., Boskou, D., Kanellaki, M., & Koutinas, A. A. (2001). Effect of carbohydrate substrate on fermentation by kefir yeast supported on delignified cellulosic material. *Journal of Agricultural and Food Chemistry*, *49*, 658–663.

Athanasiadis, I., Paraskevopoulou, A., Blekas, G., & Kiosseoglou, V. (2004). Development of a novel whey beverage by fermentation with kefir granules. Effect of various treatments. *Biotechnology Progress*, *20*, 1091–1095.

Bekatorou, A., Koutinas, A. A., Kaliafas, A., & Kanellaki, M. (2001). Freeze-dried *Saccharomyces cerevisiae* cells immobilized on gluten pellets for glucose fermentation. *Process Biochemistry*, *36*, 549–557.

Bekatorou, A., Koutinas, A. A., Psarianos, K., & Kanellaki, M. (2001). Low-temperature brewing by freeze-dried immobilized cells on gluten pellets. *Journal of Agricultural and Food Chemistry*, *49*, 373–377.

Belem, M. A. F., & Lee, B. H. (1999). Fed-batch fermentation to produce oligonucleotides from *Kluyveromyces marxianus* grown on whey. *Process Biochemistry*, *34*, 501–509.

Berni, J.-F., & Hennebert, G. L. (1991). Viability and stability of yeast cells and filamentous fungus spores during freeze-drying: Effects of protectants and cooling rates. *Mycologia*, *83*, 805–815.

Blanquet, S., Garrait, G., Beyssac, E., Perrier, C., Denis, S., Hébrard, G., et al. (2005). Effects of cryoprotectants on the viability and activity of freeze dried recombinant yeasts as novel oral drug delivery systems assessed by an artificial digestive system. *European Journal of Pharmaceutics and Biopharmaceutics*, *61*, 32–39.

Boss, E. A., Filho, R. M., & Vasco de Toledo, E. C. (2004). Freeze drying process: Real time model and optimization. *Chemical Engineering and Processing*, *43*, 1475–1485.

Champagne, C. P., Mondou, F., Raymond, Y., & Roy, D. (1996). Effect of polymers and storage temperature on the stability of freeze-dried lactic acid bacteria. *Food Research International*, *29*, 555–562.

Chen, H. C., Lin, C. W., & Chen, M. J. (2006). The effects of freeze-drying and rehydration on survival of microorganisms in kefir. *Asian–Australian Journal of Animal Sciences*, *19*, 126–130.

Garrote, G. L., Abraham, A. G., & De Antoni, G. L. (1997). Preservation of kefir grains, a comparative study. *Lebensmittel–Wissenschaft und Technologie*, *30*, 77–84.

Gehrke, H.-H., Pralle, K., & Decker, W.-D. (1992). Freeze-drying of microorganisms-influence of cooling rate on survival. *Food Biotechnology*, *6*, 35–49.

González-Siso, M. I., Freire-Picos, M. A., Ramil, E., González-Domínguez, M., Rodríguez Torres, A., & Cerdán, M. E. (2000). Respirofermentative metabolism in *Kluyveromyces lactis*: Insights and perspectives. *Enzyme and Microbial Technology*, *26*, 699–705.

González-Siso, M. I., Ramil, M. I., Cerdán, M. E., & Freire-Picos, M. A. (1996). Respirofermentative metabolism in *Kluyveromyces lactis*: Ethanol production and the crabtree effect. *Enzyme and Microbial Technology*, *18*, 585–591.

Hecker, M., & Volker, U. (2001). General stress response of *Bacillus subtilis* and other bacteria. *Advances in Microbial Physiology*, *44*, 35–91.

Hosseini, M., Shojaosadati, A. A., & Towfighi, J. (2003). Application of a bubble-column reactor for the production of a single cell protein from cheese whey. *Industrial and Engineering Chemistry Research*, *42*, 764–766.

Iconomopoulou, M., Becatorou, A., Kanellaki, M., Athanasiadis, I., Kioseoglou, V., Blekas, G., et al. (2001). *SCP production using kefir yeast from whey liquid effluent of dairy industry. In 7th international conference on environmental science & technology, 3–6 September 2001.* Ermoupolis, Syros Island, Greece, pp. 350–355.

Iconomopoulou, M., Kanellaki, M., Psarianos, K., & Koutinas, A. A. (2000). Delignified Cellulosic Material supported biocatalyst as freeze-dried product in alcoholic fermentation. *Journal of Agricultural and Food Chemistry*, *48*, 958–961.

Iconomopoulou, M., Psarianos, K., Kanellaki, M., & Koutinas, A. A. (2002). Low temperature and ambient temperature wine making using freeze dried immobilized cells on gluten pellets. *Process Biochemistry*, *37*, 707–717.

Kirk, R. S., & Sawyer, R. (1991). Dairy products I. In R. S. Kirk, & R. Sawyer (Eds.), *Pearson's composition and analysis of foods* (9th ed., pp. 530–574). Singapore: Longman Singapore Publishers Ltd.

Klein, J., & Kressdorf, B. (1983). Improvement of productivity and efficiency in ethanol production with Ca-alginate immobilized *Z. mobilis*. *Biotechnology Letters*, *5*, 497–502.

Kourkoutas, Y., Dimitropoulou, S., Kanellaki, M., Marchant, R., Nigam, P., Banat, I. M., et al. (2002). High-temperature alcoholic fermentation of whey using *Kluyveromyces marxianus* IMB3 yeast immobilized on delignified cellulosic material. *Bioresource Technology*, *82*, 177–181.

Kourkoutas, Y., Kandylis, P., Panas, P., Dooley, J. S. G., Nigam, P., & Koutinas, A. A. (2006). Evaluation of freeze-dried kefir coculture as starter in feta-type cheese production. *Applied and Environmental Microbiology*, *72*, 6124–6135.

Kourkoutas, Y., Psarianos, C., Koutinas, A. A., Kanellaki, M., Banat, I. M., & Marchant, R. (2002). Continuous whey fermentation using kefir yeast immobilized on delignified cellulosic material. *Journal of Agricultural and Food Chemistry*, *50*, 2543–2547.

Koutinas, A. A., Athanasiadis, I., Bekatorou, A., Iconomopoulou, M., & Blekas, G. (2005). Kefir yeast technology: Scale-up in SCP production using milk whey. *Biotechnology & Bioengineering*, *89*, 788–796.

Koutinas, A. A., & Kanellaki, M. (2006). *Food chemistry and technology* (in Greek). Patras, Greece: University of Patras Press.

Luis, A., Lopez, E., & Lema, C. (1993). Microflora present in kefir grains of the Galician region. *Journal of Dairy Research*, *60*, 263–267.

Mazur, P. (1970). Cryobiology: The freezing of biological systems. *Science*, *168*, 939–949.

Ndoye, B., Weekers, F., Diawara, B., Guiro, A. T., & Thonart, P. (2007). Survival and preservation after freeze-drying process of thermoresistant acetic acid bacteria isolated from tropical products of Subsaharan Africa. *Journal of Food Engineering*, *79*, 1374–1382.

Palmfeldt, J., & Hahn-Hägerdal, B. (2000). Influence of culture pH on survival of *Lactobacillus reuteri* subjected to freeze-drying. *International Journal of Food Microbiology*, *55*, 235–238.

Palmfeldt, J., Rådström, P., & Hahn-Hägerdal, B. (2003). Optimisation of initial cell concentration enhances freeze-drying tolerance of *Pseudomonas chlororaphis*. *Cryobiology*, *47*, 21–29.

Parrondo, J., Garcia, L. A., & Diaz, M. (2000). Production of an alcoholic beverage by fermentation of whey permeate with *Kluyveromyces fragilis* I: Primary metabolism. *Journal of the Institute of Brewing*, *106*, 367–375.

Pintado, M. E., Lopes Da Silva, J. A., Fernades, P. B., Malcata, F. X., & Hogg, T. A. (1996). Microbiological and rheological studies on Portugese kefir grains. *International Journal Food Science and Technology*, *31*, 15–26.

Plessas, S., Pherson, L., Bekatorou, A., Nigam, P., & Koutinas, A. A. (2005). Bread making using kefir grains as baker's yeast. *Food Chemistry*, *93*, 585–589.

Potts, M. (1994). Desiccation tolerance of prokaryotes. *Microbiology Reviews*, *58*, 755–805.

Saarela, M., Virkajärvi, I., Alakomi, H.-L., Sigvart-Mattila, P., & Mättö, J. (2006). Stability and functionality of freeze-dried probiotic bifidobacterium cells during storage in juice and milk. *International Dairy Journal*, *16*, 1477–1482.

Tal, V., Rijin, J., & Nussinovitch, A. (1997). Improvement of structural and mechanical properties of denitrifying alginate beads by freeze-drying. *Biotechnology Progress*, *13*, 788–793.

Tan, C. S., Van Ingen, C. W., Talsma, H., Van Miltenburg, J. C., Steffensen, C. L., Vlug, J. A., et al. (1995). Freeze-drying of fungi: Influence of composition and glass transition temperature of the protectant. *Cryobiology*, *32*, 60–67.

Van Dijken, J. P., & Scheffers, W. A. (1986). Redox balance in the metabolism of sugars by yeasts. *FEMS Microbiology Reviews*, *32*, 199–224.

Wang, Y. C., Yu, R. C., & Chou, C. C. (2004). Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. *International Journal of Food Microbiology*, *93*, 209–217.

Witthuhn, R. C., Schoeman, T., & Britz, T. J. (2005). Characterisation of the microbial population at different stages of kefir production and kefir grain mass cultivation. *International Dairy Journal*, *15*, 383–389.

Zourari, A., & Anifantakis, E. M. (1988). Le kefir caracteres physico-chimiques, microbiologiques et nutritionnels. Technologie de production. Une revue. *Lait*, *68*, 373–392.