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Effect of various carbohydrate substrates on the production of *kefir* grains for use as a novel baking starter

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Abstract

The propagation of *kefir* grains in a vigorously aerated, batch system, using various pure and mixed solutions of carbohydrates, was studied. From the experiments performed at 30 °C with synthetic liquid media containing single sugars, the one containing fructose (pH 5.5 and 5 g/l initial *kefir* concentration), gave the largest biomass production (20.75 g) in a 24-h experiment. When mixtures of sugars where used, the one containing glucose and sucrose at a fixed-ratio of 1:3, was found to be the most effective, yielding 27.25 g of *kefir* biomass. Kinetics of sugar bioconversions were also studied. The obtained results are important for the prediction of effective, low-cost utilization of various food-grade wastes for the production of *kefir* biomass and its possible use as a novel baking starter.

Keywords: *Kefir* grains; Carbohydrates; Biomass production; Baking starter

1. Introduction

Baker's yeast production is a large industry worldwide. Million tons of fresh baker's yeast cells (*Saccharomyces cerevisiae*) are produced annually for human consumption, using exclusively molasses as raw material (Spencer & Spencer, 1997). On the other hand, a large amount of solid and liquid wastes of the food industry, such as milk whey or citrus processing wastes, that contain respectable amounts of fermentable carbohydrates, could provide new and cheap raw materials for the yeast production industry (Anupama, 2000; Leman, Bednarski, & Tomasik, 1990). Various carbohydrate substrates, such as cane or beet molasses, containing sucrose, cheese whey containing lactose, starch-containing wastes and date waste products, have been proposed as carbon sources for baker's yeast production at the research level, but their use on the industrial scale demands the development of suitable processes (Caballero et al., 1995; Ejiofor, Chisti, & Moo-Young, 1996; Khan, Abulnaja, Kumosani, & Abou-Zaid, 1995; Nancib, Nancib, & Boudrant, 1997). For example, novel strains of baker's yeast with improved properties, developed by recombinant DNA technology (Randez-Gil, Sanz, & Prieto, 1999), and new aerobic bioreactors, are expected to enable optimal production of yeast biomass, with maximum utilization of various raw materials (Attfield, 1997; Randez-Gil et al., 1999).

Recently, the increased interest in sourdough bread production has led to the development of well-defined mixed starter cultures containing lactic acid bacteria and yeasts (Linko, Javanainen, & Linko, 1997). The use of mixed starters in baking, apart from leavening activity, displays a number of important advantages over baker's yeast, such as the formation of better flavour and extension of preservation time, through the in situ

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production of acids (lactic and propionic) or antimicrobial compounds (*bacteriocins*) (Katina, Sauri, Alakomi, & Mattila-Sandholm, 2002; Messens & De Vuyst, 2002). *Kefir* is a natural mixed culture used for centuries in the Caucasus area for the production of the traditional milk drink through lactic acid and alcoholic fermentations. Various microorganisms, sharing a symbiotic relationship, have been isolated from *kefir* microflora, including lactose-fermenting yeasts, such as *Saccharomyces kefir* and *Candida kefir*, homo-fermentative and hetero-fermentative lactobacilli, sich as *L. kefir*, mesophilic lactic acid streptococci, flavour-forming *Leuconostoc* and occasionally acetic acid bacteria (Assadi, Pourahmad, & Moazami, 2000; Macrae, Robinson, & Sadler, 1993; Simova et al., 2002). The use of *kefir* as a baker's yeast is an interesting perspective, expected to increase resistance to spoilage and produce bread of improved aroma and taste. The aim of this study was to evaluate the use of various carbohydrate substrates for *kefir* biomass production, in order to predict the possibility of an effective, low-cost utilization of various wastes of the food industry for the production of *kefir* and its possible use as a baking starter.

2. Materials and methods

2.1. Kefir culture

The *kefir* culture, isolated from Russian kefir drink, is maintained and available at the Department of Chemistry, Aristotle University of Thessaloniki, Greece (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001). *Kefir* grains were grown at 30 °C in a synthetic liquid medium containing 40 g/l of lactose monohydrate, 1 g/l of $(NH_4)_2SO_4$, 1 g/l of KH_2PO_4, 5 g/l of $MgSO_4 \cdot 7H_2O$ and 4 g/l of yeast extract (pH 5.5). For solid cultures, 2% agar was used. All media were sterilized at 130 °C for 15 min. *Kefir* biomass was obtained by successive inoculations in liquid nutrient media until 5 g of *kefir* grains were obtained. Cells were harvested by centrifugation at 4000 rpm for 10 min and were used to pitch the batch, aerated biomass production system.

2.2. Biomass production

The synthetic liquid media used for *kefir* biomass production contained 4 g/l of $(NH_4)_2SO_4$, 2 g/l of KH_2PO_4, 4 g/l of yeast extract and various concentrations of the monosaccharides glucose (*Glu*) and fructose (*Fru*), and the disaccharides sucrose (*Suc*), lactose (*Lac*) and maltose (*Mal*) (Tables 1–3). All the tested media had an initial density of 2.5 ^{0}Be (~40 g/l initial sugar concentration) and pH 5.5, which was adjusted using saturated Na_2CO_3 solution.

The apparatus used for the *kefir* biomass production consisted of a 2 l glass tower reactor, placed in an incubator set at 30 °C and connected to a compressor that supplied the system with air through an air pump. The air was sterilized by passing through a bacteriostatic filter before reaching the fermenting material. The air flow was held constant at 3 l/min. An initial amount of 5 g of *kefir* grains and 1 l of synthetic liquid medium were used in all the experiments. When the fermentations were completed (about 24 h in most cases), the produced biomass was harvested by centrifugation at 4000 rpm for 10 min, and the biomass yield was assessed as the grammes of *kefir* produced per gramme of sugar utilized (g/g). The supernatants were retained for further analysis. Various sugar bioconversion kinetics were also studied by analysing samples of the fermenting media, at frequent time intervals, for ethanol, lactic acid and residual sugar.

Table 1
Effect of various sugar substrates, containing glucose, on *kefir* biomass production, at 30 °C

Sugar	Fixed-ratio	ISC[a] (g/l)	Residual sugar (g/l)						Ethanol (% v/v)	Lactic acid (g/l)	Total biomass (g)	Biomass yield (g/g)	Conversion (%)
			Glu	*Fru*	*Suc*	*Lac*	*Mal*	*Total*					
Glu		42	–	–	–	–	–	–	1.12	8.55	17.50	0.42	100.0
Glu:*Fru*	(1:1)	42	–	–	–	–	–	–	0.62	3.66	25.95	0.62	100.0
Glu:*Fru*	(1:3)	44	–	–	–	–	–	–	1.35	6.33	15.25	0.35	100.0
Glu:*Fru*	(3:1)	44	–	–	–	–	–	–	1.94	6.71	16.39	0.37	100.0
Glu:*Suc*	(1:1)	42	–	–	0.53	–	–	0.53	0.63	8.45	17.82	0.43	98.7
Glu:*Suc*	(1:3)	42	–	–	–	–	–	–	0.76	8.70	27.25	0.65	100.0
Glu:*Suc*	(3:1)	42	–	–	–	–	–	–	0.43	7.28	25.75	0.61	100.0
Glu:*Lac*	(1:1)	43	–	–	–	4.89	–	4.89	0.37	4.89	22.40	0.59	88.6
Glu:*Lac*	(1:3)	42	–	–	–	–	–	–	1.70	7.65	21.30	0.51	100.0
Glu:*Lac*	(3:1)	42	–	–	–	0.76	–	0.76	1.89	6.65	7.40	0.18	98.2
Glu:*Mal*	(1:1)	40	–	–	–	–	19.8	19.8	0.76	8.43	10.35	0.51	50.5
Glu:*Mal*	(1:3)	40	–	–	–	–	28.9	28.9	–	10.5	7.60	0.68	27.8
Glu:*Mal*	(3:1)	40	–	–	–	–	9.71	9.71	–	10.7	15.33	0.51	75.8

[a] Initial sugar concentration.

Table 2
Effect of various sugar substrates, containing fructose, on *kefir* biomass production, at 30 °C

Sugar	Fixed-ratio	ISC[a] (g/l)	Residual sugar (g/l)					Ethanol (% v/v)	Lactic acid (g/l)	Total biomass (g)	Biomass yield (g/g)	Conversion (%)
			Fru	*Suc*	*Lac*	*Mal*	*Total*					
Fru		40	–	–	–	–	–	2.20	10.3	20.75	0.52	100.0
Fru:*Suc*	(1:1)	43	–	–	–	–	–	0.63	7.91	16.10	0.37	100.0
Fru:*Suc*	(1:3)	43	–	–	–	–	–	1.64	6.07	13.47	0.31	100.0
Fru:*Suc*	(3:1)	43	–	–	–	–	–	2.00	7.38	9.08	0.21	100.0
Fru:*Lac*	(1:1)	42	–	–	–	–	–	0.55	7.60	19.65	0.47	100.0
Fru:*Lac*	(1:3)	42	–	–	–	–	–	1.95	7.82	12.75	0.30	100.0
Fru:*Lac*	(3:1)	42	–	–	6.21	–	6.21	0.95	4.66	10.39	0.29	85.2
Fru:*Mal*	(1:1)	42	–	–	–	19.8	19.8	–	4.27	11.12	0.50	52.9
Fru:*Mal*	(1:3)	42	–	–	–	31.2	31.2	–	9.58	8.02	0.74	25.7
Fru:*Mal*	(3:1)	42	–	–	–	10.3	10.3	0.23	7.65	15.87	0.50	75.5

[a] Initial sugar concentration.

Table 3
Effect of various disaccharide substrates, on *kefir* biomass production, at 30 °C

Sugar	Fixed-ratio	ISC[a] (g/l)	Residual sugar (g/l)				Ethanol (% v/v)	Lactic acid (g/l)	Total biomass (g)	Biomass yield (g/g)	Conversion (%)
			Suc	*Lac*	*Mal*	*Total*					
Suc		42	0.55	–	–	0.55	0.51	8.71	19.75	0.48	98.7
Lac		40	–	9.45	–	9.45	0.50	8.77	13.87	0.45	76.4
Mal		40	–	–	40	40	–	9.24	6.37	–	–
Suc:*Lac*	(1:1)	42	–	–	–	–	0.64	6.39	18.60	0.44	100.0
Suc:*Lac*	(1:3)	42	–	–	–	–	0.88	6.51	19.17	0.46	100.0
Suc:*Lac*	(3:1)	42	–	–	–	–	0.84	6.68	17.72	0.42	100.0
Suc:*Mal*	(1:1)	43	–	–	19.0	19.0	0.42	6.60	10.80	0.45	55.9
Suc:*Mal*	(1:3)	43	–	–	30.3	30.3	–	10.0	9.70	0.76	29.7
Suc:*Mal*	(3:1)	43	–	–	9.52	9.52	1.15	6.30	11.85	0.35	77.9
Lac:*Mal*	(1:1)	40	–	–	19.2	19.2	–	9.99	2.07	0.10	52.1
Lac:*Mal*	(1:3)	40	–	–	30.4	30.4	0.37	8.09	5.12	0.54	23.9
Lac:*Mal*	(3:1)	40	–	–	9.78	9.78	0.40	9.12	10.58	0.35	75.6

[a] Initial sugar concentration.

2.3. Assays

Dissolved oxygen concentration, during fermentations, was determined using a Consort Multi Meter Analyser, Model C534. Ethanol, lactic acid and residual sugar were determined by high-performance liquid chromatography (HPLC) on a SHIMADZU LC-9A Liquid Chromatograph. A Shim-pack SCR-101N column (packed with a cation-exchange resin-sulphonated polystyrene-divinylbenzene copolymer), a RID-6A refractive index detector, a C-R6A Chromatopac integrator and three times distilled and filtered water as mobile phase at a flow rate of 0.8 ml/min, were used. The column temperature was 60 °C. Standard solutions of ethanol, lactic acid and sugars were prepared and determinations were done by means of standard curves. All samples were filtered using 0.22 μm pore size microfilters and 40 μl of them were injected directly into the column. Lactic acid and residual sugar concentrations were calculated as g/l and ethanol concentration was determined as % v/v (alcoholic degrees).

3. Results and discussion

The production of *kefir* biomass was studied using synthetic media containing various carbohydrates as carbon sources. To study (exclusively) the carbohydrate effect on cell growth, factors that influence biomass production, such as temperature, mineral salt concentrations and degree of aeration, were kept constant. The air flow was kept constant at 3 l/min during all experiments, and dissolved oxygen was maintained at levels as high as 6.5–7.2 mg/l, ensuring fully aerobic conditions (González Siso, Ramil, Cerdán, & Freire-Picos, 1996). The utilization of each sugar substrate was assessed by the determination of the produced biomass and residual sugar, as well as by the by-product ethanol and lactic acid formation. Kinetics of the bioconversion of selected carbohydrates were also studied. The results are given in Tables 1–3 and Figs. 1–3.

Glucose and fructose, when used as single carbon sources were totally converted by *kefir* grains, as were the majority of their mixtures (Tables 1 and 2). In the

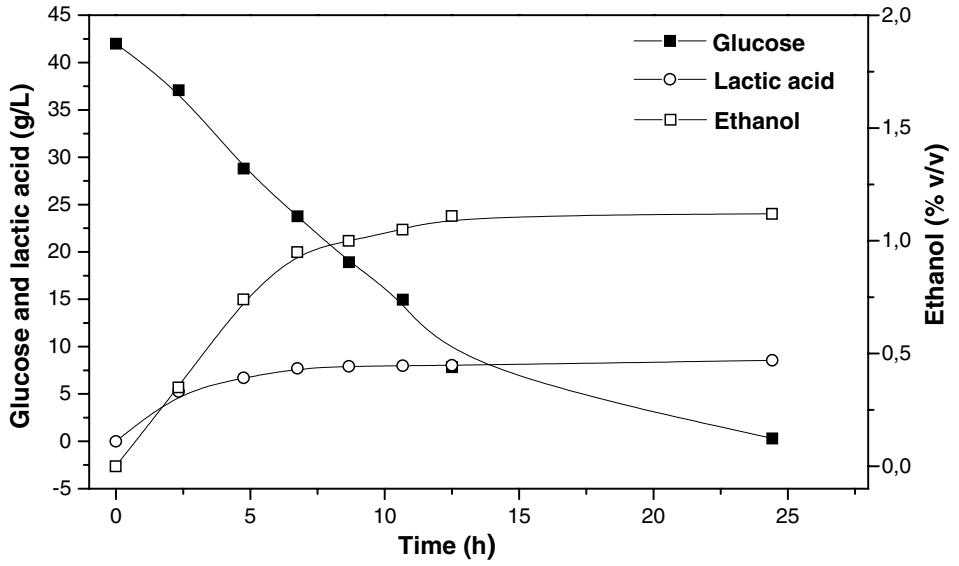

Fig. 1. Kinetics of glucose bioconversion by *kefir* grains.

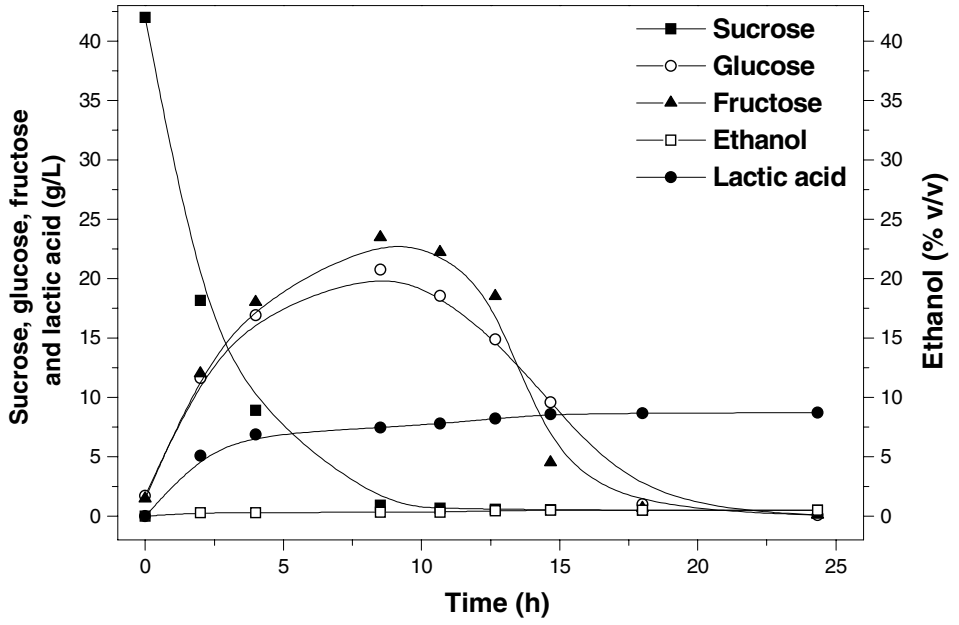

Fig. 2. Kinetics of sucrose bioconversion by *kefir* grains.

case of sucrose and lactose, only small quantities of residual sugar were observed when used, either as single carbon sources, or in mixtures with other sugars (Tables 1–3). On the other hand, when mixtures of disaccharides were used, conversions were not satisfactory, as only combinations of sucrose with lactose were totally utilized and maltose remained totally unfermented in all cases (Tables 1–3).

Specifically, biomass yields were 0.10–0.76 g/g and % conversion of sugars ranged from 0 to 100 (Tables 1–3). As far as single sugar substrates are concerned, fructose was found to be the most efficient, yielding 20.75 g of *kefir* grains, followed by sucrose (19.75 g) and glucose (17.50 g). Lactose produced less biomass (13.87 g), while maltose was not adequately utilized, yielding only 6.37 g of *kefir* grains. Among the tested mixtures of sugars, the *Glu*:*Suc* mixture, at a fixed-ratio of 1:3, produced the highest biomass (27.25 g) (Table 1). High biomass yields were also achieved by all the combinations of glucose and sucrose, by the *Glu*:*Fru* 1:1 mixture, the *Glu*:*Lac* 1:1 and 1:3 mixtures (Table 1), the *Fru*:*Lac* 1:1 mixture (Table 2) and all the combinations of sucrose and lac-

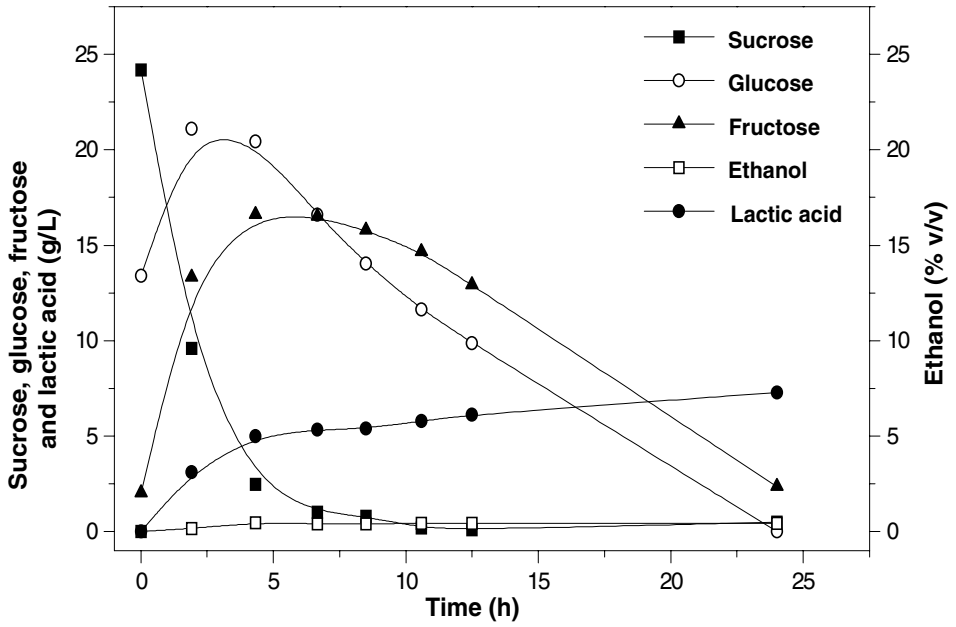

Fig. 3. Kinetics of the bioconversion of a mixture of glucose and sucrose at a fixed-ratio of 1:3, by *kefir* grains.

tose (Table 3). Ethanol concentrations at the end of fermentation were quite high in most cases (0.23–2.20% v/v). Lactic acid was also produced at relatively high concentrations (3.66–10.7 g/l). The formation of ethanol and lactic acid under aerobic conditions and high sugar concentrations is a typical characteristic of the "*sugar-sensitive*" or "*Crabtree effect-positive*" species present in *kefir*, such as yeast species (e.g. *Saccharomyces*) and the strictly fermentative (homo-fermentative or homo-lactic) members of the lactic acid bacteria (e.g. *Streptococcus*, *Lactococcus*, *Lactobacillus*).

The bioconversion kinetics, by *kefir* grains, of glucose, sucrose and the mixture of glucose and sucrose (*Glu*:*Suc*) at a fixed-ratio of 1:3, were studied during a 24-h experiment (Figs. 1–3, respectively). In the first case, glucose (42 g/l) was totally utilized, leading mainly to biomass production (17.5 g), and partially to ethanol (1.12% v/v) and lactic acid (8.55 g/l), which were produced during the first hours of fermentation and remained constant throughout the experiment (Table 1). In the case of sucrose (42 g/l), it was hydrolysed rapidly to glucose and fructose, whose concentrations reached a maximum after about 8.5 h. The monosaccharides were further metabolised, until total consumption, producing 19.75 g *kefir* biomass, 7.5 g/l lactic acid and 0.51% v/v ethanol (Table 3). Finally, during the utilization of the *Glu*:*Suc*, 1:3 mixture, the sucrose concentration was fully converted after 6.5 h. Glucose and fructose concentrations were initially increased due to their formation by sucrose hydrolysis, and glucose was converted faster than fructose, revealing a preference of *kefir* microflora for this sugar. All sugars were totally consumed, leading to the highest biomass production (27.25 g) compared to the other tested sugars. The final lactic acid concentration was 7.28 g/l and ethanol was 0.43% v/v (Table 1).

4. Conclusions and technological consideration of results

From the above results, it is obvious that various agro-industrial wastes could be effectively treated to produce large quantities of *kefir* biomass, therefore producing added value and reducing pollution. *Kefir* grains could be used in baking as baker's yeast or sourdough starter, since their unique microbial composition enables lactose utilization via lactic acid bacteria activity, and sucrose, glucose and fructose bioconversion through yeast metabolic pathways. Various wastes in proper combinations, containing the above carbohydrates, such as milk whey, molasses, citrus, starch and raisin-processing residues, could therefore be used as raw materials for *kefir* biomass production. The feasibility of industrial application is additionally supported by the fact that *kefir* grains precipitate rapidly, allowing the possibility of avoiding centrifugal separators, thus reducing cost. The technological aspects and economical feasibility of a large-scale biomass production process, the suitability for baking as well as the potential probiotic properties of the proposed mixed culture will be the focus of future investigations.

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