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Saccharomyces cerevisiae and Kefir Production using Waste Pomegranate Juice, Molasses, and Whey

Chryssa NOUSKA[1], Ioanna MANTZOURANI[1], Athanasios ALEXOPOULOS[1], Eugenia BEZIRTZOGLOU[1], Argyro BEKATOROU[2], Konstantoula AKRIDA-DEMERTZI[3], Panagiotis DEMERTZIS[3] and Stavros PLESSAS[1]

[1]Laboratory of Microbiology, Biotechnology and Hygiene, Faculty of Agriculture Development, Democritus University of Thrace, Orestiada, Greece; [2]Department of Chemistry, University of Patras, Patras, Greece; [3]Laboratory of Food Chemistry and Technology, Department of Chemistry, University of Ioannina, Ioannina, Greece

Abstract

Nouska Ch., Mantzourani I., Alexopoulos A., Bezirtzoglou E., Bekatorou A., Akrida-Demertzi K., Demertzis P., Plessas S. (2015): ***Saccharomyces cerevisiae* and kefir production using waste pomegranate juice, molasses, and whey**. Czech J. Food Sci., 33: 277–282.

The growth of *Saccharomyces cerevisiae* (baker's yeast) and kefir was studied in substrates containing pomegranate juice, molasses, and cheese whey, at various conditions such as fermentation temperature, air supply, initial sugar concentration, and substrate composition. The results showed that, in the case of kefir, the highest production yield of biomass (0.24 g/g of utilised sugar) and productivity (6.5 g/l/day) was obtained in 40/60 and 20/80% of pomegranate/cheese whey. *S. cerevisiae* grew easily on all substrates with higher cell mass yields (0.34 g/g) and productivities (13.1 g/l/day) compared to kefir, with the best results obtained at the ratio of 40/60 and 20/80% of pomegranate/molasses. These results are promising regarding the exploitation of non-conventional substrates, such as the juice from discarded pomegranate fruits of a currently significantly increasing market, for microbial biomass production.

Keywords: baker's yeast; lactic acid bacteria; cheese whey; growth; agricultural wastes; fruit

The majority of food industry liquid wastes, like cheese whey, are usually discarded since their high organic load is prohibitive for direct disposal in biological treatment plants. On the other hand, solid wastes such as fruit and vegetable wastes, brewery by-products, etc., are partially utilised as animal feeds or otherwise discarded. These wastes are rich in nutrients, and therefore their biotechnological utilisation is of great importance from both economic and environmental points of view. Specifically, single cell protein (SCP) production by microbial treatment of various agro-industrial wastes has been the subject of extensive research mainly for the production of animal feeds enriched with protein. The most common examples of food grade SCP are yeasts destined for use as food starters, yeast extract production, and as food and feed supplements (Bekatorou *et al.* 2006). However, SCP production or development of other fermentation processes employing mixed food waste substrates, without addition of synthetic nutrients, has been scarcely reported (Aggelopoulos *et al.* 2013). To explore this possibility, the use of mixed cultures of species with different carbohydrate bioconversion abilities, such as kefir, has been proposed (Harta *et al.* 2004). Kefir is a natural dairy culture consisting of a symbiotic consortium of yeasts and bacteria, used for alcoholic and lactic acid fermentation of milk in the areas around the Caucasus (Plessas *et al.* 2011). Single cultures have also been used for waste utilisation, such as *Saccharomyces cerevisiae*, which is produced worldwide mainly using molasses (Bekatorou *et al.* 2006).

Cheese whey has been extensively studied as a substrate for kefir-based dairy and baking starter culture production, as well as for ethanol and lactic acid production by fermentation with kefir, in laboratory and pilot scale operations (Plessas *et al.* 2007). Mo-

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lasses is also a renewable carbohydrate-rich substrate, ready-to-use for ethanol production (Kopsahelis *et al.* 2007). Its use for microbial growth is determined by its availability and cost, composition and absence of toxic substances and fermentation inhibitors (Bekatorou *et al.* 2006). Finally, discarded fruit and fruit industry wastes have also been proposed as raw materials for biotechnological conversion to added value products through a biorefinery approach, such as enzymes, animal feeds, organic acids, pectins, biofuels, etc. Discarded citrus fruits have been proposed as growth media for the production of starter cultures, such as kefir and baker's yeast, for applications in bread making, alcoholic, and dairy fermentations (Plessas *et al.* 2008).

The use of pomegranate for SCP production has not been reported. The juice can be obtained from discarded fruits of the recently fast-growing industry due to the known beneficial effects of pomegranate consumption, mainly its antioxidant properties that have attracted both research and commercial interest (Tezcan *et al.* 2009). Pomegranate (common name of the plant *Punica granatum* L.) is native to and widely cultivated in Asia as well as the Mediterranean region of southern Europe, Latin America, and California. The global production is about 2 250 000 t with India being the largest producer (50%). Pomegranate is also native to Greece and its cultivation is currently encouraged and funded by both public and private sectors. The aim of this study was to evaluate the use of pomegranate juice as sole substrate or in combination with molasses or cheese whey for optimised SCP production of *S. cerevisiae* (baker's yeast) or the natural mixed culture kefir.

MATERIAL AND METHODS

Microorganisms and media. Kefir grains were obtained from a commercial kefir drink. Free kefir cells were isolated at the Department of Agricultural Development of the Democritus University of Thrace as described in Plessas *et al.* (2012). In brief, the kefir grains were added to MRS broth and blended in a Stomacher Blender 400 (Seward Laboratory, London, UK). Aliquots of the supernatant were spread plated on MRS agar and cultured at 37°C. Further growth of kefir culture (from this point on referred to as "kefir" containing free cells of both lactic acid bacteria and yeasts) was done by successive cultivations in MRS broth in cotton-plugged flasks at 37°C, without agitation (static cultures). No other measurements were taken to avoid oxygen penetration into the media. The cells were harvested by centrifugation at 5000 rpm for 10 min and were used as inocula in the following fermentation experiments. For viable counts in the kefir culture (Chen *et al*. 2008), decimal dilutions in 0.1% peptone water of the kefir culture were plated on M17 agar and MRS agar (Merck) for the determination of *Lactococcus* spp. and *Lactobacillus* spp., respectively. For yeasts, yeast extract glucose chloramphenicol (YGC) agar (Merck) was used. To avoid the growth of yeasts on the bacterial plates and conversely, 200 mg/l cycloheximide was added to M17 and MRS, and 100 mg/l oxytetracycline hydrochloride was added to YGC. The plates were incubated at 25°C for 5 days for YGCA, and under anaerobic conditions (6% CO_2) at 30°C for 2 days for M17 and 4 days for MRS. The results were expressed as log colony forming units (CFU) per ml. *S. cerevisiae* was a commercial pressed (fresh) baker's yeast product manufactured by S.I. Lesaffre, Marcq-en-Baroeul, France. Fully ripe pomegranate (*Punica granatum L.*) fruits were obtained from the local market of Orestiada, Greece. They were carefully washed and cut into halves and the edible parts were separated and blended for juice extraction. The juice was then pressed manually and filtered using a cheesecloth for separation of the seed solids. The initial sugar concentration was determined by HPLC as described below, and the juice was then diluted to various concentrations with sterilised distilled water. The initial sugar concentration of the juice was on average 150 g/l (75.3 g/l fructose, 67.3 g/l glucose, and 7.3 g/l sucrose). Cheese whey was produced from commercial cow's milk by addition of 0.1 g rennin/l and incubation in a water bath at 37°C. The mixture was left to stand for 1 h for casein coagulation and cheese whey was separated by cloth filtration. The average sugar concentration of whey produced by the above procedure was 49.9 g/l (45.9 g/l lactose, 2.3 g/l glucose, and 1.7 g/l galactose). Molasses was supplied by the BG Spiliopoulos SA alcohol distillery (Patras, Greece). It contained on average 23% (wt) moisture, 77% solids, 43% total fermentable sugar (sucrose, fructose and glucose) and had pH 4.7. The proximate composition of molasses from the above supplier was described in more detail in a previous study (Aggelopoulos *et al.* 2013). Molasses were also diluted with distilled water to various sugar concentrations and were enriched with KH_2PO_4 and $(NH_4)_2SO_4$ at a concentration of 1 g/l. The initial pH was adjusted to 5.5 by the addition of 10% NaOH solution. All media were sterilised by autoclaving at 120°C for 15 minutes.

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Fermentations. Pomegranate juice, diluted with sterilised water (500 ml), and appropriate quantities of diluted molasses (in the case of *S. cerevisiae*) or cheese whey (in the case of kefir) were mixed in 1 l conical flasks to obtain the desirable initial sugar concentrations (40 and 50 g/l), and then 0.7 g (dry weight – DW) of kefir culture or *S. cerevisiae* were added. *S. cerevisiae* growth took place at 30°C and at two different air flow rates (500 and 1000 ml/min). The air was supplied to each flask using aquarium type air pumps and air spargers, for both oxygen supply and stirring, and was sterilised by passing through a bacteriostatic filter. Foaming was controlled by the addition of a few drops of antifoam if necessary. Kefir was grown at two different temperatures (30 and 35°C) without air supply or agitation (static cultures). Totally 28 different growth experiments (each carried out in triplicate) were organised using substrates of different compositions as shown in Table 1.

Analyses. Cell mass concentrations (DW/l) were determined by harvesting and weighing the cell mass produced at the end of each process. Dry cell mass was obtained by drying at 105°C for 24 hours. To eliminate the interference from solids and coloured substances of pomegranate juice, molasses and cheese whey, equal amounts of each substrate were centrifuged under the same conditions and the weight of

Table 1. Effects of initial sugar concentration (ISC) and air flow *on S. cerevisiae* growth at 30°C in various pomegranate juice/molasses substrates

Batch	Vol. ratio of pomegranate juice/molasses (ml)	Air flow (ml/min)	Initial sugar (g/l)	Produced cell mass (g DW/l)	Time (h)	Cell mass productivity (g/l/day)	Residual sugar (g/l)	Cell mass yield (g/g DW)
1	100/0	500	40 ± 2	6.7 ± 0.2	35 ± 1	4.6 ± 0.2	1.1 ± 0.05	0.17 ± 0.01
2	100/0	500	50 ± 1	7.2 ± 0.3	29 ± 2	5.9 ± 0.3	1.7 ± 0.00	0.15 ± 0.02
3	100/0	1000	40 ± 2	7.8 ± 0.3	28 ± 1	6.7 ± 0.2	1.9 ± 0.10	0.20 ± 0.02
4	100/0	1000	50 ± 2	8.6 ± 0.2	30 ± 1	6.9 ± 0.2	1.8 ± 0.10	0.18 ± 0.01
5	80/20	500	40 ± 2	7.2 ± 0.1	26 ± 1	6.6 ± 0.2	1.8 ± 0.10	0.19 ± 0.01
6	80/20	500	50 ± 2	9.1 ± 0.1	27 ± 1	8.1 ± 0.3	1.5 ± 0.10	0.19 ± 0.01
7	80/20	1000	40 ± 2	8.5 ± 0.1	33 ± 2	6.2 ± 0.2	1.7 ± 0.10	0.22 ± 0.01
8	80/20	1000	50 ± 2	8.9 ± 0.1	29 ± 2	7.4 ± 0.2	0.9 ± 0.10	0.18 ± 0.02
9	60/40	500	40 ± 1	9.7 ± 0.1	26 ± 1	8.9 ± 0.4	0.8 ± 0.05	0.24 ± 0.02
10	60/40	500	50 ± 1	9.9 ± 0.1	30 ± 2	7.9 ± 0.3	1.1 ± 0.10	0.20 ± 0.02
11	60/40	1000	40 ± 2	10.3 ± 0.2	26 ± 1	9.5 ± 0.2	0.6 ± 0.05	0.26 ± 0.01
12	60/40	1000	50 ± 2	11.4 ± 0.2	29 ± 2	9.4 ± 0.2	1.2 ± 0.10	0.23 ± 0.02
13	50/50	500	40 ± 1	10.6 ± 0.2	30 ± 2	8.5 ± 0.2	0.9 ± 0.10	0.27 ± 0.02
14	50/50	500	50 ± 2	11.4 ± 0.2	29 ± 1	9.4 ± 0.2	0.9 ± 0.05	0.23 ± 0.02
15	50/50	1000	40 ± 1	11.9 ± 0.2	29 ± 1	9.8 ± 0.2	0.8 ± 0.10	0.30 ± 0.02
16	50/50	1000	50 ± 2	11.9 ± 0.1	31 ± 2	9.2 ± 0.3	1.2 ± 0.10	0.24 ± 0.01
17	40/60	500	40 ± 2	12.3 ± 0.1	25 ± 1	11.8 ± 0.2	1.1 ± 0.10	0.32 ± 0.02
18	40/60	500	50 ± 2	12.1 ± 0.1	27 ± 1	10.7 ± 0.2	0.6 ± 0.10	0.25 ± 0.01
19	40/60	1000	40 ± 1	13.2 ± 0.1	28 ± 1	11.3 ± 0.2	0.6 ± 0.05	0.34 ± 0.01
20	40/60	1000	50 ± 2	13.4 ± 0.2	28 ± 2	11.5 ± 0.2	0.4 ± 0.10	0.27 ± 0.01
21	20/80	500	40 ± 2	12.2 ± 0.1	25 ± 1	11.7 ± 0.3	0.5 ± 0.10	0.31 ± 0.01
22	20/80	500	50 ± 2	14.9 ± 0.1	25 ± 1	14.3 ± 0.2	0.6 ± 0.05	0.30 ± 0.02
23	20/80	1000	40 ± 2	13.1 ± 0.1	24 ± 1	13.1 ± 0.2	0.9 ± 0.05	0.34 ± 0.01
24	20/80	1000	50 ± 2	14.9 ± 0.1	27 ± 1	16.8 ± 0.2	0.3 ± 0.05	0.34 ± 0.01
25	0/100	500	40 ± 2	9.8 ± 0.1	29 ± 2	11.8 ± 0.1	1.3 ± 0.05	0.30 ± 0.01
26	0/100	500	50 ± 2	11.2 ± 0.1	26 ± 1	12.1 ± 0.2	1.5 ± 0.05	0.25 ± 0.01
27	0/100	1000	40 ± 1	9.9 ± 0.1	27 ± 2	11.2 ± 0.1	1.2 ± 0.10	0.29 ± 0.02
28	0/100	1000	50 ± 3	12.1 ± 0.1	28 ± 1	14.2 ± 0.1	1.3 ± 0.10	0.29 ± 0.02

DW – dry weight

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the harvested residue was deducted from the final weight of each fermentation produced cell mass. Cell mass productivity was expressed as g DW of cell mass produced per litre and per day. Cell mass yield was expressed as g DW of cell mass produced per g of utilised sugar, and conversion was calculated using the equation: Sugar conversion (%) = [(Initial sugar – Residual sugar)/Initial sugar)] × 100. Ethanol and sugar concentrations were determined by HPLC on a Shimadzu chromatograph with a SCR-101 N stainless steel column, LC-9A pump, CTO-10A oven at 60°C and RID-6A refractive index detector. Triple distilled water was used as a mobile phase with a flow rate of 0.8 ml/min and 1-butanol was used as an internal standard. Samples of 0.5 ml of effluent and 2.5 ml of a 10 ml 1-butanol/l solution were diluted to 50 ml and 40 µl were injected directly into the column. Sugar and ethanol concentrations were calculated using standard curves and expressed in terms of g of sugar per l and % (w/w), respectively.

RESULTS AND DISCUSSION

Fermentation of pomegranate juice for SCP (S. cerevisiae and kefir) production. The growth of kefir or *S. cerevisiae* in substrates containing solely pomegranate juice or its mixture with molasses or cheese

Table 2. Effects of initial sugar concentration (ISC) and temperature on kefir growth in various pomegranate juice/cheese whey substrates

Batch	Vol. ratio of pomegranate juice/cheese whey (ml)	Temperature (°C)	Initial sugar (g/l)	Produced cell mass (g DW/l)	Time (h)	Cell mass productivity (g/l/day)	Residual sugar (g/l)	Cell mass yield (g/g DW)
1	100/0	30	40 ± 1	4.2 ± 0.3	42 ± 1	2.4 ± 0.1	1.4 ± 0.05	0.11 ± 0.02
2	100/0	30	50 ± 2	5.1 ± 0.3	34 ± 1	3.6 ± 0.2	1.7 ± 0.05	0.11 ± 0.02
3	100/0	35	40 ± 1	4.8 ± 0.2	35 ± 1	3.3 ± 0.2	1.9 ± 0.05	0.13 ± 0.01
4	100/0	35	50 ± 2	5.3 ± 0.1	37 ± 1	3.4 ± 0.2	1.8 ± 0.05	0.11 ± 0.02
5	80/20	30	40 ± 1	6.3 ± 0.3	32 ± 1	4.7 ± 0.2	1.8 ± 0.05	0.16 ± 0.01
6	80/20	30	50 ± 2	5.9 ± 0.2	36 ± 2	3.9 ± 0.1	1.5 ± 0.05	0.12 ± 0.02
7	80/20	35	40 ± 1	7.1 ± 0.3	35 ± 1	4.8 ± 0.2	1.7 ± 0.05	0.19 ± 0.02
8	80/20	35	50 ± 2	6.5 ± 0.2	37 ± 1	4.2 ± 0.2	1.5 ± 0.05	0.13 ± 0.01
9	60/40	30	40 ± 2	6.8 ± 0.2	33 ± 1	4.9 ± 0.1	1.3 ± 0.05	0.18 ± 0.01
10	60/40	30	50 ± 2	6.9 ± 0.2	31 ± 2	5.3 ± 0.2	1.2 ± 0.05	0.14 ± 0.01
11	60/40	35	40 ± 1	7.4 ± 0.1	31 ± 1	5.7 ± 0.1	1.3 ± 0.05	0.19 ± 0.02
12	60/40	35	50 ± 2	7.4 ± 0.1	32 ± 1	5.6 ± 0.2	1.2 ± 0.05	0.15 ± 0.02
13	50/50	30	40 ± 2	7.6 ± 0.1	31 ± 1	5.8 ± 0.2	1.3 ± 0.05	0.19 ± 0.02
14	50/50	30	50 ± 2	8.9 ± 0.1	33 ± 2	6.5 ± 0.2	1.4 ± 0.05	0.18 ± 0.02
15	50/50	35	40 ± 1	8.3 ± 0.1	35 ± 1	5.7 ± 0.2	1.5 ± 0.05	0.22 ± 0.01
16	50/50	35	50 ± 2	8.5 ± 0.1	32 ± 1	6.4 ± 0.1	1.3 ± 0.10	0.17 ± 0.01
17	40/60	30	40 ± 2	7.5 ± 0.1	34 ± 1	5.3 ± 0.2	1.2 ± 0.15	0.19 ± 0.01
18	40/60	30	50 ± 2	9.9 ± 0.1	32 ± 1	7.4 ± 0.1	1.0 ± 0.10	0.20 ± 0.02
19	40/60	35	40 ± 1	8.5 ± 0.1	32 ± 1	6.4 ± 0.2	1.0 ± 0.10	0.22 ± 0.01
20	40/60	35	50 ± 2	10.1 ± 0.2	32 ± 2	7.6 ± 0.1	1.0 ± 0.10	0.21 ± 0.01
21	20/80	30	40 ± 2	8.7 ± 0.2	31 ± 1	6.7 ± 0.1	1.2 ± 0.10	0.22 ± 0.01
22	20/80	30	50 ± 2	9.8 ± 0.2	33 ± 1	7.1 ± 0.1	1.1 ± 0.10	0.20 ± 0.02
23	20/80	35	40 ± 2	9.2 ± 0.2	34 ± 1	6.5 ± 0.1	1.1 ± 0.10	0.24 ± 0.01
24	20/80	35	50 ± 1	10.1 ± 0.2	34 ± 1	7.1 ± 0.2	1.1 ± 0.10	0.21 ± 0.01
25	0/100	30	40 ± 1	9.5 ± 0.2	34 ± 2	6.7 ± 0.2	1.1 ± 0.05	0.24 ± 0.01
26	0/100	30	50 ± 2	9.7 ± 0.1	29 ± 1	8.0 ± 0.2	1.1 ± 0.05	0.19 ± 0.01
27	0/100	35	40 ± 1	8.2 ± 0.2	29 ± 2	6.8 ± 0.2	1.4 ± 0.05	0.21 ± 0.01
28	0/100	35	50 ± 1	9.3 ± 0.2	30 ± 2	7.0 ± 0.2	1.5 ± 0.05	0.19 ± 0.01

DW – dry weight

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whey, respectively, was evaluated. Cheese whey and molasses are widely used as substrates for microbial growth and SCP production. Pomegranate juice is proposed (a) for the utilisation of discarded fruit of the expanding pomegranate-based products industry, (b) its potential beneficial effect on the nutritional value of the produced cell mass, and (c) to evaluate the effect on microbial growth as substrate without the need of extra nutrients (e.g. yeast extract). In order to study the effect of these substrates on cell growth, factors such as added mineral salt concentrations, pH and temperature were kept constant. During kefir cultivation, neither agitation nor air supply was applied in order to allow the slow growth of lactic acid bacteria. Specifically, the culture contained 9.85 ± 0.08, 7.61 ± 0.05, and 4.51 ± 0.05 log CFU/ml of *Lactobacillus* spp., *Lactococcus* spp., and yeasts, respectively. In the case of *S. cerevisiae* two air flow rates were applied (500 and 1000 ml/min). In both cases the effect of the initial sugar concentration was studied (40 and 50 g/l). The utilisation of each substrate was assessed by the determination of the produced cell mass and residual sugar, as well as by the determination of cell mass productivity and yield (Tables 1 and 2).

***S. cerevisiae* growth in pomegranate juice and molasses.** Higher cell mass concentrations (13.1 and 13.2 g/l), productivities (11.3 and 13.1 g/l/day), and yield (0.34 g/g sugar) were observed when pomegranate juice and molasses were used at ratios 40/60 and 20/80, respectively, at a 1000 ml/min air flow rate and initial sugar concentration 40 g/l (Table 1). It is noteworthy that when molasses and pomegranate juice were used as single substrates (Table 1, batches 1–4 and 25–28, respectively), pomegranate juice was efficient and comparable with molasses, which is the traditional substrate used for commercial baker's yeast production. The obtained cell mass yields were quite high compared to other data found in the literature (Table 3) with low levels of residual sugar (< 1.5 g/l). Therefore, the use of pomegranate juice was shown to be effective as a growth substrate for baker's yeast production.

Kefir growth in pomegranate juice and cheese whey. For kefir growth, two fermentation temperatures were examined (30 and 35°C) due to the multispecies character of kefir. No air was supplied to allow the growth of lactic acid bacteria. The results (Table 2) showed that pomegranate juice could be effectively added to cheese whey-based kefir growth media. Specifically, higher cell mass concentration (8.5 and 9.5 g/l), productivity (6.4 and 6.7 g/l/day) and yield (0.22–0.24 g/g) were observed when pomegranate juice and cheese whey were used at ratios 40/60 or 20/80 at 35°C and initial sugar concentration 40 g/l (Table 2). Comparing cheese whey and pomegranate juice as single substrates (batches 1–4 and 25–28, respectively) it was observed that pomegranate juice was more effective than cheese whey. Residual sugar was low in almost all the studied fermentation batches (Table 2). The positive effect on kefir growth may be due to the requirements that lactic acid bacteria have for trace elements and vitamins found in pomegranate juice as other researchers have demonstrated. Therefore, no addition of such nutrients was needed for the enrichment of the substrates (Akpinar-Bayizit 2010). The obtained cell mass yields were quite satisfactory compared to other data in the literature as shown in Table 3. In both cases, *S. cerevisiae* and kefir, very low ethanol concentrations were observed (~0.1% v/v). In the case of kefir,

Table 3. Cell mass productivities and yields obtained from the literature for batch type propagation in various substrates

Raw material	Microorganism	Biomass productivity (g DW/l/day)	Biomass yield (g/g utilised sugar DW)	Reference
Molasses	*S. cerevisiae* (I)	2.7	nr	Khan *et al.* (1995)
	S. cerevisiae (II)	2.2	nr	Khan *et al.* (1995)
	Candida utilis	5.8	0.67	Lee & Kim (2001)
	Candida utilis	51.6	0.36	Lee & Kim (2001)
Glucose	*Saccharomyces* sp. LK3G	13.4	0.20	Konlani *et al.* (1996)
	Candida krusei SO1	16.3	0.25	Konlani *et al.* (1996)
Molasses contaminated with lead	baker's yeast	4.8	nr	Skountzou *et al.* (2003)
Molasses and orange peel as promoter	baker's yeast	14.9	0.22	Plessas *et al.* (2007)

DW – dry weight; nr – not referred

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lactic acid production was observed at concentrations up to 1 g/l, which is justified by the presence of oxygen in the medium and mutually interacting consortium of kefir yeasts and lactic acid bacteria.

CONCLUSIONS

Pomegranate juice is a good source of nutrients and can be added to cheese whey or molasses to produce effective *S. cerevisiae* (baker's yeast) or kefir growth media, respectively, without addition of extra nutrients (e.g. extracts and vitamins). The combination of pomegranate juice from discarded fruit with such major wastes of the food industry can facilitate their utilisation as microbiology media, thus imparting environmental benefits and creating added value. The technological aspects and economic feasibility of large-scale cell mass production and the potential probiotic properties of the proposed mixed culture should be the focus of future investigations.

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Corresponding author:

Asst Prof Dr Stavros Plessas, Democritus University of Thrace, Faculty of Agriculture Development, Laboratory of Microbiology, Biotechnology and Hygiene, 193 Pantazidou str. GR-68200, Orestiada, Greece; E-mail: splessas@agro.duth.gr