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Upgrading of discarded oranges through fermentation using kefir in food industry

Stavros Plessas [a], Dionysios Koliopoulos [a], Yiannis Kourkoutas [a], Constantinos Psarianos [a], Athanasios Alexopoulos [b], Roger Marchant [c], Ibrahim M. Banat [c], Athanasios A. Koutinas [a,*]

[a] *Food Biotechnology Group, Section of Analytical Environmental and Applied Chemistry, Department of Chemistry, University of Patras, GR-26500 Patras, Greece*
[b] *Laboratory of Food Microbiology, Biotechnology and Hygiene, Faculty of Agricultural Development, Democritus University of Thrace, 193 Pandazidou Str., Orestiada, GR-68200, Greece*
[c] *School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK*

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Abstract

Upgrading of orange pulp suspension by enrichment with kefir was investigated and the effects of initial sugar concentration, pH, aeration and the addition of molasses on kefir growth were monitored. Higher maximum growth rate μ_{max} for kefir was observed when initial sugar used was 75 g/l. Both 75 g/l and 90 g/l initial sugar contents of kefir resulted in higher final biomass concentration and daily biomass productivity. At an initial sugar concentration >75 g/l, however, high residual sugar and ethanol concentrations were observed. Air flow rate and pH affected kefir's μ_{max}, while the addition of molasses had no effect on growth. Potential application of upgraded orange pulp in bread-making was examined. Bread produced by immobilized kefir on orange pulp had an improved aromatic profile in comparison with bread produced by baker's yeast, and preliminary sensory evaluation of the produced bread was acceptable.

Keywords: Orange pulp; Immobilization; Kefir; Aerobic fermentation; Sourdough bread

1. Introduction

Exploiting waste residues from the citrus industry has been a subject of interest. Enzymatic or alkaline treatments of orange pulp were proposed for animal feed production (Tripodo, Lanuzza, Micali, Coppolino, & Nucita, 2004) and lemon pulp was used for SCP and crude pectinase production (De Gregorio et al., 2002). Orange pulp extracts have also been utilized as a carbon source in continuous production of single cell protein, using the microorganism *Geotrichum candidum* (Ziino et al., 1999). The most common exploitation of citrus wastes is in the application of solid state fermentation to increase protein and decrease moisture content, leading to a commercial process for the production of feed for dairy cattle (Scerra, Caridi, Foti, & Sinatra, 1999).

Kefir yeast is a known culture, fermenting milk to produce the traditional Russian drink "kefir" with low alcohol content. It is a mixed culture consisting of various yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia*) and various bacteria of the genus *Lactobacillus* (Luis, Lopez, & Lema, 1993). Yeast and lactic acid bacteria co-exist in a symbiotic association (Beshkova, Simova, Simov, Frengova, & Spasov, 2002; Koroleva, 1988; Vedamuthu, 1982). This mixed culture is able to utilize lactose and therefore, whey could be used as a raw material for kefir biomass production (Koutinas, Athanasiadis, Bekatorou, Iconomopoulou, & Blekas, 2005). Kefir biomass has also been proposed for use as baker's yeast in baking (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005) and the

* Corresponding author. Tel.: +30 2610 997104; fax: +30 2610 997105.
E-mail address: A.A.Koutinas@upatras.gr (A.A. Koutinas).

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functional properties of single cell protein produced by kefir were also extensively studied (Paraskevopoulou et al., 2003). Baker's yeast, on the other hand, is widely used in the food industry for many applications, especially in baking. Many researchers have studied and proposed a variety of methods for baker's yeast production, using food wastes (Ejiofor, Chisti, & Moo-Young, 1996; Khan, Abulnaja, Kumosani, & Abou-Zaid, 1995; Lee & Kim, 2001; Lotz, Frohlich, Matthes, Schugerl, & Seekamp, 1991).

The aim of the present study, therefore, was to investigate (i) potential upgrading of discarded oranges by enrichment with kefir and (ii) the suitability of the immobilized kefir to produce bread with an acceptable flavour.

2. Materials and methods

2.1. Microorganisms and fermentation media

Kefir yeast, a commercial product usually used to produce kefir drink and available at the Department of Chemistry at Patras University, was used. Kefir biomass was produced by aerobic fermentation (3 l/min air supply) in 1 l of liquid nutrient medium containing 20 g/l of glucose, 20 g/l of lactose, 1 g/l of $(NH_4)_2SO_4$, 1 g/l of KH_2PO_4, 10.2 g/l of $MgSO_4 \cdot 7H_2O$ and 4 g/l of yeast extract at 30 °C. The media was sterilized at 120 °C for 15 min. After aerobic growth, kefir culture consisted of approximately 10^{10}–10^{11} cfu/ml total aerobic count, 10^6–10^7 cfu/ml of yeasts and lactobacilli and 10^8 cfu/ml of lactococci. The produced biomass (small grains of approximately 0.5 mm diameter) was harvested by centrifugation at 5000 rpm for 10 min to yield pressed kefir biomass. Baker's yeast was a commercial *Saccharomyces cerevisiae* strain obtained in the form of pressed blocks (70% w/w moisture), manufactured by S.I. Lesaffre, France.

Molasses was produced by dilution with distilled water to obtain a sugar concentration of 20 g/l and by adding 0.1% KH_2PO_4 and 0.1% $(NH_4)_2SO_4$. Initial pH was adjusted to 5.5 and the mixture autoclaved.

2.2. Orange pulp suspension

Oranges were washed twice with water; the external (yellow *exocarp*) parts of skins were removed and the remainder was blended for 10 min to produce the pulp suspension. Water was added in order to adjust the initial sugar concentration and used without sterilization.

2.3. Aerobic fermentations

Orange pulp suspension (700 ml) with and without molasses, and 0.4 g (dry weight) of kefir biomass were dispensed into a glass bioreactor of 42.5 cm height and 7.4 cm internal diameter. Filtered sterile air was continuously supplied to the bioreactor, using an air compressor (Compressori D' Aria LT 50 HP 1.5). Foam was controlled using a silicon anti-foaming agent (Merck). The pH was continuously controlled using 8% (w/v) NaOH or 10% (v/v) H_2SO_4 solutions when necessary. The effects of initial sugar concentration, pH, air flow rate and addition of molasses on kefir biomass production were monitored. Samples were collected at various time intervals and analyzed for biomass concentration, residual sugar and alcohol concentration. All treatments were carried out in duplicate and the mean values are presented (max deviation for all values was approximately ±5%).

2.4. Baking

Dough was prepared by using the direct and sourdough techniques. In the direct method, 10% v/v of kefir immobilized on orange pulp was added to 500 g of flour (white flour manufactured by Hellenic Allatini Co, Greece, containing 13% protein), 300 ml of water and 4 g of salt were also added. Sourdough was prepared by mixing 500 g of wheat flour, 300 ml of water and 10% v/v (on flour basis) of kefir immobilized on orange pulp and using traditional procedures (Paramithiotis, Chouliaras, Tsakalidou, & Kalantzopoulos, 2005); an amount of 100 g of the final sourdough was used for bread-making with the addition of 500 g of flour, 300 ml of water and 4 g of salt. Dough samples were kneaded for 10 min, and then incubated for 30–40 min at 30 °C to allow initial rising. Proofing was carried out at 46 °C for 60–80 min. The loaves were then baked in an oven at 210 °C for 60 min and allowed to cool at room temperature. For comparison, commercial bread was produced by adding 2% w/w (on flour basis) wet free cells of baker's yeast to 500 g of wheat flour, 280 mL of tap water and 4 g of salt. After mixing the ingredients for 10 min, the dough was fermented at 30 °C for 30 min, proofed at 46 °C for 30 min and baked at 230 °C for 40 min. The experiments were carried out in triplicate.

2.5. Analyses

Biomass concentrations (dry weight/l) were determined using optical density calibrated against dry weight (Klein & Kressdorf, 1983). Dry biomass was obtained by drying at 105 °C for 24 h. In order to eliminate interference from solids, coloured substances contained in orange, and possibly by degradation or transfer of orange substances to the fermentation broth, samples containing equal amounts of orange suspensions collected at the same time from an identical bioreactor, using exactly the same fermentation conditions were used as blanks.

Biomass productivity was expressed as grams (dry weight) of biomass produced per litre per day. Biomass yield was expressed as g (dry weight) of biomass produced/g of utilized sugar and conversion was calculated using the following equation:

$$(\text{Initial sugar concen.} - \text{Residual sugar concen.})/\text{Initial sugar concen.} \times 100.$$

Maximum growth rate μ_{max} was determined by linear regression (indicated by the correlation coefficient r^2) from the plots of optical density vs. time.

Ethanol and residual sugar concentrations were determined by high performance liquid chromatography, using a Shimadzu chromatograph with a SCR-101 N 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 and 1-butanol was used as an internal standard. Samples of 0.5 ml of effluent and 2.5 ml of a 1% (v/v) solution of 1-butanol were diluted to 50 ml and 40 μl were injected directly into the column. Ethanol and residual sugar concentrations were calculated using standard curves and expressed in terms of percentage (v/v) and grammes of residual sugar per litre, respectively.

2.6. Solid phase microextraction (SPME) gas chromatography/mass spectrometry (GC/MS) analysis

The immobilized kefir on orange pulp and commercial baker's yeast were dried, separately, at 60 °C for about 24 h. Subsequently, 2 g of each sample were collected for the SPME procedure. After cooling the finished bread at ambient temperature (1–2 h), 2 g of bread crumb were selected for the SPME GC/MS analysis.

Volatile by-product compositions in dried baker's yeast, dried immobilized kefir on orange pulp and bread samples were determined using SPME GC/MS analysis. Grated samples (2 g each) were placed in 20 ml headspace vials fitted with a teflon-lined septum sealed with an aluminium crimp seal, through which the SPME syringe needle (bearing a 2 cm fibre, coated with 50/30 mm divinylbenzene/carboxen on poly-dimethyl-siloxane, bonded to a flexible fused silica core, Supelco, Bellefonte, PA, USA), was introduced. The container was then thermostatted at 60 °C for 60 min (Ruiz, Quilez, Mestres, & Guasch, 2003). The absorbed volatile analytes were then analyzed by GC/MS (Shimadzu GC-17A, MS QP5050, capillary column Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). Helium was used as carrier gas (linear velocity of 2.0 ml/min). Oven temperature was set at 35 °C for 5 min, followed by a temperature gradient of 5 °C/min to 50 °C, where it was held for 5 min and then increased by 5.5 °C/min to 230 °C. A final extension was applied at 230 °C for 10 min.The injector was operated in splitless mode. Injector and detector temperatures were 280 °C and 230 °C, respectively. The mass spectrometer was operated in the electron impact mode with the electron energy set at 70 eV. For semi-quantification of volatile compounds, 4-methyl-2-pentanol (Sigma–Aldrich, Poole, UK) diluted in pure ethanol was used as internal standard (IS) (Ruiz et al., 2003). The identification of volatile compounds was achieved by comparing the mass spectra with those in NIST107, NIST21 and SZTERP3 libraries. Quantitative determination was carried out by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio with the initial IS concentration (expressed as μg/g). The peak areas were measured from the full scan chromatograph using total ion current (TIC). Each determination was carried out in triplicate and the mean data are presented.

2.7. Microbiological analyses

Total aerobic count, and counts of yeasts, lactobacilli and lactococci in kefir culture after aerobic fermentation were determined as colony forming units (cfu/ml). Decimal dilutions were prepared from the kefir culture suspension, using 1/4 strength ringer solution, and plated on agar plates.

The following microbiological analyses were performed: (i) total aerobic counts on plate count agar (Fluka, 70188) at 30 °C for 72 h, (ii) yeasts on malt agar (Fluka, 70145) (pH was adjusted to 4.5 by a sterile solution of 10% lactic acid) at 30 °C for 72 h, (iii) lactococci on M-17 agar (Fluka, 63016) at 30 °C for 72 h, and (iv) lactobacilli on acidified MRS agar (Fluka, 69964) at 37 °C for 72 h, anaerobically (Anaerobic jar, Anerocult C, Merk). 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 log of mean colony-forming units on solid media culture plates containing between 30 and 300 colonies per gramme of cheese.

2.8. Electron microscopy

The substrate, before and after the cell immobilization, was washed and dried overnight at 30 °C. It was coated with gold in a Balzers SCD 004 Sputter Coater for 3 min and examined in a Cambridge Stereoscan 120 scanning electron microscope.

2.9. Preliminary sensory evaluation

Finished breads were allowed to cool to room temperature and a preliminary sensory evaluation was carried out by 20 non-trained testers using a taste-test local protocol based on a 1–10 preference scale, as described previously (Plessas et al., 2005). Briefly, the testers were asked to evaluate the taste and aroma, as well as the overall quality, of bread on the basis of colour of crust, elasticity and density of crumb, and volume of bread.

2.10. Experimental design and statistical analysis

The effects of initial sugar concentration, pH, air flow rate and addition of molasses on kefir biomass growth were examined. The experiments were designed and analyzed statistically by ANOVA. Results obtained by sensory evaluation were also statistically studied by one-way analysis of variance. Duncan's multiple range test was used to determine significant differences among of results (coefficients

and the ANOVA tables were computed using Statistica v5.0).

3. Results and discussion

3.1. General

Orange pulp suspension, with and without molasses, were used as substrates for kefir biomass production. The strategy adopted was to develop an environmentally friendly technological approach for the utilization and exploitation of discarded oranges and wastes of the citrus industry. The produced biocatalyst was evaluated for suitability in bread-making, in terms of both volatile compounds related to the aromatic profile of the biocatalyst and the produced bread.

It is known that high volumes of oranges and similar wastes are destroyed every year in southern Europe (Tripodo et al., 2004). Searching for an environmentally friendly technological approach for the utilization and exploitation of discarded oranges and wastes of the citrus industry is therefore, a desired activity. Upgrading of orange pulp suspension can be accomplished for use in food and animal feed production. The use of kefir culture for SCP production, using fruits as raw materials, or in aerobic fermentation, has not been reported in the literature. In addition, the production and use of such a kefir biocatalyst in baking and its effects on produced volatile compounds, in comparison with bread made with commercial baker's yeast, have not been previously examined.

3.2. Orange pulp suspension as substrate for kefir growth

Orange pulp suspension was tested as a suitable substrate for kefir growth and the effects of initial sugar concentration, pH, air flow rate and addition of molasses were monitored. The results are summarized in Table 1 and kefir growth curves and sugar consumption kinetics are presented in Figs. 1 and 2.

Initial sugar concentration significantly affected all parameters studied ($P < 0.01$) except fermentation time ($P > 0.05$). Air flow rate and pH significantly affected maximum growth rate μ_{max} ($P < 0.05$), while the addition of molasses had no effect on the kinetic parameters ($P > 0.05$).

The statistically highest maximum growth rate μ_{max} was observed when 75 g/l of initial sugar was used. Duncan's multiple range test clearly showed that the significantly highest maximum growth rate, μ_{max}, was observed at a 4.2 l/min air flow rate. Maximum growth rate, μ_{max}, was significantly higher at pH $\geqslant$ 5.0, while pH 4.5 resulted in statistically lower values. The highest final biomass concentration and daily biomass productivity were observed when 75 and 122 g/l of initial sugar were used. High amounts of ethanol were produced, up to 3.50% (v/v), although they were significantly decreased at low initial sugar concentrations ($\leqslant$75 g/l) (Crabtree effect) (González-Siso, Ramil, Cerdán, & Freire-Picos, 1996). High residual sugar was obtained at 122 g/l

Table 1
Effects of pH, initial sugar concentration (ISC), air flow rate and molasses on biomass kinetic parameters in aerobic fermentation of orange pulp suspension using kefir at 30 °C

Volume ratio (orange pulp suspension/molasses)	Air flow rate (l/min)	pH	ISC (g/l)	Fermentation time (h)	μ_{max}	Final biomass concentration (g dry weight/l)	Ethanol concentration (% vol)	Residual sugar (g/l)	Daily biomass productivity (g dry weight/l)	Biomass yield (g dry weight/g utilized sugar)	Conversion (%)
–	4.2	5.0	20	21.9^{a}	0.19^{a}	3.9^{a}	0.19^{a}	0.7^{a}	3.8^{a}	0.18^{a}	96.5^{a}
–	4.2	5.0	34	25.5^{a}	0.22^{a}	6.4^{b}	0.14^{a}	$1.3^{a,b}$	5.7^{b}	0.18^{a}	96.2^{a}
–	4.2	5.0	75	24.0^{a}	0.52^{b}	10.6^{c}	0.32^{a}	$1.8^{a,c}$	10.2^{c}	0.14^{b}	97.6^{a}
–	4.2	5.0	90	22.0^{a}	0.46^{c}	8.4^{d}	1.83^{b}	$2.4^{b,c}$	8.7^{d}	0.09^{c}	97.3^{a}
–	4.2	5.0	122	25.0^{a}	0.41^{c}	10.3^{c}	3.50^{c}	10.5^{d}	$9.5^{c,d}$	0.09^{c}	91.4^{b}
–	4.2	4.5	20	25.3^{a}	0.12^{d}	3.4^{a}	0.13^{a}	1.2^{a}	2.8^{a}	0.16^{a}	94.0^{a}
–	4.2	5.5	20	24.2^{a}	0.16^{a}	3.9^{a}	0.15^{a}	0.7^{a}	3.5^{a}	0.18^{a}	96.5^{a}
–	4.2	6.0	20	21.8^{a}	0.17^{a}	3.7^{a}	0.13^{a}	0.5^{a}	3.6^{a}	0.17^{a}	97.5^{a}
–	2.5	5.0	20	24.3^{a}	0.11^{e}	2.9^{a}	0.55^{a}	0.3^{a}	2.5^{a}	0.13^{a}	98.5^{a}
–	3.3	5.0	20	23.1^{a}	0.12^{e}	3.1^{a}	0.38^{a}	0.2^{a}	2.8^{a}	0.14^{a}	99.0^{a}
6:1	4.2	5.0	20	26.5^{a}	0.16^{a}	3.6^{a}	0.45^{a}	0.3^{a}	2.9^{a}	0.16^{a}	98.5^{a}
5:2	4.2	5.0	20	25.5^{a}	0.18^{a}	3.7^{a}	0.29^{a}	0.4^{a}	3.1^{a}	0.17^{a}	98.0^{a}
4:3	4.2	5.0	20	26.0^{a}	0.18^{a}	3.9^{a}	0.55^{a}	0.4^{a}	3.2^{a}	0.18^{a}	98.0^{a}

(–): No molasses was added to the orange pulp suspension; statistical differences for various treatments within a column are shown with different letters in superscript.

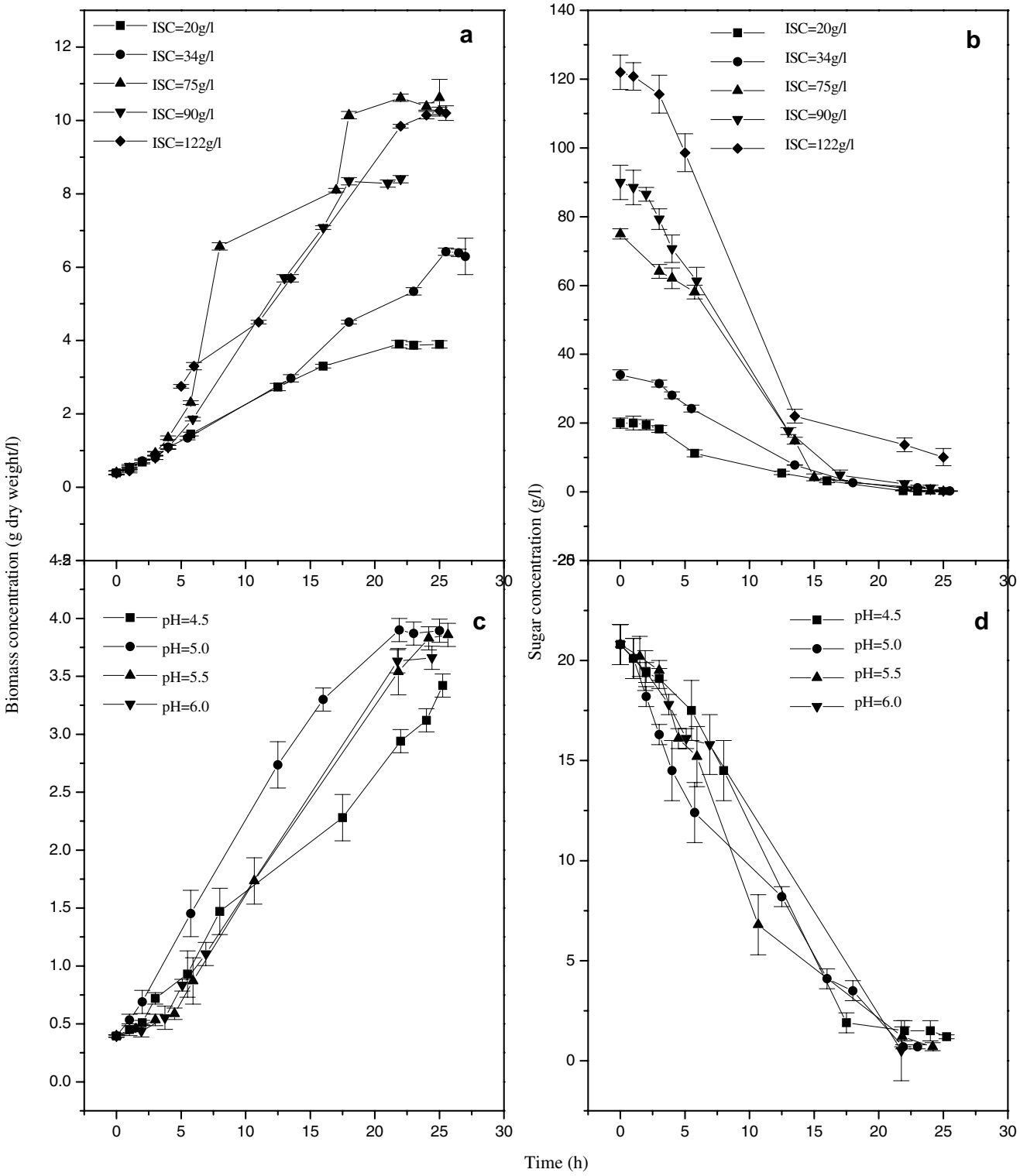

Fig. 1. Effect of ISC and pH on biomass concentration during aerobic fermentation of orange pulp suspension using kefir (a) pH 5, air flow rate = 4.2 l/min; (b) pH 5, air flow rate = 4.2 l/min; (c) ISC = 20 g/l, air flow rate = 4.2 l/min; (d) ISC = 20 g/l, air flow rate = 4.2 l/min.

initial sugar content and, thus low biomass yield (0.09 g/g) was reported. Although a statistically lower value for conversion was observed when the highest initial sugar concentration was used, it still remained at high levels (91.4%).

Part of the produced biomass was immobilized on orange pulp, as shown by the electron micrograph (Fig. 3). The produced immobilized biocatalyst may be employed for many applications in the food industry, as a value-added starter culture for baking (Plessas et al., 2005), alcohol production (Athanasiadis, Boskou, Kanellaki, Kiosseoglou, & Koutinas, 2002; Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001) and whey fermentation

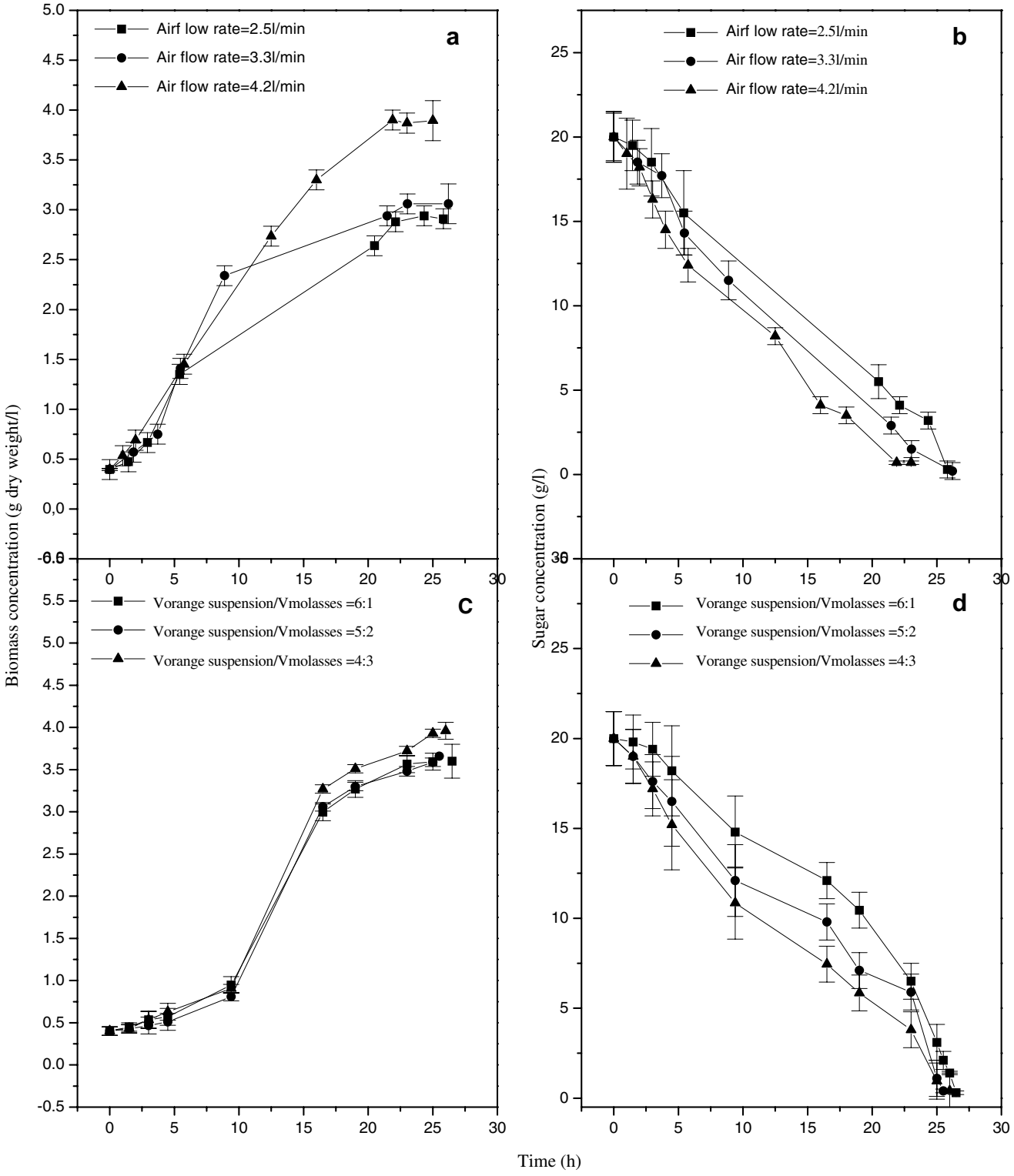

Fig. 2. Effect of air flow rate and addition of molasses on biomass concentration during aerobic fermentation of orange pulp suspension using kefir (a) pH 5, ISC = 20 g/l; (b) pH 5, ISC = 20 g/l; (c) ISC = 20 g/l, pH 5, air flow rate = 4.2 l/min; (d) ISC = 20 g/l, pH 5, air flow rate = 4.2 l/min.

(Athanasiadis, Paraskevopoulou, Blekas, & Kiosseoglou, 2004), taking into account that whey is also a waste of negligible cost, creating a major disposal problem for the dairy industry. Likewise, upgraded orange pulp with kefir yeast could be also used as an enriched animal feed.

3.3. Aroma-related compounds

In order to investigate the suitability of kefir yeast for food production, the aromatic profile of kefir, produced by aerobic fermentation of orange pulp, was compared to

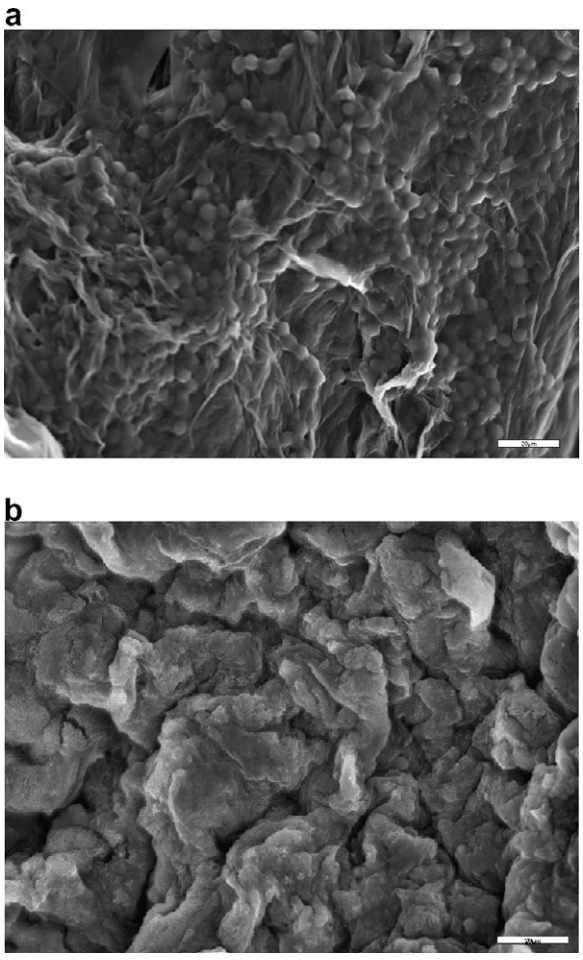

Fig. 3. Electron micrographs showing immobilized cells of kefir on orange pulp (a) and orange pulp before the immobilization (b).

Table 2
Aroma-related compounds isolated in dried baker's yeast produced by aerobic fermentation of molasses and dried immobilized kefir on orange pulp produced by fermentation of diluted orange pulp suspension

Kovats index	Compound	Baker's yeast (μg/g)	Immobilized kefir (μg/g)
Alcohols			
<800	Methanol	2.45[a]	1.93[a]
905	Ethanol	12.26[a]	12.94[a]
1012	Isobutyl alcohol	0.49[a]	2.46[a]
1045	1-Butanol	0.92[a]	8.84[a]
1120	Isoamyl alcohol	0.19[a]	0.25[a]
1550	2,3-Butanediol	0.29[a]	1.22[a]
1811	2-Phenyl-ethanol	0.06[a]	2.46[a]
Esters			
<800	Methyl acetate	1.33[a]	0.60[a]
866	Ethyl acetate	n.d.	0.80[a]
1118	Ethyl hexanoate	n.d.	0.51[a]
1288	Methyl octanoate	n.d.	0.95[a]
1315	Ethyl octanoate	n.d.	1.86[a]
1385	Ethyl nonanoate	n.d.	2.21[a]
1423	Methyl decanoate	1.32[a]	n.d.
1563	Ethyl decanoate	n.d.	5.07[a]
1616	Methyl 4-methyl-pentanoate	1.19[b]	n.d.
1725	2-Phenylethyl acetate	0.48[a]	1.67[a]
1631	Methyl dodecanoate	n.d.	0.14[a]
1649	Ethyl undecanoate	n.d.	0.63[a]
Organic acids			
1524	Isobutyric acid	n.d.	Tr.[b]
1531	Isocaproic acid	n.d.	3.27[b]
1900	Hexanoic acid	n.d.	1.25[a]
>2000	Octanoic acid	n.d.	0.51[a]
Carbonyl compounds			
<800	Acetaldehyde	n.d.	0.82[a]
Other compounds			
1337	2,6-Dimethyl-pyrazine	0.09[a]	n.d.

n.d.: None detected.
Tr.: Compounds <0.01 μg/g are indicated by Tr. (traces).
[a] Positive identification from MS and retention times.
[b] Positive identification from MS data only.

that of commercial baker's yeast, using the SPME GC/MS technique. Results of analysis of the aroma-related compounds detected in the dried baker's yeast and immobilized kefir on orange pulp are presented in Table 2. Specifically, 21 compounds, among which 10 esters were identified in kefir yeast, and 12, among which four esters, were in baker's yeast. The higher number of esters in kefir yeast was considered as positive for successful application of the proposed yeast in food production, showing a potential positive effect in the flavour character of the final product.

Subsequently, for the final evaluation of kefir yeast's suitability in baking, bread produced by the direct and sourdough method were compared to breads produced by commercial baker's yeast by the direct method, in terms of volatile compounds. The results of the analysis are presented in Table 3. The most important compounds identified were esters, organic acids, alcohols and carbonyl compounds. However, the presence of certain volatiles in bread does not necessarily imply positive contribution to the overall aroma. In order to correlate the occurrence or amounts of volatile compounds with pronounced aroma notes or a more intense overall aroma, extended knowledge of the key aroma compounds among the bulk of odourless volatiles is a prerequisite for flavour characterization (Hansen & Schieberle, 2005).

In bread produced by the direct bread method, compounds such as benzyl alcohol, 2-furanmethanol, 2-nonenal and benzaldehyde were detected. These compounds are considered important for bread flavour (Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001; Kirchhoff & Schieberle, 2002). On the other hand, bread produced by the sourdough method contained higher numbers of volatile compounds. It is also noteworthy that increased numbers of esters were detected in bread produced by the sourdough method. Esters are known for their positive impact on bread aroma (Maga, 1974).

Ethanol was the dominating volatile compound in all cases, as yeasts and heterofermentative lactic acid bacteria are primary contributors. Many fusel alcohols were detected, which could be the result of the metabolism of aminoacids or carbohydrates (Levesque, 1991). Higher amounts of 2-phenyl-ethanol were recorded in breads

Table 3
Aroma-related compounds isolated in bread produced by baker's yeast and by immobilized kefir on orange pulp, using the direct and sourdough methods

Kovats index	Compound	Baker's Yeast (direct method) (μg/g)	Immobilized kefir (direct method) (μg/g)	Immobilized kefir (sourdough method) (μg/g)
Alcohols				
905	Ethanol	1040[a]	1029[a]	1080[a]
1012	Isobutyl alcohol	0.42[a]	0.39[a]	0.46[a]
1120	Isoamyl alcohol	0.82[a]	1.55[a]	2.59[a]
1159	1-Pentanol	n.d.	0.12[a]	0.19[a]
1257	1-Hexanol	0.27[a]	0.27[a]	0.55[a]
1381	1-Heptanol	0.15[a]	0.05[a]	0.09[a]
1420	3-Octen-1-ol	n.d.	n.d.	0.02[a]
1526	2-Furanmethanol	0.55[a]	0.63[a]	0.65[a]
1807	Benzyl alcohol	0.57[a]	0.78[a]	0.83[a]
1811	2-Phenyl-ethanol	0.25[a]	0.61[a]	1.13[a]
Esters				
<866	Ethyl acetate	n.d.	0.03[a]	2.08[a]
1107	Butyl acetate	1.11[a]	1.02[a]	1.61[a]
1162	Hexyl acetate	n.d.	0.21[a]	0.61[a]
1238	Ethyl heptonate	n.d.	n.d.	0.05[b]
1315	Ethyl octanoate	0.08[b]	0.05[b]	0.73[b]
Organic acids				
1452	Acetic acid	Tr.[a]	Tr.[a]	0.11[a]
1615	Lactic acid	n.d.	Tr.[b]	0.02[b]
1937	Hexanoic acid	Tr.[b]	Tr.[b]	Tr.[b]
Carbonyl compounds				
<800	Acetaldehyde	n.d.	n.d.	0.02[b]
886	2-Methyl-butanal	0.03[a]	0.02[a]	0.04[a]
<935	2,3-Butanedione	n.d.	Tr.[a]	0.01[a]
1002	Hexanal	0.08[a]	0.12[a]	0.11[a]
1071	2-Heptanone	0.03[a]	Tr.[a]	Tr.[a]
1410	Furfural	0.30[a]	0.99[a]	0.88[a]
1458	Benzaldehyde	0.44[a]	1.03[a]	0.95[a]
1467	2-Nonenal	0.19[b]	0.04[b]	0.23[b]
Other compounds				
1021	2-*n*-Butyl furan	n.d.	n.d.	Tr.[b]
1128	2-Pentyl-furan	n.d.	Tr.[b]	Tr.[b]
1655	Octahydro-2,3-bifuran	n.d.	Tr.[b]	Tr.[b]
1715	Dihydro-5-pentyl-2(3H)-furanone	n.d.	n.d.	Tr.[b]

n.d.: None detected.
Tr.: Compounds <0.01 μg/g are indicated by Tr. (traces).
[a] Positive identification from MS and retention times.
[b] Positive identification from MS data only.

produced by immobilized kefir on orange pulp compared to bread produced by baker's yeast (Table 3). Production of 2-phenyl-ethanol has been related to the association of lactic acid bacteria and yeasts (Gobbetti, 1998). 1-Pentanol, identified in bread samples, produced by immobilized kefir on orange pulp, and 3-octen-1-ol, detected in bread produced by the sourdough method, have been identified in soya bread (Frasse, Lambert, Richard-Molard, & Chiron, 1993). The presence of ethyl acetate only in bread produced by immobilized kefir on orange pulp has been linked with lactic acid bacteria metabolism (Damiani et al., 1996; Gobbetti, 1998). In addition, ethyl acetate was not detected in dried baker's yeast (Table 2). The increased amounts of acetic acid reported in bread produced by immobilized kefir on orange pulp, using the sourdough method, could be attributed to heterofermentative lactic acid and acetic acid cultures (Gobbetti et al., 1995; Hansen, Lund, & Lewis, 1989) probably present in kefir microflora (Garrote, Abraham, & De Antoni, 1997; Pintado, Lopes Da Silva, Fernades, Malcata, & Hogg, 1996; Witthuhn, Schoeman, & Britz, 2005). 2,3-Butanedione (diacetyl), identified only in bread samples produced by immobilized kefir on orange pulp, was considered among the most important odour compounds in French bread dough (Frasse et al., 1993) and in sourdough (Gobbetti, 1998), providing a butter-like note (Frasse et al., 1993). It is obtained from pyruvate and citrate metabolism and its production is mainly due to the activity of lactic acid bacteria and, more specifically, *Lactococcus lactis* (Welsh, Murry, & Williams, 1989). Even though the greatest amounts of aroma substances are produced during baking (Spicher, 1983), sourdough fermentation is essential for achieving an acceptable flavour (Rothe & Ruttlof, 1983).

It is obvious, from the above results, that a plethora of aroma compounds is formed due to the action of immobilized kefir yeast. However, it is yet difficult to interpret the relationships between the microbial associations and the chemical compounds, due to the high complexity of microbial interactions.

3.4. Preliminary sensory evaluation

Bread samples produced by immobilized kefir on orange pulp by the direct and sourdough method were compared to bread samples produced by baker's yeast by the direct method, for aroma, taste and overall quality. The results of the sensory evaluation are summarized in Table 4. The kind of yeast used and the production method significantly affected ($P < 0.01$) the preference of the tasters during aroma and taste evaluation, but not overall quality ($P > 0.05$). Bread samples produced by immobilized kefir on orange pulp were accepted by the panel. The well-known improved quality of bakery products produced by the sourdough method (Hansen & Hansen, 1994) was confirmed, as bread produced by immobilized kefir on orange pulp by the sourdough method scored significantly higher values for aroma and taste than did bread samples produced by the direct method. However, no significant differences were observed between bread samples produced by immobilized kefir on orange pulp and baker's yeast, by the direct method.

Table 4
Preliminary sensory evaluation of bread produced by baker's yeast and immobilized kefir on orange pulp using the direct and sourdough methods (Score 1: unacceptable, 10: excellent)

	Direct method		Sourdough method
	Baker's Yeast	Immobilized kefir	Immobilized kefir
Aroma	7.4 ± 0.98	7.4 ± 0.33	8.5 ± 0.72
Taste	8.0 ± 0.68	8.0 ± 0.41	8.7 ± 0.59
Overall quality	8.1 ± 0.82	8.1 ± 0.95	8.5 ± 0.43

3.5. Technological consideration

The use of whole orange as a raw material for fermentation, using the mixed culture of kefir has a great potential in production of a livestock feed, for animals, at low cost, because kefir contains various lactic acid bacteria and the raw material is an agroindustrial waste. Nowdays, a very nutritive livestock feed, based on *S. cerevisiae* biomass, is employed with a market price about 0.5 Euros/kg. It is estimated that the production cost of the proposed livestock feed will be less. The quality of produced meat and milk may be improved, due to the improved aromatic profile and availability of more vitamins in orange suspension. Likewise, it could also be used as additive in baking and in breakfast cereals, since consumption of kefir has been related to a variety of health benefits (Cevikbas et al., 1994; Liu & Lin, 2000; Rodrigues, Gaudino Caputo, Tavares Carvalho, Evangelista, & Schneedorf, 2005), and it could also provide antioxidative properties due to the flavonoid and ascorbic acid contents of oranges (Del Caro, Piga, Vacca, & Agabbio, 2004). Reported yields are at relatively high levels and the fermentation time could be accepted for industrial production. Higher yields and productivity during scale-up of the process are expected.

4. Conclusions

Cell growth of kefir, using agroindustrial citrus wastes, has a great potential. Immobilized kefir on orange pulp was successfully used as a suitable yeast for bread production, with improved volatiles profile. The immobilized biocatalyst may also be used in animal feed production with improved nutritional properties.

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References

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 Agriculture Food Chemistry, 50*(25), 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 materials. *Journal of Agriculture Food Chemistry, 49*(2), 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*(4), 1091–1095.

Beshkova, D. M., Simova, E. D., Simov, Z. I., Frengova, G. I., & Spasov, Z. N. (2002). Pure cultures for making kefir. *Food Microbiology, 19*(5), 537–544.

Cevikbas, A., Yemni, E., Ezzedenn, F. W., Yardimici, T., Cevikbas, U., & Stohs, S. J. (1994). Antitumoural, antibacterial and antifungal activities of kefir and kefir grain. *Phytotherapy Research, 8*, 78–82.

Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M. S., & Rossi, I. (1996). The sourdough microflora. Characterization of hetero- and homofermentative lactic acid bacteria, yeasts and their interactions on the basis of the volatile compounds produced. *Lebensmittel-Wissenschaft und Technologie, 29*, 63–70.

De Gregorio, A., Mandalari, G., Arena, N., Nucita, F., Tripodo, M. M., & Lo Curto, R. B. (2002). SCP and crude pectinase production by slurry-state fermentation of lemon pulps. *Bioresource Technology, 83*(2), 89–94.

Del Caro, A., Piga, A., Vacca, V., & Agabbio, M. (2004). Changes of flavonoids, vitamin C and antioxidant capacity in minimally processed citrus segments and juices during storage. *Food Chemistry, 84*(1), 99–105.

Ejiofor, A. O., Chisti, Y., & Moo-Young, M. (1996). Culture of *Saccharomyces cerevisiae* on hydrolyzed waste cassava starch for production of baking-quality yeast. *Enzyme Microbial Technology, 18*(7), 519–525.

Frasse, P., Lambert, S., Richard-Molard, D., & Chiron, H. (1993). The influence of fermentation on volatile compounds in French bread dough. *Food Science and Technology, 26*(2), 126–132.

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.

Gobbetti, M. (1998). The sourdough microflora: Interactions of lactic acid bacteria and yeasts. *Trends Food Science Technology, 9*(7), 267–274.

Gobbetti, M., Simonetti, M. S., Corsetti, A., Santinelli, F., Rossi, I., & Dimiani, P. (1995). Volatile compound and organic acid productions by mixed wheat sour dough starters: Influence of fermentation parameters and dynamics during baking. *Food Microbiology, 12*, 497–507.

González-Siso, M. I., Ramil, E., Cerdán, M. E., & Freire-Picos, M. A. (1996). Respirofermentative metabolism in *Kluyveromyces lactis*: Ethanol production and the Crabtree effect. *Enzyme Microbial Technology, 18*(8), 585–591.

Hansen, Å., & Schieberle, P. (2005). Generation of aroma compounds during sourdough fermentation: Applied and fundamental aspects. *Trends in Food Science Technology, 16*(1-3), 85–94.

Hansen, B., & Hansen, Å. (1994). Volatile compounds in wheat sourdoughs produced by lactic acid bacteria and sourdough yeasts. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung, 198*(3), 202–209.

Hansen, Å., Lund, B., & Lewis, M. J. (1989). Flavour of sourdough rye bread crumb. *Lebensmittel-Wissenschaft und Technologie, 22*, 141–144.

Khan, A. J., Abulnaja, O. K., Kumosani, A. T., & Abou-Zaid, A-A. A. (1995). Utilization of Saudi date sugars in production of baker's yeast. *Bioresource Technology, 53*(1), 63–66.

Kirchhoff, E., & Schieberle, P. (2001). Determination of key aroma compounds in the crumb of a three-stage sourdough rye bread by stable isotope dilution assays and sensory studies. *Journal of Agriculture Food Chemistry, 49*(9), 4304–4311.

Kirchhoff, E., & Schieberle, P. (2002). Quantitation of odor-active compounds in rye flour and rye sourdough using stable isotope dilution assays. *Journal of Agriculture Food Chemistry, 50*(19), 5378–5385.

Klein, J., & Kressdorf, B. (1983). Improvement of productivity and efficiency in ethanol production with Ca-alginate immobilized Z. mobilis. *Biotechnology Letters, 5*(8), 497–502.

Koroleva, N. S. (1988). Starters for fermented milks. Section 4: kefir and kumys starter. In: *Bulletin of the IDF 227, chapter 2. international dairy federation*, Square Vergotte, Brussels, Belgium.

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*(7), 788–796.

Lee, B-K., & Kim, J. K. (2001). Production of *Candida utilis* biomass on molasses in different culture types. *Aquacultural Engineering, 25*(2), 111–124.

Levesque, C. Etude des potentialités de la souche S47 de *Saccharomyces cerevisiae* à produire des alcools supérieures à partir de composés organiques identifiés et des précurseurs de la farine. Thése d' Université Nantes, 1991.

Liu, J. R., & Lin, C. (2000). Production of kefir from soymilk with or without added glucose, lactose or sucrose. *Journal of Food Science, 65*, 716–719.

Lotz, M., Frohlich, R., Matthes, R., Schugerl, K., & Seekamp, M. (1991). Baker's yeast cultivation on by-products and wastes of potato and wheat starch production on a laboratory and pilot-plant scale. *Process Biochemistry, 26*(5), 301–311.

Luis, A., Lopez, E., & Lema, C. (1993). Microflora present in kefir grains of the Galician region. *Journal of Dairy Research, 60*(2), 263–267.

Maga, J. A. (1974). Bread flavor. *CRC Critical Reviews in Food Science and Nutrition, 5*, 55–142.

Paramithiotis, S., Chouliaras, Y., Tsakalidou, E., & Kalantzopoulos, G. (2005). Application of selected starter cultures for the production of wheat sourdough bread using a traditional three-stage procedure. *Process Biochemistry, 40*(8), 2813–2819.

Paraskevopoulou, A., Athanasiadis, I., Kanellaki, M., Bekatorou, A., Blekas, G., & Kiosseoglou, V. (2003). Functional properties of single cell protein produced by *kefir* microflora. *Food Research International, 36*(5), 431–438.

Pintado, M. E., Lopes Da Silva, J. A., Fernades, P. B., Malcata, F. X., & Hogg, T. A. (1996). Microbiological and rheological studies on Portuguese kefir grains. *International Journal of 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*(4), 585–589.

Rothe, M., & Ruttlof, H. (1983). Aroma retention in modern bread production. *Die Nahrung, 27*(5), 505–512.

Rodrigues, K. L., Gaudino Caputo, L. R., Tavares Carvalho, J. C., Evangelista, J., & Schneedorf, J. M. (2005). Antimicrobial and healing activity of kefir and kefiran extract. *International Journal of Antimicrobial Agents, 25*, 404–408.

Ruiz, J. A., Quilez, J., Mestres, M., & Guasch, J. (2003). Solid-phase microextraction method for headspace analysis of volatile compounds in bread crumb. *Cereal Chemistry, 80*(3), 255–259.

Scerra, V., Caridi, A., Foti, F., & Sinatra, M. C. (1999). Influence of dairy *Penicillium* spp. on nutrient content of citrus fruit pulp. *Animal Feed Science Technology, 78*(1–2), 169–176.

Spicher, G. (1983). Baked goods. In H. J. Rehm & G. Reed (Eds.). *Biotechnology* (vol. 5, pp. 1–80). Germany: Weinheim, Verlag Chemie.

Tripodo, M. M., Lanuzza, F., Micali, G., Coppolino, R., & Nucita, F. (2004). Citrus waste recovery: A new environmentally friendly procedure to obtain animal feed. *Bioresource Technology, 91*(2), 111–115.

Vedamuthu, E. R. (1982). Fermented milks. In A. H. Rose (Ed.), *Economic microbiology* (pp. 199–226). Washington: Academic Press.

Welsh, F. W., Murry, W. D., & Williams, R. E. (1989). Microbiological and enzymatic production of flavour and fragrance chemicals. *CRC Critical Reviews in Biotechnology, 9*, 105–169.

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.

Ziino, M., Lo Curto, R. B., Salvo, F., Signorino, D., Chiofalo, B., & Giuffrida, D. (1999). Lipid composition of *Geotrichum candidum* single cell protein grown in continuous submerged culture. *Bioresource Technology, 67*(1), 7–11.