Food Chemistry 145 (2014) 710–716

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production

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ARTICLE INFO

Article history:
Received 8 February 2013
Received in revised form 13 July 2013
Accepted 20 July 2013
Available online 29 July 2013

Keywords:
single cell protein
solid state fermentation
ε-pinene
food industry wastes

ABSTRACT

Growth of selected microorganisms of industrial interest (*Saccharomyces cerevisiae, Kluyveromyces marxianus* and kefir) by solid state fermentation (SSF) of various food industry waste mixtures was studied. The fermented products were analysed for protein, and nutrient minerals content, as well as for aroma volatile compounds by GC/MS. The substrate fermented by *K. marxianus* contained the highest sum of fat and protein concentration (59.2% w/w dm) and therefore it could be considered for utilisation of its fat content and for livestock feed enrichment. Regarding volatiles, the formation of high amounts of ε-pinene was observed only in the SSF product of kefir at a yield estimated to be 4 kg/tn of SSF product. A preliminary design of a biorefinery-type process flow sheet and its economic analysis, indicated potential production of products (enriched livestock feed, fat and ε-pinene) of significant added value.

1. Introduction

Many efforts have been recently made to exploit waste biomass through a biorefinery approach, for the recovery or production of value added products such as single-cell protein (SCP), enzymes, animal feeds and feed additives, biofuels and various other bulk chemicals and products (Buschke, Schafer, Becker, & Wittmann, 2013; Kosseva, 2013; Mathews, Tan, Moore, & Bell, 2011). Regarding wastes from the food industry, the interest has mainly focused on bioconversion of the liquid wastes from the dairy and sugar industries (whey and molasses, respectively), and solid wastes such as brewer's spent grains (BSG) and malt spent rootlets (MSR), and vegetable and fruit processing wastes (mainly potato and citrus) (Bampidis & Robinson, 2006; Kamat, Khot, Zinjarde, RaviKumar, & Gade, 2013; Kosseva, 2013; Koutinas et al., 2009; Nitayavardhana & Khanal, 2010; Redwood, Orozco, Majewski, & Macaskie, 2012). Microorganisms that have been used for this purpose include yeasts, fungi, bacteria and algae (Anupama & Ravindra, 2000; Buschke et al., 2013; Ferreira, Lennartsson, Edebo, & Taherzadeh, 2013; Hasunuma et al., 2013; Kumar, Gupta, Kumar, Sahoo, & Kuhad, 2013). However, for the exploitation of food industry wastes by a biorefinery approach, the use of mixed waste substrates has been scarcely reported (Aggelopoulos, Bekatorou, Pandey, Kanellaki, & Koutinas 2013). The bioconversion of mixed waste substrates has several advantages such as (i) reduction of transportation and disposal costs, (ii) possibility to develop completer fermentation feedstocks without need for extra nutrients, (iii) ability of low-capacity food production plants to exploit their wastes by supplying them to a central food waste treatment plant, (iv) improvement of the nutritional value of livestock feeds, and (v) production of a variety of products in a biorefinery manner.

To explore this possibility various microorganisms with different carbohydrate bioconversion potential, such as kefir, have been proposed (Harta et al., 2004). Kefir is a natural mixed dairy culture consisting of symbiotic consortia of yeasts and bacteria embedded in a polysaccharide matrix (kefiran), used for the production of a milk beverage by both alcoholic and lactic acid fermentation (Beshkova, Simova, Frengova, Simov, & Dimitrov, 2003; Plessas, Alexopoulos, Voidarou, Stavropoulou, & Bezirtzoglou, 2011). Cheese whey, has been extensively studied as substrate for kefir-based dairy and baking starter cultures production, as well as for ethanol and lactic acid production by fermentation with kefir, in laboratory and pilot scale operations (Koutinas, Athanasiadis, Bekatorou, Iconomopoulou, & Blekas, 2005, 2007, 2009; Plessas et al., 2007). Regarding single cultures, the yeast *S. cerevisiae* is one of the most important yeasts in food and beverage production, and is produced worldwide in media containing molasses (Bekatorou, Psarianos, & Koutinas, 2006). Molasses, is also a renewable carbohydrate-rich substrate, ready-to-use for ethanol production (Kopsahelis, Agouridis, Bekatorou, & Kanellaki, 2007). It contains 45–55% fermentable sugars and its use for SCP production is

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http://dx.doi.org/10.1016/j.foodchem.2013.07.105

determined by its availability and cost, composition and absence of toxic substances and fermentation inhibitors (Bekatorou et al., 2006). The yeast *K. marxianus* also has several characteristics that make it attractive for industrial production, such as ability to grow on a wide variety of inexpensive carbon sources with few additional nutrient requirements (Banat, Nigam, Singh, Marchant, & McHale, 1998; Lane & Morrissey, 2010; Singh, Nigam, Banat, Marchant, & McHale, 1998).

Amongst process types for bioconversion of food industry wastes, solid state fermentation (SSF) has been extensively studied with thousands of publications describing various types of bioreactor designs, process conditions and microorganisms for the production of various value added products like SCP, feeds, enzymes, ethanol, organic acids, B-complex vitamins, pigments, flavours, etc. (Couto & Sanroman, 2006; Singhania, Patel, Soccol, & Pandey, 2009; Barrios-González, 2012; Kosseva, 2013). The production of aroma compounds by SSF is an interesting application as it has been shown that higher yields or better product characteristics can be obtained by SSF. *S. cerevisiae*, *K. marxianus* and kefir species have been evaluated for this purpose (Beshkova et al., 2003; Couto & Sanroman, 2006; Feron, Bonnarme, & Durand, 1996; Medeiros, Pandey, Freitas, Christen, & Soccol, 2000). The production of food additive flavours by bioconversion of food grade substrates is recently gaining more attention, due to better consumer acceptance compared to synthetic production or extraction processes as well as avoidance of drawbacks such as formation of mixtures of isomers and difficulty to control availability and quality of plant sources (Couto & Sanroman, 2006; Janssens, De Pooter, Schamp, & Vandamme, 1992).

The aim of this study was to optimise SSF processes for the treatment of mixed solid and liquid food industry wastes using selected *S. cerevisiae* and *K. marxianus* strains and the natural mixed culture kefir, for the production of various products in a biorefinery approach.

2. Materials and methods

2.1. Materials

Cheese whey was obtained from the Agricultural Cooperatives Union of Kalavryta (Achaia province, Greece). It contained 5.2% w/v lactose and 6.5% w/w dry matter and (% w/w dry matter) 14.2 crude protein, 3.8 fat, 1.9 ash, and had pH 4.2 (Aggelopoulos et al., 2013). Molasses was supplied by the BG Spiliopoulos SA alcohol distillery (Patras, Greece). It contained 77.2% w/w dry matter and (% w/w wet weight basis) 7.3 crude protein, 42.5 fermentable sugar, 7.5 ash and had pH 4.7 (Aggelopoulos et al., 2013). BSG and MSR were supplied by the Athenian Brewery SA (member of the Heineken NV group, Patras, Greece). BSG contained 73.8% w/w moisture and (% w/w dry matter) 24.0 crude protein, 4.2 ash, 6.2 fat, and 2.6 fermentable sugars. MSR contained 87.1% w/w dry matter and (% w/w dry matter) 31.1 crude protein, 6.8 ash, and 4.4 fat (Aggelopoulos et al., 2013). Potatoes and oranges were obtained from a local market.

2.2. Microorganisms

The kefir culture was isolated from commercially available kefir grains (Meliton SA, Thessaloniki, Greece). It was grown at 30 °C in synthetic medium consisting of (% w/w): 4 lactose (Fluka, Buchs, Switzerland), 0.4 yeast extract (Fluka), 0.1 $(NH_4)_2SO_4$ (Fluka), 0.1 KH_2PO_4 (Fluka) and 0.5 $MgSO_4{\cdot}7H_2O$ (Fluka). The pH of the synthetic medium was adjusted to 5.6. The psychrotolerant and alcohol resistant yeast strain *S. cerevisiae* AXAZ-1, isolated from the Greek agricultural area (Argiriou et al., 1996), was grown at 30 °C in a medium consisting of (% w/w): 4 glucose, 0.4 yeast extract, 0.1 $(NH_4)_2SO_4$, 0.1 KH_2PO_4 and 0.5 $MgSO_4{\cdot}7H_2O$. The thermotolerant yeast *K. marxianus* IMB3 was obtained from the University of Ulster (Kourkoutas et al., 2002) and it was grown in the same media as *S. cerevisiae* AXAZ-1. All media were sterilized by autoclaving at 120 °C and 1.5 Atm for 15 min prior to use.

2.3. Preparation of the substrates

The external (yellow exocarp) parts of orange skins were removed and then the whole remaining fruit was blended for 10 min. The blended product hereinafter referred to as "orange pulp" (including the juice) was diluted with water at a ratio of 1:1 (by weight). Potato pulp was prepared in the same manner and was diluted with water at a ratio of 1:2 (by weight). Orange pulp contained 82.9% w/w moisture, 2.9% w/w (dry matter) crude protein, 8.3% w/w (wet weight) fermentable sugar, and had pH 4.1. Potato pulp contained 82.8% w/w moisture, 2.5% w/v crude protein, 0.9% w/v fermentable sugar, and had pH 5.5 (Aggelopoulos et al., 2013). Molasses was used after dilution with water to a density of 4 Be. Cheese whey, BSG and MSR were used without any pretreatment.

2.4. SSF of mixed food industry wastes

The mixed substrates used for SSF consisted of: (1) for kefir, 30 ml orange pulp, 10 ml molasses, 10 ml potato pulp, 100 ml whey, 50 ml distilled water, 60 g BSG and 25 g MSR, (2) for *S. cerevisiae* AXAZ-1, 100 ml orange pulp, 50 ml molasses, 50 ml distilled water and 70 g BSG, and (3) for *K. marxianus* IMB3, 100 ml orange pulp, 10 ml molasses 10 ml potato pulp, 30 ml whey, 50 ml distilled water and 80 g BSG. The amount of BSG and MSR was chosen so that the mixed substrates to have 70–80% w/w moisture content. The pH was adjusted to 5.5 by a 2 N NaOH solution for *S. cerevisiae* and kefir and to 7.0 for *K. marxianus*. The substrates were sterilized by autoclaving for 15 min at 120 °C and 1.5 Atm. After cooling, they were spread into petri dishes, inoculated with 1 g of harvested culture and incubated for 4 days at 30 °C without any agitation.

2.5. Assays

Fat content (% w/w dry matter) were determined by the Soxhlet method (AOAC., 1995) after drying at 105 °C for 24 h. Total nitrogen expressed as % crude protein on dry weight basis was determined by the Kjeldahl's procedure. Metals Ca, Mg, Fe and Cu, after ashing at 550 °C and dilution with concentrated sulphuric acid, were determined on an AA-6500 Series Atomic Absorption Spectrometer (Shimadzu Corporation).

2.5.1. Determination of fatty acids

Fatty acids were determined by gas chromatography (GC) after transesterification of glycerides to methyl esters of fatty acids. Specifically, in a 200 ml spherical flask 1 g of the fat that was extracted by the Soxhlet method and then 15 ml of a mixture containing methanol and benzene (3:1) and 0.2 g *p*-toluenesulfonic acid were added. The flask was connected with a vertical condenser and heated in a water bath at 100 °C for 2 h. After cooling the content was transferred to a 250 ml separation funnel and 100 ml of distilled water were added. Extraction was carried out twice with 50 ml petroleum ether. Sodium sulphate was added (0.7 g) to dry the solution and the solvent was removed using a rotary evaporator. The residual liquid was diluted with 5 ml toluene and 2 μL were injected in the injection port of a Shimadzu Gas Chromatograph GC-8A (Kyoto-Japan) with a FFAP type stainless steel column (3 m). The carrier gas was N_2 at 20 mL/min. The injection port and

Table 1
Fat, crude protein and minerals composition of food industry waste mixtures after SSF.

Microorganism	Conditions	Substrate composition	Fat %wt. dm	Protein %wt. dm	Ca mg/kg	Mg mg/kg	Fe mg/kg	Cu mg/kg
S. cerevisiae AXAZ-1	T = 30 °C, pH 5.5	100 ml OP, 50 ml M, 70 g BSG, 50 ml H_2O	12.9	38.5	96.8	771.1	2.3	17.4
K. marxianus IMB3	T = 30 °C, pH 7	100 ml OP, 10 ml M, 80 g BSG, 50 ml H_2O, 30 ml W, 10 ml PP	25.5	33.7	92.7	454.4	0.9	17.4
Kefir	T = 30 °C, pH 5.5	30 ml OP, 10 ml M, 60 g BSG, 50 ml H_2O, 100 ml W, 10 ml PP, 25 g MSR	12.3	23.6	85.6	332.2	25.1	22.5

dm: dry matter; OP: orange pulp; PP: potato pulp; M: molasses; W: whey; BSG: Brewer's spent grains; MSR: malt spent rootlets.

Table 2
Higher fatty acid methyl esters in transesterified fat extracted from food industry waste mixtures after SSF treatment with *K. marxianus* IMB3.

Fatty acid methyl ester	Concentration (g/100g fat)
Methyl palmitate	85.7
Methyl oleate	3.6
Methyl linoleate	9.0

FID detector temperatures were 250 °C and the initial column temperature was 160 °C programmed to rise to 250 °C with a rate of 6 °C/min. All assays were carried out in triplicate and the mean data are presented.

2.5.2. Determination of volatiles by SPME GC/MS

The composition of headspace volatile compounds of the treated substrates after SSF was analysed by solid phase micro extraction (SPME) GC/MS. The fibre used for the absorption of volatiles was a 50/30 μm DVB/Carboxen/PDMS StableFlex for manual holder (Supelco, Bellefonte, PA, USA). The conditions of headspace-SPME sampling used were as follows: 5 g of sample were transferred to a 20 ml glass vial sealed with a rubber septum. The contents were stirred for 5 min at 60 °C and the fibre was then exposed to the headspace for 45 min. GC/MS analysis was performed on a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer. Injection was performed in splitless

Table 3
Headspace volatile compounds identified by SPME GC/MS in the raw substrates.

Compound	OP mg/kg	M mg/kg	BSG mg/kg	MSR mg/kg	PP mg/kg	W mg/kg	KI mg/kg	KI_{Ref}	Odour
Esters									
Pentanoic acid-1-methylethyl ester	nd	22.0	nd	nd	23.6	nd	1079	980	Candy, strawberry
Acetic acid 2-phenylethyl ester	21.6	nd	nd	nd	nd	nd	1807	1847	Sweaty, rosy, honey
Total	21.6	22.0	0	0	23.6	0			
Alcohols									
2,6-Dimethyl-2-heptanol	827.6	434.8	103.8	102.2	520.2	2.13	980	1023	Citrus
2-Hexanol	7849.4	nd	nd	22.4	52.8	nd	1168	1176	Fruity, wine
3-Heptanol	8.2	nd	nd	3.8	nd	nd	1184	1230	Banana, minty, coffee
1-Octen-3-ol	nd	nd	1.0	nd	nd	nd	1440	1438	Mushroom, herbal
Phenylethyl alcohol	82.6	nd	nd	1.6	nd	0.4	1991	1900	Sweaty, fruity
Total	8767.8	434.8	104.8	130.0	573.0	2.53			
Carbonyl compounds									
2-Hexanone	164.4	nd	22.4	22.4	69.6	42.8	1069	1082	Green
2-Heptanone	nd	nd	16.0	16.8	47.4	27.2	1116	1113	Banana
4-Methyl-3-penten-2-one	nd	nd	nd	18.2	nd	nd	1129	1152	Vegetable
5-Nonanone	292.0	205.2	40.2	44.8	nd	195.8	1145	1153	–
3-Heptanone	93.8	45.8	12.2	9.2	16.6	10.8	1084	1160	Grape
2-Methyl-4-heptanone	108.8	54.0	14.0	11.4	81.4	49.4	1129	1213	Fruity
2-Octenal	nd	nd	nd	nd	16.2	nd	1432	1432	Walnut
Benzaldehyde	nd	nd	2.0	nd	nd	nd	1530	1525	Bitter, almonds
Benzaldehyde-2,4 dimethyl	nd	2.2	nd	nd	nd	nd	1727	1710	Cherry, almond, vanilla
2,4-Decadienal	nd	nd	nd	nd	14.4	nd	1760	1753	Citrus, orange, grapefruit
β-Damascedone	nd	2.8	nd	nd	nd	nd	1812	1805	Floral, sweaty, fruity
Total	659.0	310.0	106.8	122.8	245.6	326.0			
Organic acids									
Acetic acid	nd	nd	332.4	nd	nd	nd	1478	1435	Vinegar
Total	0	0	332.4	0	0	0			
Miscellaneous									
Dimethyl ether	2954.8	nd	nd	nd	nd	nd	872	890	Sweaty
Cyclohexane-1,1,3,5-Tetramethyl	155.8	89.2	41.4	17.6	59.2	95.6	893	932	–
p-Xylene	98.2	33.2	19.4	nd	56.4	36.6	1121	1127	Cold meat fat
Benzene-1,2,3-trimethyl	nd	nd	90.6	nd	nd	nd	1180	1048	Aromatic
1,4-Dimethoxy benzene	nd	12.6	nd	nd	nd	nd	1104	1163	Green
Benzene-1-ethyl-2-methyl	nd	nd	nd	nd	18.6	nd	1180	1257	Sweaty, pungent
Caryophyllene	nd	nd	13.6	nd	nd	nd	1589	1594	Woody, spicy
α-Caryophyllene	nd	nd	35.0	nd	nd	nd	1664	1663	Oily, fruity, woody
Valencene	32.8	nd	nd	nd	nd	nd	1711	1713	Pepper, orange
δ-Cadinene	nd	nd	2.2	nd	nd	nd	1749	1749	Thyme, herbal
Hexamethyleneimine	25.0	34.2	7.4	6.4	38.2	30.4	1050	MS	Pepper
Benzene-1,3,5-trimethyl	861.2	639.0	nd	105.0	700.4	4.6	1181	1143	Herbaceous, aromatic
Total	4127.8	808.2	209.6	129.0	872.8	167.2			

nd: not detected; OP: orange pulp; PP: potato pulp; M: molasses; W: whey; BSG: Brewer's spent grains; MSR: malt spent rootlets.

Table 4
Headspace volatile compounds identified by SPME GC/MS in the mixed substrates after SSF treatment by *S. cerevisiae* AXAZ-1, *K. marxianus* IMB3 and kefir.

Compound	*S. cerevisiae* mg/kg	Kefir mg/kg	*K. marxianus* mg/kg	KI	KI_{Ref}	Odour
Esters						
Ethyl acetate	nd	nd	12.4	886	885	–
Pentanoic acid-1-methylethyl ester	nd	100.2	6.6	1079	980	Candy, strawberry
Total	0	100.2	19.0			
Alcohols						
Ethanol	135.6	245.6	91.4	937	936	–
2,6-Dimethyl-2-heptanol	35.8	549.6	159.8	980	1023	Citrus
1-Octanol	10.2	nd	nd	984	1071	Herbal, toast bread
Phenylethyl alcohol	31.2	764.0	36.2	1991	1900	Sweaty, fruity
Total	212.8	1559.2	287.4			
Carbonyl compounds						
2-Hexanone	1.2	201.4	29.0	1069	1082	Green
3-Heptanone	19.0	744.6	7.8	1084	1160	Grape
2-Heptanone	20.6	241.0	21.2	1116	1113	Banana
2-Methyl-4-heptanone	926.8	533.2	9.4	1129	1213	Fruity
5-Nonanone	51.8	531.4	59.6	1145	1153	–
Total	1019.4	2251.6	127.0			
Organic acids						
Valeric acid	6.4	nd	nd	1673	1720	Sweaty, pungent, cheesy
Total	6.4	0	0			
Miscellaneous						
Cyclohexane-1,1,3,5-tetramethyl	31.0	945.4	34.6	893	932	–
p-Xylene	17.8	517.2	23.6	1121	1127	Cold meat fat
Benzene-1,3,5-trimethyl	175.4	3238.0	242.6	1181	1143	Herbaceous, aromatic
α-Caryophyllene	3.4	nd	nd	1664	1663	Oily, fruity, woody
Valencene	7.0	nd	8.4	1711	1713	Pepper, orange
Hexamethyleneimine	8.6	nd	10.4	1050	MS	Pepper
ε-Pinene	nd	4208.0	nd	1124	MS	Woody, pine
Total	243.2	8908.6	319.6			

nd: not detected.

mode. Separation of compounds was performed on a capillary column (Supelco CO Wax-10 60 m, 0.32 mm i.d., 0.25 μm film thickness). The oven temperature was held at 40 °C for 5 min, then programmed to rise to 110 °C by 10 °C/min, then to 180 °C by 2 °C/min, and finally to 250 °C by 10 °C/min where it was held for 6 min. Electron impact mass spectra were recorded at 70 eV ionisation energy in the 30–400 m/z mass range. The identification was carried out by comparing the retention times and mass spectra of volatiles to those of pure compounds (Sigma–Aldrich, Poole, UK), by mass spectra obtained from NIST107, NIST21 and SZTERP libraries, and by determining the Kovats' retention indexes and comparing with those reported in the literature (Mallouchos, Loukatos, Bekatorou, Koutinas, & Komaitis, 2007). Kovats' retention indexes were determined by injection of a standard mixture containing the homologous series of normal alkanes (C8–C24) in pure hexane under exactly the same experimental conditions, as described above. Semi-quantification, expressed as mg/kg sample, was carried out using 4-methyl-2-pentanol (Sigma–Aldrich, Poole, UK) as the internal standard. Each determination was carried out in triplicate and the mean values are presented.

3. Results and discussion

The growth of selected microorganisms of industrial interest (kefir, *K. marxianus* and *S. cerevisiae*) by SSF of substrates composed of various common food industry wastes (whey, molasses, brewer's solid wastes, orange and potato residues), was studied. The selected strain *S. cerevisiae* AXAZ-1 is a psychrotolerant and alcohol resistant yeast extensively studied for alcoholic fermentation processes, such as winemaking, brewing, distillates and ethanol production (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). The thermotolerant lactose fermenting yeast *K. marxianus* IMB3 and the mixed dairy culture kefir have been previously studied for the exploitation of cheese whey (Koutinas et al., 2005, 2007, 2009; Kourkoutas et al., 2002). The optimum composition of the waste mixtures used, as well as the fat, crude protein and mineral content of the treated substrates after SSF are shown in Table 1.

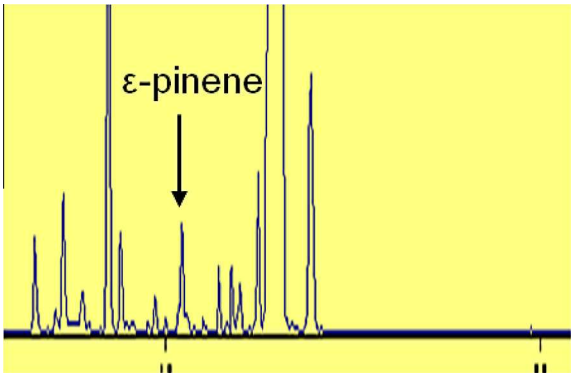

Fig. 1. GC/MS chromatograph showing the peak of ε-pinene produced by SSF of mixed food waste substrates with kefir.

3.1. Nutritional value of the SSF products for potential use as livestock feeds

An estimation of nutritional value of the SSF products as potential livestock feeds was done taking into account their composition in fat, protein and nutritive minerals. The fermented products can be considered for use as protein enriched livestock feeds as they contained protein in the range 23.6–38.5% depending on the

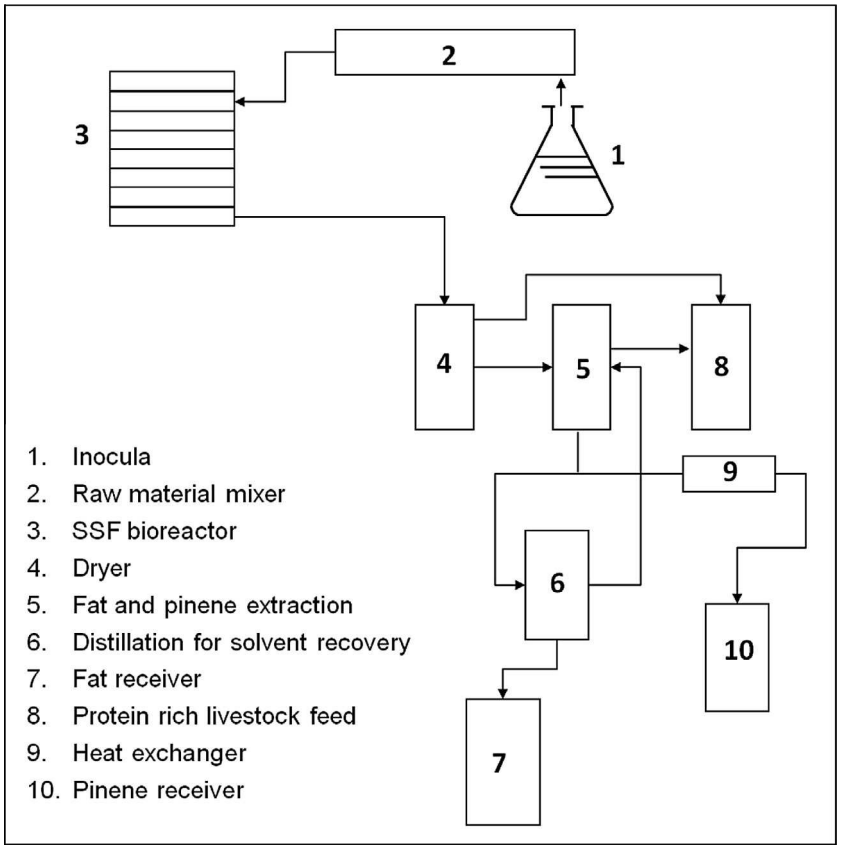

Fig. 2. Process flow sheet for protein enriched livestock feed, fat and ε-pinene production, in a biorefinery approach, by SSF of mixed food waste substrates with *K. marxianus* and kefir.

microorganism used (Table 1). Amongst the three microorganisms, the maximum sum of fat and protein content was obtained by SSF treatment of the substrates with *K. marxianus*. The fermented product with the highest protein content (38.5% w/w on dry weight basis) was the one prepared with *S. cerevisiae* AXAZ-1. Both this value and that obtained by *K. marxianus* IMB3 (33.7%) are almost double than these of the corresponding substrates before treatment (20.9–22.9%) and higher than those that have been reported for other fermented animal feed products such as barley, dried citrus pulp and fermented dried apple residue (Bambidis and Robinson, 2006; Joshi & Sandhu, 1996; Mathot et al., 1992). The maximum fat content obtained (25.5%) was that of the fermented product of *K. marxianus* (Table 1). This, in combination with the low fat content of the substrate itself (3.9%), shows a potential use of *K. marxianus* for protein/lipid enriched livestock feeds from food industry wastes or for fat extraction for other uses. GC analysis of fatty acid methyl esters in fat extracted from the substrates treated with *K. marxianus* and transesterified with methanol, showed palmitate, oleate, and linoleate as the major fatty acids (Table 2) as also shown in previous studies (Al-Shabibi & Younis, 1984; Cottrell, Kock, Lategan, Botes, & Britz, 1985). Regarding minerals (Table 1), no significant differences for Fe, Cu and Ca contents were observed amongst the SSF products. For Mg on the other hand, much higher concentrations (332–771 mg/kg) were found in the fermented substrates, with the *S. cerevisiae* product containing the highest concentration followed by *K. marxianus*. To conclude, the results showed that the product of SSF with *K. marxianus*, compared with that of *S. cerevisiae* and kefir, contained the highest amounts of the major nutrients fat and protein and considerable amounts of minerals, and can be considered a rich nutritious substrate for potential use as livestock-feed.

3.2. Evaluation of results based on the biorefinery concept

The maximum fat content (25.5%) obtained by treatment of the mixed waste substrates by *K. marxianus* under the given conditions, is a promising result for utilisation of this species for both lipid and protein production. Apart from a protein rich livestock feed obtained by fermentation of cheap waste materials, the high fat content could be also be recovered, e.g. by extraction with an organic solvent. The analysed fat consisted of 85.7% saturated palmitate and 12.6% unsaturated fatty acids oleate and linoleate (Table 2). Its exploitation could include, amongst other applications, the production of biodiesel, after suitable mixing with petroleum diesel or with biodiesel with high cetane number (Wang, Yu, He, & Liu, 2012). The saturated fatty acid methyl esters, such as methyl palmitate, although they have several disadvantages for biofuel production, they can be used to produce biodiesel blends with positive influence over air and noise emissions (Redel-Macias, Pinzi, Ruz, Cubero-Atienza, & Dorado, 2012).

The headspace SPME GC/MS analysis of the raw materials and the substrates after SSF (Tables 3 and 4), showed that in the case of *K. marxianus* the ester contents were similar, whilst alcohols were reduced by about 70–80%. Kefir led to higher ethanol concentration than *S. cerevisiae* and *K. marxianus* possibly due to lactic acid bacteria metabolism including transamination, oxidative deamination and decarboxylation reactions of amino acids (Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005; Katechaki, Panas, Kourkoutas, Koliopoulos, & Koutinas, 2009). Carbonyl compounds were reduced by about 90% and miscellaneous other compounds by 95%. Furthermore, the GC/MS analysis showed that the substrates treated with kefir contained 4208.0 mg/kg ε-pinene (Table 4, Fig. 1). Its concentration was 38% of total identified

Table 5
A preliminary economic evaluation of cost and product values of a potential biorefinery (Fig. 2) for the production of protein enriched livestock feed, fat and ε-pinene by SSF of food waste mixtures with *K. marxianus* and kefir.

	Cost (€/tn)					Product value (€)		
Microorganism	Raw material	Labour	Energy	Consumables (including electricity)	Total	Livestock feed	Fat	ε-Pinene
K. marxianus	270	120	70	50	510	750	200	–
Kefir	150	120	100	50	420	1000	–	400

volatiles, accounting for 0.42% of the total mass of the treated substrate, whilst it was absent in the raw materials. Likewise, this compound could be recovered by solvent extraction, membrane technologies, suitable adsorbents, etc. (Feron et al., 1996; Janssens et al., 1992), and be exploited as a flavouring product of high added value. The production of aroma compounds such as monoterpene alcohols and isoamyl acetate by fermentation with *K. lactis* and *K. marxianus* has been previously reported (Medeiros et al., 2000). Moreover, fragant compounds in fruit, including linalool, α-terpineol, 2-phenylethanol, α- and γ-terpinene and α-pinene, are usually bound in glycoside forms and are naturally revealed during maturation by endogeneous enzymes identified to β-glucosidases. Therefore, production of such compounds by fermentation of raw materials containing bound aroma precursors can by related with microbial β-glucosidase activity.

3.2.1. Proposed biorefinery process flow sheet

In the frame of a biorefinery approach, three products can be produced with the proposed SSF processes from food wastes: (i) protein enriched livestock feed, (ii) microbial lipids and (iii) ε-pinene. Fig. 2 illustrates a proposed process flow sheet of the production of these products. The inocula of *K. marxianus* or kefir are produced in vessel (1) and then added in the raw materials mixing machinery (2). The mixture is then supplied to the SSF bioreactor (3). The fermented product is dried in the drying chamber (4) and then the fat and ε-pinene are solvent extracted in unit (5). The protein rich livestock feed is collected in tank (8), whilst the extract is supplied to the distillation column (6) for solvent recovery and ε-pinene separation, which is liquefied in the heat exchanger (9) and collected in tank (10) as a crude product with other volatile impurities. The fat is collected in receiver (7). The above process can be carried out for ε-pinene and fat production separately with the livestock feed being a common product.

3.2.2. Preliminary cost analysis

Table 5 provides a cost analysis of the products that could be produced by the proposed biorefinery using *K. marxianus* or kefir. This analysis took into account the raw materials, labour, energy and consumable costs in €/tn of fermented product. The results showed that the lower total cost would be that of kefir. The prices of the products are close to those of similar commercial products currently found in the market. Regarding ε-pinene, it is a product with similar properties with the α-pinene and β-pinene. An indicative β-pinene price for 10 g of 99% purity is 166 € and 144 € for 100 g of α-pinene of the same purity. Therefore, the biotechnological production of ε-pinene could reduce these prices. Taking into account a price for crude ε-pinene of 100 €/kg, the total value produced would be 1400 €/tn of fermented product, in contrast to the 950 €/tn of the obtained *K. marxianus* products. So, increasing the ε-pinene purity to 99% the profit could be extremely increased.

4. Conclusions

SSF of mixture of food industry wastes with selected microorganisms of industrial interest can lead to simultaneous production of a protein enriched livestock feed, fat and ε-pinene based on the biorefinery concept. Amongst the tested stains, *K. marxianus* IMB3 led to the highest yield of protein and fat in the treated substrate, making it more suitable for feed enrichment as well as for exploitation of its fat content, compared with *S. cerevisiae* and kefir. Kefir on the other hand, produced a significant amount of the aroma compound ε-pinene, giving the possibility to produce 4 kg of this value added product per ton of treated substrate.

Acknowledgement

This work was supported by the Research Committee of the University of Patras, Greece (Program K. Karatheodori 2010, Project No. C916).

References

Aggelopoulos, T., Bekatorou, A., Pandey, A., Kanellaki, M., & Koutinas, A. A. (2013). Discarded oranges and brewer's spent grains as promoting ingredients for microbial growth by submerged and solid state fermentation of agro-industrial waste mixtures. *Applied Biochemistry and Biotechnology, 170*, 1885–1895.

Al-Shabibi, M. M. A., & Younis, N. A. J. (1984). Fermentation of whey filtrate by *Kluyveromyces fragilis*: The effect on cell lipids. *Canadian Institute of Food Science and Technology Journal, 17*, 117–120.

Anupama, X., & Ravindra, P. (2000). Value-added food: single cell protein. *Biotechnology Advances, 18*, 459–479.

AOAC (1995). *Official methods of analysis of AOAC international* (16th ed.) (Vol. II). Arlington: AOAC.

Argiriou, T., Kaliafas, A., Psarianos, K., Kanellaki, M., Voliotis, S., & Koutinas, A. A. (1996). Psychrotolerant *Saccharomyces cerevisiae* strains after an adaptation treatment for low temperature wine making. *Process Biochemistry, 31*, 639–643.

Bambidis, V. A., & Robinson, P. H. (2006). Citrus by-products as ruminant feeds: A review. *Animal Feed Science and Technology, 128*, 175–217.

Banat, I. M., Nigam, P., Singh, D., Marchant, R., & McHale, A. P. (1998). Review: ethanol production at elevated temperatures and alcohol concentrations. Part I. Yeast in general. *World Journal of Microbiology and Biotechnology, 14*, 809–821.

Barrios-González, J. (2012). Solid-state fermentation: Physiology of solid medium, its molecular basis and applications. *Process Biochemistry, 47*, 175–185.

Bekatorou, A., Psarianos, C., & Koutinas, A. A. (2006). Production of food grade yeasts. *Food Technology and Biotechnology, 44*, 407–415.

Beshkova, D. M., Simova, E. D., Frengova, G. I., Simov, Z. I., & Dimitrov, Zh. P. (2003). Production of volatile aroma compounds by kefir starter cultures. *International Dairy Journal, 13*, 529–535.

Buschke, N., Schafer, R., Becker, J., & Wittmann, C. (2013). Metabolic engineering of industrial platform microorganisms for biorefinery applications-optimization of substrate spectrum and process robustness by rational and evolutive strategies. *Bioresource Technology, 135*, 544–554.

Cottrell, M., Kock, J. L. F., Lategan, P. M., Botes, P. J., & Britz, T. J. (1985). The long-chain fatty acid compositions of species representing the genus *Kluyveromyces*. *FEMS Microbiology Letters, 30*, 373–376.

Couto, S. R., & Sanroman, M. A. (2006). Application of solid-state fermentation to food industry-a review. *Journal of Food Engineering, 76*, 291–302.

Feron, G., Bonnarme, P., & Durand, A. (1996). Prospects for the microbial production of food flavours. *Trends in Food Science & Technology, 71*, 285–293.

Ferreira, J. A., Lennartsson, P. R., Edebo, L., & Taherzadeh, M. J. (2013). Zygomycetes-based biorefinery: Present status and future prospects. *Bioresource Technology, 135*, 523–532.

Gobbetti, M., De Angelis, M., Corsetti, A., & Di Cagno, R. (2005). Biochemistry and physiology of sourdough lactic acid *bacteria*. *Trends in Food Science & Technology, 16*, 57–69.

Harta, O., Iconomopoulou, M., Bekatorou, A., Nigam, P., Kontominas, M., & Koutinas, A. A. (2004). Effect of various carbohydrate substrates on the production of kefir grains for use as a novel baking starter. *Food Chemistry, 88*, 237–242.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K. Y., Ishii, J., & Kondo, A. (2013). A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. *Bioresource Technology, 135*, 513–522.

Janssens, L., De Pooter, H. L., Schamp, N. M., & Vandamme, E. J. (1992). Production of flavours by microorganisms. *Progress Biochemistry, 27*, 195–215.

Joshi, V. K., & Sandhu, D. K. (1996). Preparation and evaluation of an animal feed byproduct produced by solid-state fermentation of apple pomace. *Bioresource Technology, 56*, 251–255.

Kamat, S., Khot, M., Zinjarde, S., RaviKumar, A., & Gade, W. N. (2013). Coupled production of single cell oil as biodiesel feedstock, xylitol and xylanase from sugarcane bagasse in a biorefinery concept using fungi from the tropical mangrove wetlands. *Bioresource Technology, 135*, 246–253.

Katechaki, E., Panas, P., Kourkoutas, Y., Koliopoulos, D., & Koutinas, A. A. (2009). Thermally-dried free and immobilized kefir cells as starter culture in hard-type cheese production. *Bioresource Technology, 100*, 3618–3624.

Kopsahelis, N., Agouridis, N., Bekatorou, A., & Kanellaki, M. (2007). Comparative study of delignified and non-delignified brewer's spent grains as yeast immobilization supports for alcohol production from molasses. *Bioresource Technology, 98*, 1440–1447.

Kosseva, M. R. (2013). Recovery of commodities from food wastes using solid-state fermentation. *Food Industry Wastes*, 77–102 [Chapter 5].

Kourkoutas, Y., Bekatorou, A., Banat, I. M., Marchant, R., & Koutinas, A. A. (2004). Immobilization technologies and support materials suitable in alcohol beverages production: A review. *Food Microbiology, 21*, 377–397.

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.

Koutinas, A. A., Papapostolou, H., Dimitrellou, D., Kopsahelis, N., Katechaki, E., Bekatorou, A., et al. (2009). Whey valorisation: A complete and novel technology development for dairy industry starter culture production. *Bioresource Technology, 100*, 3734–3739.

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

Koutinas, A. A., Athanasiadis, I., Bekatorou, A., Psarianos, C., Kanellaki, M., Agouridis, N., et al. (2007). Kefir-yeast technology: Industrial scale-up of alcoholic fermentation of whey, promoted by raisin extracts, using kefir-yeast granular biomass. *Enzyme and Microbial Technology, 41*, 576–582.

Kumar, S., Gupta, R., Kumar, G., Sahoo, D., & Kuhad, R. C. (2013). Bioethanol production from *Gracilaria verrucosa*, a red alga, in a biorefinery approach. *Bioresource Technology, 135*, 150–156.

Lane, M. M., & Morrissey, J. P. (2010). *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. *Fungal Biology Reviews, 24*, 17–26.

Mallouchos, A., Loukatos, P., Bekatorou, A., Koutinas, A. A., & Komaitis, M. (2007). Ambient and low temperature winemaking by immobilized cells on brewer's spent grains: Effect on volatile composition. *Food Chemistry, 104*, 918–927.

Mathews, J. A., Tan, H., Moore, M. J. B., & Bell, G. (2011). A conceptual lignocellulosic 'feed+fuel' biorefinery and its application to the linked biofuel and cattle raising industries in Brazil. *Energy Policy, 39*, 4932–4938.

Mathot, P., Debevere, C., Walhain, P., Baudart, E., Théwis, A., & Brakel, J. (1992). Composition and nutritive value for rats of *Aspergillus niger* solid fermented barley. *Animal Feed Science and Technology, 39*, 227–237.

Medeiros, A. B. P., Pandey, A., Freitas, R. J. S., Christen, P., & Soccol, C. R. (2000). Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology. *Biochemical Engineering Journal, 6*, 33–39.

Nitayavardhana, S., & Khanal, S. K. (2010). Innovative biorefinery concept for sugar-based ethanol industries: Production of protein-rich fungal biomass on vinasse as an aquaculture feed ingredient. *Bioresource Technology, 101*, 9078–9085.

Plessas, S., Alexopoulos, A., Voidarou, C., Stavropoulou, E., & Bezirtzoglou, E. (2011). Microbial ecology and quality assurance in food fermentation systems. The case of kefir grains application. *Anaerobe, 17*, 483–485.

Plessas, S., Trantallidi, M., Bekatorou, A., Kanellaki, M., Nigam, P., & Koutinas, A. A. (2007). Immobilization of kefir and *Lactobacillus casei* on brewery spent grains for use in sourdough wheat bread making. *Food Chemistry, 105*, 187–194.

Redel-Macias, M. D., Pinzi, S., Ruz, M. F., Cubero-Atienza, A. J., & Dorado, M. P. (2012). Biodiesel from saturated and monounsaturated fatty acid methyl esters and their influence over noise and air pollution. *Fuel, 97*, 751–756.

Redwood, M. D., Orozco, R. L., Majewski, A. J., & Macaskie, L. E. (2012). An integrated biohydrogen refinery: Synergy of photofermentation, extractive fermentation and hydrothermal hydrolysis of food wastes. *Bioresource Technology, 119*, 384–392.

Singh, D., Nigam, P., Banat, I. M., Marchant, R., & McHale, A. P. (1998). Review: Ethanol production at elevated temperatures and alcohol concentrations. Part II. Use of *Kluyveromyces marxianus* IMB3. *World Journal of Microbiology and Biotechnology, 14*, 823–834.

Singhania, R. R., Patel, A. K., Soccol, C. R., & Pandey, A. (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal, 44*, 13–18.

Wang, L. B., Yu, H. Y., He, X. H., & Liu, R. Y. (2012). Influence of fatty acid composition of woody biodiesel plants on the fuel properties. *Journal of Fuel Chemistry and Technology, 40*, 397–404.