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Yeast flavour production by solid state fermentation of orange peel waste

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ABSTRACT

Consumer demand for natural products and the requirement for eco-friendly processes promote development of innovative processes for flavour synthesis *via* biotechnology. In this direction, cultivation of selected industrial yeast strain under solid state fermentation of orange peel (OP) was studied. For this purpose, autoclave sterile OP for the elimination of D-limonene and natural microflora was evaluated with regard to yeast viability, nutrient consumption and cell ability to produce flavour active compounds. Non-sterile OP was also used to follow pros and cons of the sterilization process. Yeast cells showed better growth performance under sterilized process conditions, than under non-sterilized ones. In the first case, the enhanced *de novo* synthesis of "fruity" esters was demonstrated (48.7, 25.2, 9.3, 6.3 and 4.5 mg/kg of fermented OP for isoamyl acetate, ethyl dodecanoate, decanoate, octanoate and phenyl ethyl acetate, respectively, after 72 h). Yeast cells exhibited accelerated synthesis of ethyl hexanoate (154.2 mg/kg OP at 48 h). Biotransformation of naturally occurring aroma compounds by yeast may be considered in this process. The proposed process, resulting in high yields of industrially important volatile aroma esters (total of ~250 mg/kg OP), could be applied to a sustainable biorefinery for the valorization of OP waste.

1. Introduction

Flavour-active compounds contribute significantly in the organoleptic properties of many food products. In terms of value, the flavour market accounts for more than 50% of the global market for flavours and fragrances, which is expected to grow from US$ 21.4 billion in 2013 to US$ 25 billion in 2018 [1]. Nowadays, most flavours are either extracted from plant sources or synthesized by chemical means. The rising concern of consumers about natural products and the requirement for eco-friendly processes definitely encourage research and development of alternative processes for flavour synthesis *via* biotechnology. This aspect has been strengthened by the latest EC legislation [2], according to which natural flavours include biotechnology-derived products based on microorganisms, plant cell cultures and enzymes [3–6].

Adoption of microbial technology for flavour production by industry is hindered by high manufacturing costs involved. To build a sustainable and an economically competitive microbial process, the use of expensive substrates, the low productivity and the high pre-treatment and downstream processing costs must be overcome. In this direction, the production of flavour compounds from agro-industrial wastes with negligible or even no-cost, such as orange peels, is an interesting approach. Orange peel (OP) is the major solid waste generated by the fruit processing industry. Its use as a potential substrate for the *de novo* synthesis of isoamyl acetate, phenylethyl acetate and ethyl esters (hexanoate, octanoate, decanoate and dodecanoate) by *Saccharomyces cerevisiae* in liquid fermentation has been recently determined [7,8]. Its notable activity was mainly due to its high level of fermentable carbohydrates, *i.e.* coming from naturally occurring simple sugars (glucose, fructose) and polysaccharides (cellulose, hemicellulose, pectin) after being hydrolysed, along with amino nitrogen. Amongst the various types of microbial processes to convert solid wastes into value-added compounds, the use of solid-state fermentation (SSF) as a means to improve cost effectiveness of these processes and its application for the production of aroma compounds has been recommended. As examples, cassava bagasse, sugarcane bagasse, apple pomace, soya bran and coffee husk have been evaluated for this purpose by cultivating different microorganisms (Table 1). SSF is a process carried out in a solid matrix with sufficient moisture content for microbial growth and metabolism requirements but almost no free water in the system [18]. Due to the limited amount of water, capital and operating costs are

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Table 1
Agro-industrial wastes used for the flavour-active compound production by solid state fermentation.

Microorgansims	Substrates	Flavour active compounds	References
Ceratocystis fimbriata	Cassava bagasse, wheat bran, sugarcane bagasse	Acetaldehyde, 3-methyl butanol, 3-methylbutyl acetate, ethyl acetate, ethyl propionate	[9]
Rhizopus oryzae	Cassava bagasse, soybean meal, apple pomace	Acetaldehyde, 3-methyl butanol, 1-propanol, ethyl acetate, ethyl propionate	[10]
Kluyveromyces marxianu	Cassava bagasse, giant palm bran	Isoamylic alcohol, ethyl acetate, propyl acetate, butyl acetate, ethyl propionate, ethyl isobutyrate, isoamyl acetate	[11]
Ceratocystis fimbriata	Coffee husk	Isopropanol, ethyl acetate, ethyl isobutyrate, isobutyl acetate, isoamyl acetate, ethyl-3-hexanoate	[12]
Moniliella suaveolens, *Trichoderma harzianum*, *Pityrosporum ovale*, *Ceratocytis oniliformis*	Linseed cake, castor oil cake, olive press cake, sunflower cake	δ-and γ-decalactone	[13]
Ceratocystis fimbriata	Mixture of citric pulp and soya bran, sugarcane molasses, soya molasses	Isoamyl acetate	[14]
Aspergillus niger, metabolically engineered *Escherichia coli*	Cereal or maize bran, sugar beet pulp	Vanillin	[15]
S. cerevisiae, *K. marxianus*, Kefir culture	Mixed solid and liquid food industry wastes (*i.e.* cheese whey, molasses, brewer's spent grains, malt spent rootlets, orange and potato pulp)	ϵ-pinene	[16]
Trichoderma viride	Sugarcane bagasse	6-pentyl-α-pyrone	[17]

reduced as a result of lower working volumes per product yield and process wastewater as well as lower energy costs for sterilization and stirring [18–22]. Moreover, SSF of agro-industrial wastes simulates the natural environment of many microorganisms offering high productivity rates, higher product stability and lower extent of catabolite repression [19]. Despite the fact that SSF remains a sustainable approach for the production of natural aroma compounds using various agro-industrial residues, only the work of Rossi et al. [23] has focused on the utilization of citric pulp for the production of aroma volatiles by *Ceratocystis fimbriata* in solid-state cultures. According to their results, citric pulp supplemented with soya bran, sugarcane molasses and mineral saline solution produced a strong fruity aroma.

Following our previous works, where the production of volatile bio-esters by a commercial wine yeast strain (Vitilevure MT) was studied in submerged fermentation (SmF) using OP complemented with a nutritive medium containing glucose, yeast extract and salts [8] or OP hydrolysate [7], in this work the potential of such a waste as a substrate for flavour-active compounds production by SSF using the same microorganism was investigated. In such a case, the SSF would be preferred to SmF if it could provide several advantages such as higher productivity and lower pre-treatment, downstream processing and waste disposal costs. Since elimination of autoclave sterilization is expected to reduce the overall cost of the final products, the effect of non-sterile conditions on process parameters such as cell viability and nutrient assimilation was also assessed. Gas chromatography (GC–MS, GC–FID) analysis was employed to monitor changes in the composition of flavour-active compounds during the fermentation process.

2. Materials and methods

Fresh Washington Navel oranges were purchased from the local market. The peel (white mesocarp and orange–yellow exocarp), remained after the extraction of the juice, was sliced into small pieces and grounded with an electric mill (Braun 4240, Germany).

2.1. Compositional analysis

Orange peel moisture content was determined by gravimetric analysis after drying at 105 °C to constant weight. Key nutrients in the different fractions of OP were quantified as described by Mantzouridou and Paraskevopoulou [8].

2.2. Yeast strain and inoculum preparation

A commercial wine yeast strain, Vitilevure MT (*S .cerevisiae*) that expresses and enhances varietal aromas very well was employed in this study. The selected yeast was provided in dry form by a Greek wine industry (Tsantalis S.A., Chalkidiki, Greece). Cells were activated by adding the appropriate quantity of distilled water (1:10, w/v), followed by periodical stirring inside a water bath (35–37 °C)

Table 2
Key nutrient composition analysis of raw and fermented orange peel (OP) in the sterile and non-sterile trials.

Nutrient	Value[b] (% on dry basis)			
	Raw OP		Fermented OP[c]	
	Non sterile	Sterile	Non sterile	Sterile
Alcohol-soluble carbohydrates[a]	26.70 ± 0.60^{C}	28.90 ± 2.10^{D}	20.09 ± 0.31^{B}	1.73 ± 0.05^{A}
Alcohol-insoluble solids	8.67 ± 0.11^{C}	7.35 ± 0.86^{B}	5.92 ± 0.08^{A}	7.88 ± 0.12^{B}
EDTA-soluble solids	18.46 ± 0.51^{B}	18.00 ± 2.33^{B}	11.58 ± 0.33^{A}	17.41 ± 0.62^{B}
Water unextractable polysaccharides[a]	15.83 ± 0.25^{B}	13.78 ± 1.65^{A}	14.00 ± 0.28^{A}	$14.96 \pm 0.32^{A,B}$
Crude protein	4.25 ± 0.56^{A}	$4.70 \pm 0.32^{A,B}$	5.23 ± 0.16^{B}	9.72 ± 0.55^{C}
Fat	1.22 ± 0.10^{A}	1.28 ± 0.08^{A}	1.30 ± 0.02^{A}	1.36 ± 0.05^{A}

[a] Expressed on a glucose equivalent basis.
[b] Mean value of three independent measurements ± standard deviation.
[c] After 5 days of the fermentation process; different capital letters were used to label significantly different values in the same row.

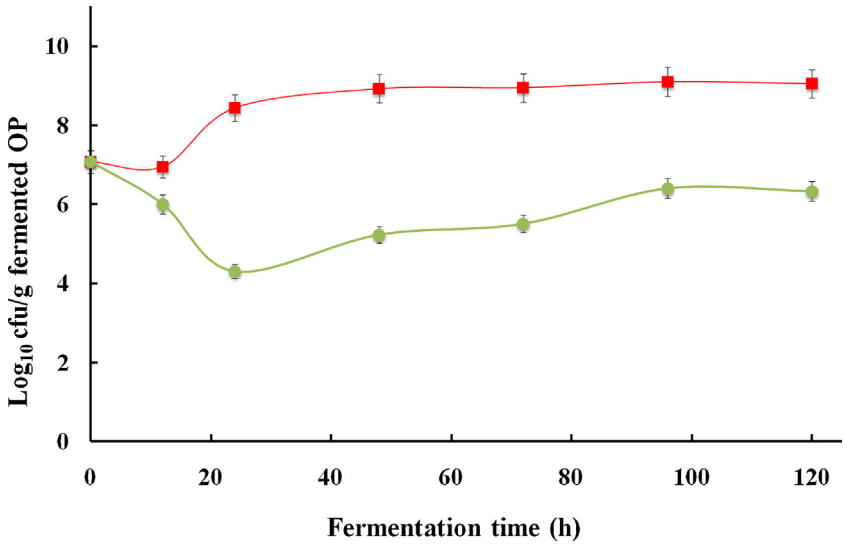

Fig. 1. Growth kinetics of *S. cerevisiae* in sterile (red squares) and non-sterile (green circles) orange peel (OP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for 15–20 min. For inoculum preparation 2 mL of the cell suspension were transferred in 100 mL of a synthetic medium with the following composition (g/L): 20, glucose (Panreac, Barcelona, Spain); 10, soy peptone (Lab M Ltd., Lancashire, UK); 5, yeast extract (Merck, Darmstadt, Germany). The culture was incubated on a rotating shaker at 200 rpm, 30 °C to a final OD_{600} value of approximately 2. Then, yeast cells were collected by centrifugation (3000 × *g*, 10 min), washed and resuspended in a sterile saline solution (9 g/L NaCl) (Merck) to reach a concentration of 1×10^9 colony forming units cfu/mL.

2.3. Solid state fermentation

Experiments were conducted in 250 mL Erlenmeyer flasks containing 55 g of milled samples of OP (wet weight basis) corresponding to loading of 60% of the flask working volume. The material was enriched with the following nutrients (g/kg substrate): 0.4, yeast extract; 1.0, $(NH_4)_2SO_4$; 1.0, KH_2PO_4; 5.0, $MgSO_4$. All salts were from Merck. The substrate was sterilized by autoclaving at 120 °C for 15 min unless otherwise stated. After cooling, the substrate (pH 6.0) was inoculated with 1.0×10^7 cfu/g and incubated at 25 °C for 5 days. The volume of the liquid phase (nutrient solution) and inoculum added to the substrate was chosen to reach final water content of 75% (w/w) from initial water content of 72.3% (w/w). This value is within the range of values reported in literature for ethanol production by SSF of other solid wastes using *S. cerevisiae* [24,25]. At specific time intervals (0, 12, 24, 48, 72, 96 and 120 h), the whole content of a flask was removed and subjected to further analysis. Also, the enriched OP was allowed to undergo spontaneous (uninoculated) fermentation by indigenous microflora for 72 h. All fermentation experiments were carried out in triplicate.

2.4. Yeast enumeration

10 g sample was transferred in peptonized water (90 mL) and homogenized with stomacher for 1 min at room temperature. The homogeneous mixture was used as stock solution for making appropriate decimal dilutions in peptonized water and plated on Rose Bengal Chloramphenicol agar (RBCA; Oxoid CM 549

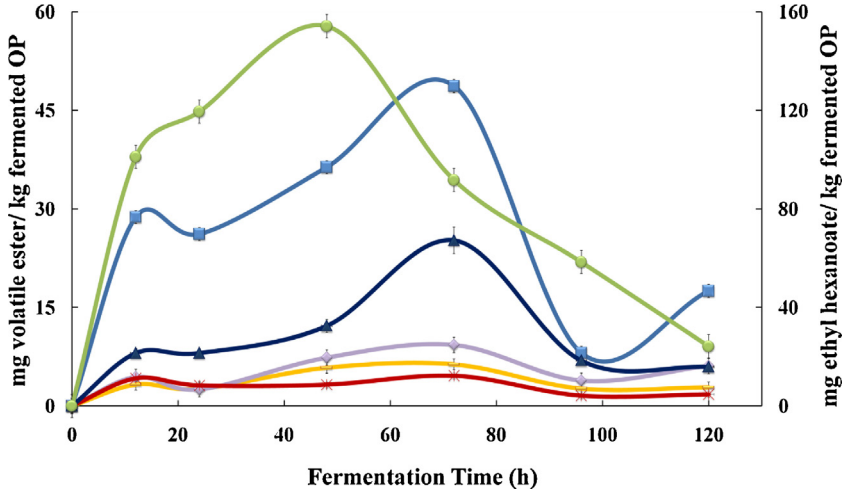

Fig. 2. Kinetics of the *de novo* synthesis of isoamyl acetate (blue squares), phenyl ethyl acetate (red stars), ethyl hexanoate (green circles), ethyl octanoate (orange dashes), ethyl decanoate (purple diamonds) and ethyl dodecanoate (dark blue triangles) by solid-state fermentation of sterile orange peel (OP) using *S. cerevisiae*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Supplemented with SR 78, Oxoid Ltd., Hants, United Kingdom). Cultures were incubated for 48 h at 30 °C to determine yeast population. Results were expressed in $\log_{10}$ cfu/g of substrate.

2.5. Optical microscopy

Microbial observation of individual colonies appearing on the Plate Count Agar (Merck 1.05463) and RBCA after plating the appropriately diluted homogeneous fermented samples and incubation at 25 °C for 48 h was conducted by using a Zeiss Axiolab reflected light microscope (100× magnitude) equipped with a Canon Power Shot G 2 mm (Canon, Tokyo, Japan) photographic camera.

2.6. Determination of residual carbon content

Aqueous extract was obtained from 5 g sample by addition of distilled water up to a 5:1 (v/w) ratio. The extraction was assisted by mechanical agitation using an Ultra Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) for 30 s, and centrifugation at 4500 rpm for 10 min to remove the solid particles. Residual sugars were determined in the aliquots of the aqueous extract spectrophotometrically by the method of Dubois et al. [26].

2.7. Isolation of volatiles

Flavour-active compounds were extracted from 5 g sample with the addition of dichloromethane up to a 2:1 (v/w) ratio (shaking for about 2 h at 20 °C). The organic fractions were dried over anhydrous Na_2SO_4 and concentrated initially to 2 mL in a Vigreux column and then under a slow nitrogen stream up to a final volume of 500 μL. The crude extracts were kept at −18 °C until further analysis. Final extracts were analysed both qualitatively and quantitatively as described by Mantzouridou and Paraskevopoulou [8]. An Agilent 6890A gas chromatograph (USA) equipped with MSD 5973 mass spectrometer (MS) as well as an Agilent 6890A gas chromatograph equipped with a flame ionization detector (FID) were used. Volatile compounds were separated on a HP-FFAP column (25 m × 0.20 mm i.d., film thickness 0.30 μm) with helium as carrier gas (2 mL/min). The oven temperature was kept at 40 °C for 2 min and then raised to 230 °C at a rate of 10 °C min^{-1} (4 min). The injector and the transfer line temperatures were set at 230 and 240 °C, respectively. The injection volume was 2 μL (split ratio 100:1). The MS was operated in the EI mode at 70 eV, scanning the range 35–350 m/z at a scan rate of 2 scans s^{-1} and the ion source temperature was 230 °C. The identification of the compounds were conducted by comparison of their retention times and mass spectra with those of available authentic standards and with those recorded in NIST library (Version 2.0d, 2005). The concentration of isoamyl acetate, phenylethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate and limonene was measured by comparison with calibration curves made with the reference compounds (supplied by Fluka, Munich, Germany) under the same conditions. Each sample was analysed in triplicate.

In need of validation before application, the extraction protocol was inspected with regard to repeatability of the method and recovery of the six esters using 5 g "sterile OP fermented for 48 h" as a matrix. The recovery was examined at two concentration levels of addition (2 mg/L and 40 mg/L) for each ester standard. A recovery ranging from 88.4 to 103% ($n = 5$) and a coefficient of variation of the measurement less than 7.43% ($n = 5$) confirmed that the extraction method used was accurate and convenient for quantitative analysis.

2.8. Pectin extraction

Pectin was extracted from OP using ethylenediamine tetra acetic acid (EDTA) (Sigma–Aldrich, Milan, Italy) in acidic environment (pH 1.5). Specifically, OP was treated with 0.05 M EDTA (prepared by dissolving 1.46 g of EDTA powder into 100 mL of distilled water and adjusting the pH to 1.5) at a ratio 1:20. The extraction took place at 80 °C for 20 min. Pectin was then precipitated and separated from the solution by mixing 15 mL of the extracted solution with 25 mL of heated 95% ethanol (Sigma–Aldrich). The mixture was heated in a water bath at 80 °C under vigorous mixing. After centrifugation for 15 min at 4500 rpm the supernatant was discarded and the sediment was further washed with ethanol and centrifuged repeatedly until there was no sugar left. Five percent 1-naphthol (Sigma–Aldrich), dissolved in ethanol, was used to detect the presence of sugars (appearance of purple colour indicates positive reaction). The recovered pectin was determined gravimetrically after drying at 105 °C for 4 h.

2.9. Extraction of phenolic compounds and determination of total phenol content

The phenolic compounds were extracted from the peels after tissue rupture by freezing and thawing, using liquid nitrogen, and then by manual grinding. For the extraction, 2 g of orange peel powder and 16 mL of ethanol (80% w/v) were transferred in a 50 mL centrifuge tube and placed in a shaker incubator at room temperature (200 rpm, 18 h). The mixture was then centrifuged at 1000 × g for 5 min at 4 °C. The obtained aqueous organic extract was condensed to 1 mL by a rotary evaporator at 40 °C under vacuum and resuspended in a final volume of 10 mL in deionized water. Total phenol content of OP extracts was estimated by using the Folin–Ciocalteau assay at 750 nm with gallic acid (GA) (Sigma–Aldrich) as reference [27].

2.10. Carotenoid extraction and determination of total carotenoid content

The carotenoids were extracted from OP after tissue rupture by freezing and thawing, using liquid nitrogen, and then by manual grinding. Carotenoids were extracted with acetone (Chem-Lab., Zedelgen, Belgium) using a shaker incubator (200 rpm, 30 min, 30 °C). The acetone/treated OP ratio was 1:1, v/v. The coloured organic layers from three successive extractions were mixed and centrifuged at 10,000 × g for 10 min, passed through a water-free Na_2SO_4 (Sigma–Aldrich) layer, and rotary-evaporated to dryness. Crude extract was dissolved in n-hexane. The total carotenoid content estimated as β-carotene was quantified spectrophotometrically at 450 nm by using a five-point standard calibration curve using β-carotene (Sigma–Aldrich).

2.11. L-ascorbic acid extraction and determination

For L-ascorbic extraction, frozen pulverised OP peels (2 g) were mixed with 20 mL of the extraction solution (3% metaphosphoric acid (Sigma–Aldrich) –8% acetic acid (Chem-Lab.) and the preparation was subjected to intensive agitation (in ice and darkness) with a four blade blender (Braun ZK100, Kronberg im Taunus, Germany) for 1 min. The mixture was centrifuged at 10,000 × g for 10 min at 4 °C. This procedure was repeated three times and the resulting supernatants were mixed together. Several precautions were taken in order to perform all the operations under reduced light and at 4 °C temperature. Determination of L-ascorbic acid in OP extracts was performed according to the AOAC's official titrimetric method (AOAC method 967.21) [28].

2.12. Statistical analysis

Statistical differences in compositional data of raw and fermented OP in the sterile and non-sterile trials were found by

Table 3
Yield and productivity of volatile esters produced by spontaneous and inoculated solid-state fermentation of orange peel (OP) in the sterile and non-sterile trials.

	Yield[a] (mg/kg fermented OP)			Productivity[a,b] (mg/kg h)		
	Non-sterile		Sterile	Non-sterile		Sterile
Volatile ester	Spontaneous	Inoculated	Inoculated	Spontaneous	Inoculated	Inoculated
Isoamyl acetate	–	5.76 ± 0.42 ^{A}	49.50 ± 3.95^{B}	–	0.08 ± 0.006 ^{A}	0.69 ± 0.055 ^{B}
Phenyl ethyl acetate	–	2.59 ± 0.22 ^{A}	5.05 ± 0.42 ^{B}	–	0.04 ± 0.003 ^{A}	0.07 ± 0.0058 ^{B}
Ethyl hexanoate	–	12.49 ± 0.90 ^{A}	155.2 ± 12.3 ^{B}	–	0.26 ± 0.019 ^{A}	3.23 ± 0.26 ^{B}
Ethyl octanoate	–	4.33 ± 0.33 ^{A}	7.10 ± 0.63 ^{B}	–	0.06 ± 0.0046 ^{A}	0.10 ± 0.0087 ^{B}
Ethyl decanoate	1.31 ± 0.11 ^{A}	10.41 ± 0.75 ^{B}	9.90 ± 0.65 ^{B}	0.02 ± 0.0015 ^{A}	0.15 ± 0.010 ^{B}	0.14 ± 0.009 ^{B}
Ethyl dodecanoate	0.21 ± 0.02 ^{A}	4.22 ± 0.30 ^{B}	26.52 ± 1.90 ^{C}	0.003 ± 0.0028 ^{A}	0.06 ± 0.0042 ^{B}	0.37 ± 0.026 ^{C}

[a] Mean value of three independent measurements ± standard deviation.
[b] After 72 h for isoamyl acetate, phenyl ethyl acetate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate and after 48 h for ethyl hexanoate. Different capital letters were used to label significantly different values in the same row for yield and productivity columns.

one-way ANOVA, followed by the Duncan's test ($p < 0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Mean values of three independent experiments are shown in Tables 2–4, and in Figs. 1 and 2. Error bars represent the standard deviation (SD) of the mean value. Different letters were used to label significantly different values in the same row.

3. Results and discussion

3.1. Growth characteristics of S. cerevisiae during SSF of autoclave sterile and non-sterile OP

The main limitation of OP usage as a fermentation feedstock is the presence of D-limonene found in the peel oil at a percentage higher than 90% (w/w). The strong inhibitory effect of this compound towards yeast growth has been attributed to disruption of cellular membrane and H^+ and K^+ transport energized by glycolysis [29]. Thus, protecting the yeast against this risk factor helps in preventing failure of the fermentation process. In view of the above, in the first series of experiments, evaluation of the necessity of autoclave treatment of OP for the elimination of yeast-inhibiting volatile substances, mainly D-limonene, and natural microflora present in this material took place. To achieve this goal preliminary experiment involved SSF of non-sterile and autoclave sterile OP inoculated with Vitilevure MT. The kinetic data obtained are presented in Fig. 1.

According to the growth pattern of yeast cells under non-sterile conditions, a 3-log decrease in the initial microbial population within 24 h of SSF occurred (Fig. 1). Over the same cultivation period, it was observed that yeast cells remained viable in populations of more than 8-$\log_{10}$cfu/g substrate in the process with the autoclave sterile OP. To better understand the effect of autoclave sterilization process on OP, the amount of limonene in raw and pretreated OP was determined. The estimated values (0.52 ± 0.02 *vs* 0.20 ± 0.01%, v/w of raw and autoclave sterile matrix) indicated that the loss of limonene observed during the autoclave sterilization of OP was rather high (62%) due to its evaporation. The inhibitory effect of limonene has been reported in chemically defined liquid media at very low levels (0.01–0.20%, v/v) [30,31]. A greater minimum inhibitory concentration of limonene (0.28%, v/w) was found in the study of Wilkins et al. [29], in which satisfactory ethanol yields could be obtained from citrus peel waste after saccharification and fermentation.

Noticeably, after 24 h of cultivation, in both cases, yeast cells could overcome the inhibitory effect of limonene even though the rate of growth in log phase under non-sterile conditions was strongly restricted with a negative influence in the population dynamics of yeast. As shown in Fig. 1, maximum yeast population size of around 5 log cfu/g substrate was achieved within 48 h of SSF of non-sterile OP, less than that in the sterilized process (9 log cfu/g), which meant that yeast cells grow under less favoured conditions in the first case. An important observation is that yeast cells remained alive until the end of the fermentation process (up to 120 h) under either sterile or non-sterile conditions. Together with previous findings [8,32], this evidence strengthens the suitability of OP for SSF processes, where the capacity of microbial cells to colonize the solid matrix is vital, through the natural adhesion of yeast cells to the substrate. The latter has been proposed to result from hydrogen bonding with hydroxyl groups between the surface of the cellulosic material and the yeast cell wall [33].

As shown in Table 2, a slight, although significant, decrease in the content of alcohol-insoluble solids and water unextractable polysaccharides was accompanied by a proportional increase of the alcohol-soluble carbohydrate level. This implies that part of the cell wall components, particularly pectin and cellulose, in the OP was hydrolysed to reducing sugars after treatment with heating by autoclaving. At the end of the bioprocess under sterile conditions, simple sugars (*i.e.* glucose, fructose and sucrose), which constitute the alcohol-soluble fraction of OP, were almost exhausted (consumption of 94%). These are readily available carbon sources for yeast growth. Glucose and fructose were the only sugars detected in the fermented substrate with the former being in slight excess (data not shown).

No other carbohydrate fractions changed significantly due to inability of yeast cells to utilize cell wall polysaccharides (*i.e.* pectin, hemicellulose and cellulose). Noticeable, crude protein content doubled compared to the level in the corresponding raw substrate before fermentation (9.72 *vs* 4.70%, respectively). This finding fits well with those in a recent study concerning the composition of the post-SSF food waste mixtures (orange pulp, potato residues molasses, brewer's solid wastes, and whey) using *Kluyveromyces marxianus* and *S. cerevisiae* [16]. In the above mentioned study it was stressed the potential of SSF to improve the nutritional value

Table 4
Amount[a] of added-value products obtained from 1 kg fermented[b] orange peel (OP).

	Volatile aroma esters[c] (mg)	Pectin (g)	Total polyphenols (mg equiv GA)	Carotenoids (mg)	L-ascorbic acid (mg)
1 kg fermented peels	253 ± 19	19 ± 2	3009 ± 245	376 ± 38	153 ± 12

[a] Mean value of three independent measurements ± standard deviation.
[b] After 72 h of fermentation process.
[c] After 72 h for isoamyl acetate, phenyl ethyl acetate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate and after 48 h for ethyl hexanoate.

of food industry wastes for animal feed. This was not the case in the non-sterilized process since significantly lower cell population size was measured that coincided with lower sugar consumption (24.76%). However, there were decreases in the amounts of cell wall polysaccharides concentrated in the alcohol-insoluble and EDTA-soluble solids (mainly pectins), indicating degradation capability of indigenous microflora of OP. Microscopic observation revealed the presence of bacterial colonies (rod and cocci) in the non-sterile fermented OP. This result is in line with that reported by Attyia and Ashour [34] indicating the presence of rod and cocci, gram negative and non spore forming bacterial strains and *Streptobacilli* strains in the microflora of fermented OP under natural environmental conditions. On the other hand, microscopic observation revealed that in the fermented sterile OP the colonies were yeasts.

Overall, the better growth performance of yeast cells during SSF of autoclave sterile OP as compared to that on the non-sterile matrix shown here stresses the need limonene to be reduced prior to fermenting the peel waste using *S. cerevisiae*. In this direction, steam explosion has been also proposed as an effective pretreatment for citrus peel waste to remove D-limonene for subsequent enzymatic hydrolysis and sugar fermentation for making ethanol [29,35], anaerobic digestion for methane production [36] and the successful production of succinic acid [37]. Thus, in the next part of our study, autoclave sterile OP was used as a substrate to examine the ability of this strain to synthesize flavour-active compounds *via* SSF under microbially controlled environment.

3.2. Yeast flavour production during SSF of autoclave sterile OP

Our results demonstrate that esters were quantitatively the major group of flavour active compounds produced throughout the SSF of autoclave sterile OP. In total, six esters were found as newly formed compounds in the system, namely isoamyl acetate ("banana"), phenylethyl acetate ("rose-like, honey, fruity" aroma), ethyl hexanoate ("anise, apple-like, strawberry"), ethyl octanoate ("sour apple, pineapple"), ethyl decanoate ("grape-like") and ethyl dodecanoate ("sweet, cream-like"). The results in Fig. 2, revealed that from the quantitative point of view isoamyl acetate, ethyl hexanoate and ethyl dodecanoate were the most important esters. Isoamyl acetate and ethyl dodecanoate showed increased total maximum production of 48.7 and 25.2 mg/kg OP after 72 h of the process, above their corresponding odour thresholds reported in the literature (0.3 and >1.0 mg/L in water, respectively) [38]. In the case of ethyl hexanoate, yeast cells exhibited accelerated synthesis of the above ester reaching 154.2 mg/kg OP at 48 h exceeding its odour threshold (0.005 mg/L). Regarding phenyl ethyl acetate, ethyl octanoate and ethyl decanoate, their maximum concentrations were reached after 72 h and were 4.5, 6.3 and 9.3 mg/kg OP, respectively, being present at levels higher than their corresponding odour thresholds (0.02, 0.07 and 0.50 mg/L). Further incubation of yeast cells resulted in a dramatic decrease in the amount of volatile esters until the final sampling point (120 h) probably due to the activity of the enzymes involved in ester hydrolysis and/or evaporation rates [39]. One of the most important factor affecting the *de novo* synthesis of volatile esters during SSF of OP is the sufficient colonization of yeast on the cellulosic matrix that contains high concentration of substrates for volatile ester synthesis such as glucose and amino acids (*i.e.* leucine and phenylalanine) [40]. Since these molecules come very close to yeast cells, their uptake is expected to be enhanced [33] resulting in improved synthesis of precursors, acetyl-CoA and higher alcohols, as well as of alcohol acetyltransferases encoded by the genes *ATF1* and *ATF2* through the Ras/cAMP/PKA nutrient signalling pathway and the "fermentable growth medium-induced" pathway [41]. The link between available carbon catabolism and medium-chain fatty acid formation *via* the enhancement of fatty acid synthesis may also explain the enhanced synthesis of ethyl esters during SSF of OP. Moreover, oxygen limitation in the micro-environment present in the solid matrix without forced aeration and/or agitation is expected to restrict unsaturated fatty acid synthesis in yeast cells, with a corresponding increase in long-chain saturated fatty acid accumulation. Concurrently, there is an inhibition of acetyl-CoA carboxylase activity resulting in acyl-CoAs release from the cytosolic fatty acid synthase complex and an increase of the intracellular pool of medium-chain fatty acyl-CoAs. The latter can then be converted to the corresponding esters [5].

To examine the exact merits of the above process for flavour production, additional experiments were carried out to highlight qualitative and quantitative differences between flavour-active compounds associated with non-inoculated (spontaneous) and yeast-inoculated fermentation under non-sterile and sterile conditions. As it is depicted in Table 3, native microorganisms present in non-sterile OP did not synthesize flavour compounds except than ethyl decanoate and dodecanoate in very low amounts after 72 h. Also, the yield and productivity of the target compounds differed significantly (except for the case of ethyl decanoate) between sterile and non-sterile conditions of the inoculated fermentation within the optimum time frame provided by the kinetic study (72 h for all the *de novo* synthesized esters and 48 h for ethyl hexanoate). These observations are in line with yeast growth performance (Fig. 1). To address the fact that steam sterilization further enhances manufacturing costs, the main advantage by using this step is that 62% of the limonene content of OP was released and may possibly be recovered by condensation of vapour outlet and sold as a high value co-product. This is a strategy that is followed to improve the economic feasibility of the peel-to-ethanol process with concomitant benefits (a) the improvement of the ability of yeast cells to grow and (b) the reduction of the Volatile Organic Compound emissions that help the citrus processors to meet the federal mandates [42].

By comparing the results of this study with those derived from the SmF of orange peel after dilute acid hydrolysis using Vitilevure MT [7], both qualitative and quantitative differences in the volatile ester profiles were found. Noticeably, ethyl hexanoate and isoamyl acetate formation was enhanced *via* SSF, whereas the synthesis of these compounds was restricted *via* SmF of OP hydrolysate using free yeast cells. It seems that SSF overcomes limitations associated with the reduction of hexanoic acid production by liberated inositol from phytic acid hydrolysis in acid medium by repressing FASI (β-subunit of fatty acid synthetase gene) expression [43] and the loss of amino acids as precursors due to some degree of degradation during hydrolysis. Noticeably, by the SSF, maximum yield and productivity of ethyl hexanoate (Table 3) surpassed the respective values in SmF of OP hydrolysate using immobilized cells by 15 and 23 folds, respectively [7]. Except for the case of phenylethyl acetate, maximum yields of ethyl octanoate, ethyl decanoate and ethyl dodecanoate in fermented OP were comparable to those previous reported in orange peel hydrolysate using either free or immobilized cell system [7] resulting also in comparable productivity values. The above findings in combination with the contribution of the hydrolysis step to the fixed capital investment and manufacturing costs [46] strengthen the suitability of SSF for the exploitation of heat-treated OP in the direction of yeast flavour production.

Additionally to the *de novo*-synthesized flavour-active compounds, the GC/MS analysis revealed that the total amount of terpenic compounds initially present in the matrix decreased (~28%), probably due to their evaporation. D-limonene was found to be the most abundant one. From the industrial point of view, a 3.5 fold increase in the content of α-terpineol in the fermented matrix with regard to that in the raw OP should be pointed out. This observation along with the concomitant disappearance of geraniol strengthens previous findings concerning the biotransformation of monoterpene alcohols by yeast cells [44]. Additionally, according

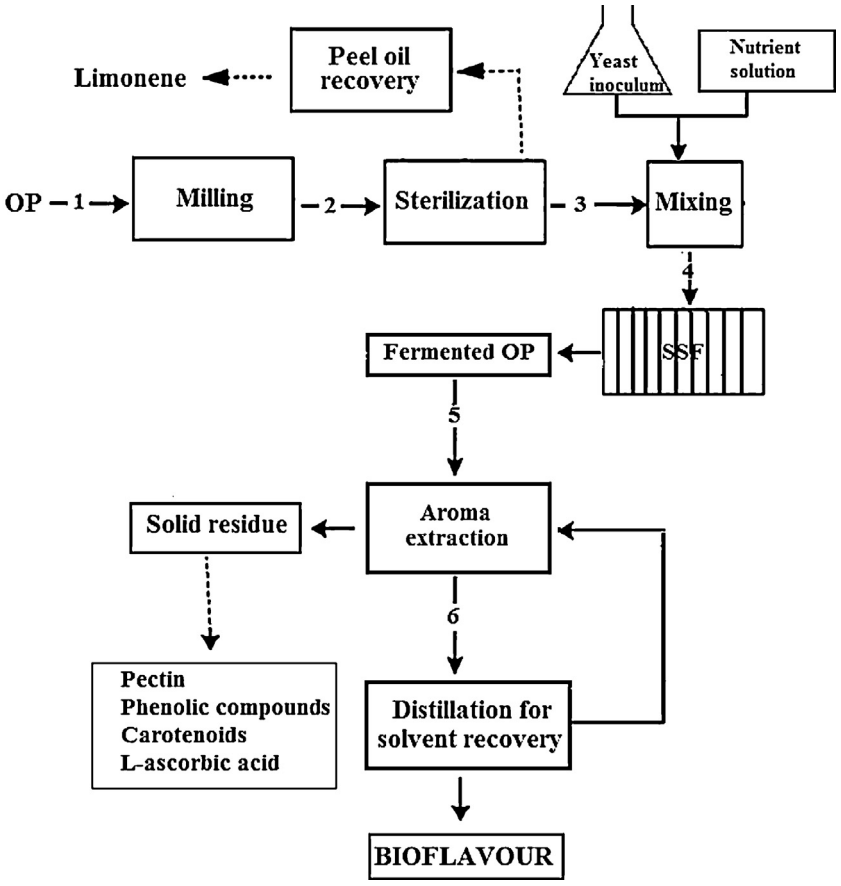

Fig. 3. Process flow diagram for volatile ester production by solid-state fermentation (SSF) of sterile orange peel (OP) using *S. cerevisiae*.

to a recent study [16], enzymatic liberation of glycosidically bound flavour compounds found in the matrix by the activity of yeast β-glucosidase could also contribute to the increase of α-terpineol and α-pinene in the fermented OP.

3.3. Proposed process flow diagram

According to the Agriculture Ministry of Greece, oranges account for 80% of the citrus fruits used for juice making in which only 1/3 of the fruit is exploited and the rest is disposed or sold as waste. The solid wastes of orange juice making (mainly peels and pulp) are estimated about 160,000 tons per year and are sold at a zero to minimum cost for animal feed [45]. In view of the above, the production of value added products such as yeast aroma compounds offers new perspective on the environmentally friendly exploitation of OP waste. In Fig. 3 a proposed flow diagram of the process for yeast flavour production by SSF of OP waste is illustrated. The raw material is milled (1), sterilized (2) and then mixed with inoculum and nutrient solution (3). The mixture is then fed into the SSF bioreactor (4). At the end of the fermentation process, the aroma compounds are separated using solvent-assisted extraction in a unit (5). The solid residue is collected, whilst the extract is supplied to the distillation column (6) for bioflavour isolation and the recovery of solvent for re-use. The proposed valorization route is expected to create an additional source of input with a concomitant decrease in disposal expenses. The technical and economic benefits of the proposed valorization route are expected to increase if recovery of more than one target compound is designed [46,47]. This is the case of limonene from peel oil/water vapour generated during steam pretreatment, offsetting some of the production costs [42]. Long-term commercialization of the proposed process can benefit from the recovery of more value-added products present in the OP such as pectin, phenolic compounds, carotenoids and L-ascorbic acid [48,49,50]. In an attempt to recover the above mentioned compounds from OP before fermentation, each treatment caused some loss of fermentable sugars (glucose, fructose, saccharose) and of crude protein (up to 30% and 50%, respectively), weakening the dynamic of OP for volatile ester synthesis. However, fermented OP could still be used as a source of pectin, phenolic compounds, carotenoids and L-ascorbic acid increasing the potential return for the proposed process. As it is summarized in Table 4, from 1 kg of fermented OP, it was obtained 19 g of pectin, 3 g of polyphenols, 376 mg of carotenoids and 153 mg of L-ascorbic acid. Noticeably, except for the loss of L-ascorbic acid (503 *vs* 153 mg/kg before and after fermentation, respectively), no other losses were observed after the fermentation process.

4. Conclusions

The results of this study point out for the first time the feasibility of SSF of OP waste for the production of yeast volatile esters with "fruity-like" character and of high industrial importance. Compared to previous results obtained in SmF of orange peel hydrolysate, the SSF proved to be very adaptable and did not need any treatment besides sterilization. During SSF the yeast showed good growth performance and increased ability to consume the available nutrients and remain alive over the whole fermentation process. SSF also ensured high yields of volatile aroma esters (total concentration of 250 mg/kg of fermented OP). Our findings seem encouraging towards the direction of scale up in bioreactors that should be carried out together with respective provision of process economics.

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References

[1] IAL Consultants, An overview of the global flavours and fragnances Market, 9th Edition (2013) cited in http://www.ialconsultants.com
[2] EU Regulation No 1334 (2008) Official J EU 354/34.
[3] S. Dubal, Y. Tilkari, S. Momin, I. Borkar, Biotechnological routes in flavour industries, Adv. Biotechnol. 3 (2008) 20–31.
[4] M. Longo, M. Sanromán, Production of food aroma compounds: microbial and enzymatic methodologies, Food Technol. Biotechnol. 44 (2006) 335–353.
[5] S.M.G. Saerens, F.R. Delvaux, K.J. Verstrepen, J.M. Thevelein, Production and biological function of volatile esters in *Saccharomyces cerevisiae*, Microb. Biotechnol. 3 (2010) 165–177.
[6] A. Braga, D.P. Mesquitaa, A.L. Amarala, E.C. Ferreiraa, I. Belo, Aroma production by *Yarrowia lipolytica* in airlift and stirred tank bioreactors: differences in yeast metabolism and morphology, Biochem. Eng. J. 93 (2015) 55–62.
[7] S. Lalou, F. Mantzouridou, A. Paraskevopoulou, B. Bugarski, S. Levic, V. Nedovic, Bioflavour production from orange peel hydrolysate using immobilized *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 97 (2013) 9397–9407.
[8] F. Mantzouridou, A. Paraskevopoulou, Volatile bio-ester production from orange pulp-containing medium using *Saccharomyces cerevisiae*, Food Bioprocess Technol. 6 (2013) 3326–3334.
[9] P. Christen, J.C. Meza, S. Revah, Fruity aroma production in solid state fermentation by *Ceratocystis fimbriata*: influence of the substrate type and the presence of precursors, Mycol. Res. 101 (1997) 911–919.
[10] A. Bramorski, P. Christen, M. Ramirez, C.R. Soccol, S. Revah, Production of volatile compounds by the edible fungus *Rhizopus oryzae* during solid state cultivation on tropical agro-industrial substrates, Biotechnol. Lett. 20 (1998) 359–362.
[11] A. Medeiros, A. Pandey, R.J.S. De Freitas, P. Christen, C.R. Soccol, Optimization of the production of aroma compounds by *Kluyveromyces marxianus* in solid-state fermentation using factorial design and response surface methodology, Biochem. Eng. J. 6 (2000) 33–39.
[12] M. Soares, P. Christen, A. Pandey, C. Soccol, Fruity flavour production by *Ceratocystis fimbriata* grown on coffee husk in solid-state fermentation, Process Biochem. 35 (2000) 857–861.
[13] G. Laufenberg, B. Kunz, M. Nystroem, Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations, Bioresour. Technol. 87 (2003) 167–198.
[14] S.C. Rossi, L.P.S. Vandenberghe, B.M.P. Pereira, F.D. Gago, J.A. Rizzolo, A. Pandey, et al., Improving fruity aroma production by fungi in SSF using citric pulp, Food Res. Int. 42 (2009) 48–484.
[15] J.C. Bicas, J.L. Silva, D.A. Paula, G.M. Pastore, Biotechnological production of bioflavors and functional sugars, Ciênc. Tecnol. Aliment. 30 (2010) 7–18.
[16] T. Aggelopoulos, K. Katsieris, A. Bekatorou, A. Pandey, I.M. Banat, A.A. Koutinas, Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production, Food Chem. 145 (2014) 710–716.
[17] H.H.M. Fadel, M.G. Mahmoud, M.M.S. Asker, S.N. Lotfy, Characterization and evaluation of coconut aroma produced by *Trichoderma viride* EMCC-107 in solid state fermentation on sugarcane bagasse, Electron. J. Biotechnol. 18 (2014) 1–5.
[18] S.G. Dastager, Aroma Compounds, in: P.S. Nigam, A. Pandey (Eds.), Biotechnology for Agro-Industrial-Residues-Utilisation, Springer, US, 2009, pp. 105–127.
[19] R. Singhania, A. Patel, C. Soccol, A. Pandey, Recent advances in solid-state fermentation, Biochem. Eng. J. 44 (2009) 13–18.
[20] P. Nigam, A. Pandey, Solid-state Fermentation Technology for Bioconversion of Biomass and Agricultural Residues, in: P.S. Nigam, A. Pandey (Eds.), Biotechnology for Agro-Industrial-Residues-Utilisation, Springer, US, 2009, pp. 105–127.
[21] A. Pandey, C. Soccol, D. Mitchell, New developments in solid state fermentation: i-bioprocesses and products, Process Biochem. 35 (2000) 1153–1169.
[22] L. Thomas, C. Larroche, A. Pandey, Current developments in solid-state fermentation, Biochem. Eng. J. 81 (2013) 146–161.
[23] S.C. Rossi, L.P.S. Vandenberghe, B.M.P. Pereira, F.D. Gago, J.A. Rizzolo, A. Pandey, C.R. Soccol, A.B.P. Medeiros, Improving fruity aroma production by fungi in SSF using citric pulp, Food Res. Int. 42 (2009) 484–486.
[24] M. Afifi, T.M.A. El-Ghany, A. Abboud, T.M. Taha, K.E. Ghaleb, Biorefinery of industrial potato wastes to ethanol by solid state fermentation, Res. J. Agric. Biol. Sci. 7 (2011) 126–134.
[25] D. Chatanta, C. Attri, K. Gopal, M. Devi, G. Gupta, T. Bhalla, Bioethanol production from apple pomace left after juice extraction, Internet J. Microbiol. 5 (2007) 5.
[26] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[27] T. Gutfinger, Polyphenols in olive oils, J. Am. Oil Chem. Soc. 58 (1980) 966–968.
[28] AOAC official method 967.21, Ascorbic acid in vitamin preparations and juices, 2,6-dichloroindophenol titrimetric method AOAC official method of analysis, 45.1.14, (2005).
[29] M.R. Wilkins, W.W. Widmer, K. Grohmann, Simultaneous saccharification and fermentation of citrus peel waste by *Saccharomyces cerevisiae* to produce ethanol, Process Biochem. 42 (2007) 1614–1619.
[30] M. Pourbafrani, F. Talebnia, C. Niklasson, Protective effect of encapsulation in fermentation of limonene-contained media and orange peel hydrolyzate, Int. J. Mol. Sci. 8 (2007) 777–787.
[31] M.R. Wilkins, L. Suryawati, N.O. Maness, D. Chrz, Ethanol production by *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in the presence of orange-peel oil, World J. Microbiol. Biotechnol. 23 (2007) 1161–1168.
[32] S. Plessas, A. Bekatorou, A.A. Koutinas, M. Soupioni, I.M. Banat, R. Marchant, Use of *Saccharomyces cerevisiae* cells immobilized on orange peel as biocatalyst for alcoholic fermentation, Bioresour. Technol. 98 (2007) 860–865.
[33] A. Koutinas, V. Sypsas, P. Kandylis, A. Michelis, A. Bekatorou, Y. Kourkoutas, C. Kordulis, A. Lycourghiotis, I.M. Banat, P. Nigam, R. Marchant, M. Giannouli, P. Yianoulis, Nano-tubular cellulose for bioprocess technology development, PLoS One 7 (2012) e34350, http://dx.doi.org/10.1371/journal.pone.0034350.
[34] S. Attyia, S. Ashour, Biodegradation of agro-industrial orange waste under solid state fermentation and natural environmental conditions, Egypt J. Biol. 4 (2002) 23–30.
[35] W. Widmer, W. Zhou, K. Grohmann, Pretreatment effects on orange processing waste for making ethanol by simultaneous saccharification and fermentation, Bioresour. Technol. 101 (2010) 5242–5249.
[36] G. Forgács, M. Pourbafrani, C. Niklasson, M.J. Taherzadeh, I.S. Hováth, Methane production from citrus wastes: process development and cost estimation, J. Chem. Technol. Biotechnol. 87 (2012) 250–255.
[37] E. Uckun Kiran, A.P. Trzcinski, Y. Liu, Platform chemical production from food wastes using a biorefinery concept, J. Chem. Technol. Biotechnol. (2014), http://dx.doi.org/10.1002/jctb.4551.
[38] T. Cserháti, Chromatography of Aroma Compounds and Fragrances, Springer-Verlag, Berlin Heidelberg, 2010.
[39] S.M.G. Saerens, F. Delvaux, K.J. Verstrepen, P. Van Dijck, J.M. Thevelein, F.R. Delvaux, Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation, Appl. Environ. Microbiol. 74 (2008) 454–461.
[40] V.A. Bampidis, P.H. Robinson, Citrus by-products as ruminant feeds: a review, Anim. Feed Sci. Technol. 128 (2006) 175–217.
[41] K.J. Verstrepen, S.D.M. Van Laere, B.M.P. Vanderhaegen, G. Derdelinckx, J. Dufour, I.S. Pretorius, J. Winderickx, J.M. Thevelein, F.R. Delvaux, Expression levels of the yeast alcohol and ATF2 control the formation of a broad range of volatile esters, Appl. Environ. Microbiol. 69 (2003) 5228–5237.
[42] W. Zhou, W. Widmer, K. Grohman, Economic analysis of ethanol production from citrus peel waste, Proc. Fla. State Hortic. Soc. 120 (2007) 310–315.
[43] K. Furukawa, T. Yamada, H. Mizoguchi, S. Hara, Increased ethyl caproate production by inositol limitation in *Saccharomyces cerevisiae*, J. Biosci. Bioeng. 95 (2003) 448–454.
[44] A. King, J.R. Dickinson, Biotransformation of monoterpene alcohols by *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis*, Yeast 16 (2000) 499–506.
[45] K.I. Israelidis, Exploitation of solid argoindustrial wastes in: Report for National Agricultural Research Foundation, 2013.
[46] M. Lohrasbi, M. Pourbafrani, C. Niklasson, M.J. Taherzadeh, Process design and economic analysis of a citrus waste biorefinery with biofuels and limonene as products, Bioresour. Technol. 101 (2010) 7382–7388.
[47] K. Grohman, R. Cameron, Y. Kim, W. Widmer, G. Luzio, Extraction and recovery of pectic fragments from citrus processing waste for co-production with ethanol, J. Chem. Technol. Biotechnol. 88 (2013) 395–407.
[48] G. Aravantinos-Zafiris, V. Oreopoulou, C. Tzia, C.D. Thomopoulos, Utilisation of orange by-products-orange peel carotenoids, J. Sci. Food Agric. 59 (1992) 77–79.
[49] R.C. Rivas-Cantu, K.D. Jones, P.L. Mills, A citrus waste-based biorefinery as a source of renewable energy: technical advances and analysis of engineering challenges, Waste Manage. Res. 31 (2013) 413–420.
[50] M. Boukroufa, C. Boutekedjiret, L. Petigny, N. Rakotomanomana, F. Chemat, Bio-refinery of orange peels waste: a new concept based on integrated green and solvent free extraction processes using ultrasound and microwave techniques to obtain essential oil, polyphenols and pectin, Ultrason. Sonochem. 24 (2015) 72–79.