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Production of flavor compounds from olive mill waste by *Rhizopus oryzae* and *Candida tropicalis*

Onur Guneser[a,*], *Asli Demirkol*[b], *Yonca Karagul Yuceer*[b], *Sine Ozmen Togay*[c], *Muge Isleten Hosoglu*[b], *Murat Elibol*[d]

[a] *Usak University, Engineering Faculty, Department of Food Engineering, Usak, Turkey*
[b] *Canakkale Onsekiz Mart University, Engineering Faculty, Department of Food Engineering, Canakkale, Turkey*
[c] *Uludag University, Agricultural Faculty, Department of Food Engineering, Bursa, Turkey*
[d] *Ege University, Engineering Faculty, Department of Bioengineering, Izmir, Turkey*

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ABSTRACT

The purpose of this study was to investigate the production of flavor compounds from olive mill waste by microbial fermentation of *Rhizopus oryzae* and *Candida tropicalis*. Olive mill waste fermentations were performed in shake and bioreactor cultures. Production of flavor compounds from olive mill waste was followed by Gas Chromatography–Mass spectrometry, Gas chromatography- olfactometry and *Spectrum Sensory Analysis*®. As a result, 1.73-log and 3.23-log cfu/mL increases were observed in the microbial populations of *R. oryzae* and *C. tropicalis* during shake cultures, respectively. *C. tropicalis* can produce a higher concentration of D-limonene from olive mill waste than *R. oryzae* in shake cultures. The concentration of D-limonene was determined as 185.56 and 249.54 μg/kg in the fermented olive mill waste by *R. oryzae and C. tropicalis* in shake cultures respectively. In contrast, *R. oryzae* can produce a higher concentration of D-limonene (87.73 μg/kg) D-limonene than *C. tropicalis* (11.95 μg/kg) in bioreactor cultures. Based on sensory analysis, unripe olive, wet towel, sweet aromatic, fermented aromas were determined at high intensity in olive mill waste fermented with *R. oryzae* meanwhile olive mill waste fermented with *C. tropicalis* had only a high intensity of unripe olive and oily aroma.

Introduction

The food and agricultural industries produce annually million tons of waste, resulting from production and consumption of food, peels, pulps, aqueous residues and others, many of which raise serious disposal issues and, consequently, considerable costs to various industries.[1] Therefore, using agro-wastes is the most popular aspect in biotechnological processes for production of high value-added products in terms of the reducing production cost.[2,3] Recently, agro-wastes have been focused in biotechnological flavor production by using microbial fermentation or biotransformation owing to their high amount of reusable components

* *Corresponding author.*
E-mail: onur.guneser@usak.edu.tr (O. Guneser).
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for microorganisms.[1,4–6] Several studies were conducted on the production of natural flavor compounds from agro-wastes by microbial fermentation. Cassava bagasse, sugar beet, beet molasses, coffee husk, soy bean waste, apple pomace, cacao bagasse were extensively used in these studies for the production of natural flavors by yeast or molds.[7–11] For instance, de Oliveira et al.[12] reported that 2-phenylethanol was produced from cassava wastewater by *Saccharomyces cerevsiae*, *Geotrichum fragrans* and *Kluveromyces marxianus* in the following yields order: 0.74 g/L, 0.19 g/L and 0.08 g/L. Zheng et al.[13] produced vanillin from waste residue of rice bran oil by both strains of *Aspergillus niger* CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115. In the more recently, Wilkowska et al.[14] showed that the production of esters and alcohols including ethyl acetate, isoamyl acetate, isoamyl alcohol and 2-phenylethanol from apple pomace with chokeberry and cranberry pomaces can be achieved by immobilized *K. marxianus* LOCK0024 in shake culture. Fadel et al.[15] reported that a high concentration of 6-pentyl-α-pyrone (3.62 mg/g DM) associated with coconut aroma can be produced from a sugarcane bagasse by using *T. viride* EMCC-107. Olive mill waste (OMW) and olive mill waste water (OMWW) are the most important wastes for olive oil industry in Mediterranean regions. One to 2.5 million tons was produced annually during olive oil season in Andalusia region (Spain)[16] while 200–250 thousand tons of OMW are produced in Turkey.[17] OMW is a solid phase by-product resulting from extraction of olive oil by pressure or centrifugation. OMW approximately has 25–55% water; 25–50% of fiber with a great degree of lignifications, 5–8% of residual oil, 2–6% of ash and 6–10% of nitrogen associated with the insoluble fiber fraction.[18–20] Microbial fermentation has been taken over by many researchers due to possibility of valorization of agro-wastes, low-cost production steps and the possibility of using several types of microorganisms.[21,22] From this perspective, utilization of the filamentous fungi such as *Rhizopus* species and the thermo and ethanol tolerance of *Candida* species have been widely examined in bioenergy and bioproduct industries.[23–25] When we take into the consideration the nutritional content of OMW for microbial growth, OMW might be used as raw material in the production of flavor compounds by both microorganisms via microbial fermentation. Therefore, this study focuses to investigate the production of natural flavor compounds from OMW by microbial fermentation of *Rhizopus oryzae* and *Candida tropicalis*.

Material and methods

Microorganisms and inoculum preparation

Strains of *Rhizopus oryzae* NRLL 395 and *Candida tropicalis* ATCC 665 were obtained from Department of Bioengineering, Ege University (Izmir, Turkey). Both microorganisms were grown on slant Potato Dextrose Agar (PDA) in Petri plate at 30 °C for 7 days. Then, *R. oryzae* spores and *C. tropicalis* cells were collected by washing with 0.1% (w/v) Tween 80 from agar surface, separately. The spore suspension of *R. oryzae* was filtered through two layers cheesecloth and centrifuged at 3000 rpm for 5 min. The cell suspension of *C. tropicalis* was centrifuged at 3000 rpm for 5 min. Both microbial suspensions were counted with Thoma counting chamber by using light microscope.[7,26] The suspensions contain 10^{7-8} spores or cells/mL for *R. oryzae* and *C. tropicalis*.

Experiments of shake cultures and bioreactor cultures

Initially, 200 g of OMW were weighed and grinded by using knife mill Restch GM 200 (Haan, Germany) for 15 min. Then, 2 L of OMW solution (10%, w/v) was prepared for shake cultures. The OMW solution was homogenized at 24,000 rpm by Ultraturax (IKA-WERKE GmbH, Germany). The solution was divided into six groups (300 mL) and initial pH was adjusted to 5.0, for *R. oryzae* and pH 7.0, for *C. tropicalis* by using 1 N HCl and 1 N NaOH. Initial pHs were selected based on previous studies and these pHs are optimum for microbial growth.[27,28] 30 mL of OMW solution was poured into 100 mL Erlenmeyer and tops were closed with cotton wool and aluminum foil. The OMW solutions were sterilized in an autoclave (Hirayama, Saitama, Japan) at 121 °C for 15 min and then inoculated with *C. tropicalis* and *R. oryzae* at a level of 10^{7-8} cell or spores/mL OMW suspension. The flasks then were incubated at 120 rpm for 288 h at 30 °C in a rotary incubator (Sartorius-Certomat IS, Goettingen-Germany). The control groups without microorganism were prepared by following the same procedure. Duplicate samples were prepared for each treatment.

Bioreactor cultures were conducted in a 5 L stirred tank bioreactor (STR) (Biostat A-plus®, Sartorius, Melsungen, Germany) with 4 L working volume. Fermentation conditions used for microbial growth and flavor production were based on the results obtained in shake cultures. The STR was equipped with two six-blade impellers, pH probe (Hamilton, Easyferm K8/325) and PT 100 temperature sensor. The aeration rate, agitation speed and temperature for both microbial cultures were set as 0.325 vvm, 120 rpm and 30 °C respectively.

Specific growth rate and microbial count

Spore count of *R. oryzae* and cell count of *C. tropicalis* during fermentation were determined by pour plate technique with Potato Dextrose agar (PDA).[26] The sample was taken from shake flask and STR intermittently in aseptic conditions.

Analysis of flavor compounds

Flavor compounds from fermented OMW were determined by gas chromatography–olfactometry (GCO), gas chromatography–mass spectrometry (GC–MS) and sensory analysis.

Extraction of flavor compounds

Flavor compounds in fermented and unfermented OMW were extracted by solid-phase microextraction (SPME).[29] Three grams of OMW were weighed in a 40 mL amber colored screw top vial with hole cap PTFE/silicon septa (Supelco, Bellafonte, USA) to which 1 g of NaCl was added to the vial. The vial was kept at 40 °C in a water bath (GFL, Grossburgwedel, Germany) for 20 min to equilibrate the volatiles in headspace. Then, a SPME (2 cm to 50/30 μm DVB/Carboxen/PDMS, Supelco, Bellafonte) needle was inserted into the vial. The SPME fiber was

exposed at a depth of 2 cm in the headspace of the vial for 20 min at 40 °C in water bath. Then, the sample was injected into GC–MS, immediately.[30]

Gas chromatography olfactometry (GCO) analysis

The GCO analysis was performed for 5-days fermented OMW to determine the changes in flavor profile. The GCO was conducted by HP 6890 GC (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector (FID), a sniffing port and splitless injector system. A nonpolar column (HP-5 30 m length, 0.32 mm i.d., 0.25 μm df; J&W Scientific) was used for sniffing. Column effluent was split 1:1 between FID and olfactory port using deactivated fused silica capillaries (90 cm length, 0.25 mm i.d.). Helium was used as the carrier gas. Inlet pressure was 7.07 psi, and flow 1.2 mL/min. The GC oven temperature was programmed from 40 to 230 °C at a rate of 10 °C/min, with initial hold of 5 min and final hold time of 20 min. The FID and sniffing port were maintained at the temperatures of 250 °C and 200 °C, respectively. GCO procedure was duplicated.[30] Post-peak intensity method was used for the determination of aroma intensity by using 10-point scale anchored to the left with 'not' and to the right with 'very'.[31] Sniffer had 100 h of experience with GCO technique, scale using and odor description. Aroma-active compounds were identified by comparing retention indices (RI) and odor quality of unknowns with those of references analyzed at the same experimental conditions by sniffer during GCO procedure. Retention indices were calculated using n-alkane series.[32]

Identification and quantification of flavor compounds

Flavor compounds were determined by gas chromatography–mass spectrometry (GC–MS). Nonpolar HP5 MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, Folsom, CA) was used for separation of flavor compounds. The GC–MS system consisted of an HP 6890 GC and 7895C mass selective detector (Agilent Technologies, Wilmington, DE, USA). The oven temperature was programmed from 40 °C to 230 °C at a rate of 10 °C/min with initial and final hold times of 5 and 20 min, respectively. Helium was used as the carrier gas with a constant flow of 1.2 mL/min. The Mass Spectrometry Detector (MSD) conditions were as follows: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 35–350 amu; scan rate, 4.45 scan/s.[30] Identification of the flavor compounds was based on the comparison of the mass spectra of unknown compounds with those in the National Institute of Standards and Technology[33] and Wiley Registry of Mass Spectral Data, 7th Edition[34] mass spectral databases. Quantification of flavor compounds was expressed as relative abundances of flavor compounds by Eq. (1).[35] 2-Methyl pentanoic acid and 2-methyl-3-heptanone were used as an internal standard (IS) for acidic and neutral-basic compounds, respectively.

$$\text{Mean relative abundance } (\mu g/kg) = \frac{\text{concentration of IS} \times \text{peak area of compound}}{\text{peak area of the IS}} \quad (1)$$

Sensory analysis

A roundtable discussion was conducted to determine descriptive sensory properties and changes in aroma profiles of fermented OMW versus control samples (unfermented OMW) for shake cultures.[36] Panelists were staff and graduate students in the Department of Food Engineering at Çanakkale Onsekiz Mart University. Four female and three male participated for sensory panel. Their ages ranged from 24 to 45 years. The panel received about 300 h of training during generation and definition of descriptive terms. Panelists quantified the attributes using 15-point product-specific scale anchored to the left with 'not' and on the right with 'very'.[36]

Statistical analysis

Analysis of variance (*ANOVA*) was conducted to determine the differences in the amount of flavor compounds during fermentation time in shake cultures and bioreactor cultures. *ANOVA* model[37] is shown in Eq. (2).

$$Y_{ij} = \mu + \alpha_i + e_{ij} \quad (2)$$

where Y_{ij} is the jth observation value in the ith fermentation time, μ is the general population mean, α_i is the effect of the ith fermentation, and e_{ij} represents the random error term. Tukey's Honestly significant difference (HSD) test was used for separating means; SPSS for Windows (version 15.0) was used for all statistical analyses.

Results and discussion

Microbial growth of Rhizopus oryzae and Candida tropicalis in OMW

The microbial growth of *R. oryzae* and *C. tropicalis* in OMW during shakecultures and bioreactor cultures were shown in Table 1. Maximum increase in microbial population of *R. oryzae* and *C. tropicalis* were determined as 1.73 log cfu/mL (1.29 fold) and 3.23 log cfu/mL (1.52 fold), respectively. The growths of *R. oryzae* and *C. tropicalis* came into stationary phase around 72 h in shake cultures. In stationary phase, microbial populations of *R. oryzae* and *C. tropicalis* were around 6.5 log cfu/mL and 9.0 log cfu/mL, respectively. In bioreactor cultures, it was observed that both microorganisms were attained the exponential growth phase within 24 h. The microbial population of *R. oryzae* increased around 3 log cfu/mL (3 fold) through 288 h of fermentation whereas *C. tropicalis* populations' increased 1.4 log cfu/mL (1.22 fold) at the same fermentation time (Table 1).

In the literature, there are several studies on the growth behavior of *R. oryzae* and *C. tropicalis* in solid state and submerged fermentation,[38–40] and most of researchers have revealed different results for growth behavior and biomass increase for *R. oryzae* and *C. tropicalis* on certain agrowastes. The obtained data about the microbial growth of both *R. oryzae* and *C. tropicalis* in OMW showed both microorganisms growths were different for shake cultures and bioreactor cultures unexpectedly. These differences could be attributed to

Table 1 – Growth of *R. oryzae* and *C. torpicalis* in OMW during shake cultures.

Fermentation time (h)	Microbial count ± S.E. (log cfu/mL OMW solution)	
	Shake cultures	
	R. oryzae	*C. tropicalis*
0	0.92 ± 0.08	6.15 ± 0.14
24	6.76 ± 0.65	7.97 ± 1.13
48	7.65 ± 0.01	7.61 ± 0.01
72	6.68 ± 0.01	9.21 ± 2.25
120	6.83 ± 0.04	9.38 ± 0.01
168	6.69 ± 0.01	9.05 ± 0.14
288	6.84 ± 0.03	9.28 ± 0.01
Maximum growth (log cfu/mL)	1.73	3.23
Fermentation time (h)	Bioreactor cultures	
	R. oryzae	*C. tropicalis*
0	3.47 ± 0.16	6.35 ± 0.01
24	5.0 ± 0.01	7.30 ± 0.01
48	4.76 ± 0.07	7.72 ± 0.11
72	4.57 ± 0.07	7.69 ± 0.01
96	4.72 ± 0.11	7.23 ± 0.01
120	–[a]	7.15 ± 0.14
180	5.04 ± 0.07	–[a]
288	6.47 ± 0.07	7.75 ± 0.14
Maximum growth (log cfu/mL)	3.0	1.4

[a] Microbial analysis could not conducted for this fermentation time. S.E.: standard error, cfu: colony forming unit.

the interaction of microorganism with OMW nutrients in fermentation conditions and strain of microorganism.[2] In our previous study[41] on OMW, we observed the microbial growth of *Trichoderma atroviride* in shake cultures was higher than bioreactor cultures, whereas the yeast *Torulaspora delbrueckii* acts adverse growth behavior from *T. atroviride* at the same conditions. Jin et al.[42] investigated the growth of different *Rhizopus* strains in potato, corn, wheat starch and pineapple processing waste water streams for lactic acid production. Similar to our results, an increase in fungal biomass was found to be about 2 and 4.5 fold for *R. oryzae* and *R. arrhizus* in the fermentation of all studied agrowaste. The researchers also indicated that sharp increase in biomass formation of *R. oryzae* 2062 was higher than *R. arrhizus* 36017 at 30 °C and 150 rpm in all agrowastes. Saraçoğlu and Çavuşoğlu[43] found that biomass of *C. tropicalis*' Kuen 1022 in sunflower hull hydrolysate medium (66 g/L) increased in 6–7 fold until 20 h fermentation at 30 °C; stirring at 140 rpm. Oberoi et al.[44] investigated enhanced ethanol production via fermentation of rice straw hydrolysate by *C. tropicalis* ATCC 13803 which adapted and non-adapted for a rice straw hydrolysate medium. They found that the biomass of adapted and non-adapted *C. tropicalis* ATCC 13803 increased about 3 and 2 folds during 6–18 h fermentation at 35 °C and 120 rpm, respectively and both microorganisms biomass remained stationary during 18–24 h of the fermentation.

Flavor production characteristics by Rhizopus oryzae and Candida tropicalis in OMW

Aroma active compounds of 5 days (~135 h) fermented and unfermented OMW were shown in Table 2.

In total, 17 and 13 aroma active compounds were identified in fermented OMW by *R. oryzae and C. tropicalis* respectively. Identified aroma-active compounds included acids, alcohols, aldehydes, esters, ketones, terpene, and 4 unknown compounds. Among these aroma-active compounds, unknown 1 associated with dirty-acid aroma, isovaleric acid, hexyl acetate methional, (*E*)-2-nonenal and 2,4-nonadienal were detected at higher intensities in unfermented OMW. Moreover, it was determined that there were some differences in aroma profile of unfermented OMW. These differences might be related in pH value of the OMV solution; absorption behavior of SPME fiber and sensitivity of perception of panelists during GC-O analysis

OMW fermented with *R. oryzae* had higher intensities of 2-pentanone, D-limonene and 2-phenylethanol than unfermented OMW whereas D-limonene was only found to be at higher intensity in OMW fermented with *C. tropicalis*. These GCO results revealed that 2-pentanone, D-limonene and 2-phenylethanol can be produced from fermented OMW.

Limonene is monoterpen which is one of the main compounds of citrus essential oils and 2-phenylethanol is aromatic alcohol with rose like flavor. D-Limonene and 2-phenylethanol can be found in many plant sources. Both flavor compounds are greatly used in food, perfume and cosmetic industry. 2-Phenylethanol and D-limonene are naturally produced by distillation of citrus peel and rose petals, respectively. From aspect of yeast and fungal metabolism, there are many biochemical pathways for flavor compounds. Among these pathways, production of alcohols and ester type flavor compounds was achieved by yeast via and *Acetyl Coenzyme A/Alcohol Acetyl Transferase* reaction and *Ehrlich* pathway which covered transamination, decarboxylation, oxidation

Table 2 – Aroma-active compounds of unfermented and fermented OMW with *R. oryzae* and *C. tropicalis* ($n = 2$).

RI[a]	Volatile compound	Aroma quality	Identification methods	Aroma intensity[b] (mean ± SE)			
				Control	*R. oryzae*	Control	*C. tropicalis*
598	Diacetyl	Butter	RI,O	1.25 ± 0.25	0.60 ± 0.10	4.75 ± 0.75	4.50 ± 1.50
734	**2-Pentanone**	**Oily**	**RI,MS,O**	**ND**	**1.50 ± 0.50**	**ND**	**ND**
802	Hexanal	Green grass	RI,MS,O	3.0 ± 1.0	ND	2.0 ± 0.10	ND
856	Unknown 1	Dirty, acid	RI,O	5.50 ± 0.50	5.25 ± 1.25	0.75 ± 0.75	ND
862	Isovaleric acid	Sour, fruity	RI,O	5.50 ± 0.50	4.75 ± 0.25	1.75 ± 0.25	1.50 ± 1.50
869	Sytrene	Acid, dirty	RI,MS,O	ND	ND	2.75 ± 0.25	ND
898	Methional	Boiled potato	RI,O	4.50 ± 0.50	ND	4.50 ± 1.50	3.75 ± 0.25
928	2-Acetyl-1-pyroline	Popcorn	RI,O	ND	0.50 ± 0.50	ND	ND
975	Unknown 2	Metallic	RI,O	5.75 ± 0.25	3.0 ± 0.10	4.50 ± 1.50	3.75 ± 0.25
999	Hexyl acetate	Cologne	RI,O	4.50 ± 0.50	0.75 ± 0.25	1.50 ± 0.50	0.75 ± 0.75
1040	**D-Limonene**	**Citrus**	**RI,MS,O**	**1.75 ± 0.25**	**3.00 ± 1.0**	**1.5 ± 0.50.**	**2.50 ± 0.10**
1050	Benzeneacetaldehyde	Rose, flower	RI,MS,O	2.50 ± 0.50	2.0 ± 2.0	ND	ND
1058	Unknown 3	Vegetable oil	RI,O	2.0 ± 1.0	2.0 ± 0.10	ND	ND
1077	o-Cresol	Wet towel	RI,MS,O	1.0 ± 1.0	1.50 ± 0.50	3.0 ± 1.0	2.25 ± 0.25
1095	Guaiacol	Burn sugar	RI,MS,O	ND	ND	3.50 ± 1.50	ND
1120	Unknown 4	Sour	RI,O	3.0 ± 0.10	0.50 ± 0.50	ND	ND
1138	(*E*)-2-Nonenal	Hay	RI,MS,O	5.0 ± 0.10	2.0 ± 1.0	2.50 ± 0.50	ND
1144	**2-Phenylethanol**	**Rose**	**RI,MS,O**	**2.0 ± 0.10**	**3.0 ± 0.10**	**1.0 ± 0.50**	**1.75 ± 0.25**
1157	(*Z*)-2-Nonenal	Cucumber	RI,MS,O	2.0 ± 2.0	1.0 ± 0.10	2.50 ± 0.50	ND
1158	(*E,Z*)-2,6-Nonadien-1-ol	Hay	RI,O	3.50 ± 0.50	ND	2.75 ± 0.75	1.0 ± 1.0
1186	Naphthalene	Dirty	RI,MS,O	ND	ND	0.75 ± 0.75	1.0 ± 1.0
1191	Carveol	Minty	RI,MS,O	0.50 ± 0.50	ND	ND	ND
1216	2,4-Nonadienal	Burnt oil	RI,MS,O	5.75 ± 0.25	1.50 ± 0.50	3.25 ± 0.75	2.0 ± 2.0
1268	Geraniol	Sweet, flower	RI,O	0.50 ± 0.50	1.0 ± 1.0	2.0 ± 0.10	1.0 ± 1.0
1322	(*E,E*)-2,4-Decadienal	Burnt oil	RI,MS,O	ND	ND	4.50 ± 0.50	1.0 ± 0.50
1372	α-Cubebene	Sweet	RI,MS,O	3.0 ± 1.0	ND	ND	ND

Intensity of aroma compounds marked bold increased in fermented OMW.

[a] RI: Retention indices based on HP-5 column.

[b] Aroma intensity in 5 days (~135 h) fermented and unfermented OMW. SE, standard error; ND, not detected; MS, mass spectrometry; O, odor.

and reduction of branched chain amino acids. Moreover, some methyl ketones and lactones were produced by β-oxidation of long-chain hydroxy fatty acids (e.g. ricinoleic acid) by yeast and mold. It was also known that several fungi produced most of the terpene by the *Mevalonate* pathway as found in higher plant and biotransformation reactions.[45–49] Therefore, it was concluded that 2-pentanone, D-limonene and 2-phenylethanol from OMW were produced by *R. oryzae* and *C. tropicalis* through aforementioned reactions. Several researchers have been pointed out similar approaches for the production of flavor compounds from agrowaste.[7,11,41,50] In most recently, Mantzouridou and Paraskevopoulou[50] demonstrated de novo synthesis of fruity esters as isoamyl acetate, ethyl dodecanoate, decanoate, octanoate and phenyl ethyl acetate from orange peel waste was achieved by *Saccharomyces cerevisiae* at high level. In our previous study[41] on OMW, we observed that the filamentous fungus *Trichoderma atroviride* produces 1-octen-3-ol and 2-octenol at high level, and 2-phenylethanol and menthol also can be produced by using *Torulaspora delbrueckii*.

Christen et al.[7] indicated that *R. oryzae* can produce acetaldehyde, ethanol (sweet), 1-propanol, ethyl acetate, ethyl propionate and 3-methyl butanol from Amaranth grain supplemented with mineral salt solution. In a study by Chatterjee and Bhattacharyya,[51] production of α-terpineol (floral) from α-pinene (herbal) by microbial oxidation of *C. tropicalis* MTCC 230 with a yield of 77% was achieved. We did not observe the production of acetaldehyde, ethanol, 1-propanol, ethyl acetate, ethyl propionate and 3-methyl butanol from OMW by fermentation of *R. oryzae* and α-terpineol by *C. tropicalis* by compared with the findings of previous studies.[7,51] This could be related to composition of agrowaste and fermentation conditions including aeration, temperature and fermentation scale.[52]

In fermented OMW, methoxy phenyl oxime and methyl butanoate could not be identified by GCO analysis, but we determined these compounds by GC–MS. These results can be attributed to "*odor threshold*" and "*odor recognition threshold*". Because, the concentration of volatile compounds in matrix has to be higher than both threshold values in order to identify the volatile compound by GCO technique.[53] Methoxy phenyl oxime is N-containing compound with both phenyl and methoxy groups. There is little information on flavor characteristics of methoxy phenyl oxime.[54] Some researchers identified it as contaminant come from SPME fiber. They point out that is originated from the glue that is used for SPME fiber.[55] However, the compound has been found naturally in some food products,[56–58] especially bamboo shoots and secondary metabolites of myxobacteria[59] by some researchers. Methyl butanoate which is associated fruity flavor is the ester of butyric acid. Likewise most of ester compounds, it was biosynthesized with *Ehrlich* pathway and reactions of lipase enzyme by microorganism.

Table 3 – Changes in concentration of flavor compounds in OMW during shake cultures of *R. oryzae* and *C. topicalis*.

Volatile compound	RI[a]	Aroma quality	Mean (μg/kg OMW)[b] ± SE		
			Rhizopus oryzae		
			Control[c]	72 h	288 h
2-Pentanone	682	Sweet	12.29 ± 8.72	14.16 ± 10.04	24.28 ± 3.30
Methoxyphenyloxime	926	–	61.74 ± 29.17	64.95 ± 29.73	92.26 ± 3.32
D-Limonene	1032	Lemon, citrus	40.13 ± 1.05[B]	185.56 ± 17.47[A]	17.22 ± 1.60[B]
2-Phenylethanol	1118	Rose	1.35 ± 0.96[B]	13.91 ± 6.57[A]	11.71 ± 8.30[A]
Volatile compound	RI[a]	Aroma quality	Mean (μg/kg OMW)[b] ± SE		
			Candida tropicalis		
			Control[c]	72 h	288 h
Methyl butanoate	717	Fruity	Nd	4.67 ± 0.20[A]	0.40 ± 0.20[B]
D-Limonene	1032	Lemon, citrus	119.64 ± 77.04[AB]	249.54 ± 30.82[A]	8.93 ± 6.26[B]

[A,B] Means followed by different superscript letter represent significant differences in the same flavor compound through the fermentation time.
[a] Retention index based on HP 5MS column.
[b] Mean relative abundance = (concentration of internal standard × peak area of compound)/(peak area of the internal standard).
[c] 72 h incubation without *R. oryzae* or *C. tropicalis*, Nd: not detected, SE: standard error.

Table 3 shows the changes in concentration of flavor compounds produced by *R. oryzae* and *C. tropicalis* from OMW during shake cultures.

Fermentation time had a significant effect on the concentration of volatile compounds which were produced by both microorganisms ($p < 0.05$). It was determined that concentrations of D-limonene (citrus) and 2-phenylethanol (rose) significantly increased in OMW during 72 h of fermentation of *R. oryzae*. The maximum concentrations of D-limonene and 2-phenylethanol were determined as 185.56 and 13.91 μg/kg OMW, respectively. The concentration of 2-petanone and methoxy phenyl oxime gradually increased during fermentation of *R. oryzae*. However, no significant changes were determined in the concentration of both compounds (Table 3). It was thought that this observation is related to a high standard error of results for the compounds. During the fermentation of *C. tropicalis*, the concentrations of D-limonene and methyl butanoate (fruity) significantly increased similar to the fermentation of *R. oryzae*. The maximum concentration of D-limonene and methyl butanoate was 249.54 and 4.67 μg/kg OMW at 72 h of fermentation. The concentrations of flavor compounds produced by both microorganisms decreased after 72 h fermentation.

Fig. 1 shows changes in concentration of flavor compounds produced from in OMW by *R. oryzae* and *C. tropicalis* in bioreactor cultures.

In bioreactor cultures, fermentation time had also significant effect on the amount of flavor compounds in batch fermentation ($p < 0.05$). As seen from Fig. 1A, the concentration of 2-pentanone and methoxy phenyl oxime increased in OMW during 72 h of fermentation by *R. oryzae*, while the concentration of D-limonene and 2-phenylethanol increased during 180 h of fermentation. After these times, a significant decrease in the concentration of all flavor compounds was observed throughout the fermentation ($p < 0.05$). The maximum concentration of 2-pentanone, methoxy phenyl oxime, D-limonene and 2-phenylethanol were 19.46, 34.44, 87.73 and 11.25 μg/kg OMW, respectively. In the case of fermentation by *C. tropicalis* (Fig. 1B), concentration of methyl butanoate and D-limonene were reached at maximum level in the fermentation time of 72 h and 180 h and the maximum concentration of these compounds were determined as 7.26 and 11.95 μg/kg OMW, respectively. Moreover, a significant decrease was also observed in the amounts of all volatile compounds produced by *C. tropicalis* throughout the fermentation similar to the fermentation of *R. oryzae* ($p < 0.05$).

Mantzouridou and Paraskevopoulou[50] investigated the usage potential of orange pulp for microbial flavor production by *S. cerevisiae* in under semi-anaerobic and micro aerobic conditions. The researchers observed that acetate esters as isoamyl acetate and phenyl ethyl acetate concentrations in culture media decreased after 48 h the fermentation time in microaerobic conditions whereas concentrations of ethyl hexanoate, ethyl octanoate and ethyl decanoate decreased after 24 h in the same fermentation conditions. Rossi et al.[60] investigated the producing fruity flavor by *Ceratocystis fimbriata* in solid state fermentation using citric pulp as culture media. They found that isoamyl acetate and ethyl acetate were produced as fruity flavor by selected microorganism, and both flavor concentrations have been increased during 48 h fermentation time. In our previous study[30] on microbial flavor production from tomato pomace by *Kluyveromyces marxianus*, we determined that the concentrations of isoamyl alcohol, isovaleric acid and phenyl ethyl alcohol have been gradually increased during 24, 48 and 72 h fermentation time, respectively. After these times, significant decreases in the concentrations of all volatiles were observed through 120 h fermentation. However, Moradi et al.[61] observed that a gradual increase in γ-decalactone concentration in synthetic media during 75 h fermentation time when they fed bioreactor with castor oil at a rate of 5 mL/h and used atmospheric air for aeration. When Table 3 and Fig. 1 taken into consideration, general decrease was observed in the concentration of all volatile compounds after a particular time in shake cultures and

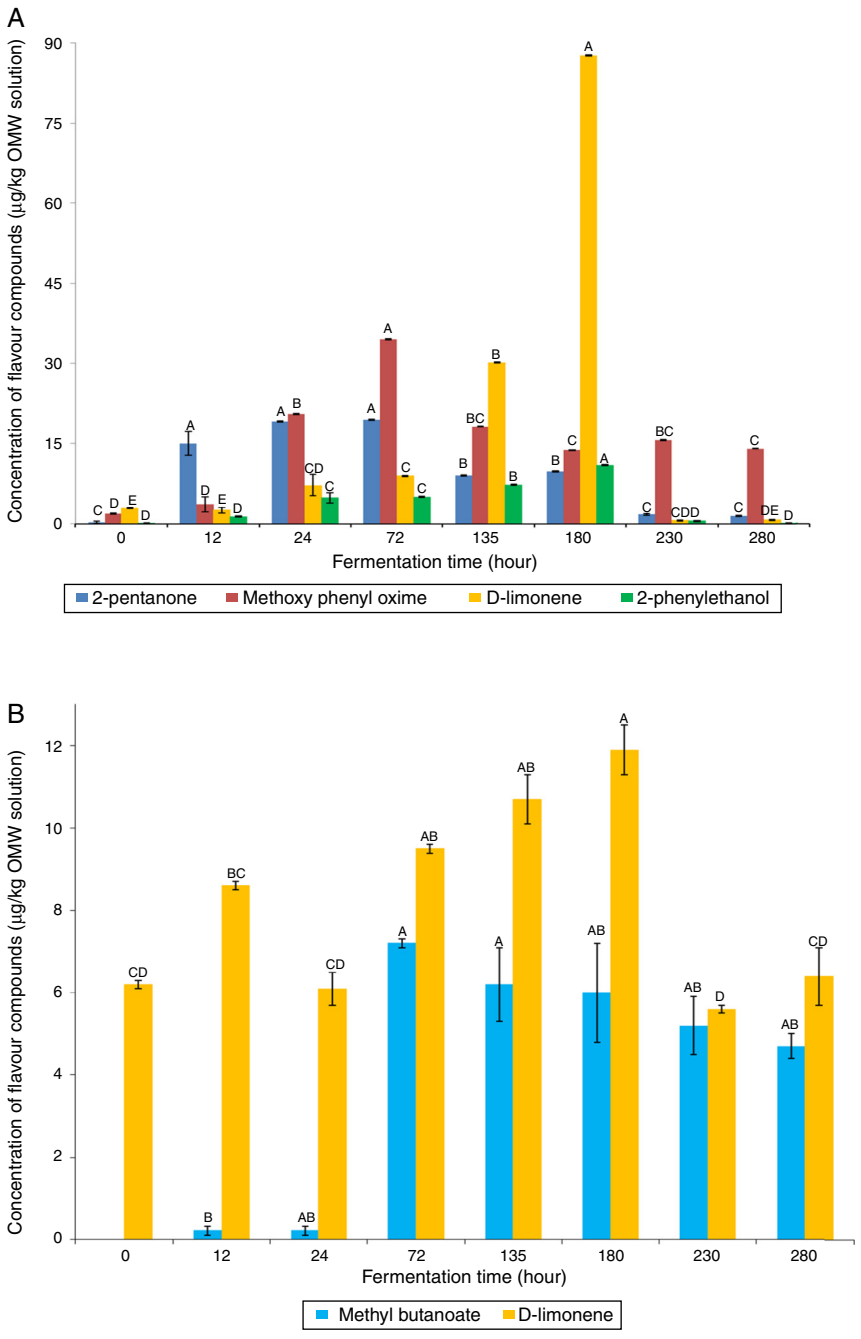

Fig. 1 – Changes in concentration of flavor compounds in OMW during bioreactor cultures of *R. oryzae* (A) and *C. tropicalis* (B). $^{A–E}$Means followed by different superscript letter on bar represents significant differences in the same flavor compound through the fermentation time.

bioreactor cultures similarly the results of previous studies.[41,50,60] This observation might be related to stripping effect which is unavoidably discharge of the flavor compounds in the shake cultures and bioreactor cultures due to flavor compounds volatilities and their dissolving behavior in culture media during the fermentation period.[62] For instance, this stripping process was modeled and investigated experimentally for the production of ethyl acetate from whey by *K. marxianus*. It was found that the stripping rate of the flavor compounds nearly independent of the stirring and was proportional to the gas flow. They pointed out the stripping rate was governed by the absorption capacity of the exhaust gas rather than the phase transfer in the bioreactor.

The productivities of flavor compounds produced both strains in bioreactor cultures have varied (Table 4). It was determined that productivity of D-limonene produced by *R. oryaze* was higher than those of *C. tropicalis*. So, it can be said the production of D-limonene from OMW by *R. oryzae* were higher than *C. tropicalis*. Moreover, 2-phentylethanol has the lowest productivity mean while similar productivity values were

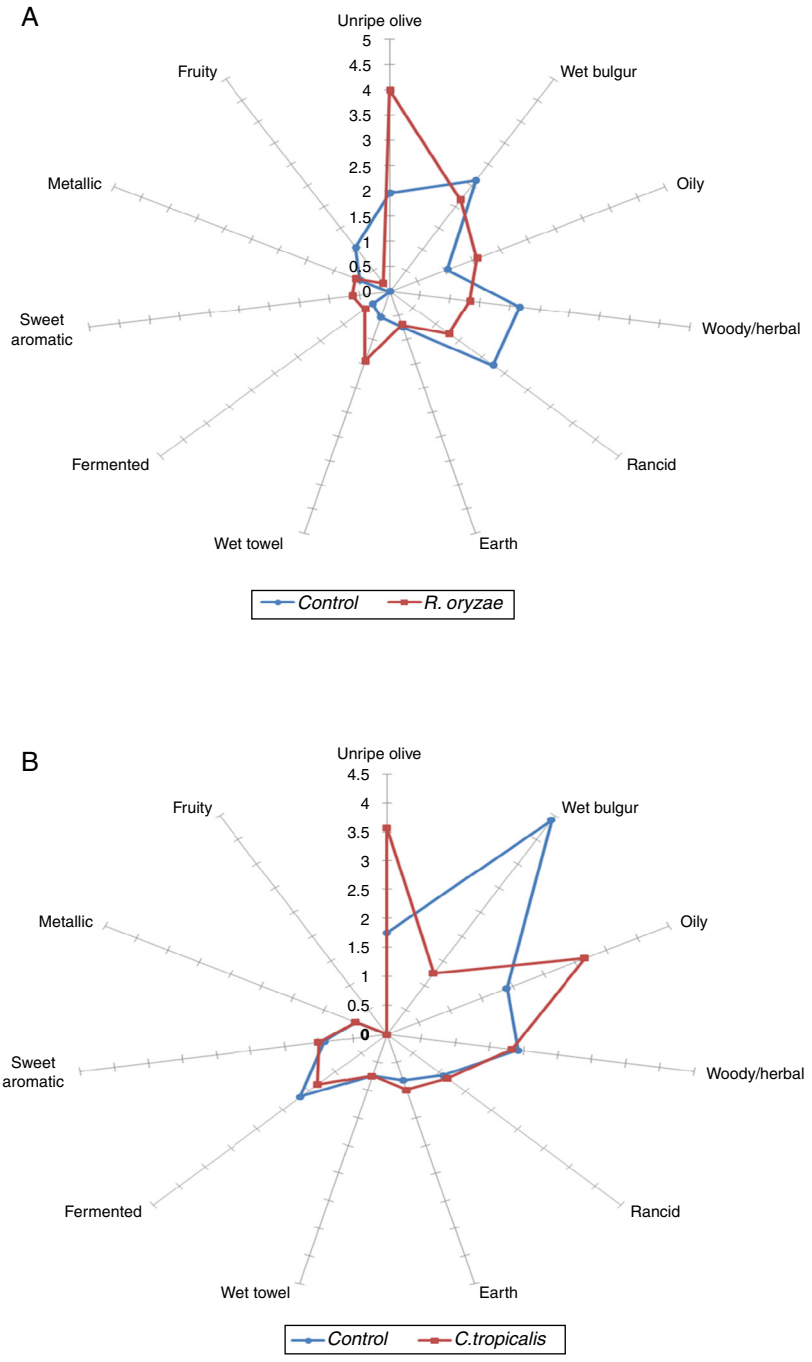

Fig. 2 – Sensory properties of OMW fermented by *R. oryzae* (A) and *C. tropicalis* (B).

observed for D-limonene and methoxy-phenyl-oxime in the case of *R. oryzae* fermentation. By comparing the productivity results of this study with previous studies, different productivity values were reported for various flavor compounds.[30,63] Mantzourido et al.[63] investigated the production of some esters from orange peel waste by solid state fermentation of *S. cerevisiae*. They found that the productivity values varied between 0.10 and 3.23 mg/kg h for some ethyl esters including ethyl hexanoate, ethyl octanoate, ethyl decanoate, ethyl dodecanoate. In a study by Guneser et al.[30] 1.41 and 5.27 μg/kg h of productivity values for isoamyl alcohol produced from tomato pomace by *K. marxianus* and *D. hansenii*, respectively. Moreover, Guneser et al.[41] reported that 8.81 μg/kg h of productivity value for 1-octen-3-ol produced from OMW by *T. atroviride*.

Sensory characteristics of OMW fermented with Rhizopus oryzae and Candida tropicalis

Interpretation of relationship between flavor compounds which are produced by biotechnological process and their sensory properties in fermented culture matrix has not been discussed in detail in the previous studies. However, this

Table 4 – The productivity of flavor compounds produced by *R. oryzae* and *C. tropicalis* in bireactor cultures.

Volatile compounds	Productivity[a] Mean (μg/kg h) ± S.E.	
	R. oryzae	*C. tropicalis*
2-Pentanone	0.26 ± 0.01	Nc
Methoxyphenyloxime	0.45 ± 0.01	Nc
D-Limonene	0.47 ± 0.01	0.06 ± 0.01
2-Phenylethanol	0.06 ± 0.01	Nc
Methyl butanoate	Nc	0.1 ± 0.01

[a] Productivity was calculated based on the maximum concentration of each volatile compounds through the bioreactor culture. Nc, not calculated.

interpretation is essential to determine the sensory quality of flavor compounds. Here, we conducted sensory analysis to evaluate the relationship between quantity of produced flavor compound and its sensory impact. Eleven aroma terms were developed by panelists for fermented OMW. Fig. 2 shows sensory characteristics of fermented and unfermented OMW.

There were significant differences between fermented and unfermented OMW in terms of oily, unripe olive and woody/herbal, wet towel, sweet aromatic ($p < 0.05$). OMW fermented with *R. oryzae* has a higher intensity in terms of unripe olive, wet towel and sweet aromatic than unfermented OMW. Woody/herbal, rancid and fruity aromas were at high intensity in unfermented OMW (Fig. 2A). Significant differences were also observed for unfermented and fermented OMW in the fermentation of *C. tropicalis*. Unripe olive and oily aromas were found to be at high intensities in fermented OMW and unfermented OMW has only a high intensity of wet bulgur aroma. Other sensory characteristics score were found to be a similar in fermented and unfermented OMW (Fig. 2B). It can be said that sweet aromatic is related to 2-pentanone, 2-phentylethanol and D-limonene while methoxy phenyl oxime are associated wet towel flavor. Moreover, unripe olive and oily flavors seem to relate to methyl butanoate and D-limonene.

Conclusion

The results show that OMW can be considered as a raw material for biotechnological production of flavors. The microbial production of 2-pentanone, D-limonene and 2-phenylethanol were achieved from OMW by fermentation of *R. oryzae*, while *C. tropicalis* produced D-limonene and methyl butanoate. In the study, the stripping effect was observed in bioreactor fermentation. Therefore, modeling of stripping effect is required for produced flavor compounds by taking into account temperature, flow rate, agitation speed and volatility in the next step for improved the productivity. Further studies should be conducted to evaluate the production potential of natural flavors from OMW by using other microorganism with biotechnological approaches.

Conflict of interest

The authors declare that they have no conflict of interest.

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