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Isolation and molecular characterization of limonene-growing microbiota capable of valorizing orange juice processing waste

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Abstract
The orange juice processing industry is among the most developed agro-industrial sectors in Greece, which accounts for more than 1.5% of the global orange juice production. Despite the increased organic matter of orange juice processing waste, its high content in essential oils, in particular of limonene, inhibits microbial growth and resists degradation, especially during anaerobic digestion applications. To enhance energy recovery during anaerobic digestion of these agro-industrial residues, an enrichment approach was employed in order to isolate microbiota capable of growing in limonene-containing medium. In this study, microorganisms capable of growing in limonene-based broth were isolated through successive enrichment steps, where limonene was served as the sole carbon and energy source. The microbial strains that were obtained through this enrichment strategy were further characterized by small subunit ribosomal RNA sequencing, placing them within the phyla *Proteobacteria* and *Firmicutes*. We can conclude that these microbial isolates possess the potential to decrease the limonene content of orange juice processing waste, contributing thus in the valorization of citrus processing wastes during both anaerobic digestion and solid-state fermentation.

Keywords: *orange juice processing waste; limonene biotransformation; biomass valorization; limonene-degrading microbiota*

1. INTRODUCTION

All around the globe, citrus, due to their taste, anti-oxidant properties, essential oils content and nutraceutical components, constitute an important parameter of a healthy, balanced diet and therefore are widely consumed either as freshly cut fruits or in the form of juice [1]. Oranges in particular, are dominant commodities of the food market all around the world, with an annual production of 68 million tons. Even though Brazil is considered to be the world's largest orange producer, in the Mediterranean region, Greece is reckoned to be among the countries with the highest orange fruit production, along with Spain, Italy, Turkey and Egypt. Interestingly, Peloponnese and Crete appear to be the leading geographical regions regarding production, while oranges are considered as a valuable sector of the country's economy [2]. The majority of these orange fruits

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are processed in order to produce orange juice, whilst half of their dry weight remained as fruit residue in liquid and solid form, which is often randomly disposed or landfilled [3].

However, the high organic content of the generated agro-industrial wastes provides an opportunity for energy recovery, especially if treated in anaerobic bioreactors, due to methane and ethanol production, as microorganisms possess the ability to induce various pectinolytic, cellulolytic and xylanolytic enzymes and thus enhance and enable the prompt biodegradation of the existing organic fraction [4-5].

On the other hand, the presence of increased limonene content, a monocyclic terpene that is naturally produced in hundreds of plants, negatively affects the microbial diversity and activity and compromises the ability of every applied system to treat the organic content of the orange juice processing waste. Limonene, known as 1-methyl-4-(1-methyl phenyl) cyclohexene, is a hydrocarbon of 10C atoms, known with the molecular formula $C_{10}H_{16}$ [6]. As a plant secondary metabolite, produced as a defensive agent against pathogens and pests, can be found either as D- or L-enantiomer, or as racemic mixture, with R-(+)-limonene being the most commonly identified form present in citrus essential oils, at a percent that can exceed 80% and in some cases, can reach 95%. The latter isomer, due to its flavor and fragrance, is used in food, cosmetic and beverage industry [7]. Nevertheless, this substance, as strong anti-microbial agent, induces adverse toxic effects to the anaerobic microorganisms, compromising their ability to successfully bio-convert organic fraction into methane [8]. Wikandari et al. [3] reported that limonene concentration as low as 400 μL/L completely inhibited anaerobic digestion at mesophilic conditions, while 423 μg/L were considered as the half maximum inhibitory concentration of limonene [9].

The most common approach regarding limonene management includes its removal, recovery and/or conversion in a less inhibitory form [8]. So far, even though some fungal species have been exploited regarding their ability to reduce limonene, there is a limited number of reports on the isolation of other microbiota, like bacteria, capable of growing at the increased limonene concentrations contained in the orange juice processing waste. Thus, the objective of this study was the isolation and molecular characterization of novel microorganisms capable of degrading the limonene contained in orange processing wastes in order to reduce its antimicrobial activity and valorize citrus residues for energy recovery.

2. MATERIALS AND METHODS

In order to isolate limonene-degrading microbiota, orange processing waste (orange peel residues) was subjected to ten-fold dilution plating using defined medium consisting of limonene as the sole carbon and energy source. However, such conventional methodological approach resulted in the isolation of microbiota that were not capable of re-growing after subculturing in fresh defined medium. This led us to the adoption of an enrichment approach, which included successive re-inoculations from the exhausted to the fresh media in order to favor microbiota that resisted the selection pressure conferred by the high limonene concentration of orange processing waste.

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2.1 Isolation of limonene-degrading microbiota

To enrich and isolate effective limonene-degrading microorganisms from orange juice processing waste, a 1/10 dilution of 10 g of orange peel residues was performed in a defined medium consisting of 13 mM Na_2HPO_4, 87 mM KH_2PO4, 10 mM $(NH_4)_2SO_4$, 0.4 mM MgSO4 and 2% v/v DL-limonene. Addition of DL-limonene was performed when the temperature of the liquid medium reached the ambient temperature. The inoculated medium was incubated at 28 ^oC in an orbital incubator (150 rpm) for a time period of 1 week. By the end of the incubation period, 1 volume of the exhausted medium was transferred to 9 volumes of the fresh defined medium (prepared as described above) and the inoculated flask was incubated for an additional period of 1 week in the orbital incubator under the same incubation conditions. A third enrichment step was also carried out to proliferate limonene-degrading microbiota. This included the performance of the same inoculation step taken place in the second enrichment procedure, i.e. addition of 1 volume of the exhausted medium to 9 volumes of the fresh defined medium (10 volumes in total), and incubation of the acclimatized microorganisms for an additional period of 1 week under the same incubation conditions (incubated at 28 ^oC and 150 rpm in a temperature controlled orbital incubator). By the end of the third enrichment step, the exhausted broth was subjected to ten-fold dilution plating. An aliquot of 0.2 mL from each dilution was spread on the appropriate solid medium. Solidified medium was prepared by using the aforementioned defined liquid medium in the presence of 1.7% w/v agar. Addition of DL-limonene was performed when the temperature of the sterile solid medium dropped down to 40-45 ^oC in order to prevent volatilization of this essential oil. A total of 40 microbial isolates were obtained by picking single colonies up from the agar surface, which were then successively subcultured twice on fresh agar plate to ensure cell purity. Purity was also examined under the Zeiss Axiostar Plus microscope.

2.2 Examination of the ability of the microbial isolates to grow in liquid medium containing limonene as the sole carbon and energy source

To ensure that the microbial isolates obtained were capable of utilizing limonene as the sole carbon source (and not the agar used as the solidified agent in Petri dishes), liquid medium consisting of the mineral salts described above in the presence of 2% v/v DL-limonene was prepared. After broth sterilization, individual colonies were inoculated into the fresh liquid medium in separate vials in order to test their efficiency to grow in limonene (as the sole carbon and energy source). All obtained isolates were inoculated at 28 ^oC in an orbital incubator (150 rpm) for a period of 1 week. The efficiency to utilize limonene was tested against uninoculated broth in a spectrophotometer at 600 nm.

2.2 Phylogenetic identification of limonene-degrading bacteria

Extraction of genomic DNA from limonene-degrading microorganisms was carried out by using the "NucleoSpin Tissue" DNA extraction kit (Macherey-Nagel, Germany). PCR products were amplified in a TP600 thermocycler (TaKaRa, Japan) through the use of the forward primer pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the reverse primer pH (5′-AAG GAG GTG ATC CAG CCG CA-3′). A reaction mixture of 25 μL was made by adding 0.5 μL of 20 ng genomic DNA, 10× buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 0.5 μM each of the forward and reverse primer and 5 U Taq polymerase

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(Jena Bioscience, Germany). Amplification was conducted by placing the PCR mixture in a PCR vial for 2 min at 94 °C. In addition, 35 thermocycles of 30 s at 94 °C, 30 s at 52 °C and 75 s at 72 °C were then performed. The PCR elongation reaction was completed by 5 min at 72 °C. The PCR amplicons were inserted into pMD20 vector (TaKaRa, Japan) by TA-ligation and the recombinant products were inserted into *Escherichia coli* DH5a cells (TaKaRa, Japan). The extraction of plasmid DNA from the recombinant clones was performed by employing the "NucleoSpin Plasmid kit" (Macherey-Nagel, Germany). All sequencing reactions were carried out at Eurofins Genomics (Germany). Recombinant amplicons were merged by the "CAP3 Sequence Assembly Program" of Huang and Madan [10]. The 16S rRNA gene amplicons were compared with their closest phylogenetic relatives by using the blastn option at NCBI platform.

3. RESULTS AND DISCUSSION

The enrichment procedure was employed in order to overcome obstacles that arisen from the fact that limonene-degrading microbiota consist of a minor part of orange processing waste community structure, since the high content in easily degradable compounds, like monosaccharides and pectin, appears to proliferate fast growers rather than the slow-growing limonene-degraders. From a total of 40 microbial isolates growing in limonene-containing plates, only 8 isolates were capable of growing in liquid medium, where limonene served as the sole carbon and energy source. This difference in the number of microbial isolates that were grown in solid and liquid medium can be explained with the fact that the remaining microbiota could resist high limonene concertation without utilizing this essential oil.

All the microbial isolates obtained after selection in liquid medium were member of *Bacteria*, as proven by both microscopic observation and identification of their phylogenetic position (through 16S rRNA gene sequencing). Molecular characterization placed the selected microbial isolates in three distinct operational taxonomic units (OTUs). The two out of three OTUs consisted of a single representative and showed high phylogenetic similarity (above 99%) with known members of the species *Acinetobacter lwoffii* (*Moraxellaceae*, *Pseudomonadales*, *Gammaproteobacteria*, *Proteobacteria*) and *Lactobacillus paracasei* (*Lactobacillaceae*, *Lactobacillales*, *Bacilli*, *Firmicutes*). On the other hand, the major OTU was comprised of six bacterial isolates, which were identical in the 16S rRNA gene to *Pseudomonas psychrotolerans*. In particular, *Pseudomonas* and *Acinetobacter* spp. are common degraders of recalcitrant compounds [11-12]. Moreover, certain *Pseudomonas* species are also specialized in the degradation of limonene, like *Pseudomonas putida* MTCC 1072 [13] and a *Pseudomonas aeruginosa* strain [14]. Lactic acid bacteria like streptococci have also possessed the potential to degrade limonene [14]. The above findings indicate that orange processing waste is a source for biotechnology and innovation since specialized microbiota can be enriched from such material to serve as biodegradation and/or biotransformation agents to food and pharmaceutical industry applications, as well as starter cultures for the valorization of limonene-rich orange processing waste via energy recovery in anaerobic digestion systems.

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4. CONCLUSIONS

This work resulted in the characterization of novel limonene-degrading microbiota isolated from orange processing waste. This manufacturing waste, which is characterized by high carbohydrate and limonene content, appears to accommodate a specialized limonene-degrading community, which, however, consists of minor constituents of the entire microbial community. Thus, an enrichment approach is the appropriate procedure to isolate novel limonene-degrading microorganisms from such wastes. Regarding the phylogeny of the characterized microbial isolates, it appears that *Pseudomonas* species are the dominant limonene degraders in such waste. This is the first repost on *Pseudomonas psychrotolerans* strains as effective limonene degraders.

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References

[1] Abd Ghafar, M.F., Prasad, K.N., Weng, K.K., Ismail, A. 2010. Flavonoid, hesperidine, total phenolic contents and antioxidant activities from citrus species. *African Journal of Biotechnology*, **9 (3)**, 326 – 330.

[2] FAO. *Citrus Fruit—Fresh and Processed, Statistical Bulletin*, 2016. Food and Agriculture Organization of the United Nations, Rome, Italy, 2017.

[3] Wikandari, R., Nguyen, H., Millati, R., Niklasson, C., Taherzadeh, M.J. 2015. Improvement of biogas production from orange peel waste by leaching of limonene. *BioMed Research International*, **2015**, 494182.

[4] Nikolaou, C., Karabagias, I.K., Gatzias, I., Kontakos, S., Badeka, A., Kontominas, M.G. 2017. Differentiation of fresh greek orange juice of the merlin cultivar according to geographical origin based on the combination of organic acid and sugar content as well as physicochemical parameters using chemometrics. *Food Analytical Methods*, **10 (7)**, 2217 – 2228.

[5] Zerva, I., Remmas, N., Ntougias, S. 2019. Diversity and biotechnological potential of xylan-degrading microorganisms from orange juice processing waste. *Water*, **11 (2)**, 274.

[6] Cao, X., Lv, Y.B., Chen, J., Imanaka, T., Wei, L.J., Hua, Q. 2016. Metabolic engineering of oleaginous yeast Yarrowia lipolytica for limonene overproduction. *Biotechnology for Biofuels*, **9 (1)**, 26.

ISBN: 978-618-5271-73-2

[7] Ravichandran, C., Badgujar, P.C., Gundev, P., Upadhyay, A. 2018. Review of toxicological assessment of d-limonene, a food and cosmetics additive. *Food and Chemical Toxicology*, **120**, 668 – 680.
[8] Ruiz, B., De Benito, A., Rivera, J.D., Flotats, X. 2016. Assessment of different pretreatment methods for the removal of limonene in citrus waste and their effect on methane potential and methane production rate. *Waste Management and Research*, **34 (12)**, 1249 – 1257.
[9] Ruiz, B., Flotats, X. 2014. Citrus essential oils and their influence on the anaerobic digestion process: An overview. *Waste Management*, **34 (11)**, 2063 – 2079.
[10] Huang, X., Madan, A. 1999. CAP3: A DNA sequence assembly program. *Genome research*, **9 (9)**, 868 – 877.
[11] Ghosh, M., Verma, S.C., Mengoni, A., Tripathi, A.K. 2004. Enrichment and identification of bacteria capable of reducing chemical oxygen demand of anaerobically treated molasses spent wash. *Journal of Applied Microbiology*, **96 (6)**, 1278 – 1286.
[12] Cortes-Tolalpa, L., Norder, J., van Elsas, J.D., Falcao Salles, J. 2018. Halotolerant microbial consortia able to degrade highly recalcitrant plant biomass substrate. *Applied Microbiology and Biotechnology*, **102 (6)**, 2913 – 2927.
[13] Chatterjee, T., Bhattacharyya, D.K. 2001. Biotransformation of limonene by *Pseudomonas putida*. *Applied Microbiology and Biotechnology*, **55 (5)**, 541 – 546.
[14] Menéndez, P., Rossini, C., Neill, S.O., Soubes, M., Heinzen, H., Moyna, P. 2000. Biotransformation of R-(+)-limonene by *Pseudomonas aeruginosa* and *Streptococcus faecalis*. *Anales des la Asociacion Quimica Argentina*, **88 (5-6)**, 79 – 82.

ISBN: 978-618-5271-73-2