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A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-*co*-hydroxyvalerate) from olive mill wastewater by *Haloferax mediterranei*

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ABSTRACT

Olive mill wastewater (OMW), a highly polluting waste from the olive oil industry, was utilized as sole carbon source for the production of polyhydroxyalkanoate (PHA) by extremely halophilic *Haloferax Mediterranei* (*H. mediterranei*) in a one stage cultivation step. *H. mediterranei* showed remarkable cell growth and tolerated the inhibitory effect of polyphenols present in medium containing 25% of OMW. *H. mediterranei* cultivation conditions were optimized in medium containing 15% OMW by investigating several parameters that affect the production of PHA. The highest polymer yield (0.2 g/L) and PHA content (43% PHA/cell dry mass) were achieved at 37 °C, 170 rpm and 22% salt concentration. Analysis of the produced PHA revealed the production of copolyester poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) containing 6.5 mol% 3-hydroxyvalerate (3HV). The production of PHBHV was observed without the need for fermentation step or adding external carbon source. The PHBHV displayed reduced melting points at 140.1 °C and 154.4 °C when compared to homopolymer polyhydroxybutyrate.

1. Introduction

The overproduction and accumulation of petroleum-based plastics have turned research efforts toward the production of biodegradable plastics. These efforts have been focused on the production of bacterial polymers, polyhydroxyalkanoates (PHAs) and in particular the homopolymer polyhydroxybutyrate (PHB) [1,2]. PHB intracellularly accumulated as carbon and energy sources by several bacteria and archaea under unfavorable conditions [3]. However, the industrial application of PHB is restricted by its poor mechanical and physical properties such as brittleness and stiffness [4]. Studies have suggested that the incorporation of other monomeric units such as 3-hydroxyvalerate (3HV) into PHB chains yields co-polymer with improved properties [5,6]. Therefore, the copolyester of 3HB and 3HV, poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) has been intensively investigated with expectations of practical use. PHBHV showed better mechanical properties when compared to PHB homopolymer and similar properties to polypropylene such as high impact resistance, toughness and flexibility [7–9].

The high production cost of PHBHV compared to that of polypropylene prevents further market penetration [10]. A cost reduction in PHBHV biosynthesis process could be obtained by using inexpensive carbon sources and developing new PHBHV accumulation process with cheap and simple purification steps. Industrial by-products such as glycerol (from the biodiesel industry), [4,11] rice bran [12], molasses [13] and cheese whey [14] have been used as cheap carbon sources for PHBHV production. The effluent of the olive oil industry, olive mill wastewater (OMW) can also be considered as a potential no-cost substrate for PHBHV production. OMW is rich in high amounts of readily consumable carbon source such as carbohydrates, lipids and volatile fatty acids which are the most direct substrates for PHBHV production [15]. Annually, high levels of OMW were recorded, particularly in the Mediterranean counties, which produced about 30 million m^3 [16]. In Jordan, there are approximately 130 olive mills serving olive plantation and generating around 200 thousand m^3 of OMW per year [17]. The OMW creates serious environmental problems such as changes in soil microbial populations, threat to surface and groundwater sources and pollution of the air through phenol and sulfur dioxide emissions [18]. Thus, their employment as feedstock for PHBHV production could represent an alternative solution for their disposal.

The production of PHAs from OMW is currently based on multi-stage processes [15,19–21]. These processes involve OMW pre-treatment, which consists of polyphenols removal, followed by an acidogenic fermentation step to obtain volatile fatty acids (VFAs).

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After that, the VFAs stream is employed for PHAs accumulation, using a pure [21] or mixed culture cell methodology [22,23]. In the pure cells methodology, high cell densities and PHAs contents were obtained. However, the selection of mixed cultures has more economic advantages than pure strains where the production process performed under non-sterile conditions [23]. The previous processes clearly showed that the OMW requires prior steps before it is utilized as sole carbon source for PHA production, due to the incompatibility between OMW medium and biological components. One approach to tackle the incompatibility between OMW and biological components is to employ extremophile organisms, which exhibit tolerance to a range of environmental stressors and provide many biotechnological applications [24–26].

The present work was dedicated to evaluating the possibility of producing PHA from OMW in a one-stage cultivation process (not multi-stage processes), using an extremely halophilic organism *Haloferax Mediterranei* (*H. mediterranei*). *H. mediterranei* was chosen as the biocatalyst due to its capability of accumulating the copolymer PHBHV from cheap carbon sources with chemical structure unrelated to 3-hydroxyvalerate (3HV), such as glycerol [11] and carbohydrates [27–29], and the fact that *H. mediterranei* is extremely resilient to contamination as the high salt concentration (2–5 M NaCl) for its optimal growth prevents almost any other organisms from replicating [30]. Moreover, *H. mediterranei* like some halophiles [31–33] could grow on in media that contains phenolic compounds such as OMW medium. Additionally, the obtained polymer can be easily recovered by hypo-osmatic shock of cells after decreasing the salinity of the external medium [34]. Finally, *H. mediterranei* has simple growth requirements, with relatively rapid doubling time in comparison to other haloarchaea [35,36]. Many studies in the literature have tested *H. mediterranei* to produce PHBHV from different carbon sources [12–14,28,37]. However, to the best of our knowledge, this work represents the first attempt to produce PHAs from OMW by employing *H. mediterranei*. In order to develop a novel culture method for the production of PHBHV from OMW, the effects of different conditions on the growth of *H. mediterranei* were investigated and the produced polymer was fully characterized.

2. Materials and methods

2.1. Chemical reagent and standards

All chemical reagents, unless stated otherwise, were purchased as analytical grade. The standard solutions used in volatile fatty acids (VFAs) analysis were prepared from concentrated formic acid (≥98%, Sigma), acetic acid (glacial) (100%, Merck), butyric acid (≥99%, Merck), pentatonic acid (≥98%, Merck), propionic acid (>99%, Merck) and hexanoic acid (≥99.5%, Merck). Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) 8 mol% PHV; natural origin, methyl (*R*)-3-hydroxybutyrate 99% and methyl (*R*)-3-hydroxyvalerate ≥98.0% were purchased from Sigma-Aldrich.

2.2. Microorganisms and growth conditions

Haloferax mediterranei DSM 1411 and *Cupriavidus necator* DSM 545 were obtained from German Collection of Microorganisms and Cell cultures (DSMZ). *Haloferax mediterranei* (*H. mediterranei*) was cultivated first in 100 ml of nutrient-rich AS-168 medium [35] containing (per liter) 200 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 2 g KCl, 3 g trisodium citrate, 1 g sodium glutamate, 50 mg $FeSO_4{\cdot}4H_2O$, 0.36 mg $MnCl_2{\cdot}4H_2O$, 5 g casamino Acids, 5 g yeast extract, pH 7.2. The culture was incubated in shaking incubator with constant shaking (170 rpm) for 3 days at 37 °C. For cultivation with OMW, three milliliter of the culture was transferred into 100 ml of a nutrient-limited MST medium [35] containing (per liter) 200 g NaCl, 20 g $MgSO_4{\cdot}7H_2O$, 2 g KCl, 1 g sodium glutamate, 37.5 mg KH_2PO_4, 50 mg $FeSO_4{\cdot}7H_2O$, 0.36 mg $MnCl_2{\cdot}4H_2O$, 1 g yeast extract and different concentrations (5, 15, 25, 50 and 75% V/V) of OMW. The culture was incubated for 4 days at 170 rpm, 37 °C. *Cupriavidus necator* (*C. necator*) was cultivated in seed mineral medium, as previously described [38].

2.3. Analytical methods and OMW characterization

Total dissolve solid (TDS), total suspended solid (TSS), total solid (TS), chemical oxygen demand (COD), total Phenols, total nitrogen (T-N), Total Kjeldhal nitrogen (TK-N), ammoniacal nitrogen (NH_4—N) and density (ρ) (at least three repetitions for each sample) were determined according to Standard Methods [39]. Volatile fatty acids (VFAs) namely acetic acid, propionic acid, butyric acid and hexanoic acid concentrations were determined using Agilent 1100 HPLC equipped with a diode array detector and 8 mm Rezex ROA-organic acid H column (Phenomenex). VFAs were eluted using 0.013 N H_2SO_4 at a flow rate of 0.6 ml/min and 35 °C operating temperature. The detection wavelength was set at 210 nm.

Fresh OMW was collected from an olive oil mill located in Madaba in central Jordan. Sample was stored in dark plastic containers at 4° C. The physicochemical characterization for OMW is described in Table 1.

2.4. Evaluation of the inhibitory effect of OMW

The inhibitory effect of OMW was evaluated by monitoring the cell growth of *H. mediterranei* in MST medium and different concentrations (percent, v/v) of 5%, 15%, 25% 50% and 75% of OMW. The culture was incubated for 4 days at 170 rpm, 37 °C. The cell growth was analyzed spectrophotometrically by measuring the optical density at 520 nm (OD_{520} nm) [40], using a Biochrom Libra S50 UV–visible spectrophotometer. Blank samples (media without culture) were performed to eliminate the matrix interference. *C. necator* the most studied PHA producer was used as reference cell to estimate the inhibitory effect of OMW. *C. necator* was cultivated in seed mineral medium with 5% to 75% of OMW. The culture was incubated for 4 days at 170 rpm, 30 °C. The cell growth was monitored by measuring the OD_{600} nm.

2.5. Optimization of PHA production

H. mediterranei was cultivated in 100 ml of nutrient-rich AS-168 medium in a 250 ml Erlenmeyer flask for 3 days with shaking at 170 rpm, 37 °C. To develop a concentrated inoculum, the

Table 1
The physicochemical characteristics of OMW.

Parameters	
pH-value	5.2 ± 0.1
EC (μS/cm)	3790 ± 57
TDS (mg/L)	17450 ± 698
TSS (mg/L)	12500 ± 813
TS (mg/L)	39920 ± 800
COD (mg/L)	58850 ± 5002
Total Phenols (mg/L)	2417 ± 12
T-N (mg/L)	544 ± 35
TK-N (mg/L)	543 ± 33
NH_4—N (mg/L)	43.7 ± 3
Acetic acid (mg/L)	6367 ± 540
Propionic acid (mg/L)	9852 ± 810
Butyric acid (mg/L)	2055 ± 180
Hexanoic acid (mg/L)	3070 ± 270
VFAs (mg/L)	21344 ± 810
ρ (g/cm^3)	0.99 ± 0.05