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Assessment of yields and properties of bioplastics production from acidified sugary wastewaters via mixed microbial cultures

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Abstract

A mixed microbial culture (MMC) of poly-hydroxy-alkanoates (PHAs) producers was developed in a draw-fill reactor (DFR) subjected to sequential nitrogen/carbon limitation stress using short chain fatty acids (SCFAs) as carbon source. The distribution of microorganisms was analyzed in the beginning of the process and after six weeks of limitation, in order to verify the domination of PHA producers in the consortium. The potential of the enhanced MMC for production of co-polymers was then evaluated in batch mode using synthetic media. The parameters tested were the C to N ratio (C/N), the propionate to butyric ratio (prop/but) and the initial pH (pH_{in}). It was shown that the type, yields and productivity of PHAs was highly affected and the optimal yield, 40% PHAs/dry cell weigh (DCW) (w/w), was achieved for a C/N of 200 (w/w), prop/but 10/90 (mol/mol) and pH_{in}, 7.5. The MMC was further assessed for the production of PHAs from acidified sugary wastewater (ASW) containing SCFAs, sugars and lactate at different ratios. PHAs were solvent-extracted from the microbial biomass; they were further analyzed in terms of their chemical composition, via ^{1}H-NMR and ^{13}C-NMR, their structure at molecular level via Attenuated total reflectance – Fourier transform infrared spectroscopy (ATR-FTIR), and their thermal properties, *via* differential scanning calorimetry (DSC). It was shown that in all cases, P(3HB-co-3HV) was produced with T_m ranging from 170,0 to 164,1 ^{o}C.

Keywords: poly-hydroxyalkanoates, co-polymers, mixed cultures, nutrients limitation, DSC, ATR-FTIR, NMR.

1. Introduction

Plastic pollution poses a major threat to the environment, due to its abundance, persistence and durability. (Da Costa et al., 2020). Thus it is of great importance to use new more environmentally friendly technologies and replace the fossil-based plastic products by bio-based equivalents. Nowadays many prokaryotic microorganisms such as bacteria and archaea are used for the production of bioplastics namely polyhydroxyalkanoates (PHAs). PHAs are aliphatic polyesters that are fully biodegradable and biocompatible (Paul et al., 2020) and bear similar material properties to various thermoplastics and elastomers. As such, they have been considered a promising replacement of conventional plastics (Mudliar et al., 2007)

In the current study a MMC of PHA producers was developed and assessed as biocalatyst for the bioconversion of acidified sugary wastewaters (ASWs) towards co-polymers.

2. Materials and Methods

2.1. MMC development and evaluation

The enriched MMC was developed using aerobic activated sludge from the Wastewater Treatment Plant of the University of Patras as seed. The enrichment was conducted as described by Ntaikou et al. (2018). To investigate the optimal conditions for high PHAs accumulation of the MMC, three sets of batch experiments were conducted during which the effect of the C to N ratio, C/N, (250, 200, 100, 50), the molecular propionate to butyric ratio, Prop/But, (10/90, 30/70, 50/50, 100/0) and the initial pH (pH_{in}) on the yields and properties of the PHAs were studied. All tests were perfromed in duplicate in 1L Erlenmeyer flasks at 600ml working volume, at 27^{o} C, 150rpm and constant agitation.

2.2. PHAs production from ASWs

For the evaluation of the MMC as a biocatalyst for PHAs production from ASWs, different effluents from a biohydrogen producing reactor (BHR) were used as carbon source. The BHR was operated continuously at mesophilic conditions with hydraulic retention time (HRT) 36h, 24h, 12h and 6h during which a sugary wastewater was fermented via a mixed acidogenic anaerobic consortium to gaseous hydrogen, SCFAs and

lactate (Kora et al., 2020). The production of PHAs from the effluents was conducted in batch mode (1L Erlenmeyer, 400ml working volume), 27° C and under constant agitation and aeration, with C/N ratio 200 using and NH_4Cl as nitrogen source.

2.3. Extraction and quantification of PHAs

The extraction of PHAs from the microbial biomass was performed according to Ntaikou et al., (2018). The extraction yield of produced polymer, $Y_{PHAs(\%)}$ was estimated gravimetrically according to the equation:

$$Y_{PHAs(\%)} = \frac{PHAs_{ext.}}{DCW} 100\%. \quad (1)$$

where $PHAs_{ext.}$ is the amount of air dried polymer extracted from lyophilized biomass expressed as DCW (dry cell weigh). The PHAs production yield per chemical oxygen demand (COD) consumed (COD_{cons}) was also determined according to the equation:

$$Y_{PHAs/COD} = \frac{[PHAs]_{ext.}}{[COD]_{cons.}} \quad (2)$$

where $[PHAs]_{ext.}$ is the estimated PHA concentration at the end of the fermentation, based on the extracted amount and $[COD]_{cons}$ is the total COD consumed throughout the fermentation.

2.4. Analytical methods

Total solids (TS) and volatile solids (VS) were determined according to Standard Methods (APHA, 1995). Soluble and total carbohydrates were quantified according to Miller (1956). Short chain fatty acids (SCFAs), ethanol and lactate were quantified as described by Antonopoulou et al. (2016).

NMR samples were prepared by dissolving them in Chloroform-d (CDCl3). The NMR spectra were recorded with a Bruker Ascend 600 spectrometer operating at a 1H Larmor frequency of 600.13 MHz. 1H and 13C NMR experiments were selected using the subsequent acquisition parameters: 6 and 12 scans and relaxation delay of 1 and 2 sec respectively. The spectra were collected at the 298,15 K temperature. Spectra were manually corrected for phase and baseline distortions using TopSpin 3.6 (Bruker BioSpin srl).

The structural analysis of the extracted PHAs and the pre- and post-extraction microbial (PreEM and PostEM respectively) biomass was evaluated via an ATR/FTIR Bruker Alpha II spectrometer equipped with a MIR DLaTGS pyroelectric detector and diamond as the Internal Reflection Element. Spectra were obtained in the spectral range 4000-400 cm^{-1} with 4 cm^{-1} resolution. Averaging was performed over 25 scans in duplicated experiments for each sample.

DSC Measurements involved a TA Instruments Q100 Differential Scanning Calorimeter equipped with a Liquid Nitrogen Cooling System (LNCS). The instrument was calibrated with Indium and Sapphire (reference standards) using 10 °C/min heating rate under Nitrogen atmosphere (50 ml/min). The sample mass was kept relatively small to minimize temperature gradients within the sample. Roughly 5 mg of powdered sample were placed in the aluminum pans for all experiments. The glass transition temperature was selected as the temperature of half devitrification and the melting points as the heat flow signal maxima.

3. Results

3.1. Development of MMC

The DFR was monitored on a daily basis in terms of pH, COD and NH_4-N consumption, optical density and PHAs accumulation. After 30 days of operation the system seemed to reach a steady state and the microbial consortium that was developed was analyzed via next generation sequencing of the 16S rRNA/18SrRNA/ITS genes of distinct regions (results not shown), via which it was found that the distribution of taxa was significantly limited compared to that of the seed culture, being dominated by the genera of *Zooglea*, *Thaurea*, *Bacteroides* and *Pseudomonas* at 29%, 7%, 6% and 4% of the overall bacterial genera, respectively.

3.2. Investigation of optimal conditions for PHAs production from the MMC

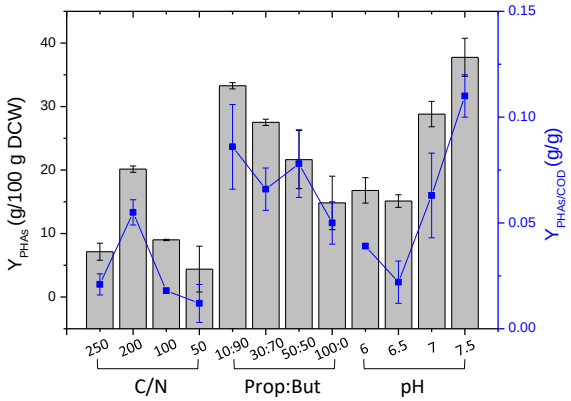

Figure 1. Estimated yields of extracted PHAs from the MMC at different conditions, after 48 h cultivation at batch mode. C/N, ratio of carbon to nitrogen, Prop:But, initial molecular ratio of propionate to butyrate concentration in the medium, pH, initial pH of the medium.

In **Figure 1**, the estimated yields of extracted PHAs from the accumulating MMC, after cultivation for 48h at the different conditions tested, are illustrated. It can be observed that the accumulation capacity of PHAs and the assimilation of total consumed COD to PHAs, exhibit similar trends. Overall, the conditions favoring the accumulation the most were C/N ~200, prop/but, 10/90 and pH_{in}=7.5. The monomeric ratio of HB and HV content in the extracted PHAs as estimated by the NMR analysis showed that the HV content was 2-3% for all samples but for the samples obtained from the cultures that propionate was used as the sole as carbon source. This could be attributed to the mechanism that is responsible for the incorporation of HV in P(3HB-*co*-3HV) co-polymer, which is based on the intracellular propionyl-CoA to acetyl-CoA ratios that is highly controlled by related enzyme properties (Yang et al, 2014).

3.3. *MMC performance during PHAs production from ASWs*

When the different ASWs there seemed to be a strong correlation between the propionate content in the substrate and the HV content in the polymer, with HV content exciding 10% in the case of 24h samples. The $Y_{PHAs/COD}$ in the case of ASWs bioconversion was quite lower than those of the synthetic feeds as shown in **Figure 2,** indicating that a high amount of the consumed COD can only be used for maintenance of the bacteria rather than PHAs biosynthesis.

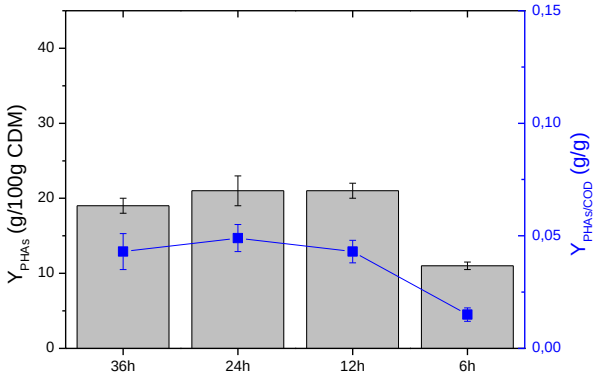

Figure 2. Estimated yields of extracted PHAs from the MMC that was cultivated using ASWs as the sole carbon source, after 48h cultivation that batch mode. 36h, 24h, 12h, 6h indicate different effluent from the H_2 producing reactor operated at the respective HRTs.

3.4. *ATR/FTIR analysis*

While PHAs accumulated in pure culture systems can be quantitatively identified easily, (Hong et al., 1999) the MMCs prove to be a more challenging task. (Arcos-Hernandez et al., 2010). The quite complex nature of the molecular structures and entities that constitute the different bacterial cells (i.e. Peptidoglycan, Techoic acid, LPS, cellular proteins, phospolipids etc.) is depicted in the absorption IR spectra shown in **Figure 3.** The extracted PHAs' spectra as well as the spectra of PostEM and the PHB reference material are also included in the same figure for comparison. Accurate assignment of the observed peaks (Baker et al., 2014,) is a hard task as certain bands can be found in the same wavenumber region and overlap. The extracted PHAs spectrum appears to be free of any biomass residues indicating the success of the extraction and subsequent purification process followed. The estimation of the yields that were presented in sections 3.2 and 3.3 was based on the PHAs masses that were extracted from the biomass via solvent extraction and purification and were quantified gravimetrically, and as such they may not reflect accurately the true bioaccumulation potential of the MMC. In order to investigate this aspect, different PreEMs and PostEMs as well as the respective extracted PHAs were furher analysed via ATR/FTIR.
The spectra acquired from different PreEMs (**Figure 4a)** indicate that there exists a correlation between the intensity of the carbonyl absorption peaks (at 1723 cm^{-1}, 1740 cm^{-1} associated with crystalline, amorphous domains respectively) to the Amide I (1642 cm^{-1}) with respect to PHA/DCW (%).

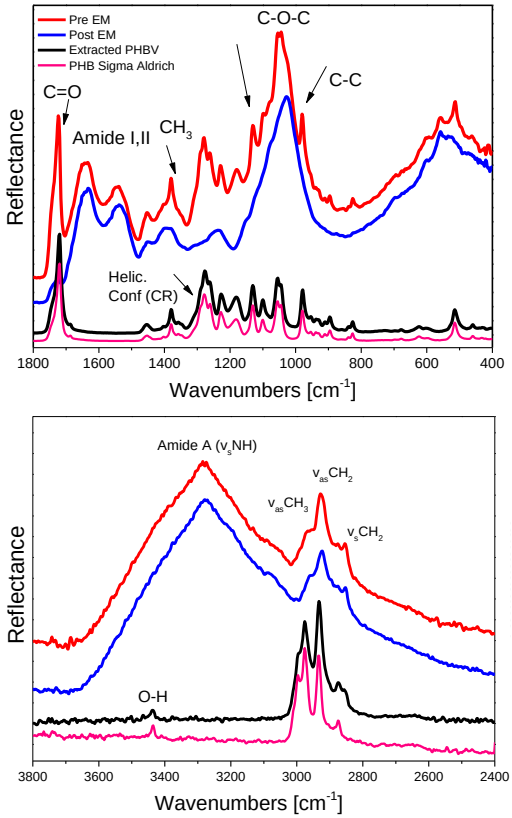

Figure 3. ATR spectra of the extraction protocol separated systems, namely PreEM, PostEM and extracted PHBV obtained for the case of ASW 12h. The spectrum of the PHB reference material is also included in the plot.

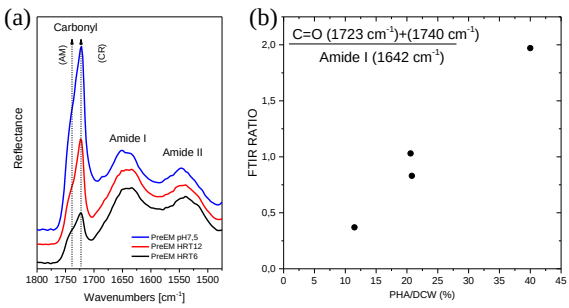

Figure 4: (a) Absorption spectra of the PreEM for systems with different accumulated ratio (PHA/DCW). (b) Correlation between spectroscopic and gravimetric methods for determining PHA accumulation.

3.5. *Thermal Analysis, DSC*

In order to extract maximum information regarding the thermodynamic properties of the samples, a specific protocol was followed for the DSC experiments.
The method employed is summarized in the following:
The thermal properties extracted from the DSC data are presented in **Table 1.** Samples possessing high T_{cc} values exhibit slower crystallization kinetics, which may indicate higher valerate content. It is well known that heterogenous polymer chains (PHBVs) of the random copolymer are structurally characterized by differences in their crystallization capabilities.

4. Conclusions

The results indicated that the propionate ratio in the synthetic medium was not reflected proportionally in the final HV content of the polymers, in contrast to the propionate in ASW, which could be attributed to the complexity of carbon sources. Nevertheless, the complex carbon sources seem to affect negatively the $Y_{PHAs/COD}$, indicating that in such cases the consumed COD is mainly used for bacteria maintenance rather than for PHAs accumulation. Valuable information can be obtained by the structural analysis of the PreEM and PostEM and the extracted polymers, which could possibly be used for the direct evaluation of the HV content of the intracellular PHAs.

Table 1. Thermal properties of extracted PHAs films produced from synthetic substrates and ASWs, extracted from the DSC data. Commercial pure PHB was also used as reference material.

Sample Name	T_{m1} (ºC)	$T_{m1'}$ (ºC)	ΔH(J/g)	T_g (ºC)	T_{cc} (ºC)	T_{m2} (ºC)	$T_{m2'}$ (ºC)
PHB Sigma Aldrich		174.6	92.0	1.7	41.9	158.0	168.4
HRT 6	-	169.0	53.2	2.7	59.3	158.4	170.0
HRT 12	-	168.8	72.4	2.6	55.8	156.8	169.0
HRT 24	-	167.6	62.0	0.9	58.0	147.4	164.0
HRT 36	-	167.5	75.7	1.3	55.9	152.7	167.0
10/90	-	166.2	88.6	2.7	50.3	156.8	167.0
30/70	133.2	163.5	77.1	2.1	56.0	154.7	165.1
pH 6	-	167.4	82.2	2.9	46.4	-	168.4
pH 6.5	131.1	160.4	87.9	0.4	52.5	146.0	162.8
pH 7	-	161.8	72.3	2.6	48.2	152.8	162.8
pH 7.5	-	161.8	82.6	2.7	49.1	153.4	163.2

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