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Waste to Bioplastic Conversion by the Moderate Halophilic Bacterium *Halomonas boliviensis*

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The main purpose of this work was to evaluate the use of a waste stream for the production of polyhydroxyalkanoates (PHA). With the anaerobic acidogenesis process it is possible to treat and revaluate complex waste into a wide combination of volatile fatty acids (VFA) with different proportions. In this study, the behaviour of the bacterium *Halomonas boliviensis* on different VFA mixtures was tested, as well as the effect of using this substrate as carbon and energy source for PHA production at flask scale. The VFA mixture that yielded the highest PHA accumulation was used to scale-up the process. At bioreactor scale, a total amount of 13 g L^{-1} of PHA was obtained, which was formed by 70 % of the copolymer P(3HB-co-3HV) with a HV fraction of 8.5 mol %.

1. Introduction

The volume of waste generated by our society is increasing every day and most of the time is not properly treated. In 2012 only 62 % of the plastic waste was recycled or directed to energy recovery processes. The environmental impact of petroleum-based plastic waste is of special importance, due to their non-biodegradable characteristics the accumulation rate of plastics in the environment is 25 Mt y^{-1}. A recent study reported that 270 Kt of plastic residues are adrift in the ocean (Ericksen et al., 2014). In other hand, several types of organic residues, obtained in huge quantities, deriving from agriculture, farming or food industry activities, could be used as substrate for different processes after a pretreatment. Organic waste can be transformed into short-chain carboxylates as intermediate feedstock chemicals, using hydrolysis and fermentation with undefined mixed cultures. The short-chain carboxylates also known as volatile fatty acids (VFA) – acetate, propionate, butyrate and valerate – are the main organic products (Agler et al. 2011).
There are several studies about the polyhydroxyalkanoates (PHA) production from acetic (Wang and Yu, 2000), propionic (Kim et al., 1992), butyric (Quillaguamán et al., 2006) and valeric (Page et al., 1992) acid, separately. However the production of a single acid, and its purification, raise the price enormously. PHA are the best alternative to petroleum-based plastics since their properties are similar, although PHA present some special characteristics like biodegradability or biocompatibility (Steinbüchel, 1992). The main drawback is the high production costs compared to petroleum-based plastics, however the use of a cheap substrate, such as VFA from anaerobic acidogenesis of waste, will help to reduce this cost (Amache et al., 2013). PHA are polyesters, which are intracellularly accumulated by the cells as carbon and energy reserves. Among them, 3-hydroxybutyrate (3HB) is the most common, but also stiff and brittle. The incorporation of co-monomers with longer carbon chains like 3-hydroxyvalerate (3HV), modify the thermomechanical properties improving its flexibility and resistance (Barham et al., 1992).
There is a vast research on the PHA production from VFA with mixed cultures. Due to their metabolic flexibility, they adapt better than pure cultures to non-sterilized complex substrates (Salehizadeh and Van Loosdrecht, 2004). Nevertheless, the main drawback of mixed cultures is the low productivity and biomass

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concentration that is possible to obtain. Meanwhile, it has been reported the highest productivity using pure cultures grown on different carbon sources (Lee et al., 1997).
The halophilic bacterium *Halomonas boliviensis* was reported as a competent PHA producer (Quillaguamán et al., 2008). The high salt content necessary for growth could allow the use of cheaper materials since no sterilization process is needed. Besides, its high tolerance to alkali could avoid the inhibition by VFA, since at a higher pH of the medium the dissociated form of the acid is the preferred uptaken species, which has a lower toxic effect than the undissociated one.
The aim of this research was to evaluate the performance of the bacterium *H. boliviensis* using VFA as carbon source for producing PHA copolymers.

2. Material and methods

2.1 Bacterial strain, maintenance and seed culture

Throughout this study, the Gram-negative, moderately halophilic bacterium *H. boliviensis* LC1 (ATCC® BAA-759™) was used. It was maintained at 4 ºC on solid HM medium (Quillaguamán et al. 2004). *H. boliviensis* was grown in seed culture medium, containing (per litre): 10 g glucose, 45 g NaCl, 1.4 g $MgSO_4 \cdot H_2O$, 0.55 K_2HPO_4, 2.3 g NH_4Cl, 0.005 g $FeSO_4 \cdot 7H_2O$, 3 g monosodium glutamate (MSG) and 15 g Tris. Inoculum was prepared by transferring cells from solid into liquid medium. Seed culture was incubated at 30 ºC for 22 h with a rotary shaking of 200 rpm.

2.2 Flask scale fermentations

Three different VFA mixtures were tested at flask scale (Table 1). Flask fermentations were performed in two stages. During the first 24 h, glucose was used as carbon source. In this first stage, culture media contained (per litre): 20 g glucose, 45 g NaCl, 4 g NH_4Cl, 2.8 g $MgSO_4 \cdot H_2O$, 2.2 K_2HPO_4, 0.005 g $FeSO_4 \cdot 7H_2O$ and 20 g MSG. Then, biomass was harvested by centrifugation and transferred to a new flask with nearly the same culture media, in which glucose was replaced by VFA mixture and NH_4Cl concentration was decreased to 0.25 g L^{-1}, in order to provoke PHA accumulation by the nitrogen limitation. The pH was adjusted to 7.5 and cultures were incubated at 30 ºC with a rotatory shaking of 250 rpm.

2.3 Bioreactor scale fermentations

Scale-up cultivations were performed in a 2 L Biostat® Bplus bioreactor equipped with pH, dissolved oxygen, temperature and foam probes, using a working volume of 1.5 L. Temperature was maintained at 30 ºC through the vessel jacket. Culture media was the same used in flask scale. Two-stage fed-batch operation was followed. During the first stage, glucose was used as carbon source until nitrogen exhaustion. The second stage took place under nitrogen limitation and the carbon source fed was a synthetic mixture of VFA. Initial aeration and agitation rates were 1 L min^{-1} and 400 rpm, respectively. The maximum air inflow rate and agitation attained were 4 L min^{-1} and 650 rpm, respectively. The pH was maintained in 7.5 by the automatic addition of 5 mol L^{-1} NaOH/HCl. Foam was controlled by the addition of antifoam A (Sigma), when it was necessary.

2.4 Quantitative analyses

Cell dry weight (CDW) or total dry weight was determined from 10 mL cell suspension samples harvested by centrifugation in a previous weighed tube, washed twice with distilled water, and freeze-dried for 24 h to constant weight. First supernatant was kept for further analysis. Residual biomass or residual cell mass (RCM) was defined as the total dry weight minus PHA. PHA content of cells, expressed as the percentage of PHA with respect to the total CDW (wt/wt), was determined by gas chromatography (GC). Cell mass was treated by acidic methanolysis as described before (Brandl et al. 1988).
The concentrations of glucose, VFA and ammonium were quantified from the sample supernatant. Glucose concentration was measured by the dinitrosalicylic acid (DNS) method (Miller, 1959). Ammonium concentration was measured following the phenol method (4500-NH_3 F) (Standard Methods APHA, 1998), using a Shimadzu UV-1800 spectrophotometer. The organic acids were measured by an HPLC (1100 Agilent with an IR detector) using an Aminex HPX-87H column (Bio-Rad), operated at 35ºC with 10 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL min^{-1}. All the analytical tests were performed in triplicate.

3. Results and discussion

It is known that pH plays an important role in anaerobic acidogenesis. It was previously demonstrated that by modifying the pH of the medium it is possible to modulate the profile of the main products obtained during the process (Zoetemeyer et al. 1982). Under acidic and neutral conditions, the main products were butyric acid, while acetic and propionic acid were the main products under basic conditions. In Table 1, the compositions of three different VFA mixtures obtained from anaerobic acidogenesis reactors are summarized. VFA 1 and 2

mixtures were taken from Horiuchi et al., 2002 and VFA 3 was obtained from Palmeiro-Sánchez T. (personal communication).

Table 1: Composition of the VFA mixtures.

VFA (% wt/wt)	VFA 1	VFA 2	VFA 3
Acetic acid	53.7	20.9	58.4
Propionic acid	32.7	1.3	24.5
Butyric acid	7.3	74.7	6.2
Ethanol	6.3	3.1	--
Valeric acid	--	--	10.9

For shake flask experiments, *H. boliviensis* was grown first on glucose during 24 h to obtain a high biomass concentration. Despite nitrogen was not in limiting concentrations, PHA was accumulated to 29.4 ± 6.1 % and only the HB monomer was detected. After 24 h, biomass was transferred to a new media with the different VFA mixtures as carbon source. A control experiment, in which VFA was replaced by glucose was also carried out. After 72 h on the new media, HB monomer was increased in all the cases, but HV was detected only with VFA 1 and 3. In Figure 1, the increment of HB, the HV production between the initial point and the 72 h and the residual biomass increment are represented. During accumulation phase, control experiment (growth on glucose) presented the highest PHB production and no copolymer was obtained. It was previously reported that acetic and butyric acid produce higher PHA accumulations than acetic and propionic acid mixtures for *Ralstonia eutropha* (Yu et al., 2002), however, in this study the opposite behaviour was observed with *H. boliviensis* (Figure 1A). VFA 1 (rich in acetic and propionic acid) reported a PHB increment 5 times higher than the obtained with VFA 2 (rich in acetic and buryric acid).

Copolymer (PHB-co-PHV) formation by the addition of precursors like propionic or valeric acid was previously studied with other microorganisms (Doi et al., 1988). With *H. boliviensis*, the PHV formation was obtained with the VFA 1 and 3 mixtures, which have both a high propionic acid concentration (Figure 1B). However the highest HV fraction (17.5 mol %) was obtained with the VFA 3 mixture, which was the only one that contained valeric acid.

Yu et al., 2002 established that *R. eutropha* preferred propionic acid for cell mass synthesis and butyric acid for polymer synthesis. However, in this study, VFA 3 reported the highest increase in cell mass (Figure 1C).

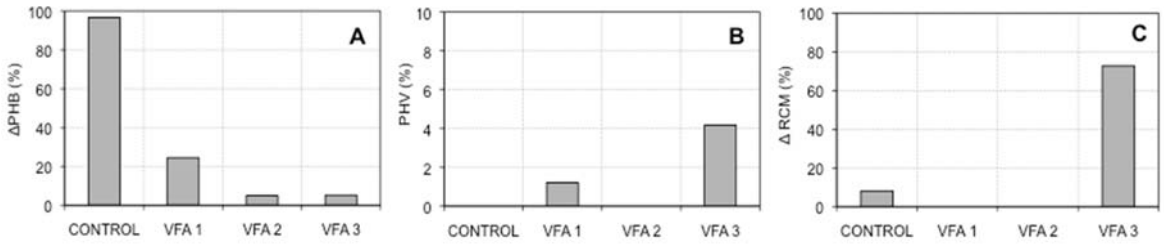

Figure 1: Increase in PHB concentration (A), accumulation of PHV (B) increase in RCM during the accumulation phase (C) with the different carbon sources tested at flask scale.

Since the VFA 3 mixture reported the highest HV fraction into the copolymer accumulation, this substrate was chosen to scale-up the process. Once more, a synthetic mixture was used during the fed-batch operation of the bioreactor in two stages. First of all, glucose was fed into the bioreactor until the nitrogen source was exhausted, then the VFA mixture started to be added to the bioreactor (Figure 2). According to Figure 2A, PHA production started from the beginning of the process. Nitrogen exhaustion took place between 21 and 24 h and then glucose feed was replaced by the VFA 3 mixture. PHA reached the highest accumulation peak (71 %) after 42 h and it was maintained constant until the end of the fermentation.

As observed in Figure 2B, the total glucose consumed during the first 27 h reached 50 g L^{-1} and no remaining glucose was detected afterwards. During the next 38 h, *H. boliviensis* used VFA as carbon source, with a total uptake of 70 g L^{-1}, from which acetic acid was the most consumed one (46 g L^{-1}), followed by propionic acid (16 g L^{-1}). These two acids were the most abundant in the VFA mixture.

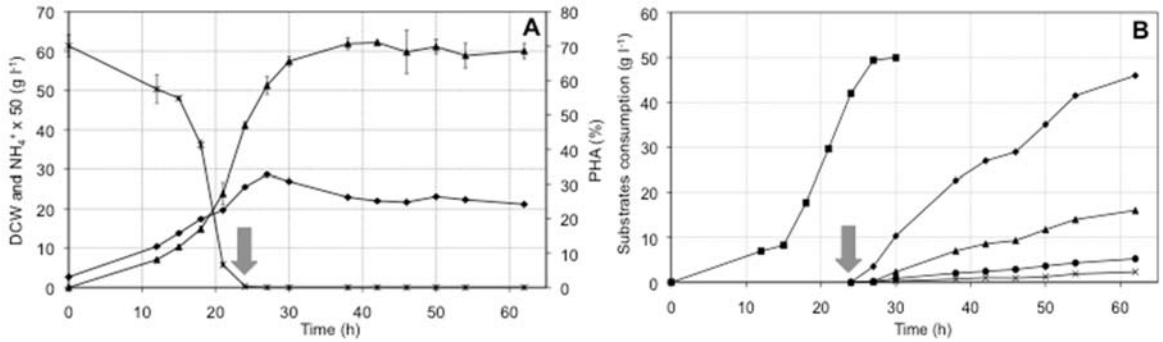

Figure 2: Time course of the PHA production at bioreactor scale. Figure A depicts the total dry cell weight production (rhombuses), PHA accumulation (triangles) and ammonia exhaustion (crosses). Figure B shows the consumption of carbon sources, glucose (squares), acetic (rhombuses), propionic (triangles), valeric (circles) and butyric (crosses) acid. Grey arrow indicates the feed switch from glucose to VFA mixture in both cases.

Copolymer P(3HB-co-3HV) was also detected at bioreactor scale. As it can be observed in Figure 3, HV monomer appeared after 24 h, in coincidence with the feed switch from glucose to VFA and increased until the end of the fermentation. Contrary to that observed at flask scale, biomass did not seem to be at optimal conditions for its own maintainance, since residual biomass decreased considerably in the presence of acids, and thus PHA concentration suffered a smaller decrease as well. Copolymer composition evolved during the time course fermentation, with the increase of the HV fraction.

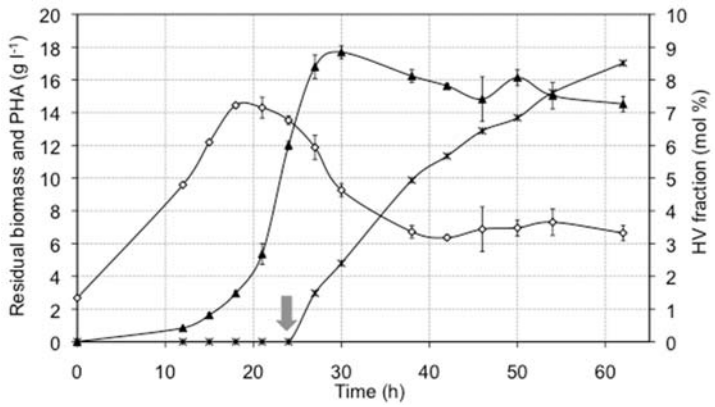

Figure 3: Evolution of the residual biomass (white rhombuses), PHA concentration (black triangle) and copolymer HV fraction (crosses) evolution during the fed-batch fermentation at bioreactor scale. Grey arrow indicates the feed switch from glucose to VFA mixture.

After 62 h of operation in fed-batch mode, 21 g L^{-1} of total biomass was obtained, from which 70 % was PHA. The type of PHA accumulated was a copolymer of P(3HB-co-3HV) with a HV content of 8.5 mol %.

Table 2 shows an overview of various PHA production studies from VFA mixtures obtained with anaerobic acidogenesis from wastes or synthetic VFA mixtures that simulate the real ones. The PHA concentration and HV fraction obtained in this study are among the highest values published so far, with the exception of the recent study from Huschner et al., 2015, that reported amazing higher results than the other studies. Their fermentation strategy is based on a high cell density process for *R. eutropha* H16, using different control techniques.

Table 2: Summary of PHA production studies from VFA mixtures as the main carbon sources at bioreactor lab scale. NG: Not given.

Microorganism	CDW (g/L)	PHA (%)	PHA (g/L)	HV (mol %)	Reference
R. eutropha	10.0	63.3	6.3	NG	Jin et al., 1999
R. eutropha	3.5	34.1	1.2	NG	Yu, 2001
R. eutropha	15.9	62.9	10.0	10.7	Ruan et al., 2003
R. eutropha	14.3	48.0	6.9	NG	Yan et al., 2003
R. eutropha	22.7	72.6	16.5	2.8	Du et al., 2004
R. eutropha	12.0	90.0	10.8	NG	Hong et al., 2009
Comamonas sp.	7.0	82.0	5.7	NG	Mumtaz et al., 2010
R. eutropha	112.0	83.3	93.3	5.6	Huschner et al., 2015
H. boliviensis	21.0	70.0	16.0	8.5	This study

Studies with mixed cultures using real VFA mixtures reported high PHA contents; such as 0.75 gPHA $gVSS^{-1}$ or 0.77 gPHA g VSS^{-1} using fermented molasses (Albuquerque et al., 2010) or fermented paper mill wastewater (Jiang et al., 2012), respectively. However, total biomass concentration (measured as volatile suspended solids, VSS) was much lower (between 5.1 and 2.4 g L^{-1}) than the values obtained in this study. Besides, the downstream process for the PHA recovery of a highly diluted stream will increase production costs.

4. Conclusions

The great flexibility of the anaerobic acidogenesis process is considered an advantage in this study, since it allow us to obtain different mixtures of acids in different proportions. Thus, according to the PHA producer used, it could be possible to adapt the carbon source composition to the metabolic requirements of the microorganism in order to obtain a specific PHA composition. In this study, it was demonstrated that the halophilic bacterium, *H. boliviensis*, is able to grow and accumulate different kinds of PHA using such a complex and toxic substrate like VFA. The presence of hydroxyvaleric fraction into the copolymer P(3HB-co-3HV) changes its properties and characteristics and this is directly related to the future application of the bioplastic. By applying a fed-batch fermentation in two stages it was possible to increase the HV fraction of the copolymer without a significance change in the total PHA content. According to the specific use of the biopolymer, the process could be stopped at any moment, depending on the required HV fraction. Our long-term objective is to produce PHA from a real VFA stream obtained from waste in an anaerobic acidogenesis reactor. This would reduce the costs of producing PHA and enlarge the types of PHA produced.

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