

Effect of the fractions of beeswax hydrolysates as carbon source in the synthesis of short and medium chain polyhydroxyalkanoates (scl-mcl-PHA)

Efecto de las fracciones de hidrolizados de cera de abeja como fuente de carbono en la síntesis de Polihidroxialcanoatos de cadena corta y media (scl-mcl-PHA)

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Abstract

This study analyzed the synthesis of short and medium chain length polyhydroxyalkanoates (scl-mcl-PHA) from fractions of beeswax hydrolysates (Hw). The fermentations were carried out in batch-fed cultures of three stages with a strain of *Cupriavidus necator*. Glucose and ammonium sulfate were used as carbon and nitrogen sources in the first and second stage of culture. During the third stage (mcl-PHA production), fresh culture medium was fed with fractions of Hw (5 g L⁻¹) as a cosubstrate, in addition of Triton X-100 (3 CMC). The accumulation of scl-mcl-PHA from the fraction of waxy alcohols of Hw (OH Hw) was 20.85 g L⁻¹ (90.19 % w/w) while from the fraction of fatty acids of Hw (AG Hw) was 5.57 g L⁻¹ (67.28 % w/w). ANOVA of one factor was performed to quantitatively compare the intracellular amount of synthesized scl-mcl-PHA. Dunnett's multiple comparisons test established the time intervals, in which *C. necator* was able to produce a higher percentage of polymer. The presence of 3-hydroxybutyrate (3HB) and 3-hydroxydecanoate (3HD) in the scl-mcl-PHA synthesized was determined by GC.

Biopolymers, *Cupriavidus Necator*, Fed batch

Resumen

Este estudio analizó la síntesis de polihidroxialcanoatos de cadena corta y media (scl-mcl-PHA) a partir de fracciones de hidrolizados de cera de abejas (Hw). Las fermentaciones se llevaron a cabo en lotes alimentados de tres etapas con *Cupriavidus necator*, usando como fuentes de carbono y nitrógeno, glucosa y sulfato de amonio en la primera y segunda etapa de cultivo. Durante la tercera etapa (producción de mcl-PHA) se alimentó medio de cultivo con fracciones de Hw (5 g L⁻¹) como cosustrato, en adición de Tritón X-100 (3 CMC). La acumulación de scl-mcl-PHA a partir de la fracción de alcoholes cerosos de Hw (OH Hw) fue de 20.85 g L⁻¹ (90.19 % p/p) mientras que a partir de la fracción de ácidos grasos de Hw (AG Hw) fue de 5.57 g L⁻¹ (67.28 % p/p). Se realizó un ANOVA de un factor para comparar cuantitativamente la cantidad de scl-mcl-PHA sintetizado. La prueba de Dunnett, estableció los intervalos de tiempo, en que *C. necator* produce mayor porcentaje de polímero. Se determinó por CG, la presencia de 3-hidroxibutirato (3HB) y 3-hidroxidecanoato (3HD) en los scl-mcl-PHA sintetizados.

Biopolímeros, *Cupriavidus Necator*, Lote alimentado

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Introduction

Polyhydroxyalkanoates (PHA) are microbial polyesters produced as energy reserve materials when there is an imbalance of nutrients (nitrogen, phosphorus, magnesium, potassium, sulfur and oxygen) and an increase in the concentration of carbon in the culture medium (González-García et al., 2013). These materials are biodegradable and also have thermoplastic and elastomeric characteristics similar to petroleum-based plastics.

Structurally, they are classified based on the number of carbon atoms in the length of the aliphatic chain of their monomers, such as short chain PHA (scl-PHA) with 3-5 carbon and medium chain PHA (mcl-PHA) with 6 - 14 carbons. (González-García et al., 2013). The bacteria producing mcl-PHA can synthesize them from fatty acids or their salts (Impallomeni et al., 2011), aliphatic alkanes, alkenes (Kim et al., 1995) or agroindustrial residues (Lerch et al., 2011). *Cupriavidus necator* (formerly *Wautersia eutropha* > *Ralstonia eutropha* > *Alcaligenes eutrophus*) PHA form with yields of 80 - 90% of accumulated polymer in relation to the weight of its biomass in dry basis (Lerch et al., 2011).

There are few reports that native *C. necator* (ATCC 17699) can synthesize mcl-PHA from fatty acids and derivatives, because their PHA synthase is strictly specific to obtain scl-PHA monomers (Slater et al., 1992). Verlinden et al., (2011), synthesized PHB from *C. necator* H16 (ATCC 17699) fed with residual frying oils, obtaining 1.2 g L⁻¹ in 72 h of culture. Jain et al., (2013); confirmed that *C. necator* H16 can synthesize copolymers such as P (3HB-co-3HHx) from crude kernel palm oil (abundant in palmitic acid), as the sole carbon source in concentrations of 5 g L⁻¹ under the limitation of nitrogen and without precursor compounds.

The polymer obtained represented 63% of the dry weight in the form of P (3HB-co-3HHx), with 4 mol% of 3HB. Regarding the carbon source, some substrates, such as beeswax, have not been reported for PHA synthesis. Beeswax is a renewable source used as an additive for ointments, creams and ointments. However, the residual waxes of unpurified bee are used for the molding of jewelry, the manufacture of adhesive tapes and insulators (León et al., 2014) that are generally discarded after their use.

Chemically, beeswax is composed of waxy esters that can be hydrolyzed with alkalis to obtain fatty acids (such as palmitate) (Buchwald et al., 2009) and long-chain monohydric alcohols (Maia and Nunes, 2013), which can be used as substrates for PHA synthesis. The objective of this work was to evaluate the yields of synthesis and incorporation of mcl-PHA monomers synthesized from separate fractions of bee wax hydrolysates (Hw), during the accumulation phase of a crop per batch fed, of three stages using *C. necator*.

Methodology

Microorganism and culture medium. *C. necator* ATCC 17699 was grown in Luria Bertani broth (LB) at 30 ° C and 150 rpm. This culture was used as a seed flask to inoculate 10% (v / v) of the reactor. The cultures were performed in triplicate in a 5 L fermentation unit (FA-5000, VICHI). The temperature was maintained at 30 ° C and the pH was controlled with 2 M NaOH and 0.47 M HCl.

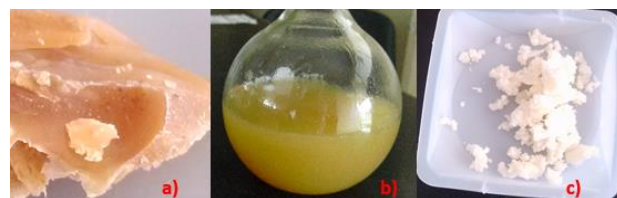


Figure 1 Fractions obtained from the alkaline hydrolysis of beeswax: a) beeswax b) fatty acids, c) waxy alcohols

Samples were taken every 3 h to quantify the concentrations of glucose, residual ammonium, biomass and PHA production. The fermentations were made from the modified method of López-Cuellar et al., (2011). To assist the bioavailability of the Hw fractions, 0.43 g L⁻¹ of Triton X-100 was added to 3 CMC (Budde et al., 2011).

Alkaline hydrolysis of beeswax

The beeswax was previously treated to alkaline hydrolysis by the method described by Wás et al., (2014). Hydrolysis was carried out by placing 20 g of beeswax at reflux (24 h at 100 ° C), 10% w / v of sodium hydroxide (Bonaduce et al., 2004) and 300 mL of a tetrahydrofuran (THF) mixture.) - methanol 1: 1 as a solvent (Wang and Advincula, 2001). Once the fractions were obtained, they were separated by filtration while still hot.

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The solid corresponded to the fraction of long-chain alcohols of waxy esters (OH Hw) (Bonaduce et al., 2004). To the remaining NaOH solution (salts of fatty acids) was added HCl (ratio 2: 1) until obtaining a pH of 6.0 (Buchwald et al., 2009), forming a yellow product corresponding to the fraction of fatty acids of the esters waxy (AG Hw) (Figure. 1). Both fractions were dried for 24 h at 105 ° C.

Analytical procedures

The concentrations of cell mass (X) and residual biomass (rX) were determined by gravimetry (Budde et al., 2011). The glucose and ammonium measurements were made using the dinitrosalicylic acid (DNS) methods (Miller 1959) and Weatherburn (1967) respectively. The amount of Hw was determined according to the method described by Quintanar-Gómez et al., (2017).

Purification of scl-mcl-PHA

The scl-mcl-PHA were purified by extraction with chloroform at reflux for 10 minutes and precipitated with cold hexane after filtering the cellular debris (Rozsa et al., 2004). The procedure was repeated three times to avoid the presence of residual cellular and residual metabolites of Hw.

Chemical characterization of scl-mcl-PHA

The scl-mcl-PHA samples were subjected to prior methanolysis to obtain the fatty acid monomers (Impallomeni et al., 2013). A Thermo Scientific GC model Trace 1310 was used, equipped with a flame ionization detector and a Thermo scientific capillary column (30 m × 0.32 mm, a film thickness of 1.0 µm). The carrier gas was He (flow of 1 mL / min). The programmed temperature ramps were: initial temperature of 80 ° C for 4 min., Followed by an increase to 260 ° C with a ramp of 8 ° C / min, staying for 23 min. Volumes of 1 µL of the organic phase were injected (10: 1 division ratio) (Rathinasabapathy et al., 2013).

Statistic analysis

The obtained values were subjected to a statistical analysis of variance (ANOVA) of a factor to quantitatively compare the intracellular amount of scl-mcl-PHA between synthetic treatments from the Hw fractions.

A Dunnett's test was complemented to analyze the difference of inter and intra-treatments means and determine the stage of highest production of mcl-PHA. The statistical software (IBM SPSS Statistics version 21.0) was used, establishing a value of $\alpha = 0.05$.

Results and Discussion

Alkaline hydrolysis of beeswax

The yield obtained for the fraction soluble in organic solvent corresponded to 28.5% grams of dry matter (% gms) of saponifiable fatty acids, which were calculated from the weight difference between the original sample and the hydrolyzate obtained.

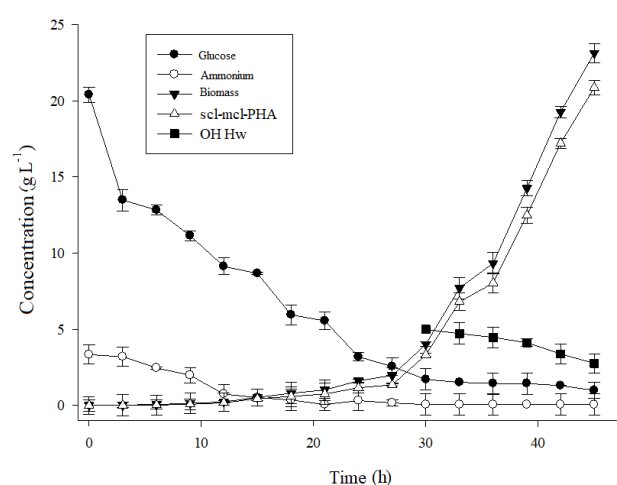


Figure 2 Consumption of glucose, ammonium, OH Hw, biomass production and scl-mcl-PHA with *C. necator*

The non-polar phase was separated by vacuum filtration, forming a white solid (Figure. 1), corresponding to the fraction of long-chain alcohols of beeswax esters (71.5% gms) (Bonaduce et al., 2004).

Synthesis of scl-mcl-PHA from fractions of OH Hw

Figure 2 shows the kinetic profile of the fermentation with the fraction of OH Hw. *C. necator* reached a concentration of 1.02 g L⁻¹, a lag phase of 15 h and a maximum specific growth rate (μ) of 0.21 h⁻¹.

The initial ammonium concentration in the culture was 3.38 g L⁻¹, with a consumption rate of 0.52 g L⁻¹ h⁻¹ (21 hours) (Khanna and Srivastava, 2007). Once the ammonium was exhausted, the first batch was fed (second stage of cultivation) with glucose and ammonium as sources of carbon and nitrogen (ratio C / N = 12).

The feeding rate was maintained at 1.46 g h⁻¹, increasing the cell density from 1.02 to 4.02 g L⁻¹ (21 to 30 h of culture respectively). The glucose and ammonium consumption rates were 0.21 and 0.13 g L⁻¹ h⁻¹. After 30 hours of culture, the residual glucose and ammonium concentrations decreased to 1.68 g L⁻¹ and 0.69 g L⁻¹ respectively (Figure. 2).

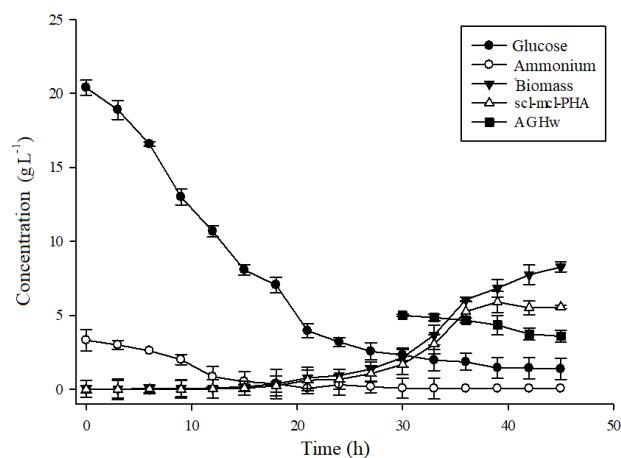


Figure 3 Consumption of glucose, ammonium, AG Hw, production of biomass and scl-mcl-PHA with *C. necator*

The second batch was fed (third stage of culture) with the fraction of OH Hw (5 g L⁻¹, C/N 200) in the presence of 0.43 g L⁻¹ of Triton X-100, obtaining a consumption rate of 0.44 g L⁻¹ h⁻¹. Shang *et al.*, (2004); reported the same consumption rate when feeding glucose and valeric acid (< 1.20 g L⁻¹) to produce P (HBHV) with *R. eutropha* NCIMB 11599. The fraction of OH Hw decreased (from 5.0 to 2.77 g L⁻¹), Seeing an increase in biomass concentration from 4.02 to 23.13 g L⁻¹ as well as the intracellular concentration of scl-mcl-PHA (from 3.32 g L⁻¹ to 20.85 g L⁻¹) during the last 15 hours of cultivation (Khanna y Srivastava, 2007).

The accumulation of OH Hw is due to residual glucose as additional co-substrate, causing the waxy carbon source to become the second source of energy. The samples collected after 42 h of culture showed 89 to 90% w / w of scl-mcl-PHA. Lee *et al.*, (2008); obtain the same values (90% w / w) by synthesizing P (3HB-co-3HV) with *C. necator* using kernel palm oil (5 g L⁻¹) and sodium propionate (5 g L⁻¹).

Synthesis of scl-mcl-PHA from fractions of AG Hw

When analyzing the incorporation of medium chain monomers from AG Hw fraction, it was observed that the concentration of biomass during the first 21 h of culture was 0.77 g L⁻¹. The lag phase was 12 hours with a μ of 0.18 h⁻¹. This concentration increased to 2.14 g L⁻¹ at 30 h of culture, after the first feeding with glucose and ammonium at a C / N = 12 ratio. The fresh medium feed flow in this stage was 2.27 g h⁻¹ with consumption rates of 2.40 g h⁻¹ and 0.51 g h⁻¹ for glucose and ammonium respectively.

After 30 h (residual ammonium consumption), the second feed was started with the AG fraction Hw. The amount of remaining residual glucose was 2.29 g L⁻¹, its consumption being observed up to a concentration of 1.36 g L⁻¹ (Figure. 3). This behavior shows that the bioavailability and the molecular size of the carbon source are factors that favor the consumption of glucose preferably to an alternative carbon source. (Lee *et al.*, 2008).

The maximum biomass concentration increased by supplying 5 g L⁻¹ of the AG Hw fraction; while the fatty acid fraction of Hw decreased in the culture medium 1.41 g L⁻¹ during the last 15 h of cultivation, with a consumption rate of 0.28 g h⁻¹. The final concentration of biomass produced during the synthesis of scl-mcl-PHA (8.27 g L⁻¹) from the fatty acid fraction of Hw, it was lower than that obtained with the waxy alcohol fraction of Hw (23.13 g L⁻¹).

When feeding the AG Hw fraction at 30 h of culture, an increase of 78% w / w to 86% w / w of scl-mcl-PHA of the generated biomass was observed, during the first 9 h of feeding of the co-substrate. Subsequent to 39 h of culture the intracellular decrease of scl-mcl-PHA was observed (Table 4). This behavior shows an adverse effect to that described by Lee *et al.*, (2008) who assume that the absence of acetyl-CoA decreases the availability of CoASH and as a consequence the production of acetoacetyl-CoA by 3-ketothiolase.

Statistical analysis of scl-mcl-PHA synthesis treatments

The contrast of equality of population variances of the scl-mcl-PHA concentrations, determined that there is a statistical difference between the synthesis yields obtained between experimental treatments. Table 1 shows that the level of significance is greater than 0.05 in the experiments carried out with the fractions of AG Hw and OH Hw.

The significance values obtained show that *C. necator* produces different amounts of scl-mcl-PHA as a function of the fraction of Hw fed as a carbon source. The treatment with OH Hw, presented the highest value of mcl-PHA, while the concentrations of polymer obtained with AG Hw were lower.

	Statistic of Levene	gl1	gl2	Next
PHA OH Hw	2.768	5	12	0.069
PHA AG Hw	2.703	5	12	0.073

Table 1 Variance homogeneity test for the quantification of scl-mcl-PHA synthesized from Hw fractions
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
gl1: degrees of freedom between groups
gl2: intra-group degrees of freedom
Sig.: significance

The ANOVA, determined that the levels of significance between classes were less than 0.05, for which the hypothesis of equality of means is rejected ($H_0: \mu_1, OH\ Hw = \mu_2, AG\ Hw$). In Table 3 (Dunnnett's chart), the comparison of means between intergroup concentrations of scl-mcl-PHA obtained with the fractions of Hw fed is made, determining that there is statistical difference, when comparing the intervals between 30 to 39 h against the 45 h of cultivation (considered as reference for obtaining the highest concentration of biomass).

This suggests the production of scl-mcl-PHA is ascending in the experiment with the fraction of OH Hw fed in the third stage of culture.

	Sum of squares	of gl	Half quadratic	F	Next
PHA OH Hw	268.944	5	53.789	328.56	0.000
Inter-groups	1.964	12	0.164		
Intra-groups	270.908	17			
Total					
PHA AG Hw	44.164	5	8.833	432.84	0.000
Inter-groups	0.245	12	0.020		
Intra-groups	44.409	17			
Total					

Table 2 One-way ANOVA for the quantification of scl-mcl-PHA synthesized with *C. necator* from fractions of Hw
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
gl: degrees of freedom
Sig: significance

The statistical analysis allowed us to infer that the fraction of AG Hw is less assimilated compared to that of OH Hw during the synthesis of the polymer. The highest value of difference of means between experiments was obtained at 39 h of culture with AG Hw. This shows that the concentration of scl-mcl-PHA decreases, as a consequence of the poor assimilation of the waxy carbon source after 39 h of culture.

Dependent variable	(I) Time	(J) Time	Difference of means (I-J)	Typical error	95% confidence interval	
					Lower limit	Upper limit
PHA OH Hw	30	45	-11.16000*	0.330	-12.1184	10.2015
	33	45	-8.91366*	0.330	-9.8721	-7.9552
	36	45	-8.21933*	0.330	-9.1778	-7.2608
	39	45	-5.41766*	0.330	-6.3761	-4.4592
	42	45	-2.35733*	0.330	-3.3158	-1.3988
PHA AG Hw	30	45	-3.88266*	0.116	-4.2210	-3.5442
	33	45	-2.51366*	0.116	-2.8520	-2.1752
	36	45	-.30500	0.116	-.6434	0.0334
	39	45	.33466	0.116	-0.0037	0.6730
	42	45	-.05866	0.116	-0.3970	0.2797

Table 3 Multiple comparisons: Dunnnett's test for the quantification of scl-mcl-PHA synthesized from fractions of Hw
PHA OH Hw: Treatment with waxy alcohols of Hw.
PHA AG Hw: Treatment with fatty acids of Hw.
*. The difference in means is significant at the 0.05 level

When comparing the values of difference of intergroup means, it is concluded that the treatment with the highest intracellular concentration of scl-mcl-PHA, was the fermentation of OH Hw at 45 h of culture (Table 3).

Characterization by GC of scl-mcl-PHA monomers synthesized with Hw fractions

The GC analysis of scl-mcl-PHA hydrolysates synthesized with OH Hw (Table 4) confirmed the incorporation of a 12 C monomer (determined as 3 HDD by 1 H NMR and 13 C by 400 MHz in a previous work (Quintanar-Gómez et al., 2017; data not shown) with a retention time of 12.5 min. The% mol of 3HDD was 1.7 in relation to that of 3HB (98.3% mol). For scl-mcl-PHA obtained from AG Hw, the retention time was 12.38 min; close to the oleic acid standard (13.0 min).

The percentage composition of each monomer was 97 mol% of 3HB and 3 mol% of 3HD (Table 4). The% mol of mcl-PHA was attributed to the previous accumulation of PHB in the second stage of cultivation, generating a metabolic deceleration effect, which determines the low assimilation of the Hw fractions (Khanna and Sirvastava, 2007). The concentration of mcl-PHA obtained was higher than that reported by Rathinasabapathy et al., (2013); who synthesized a scl-mcl-PHA, with 99.81% mol of 3HB, 0.06% mol of 3HV, 0.09 mol% of 3HHx, 0.04 mol% of 3HO using canola oil. These results confirmed that *C. necator* incorporates mcl-PHA from fractions of Hw.

Fatty acid	Holding time Reference	scl-mcl-PHA	
		OH Hw	AG Hw
Butyric acid	2.5	2.5	2.5
Valeric acid	6.25	0	0
Octanoic acid	9.4	0	0
Oleic acid	13.0	12.5	12.38
Palmitic acid	13.2	0	0

Table 4. Butyric acid
Valeric acid
Octanoic acid
Oleic acid
Palmitic acid.

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Conclusions

Cupriavidus necator assimilated the Hw fractions separately as carbon sources added during the third stage of cultivation of the batch fed. The low production of mcl-PHA was due to the metabolic deceleration caused by the residual glucose of the culture medium.

Both Hw fractions allowed to incorporate medium chain monomers, during the synthesis of scl-mcl-PHA, confirmed from the CG analysis. At the end of the synthesis processes, both residual fractions of Hw, could be separated by flocculation, to be reused again in later fermentations. These materials promise expectations when used in the biomedical field of tissue regeneration or controlled release of drugs, due to their biocompatible potential and the improvement of their chemical properties in relation to crystalline materials such as PHB.

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