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ORIGINAL PAPER

Cassava wastewater as a substrate for the simultaneous production of rhamnolipids and polyhydroxyalkanoates by *Pseudomonas aeruginosa*

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Abstract Glycerol, cassava wastewater (CW), waste cooking oil and CW with waste frying oils were evaluated as alternative low-cost carbon substrates for the production of rhamnolipids and polyhydroxyalkanoates (PHAs) by various *Pseudomonas aeruginosa* strains. The polymers and surfactants produced were characterized by gas chromatography–mass spectrophotometry (MS) and by high-performance liquid chromatography–MS, and their composition was found to vary with the carbon source and the strain used in the fermentation. The best overall production of rhamnolipids and PHAs was obtained with CW with frying oil as the carbon source, with PHA production corresponding to 39% of the cell dry weight and rhamnolipid production being 660 mg l^{-1}. Under these conditions, the surface tension of the culture decreased to 30 mN m^{-1}, and the critical micelle concentration was 26.5 mg l^{-1}. It would appear that CW with frying oil has the highest potential as an alternative substrate, and its use may contribute to a reduction in the overall environmental impact generated by discarding such residues.

Keywords Cassava wastewater · Glycerol · Polyhydroxyalkanoates · *Pseudomonas aeruginosa* · Rhamnolipids · Waste cooking oil

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Introduction

Pseudomonas aeruginosa produces glycolipidic molecules, rhamnolipids, which comprise a well-studied family of microbial surfactants, and polyhydroxyalkanoates (PHAs), which can be used as biodegradable thermoplastics. Rhamnolipids are composed of a hydrophilic portion, with one or two rhamnoses, and a hydrophobic fatty acid moiety composed of a dimer of 3-hydroxyalkanoate, the monomeric unit of PHAs (Fig. 1). In recent years, biosurfactants and PHAs have been receiving a great deal of attention owing to their low toxicity, biodegradability and relatively better environmental compatibility [22, 28]. Rhamnolipids have a wide variety of applications in the food, cosmetic, pharmaceutical, chemical and agricultural industries as anti-adhesive and antimicrobial agents, as emulsion-forming and stabilizing agents and for the cleaning of oil tankers [24]. Polyhydroxyalkanoates can be used in the manufacture of bottles, films and fibers for applications as biodegradable packaging agents [16].

The feasibility of producing PHAs and rhamnolipids simultaneously has been studied and represents one potential approach for reducing the production costs of both products [13, 20]. The recovery of PHAs requires the disruption of valuable biocatalysts of bacterial cells, the production costs of which comprise a large portion of the total cost of the fermentation process. In contrast, the production of secreted

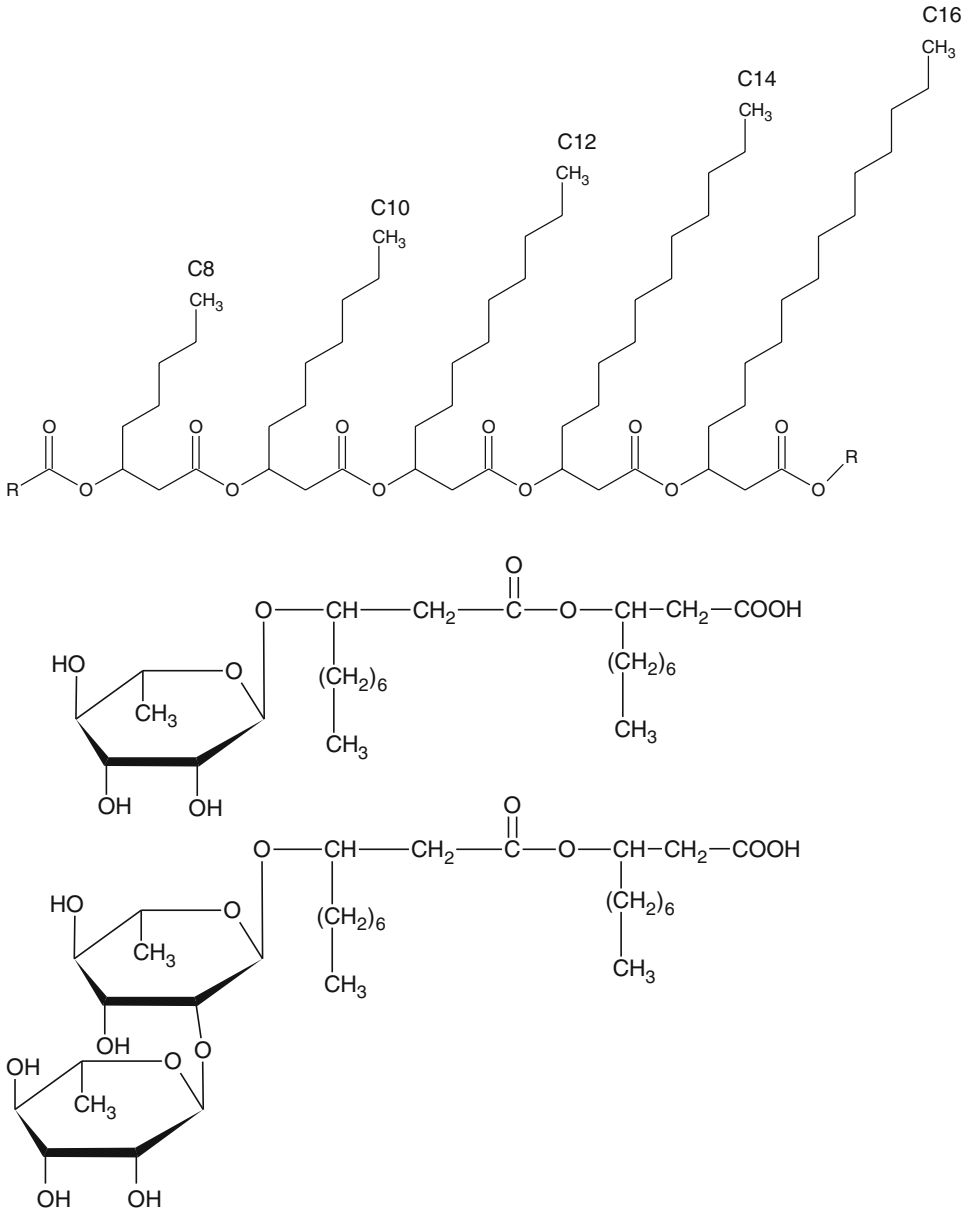

Fig. 1 General representation of polyhydroxyalkanoate (PHA) (*top*) and mono- (*middle*) and di-rhamnolipids (*bottom*)

materials, such as rhamnolipids, in combination with PHA production is expected to enhance the availability of biocatalysts because the cells can be used for the production of the secreted materials before eventual disruption to recover PHAs. In addition, conventional techniques, such as centrifugation, can be used to separate the two products [13].

The main factor that restricts the widespread use of both rhamnolipids and PHAs is that they are more expensive to produce than their synthetic counterparts. As such, different strategies must be devised and explored to reduce their production costs, such as increasing the production yields by bacterial cultures, improving the engineering aspects of the cultivation parameters, optimizing the efficiency of their biosynthesis and selecting inexpensive medium components. The choice of inexpensive raw materials is important to the overall economics of the process because raw materials account for 50% of the final product costs [17]. Apart from traditional carbon and nitrogen substrates, the spectrum of available raw materials available as "feed stocks" includes various agro-industrial by-products and waste materials, which are both inexpensive and available in large amounts [17]. With the aim of improving the economics of the production process and obtaining new compounds with different physical and chemical properties, various agro-industrial by-products or wastes have been evaluated as substrates for the production of rhamnolipids and PHAs. In particular, waste cooking oil (WCO) [12], molasses [25], vegetable oils and residues from vegetable oil refineries [21] have been shown to be excellent substrates for rhamnolipids and PHAs production.

Cassava wastewater (CW) is a carbohydrate-rich residue generated in large amounts during the production of cassava flour. Cassava is one of the six main agriculture products (wheat, rice, corn, potato, oatmeal and cassava) with an estimated production of 160 million tons per year worldwide [8]. The production of 1 ton of cassava flour generates 300 l of CW; consequently, the treatment and disposal costs of CW represent a large financial burden to the cassava flour industry as well as an environmental problem. Thereby, there is a great need for a better management of this waste. Waste cooking oil and glycerol, two sub-products from food industries and biodiesel production, are also other renewable carbon sources. Their use as feedstock in biotechnological processes is a viable alternative which can contribute towards a reduction in the production costs and also towards an increase in the economic value of these residues. Cassava wastewater has been used to produce a surfactin by *Bacillus subtilis* [23], essential oils by *Fusarim oysporum* [19] and volatile compounds by *Geotrichum fragrans* [5].

The aim of the study reported here was to evaluate the potential use of agro-industrial wastes (CW, WCO and glycerol) for the simultaneous production of PHAs and rhamnolipids. The characterization of the biosurfactants and PHAs obtained is also presented.

Materials and methods

Microorganisms

Samples (10 g) of a hydrocarbon-contaminated soil were transferred to 125-ml Erlenmeyer flasks containing 20 ml of a liquid medium composed of (g/l): yeast extract, 3.0 and peptone, 5.0, and incubated on a rotary shaker at 30°C and 200 rpm. After 24 h, 0.1 ml of the culture was plated on trypticase soy agar (TSA) plates and incubated at 30°C for 24 h, following which the cells were preserved frozen at −79°C. The microorganisms isolated in this study were *P. aeruginosa* L2-1 and *P. aeruginosa* B1-3; *P. aeruginosa* 6c and *P. aeruginosa* 7a were also used [4]. The microorganisms were identified by phylogenic analysis based on 16S ribosomal RNA sequence.

Culture conditions

Bacterial growth from a TSA slant incubated for 24 h at 30°C was scraped from the slant tube. The optical density (610 nm) of the bacterial suspension was adjusted to 0.65 (10^8 cfu ml^{-1}) with water, and 1 ml of this solution was inoculated in a 250-ml Erlenmeyer flask containing 50 ml mineral salts medium [26] and 2% (w v^{-1}) soybean WCO as a carbon source. The inoculum was incubated for 24 h at 30°C on a rotary shaker at 200 rpm, and an aliquot of 1 ml was added to the production medium. The production of biosurfactant and PHA was carried out in 125-ml Erlenmeyer flasks containing 25 ml of salts medium to which had been added 2% (w v^{-1}) of one of two carbon sources (WCO or glycerol) to be tested. The flask was incubated as described above for 120 h. The initial pH of the broth was adjusted to 6.8–6.9. Samples were collected at set time intervals and submitted for analysis. The WCO was collected from restaurants at Rio Claro, São Paulo State, Brazil and contained the following constituents (%): palmitic acid, 14.8; stearic acid, 4.51; oleic acid, 30.15; linoleic acid, 42.99; linolenic acid, 3.72, others, 3.83.

Cassava wastewater preparation

Cassava wastewate was collected from a cassava flour manufacturer in São Paulo State, Brazil and stored at −18°C until needed. The solids in the CW were removed by boiling, followed by, after cooling, centrifugation at 5000 *g* for 10 min [23]. This clarified CW was autoclaved at 121°C for 15 min and used as a growth medium. Alternatively, 2% (w v^{-1}) WCO was added to the clarified CW prior to being autoclaved. The pH of the CW was adjusted to 6.8–6.9. The supernatant was distributed into Erlenmeyer flasks containing 25 ml of CW or CW with added waste cooking oil (CWO) to be tested and incubated for 120 h at 30°C on a rotary shaker at 200 rpm. Samples were collected at set time intervals and submitted for analysis. The same clarified CW batch was used for all experiments, and its composition is summarized in Table 1. The composition of the CW was determined using the methodology described by Nitschke and Pastore [23].

Table 1 Composition of cassava wastewater used in this study

Components	Concentration
Protein (%)	0.9
Fructose (g l^{-1})	24.5
Glucose (g l^{-1})	30.1
Maltose (g l^{-1})	1.8
Nitrate (g l^{-1})	0.7
Nitrite (mg l^{-1})	0.05
Phosphorus (g l^{-1})	0.9
Potassium (g l^{-1})	3.9
Magnesium (g l^{-1})	0.5
Sodium (mg l^{-1})	23.1
Iron (mg l^{-1})	6.1
Zinc (mg l^{-1})	11.1
Manganese (mg l^{-1})	4.1
Copper (mg l^{-1})	14.1

Determination of cell dry weight

Aliquots (25 ml) were taken at regular intervals. Two 10-ml samples were centrifuged at 15,000 *g* for 10 min and the cells collected and washed with distilled water. One sample was resuspended in distilled water and then dried at 80°C for 24 h; its cell dry weight (CDW) was determined gravimetrically. The cells from the other sample were frozen and lyophilized overnight for PHA analysis. The cell-free supernatant was used for analyzing and quantifying rhamnolipid, esterase and lipase content. The experiments were conducted in duplicate.

Determination of PHAs

Intracellular PHAs were determined by the acid methanolysis method [2]. Approximately 5 mg CDW was mixed with 1 ml chloroform, 0.85 ml methanol and 0.15 ml H_2SO_4 and heated at 100°C for 160 min. Then, 0.5 ml of distilled water was added and the tube shaken vigorously for 1 min. The organic phase was dried with anhydrous sodium sulfate, and 100 µl of sample was treated with 25 µl of BSTFA [*N*,*O*-bis(trimethylsilyl) trifluoroacetamide; Supelco, Bellefonte, PA) for 3 h at 70°C. Analysis of the trimethylsilyl derivative of the 3-hydroxy fatty acid methyl esters was performed by gas-chromatography/mass–spectrometry in a electron impact (GC–MS) using a Thermo Scientific Trace GC Ultra interfaced to a Polaris Q MS and equipped with a Thermo TR-5MS (30 m × 0.25 mm × 0.25 µm) column and chemical ionization mode (Thermo Fisher Scientific, Waltham, MA). Chemical ionization mode used methane as the ionization gas. After injection at 60°C, the oven temperature was increased 4°C min^{-1} up to 240°C, and held at this temperature for 12 min. The injector and detector temperatures were 230 and 240°C, respectively. Helium was used as the carrier gas at a flow rate of 0.60 ml min^{-1}. The experiments were conducted in duplicate.

Determination of rhamnolipids

Culture samples were centrifuged at 15,000 *g* for 10 min to remove the bacteria, and 10 µl of 16-hydroxyhexadecanoic acid was added as an internal standard. All of the analyses were performed with a triple quadrupole mass spectrometer Quattro II (Micromass, Manchester, UK) equipped with a Z-spray interface using electrospray ionization in negative mode. The instrument was interfaced to a HP 1100 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a 150 × 4 mm Zorbax C_8 reverse-phase column [6]. The experiments were conducted in duplicate.

Quantification of oil, glycerol and sugar contents

For the measurement of residual oil, 5 ml of cell-free supernatant was mixed with 5 ml of hexane and centrifuged at 7500 *g* at 15°C for 15 min. After centrifugation, 3 ml of the upper phase was transferred in a pre-weighted glass cup, the hexane was evaporated at 100°C for 24 h and the residual oil was determined gravimetrically. The residual sugars in CW (glucose, fructose and maltose) and the glycerol in the cell-free supernatant were measured using a high-performance liquid chromatographic (HPLC) system (SP Thermo Separation) equipped with a Rezex Roa (Phenomenex, Torrance, CA) column. A sulfuric acid solution 0.005 mol l^{-1} was used as solvent, and the HPLC flow rate was 0.4 ml min^{-1} at 60°C. The samples were identified by comparing the retention times with the those of the sugar and glycerol standards. The experiments were conducted in duplicate.

Surface activity measurement

Measurement of the surface activity of the cell-free supernatant was performed with a du Nouy tensiometer (Fisher Scientific, Waltham, MA) using the ring method. The critical micelle concentration (CMC) was determined by measuring the surface tension of serial dilutions of the surfactant solution. The experiments were conducted in duplicate.

Determination of esterase and lipase activity

Esterase and lipase activity in the cell-free supernatant was determined by the method of Kilcawley et al. [14]. Briefly, 1.8 ml of a solution containing 0.15 *M* NaCl and 0.5% Triton X-100 in 0.1 *M* Tris–HCl buffer (pH 8.0) was preincubated at 37°C. To this solution, 200 µl of a suitable dilution of sample and 20 µl of a 50 m*M* solution of the substrate in acetonitrile (*p*-nitrophenol butyrate for esterase activity and *p*-nitrophenol palmitate for lipase activity) was added and incubated at 40°C for 30 min. The amount of *p*-nitrophenol (*p*NP) liberated was recorded at 400 nm. Activity was expressed in units, where one unit is defined as the amount of enzyme liberating 1 nmol of *p*NP under standard assay conditions. The experiments were conducted in duplicate.

Results and discussion

Growth of microorganisms in CW, glycerol and waste cooking oil as carbon sources

To find a suitable carbon source for concurrent rhamnolipid and PHA production, we compared different classes of

Table 2 Effect of the carbon source on cell growth, rhamnolipid and polyhydroxyalkanoate production by various strains of *Pseudomonas aeruginosa* after 120 h of incubation

P. aeruginosa strain	Parameters	Substrate			
		CW	CWO	WCO	Glycerol
L2-1	CDW (g l^{-1})	3.3 ± 0.1	4.2 ± 0.1	6.2 ± 0.6	3.1 ± 0.1
	Rha (mg l^{-1})	300.3 ± 18.1	609.1 ± 75.1	245.6 ± 15.1	248.7 ± 4.3
	% PHA	17.6 ± 1.3	39 ± 1.4	43 ± 2.1	4.6 ± 0.4
B1-3	CDW (g l^{-1})	0.8 ± 0.1	0.9 ± 0.2	6.3 ± 0.3	3.4 ± 0.2
	Rha (mg l^{-1})	4.1 ± 2.3	4.2 ± 1.2	256.2 ± 19.1	188.1 ± 12.1
	% PHA	1.2 ± 0.1	0.4 ± 0.1	44.2 ± 1.7	7.8 ± 0.1
7a	CDW (g l^{-1})	2.5 ± 0.2	3.1 ± 0.1	6.8 ± 0.2	3.8 ± 0.1
	Rha (mg l^{-1})	169.9 ± 20.1	129.2 ± 12.4	273.1 ± 23.1	264.2 ± 18.2
	% PHA	4.6 ± 0.2	3.4 ± 0.1	50.4 ± 1.3	16.8 ± 0.3
6c	CDW (g l^{-1})	2.8 ± 0.3	3.2 ± 0.2	6.5 ± 0.1	3.7 ± 0.1
	Rha (mg l^{-1})	176.1 ± 15.6	179.4 ± 23.9	245.4 ± 18.2	175.3 ± 23.2
	% PHA	3.8 ± 0.3	12.2 ± 0.1	48.4 ± 2.1	22.2 ± 0.4

PHA, Polyhydroxyalkanoate; CW, cassava wastewater; CWO, cassava wastewater with added waste cooking oil; WCO, waste cooking oil; CDW, cell dry weight; Rha, rhamnolipids, % PHA, % CDW

Vaues are given as the mean of duplicate experiments ± standard deviation

substrates, including one conventional (glycerol) and two unconventional substrates (WCO and cassava waste), in terms of cell growth. As Table 2 shows, all of the *P. aeruginosa* strains used in this work were able to grow well and to produce rhamnolipids and PHAs when cultivated in the various substrates tested, with the exception of *P. aeruginosa* B1-3 in CW. However, when growing in WCO and glycerol, B1-3 generated 6.3 and 3.4 g l^{-1} of biomass, respectively. Interestingly, only minimal growth was observed in the CW with added WCO, an indication that CW inhibits the growth of this strain.

For all the strains tested, the highest biomasses were obtained with WCO as the carbon source (with strain 7a achieving 6.8 g l^{-1}), while the lowest amounts of biomass were obtained with CW as the carbon source (2.5 g l^{-1} with strain 7a) (Table 2). Although there is almost twofold more carbon available in CWO than in each of WCO and CW, the final biomass obtained using the former was not larger than that obtained using either of the latter two substrates. However, the growth profile in CWO differed from that of the other substrates (Fig. 2). In glycerol, CW and WCO, maximum growth occurred during the first 48 h and the biomass remained constant thereafter until the end of the cultivation period (Fig. 2a, c, d); in contrast, in CWO, the bacterial biomass only reached a maximum after 72 h (Fig. 2b).

PHA and rhamnolipid production

Table 2 shows that for all strains, WCO was the best substrate in terms of PHA production, with between 43 and 50.4% PHA of the CDW. Using hydrophilic substrates, such as glycerol and CW, the PHA yield varied between 1.2 and 22.2% of the CDW, which is in agreement with results reported in the literature in which higher yields of PHA were obtained with hydrophobic substrates, such as WCO and other oily wastes, than with hydrophilic substrates. In some cases, the use of hydrophobic substances resulted in the production of up to 54.6% PHA of the CDW [9, 12, 20]. Using CWO, we obtained a 39% CDW yield with strain L2-1.

Rhamnolipid production showed considerable variation depending on the carbon source and strain. With glycerol as the carbon source, yields varied between 175.3 and 264.2 mg l^{-1}; when WCO was used, the yields were more constant, varying between 245.6 and 273.1 mg l^{-1}. Large variations were also observed with CW (169.9–300.3 mg l^{-1}), but the larger variations in rhamnolipid production were in CWO, with yields of between 129.2 and 609.1 mg l^{-1}. As for PHA production, rhamnolipid production is generally higher when the substrate is hydrophobic than when it is hydrophilic [21]. However, hydrophilic substrates may be more advantageous because the analytical and downstream production problems related to the presence of residual oil in the culture broth are eliminated.

Our results reveal that both glycerol and CW are potential alternative substrates for rhamnolipid production, while WCO and CWO could be used for the concurrent production of rhamnolipids and PHAs. With CWO as the carbon source, strain L2-1 showed reasonable growth (4.2 g l^{-1}), with a good yield of PHAs (39% CDW) and an excellent yield of rhamnolipids (609.1 g l^{-1}) (Table 2).

Time profile of rhamnolipids and PHA production

The time profiles of rhamnolipid and PHA production were determined with one strain, L2-1, in order to be able to correlate product synthesis and growth phase. As shown in Fig. 2, PHA synthesis in each medium was found to occur during active cell growth, during the first 48 h of culture, except in

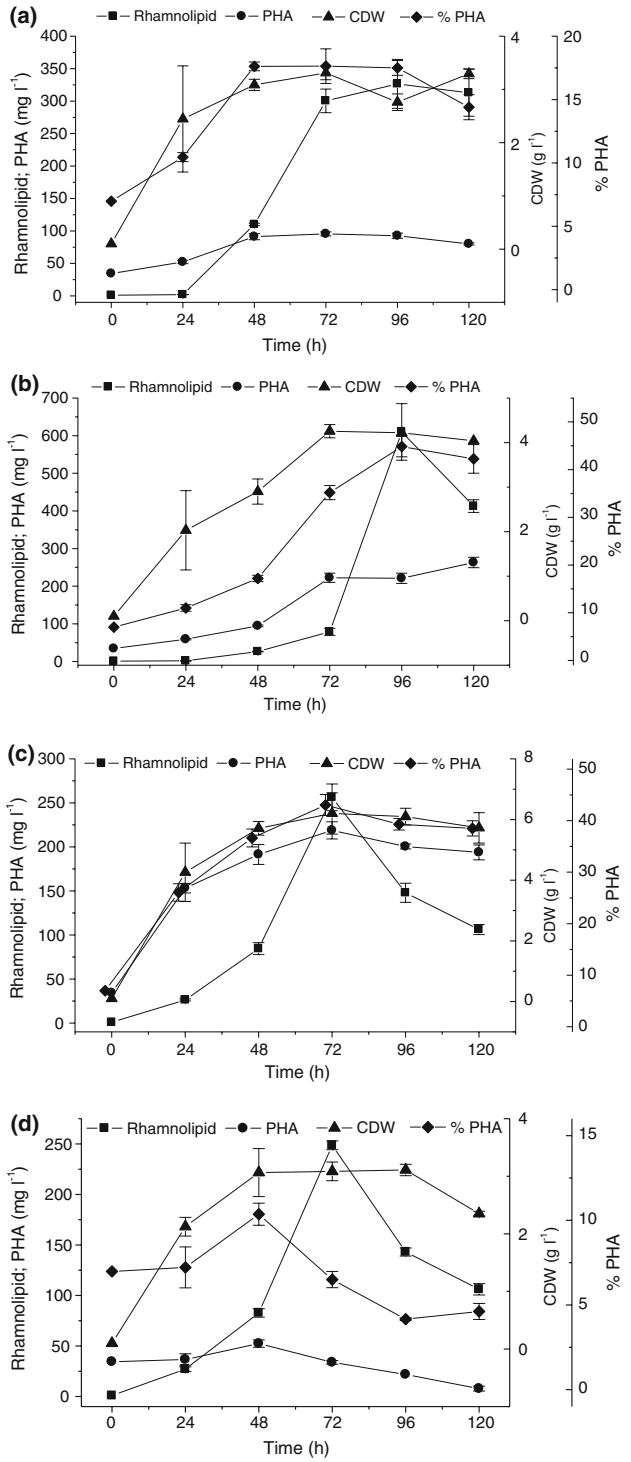

Fig. 2 Time course of rhamnolipid and PHA production by *Pseudomonas aeruginosa* L2-1 cultivated in cassava wastewater (**a**), cassava wastewater with added waste cooking oil (**b**), waste cooking oil (**c**), glycerol (**d**). *CDW* Cell dry weight

medium containing CWO, where cell growth was extended until 72 h. For rhamnolipid production, the bulk of production peaked around 72 h in all media except that containing CWO, in which the maximum production occurred at 96 h.

Carbon consumption of strain L2-1 is shown in Table 3. When CW was used as the carbon source, glucose and fructose were exhausted after 48 h of cultivation, while when glycerol was the carbon source, these were depleted only after 72 h. With the addition of WCO to the CW, sugar consumption slowed down compared to CW alone, and some residual sugars were still present after 72 h. However, the overall rate of disappearance of oil in the medium containing CWO was considerably reduced compared to that containing WCO alone (Table 3), suggesting that hydrophilic substrates, such as sugars, are assimilated more rapidly than oil as a carbon source.

In CW, strain L2-1 produced PHAs during the active cell growth, and production stopped after 48 h, at which time the carbon source was exhausted (Fig. 2a, Table 3). In WCO, PHA production was observed during and after growth, up to 72 h, at which time there was still some residual carbon source. In CWO, PHA production occurred only during the growth phase, which lasted before 72 h.

Working with *P. aeruginosa* strain IFO3924 and using glucose as the carbon source, Hori et al. [13] found that the synthesis of both PHAs and rhamnolipids started after the growth phase of the bacterium and that the products reached a maximum when the carbon source was exhausted. The accumulation of PHA reached 10% of CDW in 36 h, followed by its rapid degradation. Similar results were obtained by Koller et al. [15] using *P. hydrogenovora* with hydrolyzed whey permeate as the carbon source: they also observed that their strain started to accumulate PHAs after the growth phase. The differences in the time profile of PHA production in our present study and those reported in the literature could be due to the growth conditions, the media and the different *Pseudomonas* species or strain used.

One of the roles attributed to biosurfactants is to allow microorganisms to grow on water-immiscible substrates by reducing the surface tension of the phase boundary, thus making the substrate more readily available for uptake and metabolism [10]. In CWO, the large amount of rhamnolipids produced after 72 h occurs within the stationary growth phase, which is in agreement with the fact that biosurfactants are secondary metabolites and could be used to help disperse and solubilize the considerable amount of residual oil still present after 72 h. Nevertheless, the experiment with WCO alone shows that there must be an additional mechanism of oil uptake in addition to rhamnolipid solubilization, such as the expression of lipolytic enzymes.

Time profile of lipase and esterase production

When WCO and CWO were used as the carbon source, strain L2-1 produced lipases and esterases that degraded the triglycerides into bioavailable fatty acids and glycerol

Table 3 Carbon source consumption by *P. aeruginosa* strain L2-1 growing in cassava wastewater, cassava wastewater with added waste cooking oil, waste cooking oil and glycerol

Time (h)	Substrate						
	CW		CWO			WCO (g l^{-1})	Glycerol (g l^{-1})
	Glucose (g l^{-1})	Fructose (g l^{-1})	Glucose (g l^{-1})	Fructose (g l^{-1})	WCO (g l^{-1})		
0	24.6 ± 1.5	30.1 ± 1.5	24.5 ± 1.3	30.1 ± 1.5	20.0 ± 0.1	20 ± 0.1	20 ± 0.1
24	9.8 ± 1.6	13.5 ± 1.2	17.0 ± 0.5	18.7 ± 0.9	17.6 ± 1.4	10.0 ± 1.1	13.1 ± 0.4
48	2.0 ± 1.0	0	5.2 ± 0.1	4.5 ± 1.0	16.4 ± 0.6	5.9 ± 1.4	7.5 ± 0.8
72	0	0	3.4 ± 0.2	3.2 ± 0.0	13.9 ± 0.9	5.3 ± 1.1	1.1 ± 0.6
96	0	0	2.2 ± 0.3	0	9.2 ± 1.1	4.9 ± 0.8	0
120	0	0	2.0 ± 0.1	0	2.4 ± 1.4	0.5 ± 0.1	0

CW, Cassava wastewater; CWO, cassava wastewater with added waste cooking oil; WCO, waste cooking oil

Vaues are given as the mean of duplicate experiments ± standard deviation

(Fig. 3). In parallel with the higher biomass observed in WCO relative to CWO, maximal lipase and esterase activities were higher in WCO (esterase and lipase: 90 and 83 U ml^{-1} of culture, respectively) than in CWO (esterase and lipase: 76 and 41 U ml^{-1} of culture, respectively). The time course of esterase and lipase production for *P. aeruginosa* L2-1 is given in Fig. 3. When WCO was the carbon substrate (Fig. 3a), lipase and esterase activities were at a maximum at 48 h of growth (lipase and esterase: 83 and 90 U ml^{-1} of culture, respectively). With CWO as the substrate, maximum lipase and esterase activities occurred at 72 h of growth (lipase and esterase: 41 and 76 U ml^{-1} of culture) (Fig. 3b). These results are in agreement with the consumption of the carbon sources as shown in Table 3. In the medium containing WCO, with the sole carbon source was insoluble, the bacteria need to hydrolyze the triglycerides into more free fatty acids for growth; when CWO was the carbon source, the bacteria initially used the sugars contained in the CW prior to producing esterases and lipases to access the triglycerides.

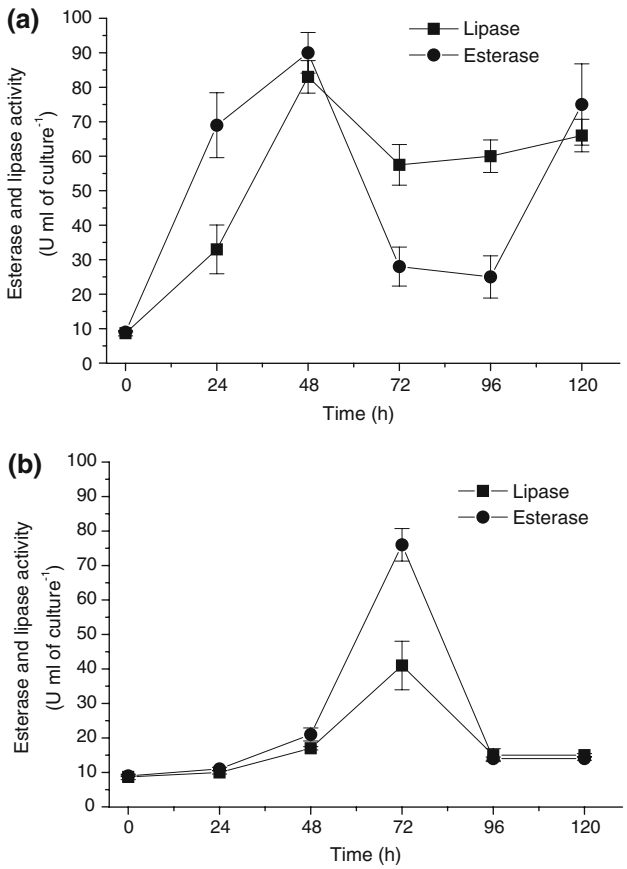

Fig. 3 Time course of esterase and lipase production by *P. aeruginosa* L2-1 cultivated in waste cooking oil (**a**) and in cassava wastewater with added waste cooking oil (**b**)

Characterization of PHA monomers

The composition of PHA monomers produced by strain L2-1 was investigated in relation to the carbon source used. It is known that the nature of the monomers produced vary according to the metabolic pathway involved with the specific carbon source used in the culture medium. When carbohydrates are used, *P. aeruginosa* accumulates C_{10} as the predominant monomer, and this monomer is derived from the de novo fatty acid biosynthesis pathway [29]. However, when fatty acids are used as the carbon source, PHA synthesis proceeds mostly from the beta oxidation pathway [18].

The composition of the PHA monomers produced by strain L2-1 varied with the substrate, with the chain length (between C_8 and C_{16}) and the unsaturation level of the monomers showing considerable variation (Table 4). Our analysis revealed the absence of PHA monomers with a carbon chain shorter than C_8. When glycerol was used as the sole carbon source, the main PHA monomers were $C_{10:0}$ (62.8%), $C_{8:0}$ (17.8%) and $C_{12:0}$ (15.9%), and the total proportion of unsaturated monomers was 1.7% ($C_{12:1}$, $C_{14:1}$ and $C_{14:2}$). The composition of PHA monomers was very

Table 4 Accumulation and composition of PHA produced by *P. aeruginosa* strain L2-1 after 120 h of incubation with different carbon substrates

Substrate	PHA (mg l^{-1})	Relative amount of monomers in purified PHA (%)							
		$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{12:1}$	$C_{14:0}$	$C_{14:1}$	$C_{14:2}$	$C_{16:0}$
CW	99.8 ± 3.1	16.2 ± 0.4	64.9 ± 1.2	17.1 ± 2.3	1.1 ± 0.8	0.5 ± 0.1	0.1 ± 0.1	0.08 ± 0.01	0.05 ± 0.02
CWO	202.4 ± 12.1	26.4 ± 0.3	50.6 ± 1.8	15.7 ± 3.1	2.2 ± 1.1	1.8 ± 0.3	1.7 ± 0.2	1.1 ± 0.2	0.1 ± 0.01
WCO	218.8 ± 9.7	37.5 ± 0.3	42.1 ± 2.1	13.2 ± 1.6	2.2 ± 0.4	2.1 ± 0.6	1.6 ± 0.2	1.1 ± 0.5	0.07 ± 0.02
Glycerol	52.4 ± 3.6	17.8 ± 0.1	62.8 ± 2.4	15.9 ± 2.7	0.9 ± 0.1	1.6 ± 0.3	0.3 ± 0.1	0.4 ± 0.2	0.1 ± 0.03

CW, Cassava wastewater; CWO, cassava wastewater with added waste cooking oil; WCO, waste cooking oil

Vaues are given as the mean of duplicate experiments ± standard deviation

Table 5 Effect of the carbon source on relative abundance (%) of the monorhamnolipid ($RhaC_{10}C_{10}$) and dirhamnolipid ($RhaRhaC_{10}C_{10}$) obtained by various strains of *P. aeruginosa* after 120 h of incubation

P. aeruginosa strain	Substrate							
	CW		CWO		WCO		Glycerol	
	$RhaC_{10}C_{10}$	$RhaRhaC_{10}C_{10}$	$RhaC_{10}C_{10}$	$RhaRhaC_{10}C_{10}$	$RhaC_{10}C_{10}$	$RhaRhaC_{10}C_{10}$	$RhaC_{10}C_{10}$	$RhaRhaC_{10}C_{10}$
L2-1	32.16	38.06	19.61	59.15	25.81	47.43	21.01	42.6
B1-3	29.92	46.13	26.66	46.66	25.56	40.86	18.06	48.21
6c	31.06	35.75	43.09	29.39	32.27	42.02	20.83	44.58
7a	30.78	37.11	40.96	33.18	30.46	37.12	14.54	48.97

CW, Cassava wastewater; CWO, cassava wastewater with added waste cooking oil; WCO, waste cooking oil

similar when CW was the carbon source, with $C_{10:0}$, $C_{8:0}$ and $C_{12:0}$ representing 64.9, 16.2 and 17% of the total, respectively. The unsaturated monomers represented only 1.3% of the total composition. Thus, a very similar composition of PHA monomers resulted when glycerol or CW was the sole carbon source, indicating that the biosynthesis of PHAs was very similar when these two water-soluble carbon sources were utilized and that it proceeded through the de novo fatty acid pathway. When WCO was the carbon source, the total amount of shorter chain PHA monomers ($C_{8:0}$) increased to 37.5% of the total, while the $C_{10:0}$ and $C_{12:0}$ monomers represented 42.1 and 13.2% of the total, respectively; the proportion of unsaturated monomers increased to 4.8%. These results reveal that the composition of PHAs accumulated by *P. aeruginosa* depends on the carbon source, the metabolic routes involved and the PHA synthase, whose two genes are involved and which have been characterized [18].

The shift to shorter chain length monomers and the increase in the proportion of unsaturated monomers observed in medium containing WCO compared to those containing water-soluble carbon sources are in agreement with the results reported by others. Haba et al. [12] and Marsudi et al. [20], working with other *P. aeruginosa* strains grown in vegetable oils or oily wastes, observed PHA compositions of $C_{10:0}$ (43–48%), $C_{12:0}$ (11–15%) and $C_{8:0}$ (10–38%), with a relatively high proportion of unsaturated monomers (5–15%). The origin of these unsaturated monomers was studied by Tan et al. [30] in *P. putida* PGA1, using saponified palm kernel oil (SPKO) (a mixture of saturated and unsaturated fatty acids), myristic acid (a saturated fatty acid), lauric acid (a saturated fatty acid) and oleic acid (an unsaturated fatty acid). The lauric and myristic acid-derived PHAs contained only saturated monomers, while SPKO- and oleic acid-derived PHAs contained saturated and unsaturated monomers. Thus, the presence of unsaturated fatty acids in the carbon source could explain the increased proportion of double bounds in the PHA monomers we observed in cells grown on WCO compared to glycerol (Table 4). The relatively high level of unsaturation in PHA monomers is characteristic of the β oxidation pathway when the fatty acid used as carbon source contains a high proportion of unsaturation. When CWO was used, the PHA composition was intermediate between the CW and the frying oil, indicating that both metabolic pathways were involved.

Characterization of rhamnolipids

The bacterial isolates were able of synthesize rhamnolipids with all of the substrates analyzed, although rhamnolipid production with strain B1-3 in medium containing CW or CWO was minimal. The most abundant rhamnolipids were $RhaC_{10}C_{10}$ and $RhaRhaC_{10}C_{10}$; all other homologues were

Table 6 Structure and relative abundance (%) of rhamnolipids obtained from *P. aeruginosa* L2-1 after 120 h of incubation

Rhamnolipid (%)	Substrate			
	CW	CWO	WCO	Glycerol
$RhaC_8C_8$	0.07	0.27	ND	ND
$RhaC_{10}C_8$	2.83	5.34	1.52	4.29
$RhaC_{10}C_{10}$	32.16	19.61	25.81	21.01
$RhaC_{10}C_{12}$	0.32	1.16	5.79	6.78
$RhaC_{12}C_{12}$	ND	ND	0.45	0.05
$RhaRhaC_{10}C_8$	6.9	5.71	2.94	8.59
$RhaRhaC_{10}C_{10}$	38.06	59.15	47.43	42.6
$RhaRhaC_{10}C_{12}$	8.29	0.48	6.68	7.03
$RhaRhaC_{12}C_{12}$	ND	ND	0.98	0.7
$RhaC_{10}C_{10:1}$	0.27	0.59	0.41	0.29
$RhaC_{10}C_{12:1}$	4.93	1.36	4.02	3.49
$RhaC_{12}C_{12:1}$	ND	ND	0.04	ND
$RhaRhaC_{10}C_{10:1}$	0.31	0.56	0.2	0.44
$RhaRhaC_{10}C_{12:1}$	5.44	5.72	3.31	3.53
$RhaRhaC_{12}C_{12:1}$	0.38	ND	0.42	0.58

CW, Cassava wastewater; CWO, cassava wastewater with added waste cooking oil; WCO, waste cooking oil; ND, not detected

present as minor components. The dirhamnolipid $RhaRhaC_{10}C_{10}$ was predominant in all substrates evaluated, with the exception of CWO, where the monorhamnolipid $RhaC_{10}C_{10}$ was predominant with strains 6c and 7a (Table 5). The highest $RhaRhaC_{10}C_{10}/RhaC_{10}C_{10}$ ratio was obtained with strain L2-1 in medium containing CWO. Table 6 shows the relative abundances of all the rhamnolipids produced by strain L2-1 in all of the substrates studied. Contrary to what was observed for PHA synthesis, the proportion of unsaturated rhamnolipids did not vary between glycerol, CW and WCO (Table 6). A number of authors have reported that monorhamnolipids were predominant components of their rhamnolipid mixtures [21, 27], whereas most reports have described the dirhamnolipids as the main components present [1, 7, 13]. The differences in the proportions of the various rhamnolipids identified our study and those reported in the literature can be explained by the rhamnolipid isolation and analysis procedures, the growth conditions, the medium used, the age of culture and/or the fact that different *P. aeruginosa* strains or species were used. Among the various substrates tested, the surfactant extracted from CWO shows the lowest surface tension, 30 mN m^{-1} and the lowest CMC, 26.5 mg l^{-1}.

Conclusion

The *P. aeruginosa* strains used in this study were able to produce rhamnolipids and PHA monomers in medium containing either CW, CWO, glycerol or WCO as the carbon substrate. To the best of our knowledge, this is the first study that describes the use of CW as a substrate for the production of rhamnolipids and PHAs. The results presented show that the composition of the rhamnolipids and PHAs produced vary with both the carbon source and with the strain utilized. Strain L2-1 in combination with CWO as the carbon source yielded high levels of rhamnolipids and PHAs compared to that obtained with the other carbon substrates. The simultaneous production of both biomolecules can contribute to a reduction in production costs and environmental problems related to waste discharge as well as to increases in the economic value of CWO.

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