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Conversion of Agro-industrial Wastes by *Serratia marcescens* UCP/WFCC 1549 into Lipids Suitable for Biodiesel Production

Dayana Montero-Rodríguez*[ab], Rosileide F. S. Andrade[b], Roberto A. Lima[b], Grayce K. B. Silva[b], Daylin Rubio-Ribeaux[ab], Thayse A. Silva[b], Hélvia W. C. Araújo[c], Galba M. Campos-Takaki*[b]

[a]Center of Biological Sciences, Federal University of Pernambuco, 50670-420, Recife, Pernambuco, Brazil.
[b]Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, 50050-590, Recife, Pernambuco, Brazil.
[c]Chemistry Department, State University of Paraíba, 58429-500, Campina Grande, Paraíba, Brazil.
dayimontero87@yahoo.es

Biodiesel has been becoming one of the most promising biofuels for global fuels market in recent years, due to the depletion of global petroleum and its increasing price. Researchers exploit oleaginous microorganisms as an alternative for biodiesel production. Currently, they are focused on reducing the production costs and searching waste materials as substrates. This study aimed to investigate the ability of *Serratia marcescens* UCP/WFCC 1549 to produce lipids using agro-industrial residues (cassava wastewater and waste vegetable oils), either alone or as additives of Luria Bertani (LB) medium. *S. marcescens* demonstrated that have good ability for growth in all media employed, mainly in LB medium supplemented with waste vegetable oils. However, the best results for lipids production were obtained in media consisting only by wastes, with values higher than 40% of lipids in biomass. In media comprising only residues, more balanced profiles of fatty acid methyl esters (FAMEs) were found, in terms of the proportion of saturated, mono-unsaturated and poly-unsaturated fatty acids (SFAs, MUFAs and PUFAs, respectively). The best result was obtained in lipids produced in medium containing 6 % cassava wastewater (CW) and 7.5 % waste soybean oil (WSO), which had the highest percentage of MUFAs (48.09 %), in accordance with the standards for biodiesel quality. In addition, a high content of oleic acid (46.82 %) was achieved in this medium, showing *S. marcescens* UCP/WFCC 1549 as an oleaginous microorganism that can be used as potential feedstock for producing good quality biodiesel. Also, this work demonstrated the suitability of CW and waste vegetable oils for microbial lipids production.

1. Introduction

In recent years, energy high prices, energy security, protecting the environment and concerns about petroleum supplies have attracted great attention and led to strong efforts to find a renewable biofuel (Leiva-Candia et al., 2014). However, crude fossil oil has a limited and finite supply, which could run out during this century depending upon the extent to which this energy source continues to be used to meet the increasing demands for energy. Therefore, if plant biomass can be increasingly exploited, this will increase energy security, thereby reducing the dependency on crude oil (a non-renewable). There are at least three distinct advantages of a biorefinery using renewable feedstocks for production of bioenergy, biofuels and biochemicals, compared to chemical refining of petrochemical feedstocks, namely: energy security, prevention of climate change and rural development (Cherubini, 2010).

One of the most promising renewable biofuels is biodiesel, which is a mixture of fatty acid methyl esters (FAMEs) obtained by the transesterification of triglycerides (in most cases, vegetable oils and animal fats) with methanol or ethanol. Biodiesel is biodegradable, nontoxic and has properties similar to conventional diesel (Liang and Jiang, 2013).

However, the cost of biodiesel is high due to the high cost of the raw materials (about 70–75% of the total cost). A cheaper raw material for biodiesel production would help to reduce the total cost (Cheirsilp and

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Louhasakul, 2013). Much attention has been paid to the development of microbial oils and it has been found that many microorganisms, such as microalgae, yeasts, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions (Subramaniam et al., 2010). Researchers exploit oleaginous microorganisms for biodiesel production due to their short life cycle, less labor being required, and their being less affected by location and easier to scale up, compared with other biodiesel sources Therefore, microbial lipids are now of interest as promising potential feedstock for biodiesel production (Kakkad et al., 2015).
On the other hand, modern society produces a high quantity of waste materials through activity related to industries, forestry, agriculture and in municipal environments. The enormous costs associated with treating these residues using conventional treatment methods have been a major concern for those who generate wastes and responsible municipal authorities (Kaur et al., 2014). The high content of fats, oils and other nutrients in these wastes make them interesting and cheap raw materials for industries involved in useful metabolite production. The use of such residual materials serves a dual role, namely that of generating a usable product and that of reducing waste disposal (O'Callaghan, 2015).
Cassava wastewater (CW) is a yellowish liquid obtained from cassava during the cassava flour manufacturing process, and is rich in many nutrients such as potassium, nitrogen, magnesium, phosphorus, calcium and sulphur. Currently, CW is discharged into rivers or released on soil without any kind of treatment, thus causing damage to the environment and human health (Ayansina et al., 2015). Similarly, large amounts of waste frying oils (WFO) are produced from restaurants, catering establishments and food industries every year and discarding them has serious consequences including that of contaminating natural reserves of water (Toralles et al., 2015). These residues have a high nutrient content and have been used as alternative substrates for several biotechnological processes (Montero-Rodríguez et al., 2015).
Industrial wastes have been used as substrates to cultivate oleaginous microorganisms so as to produce lipids (Pirozzi et al., 2015). Thus, this study sets out to investigate the potential of *Serratia marcescens* UCP/WFCC 1549 for lipid production using CW and WFO as substrates.

2. Materials and methods

2.1 Microorganism

The bacterium *Serratia marcescens* UCP/WFCC 1549 was kindly supplied from the Culture Collection of the Nucleus of Research in Environmental Sciences and Biotechnology, Catholic University of Pernambuco, Recife, state of Pernambuco, Brazil. The strain is registered in the World Federation for Culture Collection (WFCC). The microorganism was maintained in Luria Bertani (LB) solid medium (tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L and agar 15 g/L) at 5 °C. For pre-culture, the strain from the 24-h culture on LB medium was transferred to 50 mL of LB broth and maintained in an orbital shaker at 150 rpm during 18 h at 28 ºC, to obtain the seed culture.

2.2 Materials

All chemicals were of reagent grade. Cassava wastewater (CW) was obtained from an industry food in the municipal district of Carnaíba, state of Pernambuco, Brazil. Waste corn oil (WCO) and waste soybean oil (WSO) were kindly supplied from a local restaurant in the city of Recife, state of Pernambuco, Brazil. The residues were stored according to the suppliers' recommendations and used without any further processing.

2.3 Culture conditions

This study tested the following different culture media for lipid production: LB medium, LB medium supplemented with 7.5 % WSO and LB medium supplemented with 7.5 % WCO. Also, were tested two alternative media consisting of distilled water supplemented with 6.0 % CW and 7.5 % WSO and distilled water supplemented with 6.0 % CW and 7.5 % WCO. The pH of the media was adjusted to ±7.0 and they were sterilized by autoclaving. One percent aliquots (v/v) of the seed culture (0.8 optical density at 600 nm, corresponding to 10^7 cells/mL), was used to inoculate 250 mL Erlenmeyer flasks containing 100 mL of sterile production medium. Cultivations were carried out in triplicate in an orbital shaker at 150 rpm for 72 h at 28 °C.

2.4 Biomass determination

To determine the biomass, the culture samples were centrifuged at 10 000 *g* for 15 min. The cell pellets were washed three times with distilled water to remove residue from the cultivation medium and were centrifuged again. Then, they were frozen and lyophilized to constant weight. Cell dry weight was determined gravimetrically.

2.5 Extraction and determination of total lipids of biomass

The lipid content of biomass was determined according to Manocha et al. (1980): 1.0 g of the lyophilized biomass was extracted with chloroform: methanol in different proportions (2:1, 1:1, 1:2, v/v). The organic

extracts were washed with sodium chloride 0.9 %, then evaporated in a vacuum and the lipid content was determined by gravimetric estimation.

2.6 Extraction and transesterification of fatty acids

Cellular lipids were converted to their fatty acid methyl esters (FAMEs) according to Dunlap and Perry (1967). Briefly, ten milligrams of lyophilized biomass were transferred to tubes containing a solution of boron trifluoride-methane (2 mL) at 14 % and benzene (2 mL) and incubated at 60 ºC overnight. Then, distilled water (2 mL) was added and samples were shaken in vortex for 5 min and centrifuged (1700 rpm, 10 min at 4 ºC). After centrifugation, the benzene was removed and evaporated in a nitrogen atmosphere. The FAMEs were re-suspended in n-hexane and subjected to reading in gas chromatography (GC).

2.7 Gas chromatography (GC)

The FAMEs were analysed by gas chromatography (GC) using a chromatograph model Agilent Technologies 7890A with automatic injector (Analytical Central - CETENE). It was equipped with a flame ionization detector (FID), fused silica capillary column HP - 5 (5 % diphenyl and 95 % dimethylpolysiloxane), 30 m x 0.25 mm. The temperature of the column oven was as follows: heating ramp - initial temperature 150 °C for 4 min; increasing at a rate of 4 °C min^{-1} until 250 °C, and remained so for 20 min. The temperature of the injector and detector was 280 °C, and helium ($1cm^3.min^{-1}$) was used as carrier gas.

The microbial FAMEs were identified by comparison of retention times with standard FAMEs (Sigma Aldrich) and were quantified by area normalization using the software supplied with the equipment.

3. Results and discussion

3.1 Production of biomass

Figure 1 shows the yield of biomass produced by *S. marcescens* in the different production media tested. The strain was able to grow in all of them, with best results in LB medium supplemented with 7.5 % WCO (3.07 g/L) and LB supplemented with 7.5 % WSO (2.20 g/L). Probably, the considerably higher carbon and nitrogen contents in these media compared with the others tested, favoured this result.

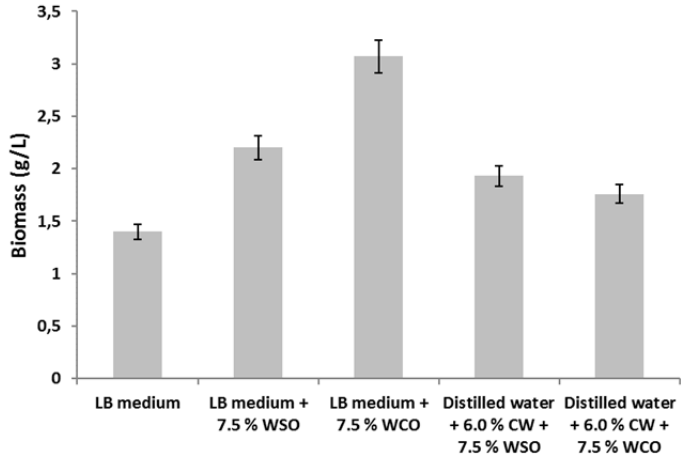

Figure 1: Biomass production on different culture media by S. marcescens UCP/WFCC 1549. (LB), Luria Bertani; (WSO) waste soybean oil; (WCO) waste corn oil; (CW), cassava wastewater.

In this study, the main strategy to achieve excellent biomass yields was to assess the substrates of WCO or WSO as product output, focusing on the appropriate use of *S. marcescens*, the nutritional balance and the use of a cheap alternative to lower the costs involved in the process of the transesterification reaction. The microorganism showed a good ability to grow in media comprising only wastes (CW and WSO or WCO), and its performance in these media was better than in LB medium. These agro-industrial residues are rich in many nutrients which are useful for microbial growth and producing metabolites with high added value, as suggested in several studies (Berger et al., 2014; Silva et al., 2014).

3.2 Production of microbial lipids

This study assessed the use of CW and vegetable WFO both alone and as LB medium additives for the production of lipids by *S. marcescens*. The percentages of total lipids obtained in each culture media tested are shown in Figure 2.

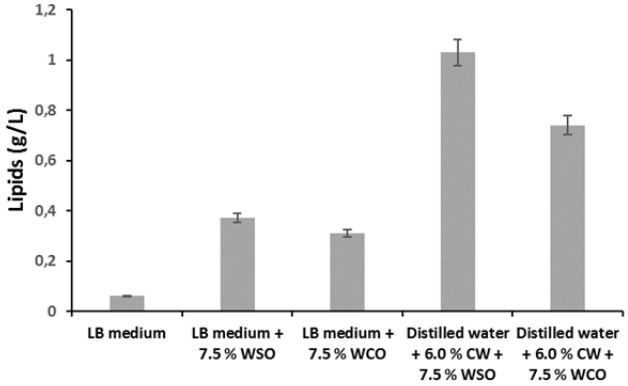

Figure 2: Total lipid yields of S. marcescens UCP/WFCC 1549 cultured in different production media for 72 h at 150 rpm and 28 °C. (LB) Luria Bertani; (WSO) waste soybean oil; (WCO) waste corn oil and (CW) cassava wastewater.

The best results were obtained in media comprising only wastes, with percentages higher than 40%, thus showing that this strain is an oleaginous microorganism. Recently, Bharti et al. (2014) reported that *Serratia* sp. produced 0.647 g of lipid/g dry cell weight in a minimal salt medium supplemented with sodium bicarbonate. In the present study, *S. marcescens* was able to produce 0.417 and 0.531 g lipid/g dry biomass, related to biomass cultured in medium containing only CW+WCO and CW+WSO, respectively. The results indicate that these agro-industrial residues are promising renewable substrates for producing lipids which would help to reduce the total costs of the process. Also, the possible conversion of organic compounds present in CW and vegetable WFO into lipids is important since this gives wastes an added value.

3.3 Biodiesel production from microbial oil

Demand for fatty acid methyl esters (FAMEs) as diesel fuel has increased significantly in recent years due to the decrease in global petroleum and the instability of oil prices (Liang and Jiang, 2013). Microbial lipids can potentially be used as raw material for biodiesel production using the commonest way to produce FAMEs in the biodiesel industry, namely base-catalyzed transesterification (Oprescu et al., 2015). However, this process has disadvantageous and undesirable reactions that can be avoided by using acid catalysts, which also produce FAMEs and increase the biodiesel yield (Thliveros et al., 2014). In this study, biodiesel from lipids of *S. marcescens* was produced by acid-catalyzed transesterification in the presence of BF_3 and methane. The results obtained are showed in Table 1.

Table 1: Percent composition of fatty acids methyl esters obtained after transesterification of lipids produced by S. marcescens UCP/WFCC 1549 cultured in different production media.

FAMEs	Production media (% FAMEs)				
	LB	LB + WSO	LB + WCO	CW + WSO	CW + WCO
Caprylic acid (C8:0)	-	-	-	1.11	0.21
Capric acid (C10:0)	-	-	-	0.43	0.18
Lauric acid (C12:0)	-	0.53	-	-	-
Myristic acid (C14:0)	13.23	3.52	1.78	0.70	0.32
Pentadecanoic acid (C15:0)	8.56	2.04	-	-	-
Palmitic acid (C16:0)	68.80	22.05	18.64	22.98	15.66
Margaric acid (C17:0)	-	0.99	-	-	0.12
Stearic acid (C18:0)	-	5.14	2.69	7.17	3.07
Oleic acid (C18:1)	-	37.12	43.99	46.82	34.46
Linoleic acid (C18:2)	-	-	-	14.39	43.36
Linolenic acid (C18:3)	9.38	27.57	32.33	2.30	1.08
Arachidic acid (C20:0)	-	-	0.56	0.97	0.73
Gadoleic acid (C20:1)	-	0.51	-	0.75	0.47
Behenic acid (C22:0)	-	-	-	1.87	0.31
Erucic acid (C22:1)	-	0.54	-	0.52	-

Considerable variations in the fatty acid profile of lipids of *S. marcescens* were observed when the medium composition was modified (Table 1). Several authors state that the substrate composition influences the FAMEs profile of microbial oils and hence biodiesel properties and quality (Canakci and Sanli, 2008; Ramos et

al., 2009). Saturated fatty acids (SFAs) content was predominant (90.59 %), in relation to unsaturated fatty acids (UFAs), in FAMEs from *S. marcescens* cultured in LB medium. In contrast, the profiles of FAMEs from microorganisms cultured in media with wastes presented a higher content of UFAs than of SFAs, as suggested by the standards for biodiesel quality (Ramos et al., 2009).

According to a report from the US Department of Energy, an ideal biodiesel should consist of more monounsaturated fatty acids (MUFAs) and less saturated and polyunsaturated fatty acids (PUFAs). High levels of PUFAs would negatively impact the oxidative stability and increase nitrogen oxide exhaust emissions, which do not suit diesel engines (Knothe, 2012). On the contrary, biodiesel derived from SFAs would have good oxidative stability, but poor fuel properties at low temperatures, which is a disadvantage in cold weather conditions. Consequently, it has been predicted that high levels of oleic acid (C18:1) and a low content of linoleic, linolenic and other PUFAs are best suited for biodiesel production (Puhan et al., 2010). In the present study, the FAMEs obtained from *S. marcescens* UCP/WFCC 1549 cultured in media containing only wastes, presented a good proportion of MUFAs: PUFAs: SFAs. The best result was achieved in medium containing CW and WSO, with a high content in MUFAs (48.09%), predominantly oleic acid and a minor content of linolenic and linoleic acids, compared to medium containing CW and WCO.

4. Conclusions

This study demonstrated the potential of the oleaginous bacterium *S. marcescens* UCP/WFCC 1549 as a sustainable candidate to be used in the production of biodiesel. This strain shown the ability to accumulate lipids using agro-industrial wastes, an attractive strategy to reduce the production costs associated with lipids production and, at same time, contribute to the reduction of environmental impact generated by the discard of residues. Also, it presented balanced profiles of FAMEs, with the best results in lipids produced in medium containing CW and WSO, with the highest percentage of MUFAs (48.09%), mainly oleic acid. These are desirable properties for a good quality biodiesel, according to international standards.

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