American-Eurasian J. Agric. & Environ. Sci., 15 (8): 1630-1636, 2015
ISSN 1818-6769

DOI: 10.5829/idosi.aejaes.2015.15.8.12756

Isolation of Microorganisms from a Swine Waste Stabilization Lake for Biodiesel Production

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Abstract: The use of fungi for oil production, aimed at the generation of biodiesel, has attracted increased interest from the global scientific community. This work aimed to isolate fungi from a swine waste stabilization lake, using several types of culture media as well as to evaluate the potential for oil production from these microbial isolates, for the purpose of biodiesel generation. Four types of culture media (GL, PDA, nutrient agar and standard count), as well as two pH ranges of the media and three dilutions were used for isolating the fungi strains. The methods of serial dilution and direct counting of CFU on the plates were used after five and nine days of incubation at 28ºC in the dark. The measurement of oil yield was performed by solvent extraction. Results exhibited that there was a microbial growth in all tested culture media. Thirteen fungal isolates showed lipid contents above 25% of their dry biomass, which characterizes them as oleaginous microorganisms. The isolates 25 (*Mucor* sp.), 26 (*Mucor* sp.), 31 (*Rhizopus* sp.) and 33 (*Rhizopus* sp.) showed the highest potential for the generation of oil and biodiesel production.

Key words: Sustainability · Lipids · Microbial oil · Oleaginous fungi

INTRODUCTION

The growing interest in the rational use of energy to meet the general needs of society is evident in areas such as industry, transportation, trade and other economic sectors of many countries. According to projections from the Energy Information Administration, worldwide energy consumption is expected to increase up to 50% between 2005 and 2030, mostly in developing countries [1, 2]. Biofuel production, especially biodiesel, has become one of the most efficient ways to diversify the energy matrix, which contributes to the preservation of the environment. Additionally, biofuel production favors economic development through the reduction of greenhouse gas emissions and the decentralization of investments, which leads to the generation of jobs and income in the rural areas. However, it is necessary to establish mechanisms for supporting the production and marketing operations of the biofuel industry. This support will only become possible through an augmentation of resources and through the interaction of public and private institutions and small farmers. Studies that demonstrate the legal, technical, environmental, social and commercial feasibility of biofuels are essential for liable assessment of this product [3].

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Certain microorganisms, such as microalgae, protozoa, fungi and bacteria can accumulate lipid in contents from 30 up to 80% of their biomass weight [4]. For a microorganism to be classified as oleaginous, it must contain a lipid content of greater than 25% (w/w) of its dry biomass [5]. Usually, the oil of such microorganisms is in the form of triglycerides, which are the main component of vegetable oils and animal fats. Therefore, microbial lipids could potentially be used as a raw material for the production of biodiesel through the reaction of transesterification with methanol in the presence of a basic catalyst. Some microbial species have a favorable potential for biodiesel due to their high lipid contents, which can comprise between 20 and 68% (w/w) of their dry mass [6, 7]. A lipid content of 85% of the dry biomass of *Mucor circinelloides* was observed which demonstrates a high potential for the production of biodiesel from this species [8].

The use of microorganisms as a source of lipids has been extensively investigated for the purpose of food and pharmaceutical additives and as ingredients of aquaculture feed [5, 9]. Microorganisms can be sources of edible oil, as they have the capacity to produce oil that is rich in polyunsaturated fatty acids. These fatty acids are useful as dietary supplements and can be used to promote child nutrition [10]. Recently, the use of oleaginous microorganisms for the production of biodiesel has been investigated but there is little information about the use of lipids from fungi for the production of biodiesel; specifically, microalgae that capture carbon dioxide and transform it into energy by using solar light were investigated. However, photosynthetic microorganisms do not grow well in reactor systems because of the need to supply light and the need for large growth areas to ensure their multiplication.

The use of microorganisms for biodiesel oil production has attracted increasing interest from the global scientific community [2]. However, until now, there has been a lack of information concerning the different fungi species that might be useful for that purpose. Moreover, the process of oil extraction, using the official methods of the Oil Chemist's Society is relatively simple [11]. In addition, biofuel-producing microorganisms can be cultivated in environments that are inhospitable for most vegetable species, such as degraded, desert and mining areas and swine waste stabilization lakes, among others. So, processing of these microorganisms does not compete with the production of food and biomass waste can be used as a microbial substrate, which adds an eco-friendly feature to the use of such biofuels. Biofuel-producing microorganisms are considerably preferable to oleaginous vegetable species, with regard to the potential for oil production. However, their use in a commercial setting requires additional studies to prove their true effectiveness relative to oleaginous plants [7]. The use of oleaginous microorganisms for the generation of biodiesel is based on their capacity to convert chemical energy of biomass into energy that is useful to humans and it is considered a clean process of energy production. Production of this type of energy does not cause a negative environmental impact or involves the production of dangerous byproducts. In addition, biodiesel production can be conducted locally near the region of demand, which minimizes the costs of the distribution system [12].

The production of swine is characterized by an intensive confinement system and a high waste production associated to it. This waste contributes to the degradation of the environment through pollution and contamination of the surface and underground water, as well as through organic pollution by the release of nitrogen and pathogenic microorganisms, air pollution and a high incidence of insects [7]. However, swine waste may be an excellent substrate for the growth of microorganisms that are of great interest for biofuel production. In the Southeast region of Goiás, Brazil, industrial swine farming generates a large amount of waste, which has been causing a negative impact on the environment. One way of mitigating this problem would be the use of swine waste stabilization lakes for the production of oleaginous fungi for the generation of biodiesel.

Therefore, the present work mainly aimed to isolate fungi from a swine waste stabilization lake, using several types of culture media as well as to evaluate the potential for oil production from the microbial isolates, for the purpose of biodiesel generation.

MATERIALS AND METHODS

This study was carried out in the "Laboratório de Microbiologia Agrícola", located at "Instituto Federal Goiano – Campus Rio Verde", Goiás, Brazil.

Fungi Isolation: Isolates of fungi were obtained from a swine waste stabilization lake, situated at the Swine Farming Sector of the institution. For the isolation, 10 mL swine waste samples were mixed with 90 mL of a saline solution (0.85%) followed by serial dilution of the samples up to 10^{-3}. From each dilution, 200 µL was transferred to

Table 1: Culture media and pH values used for the isolation of fungi from a swine waste stabilization lake, in Rio Verde, Goiás, Brazil

	Treatments	
Culture media	Media pH[1]	Sample pH[2]
Glucose and yeast extract (GL)	6.20	8.71
Potato dextrose agar (PDA)	5.40	8.70
Nutritive agar	6.80	8.73
Standard counting	7.00	8.73

[1]adjusted value following the recommendation of authors for the culture medium

[2]adjusted value to reach the mean detected values in the swine waste stabilization lake

a sterilized Petri dish, immediately followed by the culture media (Table 1). The media were warmed to 45ºC prior to being added to the dishes. The 10^{-1}, 10^{-2} and 10^{-3} dilutions were used for the isolation and counting of the microorganisms. Specifically, the number of microorganisms was determined by the direct counting of the dishes, in triplicate, after five and nine days of incubation at 28°C in the dark.

A completely randomized design (CRD) was used, with a factorial scheme of 4 x 3 x 2 (four culture media, three dilutions for each dish and two pH ranges), in triplicate. The culture media tested were: GL (glucose, 10g L^{-1}; yeast extract, 2g L^{-1}; agar, 15g L^{-1}) [13], potato dextrose agar (PDA), nutrient agar and standard counting. Two ranges of pH were used; one was the recommended pH for each type of culture medium (GL, pH = 6.2; PDA, pH = 5.4; Nutritive agar, pH = 6.8; Standard counting, pH = 7.0) and the other was similar to the pH values detected in the swine waste stabilization lake (pH = 8.7).

Oil Extraction and Composition: Twenty-six fungal isolates were chosen and they were presumed to be from different species, based on their morphology. The oil content of the fungal isolates was determined by Soxhlet extraction [14]. Briefly, 10 g of dry fungal biomass from each isolate were macerated, separated and transferred to a Soxhlet extraction device. About 250 mL of hexane was added (w/v ratio of 1 to25) and the mixture was kept under reflux at 60 ºC, for eight hours. The solvent was distilled under reduced pressure in a rotary evaporator and the oil percentage content was determined relative to the biomass weight.

Oil composition was determined by GC-EM. The isolates obtained in the study were stocked in a laboratory (in test tubes containing the appropriate isolation medium) at room temperature. The five fungal isolates with the highest oil contents were identified based on the morphological characteristics of vegetating and reproductive structures, which were observed in the micro culture using a microscope. These characteristics were used to evaluate the isolates in combination with identification keys [15, 16, 17].

Oil Transesterification: To produce biodiesel it was used 0.5 g of oil alone; 0.05 g of dry methanol; and 0.025 g of dry KOH and was stirred for 2 h at a temperature of 65°C [18]. After this process, the material was washed with a 0.05% solution of H_3PO_4 (v:v) to be removed and that excess hydroxyl and biodiesel has been separated in separatory funnel. The final product (biodiesel) was washed with hexane and $MgSO_4$ then biodiesel was recovered by rotary vacuum evaporator and maintained for HPLC analysis [19].

RESULTS AND DISCUSSION

Fungi Isolation: Fungi were isolated in the GL and PDA media in both of the pH ranges (Table 2). Similarly, Serrano-García et al. obtained four different fungal isolates (*Absidia* sp., *Aspergillus* sp., *Penicillium* sp. and *Rhizopu*s sp.) from swine waste [20]. At a neutral pH, roughly 95.000 CFU were detected and, in the acid pH, about 450 CFU were detected. Similar results using GL media has already been reported [13].

In this study, the pH did not influence the isolation capacity of the culture media, a fact that was also observed by other researchers [21, 22]. A total of 26 isolates of fungi were obtained. The isolates that had oil content were characterized as oleaginous, i.e., above 25% of their dry biomass, were the isolates numbered 4, 7, 9, 22, 24, 25, 26, 31, 32 and 33 (Table 4). Among these, four isolates (25, 26, 31 and 33) were selected to evaluate its use for biodiesel production based on its highest lipid content and diverse morphology.

Fungal Morphology and Oil Characterization: The observation of vegetative and reproductive structures demonstrated that the isolates belonged to two different genera identified as *Mucor* and *Rhizopus*. The isolates which showed the highest potential for the generation of oil and the production of biodiesel were 25 (*Mucor* sp.), 26 *(Mucor* sp.), 31 (*Rhizopus* sp.) and 33 (*Rhizopus* sp.) (Fig. 1A, B, C and D). Fungi of the genera *Rhizopus* and *Mucor* have potential for production of biomass and lipid contents of 20 and 25%, respectively [5, 23, 24]. In other studies *Mucor* sp. had a lipid content of 27.57% and a growth rate of two days for cultivation, which may be of interest for the production of biomass [25]. Lipid contents of 18% up to 57% have been observed in *Aspergillus* sp. [25, 26, 27].

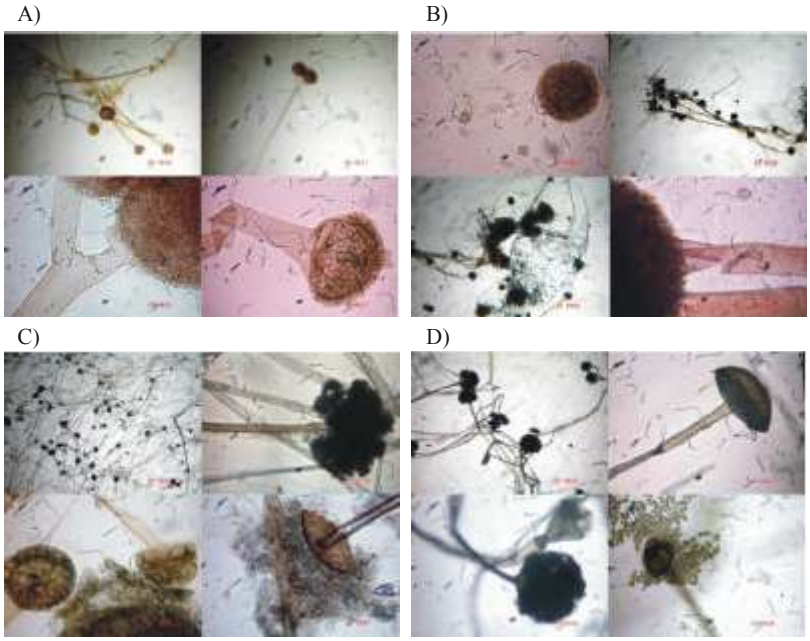

Fig. 1: Fungal isolate 25 (*Mucor* sp.) (A), 26 (*Mucor* sp.) (B), 31 (*Rhizopus* sp.) (C) and 33 (*Rhizopus* sp.) (D), obtained from a swine waste stabilization lake, in Rio Verde, Goiás, Brazil

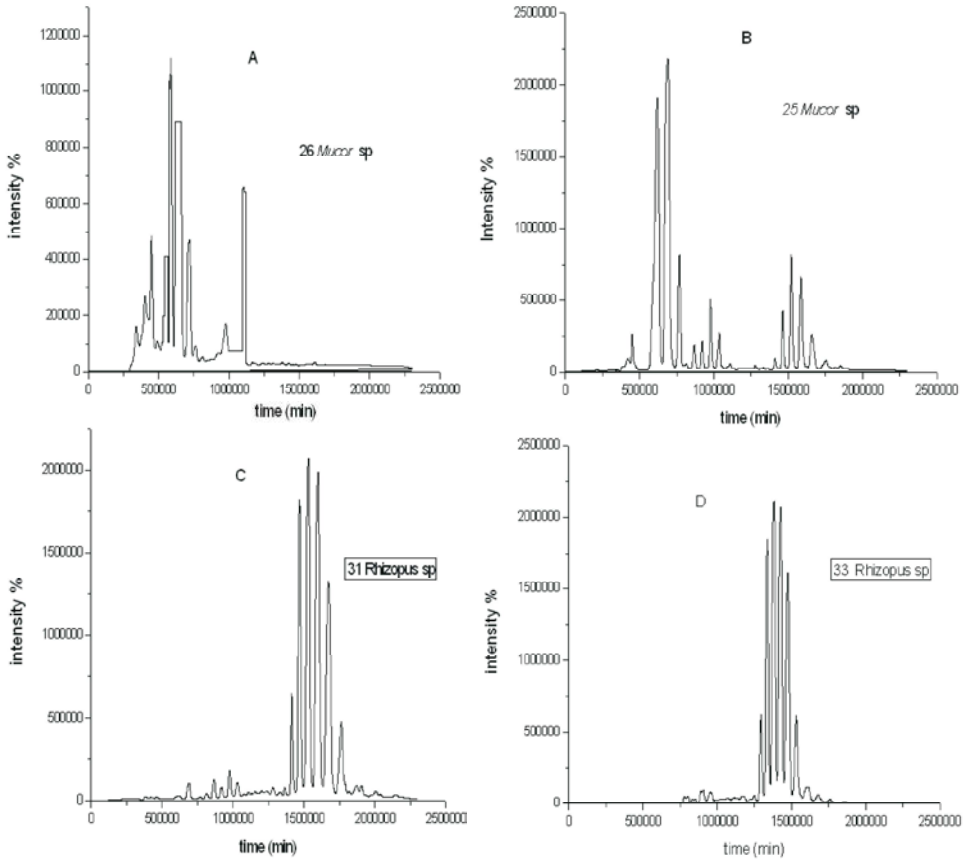

Fig. 2: High performance liquid chromatography for the oil samples from the fungi isolates: *Mucor* sp. (26) (A), *Mucor* sp. (25) (B), *Rhizopus* sp. (31) (C) and *Rhizopus* sp. (33) (D), obtained from a swine waste stabilization lake, in Rio Verde, Goiás, Brazil

Table 2: Presence (+) and absence (-) of fungi in a swine waste stabilization lake in Rio Verde, Goiás, Brazil, isolated with four types of culture media in two pH ranges

Culture Medium	pH	Fungi
Glucose and yeast extract (GL)	Sample	+
	Medium	+
Potato dextrose agar (PDA)	Sample	+
	Medium	+
Nutritive agar	Sample	-
	Medium	-
Standard counting	Sample	-
	Medium	+

Table 3: Composition analysis of the fatty acids from isolate 25 and 26 (*Mucor* sp.), 31 and 33 (*Rhizopus* sp.), obtained from a swine waste stabilization lake in Rio Verde, Goiás, Brazil

Fatty acid		Isolate 25	Isolate 26	Isolate 31	Isolate 33
Palmitic acid	16:0	14.8	14.3	16.0	14.5
Palmitoleic acid	16:1	0.9	0.7	0.5	0.7
Margaric acid	17:0				0.1
Stearic acid	18:0	18.5	16.9	18.8	14.9
Oleic acid	18:1	39.5	42.2	45.8	42.6
Linoleic acid	18:2	16.6	18.3	14.0	17.7
Linolenic acid	18:3	3.6			
Arachidic acid	20:0	1.3	1.4	1.5	1.0
Gadoleic acid	20:1	0.3			
Behenic acid	22:0	1.8	1.5	1.1	1.0
Erucic acid	22:1	0.3	0.3		0.1
Lignoceric acid	24:0		2.9	1.7	2.4
Nervonic acid	24:1	2.3			
Cerotic acid	26:0		1.4	0.6	
Melissate acid	30:0		0.8		1.3

Table 4: Oil mass and oil content of fungal isolates of interest for the generation of oil and biodiesel production obtained from a swine waste stabilization lake in Rio Verde, Goiás, Brazil

Isolate	Oil content (%)
4	47.13
7	25.41
9	34.48
19	20.09
22	30.51
24	32.3
25	44.41
26	73.04
31	46.14
32	39.79
33	42.76
34	20.93

Table 3 shows the profile of fatty acids extracted from the fungal isolates. For all the isolates the main fatty acid found was oleic acid, followed by stearic and linoleic acid. Similar results were found with *Mucor circinelloides* species [8]. Microbial oils generally differ from vegetable oil because they are rich in polyunsaturated fatty acids [28].

Biodiesel production from fungi has advantages compared to production by microalgae, as microalgae require the presence of solar light. Moreover, one of the techniques for microalgae culture, which aims at the generation of oil for biodiesel, consists of enclosing them in tubes and it is necessary to supply energy for stirring the culture media and to ensure their exposure to sunlight. Producing biodiesel from microalgae enclosed in tubular bioreactors is not practical from an environmental point of view and the culture of micro algae in open lakes is not a solution either. Although the production of greenhouse gases is lower, the water from lakes tends to evaporate, which causes a high consumption of water and the amount of biodiesel produced is low. In fact, the amount of greenhouse gases generated in the production of biodiesel from microalgae is about four times higher than the amount of greenhouse gases produced by conventional diesel production [29]. Future studies are necessary to evaluate the amount of oil produced by the fungal isolates found in this study.

Description of Biofuel Production: Biodiesel was characterized by HPLC (Shimadzu CTO-20), using UV-VIS detector (ë = 205 nm) and Shim-pack VP-ODS (C-18, 250 mm 4.6 mm id) column [30] to quantify the conversion of mono, di and triglycerides. Analysis time took 25 min and peaks characterized by time intervals where 0-5 min monoglycerides, alkyl esters 5-7,5, 7,5-10 diacylglycerols and triacylglycerols 10 to 25 min [19].

The results obtained by HPLC showed that the fungi *Mucor* sp. (sample 26) had a higher yield in the transesterification process (Fig. 2), the conversion into alkyl esters by basic catalysis was approximately 73% as *Mucor* sp. (25), *Rhizopus* sp. (31) and *Rhizopus* sp. (33) had a yield of 51.7; 7.9; 6.1 and 7.3 respectively (Fig. 2 A, B, C and D). Samples of the fungus *Mucor* sp. showed higher yield in the conversion of esters, due to low concentration of acidic compounds in the oily fraction, since the fungus *Rhizopus* sp. showed a yield of conversion of esters low due to low yield of oil production and due to presence of fatty acids. Finally, despite the oil content of the fungus *Rhizopus* sp. have been high, the conversion into alkyl esters was low due to high concentration of fatty acids in the constitution of the sample.

Biofuels are renewable energy sources, derived from agricultural products such as sugar, oleaginous plants, forest biomass and other sources of organic matter. In some cases, biofuels can be used in an isolated way, while in other cases; they are added to conventional fuels.

Some examples of biofuel products are biodiesel, ethanol, methanol, methane gas and vegetable coal [31]. The yield and quality of a transesterification reaction will depend only on the conditions of the reaction medium and the chemical quality of the oil. An example of this is the presence of water in the reaction, since this will reverse the esters in mono, di and triglycerides [18]. The chemical composition of the oil is another factor that directly influences the quality and quantity of esters produced, depending on the catalyst used. In fact, oils composed of polyunsaturated fatty acids have low oxidative stability and threaten the viability of the esters produced in the transesterification process. This fact is justified by the presence of more than three unsaturations in the fatty acid chain [32]. So overall, oils composed of short chain fatty acids from 12 to 16 carbons, which are saturated or monounsaturated are best suited for biofuel production by transesterification. This fact is justified by the ease of breaking the carbon chain for processing of composite than the triacylglycerol molecular mass. Furthermore, the use of renewable fuels reduces emissions of pollutants in atmospheric air.

CONCLUSIONS

There was a microbial growth in all culture media tested. Thirteen fungal isolates showed lipid contents above 25% of their dry biomass, which characterizes them as oleaginous microorganisms. The fungal isolates 25 (*Mucor* sp.), 26 (*Mucor* sp.), 31 (*Rhizopus* sp.) and 33 (*Rhizopus* sp.) showed the highest potential for the generation of oil and biodiesel production.

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