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Biodiesel production from microbial whole cell biocatalyst

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Abstract

Enzymatic mode of biodiesel production has recently gained a special attention among researchers because it eliminates the disadvantages of alkaline process such as end product recovery, waste water treatment, energy intensive and etc. Recently, considerable attention has paid in the direct use of intracellular lipase as a whole-cell biocatalyst (i.e immobilization of the enzyme secreting microbial cells) for biodiesel production where immobilization was carried out at the of microbial cell cultivation. The present investigation is focused on to establish appropriate conditions for immobilized whole cell biocatalyst for the production of biodiesel through transesterification. Fungi strains were isolated from wastes generated from oil industries and screened for the production of extracellular lipolytic enzymes and the most productive strain B2 was identified as *Aspergillus sp*. for further applications.

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Introduction

Eco dreamers have long hoped for a way to drive around without contributing to global warming, but the slow pace of progress in alternative fuel technologies has kept that vision from materializing (Akoh *et al.,* 2007). Vegetable oils have attracted attention as a potential renewable source for the production of an alternative for petroleum based diesel fuel due to the diminishing petroleum reserves and environmental consequences of exhaust gases from diesel engines (Vicente *et al.,* 1998). Methyl and ethyl esters of fatty acids, better known as biodiesel, are nontoxic, biodegradable, and an excellent replacement for petroleum diesel (Vangerpen *et al.,* 2004).

Transesterification of vegetable oils and animal fats for the production of fatty acid alkyl esters in a well established industrial process. The conventional biodiesel technology involves the use of an inorganic base or acid catalyst at or near the boiling temperatures of the triglyceride alcohol mixture. The removal of catalyst is through neutralization and eventual separation of salt from the product esters, which is difficult to achieve (Ban *et al.,* 2001). Enzymatic transesterification of triglycerides offers an environmentally more attractive option to the conventional process. However, the high cost of the enzymes often makes the enzymatic processes economically unattractive. The key step in enzymatic processes lies in successful immobilization of the enzyme which will allow for its recovery and reuse (Balcao *et al.,* 1996). However, biodiesel is still far more expensive than conventional petroleum-derived diesel due to the higher feedstock and processing costs. By creating a continuous process it may be possible to reduce the production cost and hence lower the overall cost of biodiesel, making the price of biodiesel more competitive. Utilizing immobilized whole cell lipase biocatalysts can substantially reduce cost by avoiding isolation, purification and immobilization of extracellular lipase (Fukuda *et al.,* 2009; Xiao *et al.,* 2009). Among the established whole-cell biocatalyst systems, filamentous fungi have been identified as robust candidates (Fukuda *et al.,* 2008). For example, it has been shown that *Rhizopus oryzae* when immobilized as whole cells on porous biomass support particles (BSPs) can efficiently catalyze transesterification for biodiesel production in both solvent-free and solvent reaction systems (Li *et al.,* 2007). Consequently, researches on immobilized whole-cell biocatalytic techniques have become more attractive as it offers potential feasibility for the industrialization of enzymatic biodiesel production. In this study, we investigate the application of a fungal isolate as a whole-cell biocatalyst for enzymatic mediated biodiesel production. The work focuses on the parameters affecting the enzyme production. Additionally in order to improve the stability of the whole-cell lipase in repeated batch operation was investigated.

Material and methods

Isolation of fungi

Oil contaminated soil samples were obtained from oil spilled areas of various oil industries around Madurai City, Tamil Nadu. Soil sample was serially diluted and plated on Sabouraud Dextrose agar medium. The plates were incubated at 37 °C for 3 days. The isolates were sub cultured and used for further studies.

Screening

Each pure fungal isolate was screened for its lipolytic activity using LBT (Luria - Bertani Tributryin) screening method. The isolates were also inoculated on LBT agar medium and incubated at 37 °C for 3 days. The appearance of clear zone indicates the presence of lipase activity.

Lipase production

High lipolytic fungal strain was inoculated in production medium contained (g/L): NaH_2PO_4 12, KH_2PO_4 2, $MgSO_4 \cdot 7H_2O$ 0.3 and $CaCl_2$ 0.25. Peptone at 1 % and coconut oil at 2 % were used as nitrogen and carbon sources, respectively. The initial pH was adjusted to 6.5 and was further incubated at room temperature at 127 rpm for 5 days.

Lipase assay

The enzyme activity was assayed and estimated using culture filtrate. The enzyme activity was performed using paranitrophenyl palmitate (pNPaplmitate) as substrate. One unit was defined as the amount of enzyme liberating 1 µmol of *p*-nitrophenol per minute at 37 °C, under the conditions of the assay.

Fatty acid profile of non edible oils

Fatty acid composition was determined using Gas Chromatography (HP 6890). The Fatty Acid Methyl Esters were thrice extracted from the mixture with redistilled n hexane. The content was concentrated to 1 µl for gas chromatography analysis and 1 µl was injected into the injection port of GC. Carrier gas was Nitrogen, Inlet temperature was 250°C, column dimension 30m × 0.25mm × 0.25 µm. Initial temperature is 60°C, first ramping at 12°C for 2 min, while second ramping at 15°C for 8 min. Detector temperature was 320°C

Characterisation of the non edible Oil

Acid value (ASTM D 664 – 07), Iodine value (ASTM D 1959 – 97), Saponification value (ASTM D 94 – 07), Peroxide value AOAC (1990), Kinematic Viscosity (*Redwood viscometer - Deep vision instrument*) and Calorific value (ASTM D 240 – 09) were studied.

Preparation of spore suspension

The organism was subcultured on Sabouraud Dextrose agar medium and incubated for 72 h at 37 °C. The spore crop of each slant was scrapped into 5 ml sterile water and shaken well with sterile glass beads on a rotary shaker for 30 min to break the spore's chain and to make a uniform suspension. This suspension was filtered through sterile cotton to remove the hyphal filaments. This spore suspension (5×10^6 spores per ml) was used for preparing the beads.

Immobilization on polyurethane foam (Biomass Support Particles, BSP)

The polyurethane foam material used for the immobilization studies had a porosity of 100 - 500 µm. It was cut into 1cm² pieces (Kuhad *et al.,* 2004) and washed with distilled water.

Erlenmeyer flasks containing 150 mL of sterilized basal medium with polyurethane foam pieces (30 no) at an initial pH, with the preferred carbon source and then sterilized at 121°C for 15 min (Kuhad *et al.,* 2004). This was aseptically inoculated by transferring the fungal spores (5×10^6 spores / ml). It was then incubated in a reciprocal shaker at 150 rpm at 37 °C. The fungal cells were thus spontaneously immobilized within the polyurethane foam during cultivation. The polyurethane foam was then separated from the culture broth after incubation. After washing five times in tap water, the polyurethane foams were stored at 20 °C, and then dried in a freeze dryer for 24 h. Immobilized cells with a water content of approximately 2–3 percent dry weight were used as the biocatalyst for the biodiesel production.

Glutaraldehyde (GA) treatment of immobilized cells

GA treatment was carried out by adding 0.1–1.0 vol.% of GA solution to immobilized cells separated from the culture broth, before incubation at 25°C for 1 h. The GA-treated cells were then separated from the solution by filtration and shaken in phosphate buffer (0.1M, pH 6.5) for 5 min at 4 1C, followed by washing with tap water for 1 min. These GA-treated cells were then stored at -20°C and were dried to approximately 2–3% water content in a freeze-drier for 24 h before use.

Whole cell catalyzed biological transesterification reaction

The transesterification reaction was carried out in a 50ml screw-cap bottle at 35°C on a reciprocal shaker (130 rpm). The compositions of the reaction mixtures were as follows: oils 5 g, tert-butanol, methanol, 0.1M phosphate buffer

(pH 6.8 ± 0.2, used to adjust water content) and whole cell. For solvent-free system, methanol was added into the reaction mixture at 0, 24, 48 h, respectively. Fifty microlitre samples were taken from

the reaction mixture at every 12 h interval centrifuged to obtain the upper layer and analyzed. After one batch, the whole cell was filtrated and added directly into a new batch of reaction mixture.

Reusability of Immobilized wholecell in Transesterification

Repeated batch Transesterification was conducted with immobilized cells by running the reaction for 96 h per batch. At the end of each batch the immobilized cells were separated and thoroughly washed with sterile water, a fresh reaction mixture was added and the transesterification was continued.

Analysis methyl esters

The quantitative analysis of methyl ester were analysed using Fourier Transform Infra Red (Perkin-Elmer- Paragon IR spectrophotometer), Gas Chromatography (HP 6890) and ^1H Nuclear magnetic resonance (NMR) Nuclear magnetic resonance (NMR) Bruker 300MHz spectrometer.

Results and discussion

Sample collection

Samples collected from five different oil industries in and around Madurai used for isolation of lipolytic organisms. Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc. This kind of oily environment may provide a good environment for isolation of lipase producing microorganisms. In this context, oil industries were chosen.

Table 1. Optimized condition for lipase production**.**

Parameter	F1
Carbon source	*Callophyllm inophyllum* Punnai oil (2%)
Nitrogen source	Peptone (1.5 %)
Salt	$MgCl_2$
Temperature (°C)	37 ± 2
pH	6.8
Incubation period (h)	60

Isolation and Screening of lipolytic fungal species

Among the isolated fungal cultures, strain B2 was found to have high lipolytic activity using LBT (Luria-Bertani Tributryin) screening method. The fungus strain B2 showed maximum zone of clearance when compared to other strains. The diameter of zone is directly proportional to the quantity of lipase produced. B2 showed 1 cm diameter zone of hydrolysis in LBT agar plate.

Table 2. Fatty acid profiles of oils selected non edible oils.

Fatty acid	*Madhuc longifolia* Mahua	*Azadirachta indica* neem oil
Myristic (C14:0)	1.0	
Palmitic (C16:0)	17.8	14.9
Stearic (C18:0)	14.0	14.4
Oleic (C18:1)	46.3	61.9
Linoleic(C18:2)	17.9	7.5
Arachidic (C20:0)	3.0	1.3
Mean molecular wt (g/mol)	870.59	874.25

Identification of Lipolytic fungi

The present fungal strain was isolated from oil spilled areas soil and identified as based on morphological and microscopical observation as *Aspergillus sp.* (Fig 1)

Table 3. Physiochemical characterises of the selected non edible oil.

Parameters	*Azadirachta indica* Neem oil	*Madhuca longifolia* Illuppai oil
Moisture content (%)	0.09 ± 0.001	0.016 ± 0.004
Free fatty acids (%)	5.03 ± 0.5	2.82 ± 0.2
Acid value (mg KOH/g)	10 ± 0.5	5.161 ± 0.2
Kinematic viscosity @ 40 °C (cSt)	35.83	55.03
Iodine value (g I_2/100g)	45. 36 ± 0.1	78.12 ± 0.3
Saponification value (mg KOH/g)	169.03 ± 0.5	126 ± 0.2
Peroxide value (meq/kg)	1.4 ± 0.05	3.2 ± 0.1
Calorific value (MJ/kg)	42.1	38.86

Optimized condition for lipase production

The optimized condition for lipase production was determined by optimization technique as represented in Table 1. The production of lipase is vastly significant in culture medium supplemented with lipids as their carbon source. It is confirmed that the

lipase activity is significantly induced by the presence of lipid substrates such as oils in the medium.

Table. 4. Suitable reaction whole cell catalyzed transesterification.

Parameter	*Azadirachta indica* (Neem oil)	*Madhuca longifolia* Illuppai oil
Amount of beads (g)	5	3
Oil/ alcohol molar ratio	1:4	1:4
Temperature (°C)	35 ± 0.2	35 ± 0.2
Water content (%)	6	8
pH	7	6.5
Reaction Time(h)	48	36
Yield	69 ± 2 %	75 ± 4 %

Fatty acid profile of non edible oils

Gas Chromatography analysis of fatty acids present in the neem oil and Mahua is displayed on Table 2.

Table 5. Comparison of wholecell transesterification with GA treatment and Non GA treatment.

Oils	Non GA treatment	GA treatment
Azadirachta indica (Neem oil)	69 %	77%
Madhuca longifolia (Illuppai oil)	75%	80%

Characterises of the non edible Oil

The results of the physicochemical analysis of the neem oil (*Azadirachta indica)* and (*Madhuca longifolia)* Illuppai oil is shown on Table 3. The acid value of the neem oil was well below this maximum limit, making the oil a good candidate for biodiesel production. The higher (> 1.5 % w/w) free fatty acid (FFA) content may cause the problems such as more catalyst needed, slow reaction rate and formation of soap instead of biodiesel and reduction in yield of biodiesel. The FFA obtained for the neem oil was 5.03 (% w/w) and Illuppai oil was about 2.82 %. Likewise the FFA for rubber seed oil was 9.6 %, coconut oil was 4.2% and palm kernel oil was 9% (Satyanarayana and Muraleedharan, 2011) The kinematic viscosity is measurement the flow resistance of the fuel. High viscosity interferes with injector operation, resulting in poor atomization of the fuel spray, and has been associated with increased engine deposits. Ordinarily, vegetable oils have higher viscosity which is reduced by transesterification processes. Iodine value an indicator of the unsaturation of the fuel, which has been linked with formation of engine deposits and problems in storing the fuel (Meher, 2006). The iodine value of neem oil was 45.36 similarly for Illuppai oil is also ranged about 78.12. The saponification value of the neem oil 169.03 mgKOH/ g oil and Illuppai oil 126 mgKOH/ g oil was found to be small, indicating high concentration of triglycerides and hence neem oil and Illuppai oil can be a suitable feedstock for the production of biodiesel. The peroxide value is a measure of the peroxides contained in the oil. The value obtained for the neem oil (1.4) is low compared to other vegetable oils indicating a lower oxidation state of the neem oil.

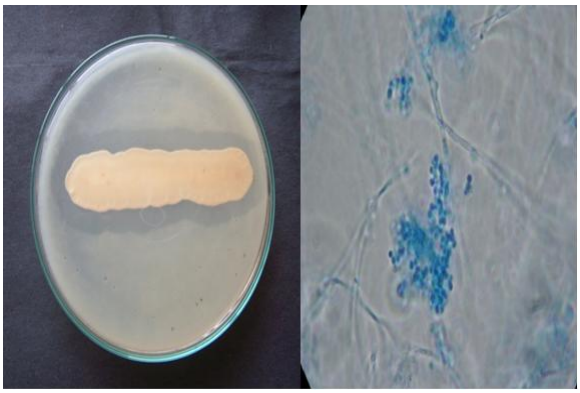


Fig. 1. Lipolytic Fungal Strain on LBT plate (A) and Microscopic observation of Fungal Spore.

Whole cell catalyzed biological transesterification reaction

From the results of single parameter experiments, reaction time, reaction temperature, substrate molar ratio, water content, and cell concentration were found to have significant effect on yield. Tamalampudi *et al.* were the first to utilize whole-cell catalyzed alcoholysis of the nonedible jatropha oil with R. oryzae cells that had been immobilized into BSPs. They found that the whole-cell catalysts performed better than the commercial enzyme Novozym 435. These results suggest that expensive

downstream processing steps for potential biodiesel production from jatropha oil can be avoided with whole-cell biocatalysts. Li *et al.* reported methyl esters production from crude and acidified rapeseed oil using whole-cells of R. oryzae IFO4697. Suitable conditions for transesterification of two different non edible oils were represented in table 4.

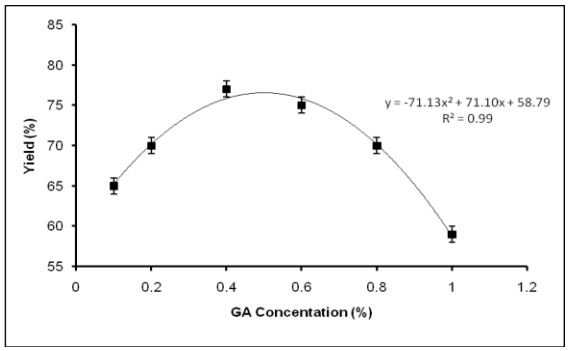


Fig. 2. Effect of GA treatment on wholecell transesterification of Neem oil.

Reusability of Immobilized wholecell in Transesterification

In order to test the stability of GA treated immobilized cell as biocatalyst were separated from the reaction mixture by filtration and directly used for the next cycle. The time of methanolysis reaction using wholecells and is kept as constant for each reaction cycle. It was found that there was almost no significant decrease in methyl esters production even after 5 batches cycle in both lipases and both can be used for repeated batches cycles.

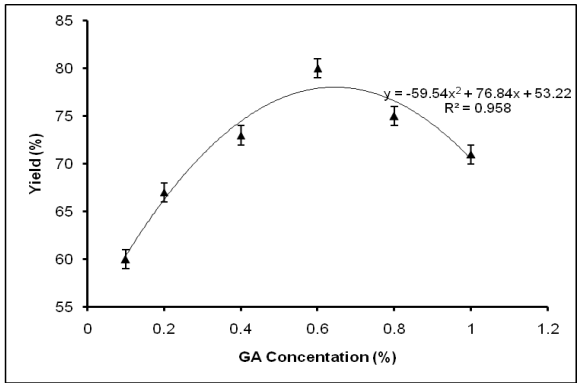
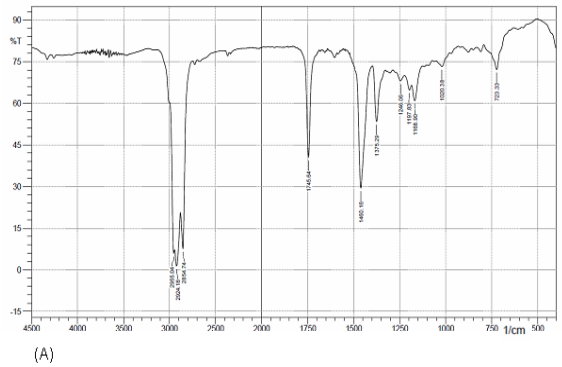


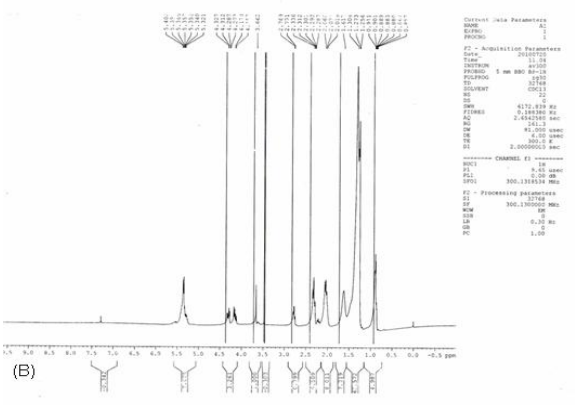
Fig. 3. Effect of GA treatment on wholecell transesterification of Illuppai oil

Analysis of oil and its methyl ester in FTIR and NMR

FT-IR refers to the fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. Molecular bonds vibrate at various frequencies depending on the elements and the type of bonds. Normally esters have vibration frequency at 1730-1750 cm^{-1}. In FTIR analysis of Mahua oil biodiesel, the peak of the band in 1743 cm^{-1}. This band conforms that ester groups are present in the biodiesel. In infrared spectrum of Illuppai oil biodiesel have vibrations at 1171.88, 1364.31, 1743.52, 2856.67 cm^{-1}, and 3006.49 cm^{-1}. These values suggest this spectrum corresponds to Mahua oil biodiesel.



(A)



(B)

Fig. 4. (A) FTIR Spectrum (B) NMR Spectrum for biodiesel.

Proton NMR provides a good probe for biodiesel since ^{1}H is the most naturally abundant and most sensitive. NMR has been used to monitor the transesterification

reaction used in the production of biodiesel and to monitor the oxidation of methyl esters in biodiesel. NMR also used to quantify blends of biodiesel and petroleum diesel. NMR is also used determine the relative amounts of identified components in biodiesel. The peaks at 5.35 ppm and 2.8 ppm are related to the ^1H located at or near the double bond(s) within the unsaturated methyl esters, 18:1 and 18:2. The sharp peak at 3.7 ppm is due to the ester methyl located next to the carbonyl carbon and the triplets around 0.9 ppm are from the terminal alkyl methyl in each of the methyl esters.

Conclusion

In this study, a newly isolated *Aspergillus* sp.strain (B2) was applied as whole-cell biocatalyst for biodiesel production from two different indigenous high FFA non edible oils via strain immobilization within BSPs. Both culture condition for lipase production and methanolysis were optimized. These findings indicate that, given the simplicity of the lipase production process and the long-term stability of lipase activity, the use of whole-cell biocatalysts immobilized within BSP s and treated with GA solution suggest a favorable means of biodiesel fuel production for industrial application.

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