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Fungal Production of Single Cell Oil Using Untreated Copra Cake and Evaluation of Its Fuel Properties for Biodiesel

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This study evaluated the microbial conversion of coconut oil waste, a major agro-residue in tropical countries, into single cell oil (SCO) feedstock for biodiesel production. Copra cake was used as a low-cost renewable substrate without any prior chemical or enzymatic pretreatment for submerged growth of an oleaginous tropical mangrove fungus, *Aspergillus terreus* IBB M1. The SCO extracted from fermented biomass was converted into fatty acid methyl esters (FAMEs) by transesterification and evaluated on the basis of fatty acid profiles and key fuel properties for biodiesel. The fungus produced a biomass (8.2 g/l) yielding 257 mg/g copra cake SCO with ~98% FAMEs. The FAMEs were mainly composed of saturated methyl esters (61.2%) of medium-chain fatty acids (C12–C18) with methyl oleate (C18:1; 16.57%) and methyl linoleate (C18:2; 19.97%) making up the unsaturated content. A higher content of both saturated FAMEs and methyl oleate along with the absence of polyunsaturated FAMEs with ≥4 double bonds is expected to impart good fuel quality. This was evident from the predicted and experimentally determined key fuel properties of FAMEs (density, kinematic viscosity, iodine value, acid number, cetane number), which were in accordance with the international (ASTM D6751, EN 14214) and national (IS 15607) biodiesel standards, suggesting their suitability as a biodiesel fuel. The low cost, renewable nature, and easy availability of copra cake, its conversion into SCO without any thermochemical pretreatment, and pelleted fungal growth facilitating easier downstream processing by simple filtration make this process cost effective and environmentally favorable.

Keywords: *Aspergillus terreus* IBB M1, copra cake, single cell oil, biodiesel, FAME, fuel properties

Biodiesel or fatty acid methyl esters (FAMEs) derived from plant, algal oils, and animal fats are considered an alternative energy source to petro-diesel. The advantages of biodiesel include its renewable and non-toxic nature, positive fossil energy imprint, good flash point, environmental compatibility, and biodegradability. Biodiesel's main economic challenge is its high feedstock/raw material cost, especially with respect to plant oils. Lipids of oleaginous fungi (single cell oil, SCO) are emerging as a promising sustainable feedstock for biodiesel production [6]. Filamentous fungi possess biotechnological advantages for SCO production, namely, their ability to use lignocellulosic carbon and pelleted growth for easier downstream processing. The use of locally available agro-industrial residues as substrates is essential to improve the process economics of microbial biodiesel [2]. A key step is the pretreatment of complex agro-residues to facilitate the use of fermentable carbohydrates with either costly commercial enzymes or hazardous chemicals requiring expensive detoxification processes. In this context, oleaginous isolates of mangrove fungi, which contribute to the intense carbon processing of this ecosystem, are ideal candidates for the conversion of untreated agro-residue into lipids in a cost-effective and eco-friendly way [3, 4].

The global production in 2013–14 of copra cake, an agro-residue obtained from dried coconut (*Cocos nucifera*) flesh

after mechanical oil expelling, was 1,931,000 tons with India being the major producer (230,000 tons) after the Philippines. The conventional application of copra cake for animal feed formulation has reduced globally owing to tightened aflatoxin B1 regulations and inferior nutritive value due to its lower protein content (20–25%) as compared with other oil cakes. This study evaluates the use of copra cake as a renewable substrate for the production of microbial SCO as a biodiesel feedstock.

The residual coconut oil cake after pressing (copra cake) was collected from a local oil mill, dried, and milled to 1 mm particle size. The oleaginous fungal strain used, *Aspergillus terreus* IBB M1, was previously isolated in our laboratory from mangrove wetlands of the Indian west coast [4]. The liquid basal medium used for fungal cultivation contained (in g/l) NaCl 15.0, $Na_2HPO_4 \cdot 2H_2O$ 5.0, KH_2PO_4 7.0, $MgSO_4 \cdot 7H_2O$ 1.5, $CaCl_2 \cdot 2H_2O$ 0.1, $FeCl_3 \cdot 6H_2O$ 0.08, $ZnSO_4 \cdot 7H_2O$ 0.01, and (in mg/l) $Co(NO_3)_2 \cdot 6H_2O$ 0.1, $CuSO_4 \cdot 5H_2O$ 0.1, $MnSO_4 \cdot 5H_2O$ 0.1. The milled copra cake (5–50 g/l) was suspended in 50 ml of basal medium (in 250 ml Erlenmeyer flasks) as the sole carbon and energy source. The flasks were inoculated with 1 ml of spore suspension ($1 - 3 \times 10^8$/ml) prepared in sterile Tween-80 (0.1% (v/v)) solution and incubated in an orbital shaker (30°C, 120 rpm). The fermented biomass was harvested at regular time intervals by vacuum filtration, and then dried in an oven until constant weight for biomass (dry weight) and SCO determination.

The extraction and gravimetric estimation of total lipids or SCO yield (milligram per gram of initial substrate) were carried out by cryo-pulverization of fermented biomass in the presence of chloroform:methanol (2:1 (v/v)). FAMEs were prepared by alkali-catalyzed transesterification of SCO (90 min; 60°C; methanol:oil (60:1); NaOH (1.5%)).The crude FAME was recovered by rotary evaporation of excess methanol, reconstituted in chloroform:methanol (2:1 (v/v)), washed repeatedly with water until neutrality, and dried over anhydrous sodium sulfate. The fatty acid composition of the FAME was analyzed according to the AOAC Method Ce 1–62, using an authentic standard (AOAC 996.06 Standard; Restek Corp., USA). The suitability of SCO as a biodiesel feedstock was evaluated by ascertaining the physicochemical fuel properties of the FAMEs. The saponification number (SN) and iodine value (IV) were estimated as per standard methods [4]. The acid number was estimated using a Kittiwake DIGI Biodiesel test kit (Kittiwake Developments Ltd., UK). The kinematic viscosity (KV), cetane number (CN), and higher heating value (HHV) were determined by using prediction models, given in Table 1.

The copra cake contained 5.5, 20, 42 and 4 (%, w/w) residual oil, crude protein, total sugars, and ash, respectively. Batch culture experiments were performed to check the effect of initial substrate concentration (5–50 g/l) on the growth of *A. terreus* IBB M1 for SCO production (Fig. 1A). The optimal substrate concentration was 10 g/l, resulting in 5.1 g/l biomass with 58 mg/g of SCO yield in 96 h. At higher copra cake concentrations (30–50 g/l), a decrease in both biomass and lipid yield was noted. The submerged fungal growth as visualized by SEM images displayed a network of fungal hyphae over the substrate particles (Fig. 1A inset). Time-course fermentation studies with 10 g/l untreated substrate showed that maximal biomass production was at 48 h (8.2 g/l), yielding 257 mg/g of SCO (Fig. 1B). The reasonably good SCO yield by *A. terreus* IBB M1 is significant, taking into account the fact that there was no thermochemical or enzymatic pretreatment of the copra cake.

Fatty acid analysis of the raw material (*i.e.*, copra cake) indicated that the major fraction was saturated fats (86.2%) whereas mono- and polyunsaturated fats were detected at 10.3% and 3.5%, respectively (Table 2). The saturated fraction was mainly composed of lauric acid (C12:0, 44.66%) and myristic acid (C14:0; 21.58%) followed by other short- and medium-chain fatty acids: palmitic (C16:0; 11.53%),

Table 1. Prediction models used for determination of biodiesel fuel properties of fatty acid methyl esters (FAMEs) derived from lipids of mangrove fungal strain *A. terreus* IBB M1 grown on copra cake.

Fuel property/quality parameter	Prediction model based on fatty acid profile
Kinematic viscosity (KV)	$\nu_{mix} = \sum(A_c \nu_c)$ [a]
Cetane number (CN)	$CN = \sum X_{ME}.CN_{ME}$ [b]
Higher heating value (HHV)	$HHV = 49.43 - [0.041(SN) + 0.015(IV)]$ [c]

[a] ν_{mix} - kinematic viscosity of sample; A_c - the relative amount (%/100) of individual FAME component; ν_c - viscosity of individual FAME component obtained from the database [5].

[b] X_{ME} - weight percentage of each methyl ester; CN_{ME} - cetane number of individual methyl ester [9].

[c] SN - Saponification number; IV - iodine value [1].

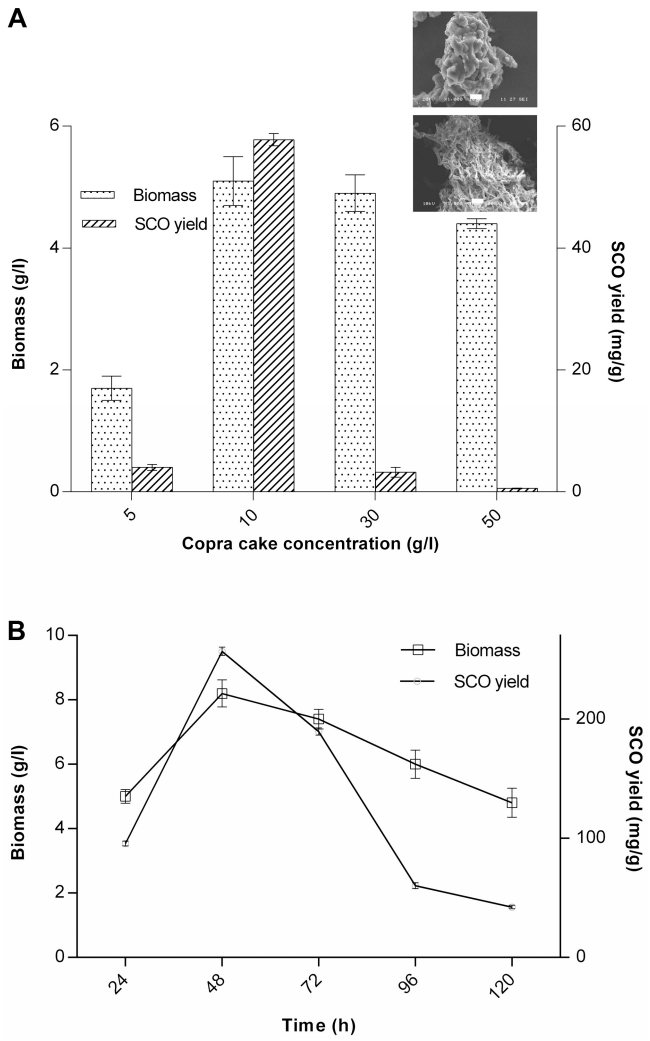

Fig. 1. Untreated copra cake as substrate for growth and lipid (SCO) production from *A. terreus* IBB M1.

(**A**) Effect of initial substrate concentration (5–50 g/l, 96 h). **Inset:** SEM images of native substrate (top) and after fungal growth (bottom). Bar indicates 10 µm. (**B**) Biomass and SCO yield on untreated copra cake (10 g/l). Results presented as the mean ± standard deviation for three replicates.

caprylic (C8:0; 3.62%), capric (C10:0; 2.19%), and stearic acids (C18:0; 2.63%). The sole monounsaturated fatty acid detected was oleic acid (C18:1n9c; 10.29%). Among polyunsaturated fatty acids, linoleic acid (C18:2n6c) was observed at 3.5% whereas other polyunsaturated fatty acids were not detected in the raw material (copra cake).

Alkali-catalyzed transesterification of the SCO obtained from the fermented biomass grown on copra cake resulted in 98% of its conversion to FAMEs (biodiesel). This was similar to that reported in our earlier study for *A. terreus* IBB M1 grown on sugarcane bagasse [3]. Thus, biodiesel conversion from SCO of fungal biomass grown on copra cake involved three steps: growth of *A. terreus* IBB M1 on raw chemically untreated copra cake to produce pelleted biomass, enabling its easy harvesting by filtration, extraction of SCO, and its transesterification to produce FAMEs for biodiesel. The FAMEs (transesterified SCO) exhibited a fatty acid profile different from that of the raw material (copra cake) (Table 2). Although the saturated fatty acids made up the major fraction of transesterified SCO, it was 25% less than that observed for the raw material (copra cake). The content of short- and medium-chain fatty acids was found to be reduced with lowering in content of lauric (C12:0) and myristic (C14:0) acids, which were detected at 15.5% and 14%, respectively. In contrast, ~5% increase each in palmitic (C16:0; 16.27%) and stearic acid (C18:0; 7.72%) content was observed in the SCO as compared with that for copra cake. Among monounsaturated FAMEs, methyl oleate (C18:1) was the major fatty acid observed at 16.57%, which was 6.3% higher than that seen in copra cake. The major polyunsaturated FAME was methyl linoleate (C18:2n6c) with 19.97% content, which is 16.47% higher than that found in copra cake. The long-chain unsaturated fatty acids (*e.g.*, with ≥4 double bonds) were not detected either in SCO or copra cake.

The fatty acid profiling of SCO obtained from *A. terreus* IBB M1 grown on copra cake suggests that the fungus has not only utilized the copra cake for its growth and lipid accumulation, as evident from the production of biomass and SCO, but also altered the fatty acid composition of the raw material, making it more suitable for biodiesel production. For example, the content of monounsaturated fatty acids was increased in SCO by 6.84% when compared with that of the raw material (copra cake). Biodiesel with high monounsaturated fatty acid content (*e.g.*, methyl oleate) is warranted as it has better characteristics with respect to ignition quality, nitrogen oxide emissions, and fuel stability [7]. As the FAMEs derived from *A. terreus* IBB M1 grown on copra cake have a higher content of methyl oleate with lesser amount of undesirable PUFAs, it was anticipated to have reasonably good fuel quality. The FAMEs were therefore analyzed for key physicochemical properties to assess their potential as biodiesel (Table 3). The density, KV, and CN were in good agreement with international (ASTM D 6751, EN 14214) and Indian (IS 15607) specifications. The predicted HHV (40 MJ/kg) was also comparable to that of the commercial soybean oil (39.95 MJ/kg)-derived biodiesel [7]. Furthermore, the absence of FAMEs having ≥4 double bonds and negligible presence

Table 2. Fatty acid profiles of copra cake and SCO obtained from *Aspergillus terreus* IBB M1 grown on copra cake[a].

Fatty acid methyl ester (%)[b]	Sample	
	Copra cake	SCO
Octanoic (caprylic) acid (C8:0)	3.62	1.84
Decanoic (capric) acid (C10:0)	2.19	1.58
Dodecanoic (lauric) acid (C12:0)	44.66	15.5
Tridecanoic acid (C13:0)	-	0.48
Tetradecanoic (myristic) acid (C14:0)	21.58	14.0
Pentadecanoic acid (C15:0)	-	0.78
Hexadecanoic (palmitic) acid (C16:0)	11.53	16.27
Heptadecanoic (margaric) acid (C17:0)	-	0.56
Octadecanoic (stearic) acid (C18:0)	2.63	7.72
Eicosanoic (arachidic) acid (C20:0)	-	0.72
Heneicosanoic acid (C21:0)	-	0.22
Docosanoic (behenic) acid (C22:0)	-	0.41
Tricosanoic acid (C23:0)	-	0.45
Tetracosanoic (lignoceric) acid (C24:0)	-	0.68
cis-Δ^{9}- Hexadecenoic (palmitoleic) acid (C16:1)	-	0.18
cis-Δ^{9}- Octadecenoic (oleic) acid (C18:1)	10.29	16.57
cis-Δ^{13}-Docosenoic (erucic) acid (C22:1)	-	0.38
cis,*cis*-$\Delta^{9,12}$-Octadecadienoic (linoleic) acid (C18:2n6c)	3.5	19.97
Alpha and gamma linolenic acid (C18:3)	-	1.11
cis-$\Delta^{11,14}$-Eicosadienoic acid (C20:2)	-	0.21
cis-$\Delta^{11,14,17}$-Eicosatrienoic acid (C20:3n3)	-	0.19
Linolelaidic acid (C18:2n6t)	-	0.18
Total saturated FAMEs	86.21	61.21
Total monounsaturated FAMEs	10.29	17.13
Total polyunsaturated FAMEs	3.5	21.66

[a]The fatty acid composition is determined as the % of the total fatty acids by GC-FID.

[b]Systematic name, (trivial name if any) followed by shorthand designation (total number of carbons : number of double bonds).

of C18:3 methyl ester meet the EN 14214 criteria. The IV and acid number are important chemical properties of biodiesel used in connection with oxidative stability. A higher acid number is observed if a fuel has undergone oxidative degradation. These values for FAMEs obtained in the present study were in accordance with international and national specifications, indicating their potential oxidative stability.

In conclusion, the conversion of raw untreated waste copra cake into suitable biodiesel feedstock was demonstrated using *A. terreus* IBB M1, as assessed from the fatty acid profile and key fuel quality parameters of the FAMEs derived from fungal SCO. The raw materials for conventional biodiesel production mainly include oils from vegetable / plants, algae, microbes, and animal fats. However, the high cost of production is due to the cost of raw material, which accounts for ~75% of the total cost [10]. Hence, the use of a cheap, renewable carbon source as substrate, especially agricultural wastes and agro-industrial residues, without any pretreatment is one of the effective ways to reduce the production costs [2, 6]. The main economical benefit offered by the use of copra cake as a substrate for oleaginous fungal growth is its low cost, renewable nature, and easy availability in India throughout the year. Moreover, a decline in global demand has been observed for copra meal as cattle feed in the last few years owing to stringent aflatoxin regulations and the availability of alternative superior oil meals such as soybean meal. In India, the introduction of attractive incentives for green fodder cultivation by the government has also affected the use of

Table 3. Biodiesel fuel properties of FAMEs derived from SCO of *A. terreus* IBB M1 culture grown on copra cake.

Fuel property/quality parameter	Value	Biodiesel standard specifications		
		ASTM D6751 (US standard)	EN 14214 (European standard)	IS 15607 (Indian standard)
Density (g/cm^3)[a]	0.80	NS	0.86–0.90	0.86–0.90
KV (40°C, mm^2/s)[b]	3.32	1.9–6.0	3.5–5.0	3.5–5.0
IV[a]	32	NS	120 max	NS
TAN (mg KOH/g)[a]	0.2	0.8 max	0.5 max	0.5 max
CN[b]	52	47–65	51 min	51 min
Concentration of linolenic acid (C18:3, %)[a]	0.1	NS	12 max	NS
FAME with ≥ 4 double bonds[a] (%)	ND	NS	1 max	NS

NS, not specified.

ND, not detected.

[a]Experimental values.

[b]Predicted values, determined as given in Table 1.

copra cake for feed preparation [8]. In this context, an alternative application for this low-cost waste copra cake as substrate for SCO production appears to be promising and offers value addition to copra cake. Furthermore, the copra cake has been used without any thermochemical pretreatment. Hence, it is an environmentally favorable process because of the use of fungal strain *A. terreus* IBB M1 possessing the ability to directly utilize insoluble components of the agro-residue for the production of lipids. High energy consumption, hazardous chemicals, and detoxification processes have thus been avoided, which are otherwise employed in conventional pretreatment methods used in the conversion of agro-residues to biofuels. Other advantages include easier, cost-effective downstream processing due to pelleted fungal growth of *A. terreus* IBB M1, and no conflict with food and land use, with the fungal growth being unaffected by light or climatic variations. Thus, this approach offers advantages over conventional biodiesel production as the growth of the fungus on raw untreated copra cake produced reasonable amounts of SCO with good FAME profiles and exhibited fuel properties similar to those of international and national specifications.

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