SYNTHESIS OF BIODIESEL FROM USED COOKING OIL USING WHOLE-CELL LIPASE PRODUCED BY SOLID STATE FERMENTATION OF *Aspergillus niger* KY401431

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ABSTRACT

Catalysis of used cooking oil (UCO) transesterification by whole-cell lipase produced by solid state fermentation (SSF) of agro-industrial wastes makes overall biodiesel synthesis greener, economic and sustainable. In the present study, *Aspergillus niger* KY401431was isolated from butter and identified based on 18S rRNA gene homology. Optimization of several biodiesel synthesis process stages including SSF conditions for *A. niger*KY401431 whole cell lipase production and UCO transesterification reaction conditions was carried out. Optimum SSF conditions, based on *A. niger* KY401431 biodiesel synthesis ability from UCO, were: a combined substrate of wheat bran (WB) and sesame meal (10/0.3, w/w),initial moisture content of 90%, initial pH of 8, inoculum size of 5x 10^7 spores/g substrate and incubation at 30°C for 6 days. Whereas, the best conditions for UCO transesterification were: 10% (w/w of UCO) biocatalyst, 3:1 methanol/UCO molar ratio with three-step additions at 0, 24 and 48h, 20% water (w/w of UCO), 30°C reaction temperature and reaction time of72 h. Under optimized process conditions, UCO conversion to fatty acid methyl esters (FAME) was improved by about 5-folds, producing a final biodiesel yield of 75.5%.

Keywords: biodiesel, whole-cell lipase, solid state fermentation, transesterification, used cooking oil, *Aspergillus niger* KY401431.

INTRODUCTION

Human existence mainly depends on the presence of energy source. Currently, fossil fuels are the main component of the world's total energy output, but they are unsustainable and responsible for the majority of global warming caused by greenhouse gas emission. Therefore, the development of alternative sources of fuel is urgently needed. Biodiesel represents a promising alternative fuel due to its nontoxicity, renewability biodegradability and low emission of sulfur dioxide, carbon monoxide and unburned hydrocarbons (Ranganathan *et al*., 1999).

Biodiesel fuel (fatty acid methyl esters, FAME) can be produced through methanolysis (transesterification) of vegetable oils oranimal fat (Kuepethkaew *et al*., 2017). Palm oil, peanut oil, castor oil, cottonseed oil, soybean oil, rapeseed oil and sunflower oilare used as biodiesel feedstock (Stoytcheva *et al*., 2011; Adachi *et al*., 2013). The cost of raw materials accounts for >85% of biodiesel cost as reported by Kuo *et al*. (2015). Therefore, to achieve sustainable low cost biodiesel production a cheap form of feedstock is preferred such as jatropha oil (non edible oil) and used cooking oil (UCO) ((Folaranmi, 2013; Chen *et al*., 2017). The accumulation of UCO from household, restaurants and industrial sources resulting in problems for wastewater treatment in the world (Pearl, 2002). Thus, conversion of UCO into biodiesel could help to reduce the production cost and solve two environmental problems, possible contamination of the water and land resources by uncontrolled disposal of used oil and production of a a carbon-neutral source of fuel (Tacias-Pascacio *et al*., 2017).

According to the catalysts used in the transesterification process, the synthesis of FAME is classified as enzymatic or chemical production (Haas *et al*. 2003). However, the chemical transesterification has several disadvantages such as high energy requirements, difficult recovery of products and byproduct, costs of disposal and the elimination of the catalyst and feedstocks must be free of water (Chen *et al*., 2017). Enzymatic transestrification using lipase offers a good solution to the aforementioned problems due to its environmental acceptance, biodegrability, easier recovery of products and insensitivity to acidity value and water content of feedstock, an essential characteristic for biodiesel production from high FFA content UCO (Lam *et al.* 2010, Tacias-Pascacio *et al*., 2017). However, the high cost of lipase is an obstacle to be applied for industrial biodiesel production. Utilizing a whole-cell biocatalyst overproducing intracellular lipase instead of commercial lipase for biodiesel production is a potential way to reduce the biocatalyst cost, because the isolation, purification and stabilization of the enzyme are not necessary (He *et al*., 2016). Recently, several researches have been published on biodiesel production using microbial lipases as a whole-cell biocatalyst (Adachi *et al*., 2013; He *et al*., 2016; Chen *et al*., 2017).

Solid state fermentation (SSF) involves cultivation of microorganisms on an insoluble substrate with enough moisture but in the absence of free-fluent water (dos Santos *et al*. 2014). The cultivation of whole-cells producing lipase under SSF of agricultural wastes is a cost effective method which contributes to reduce the cost of the enzyme preparation and consequently the biodiesel

production cost.SSF has attracted much attention in production of many enzymes, because it has several advantages over submerged fermentation (SmF) such as; use of agro-industrial residues as substrates, saving of water and energy, low production cost, high productivity, stable products and considered as eco-friendly as resolving the problem of solid wastes disposal (Pandey 2003; El-Bendary 2010). Lipases has been successfully produced by SSF of various agro-industrial residues (Kumar and Kanwar 2012) such as wheat bran (dos Santos *et al* 2014), rice bran (Hosseinpour *et al.*, 2012), soybean meal (Rigo *et al.*, 2010), almond meal (ul-Haq *et al.*, 2002), olive oil cake (Cordova *et al*. 1998), sugarcane bagasse (Cordova *et al.* 1998), mustard oil cake (Lall *et al.*, 2014), babassu oil cake (Gombert *et al.*, 1999), groundnut oil cake, gingelly oil cake (Kamini *et al.*, 1998) palm kernel oil cake and sesame oil cake (Oliveira *et al.*, 2017).

There is no available literature about the production of whole-cell lipase under solid state fermentation for biodiesel synthesis. Therefore, the present study aimed to apply *A. niger* whole-cell lipase produced under SSF conditions for biodiesel production from UCO. Optimization of several process stages including SSF

parameters and UCO transesterification reaction conditions were investigated.

MATERIALS AND METHODS

Materials
Wheat bran (WB) was purchased from local market in Egypt and oil extraction residue meals such as olive meal (OM), sesame meal(SM), pea nut meal (PNM), jatropha meal JM), linen meal (LM), cress meal (CM), wheat germ meal (WGM), black cumin meal (BCM) and gogoba meal (GM) were collected from Oil Extraction Unit at National Research Center (NRC) of Egypt. UCO was collected from local restaurants. The physicochemical characteristics of UCO (free fatty acid content, moisture content, saponification value and fatty acid profile) are presented in Table-1. Methylheptadecanoate standard was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methanol; hexane, diethyl ether and acetic acid were purchased from Merck Chemical Co. (Darmstadt, Germany). Polypeptone and medium ingredients were purchased from Fisher Scientific. All other chemicals were of analytical grade.

Table-1. Physicochemical characteristics of UCO and fatty acid composition.

Feedstock	FFA (%)	Moisture (%)	Saponification value (mg KOH/g)	Fatty acid composition (%)			
				C16:0	C18:0	C18:1	C18:2
UCO	0.16 ± 0.07	0.03 ± 0.002	192.8 ± 3.61	12.9 ± 0.28	4.7± 0.16	32.2 ± 1.04	50.2± 0.92

Microrganisms isolation and identification
Among fifteen different fungal strains used in this study (S. 1, supplementary data), thirteen strains were isolated from different seeds (beans, cotton, pea nut, wheat, soybean and maize) according to method described by Sahab *et al*. (2016). The other two strains were isolated from butter and corn oil. All fungal isolates were maintained at 4°C on PDA slants. Taxonomic identification of fungal isolates was carried out based on the shape of conidia and arrangement of spores on the mycelia (conidial ontogeny) according to Barnett and Hunter (2000), Domsch *et al*. (2007) and Samson *et al*. (2010). They were deposited at Chemistry of Natural and Microbial Product Department, NRC, Egypt.

Molecular identification of the selected isolate was performed by amplification and sequencing of its 18S rRNA genes using methods described by White *et al*. (1990). Briefly, mycelia were collected by centrifugation (at 4°C and 5000 rpm for 20 min) and DNA was extracted by using protocol of Gene Jet Plant genomic DNA purification Kit (Thermo # K0791). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo# K0221), and PCR clean up to the PCR product made by using Gene JET™ PCR Purification Kit (Thermo# K0701) in Sigma Company of Scientific Services, Egypt (www.sigma-co-eg.com). Purified DNA was subjected to PCR amplification using universal primer sets of ITS1-forward (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4-reverse (5′-TCC TCC GCT TAT TGA TAT GC-3′).

Finally, sequencing of the PCR product was performed at GATC Company (German) by using ABI 3730xl DNA sequencer and by combining the traditional Sanger technology with the new 454 technology. The sequences of 18S rRNA genes were analyzed using the BLAST searching program at the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov/BLAST/.

Screening of microorganisms for lipase hydrolysis activity and biodiesel production
The basal medium used for lipase production composed of (g/l): polypeptone 70; NaNO3 1; KH2PO4 1; MgSO4 7H2O 0.5 and olive oil 30 g in distilled water (Pazouki *et al*. 2010) Each strain was cultured in Erlenmeyer flasks (250 ml) containing 100mlof basal medium and incubated on a rotary shaker at 200rpm and 30oC for 72 h. Thereafter, fungal whole cells were separated from the broth by filtration through a filter paper. The supernatunt and whole cells were lyophilized. For each fungal strain, an equivalent amount of lyophilized supernatant or whole cells were tested for lipase hydrolysis activity and UCO transestrification yield.

Inoculum preparation
Fungal spore suspension was prepared by culturing *A. niger*KY401431 in 500 ml Erlenmeyer flasks containing 60 ml of PDA at 30 °C for a week. Spores were harvested with 30 ml of a Tween 80 solution (0.01%, w/v)

to make a final concentration of about 10^8 spores/ml. The resulting spore suspension was used as inoculum.

Solid-state fermentation (SSF)

Various industrial residues were evaluated for their potential as substrates in SSF of *A. niger* KY401431 for whole-cell lipase production and UCO transestrification. All the industrial residues were dried at 60°C for 1h, grinded and used for lipase production. Initially 10 g of WB and 6% of each oil meals as a substrate were taken individually in 250 ml Erlenmeyer conical flasks, moistened with distilled water and autoclaved for 30 min. The medium was inoculated with 1.0 ml of fungal inoculum and incubated at 30ºC for 6 days under static conditions. Each fermentation test was repeated in triplicate. The best oil industrial residue was selected (SM) and used in subsequent experiments. The fermented matter thus obtained was collected, lyophilized and used as a whole-cell biocatalyst.

Optimization of SSF parameters

Strategy adopted for the optimization was to evaluate the effect of each parameter on whole-cell lipase production and UCO transestrification yield. The parameters under investigation involved SM concentration (1.5-18%) as a substrate in presence of WB, combination of the best concentration of SM with different substrates/supports (WB, rice straw, corn cobs, potato peels, banana peels, orange peels, pea peels, beans peels and olive oil waste), initial moisture content (10-140%), inoculum size (0.5 -10 ml) and initial pH of medium (3-9).

Lipase hydrolysis activity

Lipase hydrolysis activity of the whole-cell biocatalysts was determined by how efficiently they catalyzed hydrolysis of triglycerides. One unit of lipase hydrolysis activity is defined as the release of 1 µmol of fatty acid/min. Under SSF conditions, the results are expressed in terms of units per gram of dry fermented matter (U/g) (Rigo *et al*. 2010). The hydrolysis reaction was carried out by stirring 30 ml of Tris buffer (pH 7.5) with 6 ml of UCO and 0.5 g dry weight of whole-cell biocatalyst at 40ºC for 24 h. Centrifugation at 5000 rpm for 15 min was used to separate oil from buffer and whole-cell biocatalyst. Lipase activity in the oil phase was determined by titration against 0.1N NaOH using phenolphthalein indicator. This is a modified form of the procedure described in Fukumoto *et al*. (1964). Lipase activity was determined by the following Equation1:

$$\text{Lipase activity (µmole/g cell)} = \frac{C_{NaOH} \times V_{NaOH}}{V_{titrated\ oil\ sample}} \qquad (1)$$

C: normality of sodium hydroxide V: Volume

UCO transestrification with methanol

Unless otherwise stated, methanolysis of UCO was carried out in 100 ml Erlenmeyer flasks at 40 °C with constant shaking at 200 rpm for 72 h. The reaction mixture consisted of 5 g UCO, 10 % (w/w of UCO) biocatayst, 15 % (w/w of UCO) 0.2 M Tris buffer (pH 7.5) and 4:1 methanol to UCO molar ratio (added stepwise to the reaction mixtures three times at 0, 24, 48 h reaction time). UCO was previously emulsified with Tris buffer (pH 7.5) before the addition of the biocatalyst using ultrasonication. The effect of oil/methanol molar ratio (1:3 - 1:6), methanol addition times (0, 4, 12 h; 0, 12, 24 h and 0, 24, 48 h) at different reaction times (24, 48 and 72 h), water contents (5 – 25 %) and temperature (20 – 60 °C) on biodiesel production, in terms of the FAME yield obtained, were investigated, respectively. At the end of reaction time, whole-cell biocatalyst was separated from the reaction mixture by centrifugation at 10,000 rpm for 15 min. The upper oil phase containing esters was analysed qualitatively by thin layer chromatography (TLC) and quantitatively by capillary gas chromatography (GC) (supplementary data: S. 1).

Analyses of fatty acid methyl esters (FAME)

TLC was performed on pre-coated silica gel plate (Merck, silica gel 60F-254). The plate was chromatographed for FAME with a solvent system of hexane: diethyl ether: acetic acid (80:20:1, v/v/v). The chromatograms were developed with iodine vapour.

FAME in oil phase were analyzed by a Agilent Technologies 6890N GC equipped with flame ionization detector (FID) and a fused silica capillary column (30 m× 0.32 mm × 0.25 mm). The GC temperature condition was oven temperature of 210 °C using helium as a carrier gas, flame ionization detector temperature of 250 °C and injector temperature of 250 °C. 10 mg/ml of methylheptadecanoate solution was used as an internal standard and the FAME content expressed as a mass fraction in percent was calculated by use of the Equation 2. The peak identification was made by comparing the retention time between the sample and the standard compound.

$$C = \frac{\Sigma A - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m} \times 100\% \qquad (2)$$

ΣA　　= total peak area of FAME

A_{IS}　　= peak area of internal standard (methyl heptadecanoate)

C_{IS}　　= concentration of the internal standard solution, in mg/ml

V_{IS}　　= volume of the internal standard solution used, in ml

m　　= mass of the sample, in mg

RESULTS AND DISCUSSIONS

Identification of isolated fungal stains

By comparing the colony morphology; pigmentation and microscopic characteristics of the isolates with those described in the modern identification proposed by Domsch *et al*. (2007) and Samson *et al*.

(2010), fifteen species belonging to six genera were detected. They were classified as *Alternaria tenuis, Aspergillus flavus, A. ochraceous, A. niger* strain I*, A. niger* strain II*, A. paraziticus, A. ruber, A. sydowii, A. terreus, Fusarium graminearum, F. oxysporum, Helminthosporium sativum, Pacilomyces spp., Rhizopus nigricans*and *R. oryzea.*Among them, *A. niger* strain Iisolated from butter was selected for its performance in terms of lipase hydrolysis activity and UCO transestrification yield. Moreover, molecular analysis of the selected isolate (*A. niger* strain I isolated from butter) was carried out through 18S rRNA gene to validate and confirm its morphological identification. The sequence of 18S rRNA gene was deposited in Gen Bank (www.ncbi.nlm.nih.gov) under accession number of KY401431. Analysis of 18S rRNA gene sequences using BLAST revealed that the PCR product had 98% (699/714) similarity with *Aspergillus niger* strain SAF8-EGY (GenBank accession no. KM222496.1) and 96% (721/751) similarity with *Aspergillus niger* strain C3F (GenBank accession no. LC195003.1).Based on its 18S rRNA gene sequences, the isolate was designated as *Aspergillusniger*KY401431.

Screening of fungal strains

Fifteen fungal cultures (S. 2) were screened for their abilities to produce extracellular lipase (supernatant) or intracellular lipase (whole cells) in the basal medium under SmF conditions. Then, produced lipase (extracellular lipase and/or whole-cell lipase) was applied for UCO transesterification reaction. These cultures showed large variations in their capacity to produce lipase enzyme. Thus, while some strains had nohydrolysis activity (*F. graminearum, A. ochraceous, A. terreus, Alternaria sp., Helminsosporium sativum),* the other experimental organisms showed lipase hydrolysis activities with different amounts detected in the supernatant and whole cells. Of these cultures, *A. niger*KY401431produced the highest interacellular lipase activity(whole cells) of 66 U/g.Moreover, *R. nigricans* and *R. oryza*ewhole cells showed high lipase hydrolysis activity about 57 and 53 U/g, respectively, while the other strains produced inferior amounts. Regarding UCO transesterification abilities, generally whole cell-lipase preparations in almost all cultures produce more biodiesel yield compared to the extracellular enzyme preparations. Although the maximum extracellular lipase hydrolysis activity (22 U/ml) was produced by *R. oryzea*, only *A. niger*KY401431extracellular enzyme preparation (supernatant) could perform the desired reaction producing the highest FAME yield of 15.8%.This could be attributed to the higher methanol tolerant stability of *A. niger*KY401431 extracellular enzyme compared to that of other tested cultures. However, among whole cell-lipase preparations, *R. oryzae* whole cells produced the maximum FAME yield of 11% followed by *A. Niger* KY401431whole cells (transformed about 7.4% of added UCO).The total amount of FAME produced by *A. niger*KY401431preparations, extracellular and whole-cell lipase, was 23.2%. Thus, *A.niger*KY401431 was chosen alongside this study based on its lipase activity performance as well as its superior transesterification yield.

Optimization of SSF parameters for whole cell-lipase production

This part of work focused on the optimization of lipase production by *A. niger* KY401431underSSF and simultaneous application of the produced lipase in UCO transesterification reactions for biodiesel production. The availability and cost of the substrate material are the main factors for selection of substrates in SSF (Pandey 2000). Various oil extraction residues were tested as substrates in the presence of WB as substrate/support material (Figure-1). WB was reported as the best substrate for lipase production by Kumar and Kanwar (2012) anddos Santos *et al*. (2014). Since it is rich in carbohydrates and fibers, it could be used with dual function as a carbon source and as physical support for fungal growth. As shown in Figure-1, all tested oil extraction residues supported UCO hydrolysis and transestrification using *A. niger* KY401431 whole cell-lipase to different degrees. Combination of WB and SM yielded the highest lipase hydrolysis activity of 80.6 U/g. Supplementation of SSF medium with oil extraction residues is attractive due to the fact that each residue could serve as a physical support, source of nutrient and as an inducer for the production of lipase (Oliveira *et al*., 2017). As can be observed, this combination of WB/SM led also to maximum FAME yield of 13.2% which representing a 2.3-fold increase compared to that produced using WB only (5.7 %). Also, a combination of WB/CM improved lipase hydrolysis activity and FAME yields (66.0 U/g and11.3%, respectively). Although GM resulted in a good lipase hydrolysis activity (61.7 U/g), it couldn't support UCO conversion to FAME (1.9%). Biodiesel production is depended on lipase ability to transesterify UCO triglycerides to corresponding FAME in presence of methanol (Chen *et al*., 2017). Therefore, these results may be due to the probability that GM could enhance the lipase hydrolysis activity but had a negative effect on lipase transesterification activity.
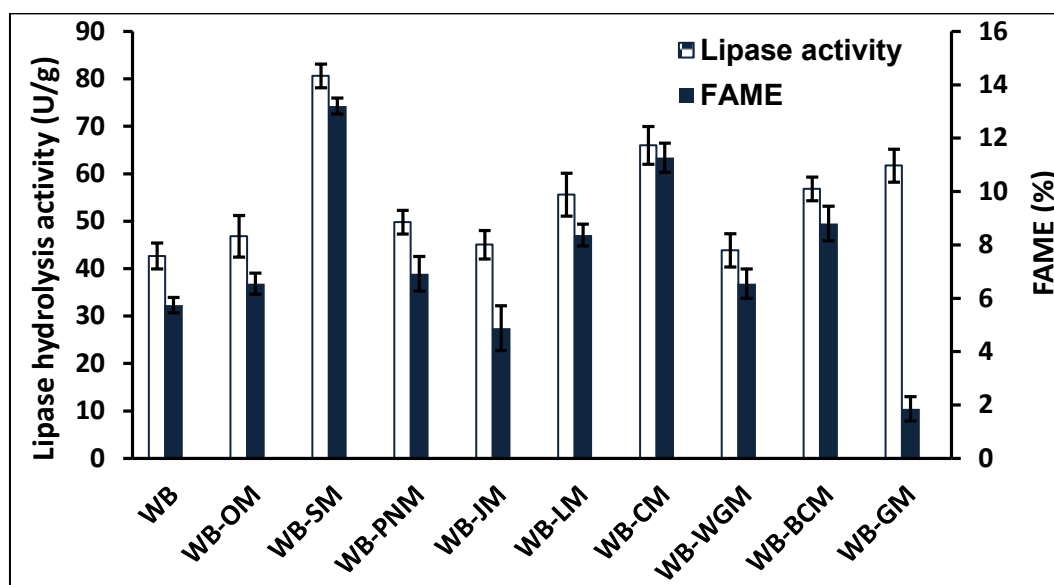
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Figure-1. Screening of different oil meals for lipase and biodiesel production by *A. niger* KY401431 whole cells. WB wheat bran; OM olive meal; SM sesame meal; PNM pea nut meal; JM jatropha meal; LM linen meal; CM cress meal; WGM wheat germ meal; BCM black cumin meal; GM gogoba meal. SSF conditions: WB and different oil meals (10/0.6, w/w) as substrate, initial moisture content 50 %, initial pH 6, inoculum 10^7 spores/g substrate at 30°C for 6 days.

Elevated lipase hydrolysis and transestrification activities in presence of SM were attributed to its high lipid content which was reported by some authors to have a positive effect on lipase production (Gombert *et al*., 1999). However, increasing lipid contents above certain limits could result in a reduction in the lipase production (Oliveira *et al*., 2017).To determine the optimal level of SM in SSF medium, various concentrations of SM in the range of 1.5 to 18%(corresponding to WB weight) were tested (Figure-2). Results revealed that maximum lipase hydrolysis activity of 101.7 U/g and FAME yield of 18.7% were achieved at 9% and 3% SM concentration, respectively. The difference in the obtained optimal SM concentration may be attributed to that SM concentration required to induce lipase hydrolysis and transesterification activities are different.
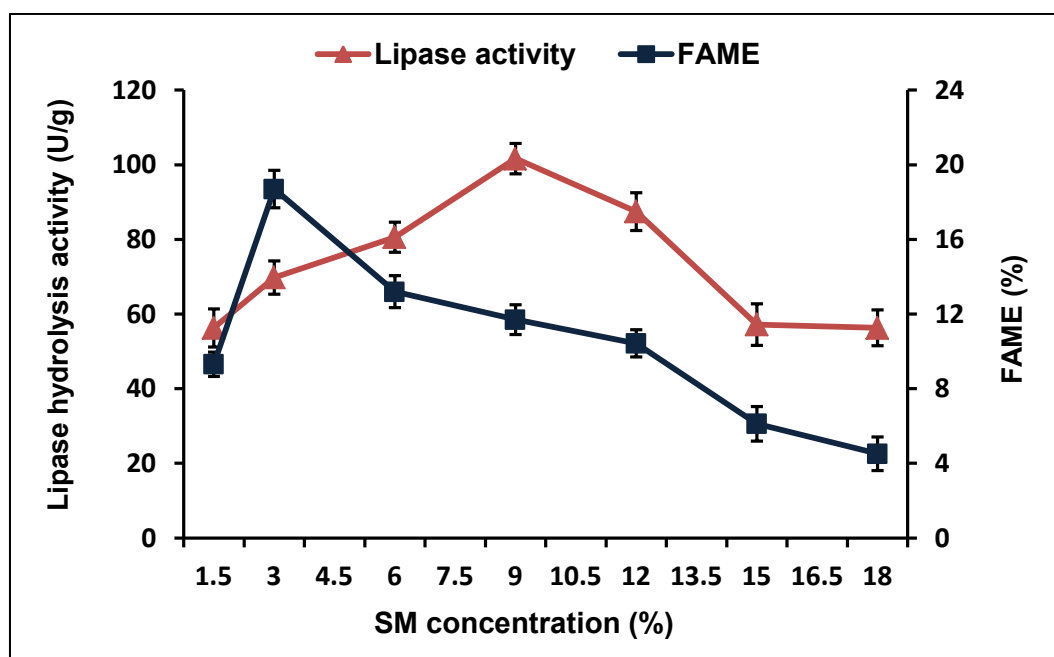

Figure-2. Effect of different concentrations of SM on lipase and biodiesel production by *A. niger* KY401431 whole cells. Control: using WB only as a substrate.

The effect of WB replacement with nine different substrates/supports on lipase hydrolysis and transesterification activities was tested in presence of 3% SM. As can be observed in Figure-3, compared with other substrates/supports WB is the best substrate/support leading to the highest lipase hydrolysis activity of 69.8 U/g and FAME of 18.7%. It is known that WB contains 60-75% carbohydrate, 9-18.6% protein, 3.9-8.1% ash, 10.4-15% fiber as reported by Onipe *et al*. (2015). Moreover, according to Oliveira *et al*. (2017), SM contains 37.1% protein, 29.1% lipids, 21.3% carbohydrates, 7% ash and 1% fiber. Therefore, the combination of WB/SM as a substrate in SSF medium almost contains all the requirements for *A. niger*KY401431 growth and lipase production. On the other hand, some tested substrates/supports resulted in significant inhibition in lipase hydrolysis activity and UCO transesterification such as banana peels (13.5 U/g and 1.9%, respectively) and orange peels (11.4U/g and 0.3%, respectively) and this could be attributed to their low nutrient content.
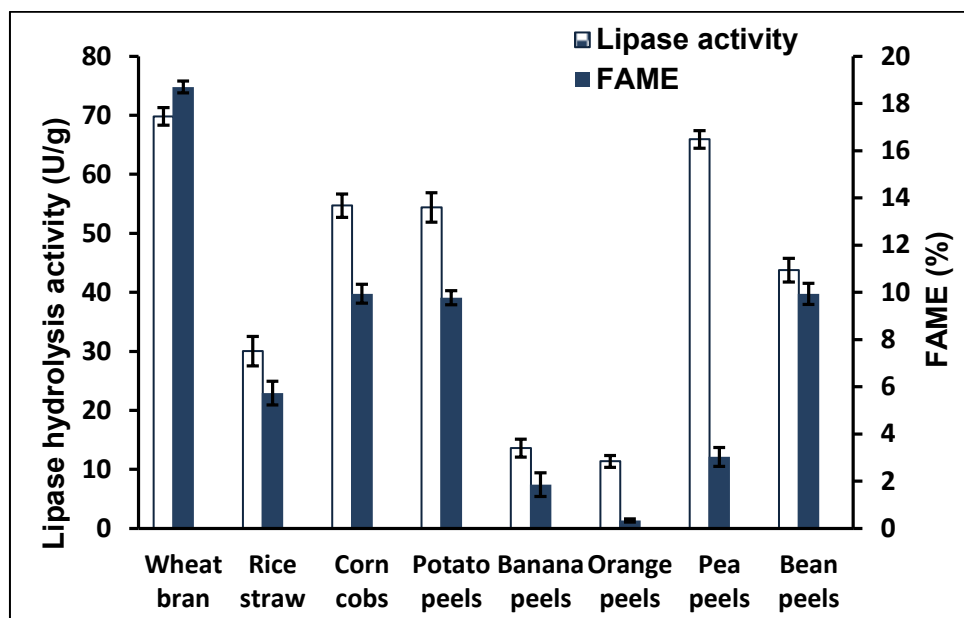

Figure-3. Effect of different substrates/supports on lipase and biodiesel production by *A. niger* KY401431 whole cells.

pH is an important factor in SSF affecting the fungal growth and lipase enzyme production. Results (S. 3) revealed that lipase was efficiently produced under a wide range of tested pH values (4-8) with the maximum hydrolysis activity at pH 8 (75.4U/g) by *A. niger* KY401431. Whereas at pH 9, lipase hydrolysis activity was significantly decreased. However, FAME yield was gradually increased with increasing initial pH reaching the maximum value of 23.7% at pH8, after that it was significantly decreased. It was reported that fungi can produce lipase enzyme over a broad pH values under SSF due to the buffering capacity of solid substrate (Sun and Xu, 2008).

Size of inoculum is an important biological factor, which determines biomass production in the fermentation process. The results presented in Figure-4 show a gradual increase in lipase hydrolysis activity by increasing inoculum size from 0.5 to 4 ml(equivalent to 5×10^6 -4×10^7 spores/g substrate, respectively), then it slightly decreased with increasing the inoculum size beyond this limit. These results are in agreement with findings of ul-Haq *et al*. (2002). However, FAME yield was significantly increased from 3.6 to 29.9%by increasing the inoculum sizes from 0.5 to 5ml (equivalent to 5×10^6- 5×10^7spores/g substrate, respectively). After that, FAME yield was significantly decreased to 12.9% upon using inoculum size of 10^8spores/g substrate. This decrease with larger inoculum size was related to decline of enzyme production due to exhaustion of nutrients available for the larger biomass (Kumar and Kanwar 2012). Thus, a balance between the proliferating biomass and available material is essential to yield maximum enzyme production (Hesseltine *et al*. 1976).
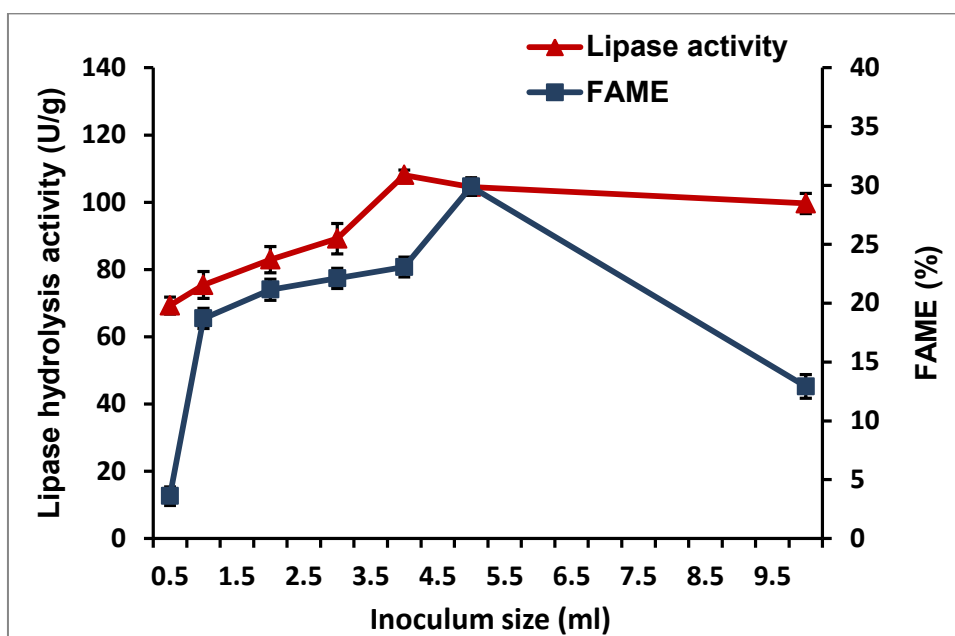
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Figure-4. Effect of inoculum size on lipase and biodiesel broduction by
A. niger KY401431 whole cells.

Moisture content level in the fermentation medium is a crucial factor in SSF process and affects the microbial growth and their products (Sun and Xu, 2008). Results illustrated in Figure-5 reveal that lipase hydrolysis activity gradually increased with increasing the initial moisture content up to 110% (153.1 U/g), then sharply decreased at 140% moisture content reaching 92.6 U/g. On the other hand, the maximum FAME yield of 34.4%was achieved at 90% initial moisture level. Lower

or higher moisture levels were accompanied by inferior FAME yields. This could be explained by the fact enzyme biosynthesis is significantly affected by high or low levels of moisture content (Sun and Xu, 2008). Low moisture content reduced nutrients solubility (Pandy, 2003). However, high moisture content decreases substrate porosity and increased stickiness, thus limiting oxygen diffusion in the substrate layer.
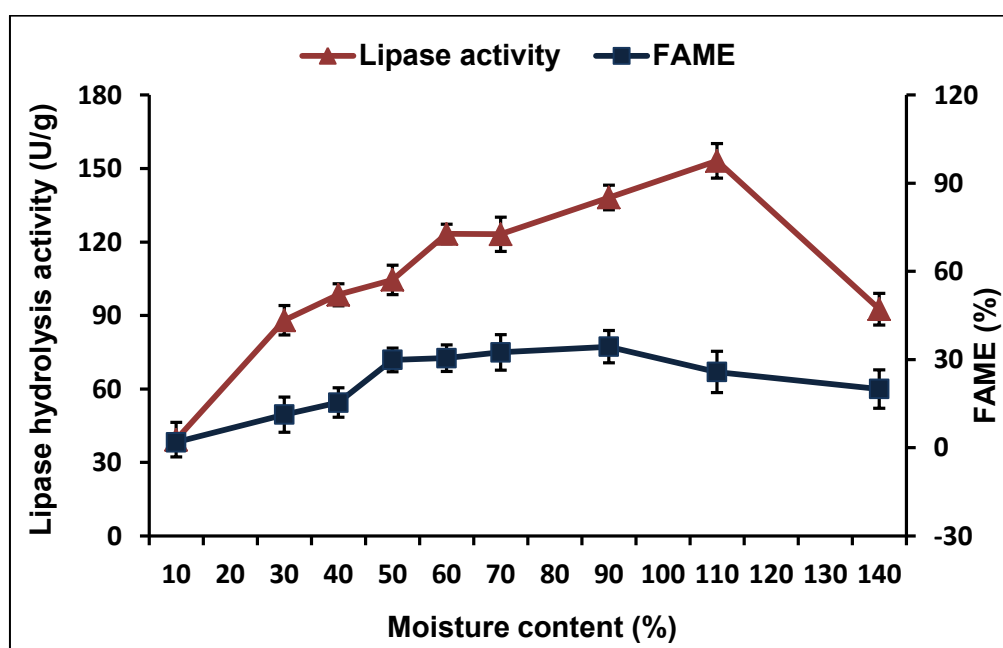

Figure-5. Effect of initial moisture content on lipase and biodiesel production by
A. niger KY401431 whole cells.

Therefore, Optimum SSF conditions for *A. niger* KY401431 cultivation based on its maximum biodiesel production ability represented by FAME yield of 34.4% were as follows: combined substrate of WB/SM (10/0.3, w/w), a moisture content of 90%, initial pH of 8, inoculum size of 5×10^7 spores/g substrate and incubation at 30°Cfor 6 days.

Optimization of the transesterification reaction using A. niger KY401431 whole cell lipase

A.niger KY401431 whole-cell lipase produced under the previously optimized SSF conditions was applied in UCO transesterification reaction for biodiesel production. Optimization of the reaction parameters was performed to achieve the maximum biodiesel (FAME) yield. For transesterification of oils, at least three molar equivalents of methanol (3:1) were necessary. To determine the optimum methanol to oil ratio, different ratios in the range of 3:1 to 6:1 were tested. As shown in Figure-6, the highest FAME conversion (46.3%) was achieved at1:3 molar ratio. A significant reduction in FAME conversion from 46.3% to 11.9% (a 3.9-fold reduction) was observed as the oil: methanol molar ratio increased from 1:3 to 1:6. According to Pazouki *et al*. (2011), when a portion of the alcohol remains insoluble (in excess) it accumulates around the active sites of lipase structure causing protein denaturation. Likewise, methanolysis of UCO by immobilized cell of *Rhizopus oryzae* PTCC5174 was achieved using methanol to oil ratio of 3:1 (Pazouki *et al*., 2010). Leelaruji *et al*. (2013) also reported the same ratio of methanol to oil for transestrification of *Jatropha curcas* oil by *Aureobasidium pullulans* var. *melanogenum* SRY 14-3 whole cells. Whereas, Talukder *et al*. (2013) found that 5:1 ratio of methanol to oil gave maximum conversion palm oil to biodiesel using *A. nomius*.
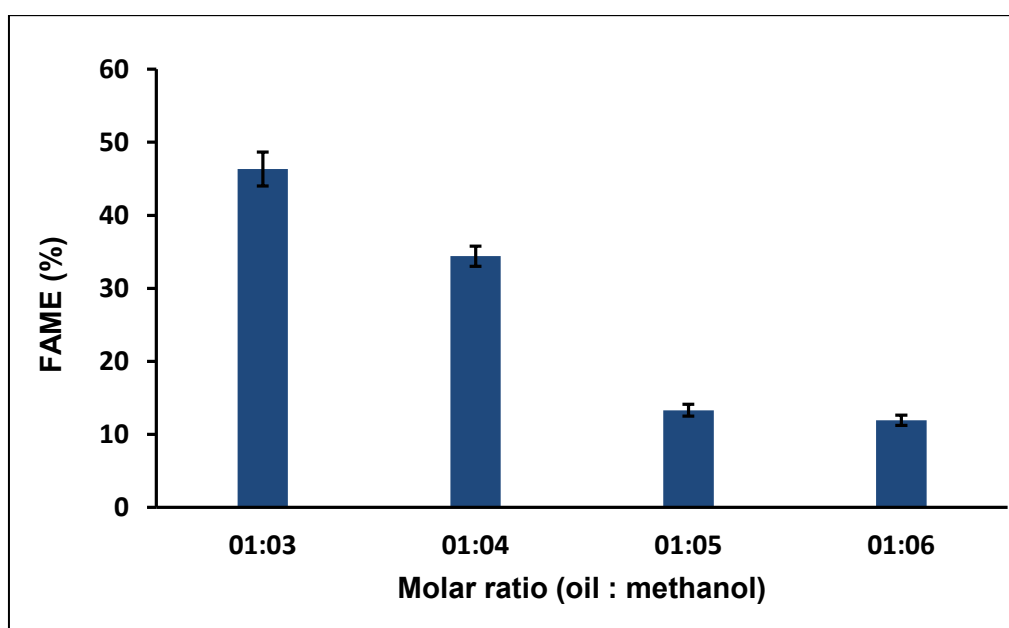

Figure-6. Effect of oil/methanol molar ratio on UCO transestrification by *A. niger* KY401431whole cells. Reaction conditions: 5 g UCO, biocatalyst 10 % (w/w of UCO), 15 % (w/w of UCO) Tris buffer (pH 7.5), three-step addition of methanol at 0, 24, 48 h, temperature 40 °C, reaction time 72 h and 200 rpm. Control: using 4:1 oil/methanol molar ratio.

The insolubility of methanol with UCO inactivates lipases (Talukder *et al*., 2013). To minimize methanol inactivation of whole-cell lipase, transesterification was carried out with stepwise additions of methanol (Adachi *et al*., 2013). The time profiles of UCO methanolysis with stepwise addition of methanol (1 molar equivalent methanol was added 3 times) at three time strategies (0, 4, 12; 0, 12, 24 and 0, 24, 48 h) were represented in Figure-7. At time of methanolysis of 0, 4, 12 and 0, 12, 24, maximum FAME yields were 9.9 and 34.3 %, respectively, after 24 and 48h, respectively. However, at longer incubation periods, FAME yields remained approximately constant. It was clear that the short time methanol addition strategy (0, 4, 12h) negatively affects FAME yields compared with other two strategies. This could be attributed to the addition of high concentration of methanol (3molar equivalents of UCO) within a short time interval (12h) leading to deactivation of enzyme (Pauzouki *et al*., 2011). However, at time of methanolysis of 0, 24, 48 hFAME yield increased gradually by time and reached the highest value of 46.32% after 72h. Pazouki *et al*. (2011) found that a maximum FAME yield of 98.4 %from UCO catalyzed by immobilized whole cells of *R. oryzae* PTCC 5174was obtained after72h using 3:1 methanol in three-step addition pattern of 0, 24, 48 h.
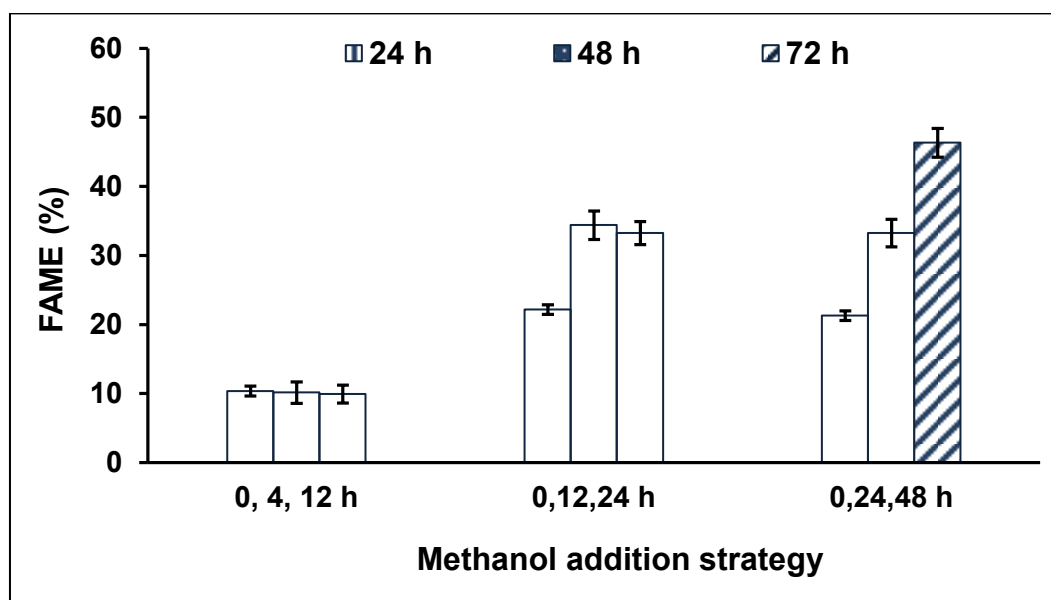
Figure-7. Time course of UCO transestrification by *A. niger* KY401431 whole cells and effect of methanol addition strategy. Reaction conditions: 5 g UCO, biocatalyst 10 % (w/w of UCO), Tris buffer (pH 7.5) 15 % (w/w of UCO), 3:1 methanol to UCO molar ratio, three-step addition of methanol at 0, 4, 12 h; 0, 12, 24 h and 0, 24, 48 h, temperature 40 °C and 200 rpm. Control: reaction time 72 h using 0, 24, 48h addition pattern.

Figure-8 illustrates the results of FAME production in the presence of different water contents (5-25% based on UCO weight). Activity of lipase depended on presence of water due to the necessity to form hydrogen bonds with water to maintain its live structure (Pauzouki *et al*., 2011). Thus oil methanolysis could be enhanced by increasing the available interfacial area between the aqueous (water) and organic (oil) phases where lipase acts (He *et al*., 2016). These view points could explain why the FAME yields increase gradually from 12 to 56% (about 4.7 fold increase) by increasing water content from 5 to 20%, respectively. However, higher concentration of water (25%) led to lower FAME yields (42%).Considering that lipases usually catalyze hydrolysis in aqueous medium, thus excess water inhibits

FAME production due to it acts as a competitive inhibitor for transesterification (Kuepethkaew *et al*., 2017). Additionally, the reaction rate would be decreased due to decreased contact between lipase and oil with large quantity of water (Kuepethkaew *et al.*, 2017). Pazouki *et al*. (2011) reported the effect of water on UCO transesterification by *R. oryzea* PTCC 5174 cells immobilized on polyurethane foam particles. In their study, the optimal water content was 15.54%. Other studies reported much lower optimal water contents likewise, 2.5% water content for biodiesel synthesis from microalgal feedstocks by immobilized *A. niger* whole-cell lipase, and 5% water content for transesterification of soybean oil by immobilized *R. oryzae* whole cells (He *et al*., 2016).
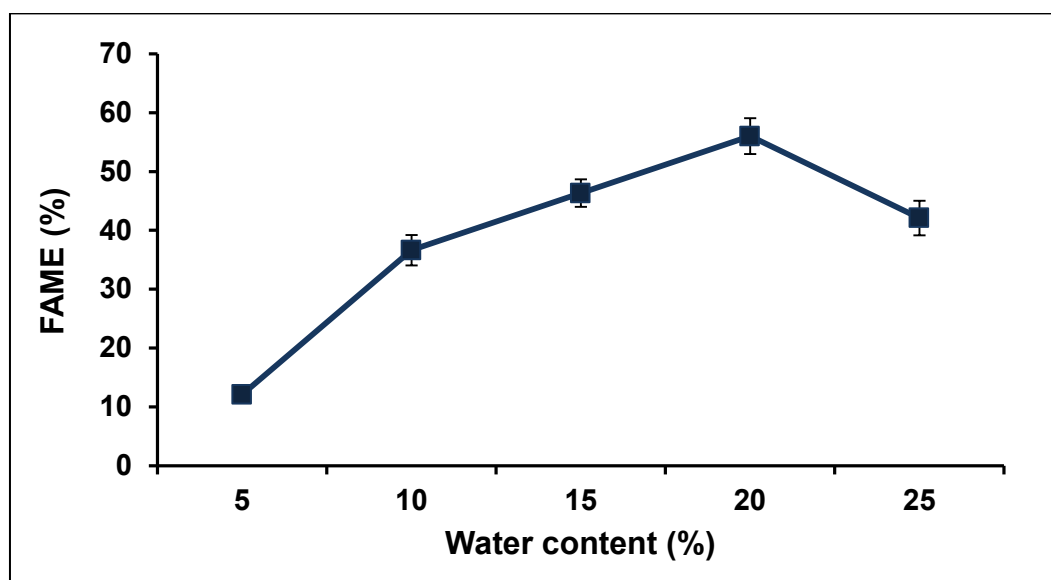
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Figure-8. Effect of water content on UCO transestrification by *A. niger* KY401431 whole cells.
Reaction conditions: 5 g UCO, biocatalyst 10 % (w/w of UCO), Tris buffer (pH 7.5) 5-25 %
(w/w of UCO), 3:1 methanol to UCO molar ratio, three-step addition of methanol at
0, 24, 48 h, temperature 40 °C, reaction time 72 h and 200 rpm.
Control: using 15 % water content.

Reaction temperature is one of the key factors that affect the efficiency of enzyme catalyzed transesterification reactions. Low temperature can slow down transesterification, while high temperature can lead to enzyme denaturation (Talukder *et al*., 2013). The temperature effect on UCO transesterification by *A. niger* whole cells has been tested over a temperature range of 25 - 45°C as shown in Figure-9. FAME yield was increased by 1.4-fold from 53.6% to 75.5% when temperature increased from 20°C to a 30°C, respectively. The maximum yield was observed at 30°C. This is in agreement with other previous studies where the optimum reaction temperature was 30°C(Marchetti *et al*., 2007; Kantak and Prabhune, 2015). However, subsequent increase in reaction temperature beyond the optimum level (30°C) led to reduction in FAME yield reached about 28.6% and 39.1 % at 50°C and at 60 °C, respectively. The observed decrease in FAME yield is most likely due to enzyme thermal denaturation (Guldhe *et al*., 2016).
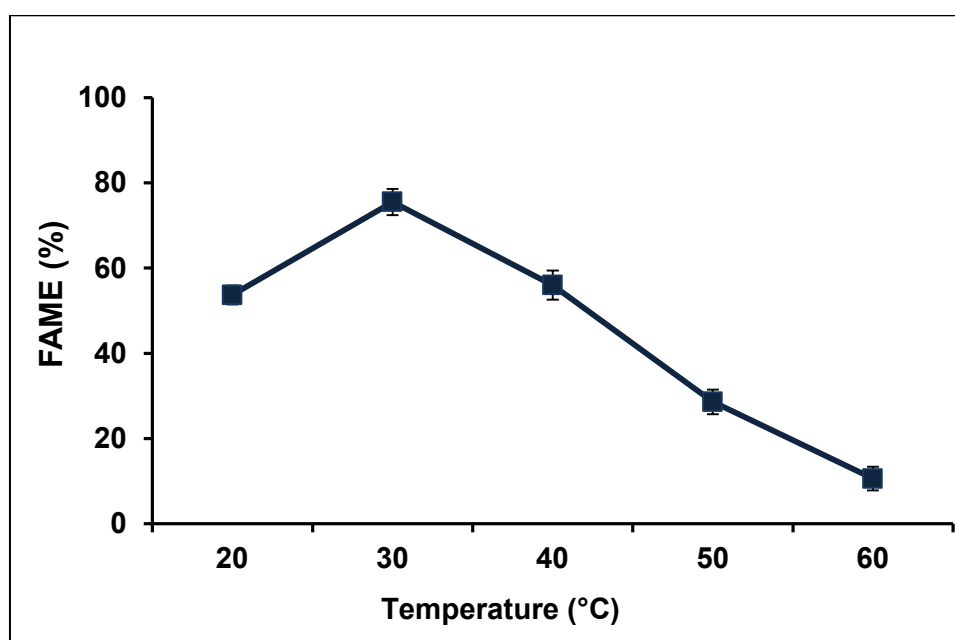

Figure-9. Effect of temperature on UCO transestrification by *A. niger* KY401431whole cells.

Therefore, the optimum reaction conditions to perform UCO transesterification by *A. niger* whole cells, reaching the maximal FAME conversion of 75.5% were as follows: 10% (wt/wt of UCO) *A. niger*KY401431 cells, 3:1 methanol/UCO molar ratio, and 20% water (wt/wt of UCO) at 30°C, 200 rpm, over 72 h and with three-step additions of methanol at 0, 24 and 48h. Other authors obtained close FAME yields (80.97%) from a microalgal feedstock (*Scenedesmus obliquus* lipids) using *A. niger* whole cells as a biocatalyst (Guldhe *et al.*, 2016). However, higher FAME yields were obtained from UCO transesterification using different whole-cell lipases likewise, 98.4% biodiesel yield using *R. oryzae* PTCC 5174 cells (Pazouki *et al.*, 2011) and 87.5% biodiesel yield using *Pseudomonas mendocina* cells (Chen *et al.*, 2017).

CONCLUSIONS

A. niger KY401431 was selected as the best fungus to support maximum conversion of UCO to FAME. Cultivation of *A. niger* KY401431 under optimized SSF conditions and using the produced whole-cell lipasein UCO transesterification under optimized reaction conditions resulted in a maximum biodiesel production of 75.7%. From an industrial technology view piont, using of *A. niger* KY401431whole-cell lipase produced by a cost effective method (SSF) and UCO would result in production of this cost-sensitive energy product with low cost and high sustainability. Also, this method would help to solve two environmental problems, revalorization of agro-industrial wastes and UCO disposal problems.

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Supplementary data

S. 1. GC analysis of FAME in reaction mixture components under optimal conditions.
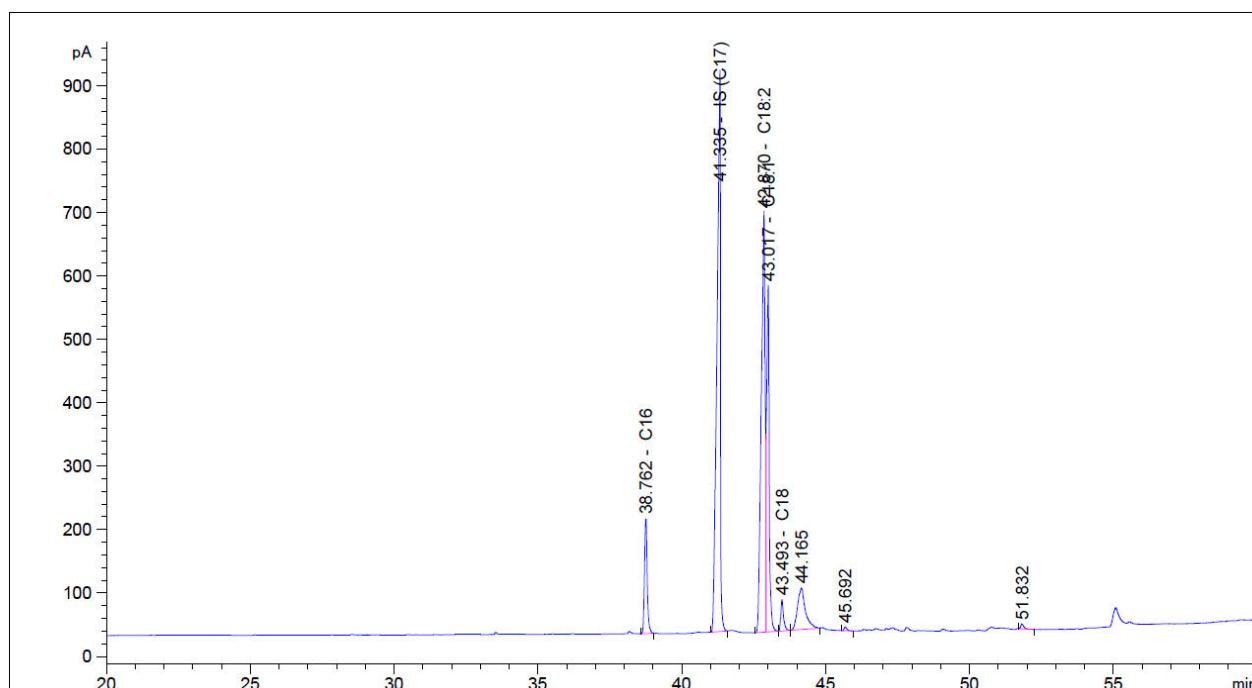
S.2. Screening of isolated fungi for lipase hydrolysis activity and FAME yield.

Microorganism	Isolation source	Whole-cell lipase		Extracellular lipase	
		Hydrolysis activity (U/g)	FAME (%)	Hydrolysis activity (U/ml)	FAME (%)
Alternaria sp.	Cotton seed	0	-	0	-
Aspergillus flavus	Peanut seed	0	-	8.7±0.4	0
Aspergillus niger I	Butter	66.9±2.8	7.4±0.3	19.1±1.4	15.8±0.6
Aspergillus niger II	Sunflower oil	4.4±0.2	2.6±0.2	17.2±0.6	0
Aspergillus ochraceous	Peanut seed	0	-	0	-
Aspergillus parasiticus	Beans seed	0	-	8.3±0.5	0
Aspergillus ruber	Beans seed	2.5±0.1	1.1±0.1	14.2±0.6	0
Aspergillus sydowi	Peanut seed	0	-	6.5±0.3	0
Aspergillus terrues	Soybean seed	0	-	0	-
Fusarium graminearum	Maize seed	0	-	0	-
Fusarium oxysporum	Peanut seed	0	0	3.3±0.1	0
Helminsosporium sativum	Wheat seed	0	-	0	-
Pacilomyces sp.	Peanut seed	6.9±0.4	0.6±0.1	15.3±0.5	0
Rhizopus nigricans	Cotton seed	57.9±2.5	4.2±0.2	10.1±0.3	0
Rhizopus oryzae	Soybean seed	53.4±2.2	11.1±0.3	22.5±0.7	0

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S.3. Effect of initial pH on lipase and biodiesel production by *A. niger* KY401431 whole cells.