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Production of Biodiesel Using Tannery Fleshing as a Feedstock via Solid-State Fermentation

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***Abstract*—**This study was initiated to evaluate and optimize the conversion of animal fat from tannery wastes into methyl ester. In the pre-treatment stage, animal fats feedstock was hydrolysed and esterified through solid state fermentation (SSF) using *Microbacterium* species immobilized onto sand silica matrix. After 72 hours of fermentation, predominant esters in the animal fats were found to be with 83.9% conversion rate. Later, esterified animal fats were transesterified at 3 hour reaction time with 1% NaOH (w/v %), 6% methanol to oil ratio (w/v %) to produce 89% conversion rate. C_{13} NMR revealed long carbon chain in fatty acid methyl esters at 22.2817-31.9727 ppm. Methyl esters of palmitic, stearic, oleic represented the major components in biodiesel.

***Keywords*—**Tannery wastes, fatty animal fleshing, trans-esterification, immobilization, solid state fermentation.

I. Introduction

THE growing demands for energy in the industrialized world and pollution problems associated with the use of fossil fuels have made obligatory, the development of alternative renewable energy sources [1], [2]. One of the alternatives considered is the use of biodiesel and it has almost all the properties required for its use as blends with the conventional fuel in compression-ignition diesel engines with minor engine modification [3], [4]. Its use leads to a decrease in CO_x, SO_x, un-burnt hydrocarbons and particulate matter emission in the combustion process. Conventionally, biodiesel is produced by the transesterification of refined/edible oils [5], [6]. But, the production cost of biodiesel is still higher than the petroleum-based diesel [7]. To overcome these disadvantages less-expensive feedstock's such as non-edible oils or animal fat should be used instead of edible oils [8]. A wide range of studies have been carried out on biodiesel production from feedstocks of low cost such as waste frying oils [9], [10] and new vegetable species [11], [12]. Recently, the so called tannery wastes - effluent from the leather tanning industry - have increasingly drawn interest for biodiesel production by the researchers [13]. Large quantity of tannery wastes is generated by leather industry annually around the world [14]. Such tannery wastes have high BOD, COD, triglycerides, phospholipids, water, odorants sterols, and other impurities such as hide scraps, skin and excess animal fats. Furthermore, its high solids concentration and acidity causes it to be unsuitable for direct discharge to water courses [15]. However, there are generally two options confronted to leather industry either to minimize the quantity of waste generated or maximizing the by-products return from the waste [16]. Tannery wastes are biodegradable to support the growth of microorganism due to the richness in fat and there is no application method to recover them [17]. The utilization of tannery wastes rich in animal fats for biodiesel production is a good alternative to recycle these wastes. According to [18], recovery of the value added products from the solid wastes has gained interest of many researchers. Biodiesel (fatty acids methyl esters), which is produced from renewable biological sources such as waste vegetable oils and animal fats, is one of such products.

Biodiesel comprises of long-chain fatty acid alkyl esters produced from vegetable oils or animal fats by transesterification of triglycerides (TGs) with lower alcohols (methanol or ethanol) [19]. During transesterification, the main problem is associated with the tannery wastes rich in animal fat as it tends to form a gel-like material due to the presence of high amount of fat [20]. A high concentration of saturated fatty acid components in animal fats from tannery wastes and low conversion of TGs are the suspects for this phenomenon. Incomplete reactions and/or reverse reactions result in formation of mono-, di-, and even TGs with relatively high melting points. These will simply form a solid or gel-like phase [21]. To address this issue, we developed a two stage process of biodiesel conversion. High conversion of TGs to unsaturated fatty acids and esters may be achieved in a first stage SSF. High purity methyl esters were prepared in a second stage transesterification process at a considerably better yield due to no more losses in the processing, absence of water and reverse reactions [22].

SSF has been defined as the fermentation process occurring in the absence or near-absence of free water [23]. In the past few years, new biotechnological innovations have identified SSF as a promising low-cost technology [24]. It has numerous advantages, including productivity and may be preferred to the submerged fermentation due to simple operational procedures, lower levels of catabolite repression and better product recovery [25]. Reports on SSF in animal fats for esterification using *Microbacterium* species via SSF for feedstock production are limited or perhaps not available. Sand silica was used as a solid matrix in SSF is the novelty in this experiment. As such, the present paper explores the utilization of the abundantly available tannery wastes for biodiesel production. To achieve this objective, controlled inoculum was isolated and identified using 16srDNA sequencing to be

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used in SSF, solid animal fats were fermented into fatty acids esters via SSF as a pre-treatment process and finally, conversion of fatty acids esters into fatty acid methyl esters (FAME) through transesterification.

II. Materials and Methods

A. Characterization of Animal Fat Feedstock

Animal fats used in the SSF are a pale whitish solid fleshing rich in fat with a mild odor. It is a readily available, inexpensive fat and a waste product of slaughterhouses at Long Lai Tannery Sdn. Bhd, Selangor, Malaysia. Moisture and ash content (%) of the animal fat were determined using the methods as described by IUPAC [26]. The percentage composition of carbon, hydrogen and nitrogen content was determined using CHNS1500 Carlo–Erba analyzer. The mineral composition was determined using Perkin Elmer 5000 atomic absorption spectrophotometer. Acetylene and air were used to maintain the temperature at 2300°C to atomize the samples.

B. Isolation of Strains and Its Culture Conditions

The controlled inoculum was isolated from tannery soak liquor (Selangor, Malaysia) and maintained in nutrient broth and LB broth. The inoculum was serially diluted and followed by plating in tri-butyrin and rhodamine B agar to procure axenic culture. Both biochemical characterizations such as sugar fermentation test and 16s rRNA sequencing were carried out for the isolates. The batch fermentation experiments for lipase production were conducted for 1g animal fat in 100ml minimal medium [0.10g/L sodium chloride (NaCl), 0.02g/L ammonium chloride (NH_4Cl), 0.85g/L dipotassium mono-hydrogen phosphate (K_2HPO_4) and 0.60g/L potassium dihydrogen phosphate (KH_2PO_4)]. The trace element solution of 1 ml, containing 0.59g/L magnesium sulphate ($MgSO_4$), 0.065g/L ferrous sulphate ($FeSO_4$), 0.038g/L cobalt chloride ($CoCl_2$), 0.029g/L manganese-dichloride ($MnCl_2$), 0.247g/L calcium chloride ($CaCl_2$) and 0.223g/L ammonium molybdate ($(NH_4)_6Mo_7O_{24}$), was also added. The media was autoclaved at 121 °C for 15 min and incubated, without agitation, at 37 °C. All experiments were carried out in duplicates and repeated three times.

C. Immobilization of Microbacterium Species

The 500 grams of silica sand was washed 3 times using tap water to remove contaminants. After washing, it was treated with 250 ml of 10% HCl for 2 hours. After treatment silica sand was washed thoroughly using distilled water to remove excess HCl and dried in Oven at 50 to 60 °C for 5 to 6 hours. The acid treated silica was used to immobilize the *Microbacterium* species via physical adsorption (ratio of sand silica to inoculum was 10:1) in SSF according to procedure described by [27]. The variables such as temperature, pH, concentration, particle size and mass, adsorption isotherms were optimized in studies (data not shown).

D. Pre-Treatment: SSF of the Animal Fat

The 250 grams of sized 0.20 cm animal fats with working height of 15 cm were inoculated with 500 grams of sand silica immobilized with *Microbacterium* species and incubated for 72 hours under 37 °C temperature, 6.5 pH with humidified air injection of 95% saturation [28]. Along with that small amount of trace elements mentioned above were added. The fermentation was carried out for 3-4 days and at suitable time intervals, culture flasks of substrates were randomly sampled and analyzed in GCMS instrument (Agilent Technologies GCMS 5973, mobile phase- methanol 2ml/ml flow rate) and separated compounds were identified using library match. These samples were used as feed stock for biodiesel production. Different animal fat sizes of 0.20, 0.4, 0.8 and 1.0 cm were investigated for the optimal lipase production.

E. Transesterification of SSF Samples Using Methanol

Transesterification process was carried out using 1L reaction flask equipped with reflux condenser, magnetic stirrer and thermometer. The process was experimented in three catalyst loadings such as (0.5%, 1.0% and 1.5% sodium hydroxide) and three alcohol-to-oil molar ratios (3:1, 6:1 and 9:1). The reaction temperature was maintained at 60 ± 1 °C for 1 to 3 hours. At first, the catalyst was dissolved into methanol and stirred magnetically. The animal fats samples from SSF reaction were introduced into the reaction flask. Once reaching reaction temperature, 0.5 % to 1.5 % catalyst added to flask and mixture was continuously stirred at 400 rpm. After completion of reaction time, the mixture was transferred to a separating funnel and allowed to stand there overnight. The lower layer was separated from upper layer. C_{13} NMR analysis was conducted for the preliminary analysis to observe the formation of biodiesel according to [29].

III. Results and Discussion

A. Animal Fat Characterization

TABLE I
Physicochemical Parameters and Mineral Composition of the Animal Fat

Parameters	Values
Moisture content (%)	78.5 ± 5.5
Ash content (%)	51.9 ± 2.5
Carbon (%)	15.728±0.296
Hydrogen (%)	0.916±0.29
Nitrogen (%)	0.025±0.003
Elements (ppm)	
Zinc	0.10±0.004
Calcium	0.709±0.5
Copper	0.01±0.008
Magnesium	0.46±0.1
Iron	1.69±0.1
Chromium	Nil
Manganese	Nil

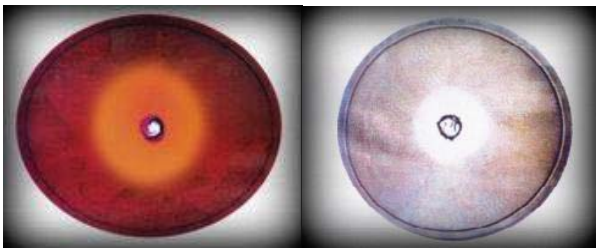
All measurements are the mean of three experimental data.

The physicochemical characteristics and mineral composition of the animal fat are presented in Table I. The moisture and ash content (%) of the animal fat were found to

be 78.5 ± 5.5 and 51.9 ± 2.5 respectively. Among the various elements studied by the atomic adsorption spectrophotometer, no elements were found higher than other elements with the absence of chromium and manganese. All the analysis was carried out on dry weight basis and the standard deviations presented are based on triplicate.

B. Characterization of Isolated Strains

Different types of bacterial strains grown on the plates were isolated based on morphological differentiation of individual colonies. In total, 5 colonies [A, B, C, D, and E] were isolated from the soak liquor. This inoculum produced lipase when tested with tri-butyrin agar and rhodamine B agar (Figs. 1 (a) and (b)). The yellow halo zone of lipolysis in tri-butyrin indicates hydrolysis of TGs present in agar. Among those, predominant strain [E] were screened and investigated because it produced the largest yellow zone and taken as lipase positive strain. The reason for yellow zone was due to release of fatty acids on lipolysis which tends to decline pH forming the yellow zone [30]. The isolated inoculum was able to ferment isoleucine, leucine and glutamate but did not utilize arginine, methionine, cysteine, lysine, histidine and serine. Production of indole was not positive and also the strain is negative for fermenting simple sugars like ribose, fructose, glucose, xylose, maltose, cellobiose, inositol and salicin. It could able to hydrolyze gelatin and esculin. The results confirmed that the isolated inoculum is peptide and lipid degrading bacterium and could hydrolyze proteins and fat rich wastes (plant and animal sources) but not carbohydrates. The morphological and physiological characters of the isolated inoculum were tabulated in Table II. The strain was identified using 16S rDNA sequencing. The Phylogeny tree showed the strain has maximum homology with the *Microbacterium* species (Fig. 2). Lipase production from *Microbacterium* species has been reported in the earlier studies [31]. The isolated species was investigated for its ability to produce lipase and ferment animal fat fleshing to esters via SSF was investigated. Also the batch experiment in liquid broth (100 ml) was conducted to know the lipase profile and the study indicated that the microbial growth and enzyme production was higher at 36 hours at the end of the log phase. The lipase activity was decreased in a late stationary phase may be due to the presence of fatty acids and proteases in the culture media (Data not shown).



(a) (b)

Fig. 1 Growth of *Microbacterium* sp. at pH 6.5 in (a) tri-butyrin agar and (b) Rhodamine B showing zone of lipolysis

TABLE II
MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF ISOLATED STRAIN FROM SOAK LIQUOR

Strains	Rhodamine B agar	Tri-butyrin agar
A	Negative	Positive
B	Positive	Negative
C	Negative	Positive
D	Negative	Positive
E*	Positive	Positive

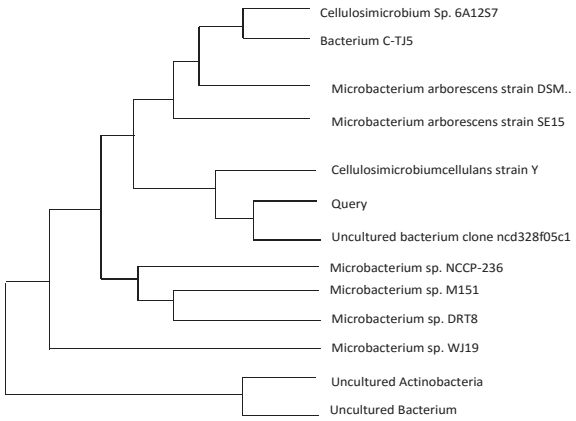


Fig. 2 Phylogenetic tree of *Microbacterium* sp. based on 16 S rDNA gene sequences

C. Strain Immobilization

Microbacterium species was immobilized on sand silica through physical adsorption. The microbial loading capacity and immobilization yield were (5.5 mg/g support and 1 mg/g support). The immobilization by physical adsorption was more effective using sand silica. The silica matrix provided the uniform mat for the bacteria to adhere and hydrolyze effectively. The colonization of the surfaces inside and outside the material resulting in a high level of contact between enzyme and substrate. As a result, hydrolysis rate was higher in the animal fat during SSF comparatively without sand silica (data not shown). Although the physical adsorption depended on the temperature, pH, substrate, and sand silica the adsorption was attributed through a hydrophilic surface of silica and thus hydrolytic efficiency and esterification was more. Moreover, thermodynamic activity of lipase is increased due to absence of water in the fermentation. The number of immobilization of microbes in SSF to treat animal fats is limited. Whereas, the immobilization of lipase on various solid supports is common in the SSF to produce enzymes. Reference [32] immobilized microbe *Thermomucor indicae on* loofah sponges (LS) as sources of the enzyme for hydrolysis via SSF and transesterification reactions for biodiesel production. Reference [33] found 2.1–4.3 times higher activity of lipase by immobilization of *Rhizomucor miehei* and *Yarrowia lipolitica* in polyurethane foams (130.5 and 90.3 U g−1, respectively).

D. Pre-Treatment Stage - SSF of Animal Fat

1) Effect of Lipase on Inoculum Size during SSF

The percentage fatty acids composition of fatty animal fleshing was determined and depicted in Table III. The fermentation characteristics of each microorganism have its own unique nutritional and physiochemical requirement. So that, it is important to characterize the fatty acid composition in order to determine its suitability as the nutrient for the fermentation. The fatty acid compositions reveal that the animal fleshing also contains considerable amount of fats. The oleic acid was predominant among the other fatty acids esters estimated 50.2% followed by stearic, palmitic, palmitoleic and meristic acids 28.7, 14.4, 5.1, 3.2% respectively. The isolate inoculum (*Microbacterium* species) was capable of producing lipase in the pH range of 6.5–7.0 (data not shown) with maximum animal fleshing degradation and enzyme production of 350–420 units/ml at pH 6.5. Increase or decrease in pH decreased the lipase production substantially and the optimum pH was 6.5. The early stages of the growth phase of microorganism and lipase activity values inclined exponentially after 24 hours and reached maximum at 36 hours at the end of the exponential phase, and reached the plateau value after 48 hours during the stationary phase and declined further down after 50 hours (Fig. 3 blue line – lipase activity, red line-growth curve). Also lipids accumulated during cell growth and maximum lipid production was achieved in the late stationary phase. Maximum amount of extracellular lipid was found at the end of 36 hours. Animal fat particles with different sizes were exhibited for hydrolysis mechanism showed that bacteria are not limited by external surface area of the particles. However, the magnitude of lipase activity was directly proportional to given fermentation period and depended upon the particle size [34]. The maximum lipase activity observed for particles size of 0.20, 0.4, 0.8 and 1.0 cm were 340–410, 280– 350, 235–300 and 200–260 units/ml, respectively which is directly proportional to rate of the esterification in SSF. The higher microbial activity and the enhanced lipase production were found to be on the animal fat fleshing of size 0.20 cm. Smaller substrate sizes (0.2 cm) provide larger surface area for microorganism to hydrolyse the substrate. Whereas too small particles resulted in particle aggregation and too larger particles had limited external surface for microorganism causing reduced hydrolytic rate. The experiment showed that 0.2 cm size of substrate was suitable for increased substrate utility henceforth 0.2 cm size of fatty animal fleshing was maintained throughout the SSF study.

TABLE III
PERCENTAGE OF FATTY ACID COMPOSITION IN RAW ANIMAL FAT

Fatty acids	Systemic Name	Formula	Amount (wt %)
Miristic	-	$C_{14}H_{28}O_2$	5.1
Palmitic	Hexadecanoic	$C_{16}H_{32}O_2$	28.7
Palmitoleic	-	$C_{16}H_{30}O_2$	3.2
Stearic	Octadecanoic	$C_{18}H3_6O_2$	14.4
Oleic	cis-9-Octadecenoic	$C_{18}H_{34}O_2$	50.2
Linoleic	cis-9,cis-12- Octadecadienoic	$C_{18}H_{32}O_2$	-

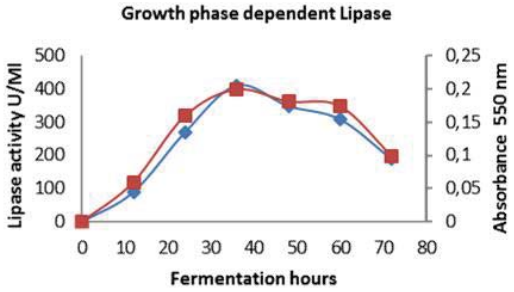


Fig. 3 Growth curve of isolated *Microbacterium* strain

2) Hydrolysis Stage of Animal Fat in SSF

The rate of hydrolysis has been described in Table IV. The acid value of the animal fat was maintained at 1.21 mg KOH $g^{-}1$. The acidity increased with hydrolysis time due to release of fatty acids and formation of esters. The maximum acidity 39.46% was observed at 72 hours with the hydrolysis rate of 83.9%. At least minimum of 10 volatile products was obtained from fermented products of animal fat at 72 h fermentation. Among those products, mostly are derived from fats. The predominant total fatty acids esters of the samples were oleic acid (C18:1n-9), palmitic acid (C16:0), and stearic acid (C18:0) and estimated to be 80% were confirmed through mass spectra. Similarly, [35] has studied the hydrolysis of animal fats by immobilized *Candida rugosa* lipase in polypropylene support. The optimal hydrolysis conditions were 0.10 kg enzyme per kilogram fat, 50% (w/v) fat, and 40°C for 24 h. The immobilized enzyme can be repeatedly used and hydrolysis rate of 90% [35] or higher can be achieved. Iodine value was found to be 42.10%

TABLE IV
RATE OF ANIMAL FAT HYDROLYSIS AND ESTERIFICATION IN SSF

Time (hrs.)	Acidity (%)	Ester Conversion (%)
24	9,43	5
36	24,46	55
48	38,63	81,69
60	33, 67	79,72
72	39,46	83,09

E. Transesterification Reaction

1) Effect of Catalyst

Transesterification process is a reversible equilibrium reaction. The mass ratio of reactants such as alcohol to oil ratio and catalyst loading and reaction time are the most significant variables to affect the conversion rate [36] (Figs. 4 (a)-(c)). It was confirmed from the experiment that fatty acids esters conversion increased from 50% to 89% with an increase catalyst loading percentage from 0.5 % to 1 wt%. In total, sodium hydroxide amount of 1.0 wt% was selected suitably for the transesterification because it was able to provide enough active sites. Further increase in catalyst ratio 1.5% shows the no improvement in conversion rate. The similar results were described by [37].

2) Effect of Methanol

Methanol/oil mass ratio from 3:1 to 6:1 shows increase in reaction rate from 65 % to 88%. Further increasing the ratio from 6:1 to 9:1, the conversion almost remained same to 88%. Therefore, the methanol/chicken oil mass ratio of 6:1 and catalyst loading of 1% was found to be an optimal value in the present process. The relationship between reaction time and the conversion rate was also further investigated. The reaction conditions were mass ratio of methanol/ oil of 6:1, reaction temperature of 344 K, the sodium hydroxide amount was 1.0 wt%.

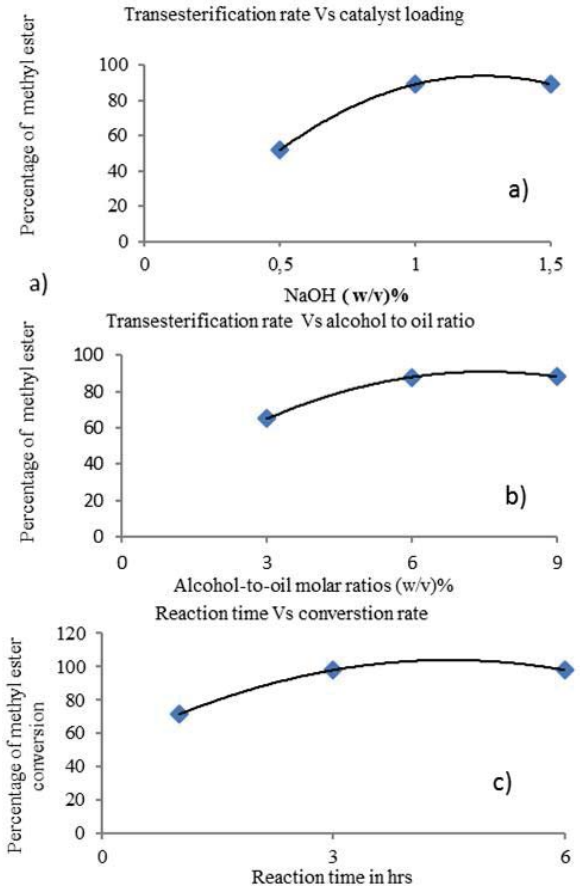


Fig. 4 Transesterification reaction: (a) Effect of catalyst on trans-esterification reaction (b) Effect of alcohol to oil ratio on trans-esterification reaction (c) Effect of reaction time on trans-esterification reaction

3) Effect of Reaction Time

The relationship between conversion and reaction time was also investigated. The experiment results showed that the reaction time had markedly significant effect on the transesterification process. The TGs conversion increased from 71.2% to 97.9% with the increase of reaction time from 1 to 3 hours at 343 K. However, further increasing reaction time, the conversion rate almost kept stable. A reaction time longer than 3 hours did not change the TGs conversion no more. This was possibly because of close equilibrium conversion achieved in the reaction [38].

After transesterification, the resulted ester contained little unreacted starting material such as TG, residual alcohol, residual catalyst and glycerol which is separated from biodiesel in the production process in trace amounts. Since transesterification is a stepwise process, monoglyceride and diglyceride formed as intermediates can also be found in biodiesel [39]. However, [40] concluded that the alkaline transesterification of beef tallow produced high quality biodiesel with a good conversion rate. The average of 0.75% fatty acids esters value was converted to biodiesel using KOH and methanol by transesterification reaction.

F. C_{13} NMR Analysis

C_{13} NMR analysis was conducted for the preliminary analysis to observe the formation of functional group desired. A representative spectrum of C_{13}NMR of the animal fat oil biodiesel showed the characteristic peaks of methylene carbons of long carbon chain in FAME at 22.2817-31.9727 ppm. Other peaks are due to the carbonyl carbon of the ester molecules of biodiesel signals at 70.374 - 77.3942 ppm. The peak around the 42.3790 ppm indicated the methoxy carbon of esters. The terminal carbon of methyl groups and methylene and methyl carbons of fatty acid moiety are at 14.1932 and 20.851 ppm respectively. Methyl esters of palmitic, stearic, oleic and represented major components. This reports that transesterification process has been successful and biodiesel (FAME) was produced.

IV. Conclusion

The present study deals with producing biodiesel from the tannery wastes rich in animal fats. After the animal fat fleshing was treated through SSF, the transesterification process was completed by using the basic catalysts. *Microbacterium* species isolated from soak liquor is valuable as a source of lipase and showed maximum lipase production after 36 hours. The analysis of aromatic groups and breakdown of fats in GCMS supported successful fermentation of animal fats. The methyl ester content was estimated to be around 89%. Therefore, the SSF system with transesterification used in this study appears to have the potential to be practically applied for industrial scale biodiesel production from tannery wastes.

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