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BIODIESEL PRODUCTION FROM SIMAROUBA DEOILED-CAKE BY TRANSESTERIFICATION AND PHYTOCHEMICAL ANALYSIS

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ABSTRACT

The preliminary phytochemical screening of simarouba deoiled cake showed the presence of various compounds belonging to carbohydrates, proteins, Terpenoids, flavonoids, saponins, alkaloids and glycosides. GC-MS analysis of the aqueous extract of simarouba oil seed cake proved the presence of these compounds. Simarouba deoiled-cake is used as a substrate for production of lipase from Aspergillus niger in MGM media. Solid state fermentation method was used to produce lipase enzyme. Partial purification of the enzyme was done by precipitation using ammonium sulphate and the pellet formed was found to have precipitated maximum at 20-60% concentration of ammonium sulphate. Lipase inhibition with aqueous extract of simarouba oil cake showed increasing trend with increasing concentration of aqueous extract and reached maximum when the concentration is 3.0 ml. Oil was extracted from simarouba deoiled cake using methanol as solvent in Soxhlet extractor. The trans-esterified product showed a clear yellow colour which was less viscous than the extracted fat. Gas Chromatographic Analysis of the biodiesel produced from deoiled cake showed a combination of different types of Methyl Esters.

KEY WORDS

Simarouba, Seed-oil-cake, Transesterification, Biodiesel, Aspergillus niger, Lipase

1. Introduction

There has been an increased exploitation of organic residues from various sectors of agriculture and industries over the past few decades. Crop residues such as bran, husk, bagasse, and fruit seeds were utilised as a potential raw material in bioprocesses as they provide an excellent substratum for the growth of microorganism supplying the essential nutrients to them [1, 2]. Their application in bioprocesses also offers advantages in bioremediation and biological detoxification of hazardous compounds and in the field of fermentation technology, has resulted in the production of bulk-chemicals and value-added products such as amino acid, enzymes, mushrooms, organic acids, single-cell protein (SCP), biologically active secondary metabolites, etc. [3].

Biodiesel production from vegetable oils remains a strong growth market in the United States, Canada and the European Union [4]. The majority of edible vegetable oils have excellent characteristics that have made them the most commonly used raw materials for biodiesel production, with the objection of their high prices [5, 6]. Oil cake represents a valuable low-cost biomass source for biodiesel manufacturing. Such low price raw material is of special interest since it will significantly reduce the production costs of biodiesel. In this way, the price of biodiesel will be in competition with petroleum based diesel [7].

India is one of the world's leading oilseeds producers. Several million tonnes of oil cake is leftover after oil extraction from the seeds annually. This large amount of oil-cake makes it the single most important agricultural byproduct in the country. However,

accumulation of such large amounts of cake in its raw form imposes a disposal and pollution burden causes serious environmental concerns despite they contained polyphenols with potential antioxidant and free radical scavenging activities [8]. Therefore, utilization of this source for energy production through a systematic method of raw material management could be significant in terms of energy cost savings and in terms of pollution control. Moreover, utilizing cake is a very good alternative in the production of environmentally friendly green bio fuel. Another key point to be noted is that the bioprocess utilising oil cakes is attractive due to relatively cheaper availability of the oilcakes throughout the year, making it even more favourable when economics is considered.

Several workers have been reported the application of oilcakes in biotechnological aspects in growth of pathogenic bacteria, fungi, and viruses, and as a substrate for production of enzymes like lipase, proteases, L-glutaminase and phytase in solid-state fermentation (SSF) or as supplement to the production medium using fungal species and for the production of antibiotics and antioxidants [9, 10].

Simarouba belongs to the family Simaroubaceae. It had also been known as paradise tree, Laxmitaru, Acetuno, a multipurpose tree that can grow well under a wide range of hostile ecological condition. It is both a source of edible oil and also biofuels. The oil can be used as cocoa butter substitute/ extenders in confectionary and bakery industry. The oil cake is valued organic manure which is a very rich source of protein [8]. Its seed contain about 40 % kernel and kernels content 55-65% oil. It was used for industrial purposes in the manufacture of soaps, detergents and lubricants etc. The oil cake being rich in nitrogen (7.7 to 8.1%), phosphorus (1.07%) and potash (1.24%) could be used as valuable organic manure. Simarouba was a rich source of fat having melting point of about 29^{0}C. The major green energy components and their sources from Simarouba were biodiesel from seeds, ethanol from fruit pulps, and biogas from fruit pulp, oil cake, leaf litter and thermal power from leaf litters, shell, and unwanted branches [8].

In this paper we will discuss on use of Simarouba deoiled-cake for production of lipase from *Aspergillus niger* and use of oil extracted from seed-cake for biodiesel production by trans-esterification method and phytochemical analysis of the extract of deoiled-cake.

2. Materials and methods

2.1 Collection of Raw Material

Simarouba deoiled-cake was collected from Biofuel Park, Madenur, Hassan (Dt), Karnataka, India. It was stored in laboratory condition for further use. Initial drying was carried out in an oven for 1 h at 105^{o}C. This much time and temperature were found to be sufficient for further processing.

2.2 Selection of fungal culture

The fungal culture used was *Aspergillus niger, maintained* on Potato dextrose agar (PDA).

2.3 Aqueous extraction

The deoiled-seed cake was dried, crushed in a mortal with pestle. The distilled water was added to the crushed cake in the ratio of 1:10 and refluxed over water bath, for 3 hrs at 80 ^{0}C using Soxhlet method. The filtrate was kept for phytochemical analysis and lipase inhibitory analysis [11].

2.4 Phytochemical Assay

The preliminary phytochemical screening of was carried out for the detection of various phytoconstituents using standard procedure [12] and Gas chromatography and Mass spectrometry (GC- MS) as recognizable proof for various phytoconstituents.

2.5 Solid substrate fermentation

Autoclaved substrate was utilized for enzyme production using fungus *Aspergillus niger.* Spore number of 1 x 10^8 was used for inoculation. The composition of media was (g/L): K_2HPO_4 1.08; $MgSO_4$.$7H_2O$ 0.5; KCl 0.5; $FeSO_4$ 0.01; Glucose 12.5; Peptone 20; Tween 80 15; Triton X100 4; Olive Oil 25; pH 6.5. 5ml of mineral growth media was added to the flasks containing 10g of Simarouba dried cake powder and inoculated with *Aspergillus niger*, incubated at 37^{0}C for 7 days on rotary shaker at 120rpm. Crude enzymes were extracted using 25ml of chilled phosphate buffer. The supernatant obtained was analyzed for lipase enzyme activity. Enzyme obtained was used for transesterification process [13].

2.6 Enzyme extraction and partial purification

After fermentation, 25 ml of chilled 0.05M phosphate buffer of pH 7.0 was added into flask containing fermented solids. Extraction of Enzyme was carried out by magnetic stirring at room temperature for 30 min followed by double layered filtration through muslin

cloth. The obtained filtrate was further centrifuged at 10000 x g for 30 min and passed through whatman filter paper no 2 and then through membrane filters with 0.45 µm porosity in order to remove cells and spores. The obtained clear supernatant was used for partial purification of extracellular enzyme. Partial purification was done by ammonium sulphate precipitation (20-100%) with three ammonium sulphate fractions of 20%, 40% and 60%. Solution allowed to stand for 30–60 minutes at 4^0C temperature and centrifuged at 10,000 rpm for 30 minutes. The formed protein pellet was resuspended in small volume of 0.05M phosphate buffer of pH 7.0 [14].

2.7 Lipase Enzyme Assay

The lipase activity was carried out by titrimetric method. 1ml of partially purified enzyme taken along with assay substrate containing 10ml of 10% olive oil mixed with 10% (w/v) gum acacia, 2ml of 0.6% calcium chloride and 5ml of phosphate buffer (pH 7)**.** The mixture of enzyme and substrate is incubated at 30°C for 60 minutes at 150 rpm in incubator shaker. 20ml of alcohol: acetone mixture in ratio of 1:1 is added to the reaction mixture. The liberated fatty acids are titrated against 0.1N NaOH using phenolphthalein indicator. End point is determined by change in colour of the mixture to light pink [15].

Lipase activity was calculated using the formula:

Lipase Activity= ΔV∗N∗1000/ V (sample)* time (60 min) U/ml

V1= Volume of Sodium Hydroxide against control flask (ml)
V2= Volume of Sodium Hydroxide experimental flask (ml)
N= Normality of Sodium Hydroxide
V (Sample) = Volume of Enzyme extract (supernatant) (ml)

2.8 Lipase inhibitory Assay

Lipase inhibitory assay was carried out as that of lipase enzyme assay with addition of various concentrations of the aqueous extract of Simarouba deoiled-cake (0.5ml to 3.5ml) to study the inhibition of the lipase.

2.9 Oil extraction

About 10g of deoiled dried cake samples were prepared and placed into a thimble in the soxhlet extractor fitted to conical flask. The 100 ml of methanol was used as an extracting solvent. The mixture was boiled for 8 hrs at 60^0 C, at the end of the extraction process, when the recycled solvent becomes clear, the extraction solvent is recovered. The oil sample, which was cooled to the room temperature, was then weighed. The amount of oil was determined from the original sample [7].

Weight of the oil recovered = {(*W*2- *W*1)/ *W*3} *100

Where, *W*1 = weight of the extraction cup
*W*2 = weight of the extraction cup + extract
*W*3 = weight of the dry olive cake sample

2.10 Transesterification and Biodiesel Production

The partially purified lipase was used as the catalysts for transesterification reactions to investigate the effect of catalyst on the fuel properties of biodiesel. The catalyst amount used was 1ml of the extract. Methanol was taken in the ratio of 1:4 with respect to oil obtained and incubated in shaker at 40^0C for 24 hours [10]. After the reaction completed, the mixture was allowed to settle in a 500-ml separation funnel for 3 hr. The ester layer, lower content of FFA was separated by gravity and located in the upper layer. Impurities, glycerol, extra methanol and undesired products were decanted. The biodiesel layer was purified by washing with distilled water for complete removal of excess methanol and any catalyst traces. The procedure was repeated until the washing water had a pH value that was similar to that of distilled water. The treated oil was then dried by gentle heating for complete water removal to obtain the refined biodiesel. The degree of oil conversion and the biodiesel yield were recorded.

2.11 Gas Chromatographic analysis

The Clarus 680 Gas chromatography (GC) was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument. The oven temperature was 60°C for 2 min followed by 300°C at the rate of 10°C min^{-1} and 300 °C, where it was held for 6 min. The mass detector conditions were: transfer line temperature 240°C; ion source temperature 240°C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments vary from 40 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2008) library.

3. Results and Discussion

3.1 Phytochemical Analysis

The phytochemical screening of aqueous extract of Simarouba deoiled-cake revealed the presence of

different class of compounds like sucrose, Dimethylsilyl tertiary-buthyl peroxide, N-Hexadecanoic acid, 1-Hexyl-2-Nitrocyclohexane, Oleic acid, Hexanedioic acid Bis (2-ethylhexyl) Ester, 1-Monolinoleoylglycerol Trimethylsilyl ether, OCTASILOXANE, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyl, 1-monolinoleoyl glycerol trimethylsilyl ether and 1,2-Bis(trimethylsilyl)benzene (Figure 1 and Table 1). Any of these primary and or secondary metabolites, singly or in combination with others could be responsible for the inhibition of activity of lipase enzyme from fungi *Aspergillus niger*. Similar reports for presence of various antioxidant substances tocopherols, tocotrienols, carotenoids, triterpenes, and polyphenols in seed cake has been reported by others also [10, 16].

Table 1: Phytochemical screening of aqueous extract of Simarouba deoiled-cake

Test for compound	Result
Carbohydrate	**+**
Proteins	**+**
Terpenoids	**+**
Flavonoids	**-**
Saponins	**-**
Alkaloids	**-**
Glycosides	**+**

Table 2: Activity of enzyme lipase

Enzyme sample	Enzyme activity (U/ml/min)
Control	**3.00**
Without Ammonium sulphate	**3.17**
Fraction 1	**3.42**
Fraction 2	**3.67**
Fraction 3	**3.50**

3.2 Partial purification, Activity and Inhibition of Lipase Enzyme from *Aspergillus niger*

Use of oil seed cake in production has also been reported in Ground nut cake and teesi oil cake for production of lipase from *Rhizopus oryzae* [17]. Partial purification of the enzyme was done by precipitation using ammonium sulphate as it is the most common method routinely used for extraction of proteins. The pellet formed was found to have precipitated maximum at 20-60% concentration of ammonium sulphate.

The activity of the partially purified lipase enzyme was carried out using Olive oil as substrate (Table 2). The enzyme reacts with olive oil and produces methyl or/and ethyl esters of fatty acids. This mixture is titrated against 0.1N Sodium Hydroxide taken in the burette. Phenolphthalein being used as indicator; the point was indicated by change in colour to pink.

The inhibitory activity of aqueous extract of the deoiled cake of simarouba against lipase from *Aspergillus niger* shows that the inhibition is found to be increases steadily with increasing concentration of extract and reached maximum when the concentration is 3.0 ml of aqueous extract (Figure 2).

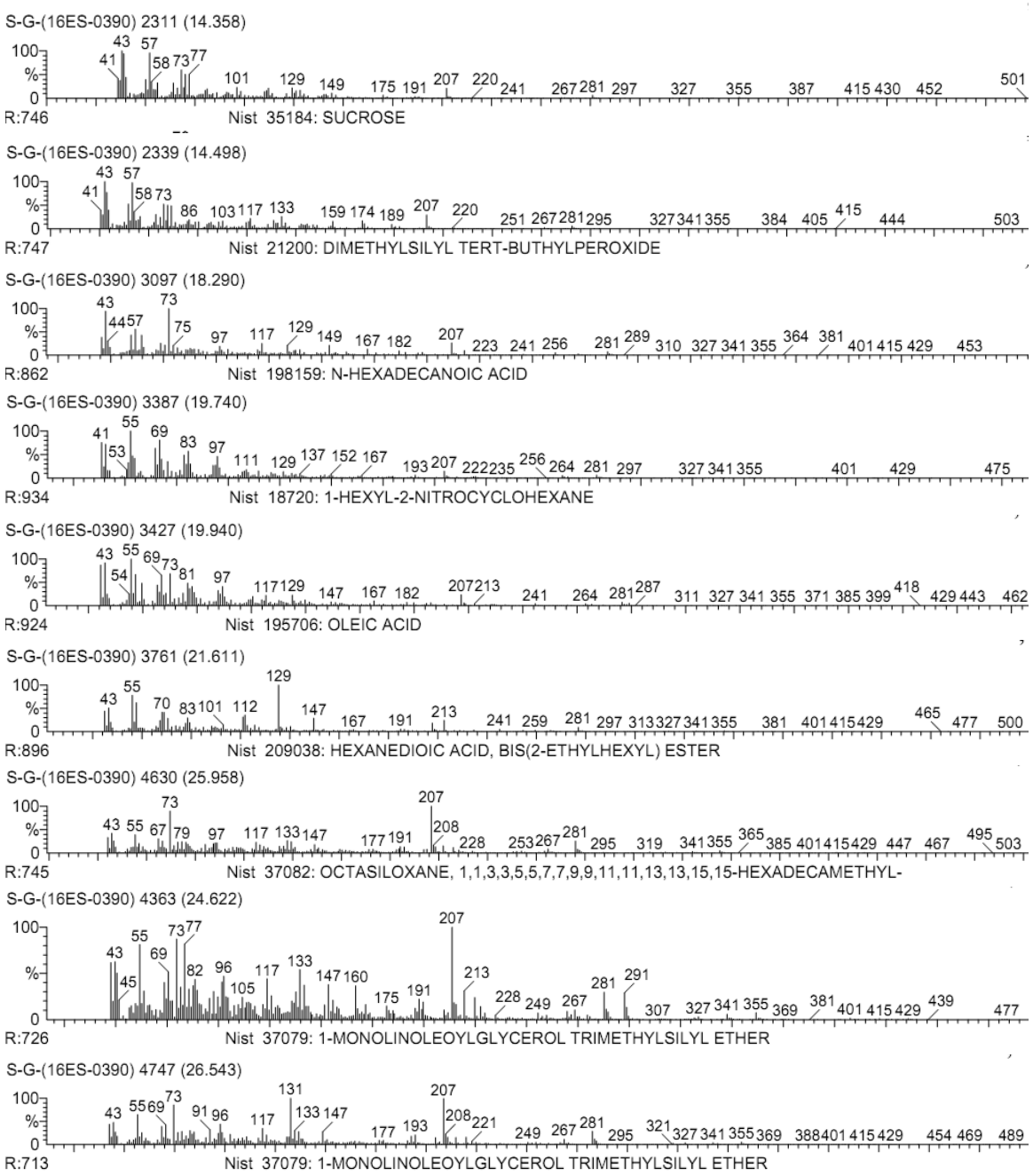

Figure 1: GC-MS analysis of aqueous extract of Simarouba de-oiled cake

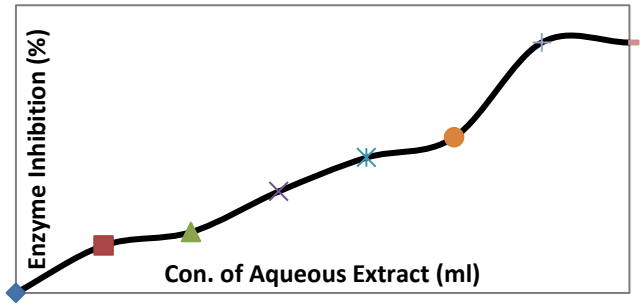

Figure 2: Inhibition of enzyme Lipase

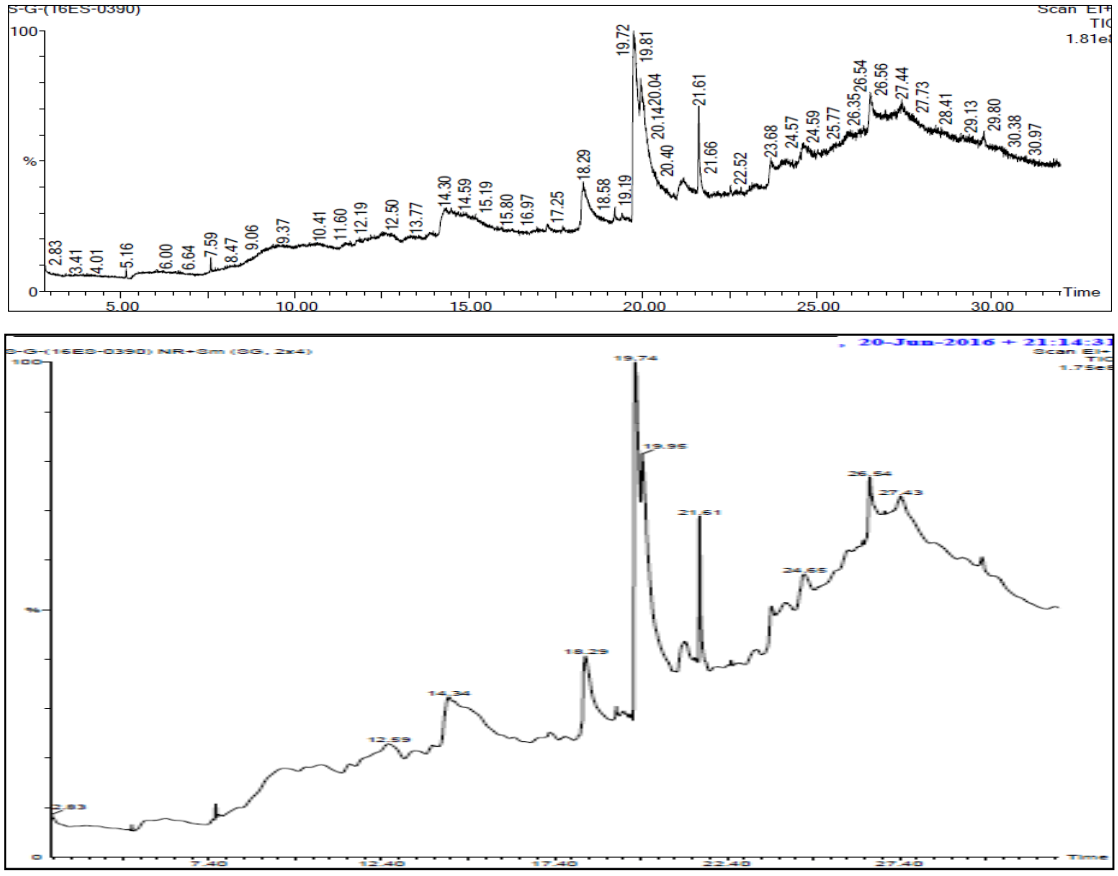

Figure 3: GC analysis of Biodiesel

3.4 Oil extraction

Oil was extracted from simarouba deoiled cake using methanol as solvent using Soxhlet extractor. The process was carried out for 5 hours in Soxhlet apparatus. For 20 g of simarouba deoiled seed cake 1.58 g of fat was obtained. There are several reports of producing biodiesel from Simarouba oil [8], there are no reports of biodiesel production from simarouba deoiled seed cake. But from other sources, it has been reported in *Madhuca indica, Shorea robusta, Pongamia glabra, Mesua ferra (Linn), Mallotus philippines, Garcinia indica, Jatropha curcas and Salvadora* [18].

3.5 Transesterification and Gas Chromatographic analysis

The transesterification was carried out by lipase enzyme from *Aspergillus niger*. The transesterified product was a clear yellow fluid which was less viscous than the extracted fat. Lipases from different sources are able to catalyze the same reaction, bacterial and fungal lipases are mostly used in biodiesel production such as *Aspergillums niger*, *Candida antarctica*, *Candida rugs*, *Chromobacterium viscous*, *Macro mashie*, *Pseudomonas cetacean*, *Pseudomonas fluorescents*, *Photobacterium lipolyticum*, *Rhizopus oryzae*, *Streptomycin sp.* etc. [12,14] Gas Chromatographic analysis of the biodiesel obtained showed a combination of different types of Methyl Esters (Figure 3).

4. Conclusion

Production of biodiesel has attracted more attention in these days to replace the non-renewable petroleum based fuels. Most of the transesterification studies have been done on edible oils like rapeseed, soybean, and sunflower etc by using NaOH or KOH catalyst and to some extent on non-edible oils like castor, Madhuca, etc. The tree borne oil like Simarouba is the most potential species to produce biodiesel in India and it also offer greater rural employment opportunity. Enzymatic of transesterification from lipase is comparatively more efficient than chemical methods and it has got many advantages like it does not require multi-step purification of end products, there is reduction in processes required for waste-water

treatment and optimal energy requirements for carrying out the process. *Simarouba glauca* seed oil has good nutritional profile and other physico-chemical properties which can be improved after refining process. Therefore, it can be used as a potential oil seed resource for edible purpose and also for bio-fuel production.

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