Transesterification of Triglycerides by Dried Biomass of Aspergillus sp.

Satnam Singh Aulakh\(^1\), N. Tejo Prakash\(^2\) and Ranjana Prakash\(^1\)*

\(^1\) School of Chemistry and Biochemistry, Thapar University, Patiala, India
\(^2\) School of Energy and Environment, Thapar University, Patiala, India

Abstract: Fungus isolate, Aspergillus sp. (RBD01), which was isolated from biocontaminated clarified butter was evaluated for its potential to transesterify used edible and non-edible oils for generation of alkyl esters, when used as biocatalyst as dry biomass. The work aimed at determining the potential of dry biomass of Aspergillus sp. (RBD01) to transesterify used cottonseed oil and non-edible oils viz., jatropha and karanj under various culture conditions. A conversion of oil (cotton seed) to ethyl ester to the extent of 84\% was obtained at reaction temperature of 35\,\degree C, with 20\% biomass and step-wise addition of ethanol at 1:5 molar ratio (oil to ethanol), within total reaction time of 36 h. Under similar conditions, transesterification of Jatropha and Karanj oils resulted in only 75 and 78.2\% ethyl ester. Further, with reference to the effect of frying on transesterification, increase in frying time decreased the extent of transesterification from 84\% to 30\%.

Key words: Alkyl ester; whole cell; Aspergillus sp.; transesterification; vegetable oils

1 Introduction

Bio- ‘Diesel’ world encompasses within itself the pioneering work of Rudolf Diesel (1858-1913) and his ever fruitful contribution. "Liquid Fuels," by Rudolf addressed vegetable oils as fuels and plant oils were actually successfully used as fuel in diesel engine by 1900. Use of vegetable oils as emergency fuel during the World War II resulted in initiation of research and development on these oils as domestic fuels in India\(^7\). Today once again, the importance of energy security and environmental concerns are acting significantly as driving forces for use of vegetable oil-based diesel fuels. In following decades, more systematic studies towards properties and application lead to development of various approaches for generation of biodiesel as an alternative fuel. The process of transesterification, which is an extensively studied protocol, is relatively a simple process and physical characteristics of the fatty acid alkyl ester produced by this process resemble very close to those of diesel fuels\(^2\), due to which it is considered the best among all the alternatives. In unmodified diesel engines, the methyl or ethyl esters of fatty acids can be burnt directly, that too with low deposit formation\(^8\)-\(^10\). It represents a fuel containing mono alkyl esters of long chain fatty acids derived from vegetable oil or animal fats\(^8\).

The catalysis of transesterification, is generally carried out by using homogeneous as well as heterogeneous catalysts. Homogenous catalysis generally done either with an acid or with a base. Acid catalysed transesterification reaction is relatively slower\(^7\) than alkali catalysis. This approach is also hindered with difficult glycerol recovery and serious environmental and corrosion related problems make their use non-practical for biodiesel production at the industrial scale\(^8\),\(^9\). The alkali catalyzed reactions also have several drawbacks such as cost and energy intensive nature, difficulty in glycerol recovery, hindrances in recovery of catalyst from the reaction, and interference of free fatty acids and water with reaction\(^8\),\(^10\). Heterogeneous (heterogeneous process where catalyst are in solid phase and reactant in liquid) catalysis such as enzymes, zeolites, clay, guanidine has recently been introduced in order to overcome or minimize the problem of homogenous catalysis\(^11\),\(^12\). However, heterogeneous base and acid catalyst(s) also have certain obvious disadvantages such as requirement of oils with low free fatty acids, anhydrous conditions, high molar ratio of alcohol to oil, high cost, etc\(^8\),\(^14\).

As a promising alternative, transesterification reaction has been attempted through enzymatic reactions involving lipases. Lipases are excellent alternatives to chemical catalysts due to the obvious advantages of the former viz. milder mild reaction conditions; specificity; reuse; immobi-
lization possibilities; improvable efficiency by genetic engineering; acceptability for new substrates; more thermostability; and eco-friendly nature\(^{20-25}\). Industrialization of biodiesel production using enzyme catalysis however has been limited due to high production and purification cost of lipase, lipase inactivation by acyl acceptors such as methanol, desorption from immobilization support, and fouling in packed bed bioreactors. These hurdles have led to pave a new path in research of biodiesel production leading towards utilization of lipase producing whole cells as catalysts which have been recently studied by a various research groups\(^{30}\).

Use of whole cell biocatalyst instead of purified lipase cuts the cost of isolation, purification and immobilization of pure lipase. Among the established whole-cell biocatalyst systems, filamentous fungi have proven to be the robust biosystems in terms of scale-up industrial applications. The successful use of Rhizopus oryzae, R. chinensis, recombinant Saccaromyces cerevisiae and most recently Aspergillus niger as whole-cell biocatalysts has recently been indicated through recent reports and reviews\(^{18}\). Our research group has earlier established the potential of a fungus isolate, Aspergillus sp. (RBD01) obtained from bio-contaminated clarified butter to grow in medium supplemented with 70% oil as main carbon source and simultaneously hydrolyze to fatty acids (FFAs)\(^1\). Studies further showed near complete transesterification of fatty acids to alkyl esters in the presence of alcohol\(^19\). The present study demonstrates the ability of the above isolate, being used as a dry biomass, to hydrolyze edible and non-edible oils and facilitate transesterification reaction generating alkyl esters; and elucidates the standardization of reaction conditions associated with above process.

2 Experimental

2.1 Material and Methods

Cottonseed oil was sourced from market. jathropa and karanj oils were obtained from Medor Biotech India Limited. Bi-ammonium hydrogen ortho-phosphate\([\text{NH}_4\text{H}_2\text{PO}_4]\) and mycological peptone, Bushnell-Hass broth (BHB), Potato dextrose agar (PDA), Potato dextrose broth (PDB) were all purchased from HiMedia, India. Other chemicals such as such as ethanol, hexane, ethyl acetate, glacial acetic acid, silica gel grade G, were sourced from SD Fine Chem., India. For \(^1\)H NMR Brucker Advance II 400 NMR spectrophotometer was used at SAIF, Panjab University, Chandigarh, India. CDCl\(_3\) and TMS were used as solvent and internal standard respectively.

2.2 Dried biomass production

Aspergillus sp. (RBD01) (MTCC5436) isolated from contaminated clarified butter\(^{19}\) sample was used as whole cell catalyst to carried transesterification of cottonseed oil. Inoculum was prepared by using \(10^7\)ml spores in 500 ml potato dextrose broth and incubating the culture flask from 120 h and at 28°C and 120 rpm. This freshly grown biomass was further used as inoculum. Dried biomass was prepared by inoculating the culture in medium that contained mineral salt medium containing magnesium sulphate (0.20 g/l), calcium chloride (0.02 g/l), monopotassium phosphate (1.0 g/l), di-potassium phosphate (1.00 g/l) and ferric chloride (0.05 g/l) in ratio of 70:30 (mineral salt medium: oil). Cottonseed oil was used as carbon source as well as lipase inducer. The medium was supplemented with mycological peptone and bi-ammonium hydrogen ortho-phosphate (0.5% w/v) as nitrogen source. After incubation of 120 h, biomass was separated from reaction mixture using Whatman filter paper and was washed with n-hexane followed by water to remove adhering oil. The biomass was air-dried overnight to remove excess water and crushed to powder in liquid nitrogen. Dried biomass, thus, obtained was stored at 4°C and used for further study.

2.3 Standardization of transesterification reaction using dried biomass

The conditions optimized to achieve maximum transesterification with dried biomass as catalyst, are outlined in the Table 1.

Transesterification reaction was standardized by varying the parameters like amount of biomass (5 – 40%) with respect to oil; temperature range (25 – 65°C); and time interval (2, 4, 8 and 12 h) of ethanol addition at 1:5 oil to alcohol molar ratio. Ethanol (3.5 ml) was divided into three parts and added step-wise at these intervals. For instant addition (0 h), 3.5 ml of ethanol (1:5 molar ratio) was added to 10 gm of oil once. Molar ratio of oil to ethanol was also varied (1:3, 1:4, 1:5 and 1:6) to achieve maximum transesterification. The reaction time was 36 h in all cases. Further, the reusability potential of biomass was examined over a series of 5 cycles. Under conditions standardized with cottonseed oil, similar experiment was performed with Jatropha and Karanj oils.

2.4 Effect of frying on transesterification reaction

Effect of frying was examined on the extent of transesterification, by generating frying oil using cottonseed oil and frying the same for 7 h as described earlier\(^{20}\). An average of 7-9 fryings were carried out each hour at 160-200°C and samples were collected at hourly interval. No fresh oil was supplemented during frying. The used oil was filtered to remove debris formed during frying and transesterification was carried out under standardized conditions for 36 h (Table 1).

2.5 Quantification of ethyl ester produced

Ethyl ester quantification by \(^1\)H NMR spectroscopy was
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Table 1 Reaction condition altering various parameters for transesterification by using dried biomass (total reaction period of 36 h)

<table>
<thead>
<tr>
<th>Steps</th>
<th>Reaction Parameters</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>10 g oil, molar ratio of oil to ethanol - 1:5, 35°C, alcohol addition at an interval of 8 h</td>
<td>Biomass percentage with respect to oil (5, 10, 20, 30 and 40%)</td>
</tr>
<tr>
<td>Step 2</td>
<td>10 g oil, 2 g biomass, molar ratio of oil to ethanol - 1:5, reaction temperature – 35°C</td>
<td>Time interval of alcohol addition (0, 2, 4, 6, 8 and 12 h)</td>
</tr>
<tr>
<td>Step 3</td>
<td>10 g oil, 2 g biomass, molar ratio of oil to ethanol - 1:5, alcohol addition at an interval of 8 h</td>
<td>Reaction temperature (25, 35, 45, 55, 65°C)</td>
</tr>
<tr>
<td>Step 4</td>
<td>10 g oil, 2 g biomass, alcohol addition at an interval of 8 h, reaction temperature – 35°C</td>
<td>Molar ratio of oil to ethanol (1:3, 1:4, 1:5 and 1:6)</td>
</tr>
<tr>
<td>Step 5</td>
<td>10 g oil, 2 g biomass, Molar ratio of oil to ethanol 1:5, reaction temperature – 35°C, alcohol addition at an interval of 8 h</td>
<td>Water content in the reaction mixture (0-50%)</td>
</tr>
</tbody>
</table>

carried using the equation proposed by Ghesti et al. (2007)\(^{21}\).

\[
\%C_{EE} = 100 \left( \frac{4 (I_{TAG} + EE) - I_{TAG}}{4 (I_{TAG} + EE) - I_{TAG} + 6 (2I_{TAG})} \right)
\]

Where

(i) \((I_{TAG})\) integration of glyceryl methylenic hydrogens at 4.25-4.35 ppm;

(ii) \((I_{TAG} + EE)\) integration of glyceryl methylenic hydrogens and -OCH\(_3\) of ethoxy hydrogens superimposed at 4.10-4.20 ppm; and

The numbers 4 and 6 in above equation are related to four glyceryl methylenic hydrogens present in TAG molecules and to six hydrogens formed in three ethyl ester products.

3 Results and Discussion

Aspergillus sp. (RBD01) grown in 30:70 (oil:mineral media) was separated, dried and powdered in liquid nitrogen so as to increase the surface area and maintain homogeneity. The powdered biomass was subjected to transesterification reaction using cottonseed oil and ethanol. Influence of various process parameters on the catalytic potential of dried biomass was further examined.

3.1 Effect of the amount of biomass

Initially the amount of biomass as catalyst percentage with respect to oil, for reaction was standardized.

It was observed that with increase in the percentage of biomass from 5-30%, resulted in increase in the yield of the ethyl ester from 15.5% to 79% ethyl ester (Fig. 1). Subsequent increase in the percentage of biomass (40%) resulted in decrease in yield of the ethyl ester (39%).

It was observed that with increase in the biomass percentage, yield of EE increased wherein maximum yield of 79% EE was obtained with 30% biomass. Further increase in biomass decreased the product yield, presumably due to increased viscosity of the reaction mixture in which homogeneous stirring couldn’t take place. The observations on the excess biocatalyst not contributing to increase in percentage conversion is also further supported by observations of Torres and Otero (2001)\(^{22}\) showing that the excess of enzyme does not necessarily increase the percentage conversion and sometimes leads to decrease in the yield of product.

3.2 Effect of time interval of alcohol addition

The observations indicated that with increase in time interval of alcohol addition, the extent of transesterification increased up to an interval of 8 h beyond which it decreased. Figure 2 shows that instant addition of alcohol resulted only 36.2% conversion of oil to ethyl ester followed by increase to 83% at 8 h interval. A yield of 36.3, 44.4 and 48.6% EE was obtained when alcohol was added at an interval of 0 (one time addition), 2 and 4 h respectively, thereby indicating inactivation of the reaction due to excess alcohol possibly due to denaturation of lipase by interfering with hydrogen bonding between water and amino acid residues\(^{21-26}\).

![Fig. 1](image_url) Extent of transesterification (ethyl ester %) using different biomass percentage 19 × 11 mm (300 × 300 DPI).
However, further increase in the time interval from 8 to 12 h between the successive alcohol additions did not enhance the ethyl ester yield. Comparatively, low yields (71% EE) were obtained when the time interval of alcohol addition was 12 h, due to reversible nature of reaction. When the time interval of the addition of ethanol is greater than 8 h, the alcohol added at initially gets completely utilized in the reaction resulting in a reverse reaction and subsequent decrease in yield of products.10, 18

3.3 Effect of temperature
Influence of different reaction temperatures on the extent of transesterification was also examined under reaction conditions as mentioned in Table 1. It was observed that yield of ethyl ester enhanced with increase in reaction temperature from 25°C (41% ethyl ester) to 35°C (80.5% ethyl ester). Further increase in the temperature from 45°C (62% ethyl ester) to 65°C (18% ethyl ester) resulted in sharp decrease in the yield of ethyl ester (Fig. 3).

At lower and upper ranges of temperature, the ester yield was less due to the fact that at lower temperature enzyme is presumably less active, whereas increasing the temperature above optimum value caused the enzyme to lose its activity as observed with Lipozyme RM-IM by Vierira et al. (2006) and Trubiano et al. (2007). Xiao and Obbard (2010) conducted experiments for determining optimal reaction temperature over a range from 25 to 50°C. At an optimum temperature of 30°C, 86.4% fatty acid methyl ester yield was achieved after 72 h. Rodrigues et al. (2008) reported that a sharp decrease in yield of ester above 40°C was likely due to thermal inactivation of the enzyme.

3.4 Effect of molar ratio of oil to alcohol
The influence of molar ratio of oil to alcohol was examined with reference to the excess of alcohol added to carry out reaction in forward direction as transesterification is reversible in nature. The reaction was carried out using 2 g biomass, 10 g oil, reaction temperature 35°C, ethanol added stepwise at an interval of 8 h, with total reaction time of 36 h.

Increase in the molar concentration of alcohol with respect to oil, enhanced the extent of transesterification and yield of ethyl ester 16% (1:3 oil: alcohol) to 81.5% (1:5 oil: alcohol). Further, increase in alcohol (1:6 oil: alcohol) concentration resulted in decrease in the yield of ethyl ester (72% ethyl ester) (Fig. 4). The decrease in activity is also presumed to be due to inactivation of enzyme by excess of ethanol. It was reported that short-chain alcohols, when added in excess can modify the hydrophilic end of the enzyme resulting in denaturation.31 When alcohol is added to the system the organic phase acquires a higher polarity and this shifts the water distribution toward the fluid phase, therefore, inhibition occurs as a result of the removal of water from the enzymatic system.32 The solubility of methanol and ethanol was found to be only 1/2 and 2/3 of the stoichiometric amount, respectively. The low rate of alcoholysis was due to the irreversible inactivation of enzyme by contact with insoluble ethanol which exists as droplets within the oil.33

3.5 Effect of water content
Transesterification using dried biomass was further carried out by varying the percentage of water content in the reaction mixture, reaction was carried out under stan-
standardized conditions. With increase in water content from 0 (81.5% ethyl ester) to 2% (83% ethyl ester) with respect to oil, no significant difference was observed in the yield of ethyl ester. However, further increase in water content from 4% to 50% resulted in decrease in the yield from 76% to 24.6% (Fig 5).

This is presumed to be due to the reduced availability of interfacial region for transesterification reaction. Xiao and Obbard (2010)28 reported that the water content in the feedstock had a variable influence on the transesterification reaction, where the highest fatty acid methyl ester (FAME) content i.e. 81.1%, was obtained with 10% water content. However in the present study increase in the extent of transesterification from 81.5% to 84% with increase in the water content (0% to 2%) was not significant.

3.6 Effect of frying

Transesterification was further carried out with cottonseed oil used for frying and collected at different time interval. Reaction was carried out using 2 g biomass under standardized conditions.

It was observed that with increase in time of frying the extent of transesterification decreases from 84% (1 h) to 30% (7 h). However from 1st frying to 3rd frying, decrease was only 10% followed by sharp decrease in the yield from 4th (50.7% ethyl ester) frying to 7th (30% ethyl ester) frying (Fig 6).

This observation was further supported by the studies of Mittelbach and Enzelsberger (1999)24; Mittelbach et al. (2000)25 and our earlier observations30, suggesting that oxidative, thermolytic and hydrolytic reactions occurring during frying process result in the formation of many potentially inhibitory unknown/unidentified compounds that affect the activity of whole cell enzymes. The observations on FFA generation due to frying indicated increase in FFA content from 0.2% to 8.3% with increase in frying time from 10 min to 7 h, during biocatalyzed transesterification, which is reversible in nature, the triglycerides and partial glycerides are first hydrolyzed to partial glycerides and FFA respectively, followed by generation of ethyl esters due to interaction between FFA and ethanol30. The yield of ethyl esters was consistently >98% with oil collected till 3 h frying, beyond which it reduced to 51% with 7 h frying oil. The reduced EE yield is presumably due to factors other than FFA influencing the transesterification reaction with used frying oil20.

3.7 Ethyl ester yield in presence of non-edible oils

Cottonseed oil yielded maximum of 84% of ethyl ester followed by jatropha (75% ethyl ester) and karanj oil (78.2% ethyl ester) when transesterified using dried biomass under standardized conditions identified with cottonseed oil (Fig 7).

We earlier reported the whole cell catalysis of jatropha and karanj oils to ethyl esters in solvent free system wherein complete conversion was obtained using active culture of A. flavus as whole cell catalyst from jatropha and karanj oils19. However, in the present study, the ethyl ester yields from non-edible oils (jatropha and karanj oils) were observably lesser than earlier findings. This is presumably due to generation of high saturated free fatty acids in the these oils during hydrolysis and simultaneous increase in the viscosity of the reaction mixture resulting in subsequent decrease in the homogenity of the reaction.
mixture and leading to the reduction of the ester yield. To best of our knowledge, there is no report on the transesterification of jatropha and karanj oil using dried biomass of Aspergillus sp. Jin et al. (2009) examined the use of a whole-cell biocatalyst, derived from R. oryzae (ATCC10260), to transesterify triglycerides, including high-FFA containing waste greases, in a water-containing system. Results showed that the biocatalyst was able to produce alkyl esters with a yield of about 75% for virgin canola oil, 80% for waste vegetable oil (0.6%-3.7% FFA) and 55% for brown grease (80% FFA) with a 72 h transesterification reaction using methanol as acyl acceptor. The reduced yield of methyl esters as observed by Jin et al. (2009) was presumed to be due to the use of methanol as reactant. Comparatively, the present study demonstrated 83, 75 and 78.2% of ethyl ester yield with ethanol as acyl acceptor using cottonseed oil, jatropha and karanj oil, in 36 h of reaction time, using dried biomass.

3.8 Repeatability of the transesterification reaction using dried biomass

The experiments carried out with 2.0 g dried biomass also indicated reproducibility in percent yield of ethyl ester [81.5% (±2.6)] under optimized conditions at various steps of the study. It was observed that under optimized condition of cell bound lipase production, the biomass produced showed nearly similar results of transesterification repeatedly.

4 Conclusion

Dried biomass of Aspergillus sp. resulted in maximum of 84% of ethyl ester, using 20% biomass, at 35°C, with stepwise addition of ethanol at an interval of 8 h, total reaction time of 36 h. With increase in moisture content in the reaction mixture decreases the extent of transesterification using dried biomass. Transesterification of frying oil collected at different time interval showed that with increase in time of frying, the extent of transesterification decreased due the formation of some unknown compounds during frying. Aspergillus sp. was proved to be very good catalyst for the biodiesel, however for industrialization the process of transesterification was need to be optimized.

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