RESEARCH ARTICLE

BIOLOGICAL TRANSESTERIFICATION OF POULTRY WASTE TO BIODIESEL USING BACTERIA ISOLATED FROM CHICKEN FEATHER

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ABSTRACT

With increase in consumption of poultry products, the waste products have been increasing in the poultry market. Disposing this waste has been a challenging task for the industry. As feathers contain 2-12% of fat, it can be used for producing biodiesel. Hence, using feathers as a source of biodiesel is also a solution to waste disposal. Lipase mediated Transesterification process for the production of biodiesel has been developed to overcome the chemical catalysts having several negative impacts on environment and downstream processing of by-products. This study aimed at, isolating bacteria from chicken feathers, extracting extracellular lipase and determining the efficiency in transesterifying the chicken fat from feathers into biodiesel. The data resulted from gas chromatography (GC) revealed the methyl esters of palmitic (C16:0), steric (C18:0), oleic (C18:1) and linoleic (C18:2) acids.

INTRODUCTION

Globally, the consumption of chicken meat has been continuously increasing. America, Asia and Europe have an approximate share of 38%, 37% and 18% respectively (Smutka et al., 2012). Forecast stated, India’s per capita consumption of poultry meat would be 2.2 kg per annum in 2014. This accounted for 16% rise since 2010, which also suggests a steady rise in consumption of poultry meat in India as per USDA International Egg and Poultry in India. Increase in consumption of chicken, increased feather waste production portraying a challenge to poultry industries to dispose waste produced. This waste can be used as source of raw material for producing biodiesel. The live bird weight comprises of feathers about 7-10%. These feathers contain 75-90% crude protein and 2-12% of fat (Kondamudi et al., 2009).

Biodiesel can be produced from the extracted fat of chicken feathers by a process known as Transesterification. Most industries manufacturing biodiesel today, make use of chemical catalysts as they provide higher conversion rate of esters under low temperature, pressure conditions and also have short reaction time. However, the major drawback is the inability to obtain pure products and by-products using chemical catalysts thereby increasing economic investments into downstream processing (Lene Fjerbaek et al., 2008; Kondamudi et al., 2009; Kumar et al., 2013). Biodiesel production using enzyme catalysts can be followed as it has several benefits including no soap formation, esterification of both free fatty acids and triglycerides in one step without need for washing, provision for higher quality glycerol, ability to handle large variation in quality of raw material and ability to work under milder conditions (Ghaly et al., 2010).

MATERIALS AND METHODS

Chemicals

Media and chemicals for isolation and lipase assay like tributyrin, teen 20, ammonium sulphate, gum acacia, NaOH were of high grades and procured from Sigma and Hi-Media Laboratories, India.

Sample Collection

For the present study, chicken feather samples were collected from slaughter houses in Yeshwanthpur and Bone mill, Hesaraghatta main road, Bangalore, India, in plastic bags for the isolation of lipase producing organisms and for the extraction of fat.

Isolation and screening of Lipase producing bacteria

For isolation of the lipase producing microorganisms, chicken feathers shredded into pieces and were soaked in sterilized water. It was then serially diluted and plated on Tween-20 media and observed for Zone of precipitation (Kumar et al., 2012). Colony showing zone of precipitation on tween 20 media was selected and subjected on to sterile tributyrin agar media containing 10gms/l Tributyrin, 10gms/l Tryptone, 5gms/l NaCl, 5gms/l yeast extract and 17gms/l agar and incubated at 37°C and checked for zone of hydrolysis after 24 hr and 48 hr. Pure culture of these isolate was obtained by repeated streaking and maintained on nutrient agar slants (Kumar et al., 2012; Prasad and Manjunath, 2012).

Identification of micro-organism

The isolate used for the study were selected on the basis of clear zone of hydrolysis on tributyr in agar media (TBA) and identified on the basis of biochemical characteristics according to Bergey’s manual of determinative bacteriology.

Extraction and of partial purification of Lipase

Lipase was extracted from the production medium using coconut oil as the substrate, Olive oil 5%, peptone 5g/ml, yeast extract 5g/ml, glucose 5g/ml, NaCl 3gm/ml and...
Extracellular lipase was extracted after an incubation time of 48 hrs, by centrifugation at 10000 x g for 30 min (Kumari et al., 2009). Partial purification done by adding 60-90% three ammonium sulphate fractions of 0-30%, 30-60% and in small increments at 4°C by constant stirring. Solution allowed to stand for 30 minutes and centrifuged at 10,000X for 30 minutes. Pellet resuspended in small volume of 0.05M phosphate buffer of pH 7.0 (Kumar et al., 2012).

**Lipase Assay**

The lipase activity was carried out by titrimetric method as per Kemper et al (2008). 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 sodium hydroxide using phenolphthalein indicator. End point is determined by change in colour of the mixture to light pink.

Lipase activity was calculated using the formula

\[
\text{Lipase Activity} = \frac{4V\cdot N\cdot 1000}{V_1(V_{\text{sample}}\cdot 60)}
\]

Unit of Activity is 1U/ml

\[
V = V_2 - V_1
\]

\[
V_1 = \text{Volume of Sodium Hydroxide against control flask (ml)}
\]

\[
V_2 = \text{Volume of Sodium Hydroxide experimental flask (ml)}
\]

\[
N = \text{Normality of Sodium Hydroxide}
\]

\[
V (\text{Sample}) = \text{Volume of Enzyme extract (supernatant) (ml)}
\]

**Extraction of Fat**

15gms of chicken feathers used for extraction of fat using hexane at 69°C for 8 hours in Soxhlet extractor. The remaining hexane solvent after extraction was evaporated and fat extracted (Kondamudi et al., 2009).

Yield percent = \[
\frac{\text{Amount of fat Extracted (gm)}\times 100}{\text{Amount of feathers used (gm)}}
\]

**Transesterification process**

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 0.2ml for 0.5ml of the extract (Hsu et al., 2001). Methanol was taken in the ratio of 1:4 with respect to oil obtained and incubated in shaker at 40°C for 24 hours (Sinnart Chongkhong et al., 2012; Kumar et al., 2013). After transesterification reaction, the mixture was kept in separating flask for 3hours for phase separation. Two distinct layers seen with, bottom layer consisting of glycerol and top layer consisting of biodiesel. The glycerin layer was separated and the ester layer was washed with distilledwater and sent for Gas Chromatographic analysis (Ertan alptekin et al., 2011).

**Gas Chromatographic analysis**

Methyl esters were analyzed by using Agilent 6890 N Gas Chromatograph with a flame-ionization detector (USA). The capillary column made of DB Wax (length 30 m, internal diameter 0.25 mm) with nitrogen as the carrier gas was used. Column oven temperature was programmed from 100(±5) °C to 240 °C (at 10 °C min⁻¹) with injector and detector temperatures at 250 and 280°C, respectively. The injection volume used was 2µl. The % molar conversion of products was identified by comparing the peak area of standard methyl oleate at the particular retention time.

**RESULTS**

**Isolation and Screening of Lipase Producing Bacteria**

When the dilution was plated onto Tween-20 agar plate, about 18 colonies were observed to have formed. Certain colonies exhibited ‘Zone of Precipitation’ the colony exhibiting maximum zone of precipitation on Tween-20 agar media was subjected to qualitative screening by streaking onto TBA for lipolytic strains, zone of hydrolysis was observed. This confirmed that the organism selected was lipase producing.

**Identification of Microorganism by Biochemical Characterization**

By biochemical characterization, the organism was identified and belonged to Pseudomonas species. When Gram’s staining was conducted, the bacteria were found to be Gram negative rods. When freshly prepared 1% N, N', N'- tetramethyl-p- phenylenediamine dihydrochloride reagent was added onto the culture plates, blue colour appeared within 10sec and it indicated that the culture were oxidize positive. With the conduction of Glucose fermentation test it was observed that the organisms were glucose negative because there was no acid production. Hence, there was no change in colour from red to yellow. Bubble formation was not observed in the Durham’s tube indicating the bacteria to be anaerobic in nature. From above results this it was concluded that the organisms belong to Pseudomonas species.

**Extraction and Partial Purification of Lipase**

The Pseudomonas was grown in production media using coconut oil as substrate. The broth wascentrifuged and the supernatant which contained extracellular lipase were isolated. Partial purification by precipitation using ammonium sulphate is the most common methodused for extraction of proteins. It is based on the principle of salting in and salting out. The amount of pellet formed was found to have precipitated maximum for 30-60% concentration of ammonium sulphate. Precipitation was also found to be maximum for the same percentage of ammonium sulphate concentration when compared to 0-30% and 60-90% in the study conducted.

**Lipase Activity Assay**

The activity of the partially purified extract was calculated using the specified formula. The activity was found to be 4U/ml. Olive oil was used as substrate for the assay. 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.

**Extraction of Fat**

Fat from chicken feathers were extracted using hexane as solvent. The process was carried out for 8 hours in Soxhlet extractor. For 10gms of chicken feathers 0.245gms of fat was obtained.

**Transesterification and GC analysis**
The transesterified product was a clear yellow fluid which was less viscous than the extracted fat. Gas Chromatographic Analysis of the biodiesel obtained showed a combination of different types of Methyl Esters as shown in the Table 1 and figure 1. On analysing individual peaks, it was identified that the fatty acid methyl esters in the biodiesel from feathers ranging from C16 to C18. Major components identified were methyl esters of palmitic (C16:0), steric (C18:0), oleic (C18:1) and linoleic (C18:2) acids. The fatty acid profile of biodiesel obtained by enzymatic transesterification shows greater amounts of oleic acid (77.31%) when compared to the amount of oleic acid by chemical transesterification (46%) (Kondamudi et al., 2009). Oleic acid, being a long chain fatty acid (C18) and having one unsaturated bond is an important component to categorize a biodiesel as a good as it not only provides high cetane index and combustion temperature to the biodiesel but also are less viscous and show higher pour and cloud points properties making the biodiesel suitable for warm and cold weather conditions.

<table>
<thead>
<tr>
<th>Table 1 Composition of Methyl esters of transesterified fat</th>
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<tr>
<td><strong>PARAMETERS</strong></td>
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<tr>
<td>Description</td>
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<tr>
<td>Fatty acid composition</td>
</tr>
<tr>
<td>Methy ester of</td>
</tr>
<tr>
<td>1  Capric acid</td>
</tr>
<tr>
<td>2  Caprylic acid</td>
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<tr>
<td>3  Caprylic acid</td>
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<tr>
<td>4  Launio acid</td>
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<tr>
<td>5  Myristic acid</td>
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<tr>
<td>6  Palmitic acid</td>
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<tr>
<td>7  Stearic acid</td>
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<tr>
<td>8  Oleic acid</td>
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<tr>
<td>9  Linoleic acid</td>
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<tr>
<td>10 Limolenic acid</td>
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<tr>
<td>11 Arachidionic acid</td>
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<tr>
<td>12 Behenic acid</td>
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<tr>
<td>13 Erucic acid</td>
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<tr>
<td>14 Linolecine acid</td>
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<td>15 Recenoic acid</td>
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Figure 1 Graph of GC analysis of Methyl Esters of Fatty Acids present in the biodiesel produced from chicken feather

**DISCUSSION**

Several studies related to production of biodiesel from chicken fat using chemical catalysts have largely aimed at optimizing steps to reduce the free fatty acids (FFA) percent in the feed stock. With reduction in the FFAs soap formation and involve lesser purification processes when compared to their isolation from other sources like plant and animal sources and hence are commonly used in bulk production (Ghaly et al., 2010; Yokel et al., 2012). Although lipases from different sources are able to catalyze the same reaction, bacterial and fungal lipases are mostly used in biodiesel production such as Aspergillus Niger, Candida Antarctica, Candida rugas, Chromo bacterium Viscous, Macro mashie, Pseudomonas cetacean, Pseudomonas fluorescents, Photo bacterium lipolyticum, Rhizopus oryzae, Streptonycin sp., and There mimics lanuginose (Yahiya et al., 1998).

It can be concluded that enzymatic method of transesterification from lipase extracted from pseudomonas bacterial isolates from feathers are comparatively more efficient than chemical methods along with other advantages of enzymatic transesterification which include non-requirement of multi-step purification of end products, reduction in processes required for waste water treatment and optimal energy requirements for carrying out the process.

**ACKNOWLEDGEMENT**

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References

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