Fuel 146 (2015) 13–19

Contents lists available at ScienceDirect

Fuel

journal homepage: www.elsevier.com/locate/fuel

# Additives improve the enzymatic synthesis of biodiesel from waste oil in a solvent free system

Kaili Nie [a,c], Meng Wang [a], Xin Zhang [a], Wenjing Hu [d], Luo Liu [a], Fang Wang [a,b], Li Deng [a,c,*], Tianwei Tan [a]

[a] Beijing Bioprocess Key Laboratory, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, PR China
[b] State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing 100029, PR China
[c] Amoy-BUCT Industrial Bio-Technovation Institute, Amoy 361022, PR China
[d] Beijing Sunpu Biochemical and Technology Co. Ltd., Beijing 100176, PR China

## HIGHLIGHTS

- Biodiesel was synthesized in solvent free system with waste cooking oil.
- Cyclodextrin was introduced as additive to enhance the biodiesel yield.
- Only 0.4 wt% lipase *Candida* sp. 99–125 was enough for 1 ton biodiesel production.
- Lipase could also be reused for 5 times.
- By the assistant function of additive, the cost of lipase was reduced to 80 CNY.

## ARTICLE INFO

*Article history:*
Received 15 October 2014
Received in revised form 10 December 2014
Accepted 22 December 2014
Available online 5 January 2015

*Keywords:*
Biodiesel
Free lipase
Cyclodextrin
*Candida* sp. 99–125
Methanolysis
Industrialization

## ABSTRACT

Biodiesel production from waste oil by lipase *Candida* sp. 99–125 assisted with cyclodextrin as additive was investigated in a solvent free system. The optimum reaction conditions was determined toward lipase dosage, agitation speed, cyclodextrin loading, water content and reuse ability of free lipase. A certain dosage of cyclodextrin showed a significant improvement of the reactions. However, the agitation speed should not be too high, to avoid breaking the weak bond between cyclodextrin and lipase. The optimal conditions were: free lipase dosage 70 U/g oil (about 0.4 wt% to the oil weight, when activity of lipase was 20,000 IU), cyclodextrin to lipase 2:1(g/g), water content 2 wt% (water/oil, g/g), and agitation speed 180 rpm. Methanol was added 30 times at 1/30 M equivalent each hour. Under the optimal conditions, the yield of FAMEs achieved 88%. After 5 cycles of reuse, no significant decrease in FAMEs yield was observed. The process has been successfully carried out at industrial scale in Shanghai, China.



## 1. Background

To subshrink fossil fuels, biodiesel is considered as a potential replacement [1–3]. The common method to transform oil into biodiesel is transesterification. Transesterification involves the reaction of triglyceride or free fatty acids (FFAs), and an acyl-acceptor. Methanol is commonly used as an acyl-acceptor because of its low cost compared with other alcohols. Biodiesel is hence commonly referred to as fatty acid methyl esters (FAMEs). The biodiesel production can be carried out by different catalytic processes [4,5] or under supercritical conditions [6,7]. The catalysts used may be classified as chemical catalysts and enzymes. Short reaction time and high yields are the advantages of chemical catalysis process; while high energy consumption, difficulties in the recovery of the catalyst and glycerol, and potential pollution of the environment are major disadvantages in chemical processes [8]. Recently, lipase-catalyzed biodiesel production has become more attractive, since the process can be carried out under mild conditions and is environmentally friendly [9–16]. *Candida* sp. 99–125 was immobilized on silk fibers in our lab [17,18], and was successfully used in enzymatic catalysis for biodiesel production [8,19–23]. However, the cost of immobilization limited its industrial implementation.

It was found that the catalytic activity of enzyme, such as α-chymotrypsin and subtilisins, in organic solvents significantly

*Abbreviations:* FFAs, free fatty acids; FAMEs, fatty acid methyl esters; CD, cyclodextrin; AV, acid value.

* Corresponding author at: Beijing Bioprocess Key Laboratory, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, PR China.
*E-mail address:* dengli@mail.buct.edu.cn (L. Deng).

http://dx.doi.org/10.1016/j.fuel.2014.12.076
0016-2361/

increased by lyophilization of the enzymes from aqueous solutions in the presence of cyclodextrins (CDs) [24–27]. It was assumed that enzyme/CD complexes were formed on co-lyophilization, probably via hydrogen bonding or hydrophilic interactions, which stabilized the enzyme structure [24]. The present paper aimed at the enzymatic production of biodiesel from waste oil with free *Candida* sp. 99–125 lipase as catalyst assisted with CD, achieving the reuse of lipase without immobilization, to reduce the process costs. Serial production conditions, such as dosage of lipase, agitation speed, amount of CD and water, were optimized to improve the prospects for further industrial operation.

## 2. Material and methods

### 2.1. Materials

Waste oil was obtained from Luming Co. Ltd. Shanghai, China. It has a high acid value at 166.6 mg KOH/g, and an average molecular weight of 350 g/mol. Table 1 showed the free fatty acid composition of the waste oil. Free lipase from *Candida* sp. 99–125 was obtained from Beijing CTA New Century Biotechnology Co. Ltd. (Beijing, China); the activity of the enzyme powder was 20,000 U/g. Methanol, with a purity of 99% was purchased from Yili Chemical Co. Ltd. (Beijing, China). CD were purchased from Aoboxing Biochemical Technology Co. Ltd. (Beijing, China).

### 2.2. Methods

#### 2.2.1. Enzyme activity assay

The hydrolytic activity of lipase was measured by titrimetric assay according to a modified olive oil emulsion method [28]. Olive oil [5%, (v/v)] was emulsified in distilled water containing 2% (w/v) of PVA in a homogenizer for 6 min at the maximum speed. The assay mixture consisted of emulsion (5 ml), phosphate buffer (4 ml, 100 mmol $l^{-1}$, pH 8.0) and lipase (1 ml, concentrated or diluted, depending on the quantity of lipase). The oil hydrolysis was incubated at 35 °C for 10 min with agitation. The reaction was stopped by adding 20 ml ethanol. The enzyme activity was determined by titration of the fatty acid released using 50 mmol $l^{-1}$ NaOH. One activity unit of lipase was defined as the amount of enzyme that released 1 mmol of fatty acid per minute under assay conditions.

#### 2.2.2. Methanolysis of waste oil

Typical methanolysis was carried out in a 1 l triple-neck flask with constant agitation at 40 °C. A condenser was used to collect the vapors methanol flow and recycle it into the reactor. If not mentioned, the reaction system contains 600 g waste oil, 2.4 g free lipase, 2.4 g additives. 4.6 g methanol was added every 2 h, with 30 h reaction time in total. For analysis, 0.5 ml sample was withdrew for acid value determination every hour. For gas chromatography analysis, another 200 μl sample was taken and centrifuged to harvest the supernatant or upper layer. Then 10 μl of the supernatant was dissolved in *n*-hexane for gas chromatography analysis. All the experiments were replicated at least three times and the results presented are the mean values for the replicated data.

#### 2.2.3. Acid value (AV) test method

Transfer the prescribed amount of reaction liquids, weighed to the nearest 0.001 g microgram level into a 250 ml Erlenmeyer flask. 50 ml 95% ethanol was added and shaken to dissolve; possibly by gentle heating. The solution was titrated with a standard KOH solution (0.1 mol $l^{-1}$) using 3 to 4 drops of thymol blue as indicator. Dark colored samples may require additional indicator to be added to the solution. Thymol blue titrates to a blue-green color. The acid value of the sample, expressed as milligrams of KOH per gram of sample was calculated, as follows:

$$\text{Acid number} = (A \times N \times 56.11)/B$$

where $A$ = alkali solution required for titration of the specimen, ml; $N$ = normality of the alkali solution; $B$ = specimen weight, g.

#### 2.2.4. Gas chromatography (GC) analysis

The methyl ester and fatty acid contents in the reaction mixture were quantified using a GC-2010 gas chromatography (Shimadzu Japan) equipped with a DB-1ht capillary column (30 m × 0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was set at 100 °C, then heated to 180 °C at 15 °C/min, followed by a slower heating rate of 10 °C/min to 230 °C, and finally to 330 °C at a heating rate of 20 °C/min, The column was maintained at 330 °C for 5 min. A programmable Temperature Vaporization (PTV) injector was used in the analysis. It was operated with an initial temperature of 280 °C, after injection the temperature ramped to 370 °C at a heating rate of 300 °C/min. The injector was held at the final temperature for 20 min. The temperature of the detector was set at 370 °C [20]. Heptadecanoic acid methyl ester purchased from Sigma was used as the internal standard.

#### 2.2.5. Circular dichroism measurement

Circular dichroism spectra were measured in 25 mM Tris–HCl buffer (pH 8.0) on a JASCO-810 spectropolarimeter (JASCO, Japan) between 180 nm and 250 nm at various temperatures. The protein concentration was 0.2 mg/ml. The secondary structure element content was estimated using the DICHROWEB application package based on the SELCON3 algorithm described by Sreerama et al. [29,30].

## 3. Results and discussion

### 3.1. Additive accessorial effect

A comparative study was carried out for free lipase catalyzed biodiesel production with and without the CD additive (Fig. 1). The initial acid value (AV) of the waste oil was as high as 166.6 mg KOH/g, the AV of reaction mixture could reflect the substrate level. Therefore, the AV was used to monitor the reaction progress. Free lipase was more sensitive to the toxicity of short chain alcohol than immobilized lipase. Without additive, the FAMEs yield could reach 68% only by using large amount free lipase (enzyme dosage was 1000 U/g waste oil). It indicated that the free lipase without additive was rapidly denatured by methanol. However, when using CD as an additive, and even at low enzyme of 80 U/g waste oil, the AV of the reaction mixture was

**Table 1**
Composition of the waste oil.

| Free fatty acids (FFAs) | | | | | | | | | Monoglycerides (MAGs) | Diglycerides (DAGs) | Triglycerides (TAGs) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C14:0 | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | C18:3 | C20:0 | C22:1 | | | |
| 1.1% | 20.9% | 1.6% | 6.2% | 32.5% | 32.9% | 3.3% | 0.9% | 0.6% | | | |
| | | | | 83.9% | | | | | 0.5% | 6.9% | 8.7% |