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Production of Biodesiel from Animal Tallow via Enzymatic Transesterification using the Enzyme Catalyst Ns88001 with Methanol in a Solvent-Free System

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Abstract

The effectiveness of enzymatic transesterification of animal fat using the experimental enzyme catalyst NS88001 with no solvent was studied. The effects of oil:alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), reaction temperature (35, 40, 45 and 50°C) and reaction time (4, 8, 12 and 16 h) on the biodiesel yield were evaluated. The highest biodiesel yield was obtained at the 1:4 molar ratios. No reactions were observed with the 1:1 and 1:2 (oil:alcohol) molar ratios and increasing the oil:alcohol molar ratio above 1:4 decreased the biodiesel conversion yield. The rate of conversion of fatty acid esters increased with increases in the reaction time. The reaction proceeded slowly at the beginning and then increased rapidly due to the initial mixing and dispersion of alcohol into the oil substrate and activation of enzyme. After dispersion of alcohol, the enzyme rapidly interacted with fatty acids esters giving a maximum conversion yield. Increasing the reaction time from 4 to 16 h increased the conversion yield of biodiesel by 114.95-65.59%. The interactions between enzyme polymer surface and substrate appears to be dependent on reaction temperature due to hydrogen bonding and ionic interactions which play an important role in maintaining the thermostability of lipase in the system. The optimum reaction temperature for the experimental enzyme catalyst (NS88001) in the solvent free system was 45°C. Increasing the reaction temperature from 40 to 45°C increased the biodiesel conversion yield while higher temperatures above 45°C denatured the specific structure of enzymes and resulted in decreased methyl esters formation. The activity of experimental enzyme catalyst NS88001 in the presence of methanol without solvent at the optimum conditions (a reaction temperature of 45°C, an oil:alcohol molar ratio of 1:4 and a reaction time of 16 h) remind relatively constant for 10 cycles and then decreased gradually reaching zero after 50 cycles.

Keywords: Animal tallow; Transesterification; Enzyme NS88001; Biodiesel; Temperature; Time; Oil:alcohol molar ratio; Solvent-free system

Introduction

The high demand for fossil fuels, their limited and insecure supply and their environmental impact prompted the search for alternative renewable fuel sources such as biodiesel from biomass materials [1-3]. Biodiesel is a renewable energy source that can be used as a fuel in compression-ignition engines instead of diesel [4,5]. It is sulfur free, non-toxic and biodegradable [6]. These characteristics make it more greener and eco-friendly than diesel [7-11].

Biodiesel can be produced from many raw materials including plant oils (jatropha, canola, coconut, cottonseed, groundnut, karanj, olive, palm, peanut, rapeseed, safflower, soybean and sunflower), animal fats (beef tallow, chicken fat, lamb fat, pig lard, yellow grease, waste cooking oil and the greasy by-product from omega-3 fatty acid production) and algae biomass [4,8,11-14]. The main component of fats and oils are triacylglycerols (triglycerides) which are made of different types of fatty acids with one glycerol (glycerine) being the backbone. The types of fatty acids present in the triglycerides determine the fatty acids profile. Fatty acid profiles from plants and animal sources are different and each fatty acid has its own chemical and physical properties which can be a major factor influencing the properties of biodiesel.

Transesterification is a chemical process used to convert oils and fats to biodiesel. A short chain alcohol is used with the feedstock to convert it to methyl esters and glycerin. The process is achieved with one of three catalysts: acid, alkali or enzyme. With an acid catalyst, the proton is donated to the carbonyl group which makes it more reactive while with a base catalyst, the proton is removed from alcohol which makes the reactants more reactive [15]. Base catalysts are widely in use by the biodiesel industry. However, both acid and alkali methods require more energy and a downstream processing step for removing the by-product glycerin. An enzymatic catalyst cleaves the backbone of the glycerol which makes the reactants more reactive, thereby giving the product without the need for a costly downstream processing step.

Objectives

The aim of this study was to investigate the effectiveness of the enzymatic transesterification process for the production of biodiesel from animal tallow using methanol in a solvent free system. The specific objectives were: (a) to study the effectiveness of experimental enzyme catalyst (NS88001) with methanol in a solvent-free system, (b) to study the effects of alcohol feedstock ratio (1:1, 1:2, 1:3, 1:4 and 1:5), reaction temperature (35, 40, 45 and 50°C) and reaction time (4, 8, 12 and 16 h) on the biodiesel yield and (c) to evaluate the usability of the enzyme.

Materials and Methods

Animal tallow

The animal rendering waste used in the study was obtained as beef tallow from the Company S.F Rendering, Centreville, Nova Scotia. Samples (10 Kg) were collected and stored at - 20°C in the Biotechnology Laboratory of Dalhousie University, Halifax, Nova Scotia. The sample material was yellowish in colour.

Chemicals and enzymes

The immobilized Lipase was an experimental enzyme catalyst (NS88001) obtained from Novozyme (Franklinton, North Carolina, USA). The chemicals used in the study included: methanol, tertrahydrofuran, N, O-Bis (Trimethylsilyl)-trifluroacetamide (BSTFA)

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and hilditch reagent. They were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The FAME standards, which included methyl myristate, methyl pentadecanote, methyl cis-11-eicosenoate, methyl all-cis-5,8,11,14,17- eicosapentaenoate (EPA), methyl erucate, methyl all-cis-7,10,13,16,19-docosapentaenoate (DPA) and methyl all-cis-4,7,10,13,16,19-docosahexenoate (DHA), were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The other FAME standard, which included methyl palmitate, methyl palmitoleate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate, were purchased from Alltech Associates, Inc. (Deerfield, Illinois, USA). The FAME standard methyl-stearidonate was purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

Experimental procedure

Purification of crude animal tallow: The animal tallow was first heated to 110°C with constant stirring at 50 rpm in a round bottom flask for one hour. During the process of melting the fats, the top layer consisting of bubbles and impurities was discarded regularly. The extracted crude oil from animal tallow filtered four times using vacuum filtration with ultra-filter paper (Whatman No.40, Fisher Scientific, Toronto, Ontario, Canada). The oil percentage was calculated as follows

$$Percent\ Oil = \left(\frac{\text{Weight of oil}}{\text{Weight of Total fat}}\right) \times 100 \quad (1)$$

Enzymatic transesterification: The enzymatic transesterification of biodiesel was carried out in order to extract fatty acid methyl esters from the animal fats by the experimental enzyme catalyst NS88001 according to the procedure described in Figure 1. Five oil:alcohol molar ratios (1:1, 1:2, 1:3, 1:4 or 1:5), four reaction temperatures (35, 40, 45 or 50°C) and four reaction times (4, 8, 12 or 16 hours) were investigated. No solvent was used.

The homogenized oil (2.3 ml corresponding or 2 g of fat) was placed into a 50 ml conical flask and heated on a hot plate (PC-620, Corning, New York, New York, USA). The experimental enzyme catalyst NS88001 (25% of the oil weight or 0.5 g) was added to the flask. The appropriate amount of alcohol (Methanol) was added based on the oil:alcohol molar ratio (1:1, 1:2, 1:3, 1:4 or 1:5). The solution was mixed using a reciprocal shaking bath (2850 Series, Fisher Scientific, Toronto, Ontario, Canada) at 200 rpm. The desired temperature (35, 40, 45 or 50°C) was selected. After the desired reaction time was completed (4, 8, 12 or 16 hours), the enzyme was filtered by vacuum filtration as recommended by Nelson et al. [19] Samples (100 µl) were taken from the mixture and analyzed using a gas chromatography system (Hewlett Packard 5890 series II, Agilent, Mississauga, Ontario, Canada). The same procedure was repeated with all oil:alcohol molar ratios, reaction temperatures and reaction times.

Determination of Biodiesel Yield: The preparation steps for the gas chromatography analysis of the biodiesel samples are shown in Figure 2. A 100 µL aliquot was taken from the transesterification process at selected time intervals (4, 8, 12 and 16 hours) and flushed with nitrogen gas in a reciprocating water bath (280 series, Fisher Scientific, Toronto, Ontario, Canada) at 45°C. A 10 mg portion of the residue was dissolved in 100 µL of tertrahydrofuran and 200 µL of BSTFA. Then, the mixture was heated in a microprocessor-controlled water bath (280 series, Fisher Scientific, Toronto, Ontario, Canada) at 90-95°C for 15 minutes.

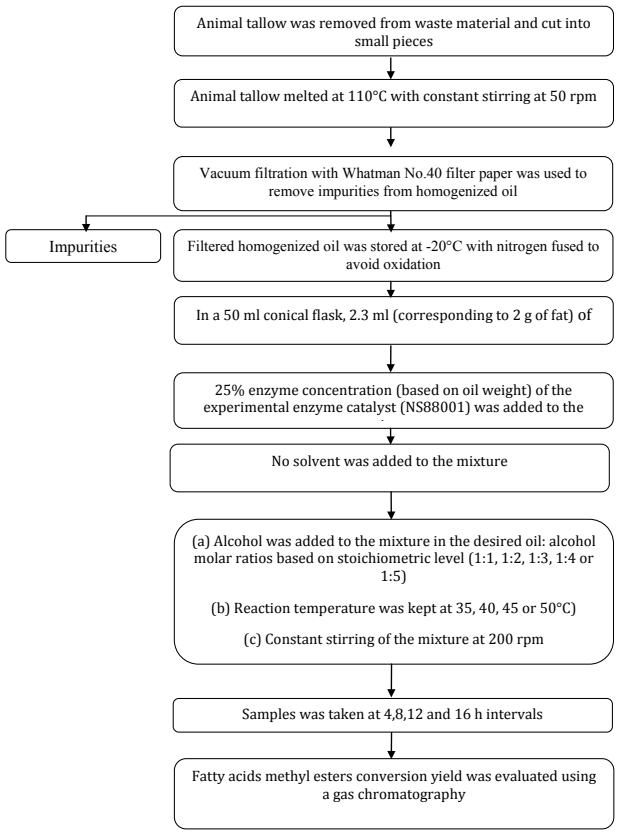

Figure 1: Enzymatic transesterification steps of animal tallow using the experimental enzyme catalyst NS88001 with methanol without solvent.

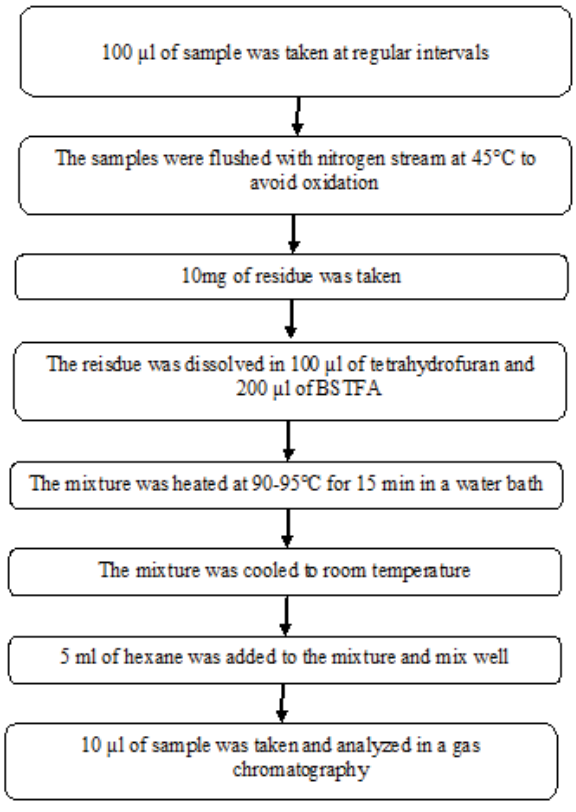

Figure 2: Biodiesel sample preparation procedure for Gas chromatography.

The sample was then cooled to room temperature for few minutes after which 5 mL of hexane was added. An aliquot of 1.5 mL mixture was transferred to the GC crimp vials and capped tightly for further analysis using GC.

An aliquot of 10 µL of the mixture was separated by fatty acid class (methyl ester, MAG, DAG and TAG) based on the carbon atom by a gas chromatography system, coupled with flame ionization detector (FID) (HP5890 Series II, Agilent Technologies, Mississauga, Ontario, Canada). An AT-FAME capillary column, 30 m in length, 0.32 mm of internal diameter and 0.25 µm film thicknesses, (Alltech Associates, Inc., Deerfield, Illinois, USA) was used for analyses. The column is a highly polar and stable bonded polyethylene glycol phase. The separated samples were injected directly into the column with the initial oven temperature of 60°C, followed by a flow rate of 20°C/min. A final temperature of 280°C was held for 10 minutes. The detection system was equipped with a flame ionization detector (FID) operating at 275°C with helium as a carrier gas at a flow rate of 0.6 mL/min. The total run time was 40 minutes.

$$\text{Conversion yield}(\text{wt }\%) = \frac{\textit{Peak area}\ \text{A} \times 100}{\sum(\text{Peak area A} + \text{Peak area B} + \ldots + \text{Peak area N})} \quad (2)$$

Statistical analyses: Statistical analyses were performed on the results using Minitab Statistics Software (Ver 16.2.2, Minitab Inc., State College, Pennsylvania, USA). Both analysis of variance (ANOVA) and Tukey's grouping were carried out.

Results

Characterization of animal tallow

Table 1 shows the composition of the animal tallow used in this study. The filtration process removed about 7.5 % of the total weight of tallow as impurities present in the animal fats. The homogenized oil was characterized by gas chromatography to identify and quantify the fatty acid composition of the tallow. Five fatty acids were identified in the animal tallow: oleic acids (44%), palmitic acids (28%), stearic acids (26%), linoleic acids (1%), and myristic acids (1%).

Enzymatic transesterification

Enzymatic transesterification by the experimental enzyme catalyst (NS88001) was carried out to investigate the effects of reaction time (4, 8, 12 and 16 h), oil:alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5) and reaction temperature (35, 40, 45 and 50°C) on biodiesel yield in a solvent free system. The results are shown in Table 2.

Table 3 shows the analysis of variance performed on the biodiesel yield data. The effects of oil:alcohol molar ratio, reaction time and reaction temperature were highly significant at the 0.001 level. All interactions (two, three and four way interactions) between the parameters were also highly significant at the 0.001 level.

Parameters	Value
Impurities (Kg)	0.375
Oil (%)	92.5
Impurities (%)	7.5
Fatty acids (wt%)	
Oleic acid	44
Palmitic acid	28
Stearic acid	26
Linoleic acid	1
Myristic acid	1

Tallow Sample Size =5 kg

Table 1: Composition of animal tallow.

The results obtained from Tukey's Grouping (Table 4) indicated that the three levels of oil:alcohol molar ratio (1:3, 1:4 and 1:5) were significantly different from one another at the 0.05 level. Two levels of alcohol: oil molar ratios (1:1 and 1:2) did not produce any biodiesel. The highest mean biodiesel yield of 80.42% was obtained with the 1:4 oil:alcohol molar ratio. The four reaction times (4, 8, 12 and 16 h) were significantly different from one another at the 0.05 level. The highest mean biodiesel yield of 49.00% was achieved with 16 hour reaction time. The three reaction temperatures (40, 45 and 50°C) were significantly different from each other at the 0.05 level. The highest mean biodiesel yield of 48.56% was obtained at the reaction temperature 45°C.

Effect of Oil:alcohol molar ratio

Figure 3 shows the effect of oil:alcohol molar ratio on the biodiesel yield using the experimental enzyme catalyst (NS88001) at different reaction temperatures and reaction times in a solvent free system. Generally, there was an increase in the biodiesel yield with increases in the oil:alcohol molar ratios from above 1:2 to 1:4 followed by decrease in the biodiesel yield with a further increase in the oil : alcohol ratios from 1:4 to 1:5 for all reaction times (4, 8, 12 and 16 h) and reaction temperatures (40, 45 and 50°C). No reaction was observed at 1:1 and 1:2 oil:alcohol molar ratios.

The biodiesel yield at the 4 h reaction time increased from 72.80 to 77.86% (6.95%), from 74.16 to 80.1% (8.00%) and from 38.4 to 58.4% (52.08%) with increases in the oil:alcohol molar ratios from 1:2 to 1:4 for the reaction temperatures of 40, 45 and 50°C, respectively. A further increase in the oil:alcohol from 1:4 to 1:5 decreased the biodiesel yield from 77.86 to 47.69% (-38.74%), from 80.1 to 59.74% (-25.41%) and from 58.4 to 32.6% (-44.17%) for the reaction temperatures of 40, 45 and 50°C, respectively. Similar trends were observed with the 8, 12 and 16 h reaction times at all reaction temperatures.

Effect of reaction time

Figure 4 shows the effect of reaction time on the biodiesel yield using the experimental enzyme catalyst (NS88001) at different reaction temperatures, oil:alcohol molar ratios and reaction times in the solvent free system. Generally, there was an initial rapid increase in the biodiesel conversion yield with increases in the reaction time during the first 4 hours followed by a slow gradual increase thereafter (between 4 and 16 h) for all reaction temperatures (40, 45 and 50°C) and the oil:alcohol molar ratios of 1:3, 1:4 and 1:5.

The biodiesel conversion yield at the 40°C reaction temperature and 4 h reaction time reached 72.80%, 77.86% and 47.69% for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reaction was observed at the 1:1 and 1:2 oil:alcohol molar ratio at the 40°C reaction temperature. Further increases in reaction time from 4 h to 16 h increased the biodiesel yield from 72.80 to 83.05% (14.95%), from 77.86 to 87.09% (11.85%) and from 47.69 to 78.97% (65.59%) the oil:alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed at the 45 and 50°C reaction temperatures for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5.

Effect of reaction temperature

Figure 5 shows the effect of reaction temperature on the biodiesel conversion yield using the experimental enzyme catalyst (NS88001) at different reaction times, reaction temperatures and oil:alcohol molar ratios in the solvent free system. There was an increase in biodiesel yield

Time Time (h)	Oil:alcohol Molar Ratio	Reaction Temperature (°C)		
		40	45	50
4	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	72.80 ± 1.46	74.16 ± 1.48	38.40 ± 1.77
	1:4	77.86 ± 1.56	80.10 ± 1.60	58.40 ± 1.17
	1:5	47.69 ± 0.95	59.74 ± 1.19	32.60 ± 0.65
8	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	77.58 ± 1.55	80.22 ± 1.60	46.60 ± 0.93
	1:4	82.46 ± 1.65	84.85 ± 1.70	67.90 ± 1.36
	1:5	75.05 ± 1.50	76.23 ± 1.52	40.10 ± 0.80
12	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	77.92 ± 1.56	82.10 ± 1.64	57.90 ± 1.16
	1:4	83.40 ± 1.67	87.67 ± 1.75	77.08 ± 1.54
	1:5	78.61 ± 1.57	78.97 ± 1.58	53.51 ± 1.07
16	1:1	Not extractable	Not extractable	Not extractable
	1:2	Not extractable	Not extractable	Not extractable
	1:3	83.05 ± 1.66	93.16 ± 1.86	62.81 ± 1.26
	1:4	87.09 ± 1.74	94.04 ± 1.88	84.19 ± 1.68
	1:5	78.97 ± 1.58	80.76 ± 1.62	70.98 ± 1.42

Table 2: Biodiesel yield (wt%) from animal tallow using 0.5 grams of experimental enzyme catalyst (NS88001) with methanol without hexane at different reaction times, oil : alcohol molar ratios and reaction temperatures.

Source	DF	SS	MS	F	P
Total	179	248498.3			
Model					
MR	4	227313.9	56828.5	57224.62	0.001
RTI	3	3989.0	1329.7	1338.95	0.001
RTE	2	6750.7	3375.3	3398.87	0.001
MR*RTI	12	3355.6	279.6	281.58	0.001
MR*RTE	8	5533.5	691.7	696.51	0.001
RTI*RTE	6	500.8	83.5	84.06	0.001
MR*RTI*RTE	24	935.5	39.0	39.25	0.001
Error	120	119.2	1.0		

DF: Degree of freedom; SS: Sum of square; MS: Mean of square; MR: Molar Ratios; RTI: Reaction Time; RTE: Reaction Temperature
R^2=99.95%

Table 3: ANOVA of biodiesel yield.

Factors	Level	N	Mean Yield (%)	Tukey Grouping
Oil:alcohol Molar Ratios	1:1	36	0.00	A
	1:2	36	0.00	A
	1:3	36	70.55	B
	1:4	36	80.42	C
	1:5	36	64.36	D
Reaction Time (h)	4	45	36.11	A
	8	45	42.06	B
	12	45	45.09	C
	16	45	49.00	D
Reaction Temperature (°C)	40	60	46.12	A
	45	60	48.56	B
	50	60	34.52	C

Groups with the same letter are not significantly different from one another at the 0.05 level.

Table 4: Tukey's grouping of the biodiesel yield.

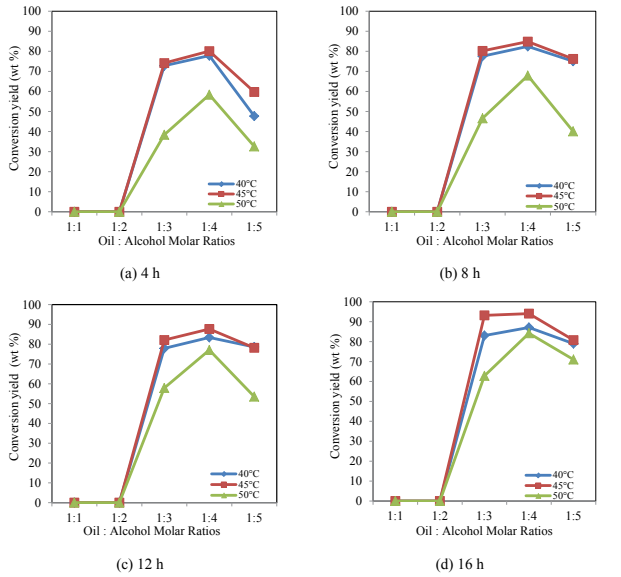

Figure 3: Effect of oil: alcohol molar ratio on the biodiesel conversion yield by the experimental enzyme catalyst (NS88001) at different reaction temperatures and reaction times without solvent.

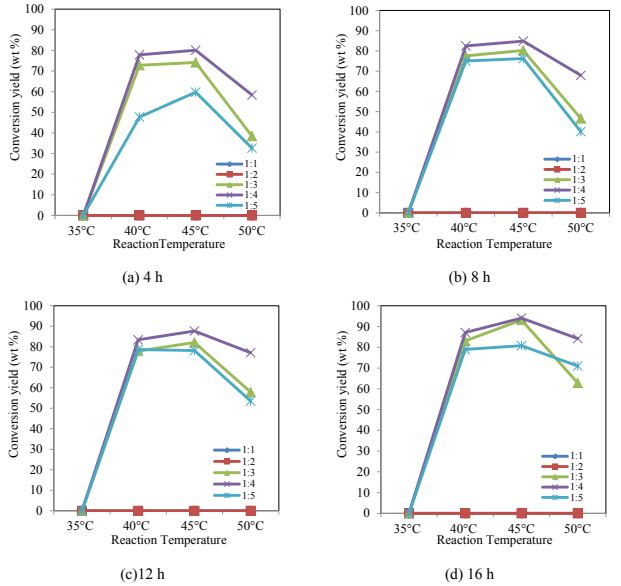

Figure 5: Effect of reaction temperature on the biodiesel conversion yield by the experimental enzyme catalyst (NS88001) at different oil: alcohol molar ratios and reaction times without solvent.

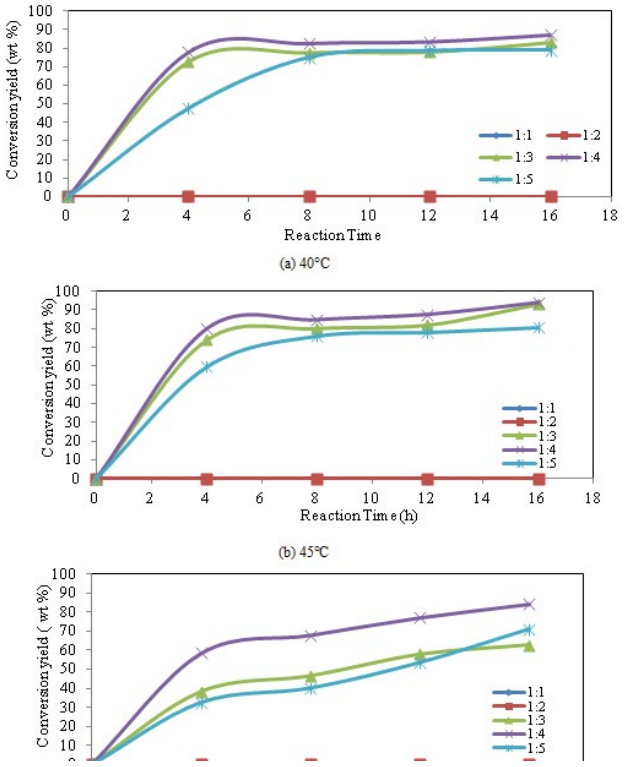

Figure 4: Effect of reaction time on the biodiesel conversion yield by the experimental enzyme catalyst (NS88001) at different reaction temperatures and oil: alcohol molar ratios without solvent (R= oil: alcohol molar ratios).

when the reaction temperature was increased from 40 to 45°C followed by a decrease in the biodiesel conversion yield when the reaction temperature was further increase from 45 to 50°C for all reaction times (4, 8, 12 and 16 hrs) and oil:alcohol molar ratios (1:1, 1:2, 1:3, 1:4 and 1:5).

The biodiesel yield at the 4 h reaction time increased from 72.80 to 74.16% (1.86%), from 77.86 to 80.10% (2.87%), from 47.69 to 59.74% (25.26%) for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reactions were observed with the 1:1 and 1:2 oil:alcohol molar ratios at the 4 h reaction time. A further increase in the reaction temperature from 45 to 50°C decreased the biodiesel yield from 74.16 to 38.40% (-48.22%), from 80.10 to 58.40% (-27.09%), from 59.74 to 32.60% (-45.76%) for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. Similar trends were observed with the 8, 12 and 16 h reaction times for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5. No reactions were observed with the 1:1 oil:alcohol molar ratio for the 8, 12 and 16 h reaction times.

Discussion

Extraction profiles of the raw material

After melting and homogenizing the animal tallow, the impurities (7.5%) were removed by filtration. The fatty acids analysis indicated that the homogenized oil contained high percentages of oleic acid (44%), palmitic acid (28%) and stearic acid (26%) as well as lower percentages of myristic acid (1%) and linoleic acid (1%). A high concentration of oleic acid improves the characteristics of biodiesel resulting in a high cetane index and combustion temperature [11]. Biodiesel produced from feedstocks containing a high level of oleic acid showed similar characteristics to these of conventional diesel [5,11]. Therefore, the biodiesel produced from oil extracted from animal tallow is expected to have good characteristics as a biofuel.

The extracted oil from animal tallow can be transformed to biodiesel by chemical or enzymatic transesterification. Watanabe et al. [16], Dorado et al. [17] and Kulkarni and Dalai [18] reported that oxidized oil can inhibit the chemical transesterification process and increase the oxidation of methyl esters. Kulkarni and Dalai [18] stated that an increase in the oxidation of methyl esters might increase the cetane number which tends to delay the ignition time in the engine. However,

Nelson et al. [19] and Watanabe et al. [16] reported that oxidation in crude tallow or oil containing high free fatty acids is a common problem and no negative effects of the oxidized oil substrate on the enzymatic transesterification process was observed. Kulkarni and Dalai [18] found that oxidized oil did not inhibit the formation of methyl esters from the methanolysis process by *Candida antarctica* lipase. Watanabe et al. [16] stated that in the enzymatic process, the oxidized substrate becomes a non-recognition site for the enzyme to bind and the process continues with the substrates which are not oxidized. The authors stated that using oxidized oil might reduce the biodiesel stability. Nelson et al. [19] reported that the stability of biodiesel can be increased by blending the biodiesel with conventional diesel especially in cold environment. In this study, enzymatic transesterification was carried out and no oxidation stability test was performed on crude tallow or oil nor was antioxidants used.

Effect of oil:alcohol molar ratios

Reports in the literature suggested that the theoretical stoichiometric oil:alcohol molar ratio of 1:3 is needed to complete the reaction in the following continuous steps: (a) conversion of triglycerides to diglycerides, (b) conversion of diglycerides to monoglycerides and (c) conversion of monoglycerides to methyl esters and glycerol [12,20,21]. In this study, increasing in the oil:alcohol molar ratio from 1:1 to 1:4 at the 4 h reaction time with no solvent increased the biodiesel yield by the experimental enzyme catalyst NS88001 by 6.95, 8.00 and 52.08% and when the oil:alcohol molar ratio was further increased from 1:4 to 1:5, the biodiesel yield was decreased by 38.74, 25.41 and 44.10% at the reaction temperatures of 40, 45 and 50°C, respectively. Similar trends were seen with all reaction times.

Kumari et al. [22] reported that the biodiesel yield increased when the oil:alcohol molar ratio was increased up to 1:4 and then decreased when the oil:alcohol molar ratio was further increased to 1:5. Chen et al. [23] reported that increasing the oil:alcohol molar ratio from 1:1 to 1:4 promoted the methanolysis reaction with waste cooking oil, but the formation of methyl esters decreased when the oil:alcohol molar ratios was increased from 1:4 to 1:5 due to an excess of methanol in the system. The decreases in the formation of methyl esters observed in these studies were similar to that observed in the present study.

The decrease in the conversion yield of methyl esters from oil at higher oil: alcohol molar ratios might be due to the presence of insoluble methanol in the reaction system which may have deactivated the experimental enzyme catalyst (NS88001). Tamalampudi et al. [24] suggested that the presence of soluble methanol would cause the active site on the surface of the lipase to be locked resulting in less access of enzyme to the surface of oil substrate. Dizge and Keskinler [25] reported that the use of excessive amount of methanol might deactivate the lipase in the reaction. Nelson et al. [19] and Bernardes et al. [26] stated that it is likely that once the maximum level of esters is formed, a further increase in number of moles of alcohol decreases the formation of methyl esters in the reaction due to enzyme inactivation. Chen et al. [23] reported that the excess methanol distorted the essential water layer needed to stabilize the structure of the enzyme. Chen and Wu [27] and Samukawa et al. [28] stated that short chain alcohols such as methanol are responsible for deactivation and inhibition of immobilized lipase. Salis et al. [29] and Al-zuhair et al. [30] reported that deactivation of enzyme occurred by the insoluble alcohol present in the reaction due to its tendency to be absorbed by the surface support matrix. They also indicated that exceeding the stoichiometric oil:alcohol molar ratio of 1: 3 ensures proper rate of reaction and leads to higher biodiesel yield but the excess amount of the alcohol might decrease the activity and distort the spatial confirmation of lipase structure and cause the lipase to deactivate.

In this study, the highest biodiesel conversion yield of 94.04% was achieved using the experimental enzyme catalyst 88001 at the 45°C reaction temperature with the 1:4 oil:alcohol molar ratio and the 16 h reaction time.

Effect of reaction time

When the reaction time was increased from 4 to 16 h at the 40°C reaction temperature, the increases in biodiesel conversion yield by the experimental enzyme catalyst NS88001 in the solvent free system were 14.95, 11.88 and 65.89% for the oil:alcohol molar ratios of 1:3, 1:4 and 1:5, respectively. No reactions were observed with the 1:1 and 1:2 oil:alcohol molar ratios. Several researchers observed similar trends from crude tallow, waste cooking oil and vegetable oil.

Nelson et al. [19] reported a maximum biodiesel yield of 83.8 % after 16 h with the 1:3 molar ratio using 25% concentration of the enzyme *Candida antarctica* (SP 435). Chen et al. [23] achieved a maximum biodiesel yield of 85.12% after 30 h with the 1:4 oil:alcohol molar ratio using 30% concentration of the immobilized enzyme *Rhizopus oryzae* and waste cooking oil as a substrate. Modi et al. [31] reported that a maximum biodiesel conversion yield of 93.4% was achieved after 8 h with the 1:4 oil:alcohol molar ratio using the enzyme *Candida antarctica* (Novozyme 435) with vegetable oil. A high biodiesel yield (94.04%) was obtained with the 16 h reaction time in this study, which indicates that NS88001 is non-regiospecific.

At the initial phase of the reaction, the enzymes, oil and alcohol appeared to be static and the reaction started when the stirring speed reached 200 rpm which increased the mass transfer between the substrate and enzyme catalyst. Formation of esters increased with increases in reaction time from 1 to 4 h. Several authors [20,32-35] reported that the rate of conversion of fatty acid esters increased with increases in reaction time and the reaction proceeds rapidly due to the initial mixing and dispersion of alcohol into the oil substrate and the activation of enzyme. After the alcohol is dispersed, it rapidly interacts with fatty acids giving a maximum conversion yield. Kose et al. [36] and Li et al. [37] reported that the initial reaction in a solvent-free system might take a longer period to activate the enzyme in the system. However, a further increase in the reaction time may decrease conversion yield due to the backward reaction of transesterification [23].

Effect of reaction temperature

In this study, when the reaction temperature was increased from 40 to 45°C at the 4 h reaction time for the experimental enzyme catalyst 88001 with no solvent, the increases in biodiesel yield were 1.86, 2.87 and 26.64 % and when the reaction temperature was further increased from 45 to 50°C, the biodiesel yield decreased by 48.22, 27.09 and 45.76% for the 1:3, 1:4 and 1:5 oil:alcohol molar ratios, respectively. No reactions were observed with the 1:1 and 1:2 oil:alcohol molar ratios at the 4 h reaction time. Similar tends were observed with other reaction times. Several researchers observed similar trends from crude tallow, waste cooking oil, canola oil and soybean oil.

Chen et al. [23] reported that the biodiesel yield increased (reaching a maximum of 87%) when the reaction temperature was increased from 30 to 40°C and then decreased when the reaction temperature was further increased from 40 to 70°C during conversion of waste cooking oil to methyl esters using Lipozyme RM IM. Dizge and Keskinler [25] reported that the biodiesel yield increased (reaching

a maximum of 85.8%) when the reaction temperature was increased from 30 to 40°C and then decreased when the reaction temperature was further increased from 40 to 70°C when converting canola oil to methyl esters using Lipozyme TL. Rodrigues et al. [38] reported that a maximum biodiesel yield of 53% was achieved at 35°C which then decreased with increases in reaction temperature above 35°C during conversion of soybean oil to methyl esters using Novozyme 435. Nie et al. [39] reported that a maximum biodiesel yield of 90% was obtained at 40°C and increasing the reaction temperature above 40°C decreased the biodiesel yield. In this study, the highest conversion yield (94.04) was obtained at 45°C which was higher than those reported in the literature.

Increasing the reaction temperature from 40 to 45°C reduced the viscosity of the oil and enhances the mass transfer between substrate and enzyme catalyst. Due to this effect, an increase in conversion yield of biodiesel was obtained. However, when the reaction temperature was further increased from 45 to 50°C the biodiesel yield decreased. A higher temperature may denature the specific structure of enzymes resulting in a decreased methyl esters formation. Denaturation of enzyme support matrix may also promote the enzyme leakage from the outer layer of the support matrix. However, the optimum reaction temperature is dependent on other parameters such as oil:alcohol molar ratio, enzyme activity, stability and type of system used. Reetz et al. [40], Kumari et al. [22] and Antczak et al. [14] reported that interactions between enzyme polymer surface and substrate appears to be dependent on reaction temperature due to hydrogen bonding and ionic interactions which play important roles in maintaining the thermostability of lipase in the system. Kose et al. [36] reported that increasing the reaction temperature over 50°C in a solvent free-system decreased the biodiesel yield of methyl esters due to inhibition of enzyme activity by higher temperature. Nie et al. [39] reported that higher temperature can give faster reaction but exceeding the optimum temperature may lead the enzyme denaturing.

Enzyme usability

In this study, the activity of experimental enzyme catalyst NS88001 in the presence of methanol without solvent at the optimum conditions (a reaction temperature of 45°C, an oil:alcohol molar ratio of 1:4 and a reaction time of 16 h) remind relatively constant for 10 cycles and then decreased gradually reaching zero after 50 cycles. Ghamgui et al. [41], Xu et al. [42], and Bernardes et al. [26] obtained similar results from immobilized Lipozyme *Thermomyces lanuginosus*, immobilized Lipozyme *Rhizomucor miehei* and immobilized *Rhizopus oryzae*. Several researchers [41,43,44] stated that repeated use of enzyme in the reaction without removing glycerol from the system might inhibit the interaction between the substrate and lipase.

Conclusions

The effectiveness of enzymatic transesterification of animal fat using the experimental enzyme catalyst NS88001 with no solvent was studied. The effects of oil:alcohol molar ratio (1:1, 1:2, 1:3, 1:4 and 1:5), reaction temperature (35, 40, 45 and 50°C) and reaction time (4, 8, 12 and 16 h) on the biodiesel yield were evaluated. The effects of oil:alcohol molar ratio, reaction time and reaction temperature on the biodiesel yield were highly significant at the 0.001 level. There were also significant interactions among the parameters at the 0.001 level. The highest biodiesel yield was obtained at the 1:4 molar ratio. No reactions were observed with the 1:1 and 1:2 oil:alcohol ratios and increasing the oil:alcohol molar ratio above 1:4 decreased the biodiesel conversion yield. The rate of conversion of fatty acid esters increased with increases in reaction time. The reaction proceeds slowly at the beginning and then increased rapidly due to the initial mixing and dispersion of alcohol into the oil substrate and activation of enzyme. After dispersion of alcohol, the enzyme rapidly interacted with fatty acids esters giving a maximum conversion yield. Increasing the reaction time from 4 to 16 h increased the conversion yield of biodiesel by 14.95-65.59%. The interactions between enzyme polymer surface and substrate appears to be dependent on reaction temperature due to hydrogen bonding and ionic interactions which play an important role in maintaining the thermostability of lipase in the system. The optimum reaction temperature for the experimental enzyme catalyst (NS88001) in the solvent free system was 45°C. Increasing the reaction temperature from 40 to 45°C increased the biodiesel conversion yield while higher temperatures above 45° C denatured the specific structure of enzymes and resulted in decreased methyl esters formation. The activity of experimental enzyme catalyst NS88001 in the presence of methanol without solvent at the optimum conditions (a reaction temperature of 45°C, an oil:alcohol molar ratio of 1:4 and a reaction time of 16 h) remind relatively constant for 10 cycles and then decreased gradually reaching zero after 50 cycles.

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