Open Journal of
Biotechnology & Bioengineering Research

Research Article

Effects of Hydrochloric Acid Concentration and Water Content on Direct Transesterification of *Physarum polycephalum* for Biodiesel Production

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Submitted: 21 December 2016; **Approved:** 17 March 2017; **Published:** 22 March 2017

Citation this article: Tran HTM, Cheirsilp B, Louhasakul Y, Stephenson SL. Effects of Hydrochloric Acid Concentration and Water Content on Direct Transesterification of *Physarum polycephalum* for Biodiesel Production. Open J Biotechnol Bioeng Res. 2017;1(1): 001-005.

ABSTRACT

The macroscopic trophic stage (plasmodium) of *P. polycephalum* lacks cell walls and produces a considerably high amount of biomass, which contains about 10% lipids and 95% of these lipids are triglycerides. The objectives of this research were first to evaluate the direct transesterification of *P. polycephalum* plasmodium into biodiesel and then to study the effects of HCl concentration and moisture content on this process. Two-step transesterification of dried plasmodium resulted in a Fatty Acid Methyl Esters (FAME) content of 105 mg. Direct transesterification of dried plasmodium yielded maximum FAME content (46 mg) at a low volume of 37% HCl (0.3 mL/0.1 g dried plasmodium) with a water content of 56.5%. To determine whether this effect came from the difference in HCl concentration or water content, additional experiments were carried out. Different HCl volumes were used whereas final water content was adjusted to be the same as 80%. FAME content increased when increasing the HCl volume from 0.3 to 0.7 mL/0.1 g dried plasmodium, but with a water content of 80% the maximum FAME content obtained was only 31.0 mg. Direct transesterification of wet biomass (with a moisture content of 80%) was then tested. The maximum FAME content obtained was 39.2 mg at 0.7 mL HCl/0.1 g dried plasmodium and a final water content of 87.4%. This indicated that water content and HCL volume interactively affected the FAME yield. Remarkably, direct transesterification of fresh plasmodium with a moisture content of about 80% yielded the highest FAME content (50 mg).

Keywords: Direct conversion; Fresh biomass; Plasmodium; Slime molds

INTRODUCTION

Traditionally, biodiesel production from microbial biomass includes two steps, which are lipid extraction and then the conversion of the extracted lipids into biodiesel through a process called transesterification. Lipid extraction from algae and other oleaginous microorganisms (yeast and bacteria) involves cell disruption, which can be done in various ways, including biological, chemical and mechanic methods [1]. However, this process is often challenging to carry out because of the rigid cell walls of these microorganisms. As a result, the lipid extraction yield is negatively influenced [2].

Direct transesterification of biomass into biodiesel (a one-step process), the conversion of biomass into biodiesel without the lipid extraction step was first applied to flaked full fat soybeans, distillers dried grains with soluble (a co-product of the production of ethanol from corn) and meat and bone meal (a product of animal rendering) [3] and algal dried biomass [4]. Recently, direct transesterification has been taken to an advanced level in which dried biomass is replaced with wet biomass for energy reduction purposes [5-9].

In term of catalyst selection for biodiesel production, bases (NaOH) and acids (H_2SO_4) are generally used. A base catalyst is used when the lipids contain little or no amount of free fatty acids to avoid saponification from taking place. However, when it comes to direct transesterification of wet biomass, acids are preferred over bases. Among the most commonly used acids are sulfuric acid and hydrochloric acid, with the latter having been found to be more productive because of having a higher affinity with water and thus disrupting microbial cell wall more effectively [8]. It should also be noted here that in order to obtain an accurate determination of the moisture content of the samples used, wet biomass has been prepared by adding a calculated amount of distilled water to completely dried biomass in order to reach particular moisture content. Actual fresh microbial biomass has not yet been investigated.

The myxomycetes are a group of primitive phagotrophic eukaryotes commonly known as slime molds. The life cycle of a myxomycete is characterized by a distinctive multinucleate trophic (feeding) stage called a plasmodium [10]. Among the myxomycetes, the plasmodium of *Physarum polycephalum* Schwein a member of the order Physarales is particularly interesting because of its rapid growth rate, high biomass production and ease of culturing. Brewer, et al. [11] successfully grew some strains of this species at a pilot scale for biomass production. In addition, as already noted, the plasmodium has no cell walls. Tran, et al. [12] first investigated the feasibility of using this plasmodium as a source of lipids for biodiesel production. They found that although the dried biomass of this myxomycete accumulated only 10% lipids, but 95% of these lipids are triglycerides and the amount of free fatty acids was too small to be detected. In another experiment, the authors also found that this myxomycete could produce a relatively high amount of biomass when grown on defatted rice bran [13]. Algae and other microbes investigated for biodiesel productions have cell walls, but there has not been any similar research carried out on microorganisms that lack cell walls. The purposes of this current research were first to evaluate the direct transesterification of *P. polycephalum* plasmodium and then to study the effects of HCl concentration and moisture content on this process. Optimized conditions were applied with actual fresh *P. polycephalum* plasmodium for comparing between direct transesterification of actual fresh biomass with the wet biomass having the same moisture content.

MATERIAL AND METHODS

Materials

Standard fatty acids were purchased from Sigma-Aldrich (USA). The strain of *P. polycephalum* used in the present study was obtained as a sclerotium from Carolina Biological Supply Company (Burlington, North Carolina, USA).

Myxomycete plasmodia preparation

Activation of *P. polycephalum* plasmodium from the sclerotium and inoculum preparation was carried out as described in Tran, et al. [13]. The plasmodium of *P. polycephalum* was cultured on nutrient agar (containing defatted rice bran as the sole carbon source) in the dark and collected after 7 days when the biomass appeared maximal.

The fresh biomass of *P. polycephalum* was lyophilized (Labconco, freeze-zone six) to a constant weight and weighed on an analytical balance (AB104-S, Switzerland) for dried cell weight determination [12].

Water content of the fresh biomass was determined using the following formula [12].

$$\text{Water content}(\%) = \frac{(\text{weight of fresh biomass} - \text{weight of dried biomass})}{\text{weight of fresh biomass}} \times 100$$

Transesterification of *P. polycephalum* plasmodial lipids

Two-step process: In order to determine the maximal fatty acid methyl esters (FAMEs), which could be produced from *P. polycephalum* dried plasmodium, a two-step process was carried out.

Total lipid extraction was carried out using the Bligh-Dyer method [14]. The extracted lipids were collected in a glass tube with tightly screwed cap, and then 3.0 mL of methanol and 0.3 mL of 98% H_2SO_4 were added. The tube was then placed in a heating block of which the temperature was set at 95°C for one hour for transesterification. The tube was then placed at room temperature for 30 minutes before FAMEs were collected [8].

Direct transesterification of wet biomass: A certain volume of 37% HCl was added to 0.15 g of dried biomass and the appropriate amount of water was added when applied. The mixture was placed at room temperature for 30 minute for water saturation, after which 3 mL of methanol:chloroform (2/1, v/v) was added. The tube was then set at 95°C for two hours for transesterification and was cooled down at room temperature for 30 minutes [8]. Afterwards, 1.0 mL of chloroform was then added for FAME separation. The chloroform layer containing FAMEs was collected to a glass vial. This process was repeated three times to make sure a complete collection of FAMEs. The solvent was evaporated. FAMEs were purified by adding 1.0 mL of hexane into the vial and followed by centrifugation of the hexane mixture. The supernatant containing FAMEs was collected into another tube. Hexane was evaporated and FAMEs were recollected by using n-heptane. A volume of 100 µL of n-heptane containing 1.0 mg of methyl heptadecanoate (C17:0) was added in the FAMEs solution to make up the final volume of 500 µL. This mixture was used for FAMEs analysis and content calculation [6].

FAME ANALYSIS

FAMEs were measured by gas chromatography (Hewlett Packard) using select biodiesel for FAME column (30 m x 0.32 mm) and a flame ionization detector with helium with a flow rate of 1.0 mL/min as a carrier gas. The initial temperature of the oven was 210°C, which was held constant for 10 minutes and then was ramped (at 20°C per minute) to 250°C and held at this temperature for another 8 minutes. The FID temperature was set at 300°C.

FAME content or amount of FAMEs obtained from 1.0 g of dried biomass was calculated using the formula given below [8].

$$\text{FAME content}(g) = \frac{\text{Weight of standard agent in sample x Sum of FAME area in GC}}{\text{Area of standard agent in GC}}$$

EXPERIMENTAL STATISTICS

All experiments were carried out in duplicate, and the results are presented as their mean values (±standard deviation).

RESULTS AND DISCUSSION

Two-step transesterification of *P. polycephalum* dried plasmodium

Fresh biomass of *P. polycephalum* was collected from 7-day-old cultures and lyophilized using a freeze-drier to a constant weight. The dried biomass was used in this study. Lipid content determination revealed that the dried biomass contained 10% of lipids. The major fatty acid compositions of *P. polycephalum* lipids are listed in table 1.

Table 1: Major fatty acid compositions of *P. polycephalum* lipids.

Common name	Abbreviation	Weight (%)
Myristic	C14:0	1.1
Palmitic	C16:0	18
Palmitoleic	C16:1	17
Stearic	C18:0	4.7
Oleic	C18:1	20
Linoleic	C18:2	33
Arachidic	C20:0	2.3
Behenic	C22:0	5.0

One gram of the dried plasmodium contains about 10% of lipids and transesterification of these lipids resulted in 105 ± 6.5 mg of FAMEs. This amount was considered as the maximum FAME content that would be achieved in this type of experiment.

Effect of different 37% HCl volumes on FAME production of *P. polycephalum* dried plasmodium

Effects of HCl volume on direct transesterification of the dried biomass was investigated in this component of the overall study. The results obtained are shown in table 2.

The increment of HCl volume significantly reduced the FAME content. However, as the concentration of HCl is 37%; therefore, it is not clear whether the effects came from the differences in HCl concentration or the water content. But this experiment generated some general information, which would be useful in case one wants to produce FAMEs directly from the dried biomass using HCl as the catalyst. It should also be noted that there was no detection of C:20 and C:22, as in the case of the two-step experiment described above.

Effect of different 37% HCl volumes on FAME production of *P. polycephalum* wet plasmodium

In this component of the study, dried plasmodium was initially placed into a tube, and then a certain HCl volume and water was added, accordingly to achieve the final moisture content of all treatments to be 80%. The obtained FAME contents are displayed in table 3.

The increase in HCl concentration had a positive effect on FAME production.

Maximum FAME content (31.0 mg) was obtained at 0.7 mL HCl/0.1 g dried plasmodium (equal to 0.008 molar). However, when

Table 2: Effect of 37% HCl volume on FAME production of *P. polycephalum* dried plasmodium.

Sample	Volume of 37% HCl (mL)	Final moisture content of sample (%)	FAME content (mg)
1	0.30 (0.189)	56.5	46.1 ± 1.7
2	0.50 (0.315)	68.4	39.2 ± 1.5
3	0.70 (0.441)	75.2	33.8 ± 1.1
4	1.00 (0.63)	81.2	29.1 ± 1.4
5	1.25 (0.79)	84.3	23.4 ± 1.1

Note: Figures in parentheses indicate the amount of water present in the corresponding acid volume. An amount of 0.15 g dried biomass was used in each treatment.

Table 3: Effects of HCl concentration on direct transesterification of *P. polycephalum* wet plasmodium.

Sample	Volume of 37% HCl (mL)	Additional water added (mL)	Final moisture content of sample (%)	FAME content (mg)
1	0.3(0.189)	0.411	80	16.4 ± 0.7
2	0.5(0.315)	0.285	80	20.3 ± 1.2
3	0.7(0.441)	0.159	80	31.0 ± 1.3
4	0.95(0.598)	0.050	80	29.1 ± 1.9

Note: Figures in parentheses indicate the amount of water presence in the corresponding acid volume. An amount of 0.15 g dried biomass was used in each treatment.

the HCl volume increased to 0.95 mL/0.1 g dried plasmodium, FAME content decreased.

The combined data presented in tables 2 and 3 indicate that moisture content seems to have a more significant effect on FAME production, since the moisture content was fixed at 80%, and the maximum FAME content obtained was 31.0 mg (Table 3). However, when the moisture content was 56%, the maximum FAME content obtained was 46.1 mg and the HCl concentration required was less than a half (Table 2).

A small experiment was carried out to test the effect of catalyst type on FAMEs production. The volume of acid sulfuric 98% was calculated to have the same molar value as that of HCl in 0.7 mL of 37% HCl, and water was added to make up the final moisture content of the sample as 80%. The FAME content of the treatment using H_2SO_4 (34.2 mg) was higher but not significantly different from that of HCl (31.0 mg).

Effects of HCl volumes on direct transesterification process using *P. polycephalum* wet plasmodium with moisture content of 80%

The moisture content of *P. polycephalum* fresh biomass (collected from 7 day old cultures) is about 80-85%. In this experiment, water was added to dried biomass to make wet biomass with a moisture content of 80%. The objective of this experiment was to test the feasibility of direct conversion of fresh biomass into FAMEs and to determine the effect of 37% HCl volumes on the process. The FAME content of each treatment is listed in table 4.

Similar to the previous experiment on direct transesterification of the wet plasmodium, the data obtained show that FAME content increased along with the increment of 37% HCl volume and reached the maximum value (39.2 mg) at 0.7 mL/0.1 g dried plasmodium. The FAME content significantly decreased when the acid volume exceeded this volume. With the presence of more water, it is understandable for the requirement of a higher acid concentration for productive cell disruption and effective transesterification, but the reason why a higher amount of acid would not be necessary to result in better FAME content is problematic. One possible explanation is that a certain ratio of acid to water would be suitable for the fatty acids dissolvent and subsequently this favours the two processes and in this experiment; such a ratio happened to be the case of the treatment 3 (Table 4).

Table 4: Effects of HCl volume on direct transesterification of *P. polycephalum* wet plasmodium with moisture content of 80%.

Sample	Water added (mL)	Volume of 37% HCl (mL)	Final moisture content of sample (%)	FAME content (mg)
1	0.6	0.3 (0.189)	84.0	20.5 ± 0.8
2	0.6	0.5 (0.315)	85.9	30.0 ± 0.4
3	0.6	0.7 (0.441)	87.4	39.2 ± 0.6
4	0.6	1.0 (0.63)	89.1	30.1 ± 1.3

Note: Figures in parentheses indicate the amount of water presence in the corresponding acid volume. An amount of 0.15 g dried biomass was used in each treatment.

When comparing the direct transesterification of dried biomass, more acid is required (0.7 mL compared to 0.3 mL) and the maximum FAME content was reduced 1.17 times (Table 2).

Tranesterification of actual fresh biomass and wet biomass having the same moisture content

Fresh plasmodium of *P. polycephalum* cultured on nutrient agar was collected after 7 days, and the moisture content was later determined as 80%. The amount of the fresh sample used for this experiment was calculated to be equal to 0.1 g dried biomass after extracting the amount of water content included in it. HCl was used with the same volume. Wet biomass with a moisture content of 80% was prepared by adding sterile distilled water to dried plasmodium. The data obtained is shown in table 5.

Table 5: Direct transesterification of wet biomass and fresh biomass of *P. polycephalum* plasmodium.

Sample type	Volume of 37% HCl (mL)	Additional water added (mL)	Moisture content (%)	FAME content (mg)
Actual fresh biomass	0.7 (0.441)	0.0	87.4	50.0 ± 0.4
Dried biomass	0.7 (0.441)	0.6	87.4	39.2 ± 0.6

Despite using the same acid concentration and having the same final moisture content of 87.4%, the actual fresh biomass resulted in 1.27 times higher FAME content than that of the wet biomass (Table 5). Especially noteworthy is the fact that this amount of FAME is even slightly higher than when using dried biomass (Table 2). This would be explained by the fact that water in the actual biomass is not in the form of free water, so the interference with HCl and the solvent would be less negative. In addition, since *P. polycephalum* plasmodium has no cell walls, lipid extraction of the fresh plasmodium would not be very different in terms of efficiency compared to the dried plasmodium.

The results indicate the feasibility of direct transesterification of fresh *P. polycephalum* plasmodium into FAMEs.

CONCLUSIONS

Direct transesterification of *P. polycephalum* dried plasmodium was found to be most effective when using a low volume of 37% HCl (0.3 mL/0.1 g dried plasmodium). On the other hand, direct transesterification of wet biomass with a moisture content of 80% increased significantly along with the increment of 37% HCl volume (within the range of 0.3-0.7 mL/0.1 g dried plasmodium). This positive effect possibly could be explained by the fact that within this range, the acid and water ratios are favoured for fatty acids dissolvent. In addition, the conversion of actual fresh plasmodium into FAME was found to be more effective than the wet biomass having the same moisture content.

ACKNOWLEDGMENTS

This research is funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.04-2015.16.

The first author also would like to thank the faculty of Agro-Industry (Prince of Songkla University, Thailand) for allowing her to use their facilities to carry out some components of the research described herein.

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