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Biodiesel production from *Jatropha curcas* oil catalyzed by whole cells of *Aureobasidium pullulans* var. *melanogenum* SRY 14-3

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The main obstacle to using lipase as a catalyst in industrial scale biodiesel production is the cost and availability of the enzyme. To overcome this obstacle, the potential of using a whole cell biocatalyst (for at least partial *in situ* lipase production) was evaluated as a means to reduce the cost of the lipase. The reaction conditions for biodiesel production via transesterification between *Jatropha curcas* (physic nut) oil and methanol when catalyzed in the presence of lipase-producing *Aureobasidium pullulans* yeast cells was investigated. The appropriate conditions for optimal biodiesel production were found to be 1:3 oil:methanol molar ratio at 30°C with constant stirring at 250 rpm. Under these conditions a maximum fatty acid methyl ester (biodiesel) production level of 71.8% was obtained after 72 h.

Key words: Lipase, *Aureobasidium pullulans*, physic nut oil, biodiesel, green energy.

INTRODUCTION

Asides the key issue of human overpopulation, the main ancillary reason for many current global environmental issues results from the level of combustion of non-renewable and rapidly depleting fossil fuels. To overcome this problem, the development of renewable and environmentally friendly energy sources, such as biodiesel, is becoming more attractive. Biodiesel is an environmentally friendly and renewable fuel that can be used directly in diesel engines without any engine modification.

Biodiesel, principally produced as fatty acid methyl esters (FAME), is a product from the transesterification reaction between triglycerides (vegetable oils or animal fat) and short chain alcohols (methanol to form FAMEs, or ethanol to form fatty acid ethyl esters) (Gupta et al.,

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Abbreviations: FAME, Fatty acid methyl esters; **CFUs,** colony forming units; **BSA,** bovine serum albumin; ***p*-NPL,** *p*-nitrophenyl laurate; ***p*-NP,** *p*-nitrophenol; **YMA,** yeast malt extract agar plates; **HPLC,** high performance liquid chromatography; **GC,** gas chromatography.

alkali, or lipase as the catalyst since supercritical methanol (or supercritical ethanol) transesterification is not commercially viable at present. However, the traditional use of an acid or alkali (chemical) catalyst has many disadvantages when compared with that enzymatically catalyzed with lipase. For example, in the presence of water or free fatty acids in the feedstock, saponification occurs when using alkali as a catalyst and this then inhibits the reaction and reduces the FAME yield by forming soap. In addition, acid or alkali catalysts lead to the production of a significant amount of contaminated wastewater from the washing stages and this is economically and environmentally costly to treat. Therefore, lipase has become attractive as a catalyst because it is a cleaner technology, producing only a little or no toxic or chemical waste and saponification does not occur in the reaction, and so potentially leads to both a high purity biodiesel production and the ability to use a wider range of feedstock including waste oils (Winayanuwattikun et al., 2011). However, the cost of immobilized lipase preparations is the main obstacle to the commercial adoption of lipase-catalyzed biodiesel production. In contrast, biodiesel production using a whole-cell catalyst, where the whole cell culture of the lipase producing microorga-nism is used to (at least partially) provide *in situ* lipase production rather than its enriched *ex-situ* produced lipase fraction, was shown to be able to reduce the cost of lipase-catalyzed biodiesel production (Fukuda et al., 2008).

Jatropha curcas (physic nut) is a multi-purpose plant that has been recommended as a potential source of oils for biodiesel production. Native to the American tropics, the physic nut (as a species at least) is adaptive and can be grown in most tropical and subtropical regions. Moreover, it is not a food plant (indeed most cultivars are poisonous) and can typically be grown in dryer and less fertile or even salty soil conditions that are unsuitable for food crop production. For this reason, it can avoid the economic and ethical issues of competition with food production, whilst it has a higher total fatty acid content than other plant oils (Parawira, 2010). Currently, physic nut oil has been shown to be able to support the production of biodiesel in large quantities, typically around 540 to 600 L/ha on poor soil. With respect to whole cell catalysis for biodiesel production, the transesterification of physic nut oil with methanol to produce FAME-based biodiesel using immobilized *Enterobacter aerogenes* as a catalyst resulted in a 94% biodiesel yield (Kumari et al., 2009), whilst immobilized *Rhizopus oryzae* gave a more than 80% biodiesel yield (Tamalampudi et al., 2008). Thus, physic nut oil appears to be a potential feedstock for enzyme produced biodiesel.

The yeast *Aureobasidium pullulans* var. *melanogenum* (Ascomycota: Dothideales) has been reported to produce lipase and to secrete it outside the cell (Yurlova et al., 1999). *A. pullulans* can grow fast, and so allows a rapid expansion of its production capacity, and can use a variety of substrates (Chi et al., 2009). In addition, *A. pullulans* has been reported as being suitable as a lipase catalyst without the need for the separation, purification and immobilization processes of the lipase that otherwise leads to a high lipase cost (Li et al., 2008). Due to the advantages of using whole-cell *A. pullulans* as a catalyst, because that it is simpler, environmentally friendlier and can reduce the cost of lipase catalyzed biodiesel production, the appropriate conditions for FAME biodiesel production from the transesterification reaction of physic nut oil with methanol catalyzed by whole-cell *A. pullulans* were evaluated in this study.

MATERIALS AND METHODS

Microorganism and chemicals

The yeast strain *A. pullulans* var. *melanogenum* SRY 14-3 (GenBank accession no. KF163060) was provided by the Biofuels Production from Biocatalysts Research Unit, Chulalongkorn University. The yeast was isolated from an oil-contaminated soil sample collected at Srichang Island, Chonburi Province, Thailand (Vitisant et al., 2010). The number of viable yeast cells, as colony forming units (CFUs), was determined from total plate counts using the spread plate method. Bovine serum albumin (BSA) was obtained from Merck and used as the standard for protein assay. The substrate for determining the lipase activity, *p*-nitrophenyl laurate (*p*-NPL), was obtained from Sigma-Aldrich, whilst the product, *p*-nitrophenol (*p*-NP), which was used to make the standard concentration curve in the assay, was obtained from Fluka.

Whole cell *A. pullulans* preparation

A culture of *A. pullulans* var. *melanogenum* SRY14-3 was established by transferring a single colony that had grown for five days on yeast malt extract agar plates (YMA; 0.3% (w/v) yeast extract, 0.5% (w/v) malt extract, 0.5% (w/v) peptone, 1% (w/v) glucose and 1.5% (w/v) agar, pH 7.0) into 50 mL of YMB (YMA without the agar) in a 250-mL Erlenmeyer flask. The yeast was left to grow at 30°C with continuous agitation at 200 rpm for 24 h and then transferred into a 500-mL Erlenmeyer flask containing 50 mL of lipase production medium (LP) [1.17% (w/v) yeast extract, 1.40% (w/v) sucrose, 1% (w/v) KH_2PO_4, 0.1% (w/v) $MgSO_4.7H_2O$, 1.44% (v/v) physic nut oil, pH 5.0]. The presence of the physic nut oil in the LP medium was to induce lipase production. The culture was incubated for 4 days at 30°C on a rotary shaker at 200 rpm and then the culture broth was filtered through a sterile cloth and clarified by centrifugation (10,000 rpm, 4°C, and 15 min) to precipitate the yeast cells. The resulting clear supernatant was used directly without additional purification for determining the lipase activity and total protein levels.

The lipase activity was determined by the enzymatic capacity to hydrolyze the *p*-NPL substrate to liberate *p*-NP. The concentration of *p*-NP was then analyzed by measuring the absorbance at 410 nm at pH 6 by a modification to that previously described (Maia et al., 2001; Marini et al., 2012). In brief, the reaction was performed for 30 min at 37°C, with 100 µL of an adequate dilution of the enzymatic sample in 650 µL of 50 mM citrate buffer (pH 6) with 100 µL of 25 mM *p*-NP in ethanol. After that, 250 µL of 1 M Na_2CO_3 was added and the mixture was centrifuged at 13,000 *g* for 5 min, the supernatant harvested and the absorbance measured at 410 nm. The absorbance value was then converted to the *p*-NP concentration by reference to the standard curve, obtained from different *p*-

NP concentrations over the range of 0 to 50 µg/mL.

One unit (U) of lipase was defined as the amount of lipase that can convert 1 µmol/min of *p*-NPL to *p*-NP at 37°C in a 30 min reaction. The total protein content was evaluated by Bradford's method (Bradford, 1976), measuring the absorbance at 595 nm, and used to calculate the specific lipase activity of the samples as well to check the purity of lipase. The standard curve of total protein content was prepared from different concentrations of calf BSA over the range of 0 to 25 µg protein.

Transesterification reactions

Transesterification reactions were carried out in 500-mL Erlenmeyer flasks and each flask contained 30 g of physic nut oil (31.8 mL; ~33 mM), various volumes of methanol (~4.19, 5.58, 6.99, 8.39 and 9.81 mL), and 50 mL of a whole cell *A. pullulans* culture pregrown in LP medium to an optical density (OD) at 600 nm of 0.2, which represents a viable yeast plate count of about 165,000 CFU/mL in LP, as the catalyst.

This then provides both lipase containing culture medium (*ex-situ* produced lipase) at the start of the reaction, and live yeast cells to produce more lipase (*in situ* produced lipase) during the transesterification reaction. The effect on the transesterification reaction, in terms of the FAME yield obtained, of varying the reaction temperature and the oil to methanol molar ratio were evaluated.

The reaction samples were collected at 0, 4, 8, 12, 16, 20, 24, 48 and 72 h after initiation of the reaction in all cases, clarified by centrifugation (13,000 rpm for 30 min) to pellet the cells, and then the supernatant was assayed for FAME content by quantitative high performance liquid chromatography (HPLC) (Shimada et al., 2002). In addition, the yeast growth rate was measured in terms of the number of yeast cells, estimated by measuring their OD at 600 nm and total plate count following the spread plate method. The effect of varying the reaction temperature (25, 30, 35, 40, 45 and 50°C) was evaluated with an oil to methanol molar ratio of 1:3, whilst the effect of varying the oil to methanol ratio (1:1, 1:2, 1:3, 1:4, 1:5, 1:6 and 1:7) was evaluated at 30°C. In all cases, reactions were performed over 72 h with continuous agitation at 250 rpm. In addition to evaluating the FAME level by HPLC, the supernatant was assayed for the residual methanol content by gas chromatography (GC), and the amount used in the reaction was determined from the initial amount added less the residual amount.

Analytical methods

FAME contents were analyzed by quantitative HPLC using a mixture of 490 µL chloroform, 10 µL sample and 10 µL eicosane as an internal standard. 2 µL of this mixture was injected per run into the HPLC equipped with an Apollo silica column 5U (250 × 4.6 mm), an Inertsil ODS-3 (7.5 mm × 4.6 mm I.D) guard column and an ELSD detector (Shimadzu LC-20A series, Japan).

The mobile phase was run at 1.5 mL/min as a 99.0 to 1.0 (v/v) binary gradient of A:B, where A was a 100: 0.2 (v/v) mixture of hexane: formic acid and B was comprised of a 85:10:10:0.1 (v/v) hexane: isopropanol: ethyl acetate: formic acid mixture (Chulalaksananukul et al., 2002).

Methanol levels were monitored by GC, where prior to analysis, 600 µL of sample was spiked with 100 µL of 1-butanol as an internal standard and then 0.3 µL injected per run into the GC (Shimadzu, Japan) with resolution over a DB5-HT capillary column (30 m × 0.32 mm I.D × 0.1 µm film thickness) with detection via a flame ionization detector and operated at a total flow rate of 47.9 mL Ar/min.

RESULTS AND DISCUSSION

Yeast growth rate and lipase specific activity in whole cell *A. pullulans* preparations

The growth rate of *A. pullulans* var. *melanogenum* SRY 14-3 was determined in terms of the number of viable cells as CFU/mL (Figure 1). *A. pullulans* had left the lag phase within the first 3 h of culture, and entered a rapid exponential growth phase from 3 to 48 h culture and then stationary and death phase from 48 and 120 h onwards, respectively. The extracellular lipase activity, in terms of the specific activity, was detected (presumed to be secreted) from 12 h of culture, which is when the yeast were in mid-exponential phase, and then rose rapidly during the exponential growth phase and continued to rise during the early- to mid- stationary phase to reach a maximal specific lipase activity of 9.7 ± 1.20 U/mg protein after 96 h of culture. Thereafter, with the yeast cells in stationary or death phase, the lipase specific activity declined sharply to a 3.6-fold lower level at 168 h culture compared to that at 96 h.

Under these conditions, the specific growth rate and generation time of *A. pullulans* were calculated to be 0.305 h^{-1} and 150 min, respectively. This specific growth rate of *A. pullulans* was better than that reported previously for *Saccharomyces* sp. (0.08 to 0.13 h^{-1}) (Taccari et al., 2012), which could potentially lead to higher activity and yield when used as catalyst.

Optimal temperature for the transesterification reaction

Temperature is one of the key factors that affect the efficiency of both enzymatic and chemically catalyzed transesterification reactions and so the yield of biodiesel obtained. For transesterification reactions using lipases as catalysts, the optimal temperature is typically in the range of 30 to 40°C (Marchetti et al., 2007), whilst for the lipase producing yeasts, the highest growth and lipase production rates were typically lower at 20 to 30°C (Kumar and Gupta, 2008). From this study, the optimal temperature of the transesterification reaction using whole *A. pullulans* cells (as well as some *ex-situ* produced lipase in the culture medium) as the catalyst was in the expected overlap at 30°C (Figure 2). This result is consistent with the optimal temperature range of lipases from other previous studies (Kumar and Gupta, 2008; Marchetti et al., 2007).

The FAME yield obtained somewhat matched the growth rate of *A. pullulans*, increasing 1.5-fold from 48% at 25°C to a maximum of 71.8% at 30°C and then declining 1.7-fold to 42% at 35°C. Higher temperatures (40 to 50°C) further reduced both the number of viable yeast cells and the FAME yield, but the latter was decreased to a less marked extent. This presumably reflects the balance between the low thermotolerance of the yeast com-

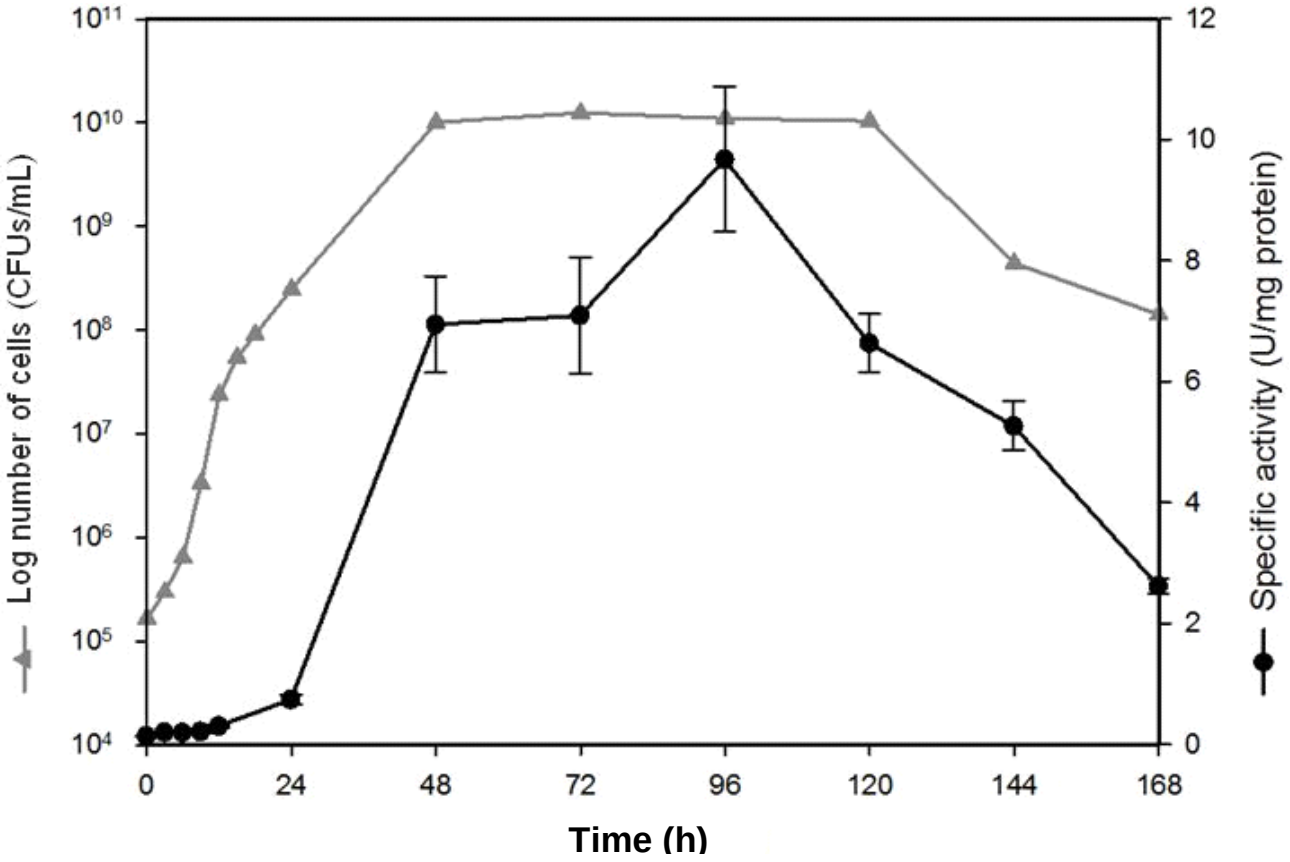

Figure 1. Growth rate (CFU/mL) of *A. pullulans* in LP media and the lipase specific activity (U/mg) in the media. Data are shown as the mean ± 1 SD, and are derived from three repeats.

pared to the lipase activity and thermostability of the enzyme (Pazouki et al., 2011). Accordingly, and from the data in Figure 1, this is to be expected if the *A. pullulans* acted as a source of *in situ* lipase production during the reaction (or at least the earlier part of it), although the alternative explanation that it simply reflects the temperature optima of the *ex-situ* produced lipase cannot be formally excluded.

Optimal oil to methanol molar ratio for the lipase-catalyzed transesterification reactions

Another important factor that affects the efficiency of transesterification reactions and the yield of biodiesel obtained is the oil to methanol ratio. Theoretically, the optimal oil to methanol molar ratio used in transesterification reactions is 1:3 because stoichometrically the reaction utilizes 1 mole of triglyceride and 3 moles of methanol to produce 3 moles of FAME and 1 mole of glycerol. However, in practice, a higher methanol:oil molar ratio usually gives a higher FAME conversion rate, but increasing the methanol:oil ratio level beyond the optimal point decreases the FAME yield obtained (Encinar et al., 2010). This is because the poorly miscible methanol starts to accumulate around the lipase structure including its active sites and denatures the protein (Pazouki et al., 2011); and also is inhibitory to the yeast viability and metabolism causing a reduced yeast growth, lipase production and secretion rate and cell viability.

However, of the tested range, oil to methanol molar ratio of 1:3 was found to give the highest FAME yield of 71.8% at 72 h (Figure 3). Decreasing the oil to methanol molar ratio to 1:1 and 1:2 decreased both the number of viable *A. pullulans* and the FAME yield obtained. In addition, the number of viable *A. pullulans* dropped dramatically (1.5×10^7-fold) when the oil to methanol molar ratio was increased from 1:3 to 1:4 and slightly declined further thereafter with higher methanol proportions. Likewise, the FAME conversion rate also fell from 71.8 to 46% (a 1.6-fold reduction) as the oil: methanol molar ratio increased from 1:3 to 1:4, but in contrast, it did not decline much further with even higher or lower methanol: oil molar ratios. The higher methanol: physic nut oil molar ratios inhibited the growth of *A. pullulans* and, presumably the rate of *in situ* lipase production and secretion and so the ability to catalyze the

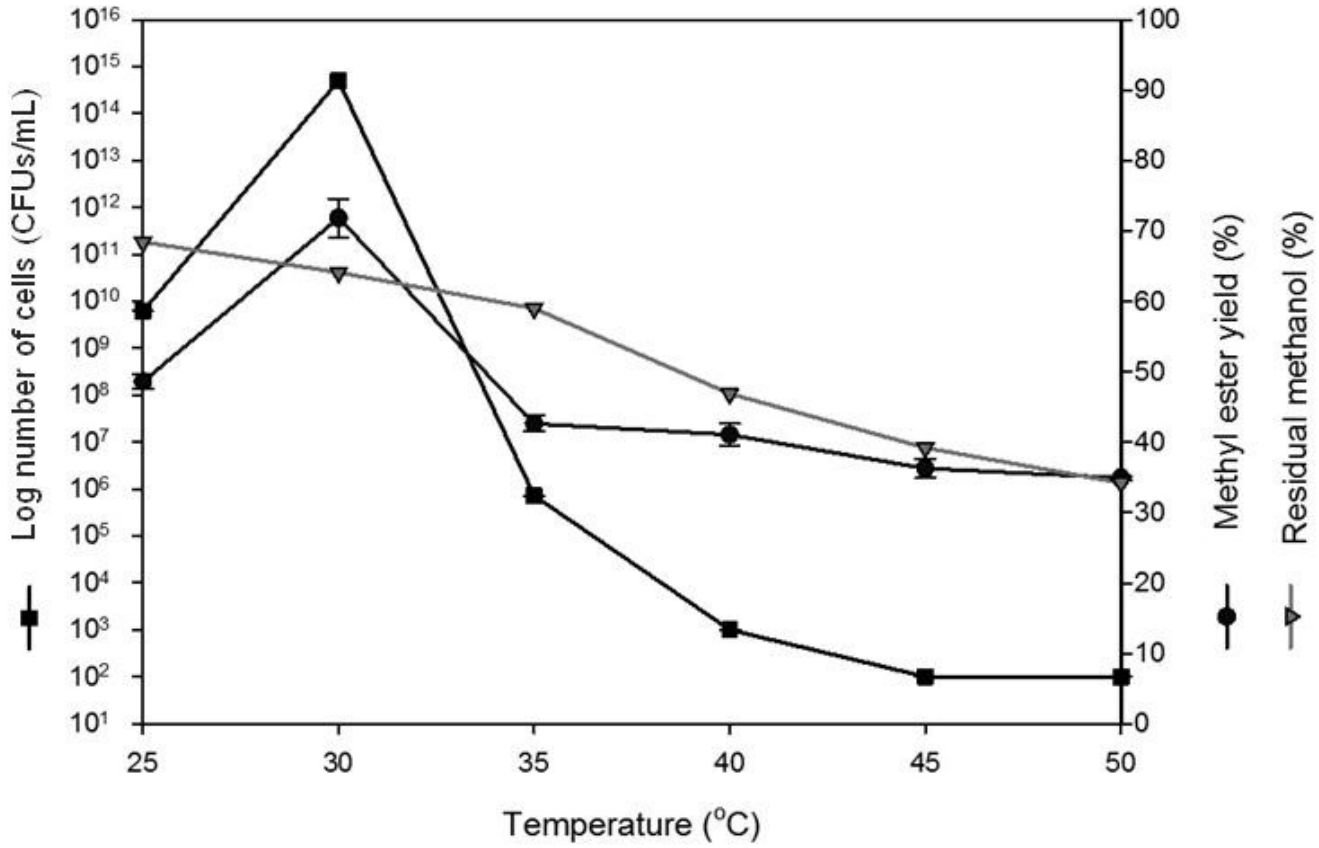

Figure 2. FAME yield (% methanol converted) obtained in the transesterification reaction of a 1:3 molar ratio of physic nut oil: methanol after 72 h with viable *A. pullulans* cells as the catalyst at different temperatures and agitation at 250 rpm, and compared with the growth of *A. pullulans* as viable CFU/mL. Data are shown as the mean ± 1 SD, and derived from triplicate experiments.

transesterification reaction, leading to the reduced FAME yield. This study shows that whole *A. pullulans* cells are promising way to produce biodiesel using the transesterification reaction in a potentially more environmentally friendly and economically viable framework. However, this method requires further improvement to increase the biodiesel yield obtained and the way to scale it up to industrial scale.

Conclusions

From this study, *A. pullulans* var. *melanogenum* SRY 14-3 was shown to produce extracellular lipase. Using LP as the medium, the highest lipase activity and specific activity obtained was 7.6 ± 0.15 U/mL and 9.7 ± 1.20 U/mg protein, respectively, after 96 h of culture. The optimal temperature for using this strain of *A. pullulans* as a whole cell catalyst for producing biodiesel by transesterification was 30°C. At lower temperatures, the reaction was slower and gave a lower yield, whilst above 30°C, the lipase was presumably denatured and so accounted for the reduced reaction rate and FAME yield. Optimal oil to methanol molar ratio of 1:3 was found for this transesterification reaction using a three-step methanol addition increasing the oil to methanol molar ratio to 1:4 and upwards to 1:7 lead to a reduced FAME yield, presumably as the lipase is denatured by the methanol. Decreasing the oil to methanol molar ratio to 1:1 or 1:2, reduced the reaction rate and so reduced the FAME yield. The highest FAME yield obtained from transesterification using the optimal temperature and oil: methanol ratio was 71.8 ± 2.8% at 72 h. Using whole cell *A. pullulans* as the catalyst would likely reduce the cost of lipase production for the transesterification reaction of physic nut oil and still give a high FAME yield, because it is an economical method that does not require the separation and purification of *ex-situ* produced lipase. However, this method requires further improvement to scale it up to industrial scale.

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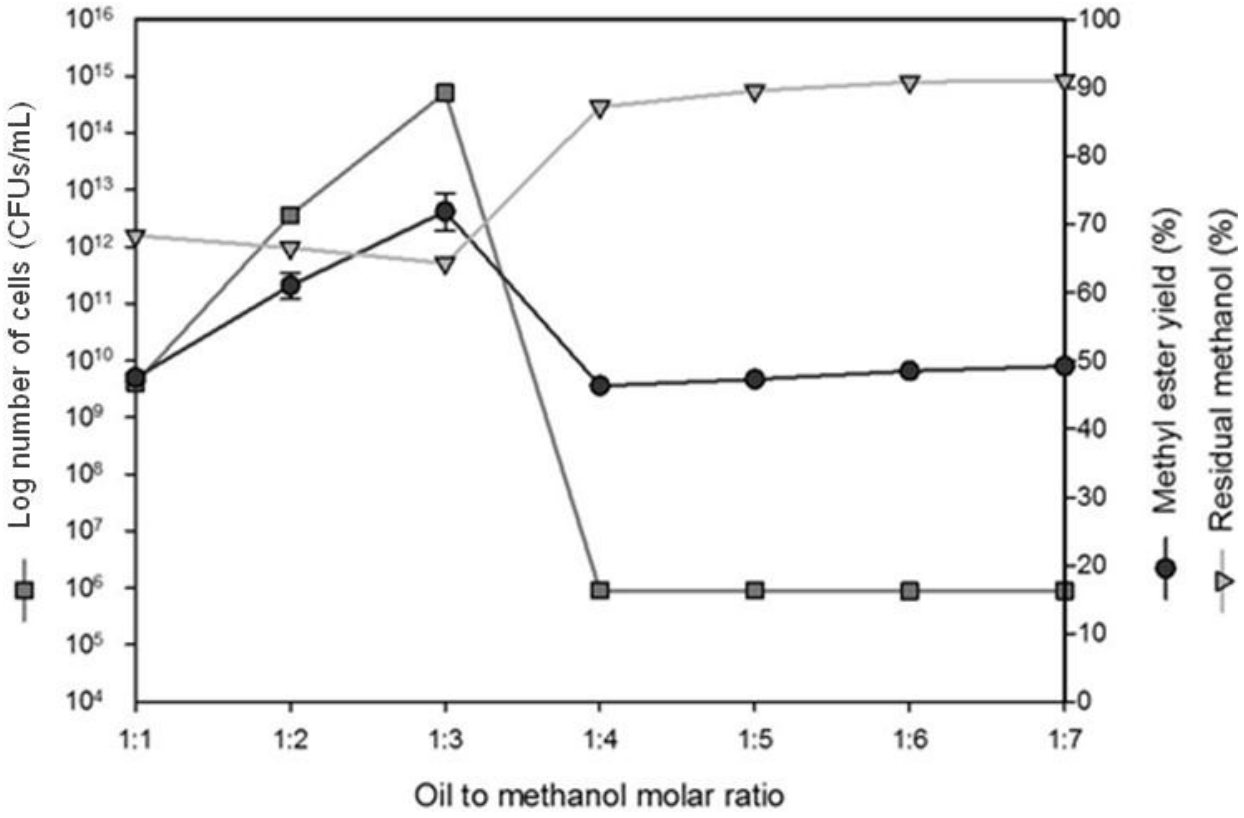

Figure 3. FAME yield (% methanol converted) obtained in the transesterification reaction with different physic nut oil: methanol molar ratios after 72 h with viable *A. pullulans* cells as the catalyst and agitation at 250 rpm, compared with the growth of *A. pullulans* as viable CFU/mL. Data are shown as the mean ± 1 SD, and derived from triplicate experiments.

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