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Biodiesel Production: Utilization of Loofah Sponge to Immobilize *Rhizopus chinensis* CGMCC #3.0232 Cells as a Whole-Cell Biocatalyst

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Rhizopus chinensis cells immobilized on loofah (*Luffa cylindrica*) sponges were used to produce biodiesel via the transesterification of soybean oil. In whole-cell immobilization, loofah sponge is considered to be a superior alternative to conventional biomass carriers because of its biodegradable and renewable properties. During cell cultivation, *Rhizopus chinensis* mycelia can spontaneously and firmly adhere to the surface of loofah sponge particles. The optimal conditions for processing 9.65 g soybean oil at 40°C and 180 rpm using a 3:1 methanol-to-oil molar ratio were found to be 8% cell addition and 3–10% water content (depending on the oil's weight). Under optimal conditions, an over 90% methyl ester yield was achieved after the first reaction batch. The operational stability of immobilized *Rhizopus chinensis* cells was assayed utilizing a 1:1 methanol-to-oil molar ratio, thus resulting in a 16.5-fold increase in half-life when compared with immobilized cells of the widely studied *Rhizopus oryzae*. These results suggest that transesterification of vegetable oil using *Rhizopus chinensis* whole cells immobilized onto loofah sponge is an effective approach for biodiesel production.

Keywords: Biodiesel, loofah sponge, *Rhizopus chinensis*, transesterification, whole cell

Introduction

Reliance on diminishing petroleum reserves has stimulated the development of alternate energy sources, such as hydrogen and alcohols [30, 31]. One such important potential substitute for conventional fossil fuels is biodiesel, which has the advantage of being nontoxic, renewable, and biodegradable [21]. Biodiesel is commonly referred to as a mono-alkyl ester, which is synthesized mainly via the transesterification of animal fats or vegetable oils that contain short-chain alcohols, such as methanol or ethanol [4, 14, 25].

Current industrial-scale biodiesel production employs chemical catalysts (alkali in particular), but this process has several drawbacks, such as a large intake of energy and methanol and a negative impact on the environment [8, 10, 36]. The use of lipase catalysts offers a number of advantages for overcoming the shortcomings of chemically catalyzed processes [10, 11, 39].

Extra- and intracellular lipases are the two major classes of enzymatic biocatalysts [2, 37]. Extracellular lipase is an enzyme isolated from microorganism broth after fermentation, and then purified [2]. Unfortunately, the extraction process of this lipase is expensive, which is an unavoidable hurdle for its development in biodiesel production [8, 23]. Intracellular lipase is an enzyme commonly employed in whole-cell processes [10]. Compared with extracellular lipase, whole-cell lipase has drawn increasing attention for its low cost, since it avoids the multifaceted processes required for extracellular lipase isolation and purification [15, 16]. Furthermore, use of whole cells as biocatalysts can provide a more stable catalytic environment for lipase

[29, 35].

Immobilization of whole cells onto matrices further reduces the cost of biodiesel production by offering reusability of the biocatalysts [6]. It has been demonstrated that porous materials like polyurethane foam [22], polyethylene plastic [6], and polyacrylamide gel [13] can immobilize lipase-producing filamentous fungi for repeated use. However, these cell supporters are polymer materials that are not biodegradable after batch reactions, thus producing potential environmental wastes and increased overall costs. Therefore, a commonly available and inexpensive material with renewability and biodegradability is required as a cell supporter for biodiesel production.

Loofah (*Luffa cylindrica*) is a bountiful and cheap vegetable because it is grown in most tropical and subtropical countries. The vegetable is often peeled and desiccated to produce a porous sponge. The sponge consists of an open fibrous support network that offers the immobilized cells immediate and sufficient contact to the surrounding aqueous medium [34]. Since the main component is lignocellulose, the sponge can be completely biodegraded via natural processes aided by microorganisms [32]. In our previous study, loofah sponge was shown to be an advantageous carrier material in whole-cell immobilization for biodiesel production [18].

Many filamentous fungi species like *Rhizopus oryzae* (*R. oryzae*) [17], *Aspergillus niger* [3], and *Mucor circinelloides* [1] have been studied as immobilized whole-cell biocatalysts for biodiesel production. Another lipase-producing fungus with an exceptional ability for catalyzing ester synthesis, *Rhizopus chinensis* (*R. chinensis*), has not been extensively investigated as a biocatalyst in whole-cell form for producing biodiesel. A previous study reported that in a solvent-free system, freely suspended *R. chinensis* whole cells can catalyze refined vegetable oil methanolysis rather efficiently, with the peak methyl ester yield reaching 86.0% [19]. However, with no immobilization, the remaining catalytic ability of this unrecyclable biocatalyst is unknown beyond the first reaction batch.

This study utilized loofah sponge particles (LSPs) in the form of whole-cell biocatalysts as biomass support particles (BSPs) in order to immobilize *R. chinensis* CGMCC #3.0232 for biodiesel production. The biocatalyst's catalytic performance and the immobilized performance of LSPs were thoroughly evaluated. Additionally, the influence of LSPs on biomass immobilization, the effects of the biocatalyst and the water content during methanolysis, and the ability to reuse the immobilized cells were examined.

Materials and Methods

Culture Medium, Microorganisms, and BSPs

R. chinensis CGMCC #3.0232 was obtained from the China General Microbiological Culture Collection Center (CGMCC; China). The basal medium consisted of the following components (reported in w/v): 3% refined soybean oil, 7% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.1% $NaNO_3$, and 0.1% KH_2PO_4. Soybean oil was obtained from a local market in Nanjing (China) and determined to have an average molecular weight of 883 g/mol. Yeast extract was obtained from Oxoid (UK). Heptadecanoic acid methyl ester was acquired from Sigma-Aldrich (MO, USA). Loofah sponge (Luokang Co., China) was cut into cubic pieces (5–7 mm) beforehand for use as BSPs and mixed with the medium in a flask after prior sterilization of all components. All of the additional reagents were certified analytical grade, and they were attained commercially.

Cell Immobilization

Flasks (250 ml), each containing 50 ml of the basal medium with differing LSP quantities (from 1.00–2.00 g), were inoculated by aseptically transferring about 1 million *R. chinensis* spores from the agar plate, and then incubated in a reciprocal shaker at 30°C and 180 rpm for roughly 72 h. Filtration was use to isolate the immobilized cells. After being washed with tap water for 0.5 min, the immobilized cells were frozen in a −80°C refrigerator, and dried in a freeze dryer (FDU-1200 model; Eyela, Japan) for about 24 h. A scanning electron microscope (JSM-7600F model; JEOL, Japan) was used to examine the biocatalyst's exterior morphology.

The net weight of the LSP-immobilized biomass was calculated as the LSP's change in weight before and after they were immobilized. The data were reproduced for three independent samples for a minimum of three trials.

Methanolysis Reaction

Methanolysis reactions were performed at 40°C in 50 ml bottles in a reciprocal shaker that was set at 180 rpm. The reaction mixture was composed of 9.65 g of soybean oil, various amounts of phosphate buffer (PB; pH 7.5, 0.1 M), and differing quantities of the dried immobilized cells (ranging 2–10%, depending on the oil's weight). A minimum of three molar equivalents of methanol are needed to successfully react the oil to the corresponding methyl esters, and the molar equivalent of methanol against 9.65 g of soybean oil was 0.35 g [7]. Subsequently, 0.35 g of methanol was respectively added at periods of 0, 24, and 48 h. At the end of each reaction, the biocatalysts were rinsed with *n*-hexane and tap water, and then dried for 24 h at 25°C for each duplicated batch.

The biocatalyst's catalytic half-life was calculated using the Arrhenius equation (Eq. (1)) and the half-time equation (Eq. (2)) by fitting to a deactivation model of first order:

$$-\ln(A/A_0) = t \times K_d \quad (1)$$

$$t_{1/2} = 0.693/K_d \quad (2)$$

in which A is the yield of methyl ester at time t, A_0 is the yield of methyl ester after the first batch reaction, t is the reaction time, K_d is the coefficient of deactivation, and $t_{1/2}$ is the catalytic half-life [18].

Gas Chromatography (GC) Analysis

Samples (100 µl) were extracted from the reaction mixture at the specified time intervals and examined by 7890A capillary GC (Agilent, USA). The GC machine contained a HP-5 capillary column supplied by Agilent with the dimensions 30 m × 0.32 mm × 0.25 µm. The samples were centrifuged for 3 min at 11,500 ×*g*, and then the top layer of the sample was isolated for GC analysis. With heptadecanoic acid methyl ester serving as the internal standard for analysis, exactly 30 µl of the sample was thoroughly dissolved in 270 µl of *n*-hexane, and then mixed with 300 µl of 1 g/l heptadecanoic acid methyl ester (*n*-hexane as the solvent). The column temperature was held at 170°C for 0.5 min, then heated at 3°C/min to 200°C, held at 200°C for 10 min, and then heated at 20°C/min to 260°C. The injector temperature was set at 250°C, and the detector temperature was set at 260°C.

Results and Discussion

Cell Immobilization

The main ingredients of loofah sponge are cellulose (55–90%), hemicellulose (8–30%), and lignin (10–23%) [33]. The combination of these ingredients forms a stable lignocellulosic material with an open structure (Figs. 1A and 1C), which offers the immobilized cells immediate and sufficient contact to the surrounding aqueous medium and thus favors nutrient diffusion and oxygen transfer for cell growth [27, 34]. Since numerous hydroxyl groups are present in lignocellulose and the main constituents of cell walls are hydrophilic polysaccharides, excessive hydrogen bonds between two surfaces result in another advantage of loofah sponge for cell adherence [26]. Comparing Figs. 1C and 1D, we can clearly see that after cultivation, a large number of *R. chinensis* mycelia were attached to the LSPs, thus signifying preferable immobilization conditions and indicating that the mycelia would not easily detach from the LSPs despite vigorous agitation. No evident cell detachment from the LSPs was detected in subsequent biodiesel production.

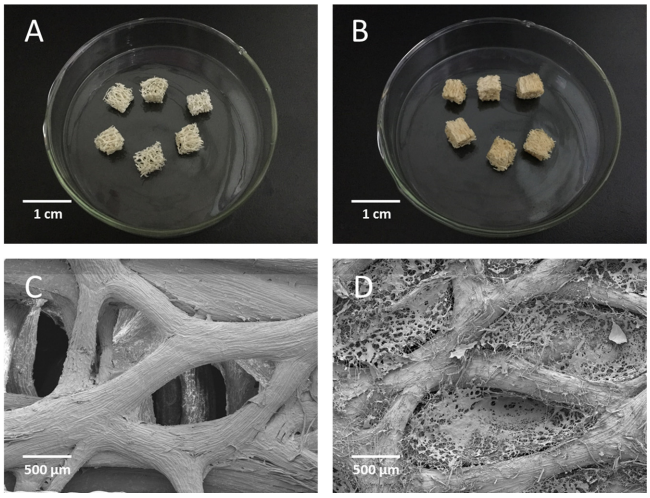

Fig. 1. Photographs and surface scanning electron microscopy images of LSPs (**A** and **C**: LSPs; **B** and **D**: LSPs with dried immobilized cells).

Effects of LSPs on Biomass Immobilization

Owing to elevated porosity and low density, the use of small amounts of LPSs can provide a large surface area for cell immobilization [33]. In order to study how cultivation cell immobilization is affected by LSP addition, varying quantities of LSPs were added to 50 ml of base medium. As displayed in Fig. 2, LSP addition caused an increase of the weight of immobilized cells. No macroscopic mycelia were observed as freely suspended biomass in the medium at the end of cultivation. This indicates that loofah sponge has a positive effect on fungi growth, and *R. chinensis* mycelia prefer to adhere to the surface of LPSs, rather than be suspended in the aqueous medium. In contrast, the concentration of immobilized cells decreased gradually with increasing LSP addition (Fig. 2). This is most likely attributable to the dispersion of fungi biomass caused by increasing LPS addition.

Continued addition of LSPs increased immobilized cells, but low cell concentration on LSPs resulted in the waste of

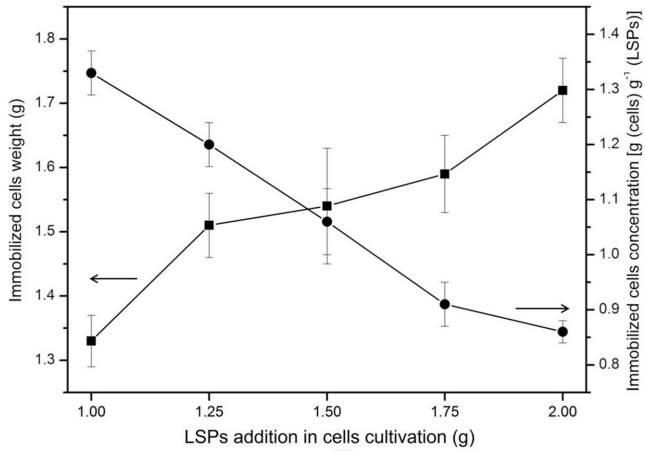

Fig. 2. Effect of LSP addition on immobilized biomass. Culture conditions: 30°C, 180 rpm for 72 h.

carriers. Although the biocatalysts with a full mycelia attachment can be harvested with a small number of LSP additions, low cell weight reflects the underutilization of nutrients in the medium, which increases costs. Based on these two considerations, 1.25–1.50 g of LSPs was utilized in successive trials.

Effects of Biocatalyst Addition on Methyl Ester Yield

The biocatalyst addition is an important parameter for methyl ester yield. In order to examine the optimum amount of biocatalyst, different amounts of dried immobilized cells were brought in to catalyze the reaction. Based on the fact that a high concentration of methanol can result in permanent lipase denaturation [8, 12, 39], 0.35 g of methanol was added stepwise three times to the reaction mixture with 10% water content (PB; pH 7.5, 0.1 M; depending on the oil's weight). As illustrated in Fig. 3, the final methyl ester yield increased after a 72-h reaction when the cell addition was increased from 2% to 8%. However, it did not keep increasing when the cell addition was increased beyond 8%, but instead decreased slightly. Higher cell additions lead to higher lipase amount and faster transformation [5], but very high cell additions can block the mass transfer [20]. Moreover, excessive enzymes could delay lipase activity [9].

Effect of Water Content on Methyl Ester Yield

The level of water is known to be a crucial parameter in a solvent-free system. In this study, the effect of water content was examined at 0%, 3%, 5%, 10%, 15%, and 20% (depending on the oil's weight) in PB (pH 7.5, 0.1 M). Lipase is an enzyme that catalyzes the reaction at the water/oil interface, so a suitable content of water results in an efficient lipase-catalyzed methanolysis reaction by supplying a sufficient number of water/oil interfaces for the reaction to take place [5, 24]. In addition, with a low water content, an insufficient amount of water would lead to the permanent inactivation of lipase, caused by denaturation of the enzyme by high concentrations of methanol [8]. Because *R. chinensis* lipase displays 1(3)-regiospecifity [28], acyl migration can spontaneously occur in any system that contains water, thus improving the final methyl ester yield in the methanolysis reaction. This might explain why the lowest methyl ester yield, 60.2%, was obtained in the absence of water after a 72-h reaction (Fig. 4). As shown in Fig. 4, the methyl ester yield peaked at around 91%, when the water content was 3–10%. A further increase in the water content from 10% to 20% decreased the yield of methyl ester. As water is one of the products in the esterification of methanol and free fatty acids, an excessive water content would inhibit the reaction's progress [24]. Additionally, the reaction efficiency would decrease owing to the obstruction of mass transfer caused by excess water content [5]. Since a higher water content prevents irreversible lipase denaturation by diluting

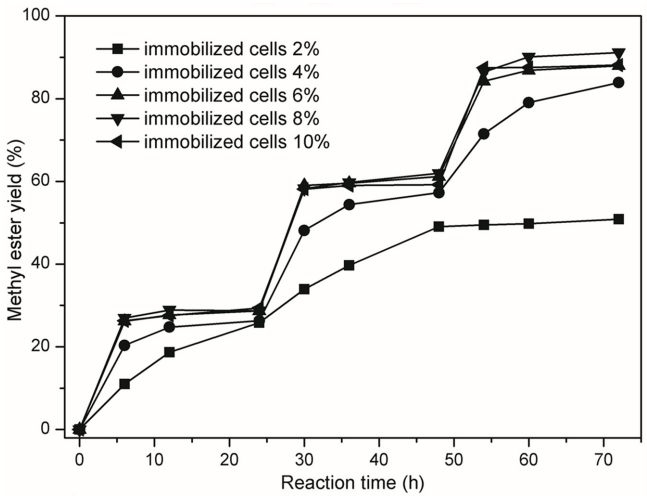

Fig. 3. Effect of biocatalyst addition on methanolysis for production of biodiesel.

Reaction parameters: 9.65 g of soybean oil, 180 rpm, 40°C, 10% water content (PB; pH 7.5, 0.1 M; depending on the oil's weight), addition of 0.35 g of methanol stepwise at periods of 0, 24, and 48 h, respectively, and varying amounts of dried immobilized cells.

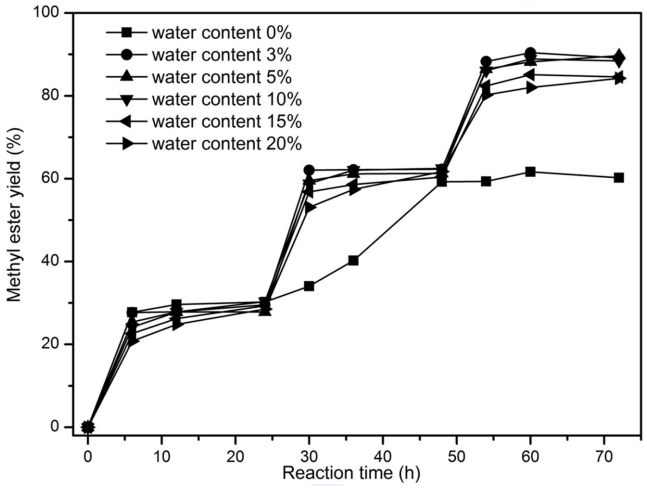

Fig. 4. Effect of water content on methanolysis for production of biodiesel.

Reaction parameters: 9.65 g of soybean oil, 180 rpm, 40°C, 8% dried immobilized cells (depending on the oil's weight), addition of 0.35 g of methanol stepwise at periods of 0, 24, and 48 h, respectively, and varying contents of water (PB; pH 7.5, 0.1 M; depending on the oil's weight).

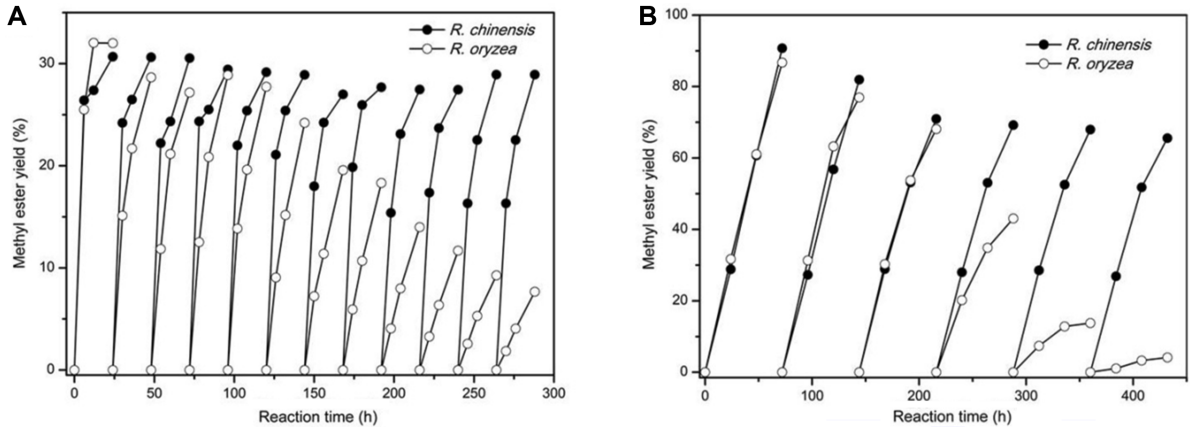

Fig. 5. Repeated methanolysis with *R. chinensis* and *R. oryzae* [18] immobilized cells.

Reaction conditions: 9.65 g of soybean oil, 180 rpm, 40°C (*R. chinensis*) or 35°C (*R. oryzae*), 8% immobilized dried cells (depending on the oil's weight), 10% water content (PB, 0.1 M, pH 7.5 (*R. chinensis*) or 6.8 (*R. oryzae*); depending on the oil's weight). (**A**) Methanolysis was performed with 0.35 g of methanol for every batch cycle. (**B**) Methanolysis was performed with addition of 0.35 g of methanol stepwise at periods of 0, 24, and 48 h, respectively, in every batch cycle.

methanol into a lower concentration throughout the reaction, 10% water content was utilized in successive biodiesel production experiments.

Reusability of LSP-Immobilized Cells

The overall cost of the process can be drastically reduced by reuse of the immobilized cells [38]. Thus, 8% of dried immobilized cells (depending on the oil's weight) was added to a mixture with 10% water content (PB; pH 7.5, 0.1 M; depending on the oil's weight), composed of 9.65 g of soybean oil and 0.35 g of methanol, for replicated reactions in each batch cycle. In our previous study, the reusability of LSP-immobilized *R. oryzae* cells was investigated by carrying out the reaction under similar conditions [18]. The results of *R. oryzae* were used as a reference for measuring the catalytic performance of immobilized *R. chinensis* cells. For immobilized *R. oryzae* cells, we determined that the methyl ester yield decreased gradually as the batch proceeded and resulted in an methyl ester yield of only 7.7% after 12 cycles of reuse (Fig. 5A). Comparatively, the catalytic performance of immobilized *R. chinensis* cells was quite stable and gave an methyl ester content in the reaction mixture of 29.6% after 12 cycles of reuse when an identical amount of immobilized *R. chinensis* cells was utilized as a biocatalyst (Fig. 5A).

The biocatalyst's catalytic stability was calculated by normalizing K_d and $t_{1/2}$ in the Arrhenius equation and fitting to the deactivation model of first order. For the immobilized *R. oryzae* cells, a K_d of 12.89×10^{-2} d^{-1} and a $t_{1/2}$ of 5 days were calculated, whereas immobilized *R. chinensis* cells had a $t_{1/2}$ of 89 days and a K_d of 0.78×10^{-2} d^{-1} (Fig. 6). Compared with immobilized *R. oryzae* cells, which have been widely studied for biodiesel production, the $t_{1/2}$ of immobilized *R. chinensis* was greater by a factor of 16.5, suggesting that *R. chinensis* is more suitable than *R. oryzae* for these applications. It has been documented that intracellular lipase leakage from the *R. oryzae* cells happens consistently under continuous operation [7]. The catalytic activity of *R. chinensis* appeared to be retained, presumably due to reduced intracellular lipase leakage.

To further compare the catalytic performance of

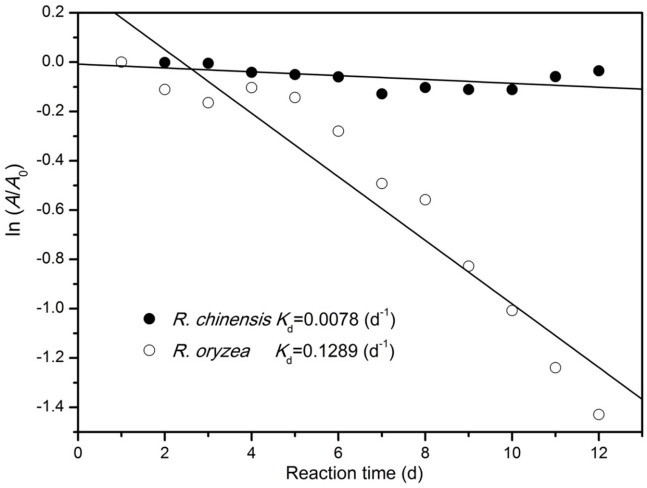

Fig. 6. Deactivation model of *R. chinensis* and *R. oryzae* [18] immobilized cells during repeated methanolysis.

Reaction parameters are as defined for Fig. 5A.

immobilized *R. chinensis* and *R. oryzae* cells, time courses of methanolysis catalyzed by immobilized *R. chinensis* cells with three stepwise additions of methanol were performed in each batch reaction for repeated reactions. As displayed in Fig. 5B, in the case of immobilized *R. oryzae* cells, a methyl ester yield of 4.1% was detected at the completion of the sixth batch cycle, which implies that the catalytic activity had substantially decreased. Comparatively, immobilized *R. chinensis* cells showed a relatively stable catalytic activity throughout the six batch cycles, with methyl ester yield reaching 65.6–90.7% in each cycle during the 72-h reaction.

He *et al*. [19] used freely suspended *R. chinensis* whole cells to catalyze the methanolysis of refined vegetable oil for biodiesel production. The reaction was carried out with three stepwise additions of methanol in a solvent-free system. Under optimal conditions, the highest methyl ester yield was found to be 86.0% after a 72-h reaction, which is lower than ours (90.7%) at the end of the first batch reaction. This is mostly attributed to abundant water/oil interfaces generated by the high hydrophilicity of loofah sponge [26], which provided sufficient reaction sites.

These findings indicate that immobilized *R. chinensis* cells have a more stable catalytic performance for catalyzing methanolysis than the widely used immobilized *R. oryzae* cells, and could be used as a whole-cell biocatalyst for practical biodiesel production. Furthermore, similarly to *R. oryzae,* loofah sponges can also be utilized in biodiesel production as a biomass supporter for immobilization of *R. chinensis* cells. Loofah sponge is renewable and biodegradable, thus making the whole process less costly and more environmentally friendly.

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