Biodiesel synthesis in a solvent-free system by recombinant *Rhizopus oryzae*: Comparative study between a stirred tank and a packed-bed batch reactor

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Keywords: *Rhizopus oryzae* lipase, biodiesel, transesterification, packed-bed reactor

Abstract

A simultaneous synthesis of biodiesel, as fatty acid methyl esters, and monoacylglycerols catalysed by the recombinant *Rhizopus oryzae* lipase immobilized by adsorption on Relizyme OD/403M is presented. The use of this 1(3)-positional specific lipase prevents the formation of glycerol as a by-product, thus avoiding its drawbacks. The synthesis was carried out in a solvent-free system and it has been studied in two different reactor systems: stirred tank and packed-bed reactor.

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Stirred tank reactor presented a high initial reaction rate and achieved a 33.6% yield, which corresponds to a value of 50.4% of the maximum yield that can be achieved with a 1(3)-positional specific lipase. In packed-bed reactor there was a smaller initial reaction rate, but it was achieved a 49.1% yield, which corresponds to a 73.6% of the maximum yield. When a second batch is performed, the yield decreased only 4% when packed-bed reactor is employed whereas a drastic decrease is observed in a stirred tank operation. Therefore, packed-bed reactor showed a best performance and minor damage to the biocatalyst.

Introduction

Fatty acid methyl esters (FAMEs), known as biodiesel, are produced by transesterification of oils and fats with short chain alcohols (mainly methanol), and have been accepted as an important alternative fuel to the current dependence to fossil fuel and also as a key to solve the depletion of these fossil sources (Ognjanovic et al. 2009). In addition, biodiesel production from transesterification of oils and animal fats almost closes carbon cycle, reducing an overall 80% of CO_2 emissions than those of fossil fuels. Moreover emissions of CO, volatile organic compounds and particulates are lower from biodiesel combustion (Van Gerpen 2005).

Currently commercial biodiesel production involves the use of strong alkalis or acid catalyst (Park et al. 2008). Recently, enzyme-catalysed transesterification using lipases has become more attractive for biodiesel production due to several advantages, such as low energy consumption and wastewater generation and also easier glycerol recovery, produced as a by-product (Fukuda et al. 2001; Nielsen et al. 2008). In addition, lipases are able to convert free fatty acids into methyl esters, owing to its esterification activity, whereas soaps are formed when alkaline catalysis is used for oils containing a free fatty acid concentration higher than 0.5% (Talukder et al. 2010). Thus, enzymatic transesterification can be carried out using waste cooking oils as substrates, which contain a higher amount of free fatty acid, removing these contaminant oils from the environment and reducing the production cost of biodiesel. The use of immobilized lipases improves enzyme stability and allows reutilization, being adsorption the most widely used method to immobilize lipase in biodiesel synthesis, because it allows a high activity (Tan et al. 2010).

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In most of the published works, lipase-catalyzed transesterification is carried out in stirred tank reactors (Robles-Medina et al. 2009), consisting of screw-capped glass vials, bottles and shaking flaks under agitation by reciprocal shakers or shaking plates (Li et al. 2010; Li et al. 2011; You et al. 2013). In any case, these laboratory reactor assemblies are very different from real reactor ones. Alternatively, only a few published works have studied transesterification in packed-bed reactors (Robles-Medina et al. 2009). It is worth notifying that different features were observed when stirred or packed bed reactors are used working with immobilized enzyme. On one hand, methanol shows a high degree of dispersion in the oil phase when a stirred reactor is used, although shear stress from the stirring can desorb the enzyme from the carrier. On the other hand, although in packed beds reactors the catalyst doesn't work under shear forces, less methanol dispersion is achieved on oil phase and the by-product glycerol can remain in the bottom of the reactor due to its high viscosity (Shimada et al. 2002; Hama et al. 2007; Tan et al. 2010; Hama et al. 2012;).

Another important key parameter in enzyme-catalyzed transesterifications is the use of organic solvents, which reduces the viscosity of the reaction mixture as well as the mass transfer problems around the enzyme (Fjerbaek et al. 2009). Also some solvents can prevent enzyme inactivation by methanol, such as t-butanol (Robles-Medina et al. 2009). However, from an industrial point of view, transesterification should be performed in a solvent free medium (Adamczak et al. 2009), to make easier final biodiesel recovery.

In this research, the simultaneous methyl esters and monoacylglycerols synthesis were studied. In order to obtain these monoacylglycerols as a by-product instead of glycerol, a 1(3)-positional specific recombinant *Rhizopus oryzae* lipase (rROL) was used, thus preventing inactivation of the biocatalyst due to glycerol, likely because it may cover biocatalyst support particles (Halim et al. 2009; Robles-Medina et al. 2009; Xu et al. 2011; Lotti et al. 2014). *Rhizopus oryzae* lipase has been previously used in other transesterification studies for biodiesel synthesis (Li et al. 2010; Canet et al. 2014; Luna et al. 2014).

Moreover, recent studies have shown that some residual compounds from biodiesel synthesis, including free fatty acids and monoacylglycerols, are responsible for lubricity of blends of biodiesel with conventional diesel and, by the way, pure biodiesel exhibit a reduced lubricity compared to the biodiesel containing these compounds (Hu et al. 2005; Caballero et al. 2009). Also, monoacylglycerols have

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increased its importance as emulsifiers, although a purification process would be necessary (Lotti et al. 2014). In addition, cleaning of glycerol of the final biodiesel is necessary, because it causes several problems, including coking of the fuel (Calero et al. 2015).

Unlike most of the published works, in which transesterifications reactions are carried out in non-real reactors systems, this study was especially focused towards flow regime of the reactor and the mass transfer problems involved in a solvent-free system working with lipase immobilized by adsorption. Therefore, a comparative study between a packed-bed and a stirred tank reactor was made using olive oil as a standard substrate.

Materials and methods

Materials

Methanol and heptane were purchased from Panreac (Barcelona, Spain) (both of high grades) and virgin olive oil from Carbonell (Córdoba, Spain). The lipase colorimetric kit used for the hydrolytic activity assay was obtained from Roche (Roche kit 11821792, Mannheim, Germany). Support particles used to immobilize the lipase, Relizyme OD/403M, was purchased from Resindion rsl (Binasco, Italy). Standards of methyl palmitate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate were purchased from Sigma-Aldrich (St Louis, USA).

Lipase

rROL was produced by the Bioprocess Engineering and Applied Biocatalysis group of Universitat Autònoma de Barcelona (UAB). This lipase was obtained by a fed-batch cultivation of a recombinant *Pichia pastoris* strain using methanol as inductor (Arnau et al. 2010). The culture broth was centrifuged and microfiltered to remove the biomass. The supernatant was concentrated by ultrafiltration with a Centrasette® Pall Filtron system equipped with an Omega membrane of 10 kDa cut-off, and subsequently dialyzed against 10 mM Tris-HCl buffer pH 7.5 and thereafter lyophilized (Guillén et al. 2011).

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Lipase hydrolytic activity and protein concentration

Lipase hydrolytic activity (UA) assay was followed in a Cary Varian 300 spectrophotometer (Varian, Palo Alto, USA) at 30°C in 200 mM Tris-HCl buffer at pH = 7.25 using the Roche lipase colorimetric kit as previously mentioned (Resina et al. 2004). Protein concentration was determined by the method of Bradford using bovine albumin as the standard (Bradford 1976). Samples were analysed in duplicate.

Immobilization on Relizyme OD/403M

rROL was immobilized on Relizyme OD/403M by adsorption as previously reported (Canet et al. 2014).

Fatty acid methyl esters analysis

Methyl esters sample concentration was analysed with a 7890A gas chromatography (Agilent Technologies), as previously reported (Canet et al. 2014).

Enzymatic reaction

Reactions in the packed-bed and stirred tank reactor were done at 30°C. In both cases 200000 UA biocatalyst were used (1 g approximately of biocatalyst) and 50 g of olive oil were employed. No water was added and methanol was fed stepwise in 7 additions of 0.985 ml each one, which corresponds to a molar ratio methanol:oil of 0.43:1 for each addition, to set a final molar ratio methanol:oil of 3:1. These conditions were determined as the best to achieve a high yield (Canet et al. 2014). When stirring speed study for stirred tank reactor and flow rate study for packed-bed reactor were carried out, it was only added one step methanol which means that a 14.3 % yield can be achieved at most.

The packed-bed reactor is a glass jacked column (column length: 160 mm; column internal diameter: 10 mm). The reaction mixture is magnetically stirred in an outside vessel, and this mixture is pumped to the column. An assembly scheme of the packed-bed is presented in Fig. 1. The stirred tank reactor consisted in glass jacked

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vessel (internal diameter: 40 mm) agitated with a propeller-shaped stirrer (stirrer diameter: 28 mm), closed by septum.

Sample analysis

To determine the amount of methyl esters by gas chromatography, samples (approximate 150 µL) of the enzymatic reactions were withdrawn at predetermined time intervals and treated as previously reported (Canet et al. 2014).

Methyl esters yield is defined as the ratio (in percentage) between methyl esters moles and the total initial fatty acids in triacylglycerols (Canet et al. 2014). Taking into account that a 1(3)-positional specific lipase is used, the maximum yield is defined as the maximum possible yield that can be achieved with this lipase.

Methyl esters initial reaction rate was calculated by the slope of the initial evolution of methyl esters concentration (approximately between the first 25 and 50 minutes).

Results and discussion

Key parameters in stirred tank and packed-bed reactors

Effect of stirring speed on stirred tank reactor

Methyl esters initial reaction rate values, as a function of stirring speed is presented in Table 1. Reynolds number (eq.1) increases when agitation is higher, which means that limitations in external mass transfer decrease.

$$\mathrm{Re} = \frac{N \cdot d^2 \cdot \rho}{\mu} \qquad (1)$$

Where N is the impeller rotational speed (s^{-1}); d is the impeller diameter (m); ρ is the fluid density ($kg \cdot m^{-3}$); µ is the fluid viscosity ($kg \cdot m^{-1} \cdot s^{-1}$)

Moreover, considering that the system works in a complete laminar regime, the external mass transfer should be low and probably limiting. Thus, as agitation is increased, methyl esters initial reaction rate should also increase (Table 1). However, working at 1500 rpm a sharp decrease in the methyl esters initial reaction rate value is

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observed, likely due to the desorption from the support particles. As it is related in the literature, the weak forces involved between the enzyme and the support in an immobilization by adsorption can be broken under not soft working conditions (Tan et al. 2010).

Effect of flow rate on packed-bed reactor

Packed-bed reactor whole system is a packed-bed connected to a stirred vessel, where the reaction mixture is pumped again to the packed-bed reactor (Fig. 1).

As can be seen in Table 2, increasing flow rate through the packed-bed reactor from 0.9 to 1.5 ml/min caused an increase in Reynolds number (eq.2) and therefore also a reduction in external mass transfer limitations.

$$\mathrm{Re} = \frac{U \cdot d_p \cdot \rho}{\mu \cdot (1 - \varepsilon)} \qquad (2)$$

where U is the linear velocity through packed-bed ($m \cdot s^{-1}$); d_p is the particle diameter (m); ε is the void fraction of the bed.

Although a higher Reynolds number should led to a higher methyl ester initial reaction rate, when flow rate is increased from 1.7 to 2.4 ml/min, methyl esters initial reaction rate decreased. This can be explained because higher flow rates promote less contact between substrate and enzyme active site, thus decreasing, in some cases, the initial reaction rate (Halim et al. 2009). When the flow rate was increased from 1.5 to 1.7 ml/min, the methyl esters initial reaction rate remained virtually the same ($6.2 \cdot 10^{-3}$-$6.1 \cdot 10^{-3}$ mol $(L \cdot min)^{-1}$), because although the flow rate of 1.7 ml/min reduced the contact between the enzyme and the substrate, this was probably compensated by a decrease in the external mass transfer limitations.

Stirred tank and packed-bed reactor performance

Comparing stirred tank and packed-bed reactor performances, it can be concluded that the required reaction times to reach an equilibrium for each addition are lower for stirrer tank reactor, 60 minutes in front of 90 minutes for packed-bed reactor, due to the higher initial reaction rate achieved in the best working conditions (Table 1

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and 2). Similar results comparing these types of reactor system for solvent-free transesterification were obtained in the literature (Xu et al. 2012).

Nevertheless, in order to study the complete performance of the stirred tank and the packed-bed reactor in transesterification reaction, 7 additions of methanol were done to achieve higher methyl esters yields.

The value of the flow rate chosen in packed bed reactor was 1.5 ml/min as the best determined (Table 2). For the stirred tank reactor, although the highest methyl ester initial rate was achieved at 1000 rpm (Table 1), it was decided to work at 500 rpm, which also produced a quite high methyl esters initial reaction rate, in order to minimize the desorption of the enzyme from the support particle.

Methanol additions were carried out when reaction started to stop and methyl esters concentration remained quite constant.

Reaction performance in stirred tank reactor

The time evolution of the methyl esters yield is presented in Fig. 2, which reached a value of 33.6% after 420 minutes (first batch), which corresponds to a value of 50.4% of the maximum yield that can be achieved with a 1(3)-positional specific lipase. Thus, the yield achieved is not close to the final possible one.

Another important aspect of the results plotted in Fig. 2 is that after each methanol addition, it can be seen graphically that the slopes or the methyl esters initial reaction rates reached are lower than that achieved in the previous one. In the latest additions it can be explained by the fact that triacylglycerols were starting to exhaust and also the approach to the equilibrium of the reaction. After the second or third addition, an increase in reaction rate was expected due to the decrease in the viscosity of the reaction mixture because methyl oleate, which is the main methyl ester obtained, has a viscosity smaller than the olive oil one, decreasing external and internal mass transfer limitations. However, this phenomenon showed not to be important enough to promote an increase in reaction rate.

In order to study the enzyme stability, a second batch was carried out and the methyl esters yield achieved was 7.7%, which means that the biocatalyst had lost practically all its activity during the first batch. This loss of activity can be attributed to the lipase desorption from the support particle during the reaction, although a low stirring speed as 500 rpm was used (Tan et al. 2010).

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Reaction performance in packed-bed reactor

The evolution of methyl esters yield along the time performed in packed-bed reactor is presented in Fig. 3. After 630 minutes the yield achieved a value of 49.1% (first batch), which corresponds to a 73.6% of the maximum yield that can be achieved. This value is clearly higher than the one achieved in the first batch in the stirred tank reactor.

In this case after the second addition, it was observed a slightly higher methyl esters reaction rate compared to the one in the first addition. In contrast with the stirred reactor behaviour, the positive effect of the reduction in the reaction mixture viscosity has as a consequence an increase of reaction rate. In the stirred tank reactor this fact was not observed, probably because of the continuous enzyme desorption from the support, which once desorbed may be inactivated. This reaction rate behaviour in the packed-bed reactor remained approximately constant until just before the fifth methanol addition, when reaction rate started to decrease.

Carrying out a second batch, a methyl esters yield of 44.8% was achieved and also a very close plot to the first batch was obtained, which means that a little enzyme loss or inactivation from biocatalyst had occurred.

On the other hand, it is also important to mention that the packed-bed tends to be slightly compacted. Thus, the value set at 1.5 ml/min slowed down to 1.3 ml/min at the end of the reaction. Although a structural material (sand) has been tested to reduce pressure drop in the packed-bed, the reactor bed tends to compact.

Conclusions

The use of 1(3)-positional specific lipase proposed in this work is able to produce simultaneously biodiesel and monoacylglycerols from olive oil, having as a future goal, the synthesis of this kind of biocombustible from waste cooking or non-edible oils. Stirred tank reactor performed a higher initial reaction rate but the turbulence of the reaction mixture was able to desorb the enzyme from the particles, thus losing its activity. Packed-bed reactor had a smaller initial reaction rate, but enzyme was not desorbed or inactivated and a similar yield was achieved after first and second batch.

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Thus, packed-bed reactor system allows keeping immobilized enzyme stability in free-solvent transesterification reactions better than stirred tank reactor, although packed-bed shows higher mass transfer limitations and bed compaction, as drawback.

Acknowledgements

This work was supported by the project CTQ2013-42391-R of the Spanish Ministry of Economy and Competitiveness. The group is member of 2014-SGR-452 and the Reference Network in Biotechnology (XRB) (Generalitat de Catalunya).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure captions

Fig.1. Scheme of the packed-bed reactor system. (1) Methanol addition gap, (2) Magnetic stirred bottle, (3) Pump, (4) Packed-bed reactor, (5) Biocatalyst, (6) Temperature water system entrances

Fig. 2. Time evolution for biodiesel synthesis in stirred tank reactor, working at 500 rpm. Arrows indicate methanol additions.

Fig. 3. Time evolution for biodiesel synthesis in packed-bed reactor system, working at 1.5 ml/min. Arrows indicate methanol additions.

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Table 1

Methyl esters initial reaction rate and Reynolds number for different impeller rotational speed in stirred tank reactor

Impeller rotational speed (rpm)	500	1000	1500
Methyl esters initial reaction rate ($mol \cdot (L \cdot min)^{-1}$), r_i	$6.6 \cdot 10^{-3}$	$8.0 \cdot 10^{-3}$	$3.2 \cdot 10^{-3}$
Reynolds number	21.6	43.2	64.7

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Table 2

Methyl esters initial reaction rate and Reynolds number for different flow rate through packed-bed reactor

Flow rate (ml/min)	0.9	1.5	1.7	2.4
Methyl esters initial reaction rate ($mol \cdot (L \cdot min)^{-1}$), r_i	$3.9 \cdot 10^{-3}$	$6.2 \cdot 10^{-3}$	$6.1 \cdot 10^{-3}$	$5.5 \cdot 10^{-3}$
Reynolds number	$1.5 \cdot 10^{-3}$	$2.5 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$	$4.1 \cdot 10^{-3}$

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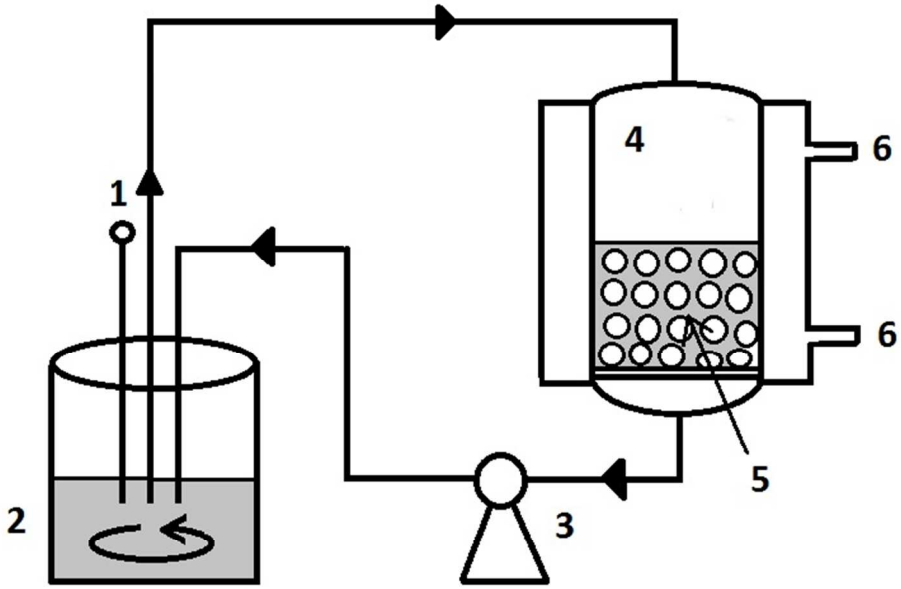

Scheme of the packed-bed reactor system. (1) Methanol addition gap, (2) Magnetic stirred bottle, (3) Pump, (4) Packed-bed reactor, (5) Biocatalyst, (6) Temperature water system entrances
208x136mm (96 x 96 DPI)

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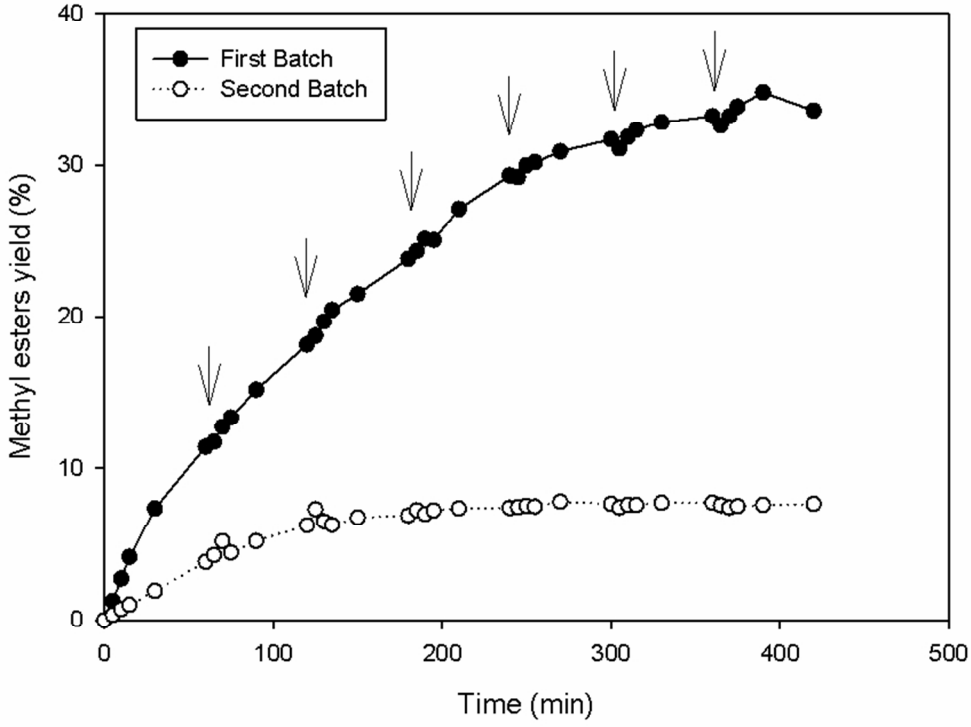

Time evolution for biodiesel synthesis in stirred tank reactor, working at 500 rpm.
148x119mm (150 x 150 DPI)

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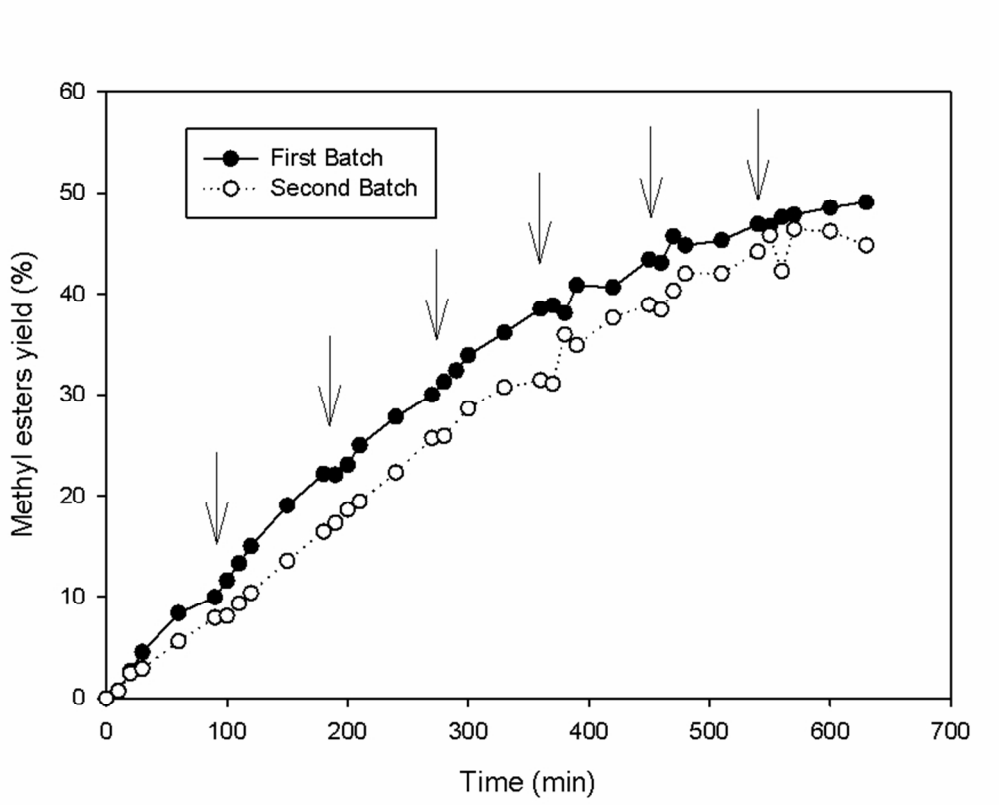

Time evolution for biodiesel synthesis in packed-bed reactor system, working at 1.5 ml/min.
148x119mm (150 x 150 DPI)

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