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Title: Lipase-catalysed transesterification: viewpoint of the mechanism and influence of free fatty acids

Article Type: Research paper

Keywords: biodiesel; lipase; transesterification mechanism; free fatty acids; lipase inactivation; Rhizopus oryzae

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Corresponding Author's Institution: UAB

First Author: Albert Canet, PhD student

Order of Authors: Albert Canet, PhD student; Kirian Bonet, PhD student; María Dolors Benaiges, Associate Professor; Francisco Valero, Dr.

Abstract: A series of lipase-catalysed transesterification experiments were carried out to study the effect of the presence of free fatty acids on synthesis reaction rate and the stability of the biocatalyst, and also to elucidate the underlying mechanism, which remains a subject of debate. Based on the results, the reaction rate and biocatalyst stability increased with increasing content in free fatty acids of the reaction mixture. Also, tests carried out with a mixture of triolein and linoleic acid revealed that the transesterification mechanism is a combination of direct alcoholysis of triacylglycerols and a two-step reaction involving hydrolysis of acylglycerols and further esterification of previously released free fatty acids. The time course of triacylglycerols and diacylglycerols revealed that the enzyme is similarly selective for both types of substrate.

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After checking that experimental error in our initial experiments is small (Canet et al 2014) and taking into account that the used lipase is not a commercial product, since it is a recombinant lipase obtained by fermentation in our laboratory with a limited production and economical limitations to produce it, the experiments in this paper have been carried out once.
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Bellaterra, 27th March2015

Professor C.P. Mitchell
Editors in chief of Biomass and Bioenergy
School of Engineering, King's College, Aberdeen, UK

Dear Sir,

I have just submitted the manuscript entitled "Lipase-catalysed transesterification: mechanism and influence of free fatty acids" by A. Canet, K. Bonet-Ragel, M.D. Benaiges and F. Valero for its submission to Biomass and Bioenergy.

The present original work has been made in the Department of Chemical Engineering at the Universitat Autònoma de Barcelona (UAB) (Barcelona, Spain). All the authors are agreeing to submit, for the first time, to Biomass and Bioenergy.

The work is a study on the effect of free fatty acids in enzymatic transesterification rate and also in enzyme inactivation. Moreover, it provides strong results that lipase transesterification is a combination of two mechanisms: a one-step reaction consisting in a direct acylglycerols alcoholysis and a two-step reaction, involving a first acylglycerols hydrolysis and secondly an esterification.

Looking forward to your news, I remain yours sincerely

Dr. Francisco Valero
Departament d'Enginyeria Química
Universitat Autònoma de Barcelona
08193-Bellaterra (Barcelona) Spain
Fax 34-935812013
e-mail: francisco.valero@uab.es

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Highlights

Free fatty acids increase lipase stability

Elucidated mechanisms transesterification

Free fatty acids increase biodiesel reaction rate

Lipase-catalysed transesterification: viewpoint of the mechanism and influence of free fatty acids

Albert Canet; Kírian Bonet-Ragel; M. Dolors Benaiges; Francisco Valero*

Departament d'Enginyeria Química, EE, Universitat Autònoma de Barcelona, Bellaterra, 08193, Barcelona

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Abstract

A series of lipase-catalysed transesterification experiments were carried out to study the effect of the presence of free fatty acids on synthesis reaction rate and the stability of the biocatalyst, and also to elucidate the underlying process, which remains a subject of debate. Based on the results, the reaction rate and biocatalyst stability increased with increasing content in free fatty acids of the reaction mixture. Also, tests carried out with a mixture of triolein and linoleic acid revealed that the transesterification is a combination of direct alcoholysis of triacylglycerols and a two-step reaction involving hydrolysis of acylglycerols and further esterification of previously released free fatty

acids. The time course of triacylglycerols and diacylglycerols revealed that the enzyme is similarly selective for both types of substrate.

Keywords: biodiesel; lipase; transesterification mechanism; free fatty acids; lipase inactivation; *Rhizopus oryzae*

Introduction

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids (FAME) with chemical and physical properties similar to those of conventional diesel, which make it a promising alternative to diesel fuel [1]. Biodiesel is obtained by transesterification of triacylglycerols — mainly vegetal fats— with short-chain alcohols such as methanol, most often in alkaly catalysis [1]. However, catalysed transesterification in a basic medium requires large amounts of energy and water for purification [1,2]. Also, the formation of soaps from substrates containing more than 0.5 wt% free fatty acids has an adverse impact on yield [2]. This makes the alkaline process unsuitable for substrates such as waste cooking oil [1]. On the other hand, acid catalysis allows oil to be treated with large amounts of free fatty acids but occurs at a much lower rate than alkaline catalysis [3].

In recent years, lipase-catalysed transesterification has become an effective alternative to basic and acid catalysis for biodiesel production. Enzyme-based transesterification uses less energy than chemically catalysed processes; also, unlike basic catalysis, it can be used with substrates containing free fatty acids [4].

Although lipases are widely known to carry out transesterification in the presence of free fatty acids [1,2,4], their effect on transesterification has scarcely been studied to date [5,6,7]. The mechanism for the reactions catalysed by lipases has been represetend by Ping-Pong models [8,9] but in the case of enzyme-based transesterification it has been explained in the light of two different viewpoints [10]. In one, lipase synthetizes esters by direct alcoholysis of triacylglycerols in a single step [8] [11,12]. The other involves hydrolysis of triacylglycerols and subsequent esterification of the resulting fatty acids [10,13,14,15].

In this work, we examined the effects of free fatty acids on the rate of the transesterification reaction and the stability of the biocatalyst. To this end, we performed series of experiments intended to elucidate whether the transesterification process involves a single step or two.

The enzyme used was recombinant lipase from *Rhizopus oryzae* obtained by culturing *Pichia pastoris* [16]. This lipase, which is a1,3-positional selective, was previously used in immobilized form on a support that was immersed in a solvent-free medium to develop an environmentally friendly process. The enzyme has also been used for transesterification in a solvent-free system [17].

Materials and Methods

Materials

High-grade methanol and heptane were purchased from Panreac (Barcelona, Spain). The oil used was virgin-grade olive oil for household use. The substrate triolein was purchased from TCI Europe N.V. (Zwijndrecht, Belgium). The other substrates (methyl oleate and methyl linoleate) were obtained from Sigma–Aldrich (St Louis, MO, USA), and so was the derivatizing reagent (MSTFA). High-grade standards of linoleic acid, stearic acid, oleic acid, linoleic acid, methyl palmitate, methyl stearate, methyl oleate, methyl linoleate, methyl linolenate, monoolein (DL-α-monoolein) and diolein (1,3-diolein) used for calibration were also supplied by Sigma–Aldrich. High-grade triolein was purchased from Acros Organics (Geel, Belgium).

The support for lipase immobilization, Relizyme OD403/S, was obtained from Resindion S.r.l. (Binasco, Italy), and the lipase colorimetric kit used to assess enzyme activity (ref. 11821792) from Roche (Mannheim, Germany).

Lipase

Recombinant *Rhizopus oryzae* lipase was produced by the Bioprocess Engineering and Applied Biocatalysis group of the Universitat Autònoma de Barcelona (UAB). The enzyme was obtained by fed-batch cultivation of a recombinant *Pichia pastoris* strain

using methanol as inductor [16]. The culture broth was centrifuged and micro-filtered to remove biomass, after which the supernatant was concentrated by ultrafiltration on a Centrasette® system from Pall Filtron (New York, USA) furnished with an Omega membrane of 10-kDa cut-off, and subsequently dialysed against 10 mM Tris-HCl buffer at pH 7.5 and thereafter lyophilized [18].

Determination of lipase activity and total protein

Lipase activity was determined in 200 mM Tris-HCl buffer at pH 7.25 at 30 °C, using the Roche lipase colorimetric kit on a Varian 300 spectrophotometer from Cary (Palo Alto, CA, USA) [19]. Total protein was determined by using the Bradford method with bovine albumin as standard [20]. Both enzyme activity and total protein were determined in triplicate.

Lipase immobilization

A volume of 100 ml of 5 mM phosphate buffer at pH 7 containing about 5000–6000 UA lipase/ml was used to dissolve lyophilized rROL under magnetic stirring at 4 °C for 1 h. The solution was then centrifuged and the supernatant mixed with 1000 mg of pre-treated support at 4 °C for immobilization under slow stirring with a roller for 7 h. Then, the solution was vacuum-filtered and the biocatalyst washed with 300 ml of the same initial phosphate buffer. Finally, the biocatalyst was dried to constant weight in a desiccator containing silica gel and stored at –20 °C until use.

The support was pre-treated by mixing 1000 mg of Relizyme OD403/S with 100 ml of water–acetone solution for 30 min. Then, the solution was vacuum-filtered and washed several times with water to completely remove the acetone.

Transesterification experiments

All transesterification reactions were carried out in 10 ml vials at 30 °C under continuous stirring in an incubator (20 mm orbital diameter, IKA KS 400 ic, Staufen, Germany). All reaction media contained 32 000 UA of biocatalyst.

The reactions used to study free fatty acids were carried out by mixing specific amounts of olive oil and oleic acid with a final weight of 8 g and adding 160 µl of methanol. For catalyst stability tests, the biocatalyst was allowed to settle at the end of the reaction and the medium removed. The vials containing the biocatalyst were stored at 4 °C until reuse.

Transesterification process was studied by using 8 g of two different mixtures of triolein and linoleic acid with 160 µl of methanol.

The time course of acylglycerols was studied by using 8 g of two different mixtures of triolein and oleic acid to which methanol was added stepwise at 30 or 60 min intervals. Seven methanol additions of 160 µl each were carried out in each experiment.

The densities of triolein, olive oil and oleic acid are very close so the final volume reached is the same for all the experiments described above.

Sample preparation and determination of methyl esters, free fatty acids and acylglycerols

Samples of the reaction mixture were withdrawn at preset intervals and passed through a PVDF filter of 0.45 µm pore size from Millipore (Billerica, MA, USA) to remove all biocatalyst. This was followed by storage at –20 °C until analysis for methyl esters, free fatty acids and acylglycerols. Analyses involved centrifuging the samples at 10 000 rpm for 3 min, withdrawing an aliquot of 10 µl from each with a micropipette, weighing on analytical balance and dilution with heptane. Weighing was required because the high viscosity of acylglycerols and also, to lesser extent, methyl esters, precluded accurate withdrawal of a given volume with a micropipette [17]. The average standard errors for the compound concentrations in each sample was 2.57%.

Methyl esters (viz., methyl palmitate, stearate, oleate, linoleate and linolenate) and free fatty acids (viz., palmitic, stearic, oleic and linoleic) were analysed on a 7890A gas chromatograph from Agilent Technologies (Santa Clara, CA, USA) equipped with a G4513A auto-sampler and a 19095N-123 INNOWAX (30 m x 0.53 mm x 1 µm), both from Agilent Technologies. The software used was Agilent ChemSation from Agilent Technologies. The initial oven temperature, 130 °C, was raised to 240 °C at 16 °C/min and held at that level this for 24 min. The temperatures of the injector and flame ionization detector were 250 and 280 °C, respectively. Helium at a constant flow rate of 3.699 ml/min was used as carrier gas. All samples were centrifuged at 10 000 rpm for 3 min prior to analysis. No further sample preparation was needed.

Mono-, di- and triolein were analysed with the same gas chromatograph and auto-sampler as the methyl esters and free fatty acids. A capillary column BD-EN14105 (10 m x 0.32 mm x 0.1 µm, Part number 123-BD01 from Agilent Technologies) was used tied to an on-column inlet with a high-temperature retention gap. The initial oven temperature, 50 °C, was held for 1 min, raised to 180 °C at 15 °C/min, then to 230 °C at 7 °C/min and 370 °C at 10 °C/min, and held for 5 min. The flame ionization detector was kept at 380 °C. Helium at a constant flow rate of 3 ml/min was used as carrier gas. Samples were prepared somewhat in accordance with European standard EN14105. 100 ml of centrifuged and diluted sample in heptane (as previously described) were derivatized with 10 µl of MSTFA at room temperature mixing for 3 min. This was followed by addition of 0.8 ml of heptane after 30 min. It should be noted that chromatographic peaks for acylglycerols are somewhat difficult to integrate because the different fatty acids combine into similar acylglycerol structures that are difficult to separate [21]. This led us to estimate a molar balance for each sample —the combined amount of triolein, diolein and monoolein should be constant in all experiments— the average error in which was estimated to be 7.27%.

Results and discussion

Figure 1.1 depicts the alcoholysis of triacylglycerols in a single step. As can be seen, an acyl donor —usually methanol— breaks the ester bond in the triacylglycerol skeleton

and causes the formation of an ester bond between the acyl donor and a hydroxyl group in the glyceride structure.

The two-step reaction (Fig. 1.2) involves hydrolysis of the triacylglycerol molecule to release a free fatty acid, followed by esterification of the fatty acid by an acyl donor. This is a cyclical process because water released in the esterification reaction hydrolyses a fatty acid moiety in a glyceride. The fact that, as stated above, lipase-catalysed transesterification can occur in the presence of free fatty acids, led us to examine their influence on methyl esters and the two-step reaction proposed for the process.

Influence of free fatty acids on transesterification

The effect of free fatty acids on transesterification was examined by using different mixtures of olive oil with variable concentrations of free oleic acid over the range 0–20%. Figure 2 shows the time course of FAME. Increasing the free fatty acid concentration increased the initial FAME production rate, albeit not proportionally; thus, the rate increased from $1.8 \cdot 10^{-4}$ mol/min in the absence of added acid to $2.6 \cdot 10^{-4}$ and $3.6 \cdot 10^{-4}$ mol/min in the presence of 5 and 20% added acid, respectively. Similar results were obtained by Li S. *et al* and Du W. *et al* [5] [7]. The fact that the FAME production rate increased with increasing amount of free fatty acids suggests that the transesterification reaction is a two-step process. However, the concentration of free fatty acids remained virtually constant except when no acid was added (Fig. 2). The increase in transesterification rate can be ascribed to a decrease in viscosity as more free oleic acid was added to the reaction mixture. With no acid added, the oleic acid concentration increased slightly as a result of the water initially contained in the biocatalyst facilitating hydrolysis of triacylglycerols by lipase.

Figure 3 illustrates the effect of the initial presence of free fatty acids on biocatalyst stability. As can be seen, stability increased with increasing initial concentration of free fatty acids. Above 10%, the biocatalyst was reusable over up to 10 cycles with a loss of activity of only about 10%; in absence the FFA, however, the catalyst lost 50% of its activity after 7 cycles and was almost inactivated after 10. The same behavior was observed by Du W. *et al* [5]. Loss of activity is widely attributed to

methanol inactivation, due to the contact of insoluble methanol with the enzyme [6,17]. Adding FFA to the reaction increases the polarity of the medium and therefore methanol becomes more soluble in the medium [6], reducing enzyme inactivation. A similar explanation is found in the literature [5], stating that the lower the value of logP, the higher lipase tolerance to methanol.

Elucidation of the transesterification process

As stated before, the concentration of free oleic acid remained constant throughout the transesterification reaction (Fig. 2). Therefore, the reaction must have occurred by direct alcoholysis of triacylglycerols (Fig. 1.1) or new oleic acid molecules coming from triacylglycerols hydrolysis, meaning that the real transesterification process is the two-step reaction (Fig. 1.2).

The fate of free fatty acids (FFA) was traced in an experiment using triolein and free linoleic acid in order to check whether FFA came from the hydrolysis of triacylglycerols since the hydrolysis of triolein would have given oleic acid as the sole fatty acid. Linoleic acid was chosen because it has the same carbon chain length as oleic acid plus an additional double bond, and also because both the ester and acid forms of the two acids can be quantified separately by gas chromatography. In addition, we assessed lipase selectivity towards oleic and linoleic acid by using an equimolar mixture of the two acids. As can be seen from Figure 4, rROL was identically selective for both acids. This result is consistent with previous reports [17].

Two different experiments using two different initial linoleic acid concentrations (10 and 20%) were performed. Figures 5 and 6 show the variation of the concentrations of oleic and linoleic acids, and their corresponding FAME, at an initial linoleic acid concentration of 10% and 20%. As can be seen, the total acid concentration remained virtually constant in both experiments. This suggests that the water molecule released when a linoleic acid molecule was esterified was rapidly used by lipase to hydrolyse triolein to a free oleic acid molecule. Also, the hydrolysis reaction was faster than the esterification reaction —otherwise, the amount of free oleic acid formed moles appeared would not have been the same as that of linoleic acid consumed and the total concentration of acid would not have remained unchanged as a result. Therefore, it can

be clearly concluded that the transesterification reaction occurs at least by the so-called two-step process (Fig. 1.2) by which water released by esterification of a free fatty acid is rapidly used to hydrolyse a triacylglycerol and produce another molecule of free fatty acid, after which the process is restarted. If this assumption is correct, the initial reaction mixture should only contain free linoleic acid and the initially formed esters should be linoleate esters mainly since virtually no free oleic acid would have yet been released. In the presence of 10% linoleic acid, however, the amount of methyl oleate at the beginning of the bioprocess exceeded that of methyl linoleate. Also, the initial reaction rate should have increased with increasing free fatty acid concentration, but the initial rate of methyl oleate formation rate was virtually the same in both experiments. Therefore, the transesterification reaction also involves direct alcoholysis of triacylglycerols.

In conclusion, lipase-catalysed transesterification is a combination of two processes, namely: direct alcoholysis of triacylglycerols in a one-step reaction and a two-step hydrolysis of triacylglycerols followed by an esterification.

One other major inference from the results with 10 and 20% linoleic acid (Figs. 5 and 6, respectively) is that the mole balance for this acid differed between the two experiments. The total initial amount of free linoleic acid should have been the combination of the final amount of free linoleic acid and that of linoleate ester. This was not the case, however, because some free linoleic acid was incorporated by the enzyme into the diacylglycerols or monoacylglycerols formed by transesterification (Fig. 7). As can be seen from Figure 6, although transesterification stopped within 30 min owing to the depletion of methanol, linoleic acid continued to be consumed and oleic acid formed.

Evolution of acylglycerols

Lipase-catalysed transesterification involves a number of compounds including triacylglycerols that are converted into diacylglycerols and then into monoacylglycerols with formation of esters. Also, the activity of lipase is known to be closely linked to the polarity of the substrate and the reaction rate to depend on its structure [22]. As a result, lipase selectivity towards acylglycerols during transesterification may change not only

because acylglycerol structure changes by effect of the conversion of triacylglycerols into mono- and dioacylglycerols, but also because these substrates differ in polarity.

This led us to examine the evolution of all species involved in the transesterification reaction including acylglycerols. The analysis of acylglycerols was complicated by the large number of chromatographic peaks given by the large variety of long-chain fatty acids present in oil. An experiment was thus performed with triolein as the sole substrate that yielded triolein, diolein and monoolein alone. The experiment was carried out in the presence and absence of free oleic acid in order to assess its potential effects on lipase selectivity towards acylglycerols. Seven different methanol additions were done corresponding to the stoichiometric molar relationship to triolein. This stepwise addition procedure was repeated at 30 and 60 min intervals to assess lipase inactivation by methanol throughout the transesterification reaction.

Figures 8 and 9 show the results obtained with methanol additions every 30 min, with 20 and 0% of initial free oleic acid, respectively. Adding methanol at 30 min intervals to a medium containing triolein but no free fatty acids (Fig. 9) led to gradual accumulation of excess alcohol not used in the reaction and to complete inactivation of the enzyme after the second addition. This was not the case in the presence of 20% of free oleic acid (Fig. 8) because the reaction was faster, so methanol accumulated to a lesser extent between additions; also, as noted earlier, the presence of oleic acid reduced the inactivation effect of methanol.

Figures 10 and 11 show the results obtained with methanol additions at 60 min intervals, with 20 and 0% of initial free oleic acid, respectively. With 20% of free oleic acid (Fig. 10), the addition interval had virtually no effect on the reaction and the FAME formation profile was independent of the rate of methanol addition, comparing it to the case of adding methanol every 30 min (Fig. 8). In the absence of free fatty acids (Fig. 11), however, adding methanol every hour prevented inactivation of the enzyme —which occurred with methanol additions at half-hour intervals (Fig. 9)— and the reaction yield was higher as a result.

As previously found, the concentration of free oleic acid remained virtually constant throughout the reaction (Fig. 2). This was also the case with these experiments (Figs. 8-11), but only between the first and second methanol addition. After that, the

concentration of free oleic acid decreased as the reaction developed. As stated above, free fatty acids reduce lipase inactivation by effect of the high polarity of methanol; therefore, a decrease in free fatty acid concentration can lead to inactivation of the enzyme. This was not the case, however, probably because diacylglycerols, monoacylglycerols and esters present in the reaction medium were more polar than triacylglycerols and hence similar to free fatty acids in their effect. It can thus be concluded that lipase stability against methanol was not specifically improved by free fatty acids, but rather by polar enough substances to buffer the high polarity of the alcohol.

No significant differences in lipase selectivity towards triacylglycerols and diacylglycerols were observed (Figs. 8-11). If rROL had been more selective for triacylglycerols than diacylglycerols, diolein and triolein would have accumulated to some extent rather than being thoroughly consumed from the reaction medium. In fact, the amount of diolein decreased even in the presence of substantial amounts of triolein.

Conclusions

One of the main advantages of lipase-catalysed over basic-catalysed transesterification is that the former process can be carried out in the presence of free fatty acids. Also, as shown here, free fatty acids increase the reaction rate and the stability of the biocatalyst. In fact, free fatty acids protect the enzyme by effect of their polarity buffering the high polarity of methanol. This also seems to be the case with other transesterification substrates such as mono- and diacylglycerols.

This study also demonstrates that transesterification is a combination of two processes, namely: direct alcoholysis of triacylglycerols and a two-step reaction involving hydrolysis of triacylglycerols followed by esterification of previously released free fatty acids.

Also, rROL is similarly selective towards tri- and diacylglycerols, so no diacylglycerol accumulation occurs during the reaction —if it does at any time, the amount of methanol to be added should be altered to avoid it and prevent inactivation of the enzyme.

Acknowledgements

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Figure Captions

Fig. 1 – Proposed transesterification processes found in literature. Subfigure 1 corresponds to one-step reaction direct triacylglycerol alcoholysis. Subfigure 2 corresponds to two-steps reaction involving a first hydrolysis and a second esterification.

Fig. 2 – Methyl esters formation and free fatty acids evolution for substrates with different amounts of free oleic acid. Dot lines and black symbols correspond to free fatty acids and solid lines to methyl esters. ◊: 20% free oleic acid, Δ: 10 % free oleic acid, □: 5 % free oleic acid, x: 2.5% free oleic acid and ○: 0% free oleic acid.

Fig. 3 – Residual yield for substrates with different amounts of free oleic acid. The yield is normalized to the first one. Empty bar: 0% free oleic acid, Solid bar: 2.5% free oleic acid, Striped bar: 5% free oleic acid, Dot bar: 10% free fatty acid and Grey bar: 20% free oleic acid.

Fig. 4 – Methyl esters formation and free fatty acids evolution. The reaction was carried out using an equimolar mixture of linoleic and oleic acid. Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

Fig. 5 – Methyl esters formation and free fatty acids evolution. The reaction was carried out using a mixture of 90% triolein and 10% free linoleic acid (in weight). Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

Fig. 6 – Methyl esters formation and free fatty acids evolution. The reaction was carried out using a mixture of 80% triolein and 20% free linoleic acid (in weight). Dot line corresponds to free fatty acids and solid line to methyl esters. Δ: linoleic and ○: oleic.

Fig. 7 – Incorporation of free fatty acids to acylglycerols.

Fig. 8 – Evolution for all the species involved in transesterification reaction. Methanol was added every half hour and the reaction medium contained initially 20% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

Fig. 9 – Evolution for all the species involved in transesterification reaction. Methanol was added every half hour and the reaction medium contained initially 0% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

Fig. 10 – Evolution for all the species involved in transesterification reaction. Methanol was added every hour and the reaction medium contained initially 20% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

Fig. 11 – Evolution for all the species involved in transesterification reaction. Methanol was added every hour and the reaction medium contained initially 0% of free oleic acid. x: triolein, ◊: diolein, □: monolein, Δ: free oleic acid and ○: methyl oleate.

Figure 1

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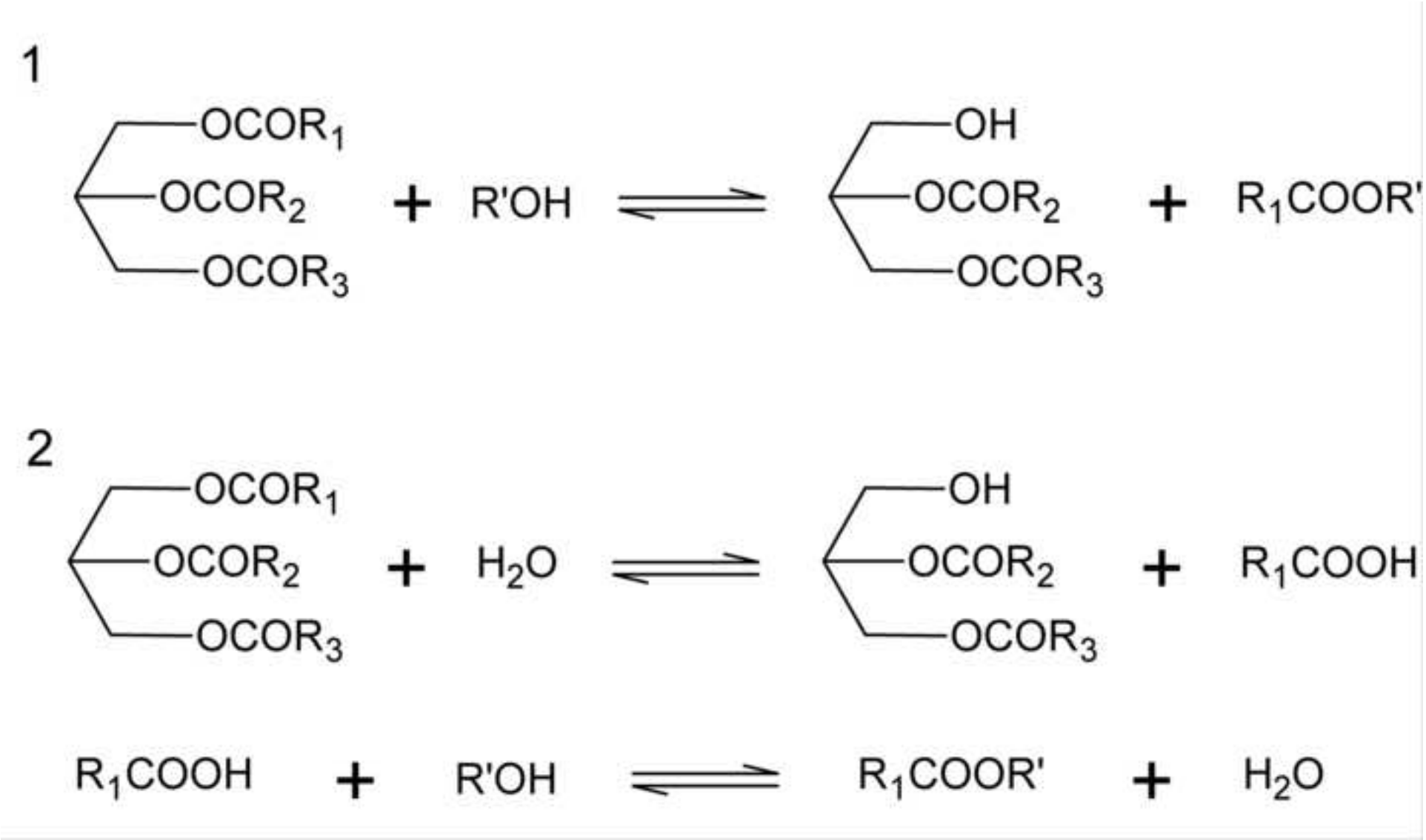

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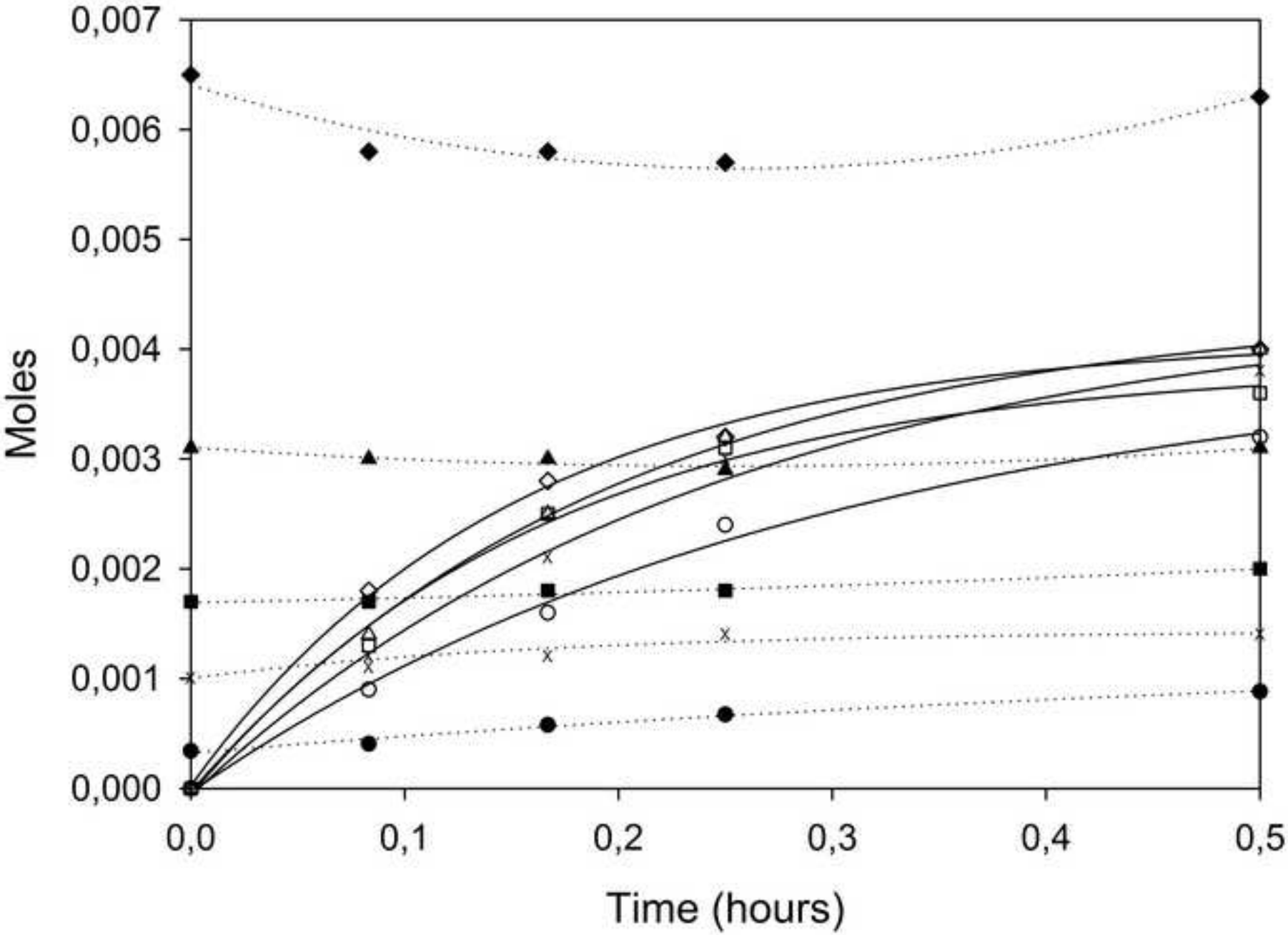

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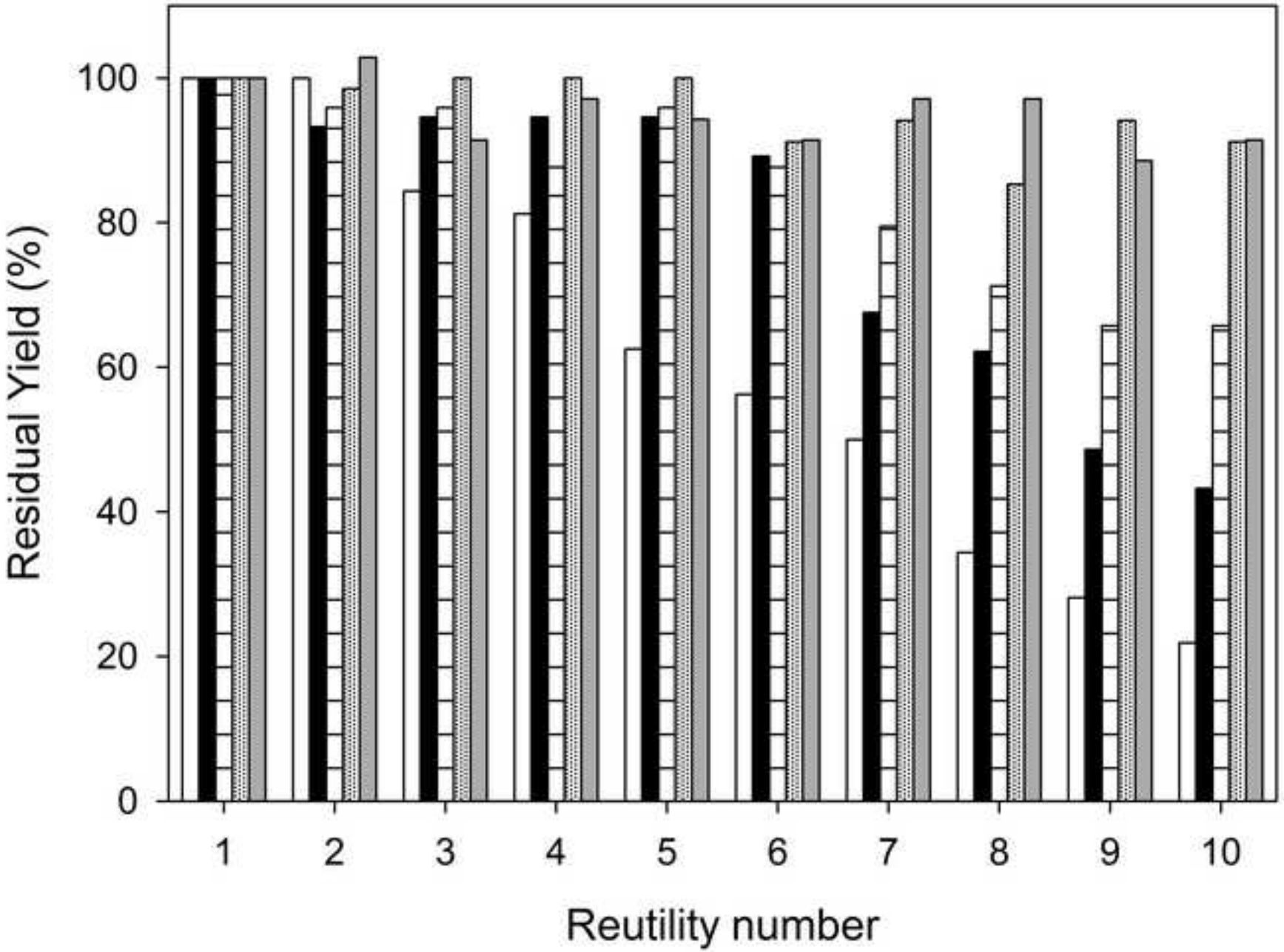

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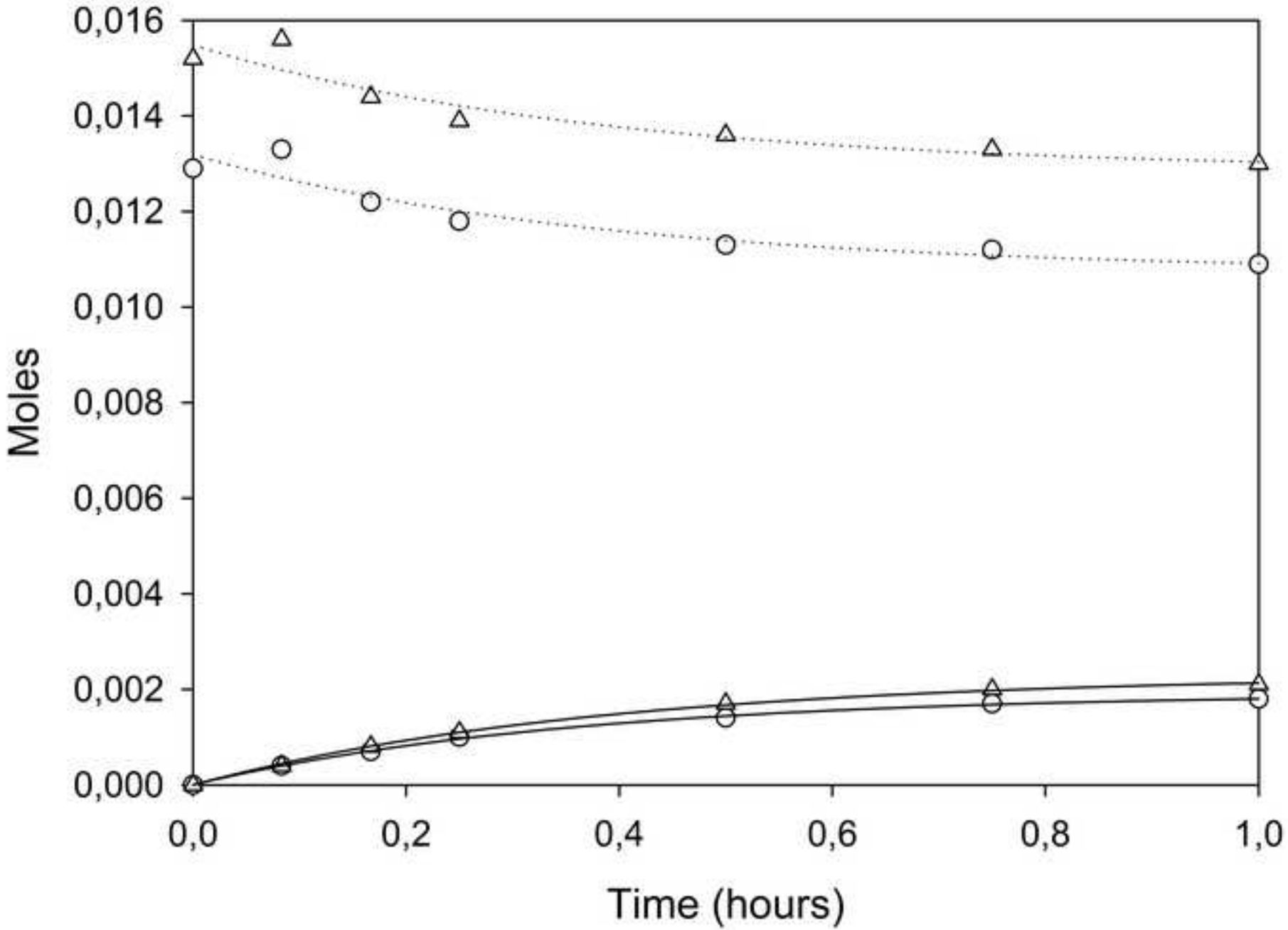

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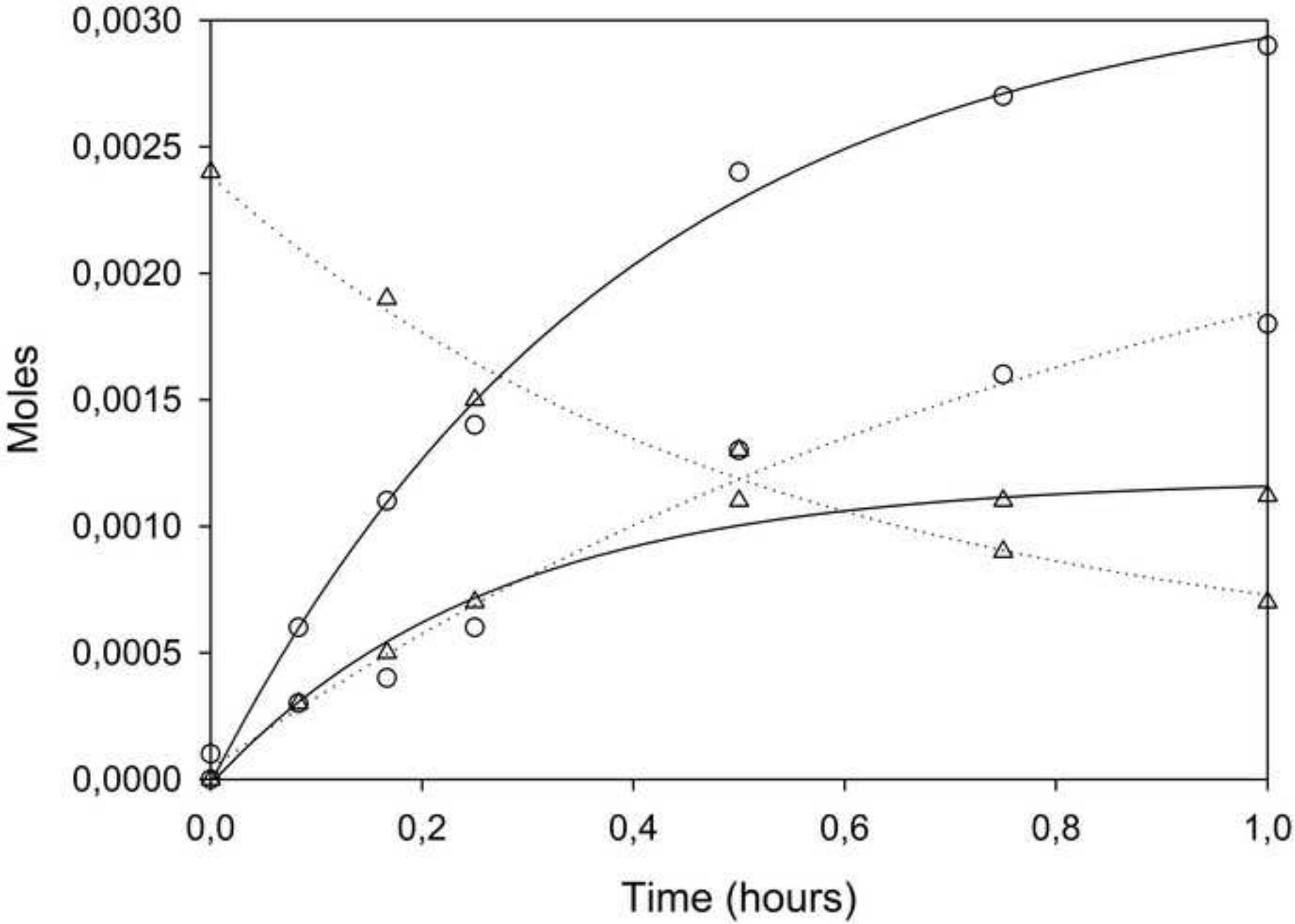

Figure 6

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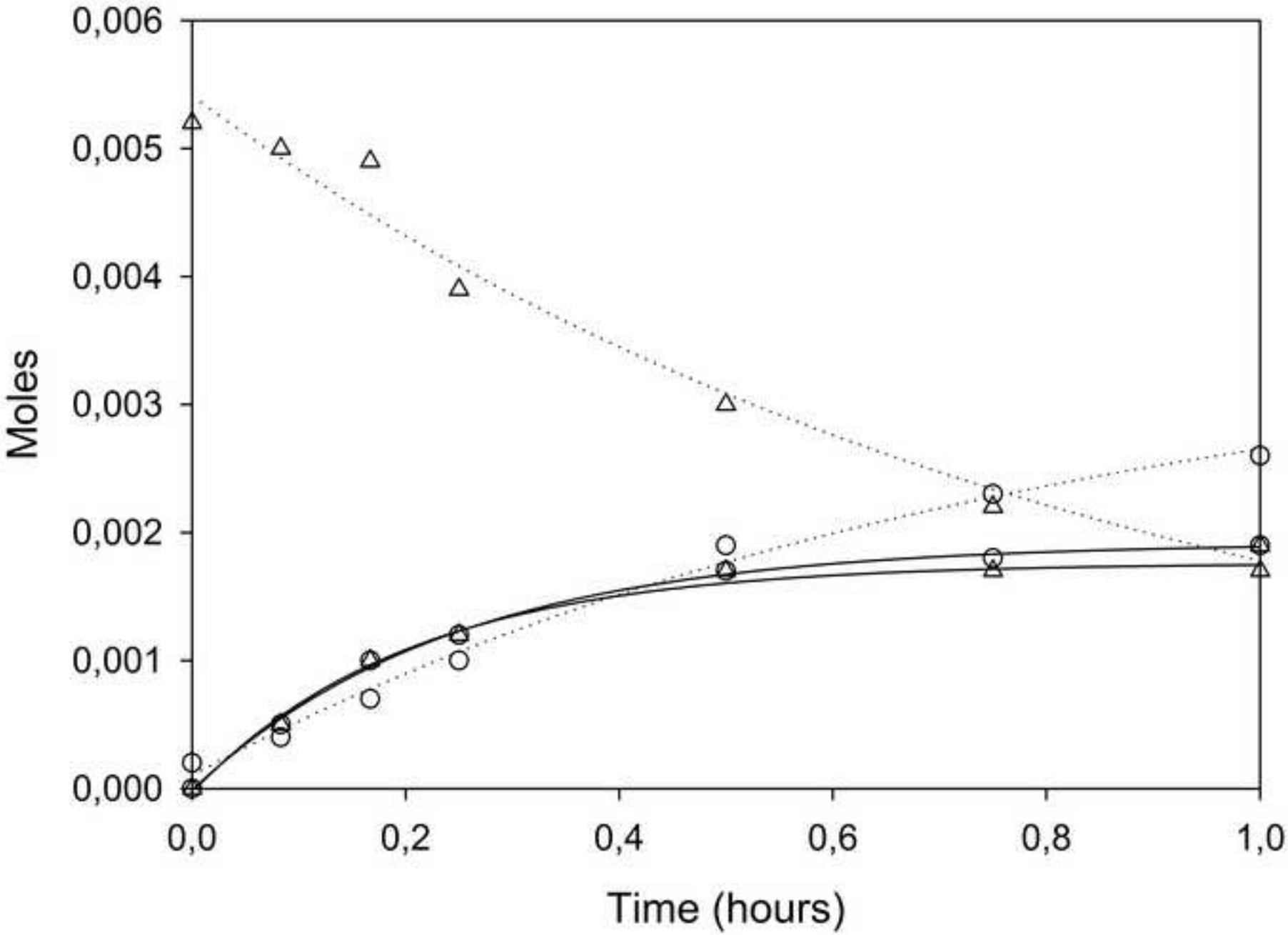

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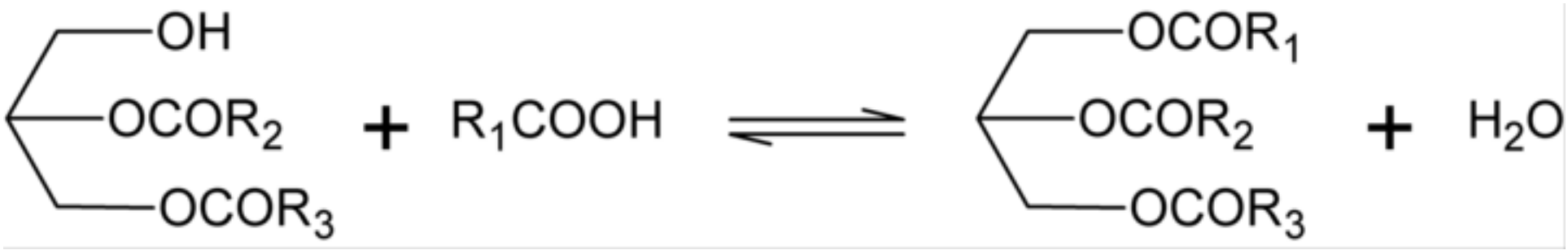

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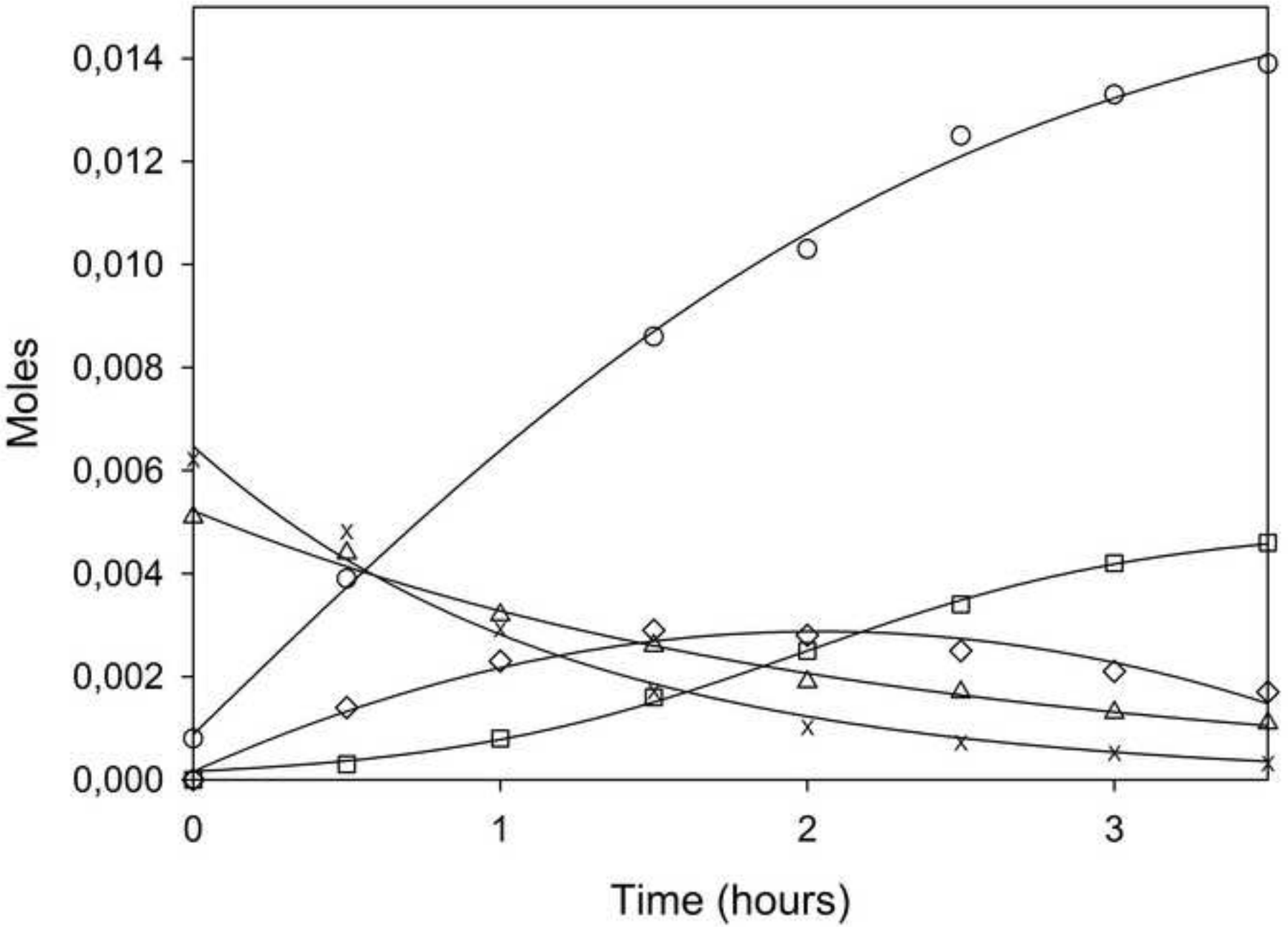

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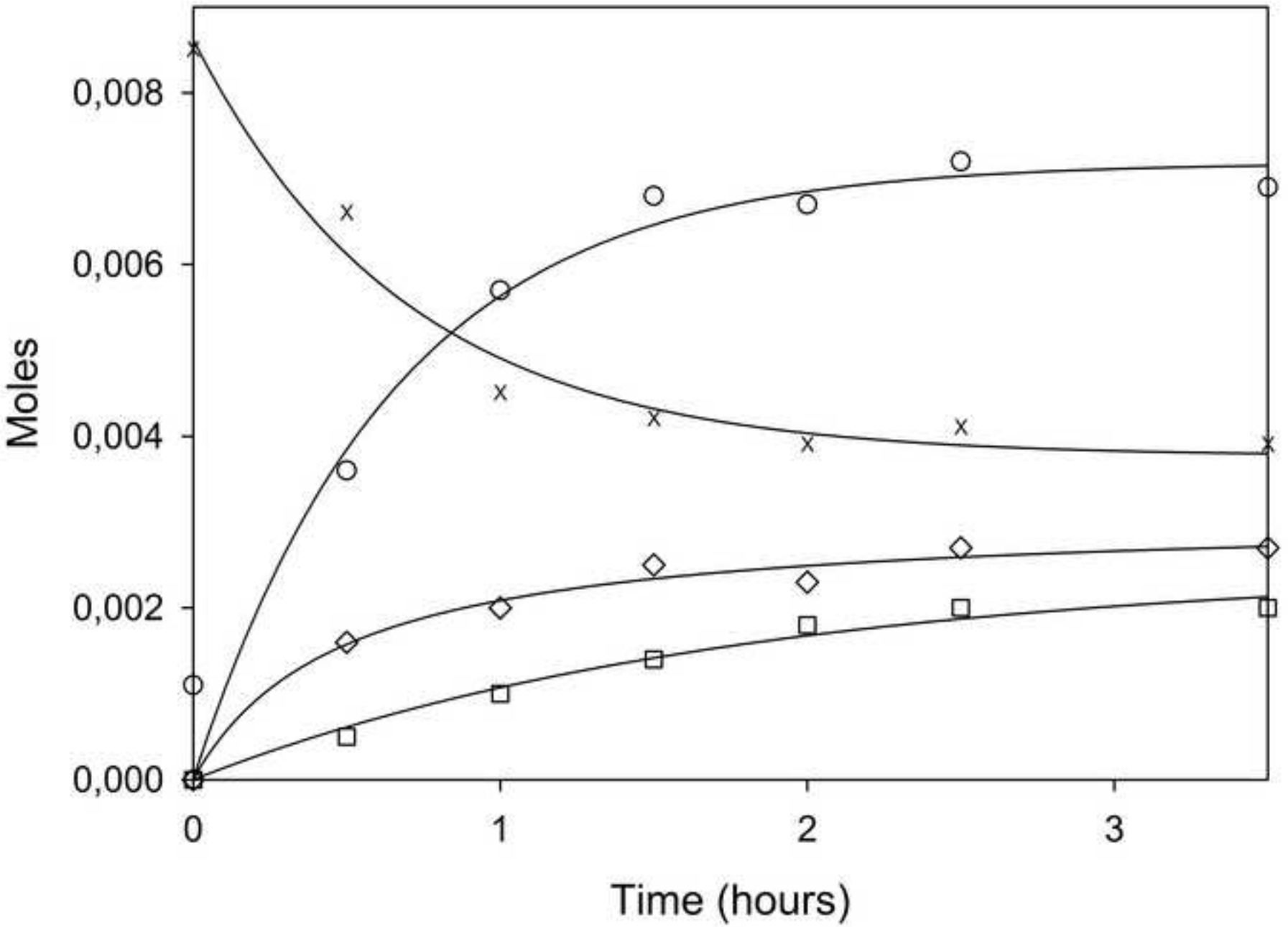

Figure 10

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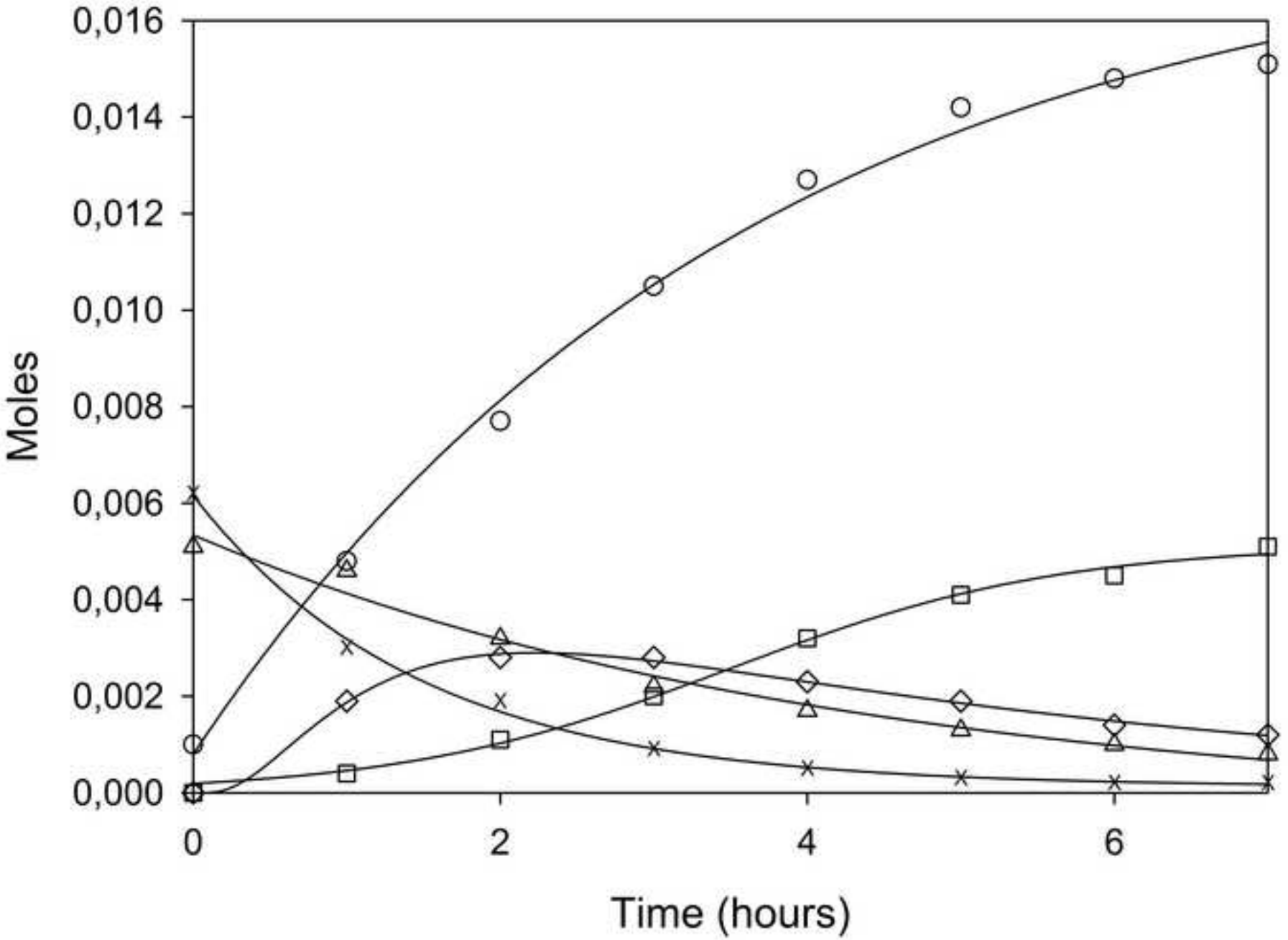

Figure 11

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