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Elsevier Editorial System(tm) for Enzyme and Microbial Technology

Manuscript Draft

Manuscript Number:

Title: Reaction strategies for the enzymatic synthesis of neopentyl glycol diheptanoate

Article Type: Research Paper

Keywords: Lipase; stepwise addition; solvent-free; neopentyl glycol diheptanoate

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Manuscript Region of Origin: SPAIN

Abstract: This work describes for the first time the green synthesis of neopentyl glycol diheptanoate in a solvent-free medium via an enzymatic pathway. The process has been carried out in an open-air reactor in order to ease water removal through evaporation and shift the chemical equilibrium towards product formation. The inhibiting effect of high concentrations of heptanoic acid has been put into evidence by a reduction of initial reaction rate when esterification was performed with stoichiometric amounts of substrates. Therefore, in this work different strategies for the stepwise addition of heptanoic acid are proposed, and best results were obtained when stoichiometric quantities of acid were divided in four equal amounts and added when previous batch was consumed. Biocatalyst Novozym® 435 concentration and temperature were optimised, giving yields higher than 95% in neopentyl glycol diheptanoate when 7.5% (w/w) and 70 °C where used. With a remaining 5% of heptanoic acid (probably caused by the monoester evaporation) the addition of neopentyl glycol led to a conversion of 99.8%. Thus, product can be used in cosmetics without further purification and can be labelled as "natural" because of its enzymatic origin.

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Murcia, June 27 2019.

Dear Prof. S. W. May,

We submit the article entitled “Reaction strategies for the enzymatic synthesis of neopentyl glycol diheptanoate" to be considered for publication in *Enzyme and Microbial Technology*.

This work describes for the first time, the enzymatic synthesis of neopentyl glycol diheptanoate by using a solvent-free process and the well-known commercial biocatalyst Novozym® 435. For this purpose, different reaction strategies have been performed, pointing out the inhibiting effect of the acid on the biocatalyst during the esterification process. This has been overcome through a stepwise acid addition.

As the synthesis has been optimized, the high conversion yields, the mild operation conditions and the fact that is unnecessary the reprocessing of the ultra-pure product are promising factors to turn economically profitable the process. This method is also environmental friendly, as the reaction media did not contained any organic solvent and little waste is generated. Therefore, the process developed can be clearly classified as a green process because it obeys many of the Green Chemistry Principles.

Yours sincerely,

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Authors' agreement

The contents represent original scientific contribution which has not been previously published, they are not under consideration for publication elsewhere and the publication is approved by all the authors and by the responsible authorities of our department. Likewise, we have understood the copyright issues and agree to the terms and agreement of the journal.

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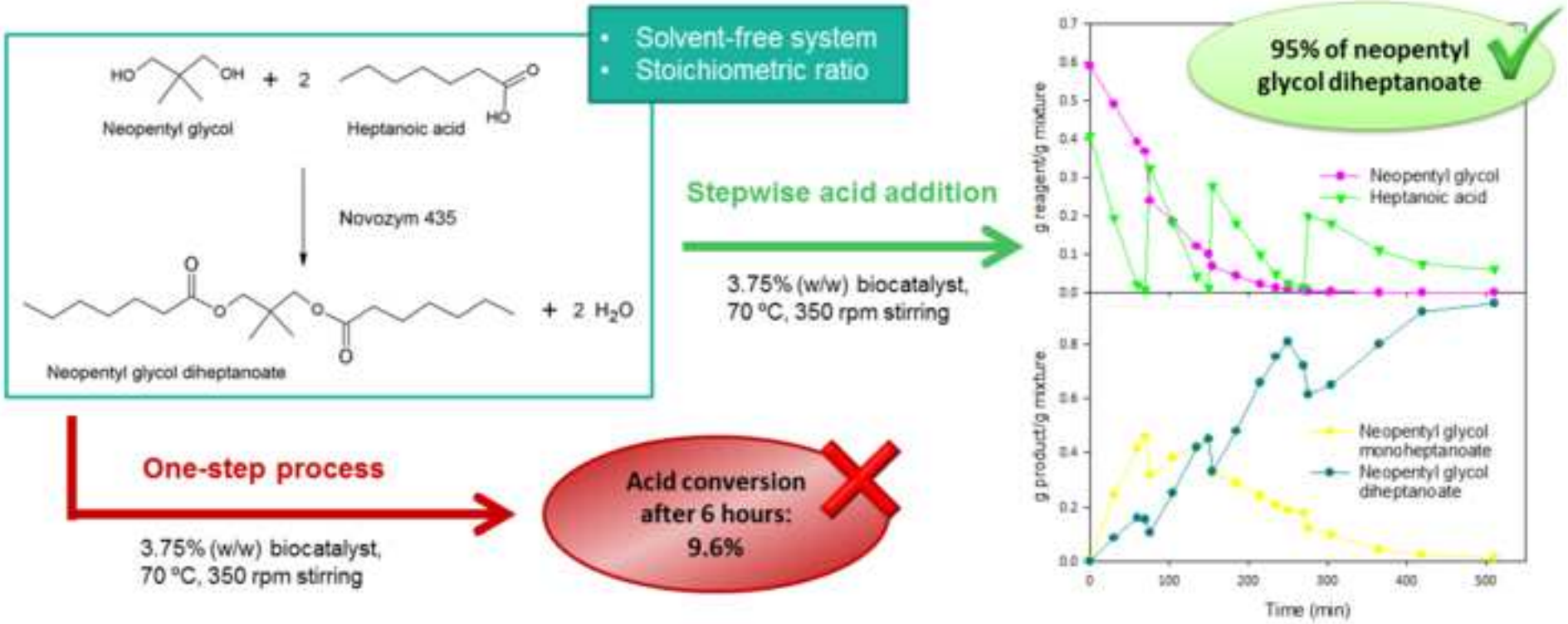
Graphical Abstract



Highlights

- The enzymatic synthesis of neopentyl glycol diheptanoate is reported for the 1st time
- Reaction rate decreases are caused by high heptanoic acid concentrations
- A stepwise acid addition strategy leads to conversions higher than 95% in 8 hours
- Conversion can be increased to 99.8% if neopentyl glycol is finally added

Reaction strategies for the enzymatic synthesis of neopentyl glycol diheptanoate

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Abstract

This work describes for the first time the green synthesis of neopentyl glycol diheptanoate in a solvent-free medium via an enzymatic pathway. The process has been carried out in an open-air reactor in order to ease water removal through evaporation and shift the chemical equilibrium towards product formation. The inhibiting effect of high concentrations of heptanoic acid has been put into evidence by a reduction of initial reaction rate when esterification was performed with stoichiometric amounts of substrates. Therefore, in this work different strategies for the stepwise addition of heptanoic acid are proposed, and best results were obtained when stoichiometric quantities of acid were divided in four equal amounts and added when previous batch was consumed. Biocatalyst Novozym® 435 concentration and temperature were optimised, giving yields higher than 95% in neopentyl glycol diheptanoate when 7.5% (w/w) and 70 ºC where used. With a remaining 5% of heptanoic acid (probably caused

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1. Introduction

A wide variety of esters are currently available. They can be of either natural or synthetic origin, with a great diversity of compositions and chemical structures that gives to them interesting properties and so, a great number of industrial uses. It is widely known that branched chemical structures cause molecules to lower their ability of crystallisation. In this sense, branched-chain esters (BCEs) show lower melting and boiling points than the related linear ones and, hence, they can be found in a liquid state for a wide range of temperatures. This specific feature makes BCEs very appropriated for their industrial application as additives, liquid lubricants or cosmetic ingredients [1].

Among BCEs, esters from neopentyl glycol (NPG) and medium or long-chain fatty acids (C8-C18) stand out [2] not only due to the above mentioned characteristics, but also because it has been proved that they possess a good biodegradability either under aerobic and anaerobic conditions [3]. In addition, such NPG esters are postulated as potential green insulating fluids because of their moderated viscosity values and great stability against oxidation [4]. Many publications dealing with the chemical synthesis of NPG esters are available, describing processes that require temperatures as high as 200 ºC and reaction times comprised between 5 and 20 hours [5,6]. In recent years, the number of research efforts with the objective of developing environmentally friendly alternatives by using lipases synthetic ability has increased. Such investigation has led to a collection of high purity NPG esters produced under mild operation conditions [2,7–10].

Among the esters obtained from NPG, neopentyl glycol diheptanoate (NPGDH) is one of the most used in cosmetic industry. Prestigious cosmetic ingredient manufacturers, such as Stearinerie Dubois or Inolex, include NPGDH in their catalogue [11,12], as its

characteristic properties make this ester an interesting replacement for the questioned volatile cyclomethicones, cyclic silicones whose environmental and health safety is now under question [13]. The great utilization of NPGDH in cosmetic industry justifies the convenience of choosing the enzymatic pathway to produce it, as it may allow the labeling of "natural" according to present requirements of cosmetic certification bodies [14]. According to the Voluntary Cosmetic Registration Program (VCRP), in 2016 NPGDH had been used as an ingredient in 337 cosmetic formulations, chiefly in skin care products and lipsticks (with the risk of being ingested). This fact evidences the need of obtaining NPGDH with high quality and purity levels [15].

To the best of our knowledge, there is no reference to the enzymatic synthesis of NPGDH in the available bibliography. This must not be attributed to a low interest in this ester, but to specific physic-chemical problems that may affect to the enzymatic pathway by lowering its reaction rate, or even preventing its development. On the one hand, short-chain acids' negative impact on lipases' enzymatic activity when they are used as the acyl donor is an issue to overcome [3,16–18]. On the other hand, it may happen that the size of the acyl acceptor can be too wide to fit in lipases' active pocket [19].

Thus, this work deals with the lipase-based esterification of heptanoic acid (HA) with neopentyl glycol (NPG) to obtain NPGDH in a solvent-free system (Figure 1). For that purpose, different stepwise additions of HA strategies have been developed. In addition, the concentration of immobilized enzyme has been optimized, in order to find a feasible process to obtain a NPGDH that can be used as a cosmetic ingredient in "natural" formulations.

(Figure 1)

2. Materials and methods

2.1 Chemicals

Novozym® 435 (*Candida antarctica* lipase B, CalB, immobilized by adsorption onto the macroporous DVB/methacrylate co-polymer resin) was kindly donated by Novozymes Spain S.A.

Heptanoic acid (97%) and neopentyl glycol (99%) were purchased from Sigma-Aldrich. Neopentyl glycol diheptanoate (99.4%) was a gift from Pronamed S.A. Other chemicals used were all analytical reagent grade.

2.2 Enzymatic synthesis

The reaction synthesis was carried out in a solvent-free medium using open-air jacketed reactors (250 mL) with an overhead stirrer equipped with a two bladed propeller at 350 rpm, which provided an axial flow. 40 g of reactants were introduced (11.43 g of NPG and 28.57 g of HA) following the reaction strategies described below. Operation temperature was set at 70 ºC and when the alcohol (melting point 130 ºC) was completely dissolved in the acid, the biocatalyst was added to the reaction mixture (in a concentration comprised between 3.75% and 7.5% w/w total substrates). Samples were taken at different time intervals (stirring stopped a few seconds before) and dissolved in absolute ethanol for gas chromatography (GC) analysis.

2.3. GC analysis

Substrates and products concentrations were analyzed by GC (7820A Agilent) equipped with a flame ionization detector (FID) and a silica capillary column (HP-5 Agilent Technologies; 30 m × 0.32 mm × 0.25 m). The injector temperature was 250 ºC, split

ratio = 2:1 and detector temperature was 300 ºC. The carrier gas used was nitrogen at a flow rate of 1 mL min^{-1}. Oven temperature was maintained at 80 °C for 1 min, increased to 120 °C with a ramping rate of 75 °C min^{-1}, held for 1 min and increased again to 290 ºC, at 20 ºC min^{-1}, temperature which was held for 3.5 min. The amount of diluted sample injected was 1 µL and total time of the analysis was 14 min. The product composition was quantified by an internal standard method with methyl myristate as the internal standard. Neopentyl glycol monoheptanoate (NPGMH) concentration in samples was evaluated by difference from the total mass injected, as this product is not commercially available.

3. Results and discussion

3.1. Heptanoic acid effect on CalB lipase activity

By reviewing the literature, it can be found several studies describing a diminution more or less pronounced of the enzymatic activity of *Candida antarctica* lipase when it is exposed to high concentrations of short-chain fatty acids [3,16–18]. Those publications have attributed this effect to an inactivation of the enzyme caused by the high polarity of such acids, which leads to the protonation of some essential residue in lipase's active site [17,18]. In this sense, Hollman et al. [17], established that acids with pK_a under 4.8 can cause the irreversible inactivation of *Candida antarctica* lipase B (CalB), as they observed that none of the acids tested with a pK_a value lower than 4.8 were esterified at a noticeable reaction rate. In contrast, when acids with pK_a over this limit were used, lipase activity depended mainly in the size and the distance between the carboxyl group substituents. The present work deals with heptanoic acid (HA), whose pK_a value is 4.8 [20] or 4.893 [21] depending on the source consulted. Even so, both values are low

enough to fear an inactivating effect of HA on the enzyme and, hence, the impossibility of the reaction to take place. Furthermore, such problem would be worsened by the fact that the stoichiometric relation of reagents for the esterification process is 2:1 (HA:NPG).

In order to confirm this hypothesis, six experiments were performed as described in section 2.2 by varying substrates molar ratio between 2:1 and 1:4 (HA:NPG), which correspond to acid concentrations ranging from 71.5 to 23.8% (w/w, referred to the reaction mixture). It is noteworthy that lower quantities of acid could not be tested, as NPG is solid under reaction conditions (70 ºC) and it must be dissolved in the HA: in this regard, over 15 minutes were required to reach an homogeneous reaction mixture when it was used the lowest amount of acid studied. Results are depicted in Figure 2, assigning a value of 100% to the highest reaction rate. As it can be seen, best results were obtained for acid concentrations comprised between 23.8 and 29.4% (w/w), i.e. for molar ratios (HA:NPG) of 1:4 and 1:3, respectively, while reaction rate dramatically decreases as the concentration of HA is augmented. Those results are in accordance with the above mentioned findings from other authors and make patent the influence of increasing acid (and H^+) concentration in the reduction of lipase's activity due to its damaging protonation [18].

Besides, it has also been described the negative effect on the enzymatic activity exerted by the NPG due to the geometric form of this branched molecule and the small size of the CalB active site [3,10]. Nevertheless, it has been demonstrated that this inevitable effect was not an obstacle for the development of the enzymatic reaction, achieving a 70% of conversion rate in an hour.

(Figure 2)

3.2. Different reaction strategies

3.2.1. Stepwise addition at constant time intervals

In the light of the previous results, it was decided to carry out a stepwise addition strategy for the HA. For that purpose, four batches of the same quantity of HA (7.14 g, the necessary amount to complete the substrates' stoichiometric ratio) were added to the reactor at regular time intervals of one hour. It was opted to select four acid additions with the purpose of not complicating the reaction strategy although the initial rate of the enzymatic reaction was inferior (61.7%) to the one obtained with the optimum molar ratio. The results obtained pointed out that the first acid addition almost completely reacted. On the contrary, in successive additions it was verified that the acid added was not totally consumed during the hour between batches, and it began to get accumulated in the reactor with the subsequent decrease in reaction rate. Finally, after 4 hours of reaction, 6.15 g of HA remained unreacted, which represents a final conversion of 78.12%.

3.2.2. Stepwise addition after acid consumption

With the aim of increasing the reaction rate, and thus, achieving higher yields, it was decided to modify the stepwise strategy, maintaining the number of acid additions and the amount added in each one, but carrying them out when reactor's acid concentration was lower than 1.5% (w/w), i.e. when the previous acid addition was almost consumed. Needless to say that this strategy requires performing the successive additions at variable time intervals, that were longer as the reaction progressed. The results obtained are shown in Figure 3, where the evolution of the substrates with time is represented at the top (NPG and HA), and for both esters at the bottom (NPGMH and NPGDH). As can be seen, both esters started to form in the beginning of the reaction, but at a

different rate. The acid additions were performed at 76, 155 and 276 minutes of reaction respectively, which explains the alcohol and the esters concentration reduction due to dilution effects. After four hours, the alcohol was completely consumed and the monoester concentration was decreasing as NPGDH was synthesized, leading to a concentration of NPGMH close to zero after 8.5 h of experiment. At that moment, the concentration of NPGDH, and thus, its purity, was 95% (w/w), with less than a 5% of HA and being negligible the monoesters presence.

(Figure 3)

3.3. Enzymatic reaction optimization

During the optimization of an enzymatic synthesis, it is important to analyze the influence of biocatalyst's concentration in the reaction medium, as normally an increase of the amount of available enzyme entails a rise in the reaction rate and a subsequent reduction processing time and energy to reach an acceptable yield [22]. So, the influence of the immobilized lipase concentration between a range of 3.75 – 7.5% (w/w, referred to total substrates) in the NPGDH synthesis has been studied.

The results obtained are given in Figure 4, where it can be confirmed that an increase in the quantity of biocatalyst present in the reactor leads to higher reaction rates, in such a way that the HA concentration decreases rapidly and the acid additions can be performed in shorter time periods. In the same vein, at the bottom of the figure it is shown that conversions close to 95% are achieved shortly after 5 hours when 7.5% of immobilized lipase is used, while 8.5 hours are necessary when biocatalyst's concentration is 3.75%.

(Figure 4)

Although the optimization of the amount of immobilized derivative saves more than 3 hours of working time, it may still be possible to improve the synthesis if the successive HA additions would be performed in order to obtain the optimum acid concentration that has been found out during the study described in section 3.1. Thereby, an experiment was carried out by using the NPG and HA quantities needed to start each addition with a concentration of HA of 23.8% (w/w), which are shown in Table 1. The results achieved are depicted in Figure 5, which compares the evolution of the HA concentration in the reactor when the additions were of equal mass and with variable mass. As it can be seen, any substantial improvement was noticeable when the variable mass strategy was used, not in the reaction speed neither in the final conversion achieved, that in both reactions was higher than 93%.

(Table 1)

(Figure 5)

In every experiment conducted it was noted that the reaction ended when the NPG and the NPGMH were completely consumed, meanwhile appreciable quantities of unreacted HA were detected. In fact, from Figure 5 it can be concluded that by the end of the experiments there are ~7% (w/w) of HA which remained unreacted, that would suppose a total of 2.45 g of HA, considering the complete content of the reactor as 35 g (taking into account the evaporated water and the collected samples). These unusual findings can be attributed to the volatility of some of the reaction compounds and its liberation to the atmosphere because all the experiments were performed in open-air reactors, allowing the elimination of the water and preventing the reversibility of the reaction in the direction of the hydrolysis. Consequently, a final addition of NPG by the end of the reaction was performed, and the conversion achieved was increased to 99.8%, what suggests that, presumably, the alcohol or the monoester is the most volatile compound

of the reacting mixture and the responsible for the presence of remaining acid in final product.

4. Conclusion

This work proves for the first time the feasibility of the stoichiometric solvent-free enzymatic synthesis of the neopentyl glycol diheptanoate through a stepwise strategy with four acid additions. Confirming the inhibiting effect of heptanoic acid on *Candida antarctica* lipase B, it has been find out that low concentrations of acid enhances the one-step process reaction rate. Despite this, it has been observed that, to a certain point, the amount of acid per batch is not a major parameter of influence on reaction rate or conversion when several additions of acid are performed, but the moment when the addition are performed is of paramount importance, as it is conditioned to the consumption of the acid previous fed to prevent its accumulation in the reactor. Thus, this work makes patent that an easy eco-friendly enzymatic process, with low energy consumption and no solvents required, can be a liable tool to cosmetic industry to obtain "natural" ingredients without the need to plunder nature.

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Acknowledgements

This work has been funded with support from the Spanish MICINN (RTI2018-094908-B-I00) and the European Commission (FEDER/ERDF). S. Ortega was beneficiary of a Torres Quevedo (PTQ-15-08015) grant from MINECO. We wish to acknowledge R. Martínez Gutiérrez from Novozymes Spain S.A., who kindly provided the biocatalyst.

Figures captions

Figure 1. Reaction scheme of the biocatalytic synthesis of NPGDH.

Figure 2. Reaction rate (%, referred to best results) for the one-step process for substrates molar ratio (HA:NPG) between 2:1 and 1:4 (3.75% w/w of Novozym® 435, 70 ºC, 350 rpm).

Figure 3. Evolution of the concentration of NPG (A, ●), HA (A, ○), NPGMH (B, ●) and NPGDH (B, ○) for the stepwise strategy with 4 acid additions at variable time intervals in the stoichiometric enzymatic synthesis of NPGDH (3.75% w/w of Novozym® 435, 70 ºC, 350 rpm).

Figure 4. Influence of the biocatalyst concentration in NPGDH production (four HA additions, 70 ºC, 350 rpm). ● 3.75%, ○ 5% and ▼ 7.5% w/w of Novozym® 435.

Figure 5. Evolution of the HA concentration in the reactor when the four HA additions were of equal mass (7.14 g, ●) and with the mass required for attaining 0.24 g HA/g mixture per addition (○) (7.5% w/w of Novozym® 435, 70 ºC, 350 rpm).

Table 1. Amounts of NPG and HA used during the experiment with a concentration of HA of 23.8% (w/w) per addition.

	Time (min)	**Amount of NPG (g)**	**Amount of HA (g)**	**Reactor' s content (g)**	**Acid concentration (% w/w)**
Initial	0	11.43	4.76	16.19	29.4
1st addition	35	-	6.35	22.54	28.2
2nd addition	85	-	8.97	31.51	28.5
3rd addition	165	-	8.49	40	21.2

Figure 1

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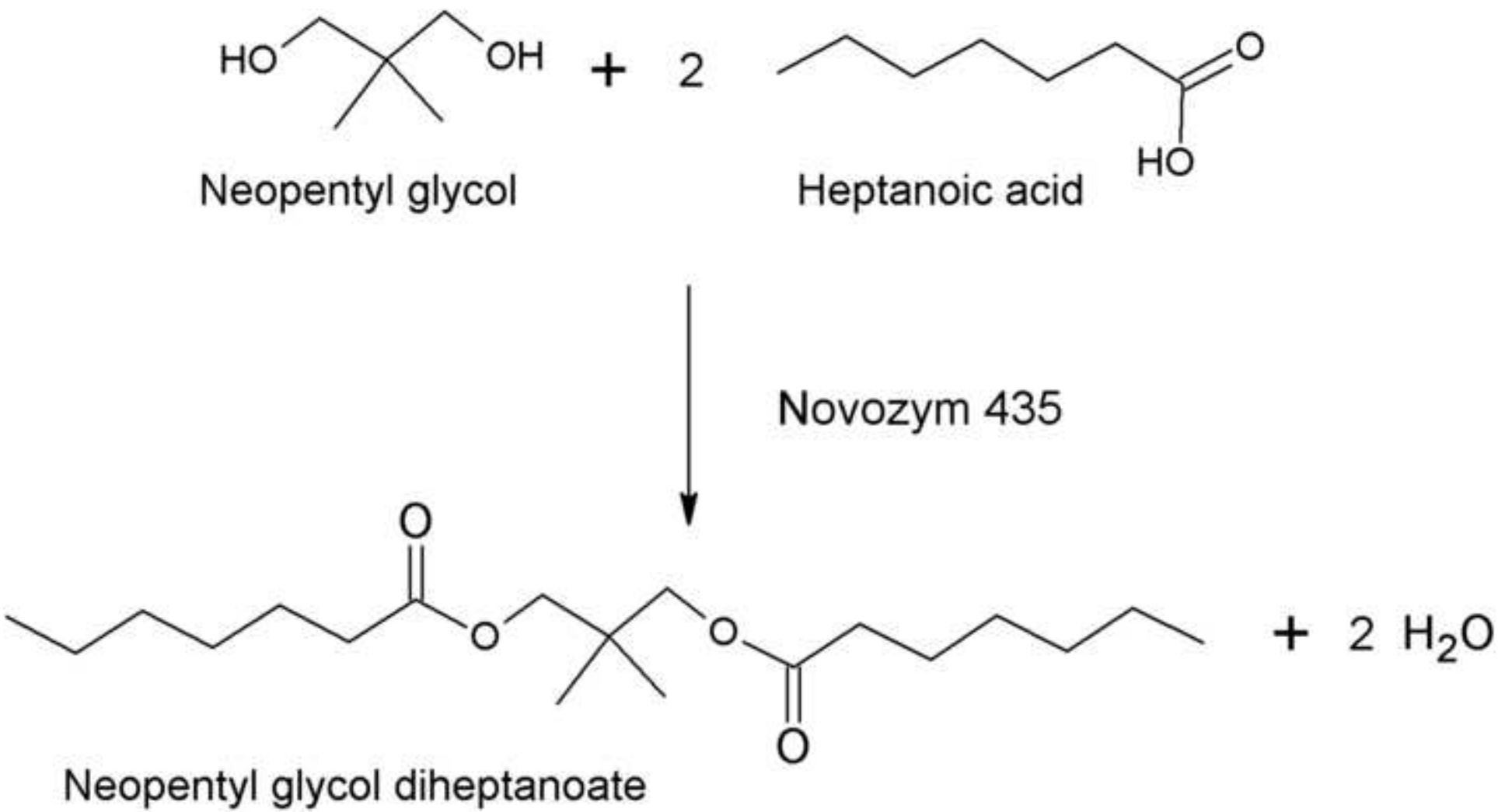

Figure 2

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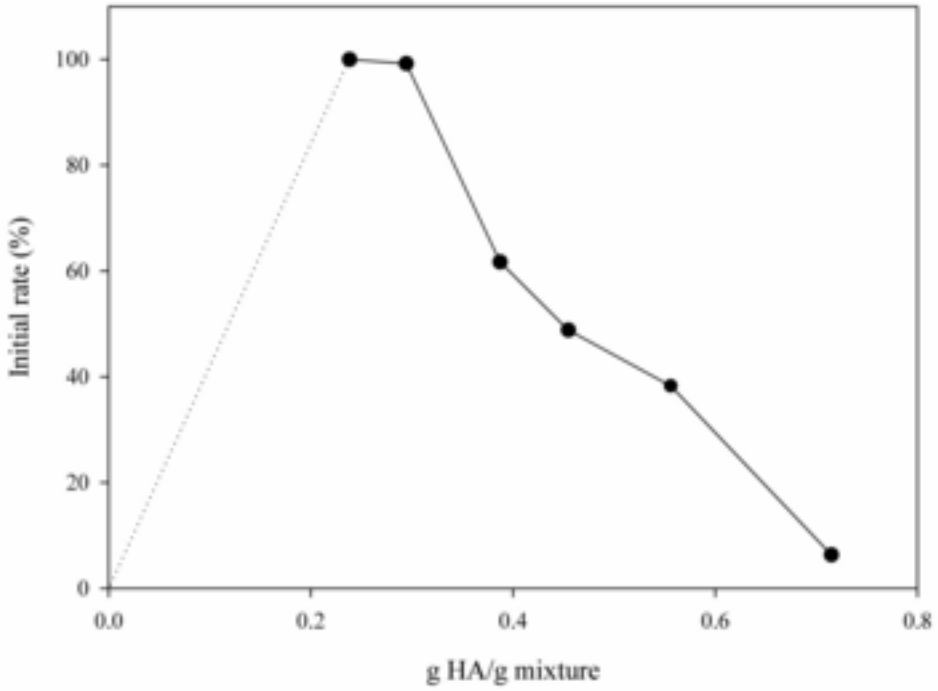

Figure 3

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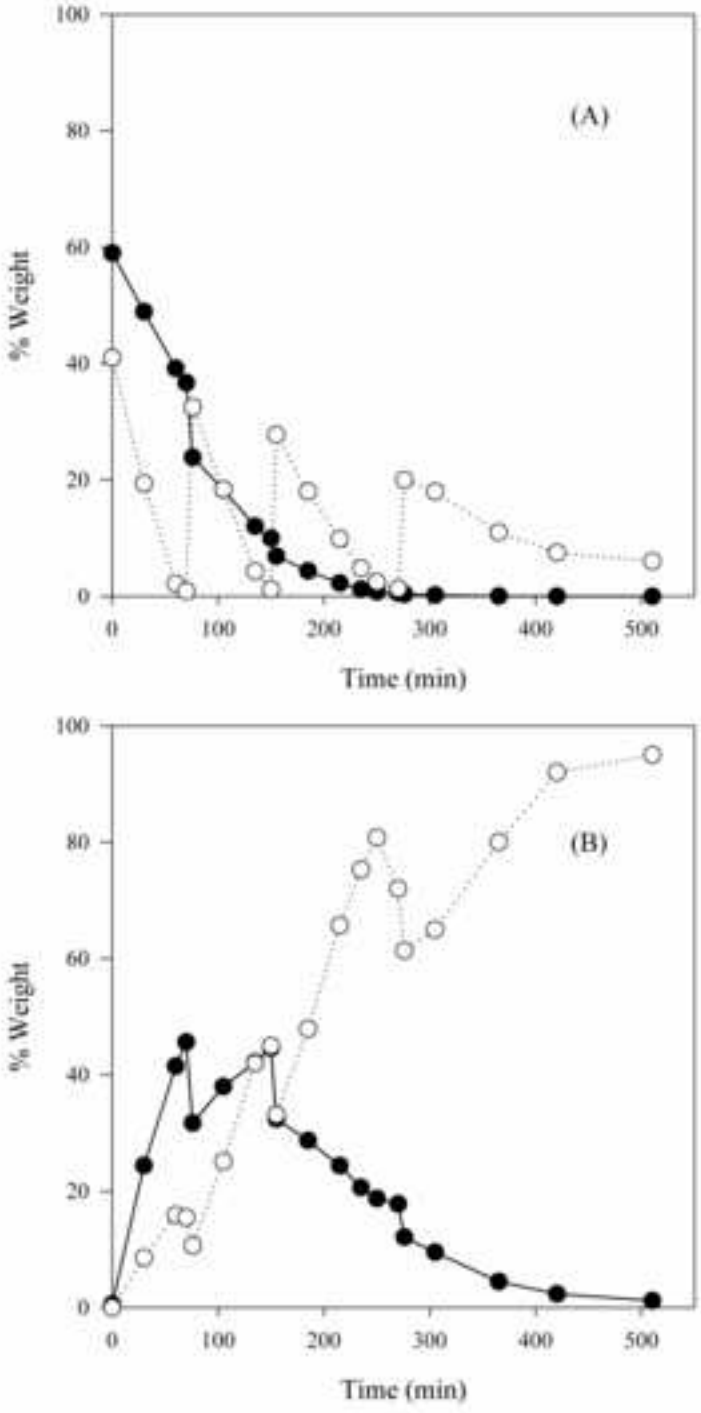

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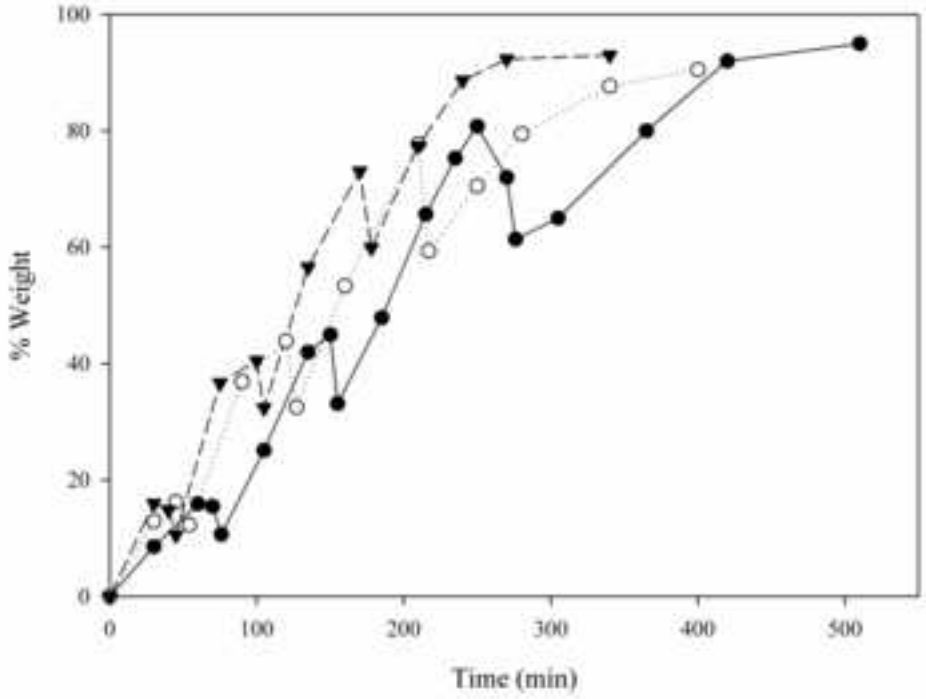

Figure 5

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