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Authors: J.L. Gómez, J. Bastida, M.F. Máximo, M.C. Montiel, M.D. Murcia, S. Ortega

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Research highlights

- We have obtained for the first time the food emulsifier E-476 enzymatically.

- Obtaining E-476 has been carried out in the absence of solvents and immobilized enzyme, fulfilling the main premises of green chemistry.

- The improved quality of the final product and the energy savings, makes this process a serous alternative for the production of PGPR (E-476).

Solvent-free polyglycerol polyricinoleate synthesis mediated by lipase from *Rhizopus arrhizus*

J.L. Gómez, J. Bastida, M.F. Máximo*, M.C. Montiel, M.D. Murcia, S. Ortega.
Chemical Engineering Department, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain.
(*) Corresponding author. Telephone number: +34-868-887367. Fax number: +34-868-884148. e-mail address: fmaximo@um.es

Other e-mail addresses:

J.L. Gómez	carrasco@um.es
J. Bastida	jbastida@um.es
M.C. Montiel	cmontiel@um.es
M.D. Murcia	md.murcia@um.es
S. Ortega	dortega@um.es

Abstract

The enzymatic biosynthesis of polyglycerol polyricinoleate (PGPR) (E-476) is described in detail for the first time. Starting from polyglycerol and polyricinoleic acid, *Rhizopus arrhizus* lipase was used as catalyst. The reaction, which is really a reversal of hydrolysis, takes place in the presence of a very limited amount of aqueous phase. No organic solvent is necessary to solubilise the substrates, which allows a reaction medium solely composed of the necessary substrates to be used.

Immobilisation of the lipase by physical adsorption onto an anion exchange resin provided good results in terms of activity, enzyme stability and the reuse of immobilised derivative. Using this immobilised derivative, PGPR with an acid value of 16 mg KOH/g was obtained, far above the

requirements of the European Commission Directive 2008/84/EC (< 6 mg KOH/g). In an attempt to force the reaction equilibrium towards the synthetic pathway, polyglycerol polyricinoleate was synthesised under controlled atmosphere in a vacuum reactor with dry nitrogen intake. This equipment allowed us to synthesise PGPR with an acid value of 4.9 mg KOH/g, which complies with the European Commission Directive and the results were entirely reproducible. This investigation represents a good starting point for using the enzymatic procedure in the industrial biosynthesis of PGPR.

KEYWORDS: Polyglycerol polyricinoleate; solvent-free; lipase; immobilised enzyme; biosynthesis.

1. Introduction

Polyglycerol esters have been used as food additives for many years. From the official point of view, food grade polyglycerol esters are divided in two classes: polyglycerol esters of edible fatty acids (E-number: E-475, also known as “PGFA”) and polyglycerol polyricinoleate (E-number: E-476, also known as “PGPR”). Polyglycerol polyricinoleate is used to maintain stable emulsions of oil and water systems with a high water content and as a viscosity modifier. In the chocolate industry, PGPR is used because it causes a noticeable reduction in the yield stress of molten chocolate. This allows chocolate to be moulded, without any air bubbles, easier coating of particulate ingredients, and the thickness of chocolate coating to be adjusted optimally. An additional property of PGPR in chocolate is its ability to limit fat bloom [1].

Known chemical methods for preparing PGPR involve autocatalytic condensation of ricinoleic acid and alkali-catalysed reaction between the condensed ricinoleic acid and polyglycerol. These procedures have the disadvantage of requiring very long reaction times, involving high energy

costs. This fact, together with the high operating temperature can adversely affect the quality of the final product because of problems related with coloration and odours, making it unsuitable for the food industry [2].

As an alternative, we propose the biotechnological production of PGPR using lipases, which act in mild reaction conditions and produce a final product more suitable for use as a food additive. The enzymatic procedure consists of two steps. First, the ricinoleic acid is polymerised to obtain the estolide [3-5] which is then esterified with polyglycerol. Figure 1 shows the chemical structure of all the species involved in the biosynthesis.

In principle, the second step of the process can be performed in a reaction system consisting of appropriate amounts of polyglycerol and estolide in the presence or absence of organic non-polar solvents. The choice of solvent in this process is a key issue from a green chemistry point of view. Supercritical carbon dioxide and ionic liquids are often referred to as green solvents, but the use of a solvent-free system (SFS) is a more attractive alternative. If the elimination of solvents is technically feasible, SFS offers significant cost savings, allows higher volumetric production than organic media and facilitates the separation of products from the unreacted substrates [6].

Although great efforts have been made to develop biotransformations with lipases, we have found no papers referring to the enzymatic production of PGPR. Previous works suggested that the esterification of polyglycerol with the ricinoleic acid estolide is successfully catalysed by *Rhizopus arrhizus* lipase [7], and so the present paper describes a detailed investigation into the enzymatic synthesis of polyglycerol polyricinoleate. In order to achieve this purpose, the following partial objectives were formulated:

- To ascertain the influence of the initial water content, enzyme concentration and reaction temperature on the enzymatic reaction rate and the equilibrium conversion.

- To obtain an immobilised derivative of *Rhizopus arrhizus* lipase with appropriate activity and stability through physical adsorption.
- To compare the behaviour of the immobilised and free enzyme in terms of PGPR production, including the reuse of the immobilised derivative.
- To carry out the enzymatic reaction in a vacuum reactor in order to shift the chemical equilibrium towards the reaction, thus obtaining an ester with an appropriate degree of esterification.

2. Materials and methods

2.1. Enzyme and substrates

Lipase (E.C. 3.1.1.3) from *Rhizopus arrhizus* (10 units/mg solid) was purchased from Fluka. One unit corresponds to the amount of enzyme which liberates 1 μmol of butyric acid per minute at pH 8.0 and 40 ºC using tributyrin as substrate. Polyglycerol-3 was kindly gifted by Solvay and polyricinoleic acid was synthesised in our laboratory following the procedure previously described [3-5].

2.2. Immobilisation support and reagents

The anionic exchange resin Lewatit MonoPlus MP 64 (supplied by Fluka) was used as immobilisation carrier. Soybean lecithin, used as support activator, was of commercial grade from Santiveri S.A., Spain. Other chemicals were of analytical grade and were used without further purification.

2.3. Immobilisation by physical adsorption

5 g of support was mixed with 50 ml of a soybean lecithin suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker overnight at room temperature. The activated support was washed with 50 ml of distilled water and then transferred to a jacketed column reactor (2.5 i.d.

and 30 cm length) equipped with a sinterised glass plate placed 5 cm from the bottom. The enzyme solution (50 ml, 10 mg/ml in acetate buffer 0.1 M, pH 5) was then added to the reactor and recirculated for 2 days at 4 ºC. The immobilised derivative was washed twice with the same buffer and stored at 4 ºC. The amount of protein initially offered and in the wash-liquid after immobilisation was determined by Lowry's procedure modified by Hartree [8], using bovine serum albumin as standard. The amount of coupled lipase was the difference between the amount of the initial enzyme added and the amount of enzyme in the wash-liquid.

2.4. Atmospheric reactor experiments

The enzymatic reaction was carried out in an open-air glass-jacketed batch reactor (250 ml total volume), maintaining the reaction temperature constant at 40 ºC. Complete mixing was achieved by means of a four-bladed propeller stirrer at 450 rpm. The reaction mixture contained 30 g of polyricinoleic acid (PR) and 2 g of polyglycerol-3 (PG), the mass ratio being PR/PG=15. This mass ratio corresponds with a molar ratio PR/PG = 3 which means that three of the five hydroxyl groups of the polyglycerol could be esterified. Unless otherwise stated, when the reaction was carried out with free lipase, 500 mg of *Rhizopus arrhizus* lipase was dissolved in 5 ml of distilled water and added to the reactor before the substrates. When the reaction was catalysed with immobilised lipase, 5 g of immobilised derivative was used and the only water in the reaction system was that soaked in the support (0.6 ml/g approximately).

2.5. Vacuum reactor experiments

For the vacuum reaction, a Parr 5100 series low pressure reactor was used. The reaction vessel (100 ml total volume) is made of glass and is equipped with a circulating water jacket to heat the vessel. The reactor head is stainless steel and accommodates the reactor controls and instrumentation. The reactor is equipped with a magnetic drive to provide an internal stirrer,

which acts as a turbine type impeller (450 rpm). The reactor top also includes a vacuum meter, an internal thermocouple, an internal cooling loop, a rupture disk, a liquid sample valve, a gas inlet valve and a gas release valve. Temperature, stirring speed and positive pressure are managed by a controller. The amount of ricinoleic acid, immobilised lipase and water in the reactor at the beginning of the reaction are the same as that reported for the open air jacketed batch reactor. The experiment was carried out at 40 ºC, and the stirring rate was kept constant at 450 rpm. The pressure was set at 160 mmHg and 74 l/h dry nitrogen was passed through the reaction mixture to facilitate water removal (the nitrogen flow was dried by passing through a silica gel column, so that its relative humidity was zero).

2.6. Measurement of the reaction extension

The acid value (AV) [9], which represents the number of milligrams of potassium hydroxide necessary to neutralise free acids in 1 g of sample, was used as an index to show the reaction extension. Here, the AV corresponds to the free carboxyl group concentration in the reaction mixture, which decreases due to the esterification of polyricinoleic acid (AV≤50) with polyglycerol-3.

2.7.- Measurement of the water content.

Water content of was measured in the reactor samples with a Karl-Fischer automatic titrator (701 KF, Metrohm), using Hydranal ® composite 5, from Riedel-De-Häen.

2.8.- Recovery of the immobilized derivative.

When immobilized derivatives were tested for reusability, the reactor content was placed in a sinterised glass filter (Pirex ®, number 0) to separate the derivative from the product. After 8 hours at room temperature the immobilized derivative was placed in the reactor for a new reaction cycle.

3. Results and discussion

As previously stated, this paper describes the enzymatic synthesis of PGPR using *Rhizopus arrhizus* lipase in a solvent free reaction medium. For the first time the acid number reached in the biosynthesis fulfilled the specification of the EU concerning to PGPR as food additive [10]. After a detailed literature search, no references were found, which might indicate, even approximately, the experimental conditions in which the reaction should be carried out. Therefore, it was necessary to start the study of the enzymatic synthesis of PGPR by establishing the optimal experimental conditions.

Polyricinoleic acid (PR), which is used as substrate in all the experiments described in this paper, was polymerised in our laboratory until it reached an acid number lower than 50 mg KOH/g. At this acid number value, the average length of the PR chains is four, which is considered as optimum for food use by most authors [3, 11]. Among the commercially available polyglycerols, Polyglycerol-3 from Solvay (PR-3) was considered the most appropriate because it produces a high performance PGPR [12]. The European Commission Directive 2008/84/EC [10] establishes an acid value (AV) lower than 6 mg KOH/g for PGPR and a hydroxyl value (HV) of between 80 and 100 mg KOH/g. HV is a measurement of the free hydroxyl groups and any reduction is concomitant with a decrease in AV because one of each is consumed when an ester linkage is formed. When the PR/PG-3 mass ratio is used is lower than 12 both requirements cannot be fulfilled because, even if all the acid groups react and AV is close to zero, too many hydroxyl groups remain unreacted (HV > 100 mg KOH/g). On the other hand when the PR/PG-3 ratio is too high, the final product will contain too many acid groups or too few hydroxyl groups. Therefore, the substrate mass ratio (PR/PG-3) in all the experiments was maintained constant at a value of 15, which means that three of the five hydroxyl groups of the polyglycerol could be esterified.

It is important to underline the dramatic influence of relative humidity on the equilibrium of this esterification process. The air conditioner/heat pump equipment installed in our laboratory stabilises the relative humidity at 70 % in summer (air conditioner) and at 20 % in winter (heat pump). As a consequence and to avoid this effect, all the experiments used to optimize an individual variable were carried out simultaneously, in an air open tank reactor when the relative humidity was 40-50 %.

3.1. Influence of initial water content

It is widely known that water is essential for the catalytic function of enzymes because it participates, directly or indirectly, in all the non-covalent interactions that maintain the conformation of the catalytic site of enzymes [13-15]. However, it has been found that the amount of water necessary for enzyme activity might be very small and, in the case of lipase, just a few layers around the enzyme surface are needed [16]. On the other hand, in esterification/hydrolysis reactions, the water content affects the equilibrium conversion of the reactions as well as the distribution of products in the media [17]. In the case of esterifications particularly, as the water content increased, lower equilibrium conversions were achieved.

In the light of the above considerations, a study on the optimal initial amount of water in the reactor was deemed necessary. For this purpose, a set of experiments was carried out with 500 mg lipase, varying the volume of water used to dissolve the enzyme from 0.75 to 10 ml of water. Since similar results were obtained in all the experiments (data not shown), 5 ml of distilled water was used in further studies when the amount of enzyme added was 500 mg, this volume of water being varied in direct proportion to the amount of lipase used.

3.2. Influence of temperature

Temperature was seen to influence the enzymatic reaction rate, enzyme stability, the velocity of water evaporation from the reaction medium and its viscosity. Therefore, the effect of the temperature on the reaction course was investigated and three experiments were carried out at 40, 50 and 60 ºC. Figure 2 shows the evolution of the acid value of the reaction mixture with reaction time. The adverse effect observed on the reaction when the experiment was carried out at the highest temperature, can be attributed to a thermal deactivation of the lipase, since it is well known that most proteins tend to denaturalize at temperatures above 50 ºC. The most common cause for the inactivation of enzymes at high temperatures is loss of the native, catalytically active conformation, i.e. thermodenaturation. Because similar acid values were achieved at 40 and 50 ºC, the first one was used in the following experiments, especially because 40 ºC has been described as the optimum temperature for *Rhizopus arrhizus* lipase [18].

3.3. Influence of the amount of enzyme

For the synthesis of polyglycerol polyricinoleate, the influence of different amounts of added lipase on the esterification reaction was studied in five experiments using 50, 100, 200, 500 and 1000 mg of lipase.

As can be seen in Figure 3, a significant improvement in the reaction extension was obtained when the amount of added enzyme was increased from 50 mg (minimum AV=22.02 mg KOH/g) to 500 mg (minimum AV=17.76 mg KOH/g). However, no additional enhancement was observed when 1000 mg of lipase was added to the reaction mixture (AV=17.69 mgKOH/g). As a consequence, 500 mg lipase was used in further experiments.

3.4. Lipase immobilisation by physical adsorption and comparison with the free enzyme.

All the experiments mentioned until now were carried out adding the native enzyme to the reaction medium. It is clear that the acid value of the synthesised PGPG did not fulfil the requirements demanded by the European Commission Directive 2008/84/EC [10]. Moreover, as shown in Figure 3, when the amount of lipase added to the reactor was increased, the reaction progressed faster and a lower acid value was reached. The differences between the acid values reached led us to think that these were not equilibrium values but that the reaction stopped because the enzyme was no longer active.

Taking into consideration the potential benefits from the use of immobilised enzymes (high activity and stability), the possibility of immobilisating *Rhizopus arrhizus* lipase by physical adsorption onto an ion exchange resin (MonoPlus Lewatit MP64) was studied. This immobilisation method was previously optimised by the authors and successfully used in PR biosynthesis with *Candida rugosa* lipase [4].

When *Rhizopus arrhizus* lipase was immobilised in the conditions considered as optimum for *Candida rugosa* lipase, an immobilised derivative containing 8.6 mg of protein per g of support was obtained with an immobilisation yield of 32.6 %. A 5 g sample of this derivative (containing 43 mg of protein) were used as catalyst for the synthesis of PGPR and, in order to compare the behaviour of the immobilised and free lipases, a similar synthesis was carried out with the same amount of protein (since the commercial preparation from FLUKA contains 26.3% of protein, 163.5 mg of commercial free enzyme were added). Figure 4 shows the results of these experiments. Two conclusions can be reached for the curves observed. First, the immobilisation provokes a moderate loss of lipase activity since the initial reaction rate (24 h) is lower when immobilised lipase is used. Secondly, immobilisation seems to stabilize the lipase activity: after 168 h the

condensation process still continues with immobilised lipase, while the reaction does not continue beyond 50 h when free lipase is used.

3.5. Reuse of the immobilised derivative

Immobilisation provides an attractive opportunity for the multiple use of the enzyme. In order to establish the reusability of our immobilised derivative (lipase), successive polymerisation reactions were carried out, examining the evolution of AV in several consecutive experiments.

Not many immobilised enzymes exhibit such good reusability as that shown in Figure 5 by *Rhizopus arrhizus* lipase. Three consecutive processes yielded an almost identical acid values after seven days of operation. Moreover, the immobilised derivative could be easily removed from the reaction medium, conventional filtration through a sinterised glass filter and gravity force being sufficient to successfully separate the derivative from the viscous product, PGPR.

3.6. Obtaining PGPR in the vacuum reactor

It was mentioned above that the European Commission Directive 2008/84/EC [10] establishes as a requirement for PGPR an acid value lower than 6 mg KOH/g. In all the experiments described until now the final acid value was far from this objective, which means that the equilibrium have to be shifted towards the esterification pathway. This can be done by using a more anhydrous medium. On the other hand, the crucial importance of the amount of water in the reaction medium is illustrated by the poor reproducibility of the processes taking place in open reactors, since this parameter is heavily influenced by seasonality and weather conditions. This is particularly important in our case, when the final purpose is the production of the additive on an industrial scale, which requires rigorous standardisation. For this reason, a high performance reactor was tested for PGPR production. The reactor is thermostated, it can work in a wide range of

pressures, and is also able to mix to the reaction medium in accordance with its high viscosity. In this reactor the amount of water can be manipulated through the pressure and the entry of dry nitrogen, making it independent of laboratory conditions.

Figure 6 shows the results of PGPR synthesis using immobilised lipase in the high performance reactor. This reactor provides a controlled atmosphere that permits the water content of the reactor medium to be adjusted. In the tested conditions, the water content in the reaction medium was stabilised at around 2000 ppm (Karl-Fisher) after 10 h (a totally anhydrous medium would lead to enzyme inactivation). In these conditions, the objective of the European Commission Directive was attained after 100 h (AV = 5.9 mg KOH/g) but even lower values can be reached at longer times (after 125 h the AV was 4.9 mg KOH/g). Although Figure 6 only depicts one curve corresponding to the vacuum reactor, several experiments were carried out in identical conditions and the same results were obtained.

Figure 6 also reveals the enormous difference between PGPR biosynthesis in atmospheric and vacuum reactors. Both esterification processes were made with the same amount of substrate, immobilised derivative and initial amount of water. In the atmospheric reactor the target was not reached even after one week, whereas in the vacuum reactor PGPR was ready after 100 h.

4. Conclusions

The biosynthesis of polyglycerol polyricinoleate (E-476) from polyglycerol and polyricinoleic acid is described in detail for the first time. The new process is catalysed by *Rhizopus arrhizus* lipase and it is carried out as a solvent free process, a feature that enhances the green-chemistry reputation that enzymatic processes already have. Moreover, the biosynthesis is catalysed by an immobilised derivative with excellent reusability, which makes the

process even more advantageous from the economic and environmental point of view.

When the biosynthesis of PGPR is carried out in an open air reactor, atmospheric humidity does not allow a sufficient degree of esterification, yielding a product with an acid value that clearly exceeds that established by the European Commission Directive for PGPR. However, when PGPR is obtained in an almost anhydrous medium (2000 ppm), the esterification equilibrium swifts towards the synthetic pathway, yielding a product that fulfils all the specifications of the European Commission.

5. Acknowledgements

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Figure Captions

Figure 1.- Chemical structure of species involved in the reaction.

Figure 2.- Influence of temperature on PGPR production using free lipase as catalyst and atmospheric reactor. Experimental conditions:

Reaction mixture, 30 g PR + 3 g PG

Enzyme, 0.5 g dissolved in 5 ml of distilled water

Temperature, (■) 40 ºC, (▲) 50 ºC and (•) 60 ºC.

Figure 3.- Influence of the amount of lipase on PGPR production using free enzyme and atmospheric reactor. Experimental conditions:

Reaction mixture, 30 g PR + 3 g PG

Temperature, 40 ºC

Enzyme, (∗) 50 mg, (■) 100 mg, (▲) 250 mg, (•) 500 mg and (×) 1000 mg of *Rhizopus arrhizus* lipase dissolved in 5 ml of distilled water.

Figure 4.- Influence of enzyme immobilisation on PGPR production using the atmospheric reactor. Experimental conditions:

Reaction mixture, 30 g PR + 3 g PG

Temperature, 40 ºC

Catalyst, (▲) 163 mg of free lipase dissolved in 5 ml of distilled water or (■) 5 g of the immobilised derivative and the amount of water soaked in the support.

Figure 5.- Reusability of the immobilised derivative in PGPR production. Experimental conditions:

Reaction mixture, 30 g PR + 3 g PG

Temperature, 40 ºC

Catalyst, 5 g of the immobilised derivative and the amount of water soaked in the support.

Reaction cycle, (■) first use, (▲) second use and (•) third use.

Figure 6.- Influence of the reactor device on the production of PGPR using immobilised *Rhizopus arrhizus* lipase. Experimental conditions:

Reaction mixture, 30 g PR + 3 g PG

Temperature, 40 ºC

Catalyst, 5 g of the immobilised derivative and the amount of water soaked in the support.

Reactor, (■) atmospheric reactor and (▲)vacuum reactor.

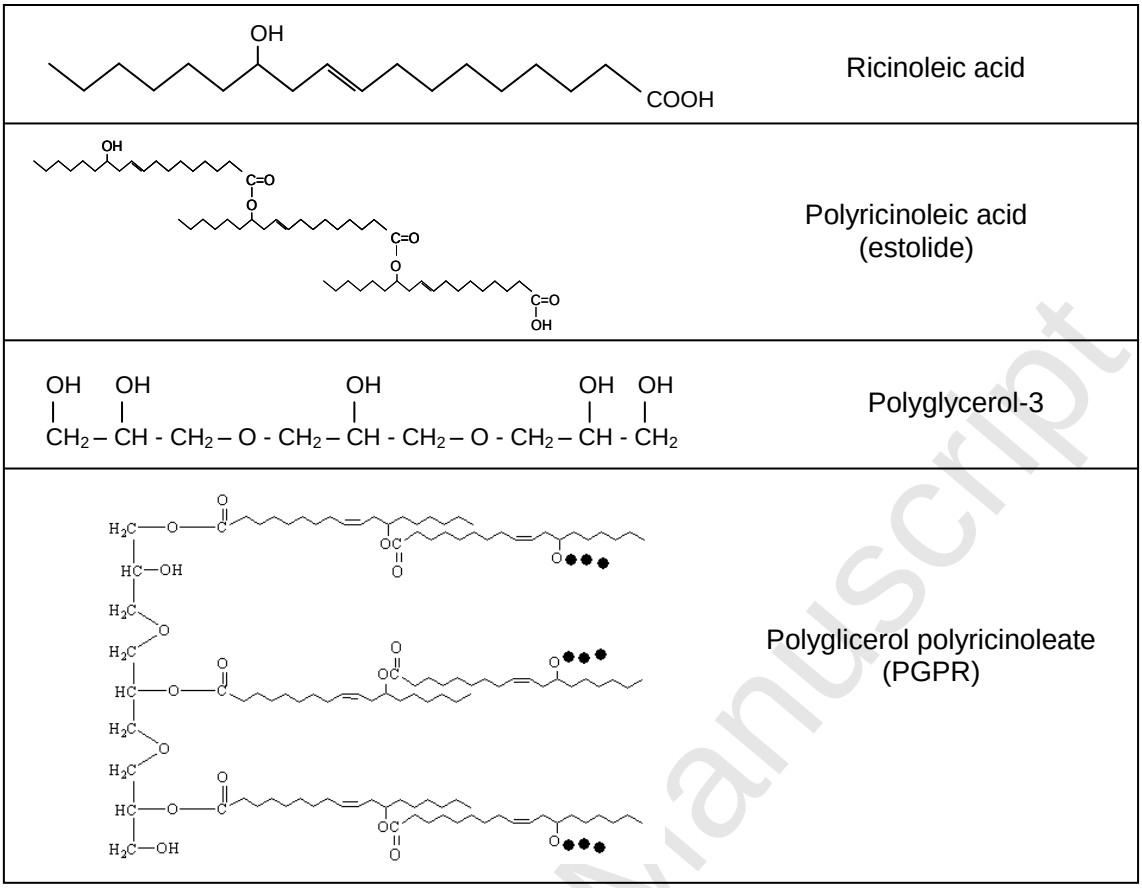

FIGURE 1

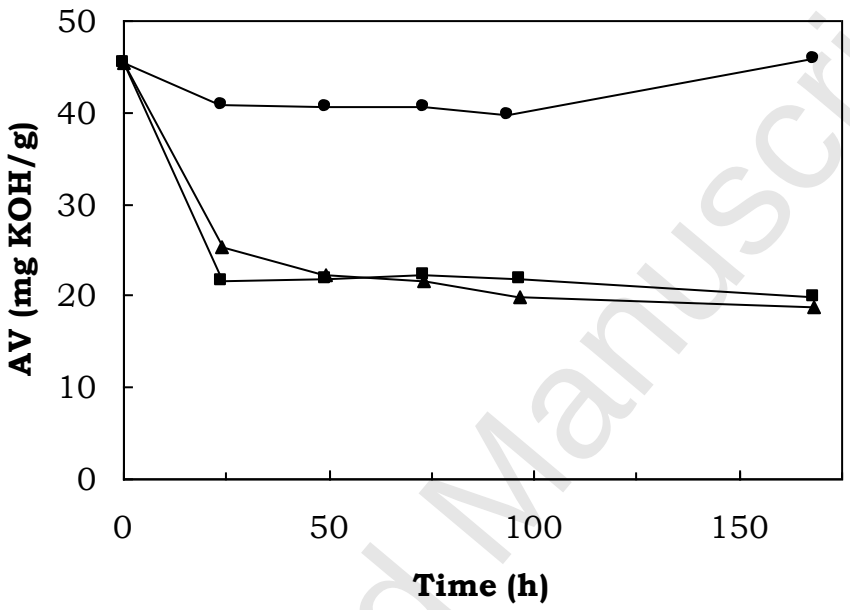

FIGURE 2

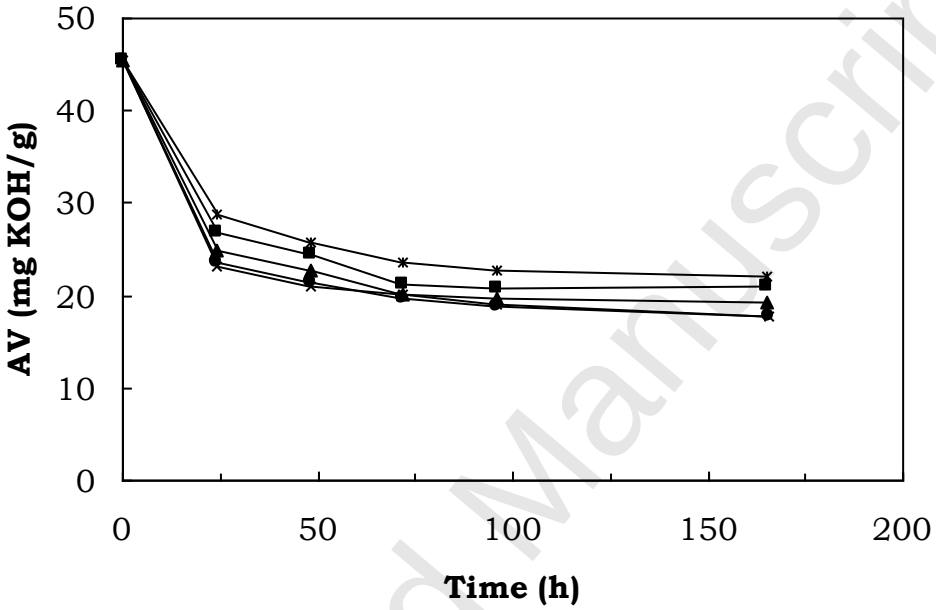

FIGURE 3

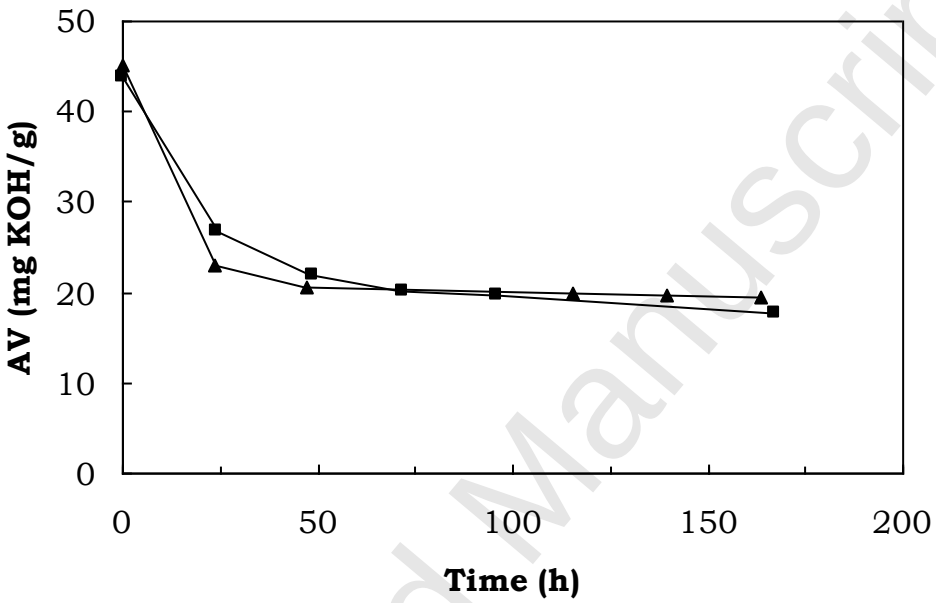

FIGURE 4

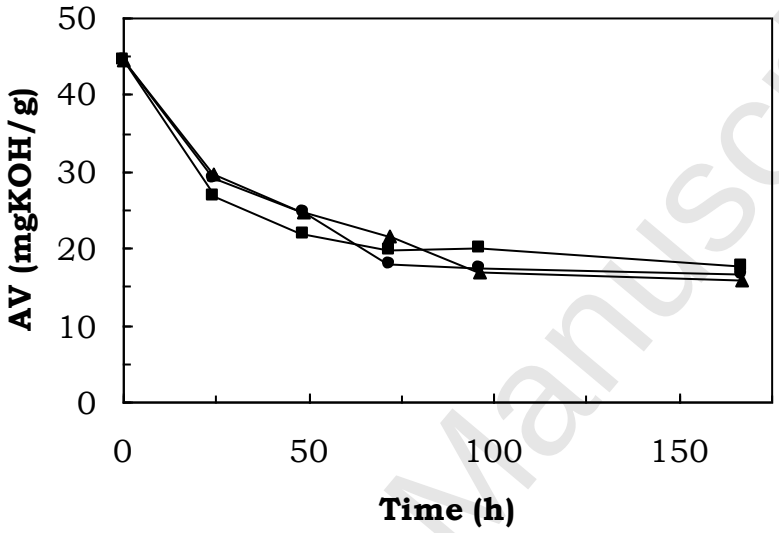

FIGURE 5

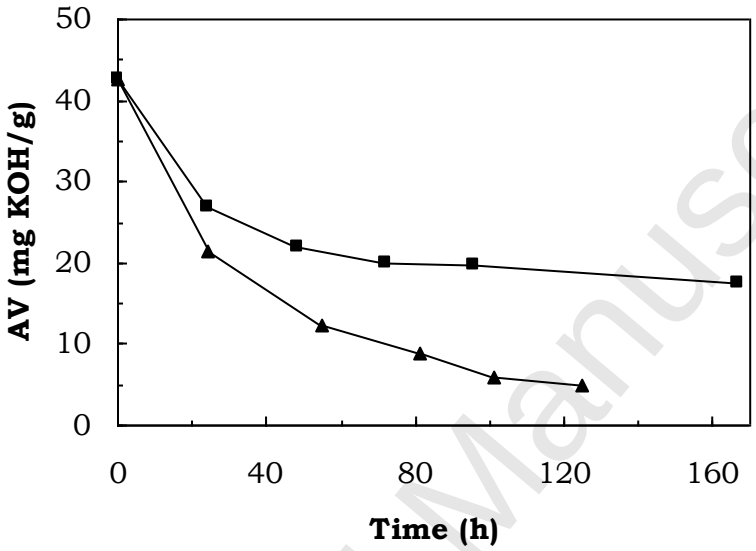

FIGURE 6