Indian Journal of Biotechnology
Vol. 4, April 2005, pp 241-245

Enzymatic hydrolysis of castor oil: An approach for rate enhancement and enzyme economy

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Received 7 October 2003; revised 12 April 2004; accepted 25 April 2004

Lipase has been used to catalyze the hydrolysis of castor oil. The effect of solvent, temperature, *p*H and the enzyme concentration on the rate of reaction has been investigated. The rate of reaction could be considerably improved by modifying the reaction protocol with the help of addition of a solvent. The amount of enzyme used for the reaction was found to increase the rate of reaction in a logarithmic relation. By optimizing the enzyme addition protocol, the total amount of enzyme required for the reaction could significantly be reduced.

Keywords: castor oil, lipase, enzyme economy, hydrolysis, solvent effect

IPC Code: Int. Cl.[7] A01N63/04; C12N9/16, 9/20, 15/09

Introduction

The biocatalysts find applications for industrial purposes because they possess high specific catalytic activity over the conventional chemical catalysts especially under milder conditions. Lipases (triacylglycerol ester hydrolase, E.C. 3.1.1.3.) are ubiquitous enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols. The microbial lipases have been used in hydrolysis of fats[1], esterification and transesterification[2,3] and resolution of chiral compounds[4]. Fatty acids and glycerol are the products of fat hydrolysis with variety of end uses as chemical intermediates[5]. Commercially accepted process for fat hydrolysis operates with superheated steam at 250°C and 50 atm[6]. This high-energy process during hydrolysis also induces polymerization and colour development and requires subsequent purification[7]. An enzymatic process obviates these drawbacks associated with the thermal process. Castor oil is the most ideal candidate for the enzymatic hydrolysis process[8]. In the present investigation, a commercial lipase was used to hydrolyse castor oil with an aim of optimizing the process conditions to get maximum yield of castor fatty acids in minimum time using as little enzyme as feasible.

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Materials and Methods

Materials

Lipase, gifted by Novo Nordisk Ltd. (Bagsvaerd, Denmark), was obtained from a genetically engineered species of *Aspergillus oryzae*. Tributryin and castor oil were purchased in Mumbai, from Loba Chemie and IPCA Chemicals and Cosmetics Ltd., respectively. Proline was purchased from Himedia Laboratories Ltd., Mumbai. All other chemicals used were of reagent grade.

Determination of Lipase Activity

Lipase activity was determined by performing controlled hydrolysis of tributryin substrate[9] (tributyrin was mixed with water using sonication to obtain a fine emulsion to which buffered enzyme was added. The resultant mixture was incubated for 10 min and the product was analyzed for the quantity of fatty acids formed). Enzyme activity was then expressed as the amount of enzyme required to liberate one μmole of fatty acid per min under the given conditions.

Reaction System without Solvent

Castor oil (1 g) was sonicated with 8 ml of water for 10 min at 60 W intensity for 5 sec on/off pulse on an ultrasonic processor. To this solution, 1 ml of enzyme solution (U i.e. 2 mg of enzyme) in phosphate buffer (*p*H-7.0) was added. The reaction mixture was incubated for different time intervals (1 to 24 hrs)

with constant stirring using a magnetic stirrer. The reaction was quenched by the addition of 20 ml ethanol to the mixture. The amount of fatty acids released by the enzyme activity was determined using titration with 0.1*M* NaOH and the percentage hydrolysis was calculated as follows:

$$\text{Percentage hydrolysis} = \frac{\text{Acid value} \times 100}{\text{Saponification value}}$$

The temperature and *p*H optima were determined by conducting the same reaction at different operating temperatures and phosphate buffer *p*H values. Sonication was optimized by varying the duration of time.

Reaction in Presence of Solvent

Various solvents like, isooctane, *n*-hexane, *n*-heptane and diethyl ether were employed to dissolve the castor oil and their effect on the rate of hydrolysis of oil was observed. Castor oil (1 g) was dissolved in solvent (1 ml) and then mixed in 7 ml of water. To this, 1 ml of enzyme solution (7 U/ml in phosphate buffer, *p*H-7.0) was added. The reaction mixture was incubated at room temperature with constant stirring, and then analyzed after the termination of reaction. The effect of solvent on enzyme activity was found by suspending the enzyme in isooctane at different time intervals and then determining the residual activity.

Effect of Enzyme Concentration

The yield of fatty acids was optimized by adding variable amounts of enzyme (3.5-175 U/gm oil) in the reaction mixture.

Effect of Intermittent Addition of Enzyme

The effect of enzyme supplementation at the time of reduced hydrolysis rate (equilibrium) was observed by adding a fixed quantity of enzyme to the reaction mixture at the end of a specified interval of time (addition at different stages of the hydrolysis reaction). The reaction was allowed to continue and the rate of hydrolysis of castor oil was determined to see the effect of intermittent addition of the enzyme.

Results and Discussion

The major parameters influencing the rates and the extent of hydrolysis were oil-water interfacial area, *p*H and temperature. The hydrolytic efficiency of enzyme was greatly influenced by the solvents like isooctane, diethyl ether, etc. and hydrotropes like proline.

Effect of Oil-Water Interfacial Area

Lipase showed interfacial activation[10,11]. The extent of interfacial area offered for the reaction depends on quality of the emulsion formed between the organic and aqueous phase. Sonication has been used in the past as a technique to get fine emulsion with maximum interfacial area[12]. Emulsion quality depends on the sonication conditions viz. time, intensity, etc. By sonicating the reaction mixture for 10 min, the amount of fatty acids released was twice than that without sonication. But for sonication above 10 min, no increase in the extent of hydrolysis was observed. This may be due to an equilibrium emulsion quality that was attained for a given intensity of sonication (60 watts/cm^2 in this case) in 10 min, and any additional sonication was unable to further improve the emulsion quality[12]. Thus, the extent of hydrolysis can be intensified by using sonication for optimized time as 10 min for the specific case considered in the work.

Effect of *p*H and Temperature on the Rate of Hydrolysis of Castor Oil

Lipase was found to be most active at optimum *p*H of 7.0, which was adjusted by addition of 0.2 *M* sodium phosphate buffer (*p*H-7.0), and the optimum temperature of 55°C. The enzyme activity fell considerably at temperatures above 55°C.

Effect of Solvent Addition

By adding 1 ml of solvent to the reaction mixture (the non-aqueous to aqueous phase ratio being 1:4), the extent of hydrolysis increased from 16% (in the absence of solvent) to 42% in first 3 hrs of reaction. However, as the reaction proceeded, the conversion values leveled off with those without solvent (Fig. 1). Thus, solvent treatment for a limited period can be beneficial to achieve the initial increase in the enzyme activity to get higher reaction rates. Various solvents like isooctane, *n*-hexane, diethyl ether, etc. gave similar effect. For all further studies, isooctane was used as the solvent. The increase in the extent of hydrolysis due to addition of solvent can possibly be attributed to prevention of product inhibition and increase in the activity of the enzyme.

The role of solvent in avoiding the product inhibition by solvation of the fatty acids produced was studied by increasing the amount of isooctane in the reaction from 1 to 5 ml, thereby increasing the capacity of isooctane to solubilize fatty acids. The hydrolysis pattern against time was compared with

that when the amount of isooctane was 1 ml. It was observed that the hydrolysis patterns in both the cases did not differ significantly and hence the theory of prevention of product inhibition due to the presence of isooctane was ruled out.

To study the effect of solvent on enzyme activation, the enzyme was exposed to isooctane for different time durations (1 to 24 hrs) and the activity was determined by the tributryin hydrolysis method. Exposure to the solvent for 3 hrs resulted into almost 2.5 times increase in the activity of enzyme (Fig. 2) than the initial activity. However, on extended exposure to the solvent (after about 9 hrs), the enzyme gradually lost its activity and was found to drop to half the initial amount in 24 hrs. Lipase was generally found to be contaminated with tightly bound lipids. The complete removal of these lipids can be achieved by the addition of solvents or certain surface-active agents[13], which seem to solubilize these lipids and decontaminate the enzyme. This and the increase in the interfacial area due to the dissolution of oil in the solvent reduced the organic phase viscosity, which appears to be the most likely reasons for the apparent increase in the activity of enzyme in the presence of solvents. On extensive exposure of the enzyme to the solvent, a deactivating effect was observed due to denaturation of the enzyme by solvents.

Effect of Enzyme Concentration

The extent of hydrolysis increased with an increase in the enzyme concentration. On increasing enzyme concentration from 7 to 175 U (in the presence of solvent and under optimum conditions of *p*H temperature and sonication), the percentage conversion obtained in 6 hrs increased from 50 to 85%. The conversion value was 74% with 7 U and 90% with 175 U for 24 hrs for the same conditions (Fig. 3). Thus, to obtain higher yields within a limited period, concentration of enzyme has to be increased. However, Fig. 3 suggests that the increase in rate of the reaction is observed during the first 6 hrs as indicated by the slope of the curves of the reaction with 7 U and 175 U of the enzyme. Thus, a strategy to increase the enzyme concentration must involve an exponential rise in the enzyme concentration and not a proportional increase. Therefore, a scheme involving the addition of the enzyme in divided parts during the course of reaction and not as the total quantity at the start of reaction was devised. This strategy promises better and efficient utilization of the enzyme by possibly reducing the overall enzyme consumption and, by avoiding the exposure of the

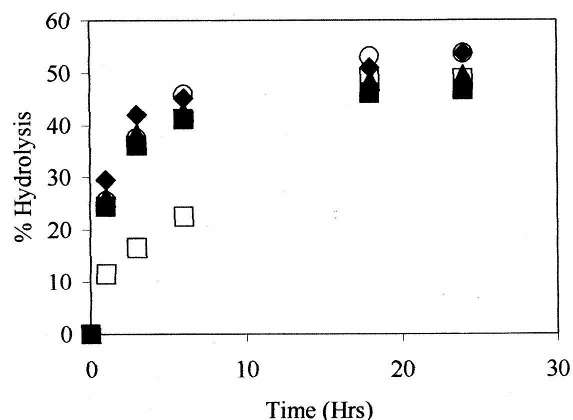

Fig. 1—Effect of various solvents on the rate of hydrolysis of castor oil. The hydrolysis of the reaction was carried out with 1ml Isooctane (υ), *n*-Hexane (ν), *n*-Heptane (σ), Diethyl ether (ο), and in the absence of any solvent where the solvent portion was replaced with 1 ml buffer (□).

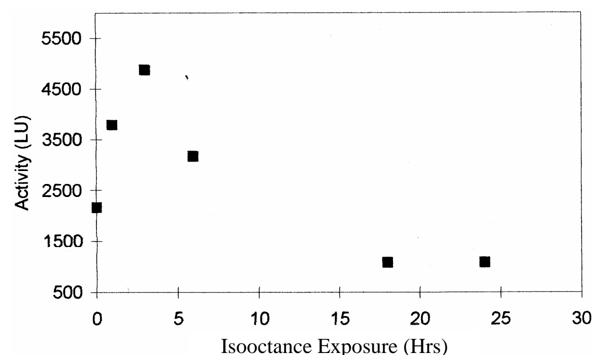

Fig. 2—Effect of isooctane on the activity of the enzyme

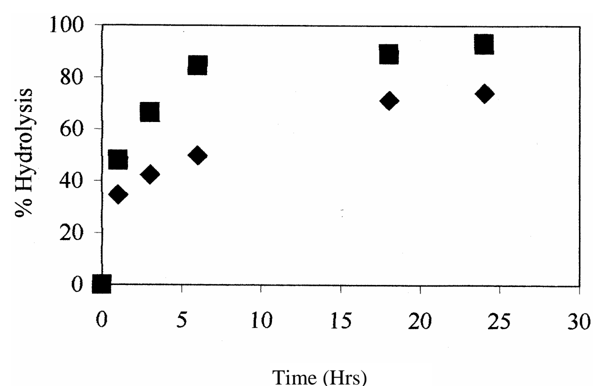

Fig. 3— Influence of amount of initial enzyme loading on the hydrolysis-rate pattern of the reaction. Legened key, 7 U/g of oil (υ) and 175 U (ν).

entire enzyme to the possible product inhibition.

Studies in Enzyme Addition during Reaction

Under the optimized conditions of *p*H (7.0) and temperature (55°C) and in the presence of solvent, for different protocols of enzyme addition (Table 1), it was observed that:

Protocol I) On the addition of 7 U/g of oil, 74% conversion was obtained at the end of 24 hrs;

Protocol II) When a single dose of 175 U of enzyme was added to start the reaction with no subsequent addition of enzyme, the conversion of about 85% was obtained in 6 hrs. However, the rate decreased with time and just 93% conversion was obtained after 24 hrs. Thus, a mere 8% enhancement in the hydrolysis was obtained in the last 18 hrs of the reaction with 25 times increase in the enzyme concentration;

Protocol III) The reaction started with 7 U/g of oil. Subsequently, 28 U were added after the first hour and 35 U were added at the end of 3, 6, and 18 hrs (total amount of enzyme added was 140 U). A net yield of 90% conversion was obtained at the end of 18 hrs. There was no significant increase in the conversion from 18 to 24 hrs;

Protocol IV) With the addition of enzyme (105 U), the conversion was similar to that obtained from protocol III. Thus, a reduction of 35 U was achieved without compromising the yield of the reaction. However, the reduction in the rate of reaction after 6 hrs was still observed. This indicated that the addition of enzyme after 6 hrs was not instrumental in improving the rate of reaction;

Protocol V) In this scheme, the reaction was initiated with 7 U and 28 U of the enzyme added at the end of 6 hrs. No subsequent addition of the enzyme was carried out. The strategy proved to be successful and about 82% conversion was observed at the end of 24 hrs. Thus, the total amount of enzyme required was brought down from 105 to 35 U with the compromise of 8% conversion, which was the difference between the per cent conversion values obtained at the end of 24 hrs from each of the schemes;

Protocol VI) In this scheme, an attempt was made to eliminate the 8% reduction in the per cent hydrolysis obtained in protocol V. When 45.5 U was added at the 6 hrs (total enzyme added was 52.5 U), the conversion value reached 90% at the end of 24 hrs, which was equal to that obtained as per protocol IV where total amount of enzyme added was 105 U. Thus, only 17.5 U of additional enzyme was required to raise conversion from 82 to 90%, which was half the amount of enzyme (35 U) required to achieve 82% hydrolysis under the same conditions.

The overall efficiency of the reaction with the least amount of the enzyme (7 U in protocol I) was highest

Table 1—Comparative % hydrolysis obtained with different protocols of enzyme addition during the course of reaction

Time (t) hrs	% Hydrolysis at different enzyme addition protocols*					
	I	II	III	IV	V	VI
0	0 (7)	0 (175)	0 (7)	0 (7)	0 (7)	0 (7)
1	34.72	48.07	34.72 (28)	34.72 (28)	34.72	34.72
3	42.43	66.47	51.27 (35)	51.27 (35)	42.43	42.43
6	49.85	84.57	68.36 (35)	68.36 (35)	49.85 (28)	49.85 (45.5)
18	71.22	89.32	89.65 (35)	89.65	77.07	83.71
24	74.18	93.18	90.85	90.04	81.85	89.53

Quantity in brackets represents the units of enzyme added at particular time

Table 2—Efficiency of the enzyme addition protocols expressed as the amount of fatty acids produced per enzyme unit per hr

Time (t) hrs	% Hydrolysis at different enzyme addition protocols*					
	I	II	III	IV	V	VI
0	0	0	0	0	0	0
1	0.36	0.07	0.36	0.36	0.36	0.36
3	0.15	0.03	0.035	0.035	0.15	0.15
6	0.086	0.02	0.012	0.012	0.086	0.086
18	0.04	0.007	0.004	0.004	0.04	0.04
24	0.032	0.006	0.002	0.0026	0.007	0.005

among all the other protocols (Table 2). This was because, even when the total amount of enzyme used in protocol VI (52.5 U) was relatively higher than the amount of enzyme used in protocol 1 (7 U) over a period of 24 hrs, the difference in yield was merely 15% (Table 2) higher in protocol VI as compared to protocol I. However, from commercial point of view, ricinoleic acid is marketed as about 87-90% hydrolysate of castor oil and hence it is necessary to achieve that yield in the hydrolysis process. In this respect, protocol VI becomes the most efficient protocol for the enzymatic hydrolysis of castor oil as it uses the least possible amount of enzyme to achieve the target of about 90% hydrolysate.

Conclusions

Under optimum operating *p*H (7) and temperature (55°C) for enzymatic hydrolysis of castor oil using lipase, presence of solvent as well as sonication improved the initial rate of the hydrolysis, however not much effect was observed over longer reaction periods. For a continuous process, the residence time can be adjusted so as to utilize the positive effects of the solvent addition and sonication. To achieve the amount of enzyme present in the reaction mixture at the end of 6 hrs was critically important to regulate the rate of hydrolysis and yield. An overall reduction (175 to 52.5 U) in the concentration of enzyme required to maximize the yield and rate of reaction can be achieved, provided an optimum intermittent addition protocol is selected (addition of 7 U initially and 45.5 U at the end of 6 hrs). The methodology followed in the present work could be used for finding the optimized enzyme addition protocols with an aim of minimizing the enzyme consumption for enzymatic reactions showing typical behaviour of product-based deactivation.

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