Enzymatic Synthesis of Estolides by a Bioreactor

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ABSTRACT: An attempt was made to synthesize ricinoleic acid estolides effectively with a bioreactor system containing immobilized lipase. The optimal water content for reaction changed during synthesis of estolide from ricinoleic acid. The water content needs to be controlled to optimize conditions for each reaction stage and to prepare estolide with a high degree of condensation. The estolide synthesis was attempted to confirm if the reaction would have proceeded to the targeted degree of condensation by controlling the reaction mixture under the optimal water content. The result showed that, with the bioreactor, the reaction could proceed rapidly. A repeated batch reaction was possible in the bioreactor. The amount of lipase used can thus be highly reduced compared with discarding it each time. The loading density of enzyme onto the carrier greatly affected enzymatic activity, with a loading level of 60 mg lipase/g carrier producing 60% more estolide per gram of enzyme than a loading level of 120 mg/g. The estolide product synthesized in the bioreactor showed no coloration during the reaction process. This fact confirmed the validity of the proposed method.

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KEY WORDS: Bioreactor, estolide, immobilized lipase, repeated batch reaction, ricinoleic acid, water content.

Estolide is a generic name for linear oligomeric polyesters of hydroxyl fatty acids wherein the carboxyl group and hydroxyl group of hydroxyl fatty acids are dehydrated to form oligomers (1). A typical example of a hydroxyl fatty acid is ricinoleic acid, a main component of castor oil fatty acids. The estolide made from ricinoleic acid (Scheme 1), hereinafter referred to as R-estolide, is a useful substance with many applications in various industries. For example, it is already used as a viscosity controller for chocolate and an emulsifier in margarine, as a cutting oil base in metal processing, and as pigment dispersant in paint, ink, and cosmetics (2).

At present, R-estolide is chemically synthesized by heating ricinoleic acid at 200°C. Owing to this high reaction temperature, problems of coloration and odors that are unsuitable for the food industry remain unsolved. The authors attempted the synthesis of R-estolide at low temperature through a lipase-catalyzed reaction to overcome these problems and clarified the following items (3): (i) R-estolide can be synthesized at low temperature by the catalytic action of lipase. The lipase from *Candida rugosa* synthesizes R-estolide most efficiently. (ii) R-estolide also can be synthesized by immobilized lipase, and activity is at least five times higher than that of free lipase. Therefore, application of an immobilized lipase to this reaction not only allows the reuse of lipase but also is an improvement in specific activity of the lipase. (iii) The water formed by the reaction should be removed from the reaction mixture so that R-estolide with a high degree of condensation can be obtained.

The present research was attempted to synthesize R-estolide efficiently in a bioreactor equipped with an ancillary water content control unit, based on the above observations. To synthesize R-estolide of a high degree of condensation rapidly by immobilized lipase, the optimal water content in the reaction mixture was identified. Furthermore, the optimal water content value was found to change during the course of the reaction.

The estolide product synthesized in the bioreactor showed no coloration during the reaction, confirming the validity of the method.

MATERIALS AND METHODS

Materials. Lipase AY from *C. rugosa*, whose utility was confirmed in the previous study (3), was provided by Amano Pharmaceutical Co., Ltd. (Nagoya City, Japan), and its specific activity was 120,000 U/g. The ceramic carrier SM-10 for immobilizing lipase, manufactured by NGK Insulators, Ltd. (Nagoya City, Japan), had an average particle size of 0.16 mm and a pore size of 20–100 nm. The lecithin used was Epicron 200S (Nippon Seber-Hegner, Tokyo, Japan). The ricinoleic acid manufactured by Itoh Oil Chemicals (Yokkaichi, Japan) contained 90% ricinoleic acid and is for sale under the commercial name of CO-FA. The components of these castor oil fatty acids and their relative contents are as follows: ricinoleic acid, 90%; oleic acid, 3–4%; linoleic acid, 3–5%; palmitic acid, 0.5–1%; stearic acid, 0.5–1.5%; dihydroxystearic acid, 0.5–1%.

Bioreactor system. The experimental apparatus for the synthesis of R-estolide, shown in Figure 1, consisted of an enzymatic reaction unit and a water content control unit. The

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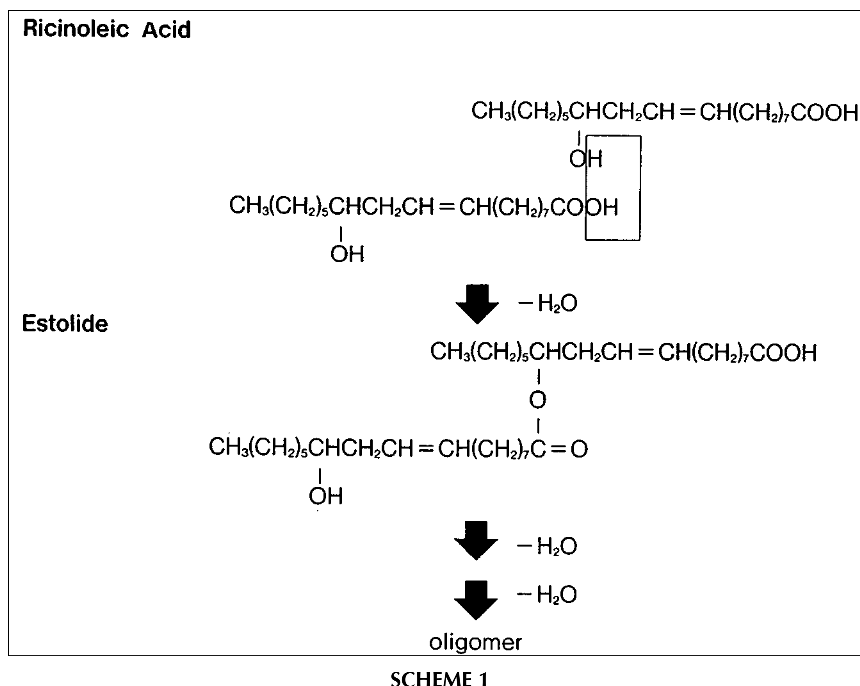

SCHEME 1

enzymatic reaction unit consisted of a reaction column, packed with immobilized lipase (inner diameter of 26 mm, adjustable length of 0–15 cm, manufactured by NGK Insulators, Ltd.); a pump for circulating the substrate in the column (variable between 0 and 10 mL/min, 880PU Jasco Corporation, Hachiouji City, Japan); a substrate tank (volume 100 mL, equipped with a water jacket, made of glass); and a constant-temperature water circulator (NTB-211, Tokyo Rika Co., Ltd., Tokyo, Japan). The substrate in the substrate tank was circulated to the column by the pump.

The water content control system was equipped with an aspirator (A3S; Tokyo Rika Co., Tokyo, Japan) for removing water from the reaction mixture, and a flash evaporator was connected with it (MF-5T, Tokyo Rika Co., Ltd.). A vacuum controller (ISCO, Shibata Kagaku Kiki Co., Ltd., Tokyo, Japan) was connected to the evaporator, and the drying rate could be controlled by adjusting the degree of vacuum. Solenoid valves, actuated by a timer, were installed in the pipes that connected the evaporator to the substrate tank and the evaporator to the aspirator. Thus, the substrate was recirculated intermittently to the evaporator by operation of a timer.

Determination of the degree of reaction. Neutralization value (NV) (4) was used as an index to show the degree of the reaction. NV corresponds to the carboxyl group concentra-

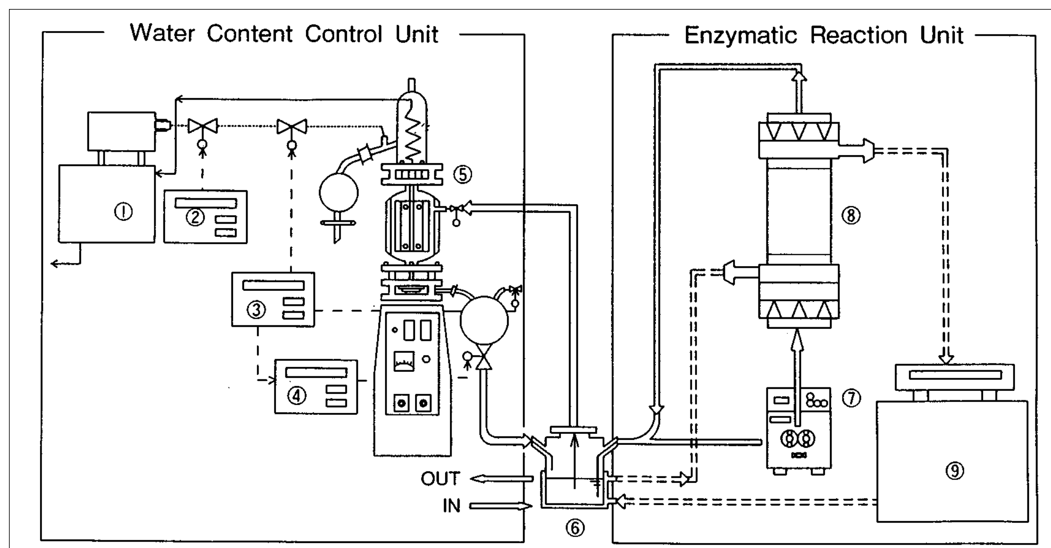

FIG. 1. Bioreactor system for estolide synthesis with water content control unit. 1) Aspirator, 2) vacuum controller, 3,4) timers, 5) flash evaporator, 6) substrate tank, 7) feed pump, 8) reaction column, 9) water bath.

tion in the reaction mixture. As the carboxyl group is consumed by the condensation of ricinoleic acid (NV, 140 to 160) during the reaction, NV is lowered, and the reaction is considered to proceed. It was also confirmed by high-performance liquid chromatography that the decrease in NV reflected the condensation of ricinoleic acid (2).

Determination of water content. The water content in the reaction solution was determined by the Karl-Fischer method with a water content meter (KF-06, Mitsubishi Kagaku Co., Ltd.).

Preparation of immobilized lipase. Sixty or 120 mg of lipase powder, together with 50 mg of lecithin, were dissolved in 1 mL of dissolved water. These two kinds of solutions were added separately to 1 g (dry base) carrier. Then, the resultant mixture was air-dried overnight to prepare the immobilized lipase preparation.

Synthesis of R-estolide by the bioreactor. Fifteen grams of the immobilized lipase (dry basis) was suspended in a small quantity of ricinoleic acid and added to the reaction column, which was previously heated to 40°C. Then, 76.5 g of ricinoleic acid was added to fill the substrate tank and was circulated through the column at a flow rate of 1 mL/min to effect the reaction. At the same time, the reaction mixture in the substrate tank was circulated through the flash evaporator under a controlled variable vacuum of 50–700 mm Hg, to effect drying. NV and water content in the reaction mixture were measured by sampling a small amount of the reaction mixture in the substrate tank.

Confirmation of the presence of minimum water content for the expression of enzymatic activity. Ricinoleic acid, previously dried to a water content of 1852 mg/kg, was circulated through the reaction column to effect the reaction. NV did not change further after 16 h, and the ricinoleic acid was then dried by the flash evaporator to observe changes for another 8 h. Then, a small portion of water was added to the substrate tank, and the change in NV was recorded again in the same manner. Drying of substrate was continuous during the operation.

Relationship between the water content of the reaction mixture and the reaction equilibrium. Water was added to the substrate tank during the reaction, and the drying was stopped. As the water content of the reaction mixture increased, the condensation reaction gradually stopped, and hydrolysis became significant. The reaction reached equilibrium when NV at the column inlet was nearly equal to the outlet value.

Determination of optimal water content at different degrees of condensation. R-estolide and ricinoleic acid of different NV (164.6, 100.5, 77.4, 63.7, and 47.4) were prepared with the experimental apparatus. Samples with different water contents were prepared by drying them or by directly adding water to them. These samples (5–15 g each) were put into vials with screw caps and held at 40°C. Then the immobilized lipase was added to the samples in amounts of 1–5% and placed in an incubator with a shaking apparatus (120 rpm). The change of water content was traced as time passed. The reaction rate was defined from the amount of water generated per gram of immobilized lipase in 1 h.

Repeated batch experiments with the bioreactor. With the bioreactor set at a vacuum of 100 mm Hg with the vacuum controller, the synthesis of R-estolide was carried out in the same manner as in the previous section. NV and water content were traced as time passed. When NV reached 40, the estolide in the reactor was replaced with fresh ricinoleic acid to keep the reaction continuous.

Color comparison of R-estolide products. The color of R-estolide products, prepared by the bioreactor, was compared with the chemically prepared products by the Gardner-Helige method (20) and by absorbance at 450 nm. Gardner-Helige is the method for estimating sample coloration by comparison with standard samples. The absorbance was measured with a spectrophotometer (UV-2200; Shimazu Seisakusho Co., Ltd., Kyoto City, Japan).

RESULTS AND DISCUSSION

Influence of the water content in the reaction mixture for the synthesis of R-estolides. NV of R-estolide, prepared by the conventional chemical method, was 40. Therefore, the targeted NV for the immobilized lipase method was also set at 40. According to our previous report (3), it was obvious that water formed by the reaction had to be removed to achieve the targeted NV in R-estolide synthesis. On the other hand, a number of reports describe that, in the enzymatic reaction in a microaqueous system, such as the R-estolide synthetic system, the enzymatic activity is highly influenced by a trace amount of water in the reaction mixture (5–15). Thus, the authors analyzed the influence of the water content of the reaction mixture on R-estolide synthesis from the following three points of view.

Presence of minimum water content for the expression of enzymatic activity. Some reports describe that, when the water content of a reaction mixture approaches 0, the enzymatic reaction rate is drastically lowered (16,17). This is explained by the requirement for a minimum water content to maintain the configuration of the enzyme in the proper form to express catalytic activity. Similar phenomena were presumed in R-estolide synthesis. Previously dried ricinoleic acid (1852 mg H_2O/kg) was circulated in the immobilized lipase column, and the change in NV was observed as time passed. No decrease in NV was observed for 16 h (Fig. 2). Because we feared that the residual water was suppressing the dehydration condensation, ricinoleic acid was thoroughly dried by flash evaporation (water content 1061 mg/kg) and subjected to reaction for a further 8 h. However, no change in NV was observed. After that, water was added to the substrate tank to increase the water content of the reaction mixture (water content 4223 mg/kg). Then, NV began to decrease, and the reaction proceeded. From these results, it appears that the minimum water content required for expressing lipase activity lies between 1800 and 4200 mg/kg for R-estolide synthesis.

The water contents reported to decrease lipase activity in transesterification of olive oil (18) and in the synthesis of

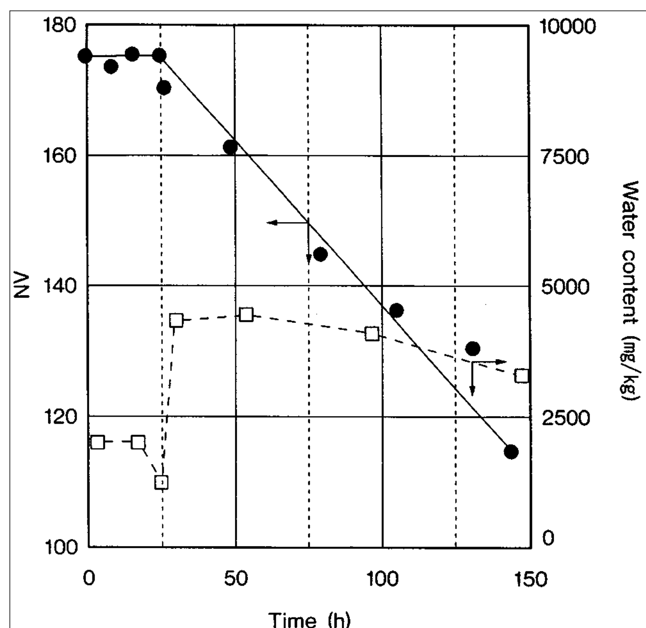

FIG. 2. Changes in neutralization value (NV) and water content during R-estolide synthesis. NV (●) and water content (□).

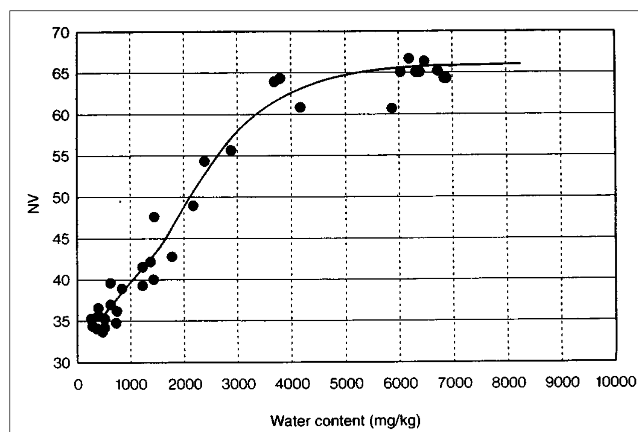

FIG. 3. Relationship between water content and NV of the reaction mixture at equilibrium. See Figure 2 for abbreviation.

triglyceride from diglyceride and fatty acid (19) are 17 and 10 mg/kg, respectively. Compared with these values, the value of 1800–4200 mg (0.1–0.23 moles)/kg obtained for the condensation of ricinoleic acid is high. It is postulated that this is because most water molecules coordinate with the polar group of ricinoleic acid, leaving few available to maintain the stearic configuration of the lipase. Approximately 3 moles of ricinoleic acid are contained in 1 kg of castor oil fatty acids that contain 90% ricinoleic acid (MW 298.5). Equivalent numbers of moles of hydroxyl and carboxyl groups are present in this material. Thus, one mole of hydroxyl and carboxyl groups possibly coordinates 0.1–0.3 mole of water. As described below, even when the water content became lower than 1000 mg/kg with the progress of the reaction, the reaction proceeded due to the decreased polarity of the bulk reaction mixture.

Relationship between the water content of the reaction mixture and reaction equilibrium. Because the R-estolide synthesis is a dehydration–condensation reaction, the reaction reaches equilibrium and stops when the water content in the reaction mixture increases by the water formed through the reaction (3). Thus, the relationship between reaction equilibrium and water content was established, and the water content that gave the targeted NV of 40 was determined.

The experimental results are shown in Figure 3. NV at the equilibrium point of the reaction increased proportionally with the water content of the reaction mixture. Dehydration–condensation occurred at a lower water content than that at the equilibrium point of the reaction, whereas hydrolysis was observed at a higher water content. To achieve the targeted NV of 40, it was necessary to adjust the water content of the reaction mixture to ≤1000 mg/kg at the final stage. In the previous experiment, the reaction did not proceed unless the water content was higher than 1800 mg/kg at the start of the reaction. However, because of the lowered polarity of the reaction mixture at the end of reaction, the water content needed to be set at 1000 mg/kg or lower in order for the reaction to proceed further at the final stage of the reaction.

Optimal water content. It was anticipated that an optimal water content will be present between the minimum water content for the enzymatic activity and the water content at the equilibrium state. In this particular reaction system, the concentrations of carboxy groups and hydroxyl groups in the reaction mixture decrease as the reaction proceeds, during which time the polarity of the reaction mixture decreases greatly. Therefore, the quantity of water molecules coordinated with the reaction mixture changes, and hence, the optimal water content is lowered. To carry out the reaction efficiently, the optimal water content was determined at each degree of condensation (Fig. 4).

In this figure, the y-axis is the water generation rate, a measure of the reaction rate. A negative value in the figure means that hydrolysis occurred and water was consumed. In a substrate with an NV of 164.6, the optimal water content for lipase reaction was about 5000 mg/kg, and a high reaction rate was observed for a wide range of water contents. In a substrate with a low NV, the range of water content in which condensation proceeds was narrowed, and the reaction rate was also decreased compared with the reaction of the substrate with a higher NV. Therefore, to get R-estolide with a high degree of condensation, the water content must be set within a narrow range. It was clarified that the optimal water content at each degree of condensation shifts to a lower value as the NV and bulk polarity become lower.

Synthesis of R-estolides at the optimal water content in the bioreactor. In accordance with the observation on water content, obtained by the previous experiments, synthesis of R-estolides was attempted in the bioreactor. The intention was to obtain R-estolide with the targeted NV of 40 in a short period by controlling the water content of the reaction mixture within the optimal water content range.

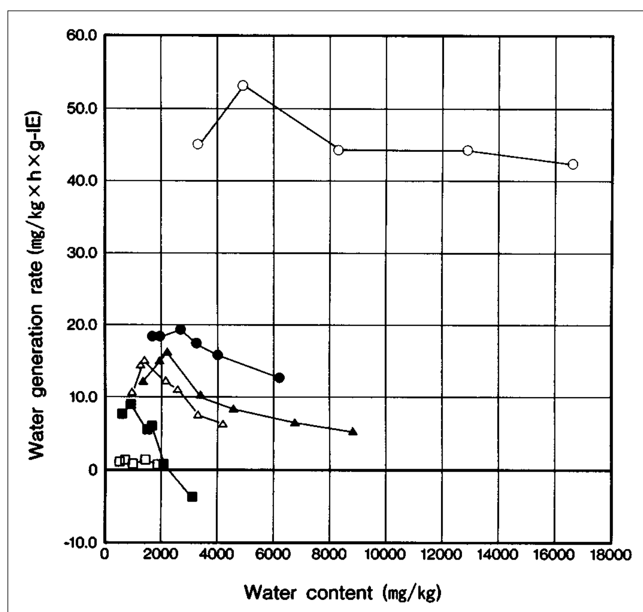

FIG. 4. Relationship between the water generation rate and water content in substrates with different NV. g-IE, grams of immobilized enzyme. NV = 164.6 (○), 115.2 (●), 100.5 (▲), 77.4 (△), 63.7 (□), and 47.4 (■). See Figure 2 for other abbreviations.

As described in the previous section for the R-estolide synthesis system, the optimal water content is different at each reaction stage. Therefore, a completely mixed type of reactor, in which the water content can be easily controlled as the reaction proceeds, is more suitable than a plug-flow type. When a completely mixed type of reactor is operated continuously, the targeted NV is only attainable at the lowest water content at which the condensation reaction rate is unfavorably small. Therefore, we felt that a batch operation in a completely mixed type of reactor was most suitable.

R-estolides were synthesized under different degrees of vacuum by using the vacuum controller connected to the bioreactor system. The water content and NV of the reaction mixture were measured as time passed. Ricinoleic acid with the same amount of water was used without special conditioning. The results are shown in Figure 5. The period required for preparing R-estolide of NV 40 varied with absolute pressure as follows: 110 mm Hg, 70 h; 310 mm Hg, 94 h; 410 mm Hg, 137 h; 760 mm Hg, not attainable. The water content reduction curve during the reaction period varied for different degrees of vacuum. A water content reduction curve close the optimal water content region was apparent when the vacuum was set at 110 mm Hg. The time for reaching the targeted NV of 40 was 70 h, approximately half of the 152 h required for vacuum under 510 mm Hg. When the water was simply evaporated under atmospheric pressure without intentional vacuum dehydration, the water content reduction curve deviated from the region of the condensation reaction and intersected the reaction equilibrium line near NV 60. As a result, NV 40 could not be reached.

The results mentioned above proved that, when R-estolide was synthesized in a reaction mixture with a water content close to the optimum, the R-estolide of the targeted degree of condensation could be synthesized in a shorter period than under other conditions.

Repeated batch experiment. Because we proved that R-estolides could be efficiently synthesized in the bioreactor system by the previous experiments, repeated batch experiments were carried out. The objective of the experiments was to search for the possibility of reducing the consumption of immobilized lipase by reusing it.

The results with immobilized lipases of different loadings

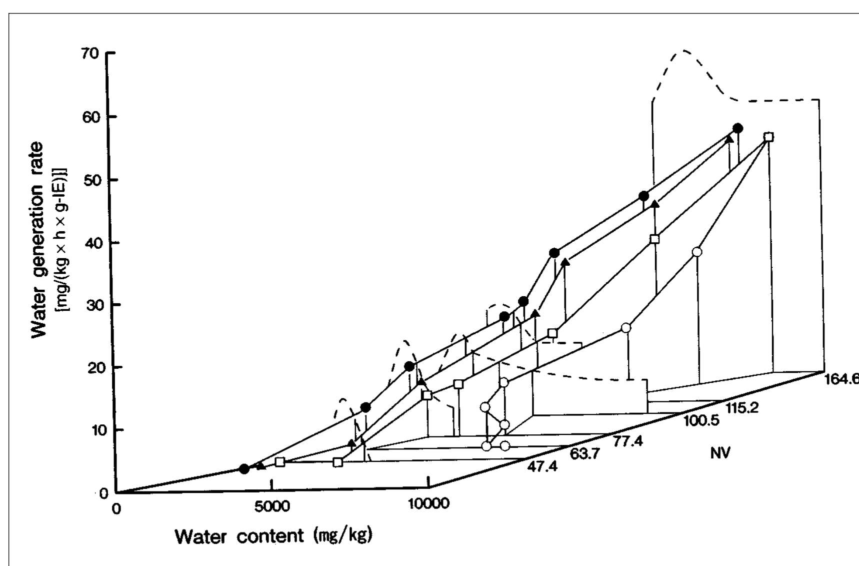

FIG. 5. Profiles of reduction in water content under different vacuum conditions. See Figures 2 and 4 for abbreviations. Absolute pressures (mm Hg) = 10 (●), 310 (▲), 410 (□), and 760 (○).

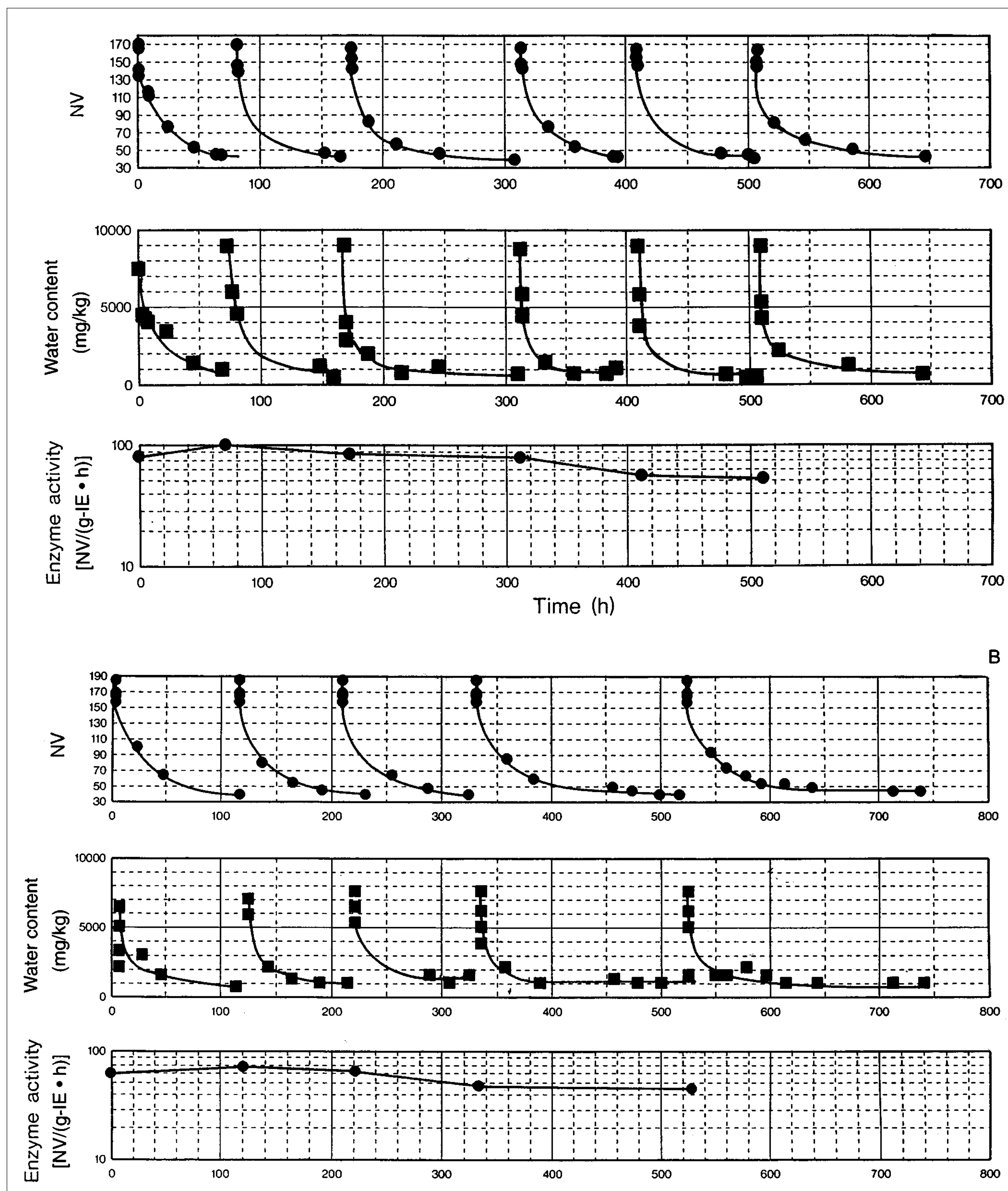

FIG. 6. Synthesis of estolides by repeated batch operation. (A) Enzyme loading = 120 mg/g carrier; (B) enzyme loading = 60 mg/g carrier. See Figures 2 and 4 for abbreviations.

Table 1
Comparison of Color of the Estolides Produced by Enzymatic Method[a]

Sample	Gardner–Helige method	Optical density at 450 nm
NV163	2	0.1106
NV138	2	0.1149
NV119	2	0.1004
NV111	2	0.1069
NV64	2	0.0709
NV47	2	0.0776
NV35	2	0.0923
NV34	2	0.1034
Chemically synthesized estolide	4–5	Over range

[a]NV, neutralization value.

(120 mg and 60 mg/g carrier) are shown in Figure 6A. The stability of the immobilized lipase was calculated from the reaction rate at the beginning of each batch operation, i.e., the decrease in NV per gram of immobilized lipase per hour.

The half life of immobilized lipase activity calculated from the reduction in enzyme activity was about 600 h and was independent of lipase loading.

For an enzyme loading of 60 mg/g, the time required for the reaction per batch increased as compared with a loading of 120 mg/g. However, in spite of the half loading of lipase, the time required per batch did not double. For example, over a 700-h time period, six reactions were possible for 120 mg/g and five reactions for 60 mg/g. In considering the fact that 76.5 g of the substrate per batch was reacted in each experiment with 15 g of the immobilized lipase, the amount of R-estolide produced per unit weight of lipase can be calculated as follows (Figs. 1 and 2).

For 120 mg/g:

$$(76.5 \text{ g/batch} \times 6 \text{ batch})/(15 \text{ g immobilized enzyme} \times 0.12 \text{ g free enzyme/g immobilized enzyme}) = 255 \text{ g estolide/g free enzyme} \quad [1]$$

For 60 mg/g:

$$(76.5 \text{ g/batch} \times 5 \text{ batch})/(15 \text{ g immobilized enzyme} \times 0.06 \text{ g free enzyme/g immobilized enzyme}) = 425 \text{ g estolide/g free enzyme} \quad [2]$$

Therefore, for a loading level of 60 mg/g, the amount of lipase used per unit weight of R-estolide is 60% of that of the 120 mg/g lipase loading level.

The authors have reported earlier (3) that the activity rate, expressed as the observed activity/(total activity used for immobilization) × 100, changed depending on the lipase loading for adsorption. Though the reaction rate can increase with the increase in lipase loading for adsorption, the activity expression rate decreases as the amount of the enzyme used per unit weight of product increases. A similar result was confirmed in this experiment.

Comparison of the coloration of R-estolide products. The color of the product was measured to clarify if the R-estolide synthesized by the bioreactor system at low temperature was improved in terms of its color compared with the product chemically synthesized at a higher temperature. The results are shown in Table 1. No product coloration due to the reaction was observed, and the R-estolide product prepared by the bioreactor system was therefore of high quality.

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