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Enzymatic Hydrolysis of Starch in Water-Immiscible Organic Solvent, Two-Phase Systems

TAKASHI MORITA[1,2] AND ISAO KARUBE*[,2]

[1]Akebono Brake Research & Development Center Ltd., Higashi Hanyu, Saitama Pref., Japan; and [2]Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153, Japan

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ABSTRACT

Enzyme-catalyzed hydrolyzations of starch by α-amylase have been studied in various two-phase systems, consisting of water and a water-immiscible organic solvent. The hydrolytic conversion of soluble starch to malto-oligosaccharides by α-amylase was greatly accelerated in 10% (v/v) water content of water-dodecane two-phase systems. However, a rapid inactivation of the enzyme has been observed in these systems. Addition of surfactant to these systems, such as polyoxyethylene (20) sorbitan monopalmitate (Tween 60) or bis(2-ethylhexyl) sodium sulfosuccinate (AOT), was effective for the enzyme stability. Effects of enzyme immobilization on the stability of α-amylase, using Ca-alginate and chitosan beads, also have been studied. The stability of immobilized enzyme was clearly enhanced in a 5–10% (v/v) water content two-phase system, whereas the free enzyme was inactivated within 41 h, remaining at a relative activity of 47–76% after 41 h of treatment. Furthermore, scanning electron micrographs (SEM) were taken to observe the effect of the two-phase system on the hydrolysis of starch. Potato starch granules have been extremely swelled and burst out in the stirred 10% (v/v) water content system, which did not contain enzymes.

Index Entries: α-Amylase; starches; enzymatic hydrolysis in water-immiscible organic media; surfactants; immobilization.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Enzyme catalysis in organic solvents has attracted much interest in recent years. The fact that enzymes and cells may be active in an organic solvent has opened a totally new area for enzyme technologists to exploit (*1*). A number of reviews presenting their importance have been published in recent years (*2–4*). The advantages of the use of such two-phase systems are especially evident when enzyme-catalyzed reactions are carried out (1) with substrates that are poorly soluble in water and (2) when water is a reagent directly involved in the reaction (*5–10*). In a two-phase system, the reaction equilibrium may be shifted favorably owing to differences in partitioning of products and substrates between the two phases.

On the other hand, the following problems might arise:

1. Enzyme can denature at the interface of the two solvents;
2. Continuous power input may be required to maintain a high surface area for mass transfer; and
3. Mass transfer may limit the observed reaction rates (*11*).

The activity of enzymes in organic solvents depends on the amount of water in the system as well as on how the water is distributed in the reaction mixture. In order to be catalytically active, the enzyme must have a surrounding bound water layer (*12*). Using organic solvents and supports with low hydrophilicity, high activities are obtained with a low amount of water addition (*13*). Furthermore, by using an organic solvent in which the enzymes are insoluble, convenient immobilization by adsorption on a solid support material is possible, and in addition, these immobilized enzymes are often more stable in these water-poor environments than in water (*14*).

On the other hand, in recent years, several kinds of surfactants have been used to obtain reverse micelles or water-in-oil (W/O) microemulsions. The system "AOT/isooctane/water" is probably the best known (*15–18*). Moreover, this system has been utilized to solubilize a variety of enzymes in organic solvents without loss of activity (*19–21*). The surfactant is oriented at the interface of the two phases, so that the hydrophilic head is in contact with the aqueous phase and the hydrophobic tail protrudes in the organic phase. Consequently, the enzyme molecules are entrapped in the reverse micelles, avoiding direct contact with the organic medium, thus potentially limiting their denaturation. The interior of such reverse micelles acts as a microenvironment for enzyme activity (*22*). Many reports described enzymatic reaction in water-immiscible organic solvents using hydrolytic enzyme (*2–4,23*). So far, only a few studies have been conducted with glycosidases (*24*).

In the present study, we investigated ways to improve the efficiency of enzymatic hydrolysis of starch by conducting the reaction in a two-phase water/dodecane emulsion system. The use of different types of

anionic, cationic, and nonionic surfactants, as well as the use of immobilized α-amylase from *Bacillus subtilis,* to make microemulsions and stabilize the enzyme activity is described.

MATERIALS AND METHODS

Enzyme

α-Amylase (1,4-α-D-Glucan glucanohydrolase, EC 3.2.1) from *B. subtilis* was purchased from Wako (Japan) as a lyophilized powder with a specific activity of 20 U/mg dry wt.

Chemicals

Soluble starch, potato starch, and standards of glucose (noted G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and maltopentaose (G5) were obtained from Wako (Japan). The anionic surfactant bis(2-ethylhexyl) sodium sulfosuccinate (AOT) was obtained from Sigma. The cationic surfactant, cetyltrimethyl ammonium bromide (CTAB), was purchased from Aldrich. The nonionic, polyoxyethylene (*20*) sorbitan monopalmitate (Tween 60) and sorbitan monooleate (Span 80) were from Merk. All surfactants were used without further purification. CHITOPEARL (BCW-3515) was purchased from Fuji-bouseki (Japan). All other chemicals and solvents used in this work were of the highest commercially available purity.

Analysis of Enzymatic Reactants and Products

The concentrations of all enzymatic reaction products were measured using high-performance liquid chromatography (HPLC) analysis. A chromatography system equipped with a Shimadzu Inc. model LC-6AD pump, a CTO-6A column oven with a 20-μL loop, and a RID-6A refractive index detector were used. The detector signal was integrated by a Shimadzu C-R6A Chromatopac. Samples were analyzed using two Asahipak GS-220H columns (4.6 × 250 mm) protected by a guard column (30 mm) with the same packing. The injected samples were eluted with water of Milli-Q quality, all at a flow rate of 0.8 mL/min. The temperature of the column was kept constant at 60°C.

Enzyme Immobilization

α-Amylase (1.5 g) was dissolved in 60 mL 3% (w/v) sodium alginate. This solution was added dropwise into a 2% (w/v) $CaCl_2$ solution, and alginate gel beads were formed. After being washed with a 0.9% (w/v) saline solution for several times, the enzyme immobilized in Ca-alginate was utilized for further experiments.

Another immobilization method for α-amylase was performed using chitosan beads. α-Amylase was dissolved in 30 mL of sodium phosphate buffer (50 m*M*, pH 6.0) and was subsequently mixed with 20 mL of wet chitosan beads for 2 h at 30°C. Subsequently, the product was washed thoroughly with the same buffer and stored at 4°C overnight in the buffer solution.

Measurement of Enzyme Activity

Both free and immobilized α-amylases were incubated at 40°C on stirring (400 rpm) in either an aqueous phosphate buffer (pH 6.0, 50 m*M*) or a 10% (v/v) water content of water-dodecane system, containing 1% (w/v) surfactant (AOT, CTAB, Span 80, or Tween 60). After incubating at different time intervals, a sample was taken and added to a 1% (w/v) soluble starch standard solution. The subsequent occurring hydrolyzation was carried out for 30 min at 40°C. To end the enzymatic reaction, each sample was boiled for 5 min, and HPLC analysis was carried out. The relative enzyme activites were calculated from the total concentration of oligosaccharides, consisting of glucose (G1) to maltohexaose (G6).

Enzymatic Reactions

α-Amylase-catalyzed reactions were conducted by adding a solution of 2–10% (w/v) of soluble starch or 10% (w/v) of potato starch to 10 mL of dodecane-containing phosphate buffer (200 m*M*, pH 6.0) solution. To some of these systems, 1% (w/v) of surfactant was added. Reactions were started by adding 4 U/mg (starch) to free α-amylase or 0.1% (w/v) of immobilized α-amylase to the above solution. All reactions were performed at 40°C, at 400 rpm using a magnetic stirrer to ensure formation of the water-dodecane emulsion systems.

Determination of the Conversion Ratio

The conversion ratio was defined as the amount of products (glucose and malto-oligosaccharides) divided by the initial amount of substrate (soluble starch or potato starch), and was calculated using an HPLC chromatogram as follows: First, the area of each product peak was converted to the concentration of monosaccharide (dividing each peak area by the obtained area of standard glucose solution). After that, the concentration of each peak of product was divided by the initial concentration of substrate in the reaction solution to determine the conversion ratio.

RESULTS AND DISCUSSION

In this study, enzymatic reactions were performed under stirring speed conditions of about 400 rpm. Such stirring led to the formation of

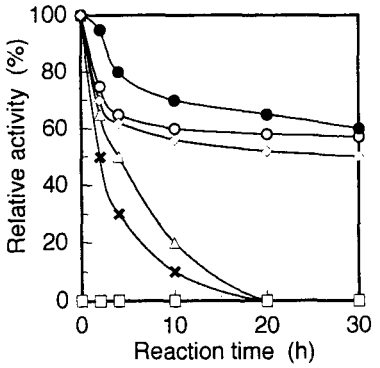

Fig. 1. Stability of α-amylase from *B. subtilis* on the hydrolysis of soluble starch in the 10% (v/v) water content water-dodecane two-phase systems, containing different kinds of surfactants.

Symbol	Water content, % (v/v)	Dodecane content, % (v/v)	Surfactant, 1 % (w/v)
○	100	—	—
×	10	90	—
■	10	90	Tween 60
◇	10	90	AOT
△	10	90	Span 80
□	10	90	CTAB

small W/O-type enzyme–substrate particles, in which solutions contained protein and substrates. In such emulsions, the properties of water in the apolar organic solvent are different from those of bulk water (*25*). For example, the polarity of the medium in which the solubilized enzyme is situated is considerably less than that of the aqueous solution (*26,27*). Furthermore, by addition of a surfactant in the biphasic solvent system, thermodynamically stable microemulsions might be easily formed and protect the enzyme from denaturation at the interface of two solvents (*21,28–30*). Below, various factors influencing the enzymatic hydrolysis of starch in two-phase organic solvent systems will be discussed.

Effect of Surfactants on the Enzyme Stability

In previous experiments, we found optimum α-amylase activity in a water-dodecane two-phase system at a water content around 2–10% (v/v) (*31*). The hydrolysis of soluble starch in water and 10% (v/v) water content water-dodecane two-phase systems, including the effect of different surfactants on the enzymatic activity, is shown in Fig. 1. Results show a rapid inactivation of the enzyme in a water-dodecane system, as well as on addition of the nonionic surfactant (Span 80). In both systems, the enzyme activity disappeared within 20 h. Addition of the cationic surfactant (CTAB) also led to catalytic inactivation of the enzyme. However,

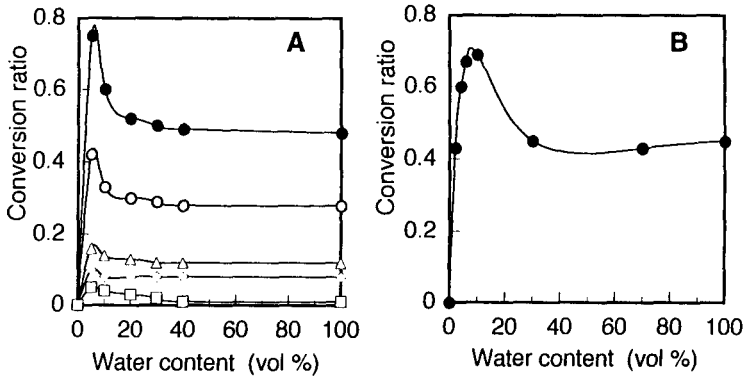

Fig. 2. Effect of water content on the α-amylase-catalyzed hydrolysis of soluble starch, containing 1% (w/v) Tween 60 in the water-dodecane system (**A**) and in the absence of any surfactants (**B**; *see* ref. *31*). The detectable products by HPLC method were glucose (□), maltose (◇), maltotriose (△), and maltopentaose (○). Total amount of these products was indicated as (●).

when anionic (AOT) and nonionic (Tween 60) surfactants were added to the systems, the enzyme was stabilized as in the aqueous system showing about 60% relative activity even after 30 h in the two-phase solvent systems. Remarkably, Tween 60 showed even better enzyme activity than in the water system.

Figure 2A shows the effect of water content on the hydrolysis of soluble starch to glucose and malto-oligosaccharides in a water-dodecane emulsion system containing 1% (w/v) Tween 60. The hydrolytic activity of α-amylase was greatly enhanced when the water content in the system was increased, showing a maximum at 5% (v/v) water. Above this concentration, the enzyme activity rapidly declined. The enzymatic reaction in the water-dodecane system without any surfactants has been determined, as shown in Fig. 2B. A similar, bell-shaped curve was obtained with respect to the water content. However, the conversion ratio of total products was lower and the concentration of water where the maximum activity occurred has shifted a little to higher water content (7.5–10% v/v). Regarding the results from Figs. 1 and 2, addition of Tween 60 improved the enzyme stability, prolonged the period for reaction termination, and consequently increased the conversion ratio. These results suggested that the enzyme in aqueous phase was protected by a surfactant membrane preventing direct contact with organic solvent, since the surfactant is located on the boundary between the apolar organic solvent and the aqueous phase. Therefore, the enzyme activity was stabilized by addition of Tween 60 and AOT in the water-dodecane system.

On the contrary, the addition of the nonionic surfactant Span 80 did not improve the stability of α-amylase in the water-dodecane system. Castanon and Wilke (*32*) have reported that addition of the nonionic surfactant Tween 80 to a reaction medium in water increased the extent of

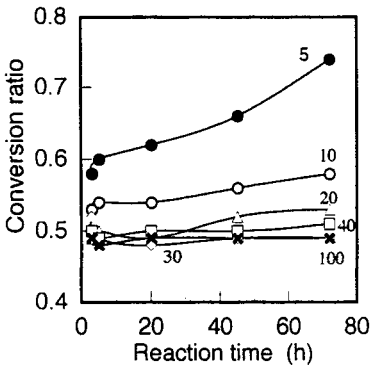

Fig. 3. Time-course of α-amylase-catalyzed soluble starch hydrolysis, containing 1% (w/v) Tween 60 in the water-dodecane, two-phase systems for various water contents (in % v/v) as indicated.

saccharification. However, Ooshima et al. (33) also showed a positive effect of addition of a nonionic surfactant on the enzymatic saccharification of cellulose in water. An apparent difference between Span 80 and Tween 60 is the value of the hydrophile-lipophile balance (HLB). The HLB values of Span 80 and Tween 60 are 5.1 and 14.9, respectively. A higher HLB value shows a higher affinity for a hydrophilic substance, and it is reported that the highest ability of protein solubilization is obtained at an HLB value of around 14. In addition, the HLB value determines the size of emulsion, and interaction between the water phase and the organic solvent. Therefore, we assume that Tween 60 with an HLB valuer of 14.9 provides a proper solubility condition and a suitable emulsion size for the stabilization of α-amylase.

Figure 3 demonstrates the time-course of soluble starch saccharification in the "water-dodecane/Tween 60" system for various water contents. With respect to the system with 5% (v/v) water, the enzyme did not seem to be inactivated, and reaction speed seemed to increase after 70 h.

Effect of Immobilization on the Enzyme Stability

Enzyme immobilizations to stabilize enzyme activity have also been studied, as a function of water content in the water-dodecane two-phase systems. Table 1 shows the relative activities of immobilized α-amylase on Ca-alginate gel beads and free α-amylase. The stability of immobilized α-amylase on Ca-alginate beads in water showed no significant differences in comparison with using free enzyme. The immobilized enzyme, however, showed an obvious stability in a 5% (v/v) water content reaction system. Furthermore, production components in the reaction aqueous phase have been analyzed by HPLC. As shown in Fig. 4, a maximal starch conversion ratio to maltopentaose (G5) was obtained at a water content of 20–40% (v/v). On the contrary, amounts of maltose (G2) and maltotriose

Table 1
Relative Activity of Immobilized α-Amylase on Ca-Alginate Gel Beads and Free α-Amylase in Different Water-Dodecane Systems

Water content, wt %	Immobilized enzyme			Free enzyme		
	Reaction time, h			Reaction time, h		
	0	17	41	0	17	41
5	92	107	76	—	—	—
10	104	82	47	146	22	0
100	100[a]	82	59	100[b]	60	60

[a]Standard relative activity of the immobilized α-amylase in an aqueous system.
[b]Standard relative activity of the free α-amylase in an aqueous system.

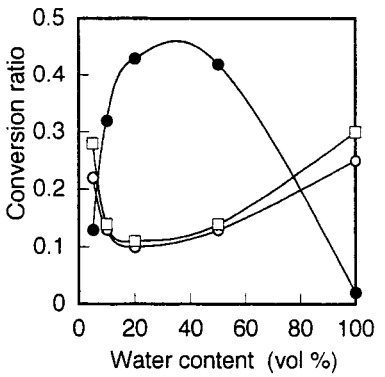

Fig. 4. Effect of water content on the α-amylase-catalyzed 2% (w/v) soluble starch hydrolysis. α-Amylase was immobilized in Ca-alginate gel beads. Maltopentaose (●); maltotriose (○), and maltose (□).

(G3) show an opposite trend when compared with G5. Using immobilized α-amylase, no production of glucose (G1) was detected, which is different from the case when using free enzyme; *see*, e.g., Fig. 2. Immobilization of α-amylase on chitosan beads was also performed. Figure 5 shows the effect of water content on the hydrolysis of 10% (w/v) soluble starch in the water-dodecane systems. A bell-shaped curve similar to that in Fig. 2 or 4 has been observed with a maximum enzymatic activity at a water content of 10–20% (v/v).

Effect of Two-phase Systems on the Structure of Starch Granules

Scanning electron micrograph (SEM) observation has been conducted to elucidate the effect of different two-phase systems on the accelerated hydration of starch. Figure 6 shows the effect of stirring in a two-phase,

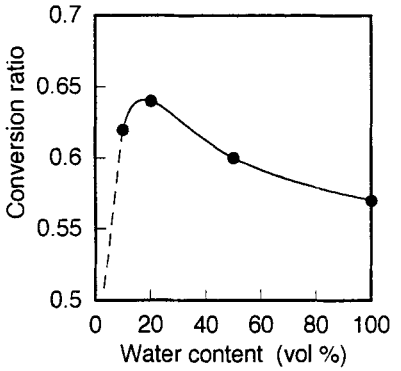

Fig. 5. Effect of water content on the α-amylase-catalyzed 10% (w/v) soluble starch hydrolysis. α-Amylase was immobilized on chitosan beads.

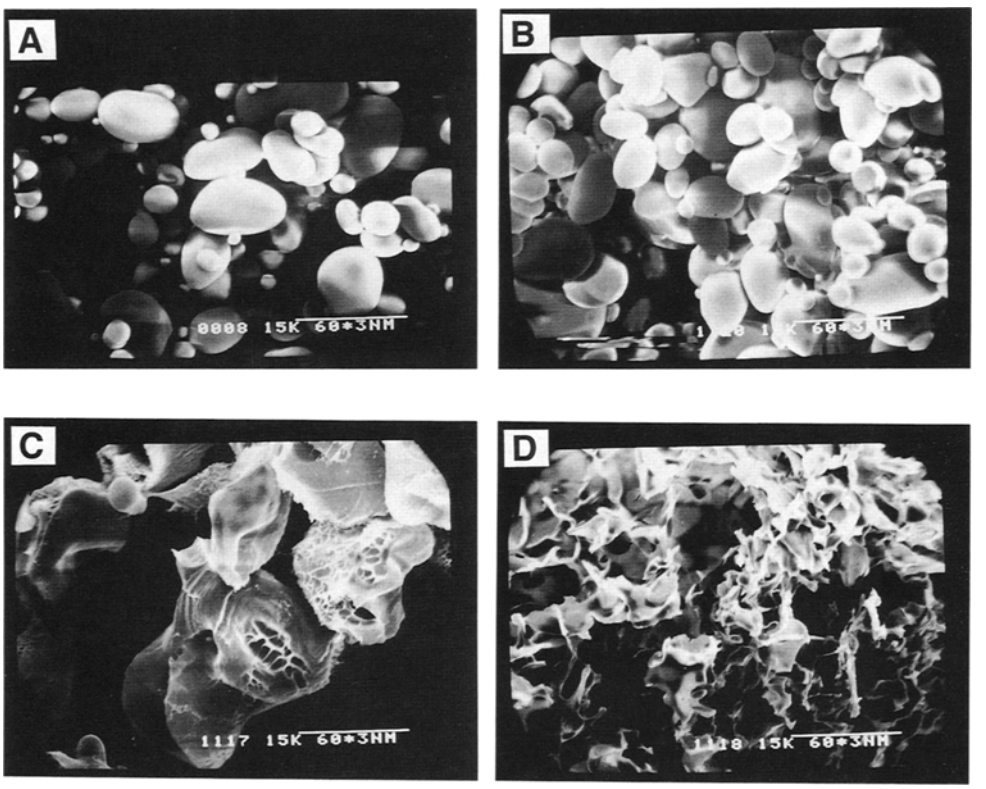

Fig. 6. SEM observation of potato starch granules, showing the effect of stirring pretreatment of starch granules on the shape of the particles in the water-dodecane system in the absence of any α-amylases. (**A**) Natural potato starch granules dried powder without any treatment; stirring pretreated; (**B**) in 100% dodecane solution; (**C**) in an aqueous buffer; (**D**) in 10% (v/v) water content water-dodecane system.

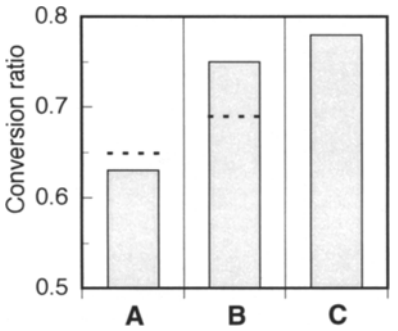

Fig. 7. Effect of stirring pretreatment, without enzyme, on the α-amylase-catalyzed soluble starch in a 10% (v/v) water content water-dodecane system. The stirring pretreatment time was 5 h. Thereafter, enzymatic reaction was performed for 9 h. Dashed line: no stirring pretreatment.

	Water content, % v/v	
Mark	Pretreatment	Enzyme reaction
A	100	100
B	10	10
C	2	10

water-dodecane system on the structure of raw potato granules in the absence of α-amylase. Potato starch granules in a dodecane solution did not change at all on stirring for 20 h (Fig. 6B). In water, the granules were partially swollen and burst-like, but kept some of their previous shape (Fig. 6C). It is obvious that in a 10% (v/v) water content water-dodecane system, the potato starch granules were extremely "exploded," resulting in an enlargement of the surface area of the granules (Fig. 6D).

In general, cereal granules are digested by amylases of plant, microbial, and animal origins both in vivo and in vitro, and degradation patterns of these granules have been extensively studied (*34–36*). Potato starch granules, on the other hand, are quite resistant to amylases, although they are hydrolyzed easily once gelatinized. Taniguchi et al. (*36*) reported that using amylase from *Bacillus circulans*, potato starch granules were degraded gradually from their surfaces, although no erosion of their internal region was observed. This might suggest that the internal part of potato starch granules is more resistant to an enzymatic attack than the surface region. Following this suggestion, adoption of a two-phase system in the enzymatic hydrolysis of raw potato starch granules could make it possible to degrade α-amylase easily because of its explaning surface area. The high conversion ratio of G5 (Fig. 4) might also be explained by this suggestion. To overcome this problem, the internal part of granules should be isolated.

Figure 7 shows the effect of stirring pretreatment on the enzymatic hydrolysis of soluble starch granules in the water-dodecane system. The

conversion ratios in water with or without stirring pretreatment were 0.63 or 0.65, respectively. The conversion ratios in a 10% (v/v) water content dodecane system with or without stirring pretreatment were 0.75 or 0.69, respectively. Moreover, when the stirring pretreatment was done in a 2% (v/v) water content system, the conversion ratio in a 10% (v/v) water content system showed a maximum of 0.78. It is apparent from Fig. 7 that the hydrolytic activity of α-amylase obviously depended on the water content of the pretreatment system and only increased in the water-dodecane two-phase system.

Throughout this article and our previous studies (*31*), one suggestion concerning the enhancement of enzymatic reactions on the saccharification of starch or other polysaccharides in a water-immiscible organic solvent is that the structure, i.e., the surface area, of the substrate might be altered favorably for the enzyme to be easily attacked. Another possibility is the phenomenon of enzyme inactivation in a water-dodecane two-phase system, which might be caused by the conformational change of the enzyme as well as the substrate. It still remains unclear why (1) a bell-shaped hydrolysis curve is obtained at a specific water content, and (2) why G5 production is greatly enhanced on the enzymatic hydrolysis of starch in a water-immiscible organic solvent two-phase system.

Further work aimed at elucidating the mechanism of the polysaccharide hydrolysis in a two-phase system solvent system is now in progress in our laboratory.

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