ISSN : 0974 - 7435 Volume 8 Issue 12

BioTechnology
An Indian Journal
FULL PAPER
BTAIJ, 8(12), 2013 [1717-1725]

Lipase-catalyzed esterification of corn-starch with oleic acid in solvent-free system

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ABSTRACT

An efficient procedure for the lipase-catalyzed esterification of starch has been established in a solvent-free medium. Corn starch was used as the acyl acceptor and oleic acid was used as the acyl donor. In order to improve the reactivity of native starch (NS), it was pretreated with NaOH/Urea hybrid solution at cool temperature below -10 °C. The esterification of starch was carried out at different initial a_w, temperatures for different times. The amount of lipase and the ratio of starch to oleic acid also had been studied. The maximum degree of substitution of starch oleate 0.229 was obtained by the presented method. The NS, pretreatment starch (PS) and starch oleate (SO) were analyzed by means of SEM techniques, FT-IR and ^{1}H NMR. © 2013 Trade Science Inc. - INDIA

KEYWORDS

Pretreatment starch;
Lipase-catalyzed;
Solvent-free system;
Starch oleate.

INTRODUCTION

To development the biodegradable and environmental coordination material has become the research hotspot with the growing concern on the global problem of environmental pollution and resource shortage. Starch is a natural, renewable and biodegradable polymer produced by many plants. It is found in roots, stalks, seeds of staple crops such as rice, corn, wheat, tapioca and potato[1,2]. It can be said, starch is the nature inexhaustible and renewable source. It is the second most abundant biomass material in nature and has already found numerous industrial applications. And starchy material itself is non-toxic, so that it is in line with the material requirements of biodegradable and environmental coordination. Therefore, the starch as a matrix material must have a very wide range of potential applications.

The hydrophilic nature of starch is the major limitation for the development of starch-based materials. Chemicals derivatization of starch has long been studied as a way to solve this problem by producing waterproof materials. Chemical modification of starch is often required to better suit its properties for specific applications. Modification of the hydroxyl groups of starch to form appropriate degree of substitution imparts thermoplasticity and water resistance to the modified starch over the unmodified one. However, the chemical reactivity and reaction efficiency of NS is usually low[3]. Reactions on starches to prepare highly substituted derivates are not easy, mainly because of the difficulty to dissolve granular starch in a suitable medium without

significant degradation and the difficulty to incorporate the fatty acid with starch[4].

Starch is a multi-crystalline polymer. The semi-crystalline structure of starch is composed of the crystalline region and the amorphous region. The dense structure of crystalline region limits the penetration of chemical reagents into the inside of starch molecules, so that resulting in low reaction efficiency and low degree of substitution of product. So research on how to properly change the structure of starch and to reduce the starch crystalline to increase its reaction efficiency both are very important study works[5,6].

Currently, the general methods of pretreatment starch include chemical methods, physical methods and enzymatic methods destined to modify the structure in the crystalline region or decrease the size of crystalline regions to increase the reactivity of starch and convert native starch into cold water soluble starch[7]. Cold water soluble corn starch was first produced by Eastman[8], by slurring corn starch in selected aqueous alcohols under the high temperature and pressure. Although the enzymatic method can effectively degrade the starch, it will take long reaction time and the enzyme activity involved in the whole reaction process influenced by many factors so that the reaction conditions must be controlled strictly; As far as the moist heat treatment, radiation, microwave, extrusion and other physical methods, the large energy consumption and the specialized equipment limited the application of them in industry; At present, the NaOH/Urea hybrid solution has been widely used to dissolve cellulose. The method does not use toxic chemicals and does not pollute the environment. The crystallinity of cellulose could be decreased after pretreated by sodium hydroxide/urea hybrid solution. A new method for preparing granular cold water swelling/soluble starches by alcoholic-alkali treatments has been studied by Seung[9]. In this paper, corn starch was pretreated at low temperatures using NaOH/Urea hybrid solution. The cold water solubility of pretreatment starch and the esterification activity were studied in-depth.

Interest in an enzymatic route to esterify starch is fairly recent and most works have been published after 2005[10], with the exception of one earlier investigation. Most enzymes work effectively in water reach environment only, but lipases are the opposite examples. It is known that lipase may be employed as effective catalyst of esterification, trans-esterification or ester hydrolysis in solvent-free system[11]. Unlike chemical esterification, enzymatic one especially in solvent-free system is an environmentally friendly method which occurs under milder conditions[12,13]. Regio-specific and stereo-specific esterification can be easily carried out using enzymes. The use of extra-cellular lipases as catalysts for esters production has a great potential. In fact, using a biocatalyst eliminates the disadvantages of the chemical process by producing very high purity compounds with less or no downstream operations[14,15].

Presented work focuses on some preliminary studies on enzymatic starch esterification with oleic acid in a solvent-free system. Firstly, native starch (NS) was pretreated with NaOH/Urea hybrid solution at cool temperature to increase its reactivity. Secondly, the pretreatment starch was esterified with oleic acid by Novozym 435 (Lipase B from Candida Antarctica immobilized on macroporous acrylic resin) in a solvent-free system. And then the effect of various kinds of reaction influencing factors on the DS had been studied to obtain the high DS of starch oleate.

MATERIAL AND METHODS

Chemicals and enzyme

Corn starch was purchased from Harbin Mei Wang Reagent Company, China. The water content was determined by drying the corn starch in a vacuum oven at 50ºC until constant weight was achieved and was 16.2% (w/w). Oleic acid was purchased from Shanghai Chemical Co., China and is of analytical grade. Novozym 435 (Lipase B from *Candida Antarctica* immobilized on macroporous acrylic resin; specific activity: 10,000 U/g) was purchased from Novozymes, Denmark.; Dimethyl sulfoxide (DMSO) purchased from Shanghai Chemical Co., China and is of chromatography grade; All the other chemicals are of analytical grade.

Methods

Starch pretreatment

The 9% aqueous solution containing NaOH/Urea at the desired ratio of 2 by weight was used as a solvent for starch. The solvent was pre-cooled to below -

10 °C. Then the starch sample in the given amount of 5% was added immediately into it. The native starch (NS) was completely dissolved within 5 min by stirring at 3000 r/min and the resultant solution came out transparent. The transparent solution of starch was neutralized with HCl (15%) until it reached neutrality. Then, starch was precipitated out from the neutral starch solution by adding 50mL ethanol drop-wise. After various durations of dropping treatment, the precipitates were washed by successive centrifugations in 95% of ethanol until no HCl existence. Where-after, they were washed with 100% of ethanol to remove water. The resulting precipitates were vacuum dried at 50 °C for 24 h.

Initial water activity control

Water activity or a_w is an important consideration for bio-catalysis in solvent-free medium. All the substrates were equilibrated to fixed water activities (a_w) over saturated salt solutions and molecular sieve (a_w <0.01) in closed container at 25□. The following salts were used: LiCl (a_w = 0.11), $MgCl_2$ (a_w = 0.33), $Mg(NO_3)_2$ (a_w = 0.54) and NaCl (a_w = 0.75)[16]. The enzyme was equilibrated in separate vessels at 25□.

General procedure for Lipase-catalyzed esterification

Reaction setup for esterification was carried out in a 25mL closed, screw-capped glass vials containing oleic acid and pretreated starch (PS) or native starch (NS), where oleic acid as the acyl donor. To conduct the reaction under the neat conditions (without solvents), a 2:1 weight ratio of oleic acid to starch is needed to provide enough solution volume to dissolve solid starch and to stir the suspended immobilized lipase. The esterification was initiated by adding immobilized lipase (Novozym 435) into each glass vial. Glass vials were placed up right on a magnetic stirrer and incubated at 55-75 °C, 50 r/min for 3-24h.

Determination of the cold water solubility (CWS)

The NS and the PS were dispersed in distilled water at a concentration of 10 mg/mL at room temperature. Stir for 15 s at 500 r/min and for 2 min at 1000 r/min to prepare the starch solution. The solution was centrifuged at 6,000 r/min for 15 min and 25mL supernatant was dried at 110□ until constant weight.

The solubility of the NS and the PS were calculated as equation (1):

$$CWS(\%) = \frac{m_1 \times 10}{m_0 \times 25} \times 100 \quad (1)$$

where m_1 is the dry weight of 25mL supernatant, g; m_0 is the dry weight of sample, g.

Determination of the degree of substitution by GC analysis

The DS indicates the average number of substitutions per anhydroglucose unit. There are three free hydroxyl groups of per anhydroglucose unit available for modification, resulting in a maximum possible DS of 3.

A small sample 30 mg of starch oleate dissolved in 1mL DMSO was mixed with 1mL of sodium methoxide (0.07 M) in methanol solution. This mixture was then heated (70 °C) under reflux for 40 min, while shaken, then cooled and 1mL of deionised water and 1 mL of n-heptane were added. The mixture was shaken for 1 min and left to settle. The top organic phase contained the methyl ester and could be removed and injected into the GC-FID (Perkin-Elmer Autosystem XL with a CP Simdist capillary column, oven set at 220 °C, the injector at 250 °C and the detector at 260 °C, flow rate of N_2 and air is 4.5mL/min and 5.5 mL/min, flow rate of tail-blowing is 5.0 mL/min).

Calculation DS of the SO

Once the methyl oleate was quantified by GC chromatograph, the average mol of acyl groups per anhydroglucose unit was calculated to give the DS of SO. DS was calculated according to the modified method of Kshirsagar[17] as equation (2).

$$DS = \frac{n \times M_1}{M_0 - n \times (M_2 - M_{H_2O})} \quad (2)$$

where n is the mol of esterifiable oleic acid; M_0 is the weight of sample, g; M_1 is the molecular weight of anhydrous glucose unit, 162; M_2 is the molecular weight of oleic acid, 282.47; M_{H20} is the molecular weight of H_2O.

SEM micrographs

SEM micrographs were obtained with a Quanta 400 scanning electron microscope (FEI Company, Hol-

land) at an accelerating voltage of 20 kV.

FTIR spectra

FTIR spectra were recorded using a Vector 33 spectrometer (Brucker Company, Germany). Potassium bromide (KBr) disks were prepared from powdered samples mixed with dry KBr in the ratio of 1:100. The spectra were recorded in a transmittance mode from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.

^{1}H NMR spectra

^{1}H NMR spectra were recorded at room temperature in DMSO-d_6 by using tetramethylsilane (TMS) as an internal reference on a Brucker DRX-400 NMR spectrometer (Germany) at 100 MHz.

RESULTS AND DISCUSSION

Exploratory experiments

A number of preliminary experiments were carried out to investigate whether the lipase catalyzed esterification of starch with oleic acid is indeed possible in solvent-free medium. The exploratory experiments were performed with lipase Novozym 435 as the catalyst at various substrate ratios and temperatures for 3-24h. At the end of reaction, the product was soaked in 95% ethanol for 10 min and washed thoroughly with 60 °C ethanol to remove un-reacted oleic acid and dried (75°C) till constant weight. The immobilized lipase was removed with 80 screen mesh. Esterification of native starch and pretreatment starch with oleic acid catalyzed by lipase under solvent-free system at the reaction condition: starch: oleic acid =1: 4, w/w; 65 °C for 24h. The DS of Pretreatment starch oleate was 0.178, but the DS of native starch oleate was only 0.001.

SEM micrographs and the cold water solubility of PS and NS

SEM micrographs in Figure 1 show the size and morphology of starch particles in the slurries. The NS (Figure 1-A) had a mean diameter of 10μm which is within the granule range of 4–15μm with a smooth surface. When starch was pretreated by NaOH/Urea, the average particle size of PS decreased to about 1μm which is within the granule range of 0.5–1.5μm. The PS particle adsorbed to agglomeration and its size distribution was uneven as shown in Figure 1-B. The smaller size of the pretreatment starch improved the degree of substitution of starch ester, that maybe due to the increase of the surface area of starch. The increase of the surface area of starch creased more opportunities for

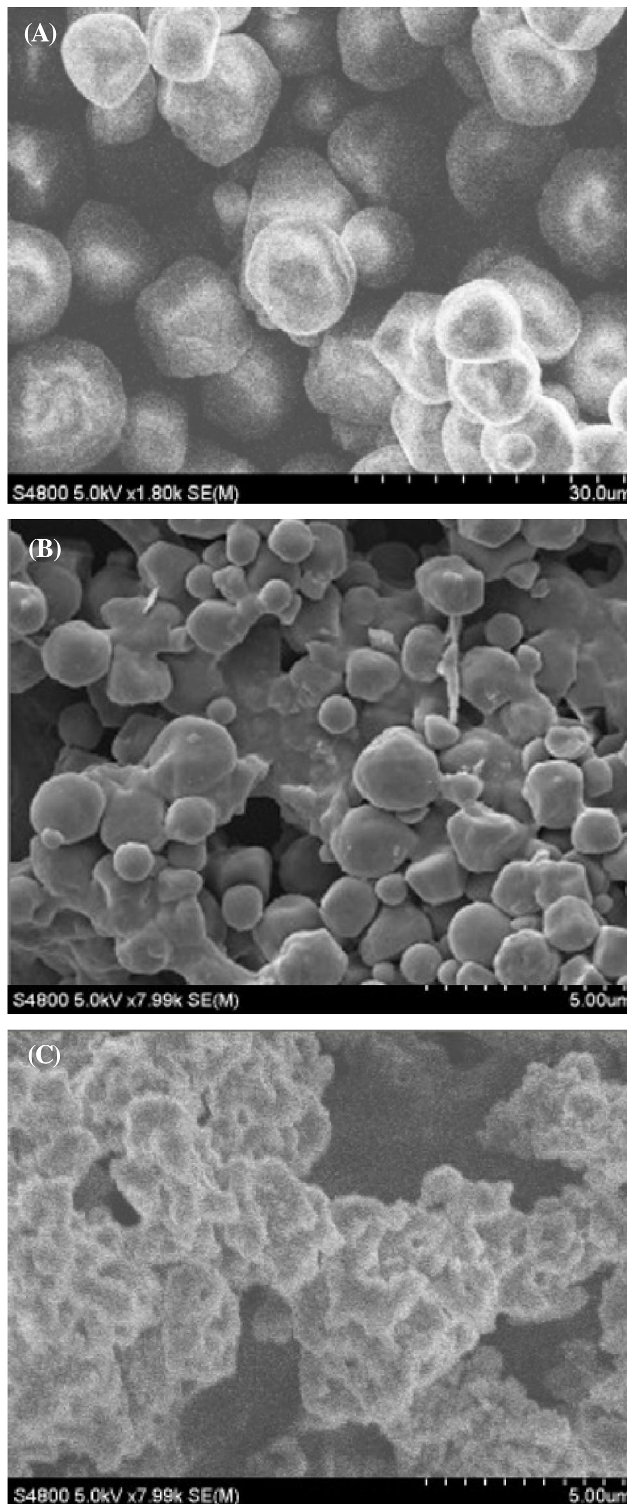


Figure 1 : SEM images of NS (A), PS (B) and SO (C)

other substrates to touch it. In comparison with PS, the particles of SO (Figure 1-C) were eroded significantly. The surface of the SO particles become rough and there were almost no smooth particle existence. It was difficult to obtain well-defined pictures of non-aggregated particles. That may be because of the heterogeneity of the size of SO particles.

Solubility is an important indicator for evaluation the physical properties of starch, it shows the water binding capacity of starch. It is related with the molecular structure of starch, particle size, and the content of amylose. The experimental results showed that the cold water solubility of PS is 96.77%, while the NS is only 0.30%. The increase of cold water solubility of PS, that may be because of the particle size become smaller as shown in Figure 1. The decrease of the particle size of PS made its specific surface area become bigger. The increase of specific surface area of PS could promote the reaction of water with starch. That is the reason why the cold water solubility of starch was improved after pretreatment by NaOH/Urea. This result further confirmed that the cold water solubility of starch is related with its particle size.

FT-IR analysis

In the NS spectrum, the characteristic peaks (954–1,184 cm^{-1}) are attributed to C–OH bond stretching. Another strong broad band due to hydroxyl bond stretching appears at 3,000–3,600 cm^{-1} (Figure 2-a and Figure 2-b) which is reduced on SO (Figure 2-c). A characteristic peak C=O bond stretching present in the starch occurred at 1,661-1,623 cm^{-1}, which is intensified on the PS (Figure 2-a and Figure 2-b). That demonstrated the molecular chain of starch had fractured after pretreatment[18]. A strong absorption band at 992 cm^{-1}, which appears at 1,024 cm^{-1}, probably due to the stretching of the C–O–C bond, was present in the spectra of the starch consistent with the earlier report by Huang[19]. The red shift of C–O–C bond stretching could weaken the hydrogen bond. An extremely broad band due to hydrogen bonded hydroxyl groups (O–H) appeared at 3,400 cm^{-1} which was attributed to the complex vibrational stretches associated with free, inter- and intramolecular bound hydroxyl groups which make up the gross structure of starch[20]. The band at 2,919 cm^{-1} is characteristic of C–H stretches. These spectra have similar profiles. The differences between NS and PS were the C=O bond stretching and the red shift of C–O–C bond stretching. There was no new peak present.

In comparison with the spectra of the PS, the major change of SO is the presence of a carbonyl C–O absorption frequency at 1,735 cm^{-1}. The strong O–H stretching band at 3,400 cm^{-1} in the SO decreased in intensity following esterification of starch with oleic acid.

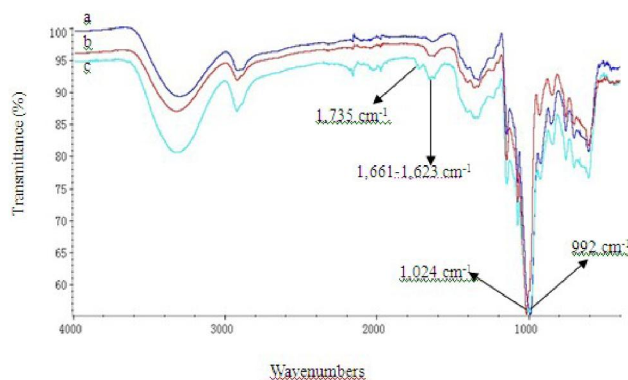


Figure 2 : FT-IR spectra of NS (a), PS (b) and SO(c) of solvent-free system

^{1}H NMR analyses

Almost all of the SO products are soluble in DMSO-d_6 except for the products with a DS higher than 0.26. These products were only partially soluble in DMSO-d_6. To improve the solubility in DMSO-d_6, one drop of TFA-d_1 was added to the mixtures [21]. Figure 3-a and Figure 3-b show the typical ^{1}H NMR spectra of NS and SO product respectively. The broad and overlapped peaks in the region δ3.3–5.6 ppm are assigned to the starch protons[22,23]. The peaks at δ 0.8–2.2ppm correspond to the aliphatic hydrogen atoms of the fatty acid chain (Figure 3-c)[24]. The absence of resonances in the olefinic region (δ 7–7.2 ppm) indicates that the products are free from unreacted oleic acid and that the work-up procedure involving thorough washing of the product with ethanol was successful.

Optimization of reaction conditions

To study the effect of reaction conditions on the DS of SO, a number of experiments were performed at various reaction temperature and initial water activity for different time. Furthermore, the effect of the ratio of starch to oleic acid and the ratio of starch to lipase were explored.

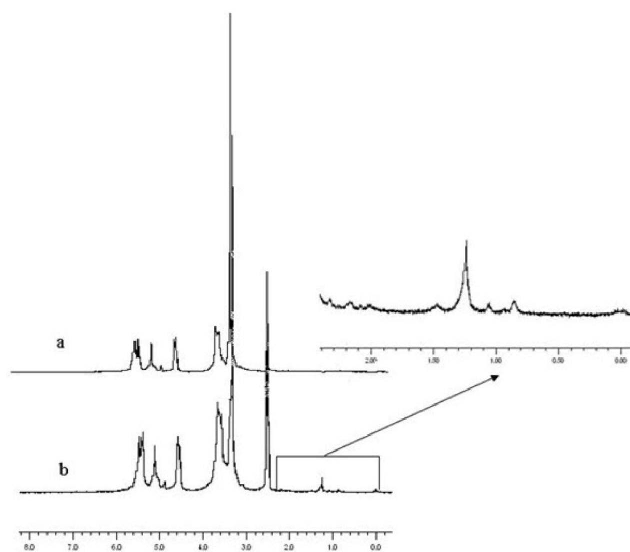
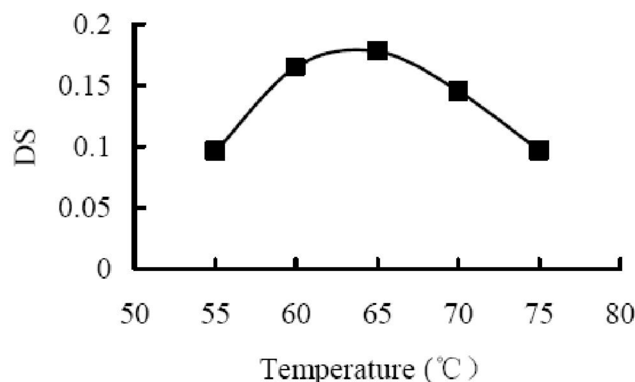


Figure 3 : ^{1}H NMR spectra of NS (a) and SO with DS of 0.08 (b)

Effect of initial water activity (a_w) on the DS of SO

Water is essential for enzymatic reaction in non-aqueous media. It is associated with maintenance of enzyme's active conformation or the "loosening up" of the rigid structure of an enzyme[25,26]. The meaning of "Essential water" is that the necessary least amount of water to maintain the catalytic activity of enzyme. Enzymes are different in their ability to retain essential water in different reaction media, therefore they are different in water requirements to express their catalytic activity in various media[27]. However, the presence of excess water in reaction media disfavors synthetic reactions while encouraging hydrolytic reactions such as hydrolysis of acylated products and acyl donors (e.g., activated esters)[28,29]. Therefore, it is particularly important to pay attention to initial water activity control in the case of lipase catalyzed esterification of starch in a solvent-free system. In this study, the lipase catalyzed esterification of starch was carried out over a wide range of a_w to see the effect of a_w on DS of SO and to determine the optimal a_w for this reaction.

As shown in TABLE 1, Novozym 435 catalyzed starch esterification with oleic acid in solvent-free system had a clear a_w dependence. When a_w value was below 0.75 in reaction media, DS of SO decreased with the increase of a_w. These results suggest that a very small amount of water could satisfy the requirement of Novozym 435 for holding essential water layer to perform its catalytic functions properly[30]. On the other hand, a_w above the optimum value allowed the enzyme completely hydrated, but the competitive hydrolysis of the products took place and hence limited the acylation. The optimal initial water activity ($a_w < 0.01$) represented the most appropriate water condition for the balance between the above-mentioned conflicts.



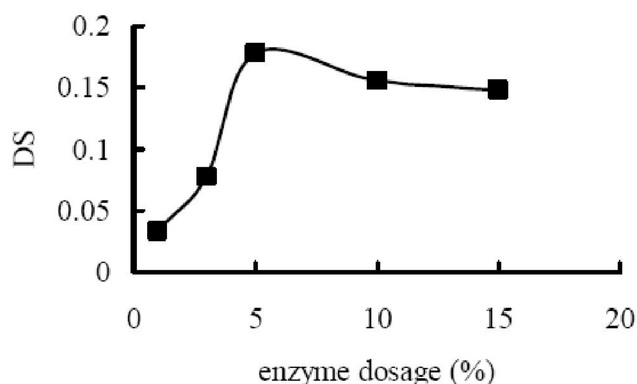
(Reaction condition: $a_w < 0.01$, starch:Oleic acid =1:4, w/w, catalyzed by 0.1g Novozym 435 lipase for 24h)

Figure 4 : Effect of reaction temperature on the DS of SO

Effect of reaction temperature on the DS of SO

The reaction temperature has a significant influence on the kinetic activity and stability of enzyme. It also affects the liquefaction of substrate, the viscosity of reaction system and the thermodynamic equilibrium of enzyme-catalyzed reactions. The maximum DS of SO (0.178) was achieved at 65□ incubation. As shown in Figure 4, the significant increase of DS with increasing temperature from 55□ to 65□ resulted from the acceleration of diffusion and intrinsic enhancement of enzyme activity. Higher temperatures can activate the substrate molecules, reduce the viscosity of reaction mixture and lead to a higher DS of SO. However, excessive temperature would lead to the deactivation of the lipase and cause a slight drop of the DS of SO. This may be due to partial lipase deactivation at higher temperature. The result was similar in the findings by most reviewed papers that Novozym 435 was optimally used at 55 ºC to 70 ºC[31]. The DS decreased slightly after 65 ºC probably caused by the vibration and movement of the enzyme molecule, which would affect the hydrogen bonds and other bonds in the lipase structure. Hence, the enzyme molecule will unfold and alter its tertiary and quaternary structure or globular structure (three-dimensional conformation). Consequently the catalytic power of lipase will be reduced, because denaturation pro-

cess has occurred. At high temperature (60-65 ºC), a higher water evaporation rate may shift the position of the equilibrium to the product side and increases the DS [32]. It also promotes collisions between enzyme and substrate molecules to result in accelerated rates of reaction. According to Novozym 435 product sheet, Novozym 435 is a heat tolerant, immobilized enzyme with a maximum activity at 70–80□. However, it is suggested that the enzyme should be used at 40–65□ for the sake of its stability. In the subsequent studies, the reaction was carried out at 65□ for SO synthesis.



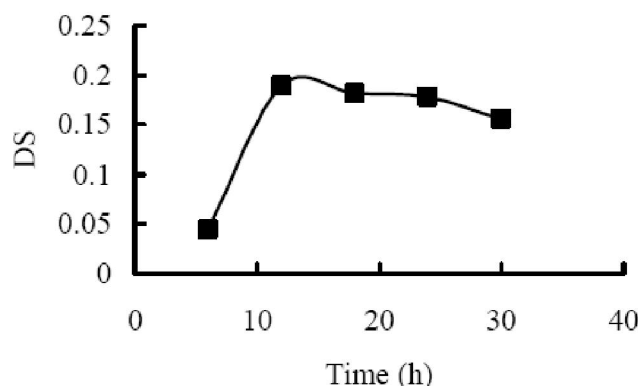
(Reaction condition: aw < 0.01, starch : oleic acid =1:4, w/w , at 65□ for 24h)

Figure 5 : Effect of enzyme dosage on the DS of SO

Effect of catalyst loading on the DS of SO

Internal diffusion problem could happen when the substrate could not reach the inner parts of the support. The effect of catalyst loading was studied from 1 to 15% (w/w of starch) under otherwise similar conditions. As shown in Figure 5, the DS of SO increased with the increase of catalyst loading. It was found that the DS of SO increased from 0.034 to 0.178 when the enzyme concentration in reaction mixture changed from 1% to 5%. Therefore the internal diffusion limitations could be minimized by increase catalyst loading. Since there was significant decrease in the DS of SO with the increase of catalyst loading from 5% to 15%, further study on the reaction parameters under the 5% catalyst loading. These results suggest that the excess enzyme did not contribute to the increase in the DS of SO. That is may be because of, at saturation point, all the substrates are bound to the enzyme and added enzyme molecule could not find any substrate to serve as a reactant.

In esterification reaction, the amount of catalyst loading will influence the total reaction time, which is required to achieve desired conversion[33]. The presence of higher amount of catalyst loading provides more active sites for acyl-enzyme complex formation and increases the probability of enzyme-substrate collision and subsequent reaction. The negative effect of further increase in enzyme concentration may be due to mass transfer limitation and poor dispersion of enzyme particles.



(Reaction condition: aw < 0.01, starch : Oleic acid =1:4, w/w , catalyzed by 0.1g Novozym 435 lipase at 65□)

Figure 6 : Effect of reaction time on the DS of SO

Effect of reaction time on the DS of SO

Time course investigation gives an insight into the performance of an enzyme as the reaction progresses. Such progress curve helps to determine the necessary shortest time for obtaining ideal DS and to enhance cost-effectiveness of the process [32]. The DS of SO produced at various time intervals was presented in Figure 6. The DS of SO increased with the increase of reaction time. Novozym 435 gave the highest DS within a reaction period of 12 h (0.189). After 12 h, the DS was relatively constant. This might be due to the reaction had achieved the reaction equilibrium. The initial reaction was rather insensitive in the solvent-free system, since the substrate-diffusion limited the esterification reaction. The rapidly increase of DS after 6 h of reaction may be due to the e miscible degree of the two substrates and the contaction level of substrates with lipase, which had all increased in an adequate value to accommodate the esterification process.

Effect of the ratio of starch to oleic acid on the DS of SO

Relative proportions of various substrates in a reaction mixture defined the physical and chemical prop-

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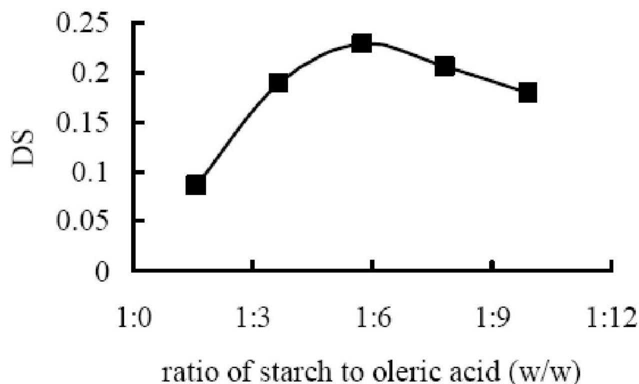
erties of a reaction system. The ratio of one substrate to another is an important parameter affecting the reaction equilibrium. The effect of ratio of starch to oleic acid on the lipase-catalyzed esterification was shown in Figure 7.



(Reaction condition: aw < 0.01, catalyzed by 0.1g Novozym 435 lipase at 65□ for 12h.

Figure 7 : Effect of the ratio of starch to oleic acid on the DS of SO

The synthesis reaction was run for various ratios of substrates in solvent-free system at 65□. Theoretically, excess oleic acid can promote the reaction equilibrium shift to starch oleate synthesis. To conduct the reaction under the neat conditions, the 1:2 ratio of starch to oleic acid is needed to provide enough solution volume (oleic acid volume) to dissolve solid starch and to stir the suspended immobilized enzyme. In our study, the starch was fixed at 2.0 g while the ratio of starch to oleic acid varied from 1:2 to 1:10. As shown in Figure 7, the ratio of acyl acceptor to donor has been shown to effect the average degree of esterification. Decrease the ratio of starch to oleic acid from1:2 to1:6 resulted in a concomitant increase of the DS of SO and reached 0.249 at the ratio of 1:6. The increase of the amount of oleic acid decreased the viscosity of reaction system and the mass transfer rate was speed up. So the DS of SO increased with the decrease of the ratio of starch to oleic acid from 1:2 to 1:6. The increase of the DS of SO with the increase of oleic acid also could be explained by a thermodynamic shift of the equilibrium in favor of the synthesis of the ester due to excess oleic acid. Also, it has been reported that the active site of lipase is accessible to the substrate through a narrow hydrophobic channel. Increase the amount of oleic acid resulted in the increase of Log P level of the medium. The hydrophobicity of reaction system was enhanced as the increase of the amount of oleic acid. The increasing hydrophobicity probably induced the conformational changes of Lipase that made the active site of the lipase to expose to the substrates.

However, further increase the amount of oleic acid beyond 12g but keeping the amount of starch unchanged would decrease the DS of SO. When the substrate ratio was decreased from 1:6 to 1:10, the DS of SO decreased from 0.249 to 0.189. The negative effect of excess of oleic acid could be due to the decrease of the concentration of enzyme, which may result in low interaction between the substrate and the enzyme.

CONCLUSION

Corn starch was successfully activated through pretreatment with NaOH/Urea/H_2O solution at low temperature below -10□. The smaller particle size of starch after pretreatment led to the greater cold-water solubility (96.77%). The esterification activity of corn starch has been significantly improved after pretreatment. In comparison with the DS of native starch oleate, the high DS (0.229) of pretreatment starch oleate could be obtained by using the investigated method under the optimum conditions: $a_w < 0.01$, starch : oleic acid = 1:6 (w/w), catalyzed by 0.1g Novozym 435 lipase at 65□ for 12h. Obtained results indicated that the investigated method may be used for obtaining high DS of other long chain fatty acid starch esters. Compared with corn starch, carbonyl band of starch ester produced by esterification had been detected by IR spectrum and ^{1}H NMR spectrum.

ACKNOWLEDGMENTS

The authors thank the National Natural Science Foundation of China (20873034, 21073050), the Scientific Research Fund of Heilongjiang Provincial Education Department (2010td04) and the Heilongjiang Provincial Funds for Distinguished Young Scientists (JC201106) for support.

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