Carbohydrate Polymers 79 (2010) 466–474

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Solvent-free lipase-catalyzed synthesis of long-chain starch esters using microwave heating: Optimization by response surface methodology

Habib Horchani [a], Moncef Chaâbouni [b], Youssef Gargouri [a], Adel Sayari [a,*]

[a] Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS, BPW 3038 Sfax, Tunisia
[b] Laboratoire de Chimie Industrielle, ENIS, BPW 3038 Sfax, Tunisia

ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 3 September 2009
Accepted 9 September 2009
Available online 13 September 2009

Keywords:
Immobilized *Staphylococcus aureus* lipase
Response surface methodology
Starch oleate synthesis
Microwave heating
Box-Behnken design

ABSTRACT

Starch is an abundant natural polysaccharide that is inexpensive, renewable, and fully biodegradable. Modification of starch O–H groups by esterification to form an appropriate degree of substitution imparts thermoplasticity and water resistance to the starch ester over the unmodified starch. Unlike chemical esterification, enzymatic esterification is an environmentally friendly method which occurs under milder conditions. A non-commercial $CaCO_3$-immobilized lipase from *Staphylococcus aureus* (SAL3) was used to catalyze the esterification reaction between oleic acid and starch in pure substrate conditions using microwave heating followed by liquid state esterification. Response surface methodology based on three variables (the reaction temperature, the amount of lipase and the molar ratio of starch/oleic acid) at three levels was adopted to optimize the experimental conditions of the starch oleate synthesis. The optimal conditions for achieving 76% conversion with a degree of substitution (DS) of 2.86 are 386 IU of immobilized lipase, a starch/oleicacid molar ratio of 0.18 during 4 h of incubation at 44 °C. The structure of the modified starch was checked by ^{13}C NMR spectroscopy, FT-IR and differential scanning calorimetry (DSC). Results showed that the α-amylolysis of the starch was significantly affected after esterification. The hydrophobic starch fatty acid esters produced may have potential industrial applications such as surface coating materials, flavoring agents in food industry and biomedicals for bone fixation and replacements.

1. Introduction

The hydrophilic nature of starch is the major limitation for the development of starch-based materials. Chemicals derivatization has long been studied as a way to solve this problem by producing waterproof materials. 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 (Sagar & Merill, 1995).

The introduction of an ester group into polysaccharide constitutes an important achievement because it resulted in modifying their original hydrophilic nature and obtaining new thermal and mechanical properties (Aburto et al., 2000). Previous works have recently reported the use of organic solvents to achieve starch solubilization followed by its esterification, to produce fully biodegradable thermoplastic materials (Fang, Fowler, Tomkinson, & Hill, 2002; Heinze, Talaba, & Heinze, 2000; Peltonen & Harju, 1996). Such compounds can replace the non-biodegradable plastics used in the plastics industry. This achievement would help to save petrochemical resources and to find out new industrial uses of starches.

The biodegradable product obtained after esterification has biomedical applications such as carriers for controlled drugs release and other bioactive agents (Malafaya, Elvira, Gallardo, San Roman, & Reis, 2001). These include starch-based biomaterials as scaffolds for the tissue engineering of bone and cartilage (Gomes, Ribeiro, Malafaya, Reis, & Cunha, 2001), materials for bone fixation (Espigares et al., 2002; Reis & Cunha, 2000; Sousa, Mano, Reis, Cunha, & Bevis, 2002). They are also used in many branches of industry as glues, adhesives and auxiliaries of a wide range of rheological and functional properties (Roper, 1996).

Recent works have described the production of esterified starches at high degree of substitution, in presence of organic solvent (Aburto et al., 1997, 1999; Fang et al., 2002). The prepared esters were synthesized by chemical gelatinization using formic acid, followed by treatment with fatty acid chlorides. Such methods rely on the use of sophisticated experimental techniques. In addition, the utilization of organic solvent is prohibited for industrial applications, especially in food industries.

Unlike chemical esterification, enzymatic one is an environmentally friendly method which occurs under milder conditions. Regiospecific and stereospecific esterification can be easily carried

* Corresponding author. Tel./fax: +216 74 675 055.
E-mail address: adelsayari@yahoo.fr (A. Sayari).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.09.003

out using enzymes. The use of extracellular 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.

The aim of this work was to synthesize starch oleate, with a high degree of substitution, using an immobilized *Staphylococcus aureus* lipase. The esterification was carried out in a solvent-free system under microwave heating and liquid state conditions. The structure and physicochemical properties of starch oleate were also checked.

2. Materials and methods

2.1. Lipases

2.1.1. Production and immobilization of lipase

The native thermoactive and alkaline lipase from a newly soil-isolated *Staphylococcus aureus* strain (SAL3) was produced according to the procedure described previously (Horchani, Mosbah, Ben Salem, Gargouri, & Sayari, 2009). After 30 h of culture, cells were removed by centrifugation and the lipase in the supernatant was precipitated by the addition of ammonium sulphate up to 65% of saturation followed by centrifugation at 2700*g* at 4 °C for 30 min. The pellet was dissolved in 20 mM sodium acetate buffer pH 5.4 containing 20 mM NaCl and 2 mM benzamidine. Then the solution was centrifuged at 4100*g* for 5 min and the supernatant containing the lipases was used for immobilization. The enzyme immobilization was made onto $CaCO_3$ as described previously (Ghamgui, Karra-Chaabouni, & Gargouri, 2004).

2.1.2. Lipase hydrolytic activity

The lipase activity was measured titrimetrically at pH 9.5 and 55 °C with a pH-Stat (Herisau, Switzerland), under standard assay conditions using olive oil emulsion as substrate (Gargouri, Julien, Sugihara, Sarda, & Verger, 1984). Activity was expressed as units per ml of enzymatic solution. One unit (IU) of lipase activity was defined as the amount of enzyme that catalyzes the liberation of 1 μmol of fatty acid per min at pH 9.5 and 55 °C.

2.1.3. Thermal stability and microwave oven heating stability of lipases

Thermostability of free and immobilized lipases was determined by incubating the enzyme at different temperatures (40–80 °C) for 60 min. Microwave radiation stability was checked for different time duration from 15 s to 30 min. The residual activity of free enzyme was determined, after centrifugation, under standard assay method.

2.2. Esterification of starch oleate

Esterification reaction was carried out, in screw-capped flasks, using microwave heating followed by liquid state esterification. Maize starch powder in 10 ml of distilled water was incubated 1 min under microwave heating until completely solubilization. Starch and oleic acid were mixed at various molar ratios (0.1–0.3) at 4 g of total weight and the reaction mixture was stirred for 5 min. To these mixtures, different amounts of immobilized SAL3 ranging from 300 to 500 IU were added according to the conditions required by an experimental design.

The mixture obtained was incubated for 2 min in a domestic microwave oven (Haier, HR-6702D; frequency, 2450 MHz; power consumption, 1150 W; maximum out power, 700 W). The radiation was given intermittently for 10 s to avoid over heating. After the microwave radiation, the reaction was carried out in shaking flask (200 rpm) at different temperatures for 4 h. A control without enzyme was run in parallel under the same conditions. After each reaction time, the enzyme was removed by centrifugation with 3200*g* for 3 min. To check the importance of the microwave heating, a second control was performed under the same conditions in presence of immobilized lipase using only microwave treatment or shaking flask. In addition the results obtained were compared to those using Novozyme 435 (Bagsvaerd, Denmark) as biocatalyst.

The residual acid content was tested by titration with sodium hydroxide 3.5 g/l using phenolphthalein as indicator and 2 ml of ethanol as quenching agent. The conversion percentage was based on the amount of acid consumed (Wu, Jaasklainen, & Linko, 1996).

2.3. Reaction products analysis

2.3.1. Degree of substitution

When the reaction was completed, removal of unesterified oleic acid was accomplished by the addition of 100 ml of pure ethanol. The precipated starch ester was then filtrated and dried at 50 °C for 2 h. The DS of esterified starch was determined using the Miladinov and Hanna method (Miladinov & Hanna, 1999).

2.3.2. Infra-red and ^{13}C NMR spectra

The evidence of esterification was controlled using FTIR NEXUS spectrophotometer (Nicoleit, Madison, WIS, USA) showing the shift of the carbonyl of carboxylic acid group to the carbonyl of ester group. The sample was mixed with KBr. FT-IR spectra were acquired after 32 scans between 4000 and 400 cm^{-1}, with spectral resolution of 4 cm^{-1}.

The Structure of the starch oleic ester was also detrmined by *^{13}C NMR* (Madison, USA). Samples were dissolved in CDCl3 containing trace amounts of tetramethylsilane, which was used as an internal chemical shift reference to indicate, in parts per million (ppm), the difference of the resonance frequency.

2.3.3. α-Amylase digestibility

The hydrolysis reaction of native or modified starch was performed using porcine pancreatic amylase (Sigma chemical, Misouri, USA). 1% of native or modified starch was resuspended in phosphate buffer (pH 7). The reaction was started by adding 400 IU of enzyme. The Erlenmeyer flasks were placed in a shaking water bath and incubated at 50 °C. Aliquots were taken after various incubation times and the degradation products were analysed by thin-layer chromatography (TLC) on silica 60 F254 previously activated at 60 °C for 30 min. The developing solvent was a mixture of chloroform/acetic acid/water (60/70/10, V/V/V). The spots were visualized after coloration with a mixture of H_2SO_4/ ethanol (5/95, V/V).

2.3.4. Differential scanning calorimetry

DSC measurements were performed with a Mettler Toledo DSC 812 (USA). About 20 mg of the dried sample were placed in closed stainless steel DSC pans and heated from 20 to 250 °C with a heating rate of 10 °C/min in a nitrogen atmosphere. The glass transition temperature (T_g) is taken as the inflection point of the increment of special heat capacity and as the peak value of the endothermal process in the DSC curves.

2.4. Experimental design

Optimization of the conversion yield was achieved by using the response surface methodology (RSM). In this work a Box-Behnken design was set up to study the empirical relationship between the conversion yield and three controlled factors namely X1: Temperature, X2: Amount of immobilized lipase, X3: Starch/oleic acid molar ratio. This part of the paper briefly discusses the principles

governing, the construction and analysis of the experimental design. The mathematical model of a three varialble Box-Behnken design can be represented by the equation:

$$\hat{y} = b_0 + b_1X1 + b_2X2 + b_3X3 + b_{11}X1^2 + b_{22}X2^2 + b_{33}X3^2 + b_{12}X_1X_2 + b_{12}X1X2 + b_{13}X1X3 + b_{23}X2X3$$

Table 1
Comparison of predicted and experimental yields of starch oleate by immobilised *S. aureus* lipase.

Experiences	Coded values			Yield of starch oleate (%)	
	X1	*X2*	*X3*	Experimental	Predicted
1	−1	−1	0	61.500	60.929
1′	−1	−1	0	63.200	60.929
2	1	−1	0	60.400	61.730
2′	1	−1	0	61.120	61.730
3	−1	1	0	65.190	63.996
3′	−1	1	0	65.000	63.996
4	1	1	0	47.600	47.817
4′	1	1	0	45.500	47.817
5	−1	0	−1	57.900	58.439
5′	−1	0	−1	55.200	58.439
6	1	0	−1	60.900	60.194
6′	1	0	−1	60.340	60.194
7	−1	0	1	60.100	60.872
7′	−1	0	1	61.200	60.872
8	1	0	1	44.600	43.739
8′	1	0	1	47.100	43.739
9	0	−1	−1	65.120	65.759
9′	0	−1	−1	67.900	65.759
10	0	1	−1	63.120	62.015
10′	0	1	−1	62.900	62.015
11	0	−1	1	59.100	60.428
11′	0	−1	1	60.120	60.428
12	0	1	1	55.190	53.325
12′	0	1	1	50.350	53.325
13	0	0	0	79.160	74.841
14	0	0	0	79.680	74.841
15	0	0	0	75.100	74.841
16	0	0	0	79.950	74.841
17	0	0	0	71.500	74.841
18	0	0	0	78.150	74.841
19	0	0	0	75.150	74.841
20	0	0	0	75.200	74.841
21	−0.4082	−0.2357	−0.1667	70.050	74.575
22	0.4082	−0.2357	−0.1667	68.900	72.896
23	0	0.4714	−0.1667	68.900	72.678
24	0	0	0.5	65.900	70.930

With $y = \hat{y} + e$. Where $\hat{y}$ is the estimated response function; y, the measured response; e, the error, b_0, b_j, b_{jk} and b_{jj}, the estimated model coeficients. X_j are the coded variables which take the levels −1 and +1 when natural variables take their low and high levels, respectively.

To estimate the model coefficients, a three variable Box-Behnken design was carried out. The corresponding matrix design with 13 experiments indicating the levels of the coded variables at each experiment is shown in Table 1. Seven supplementary experiments have been carried out in the center of the domain to estimate the pure error variance. The four last runs in the matrix design will be used to check the validity of the model.

3. Results and discussion

3.1. Immobilization and microwave radiation stability of SAL3

Immobilization of enzymes plays an important role within applied biotechnology. The main reason for immobilizing enzymes is the ability to isolate the biocatalyst from the reaction product and reuse it in order to increase its productivity. Adsorption is still the most commonly used approach because of its easy use and being the least expensive.

The determination of the best conditions of SAL3 immobilization was based on the study of the influence of the enzyme amount to be adsorbed to 1 g of $CaCO_3$, at different time courses. The results showed that the yield of immobilization increased as more lipase was loaded onto the support to reach a maximum value at 1500 IU after 60 min of incubation time which corresponds to 79% of immobilization yield (data not shown).

To investigate the effect of immobilization on the stability of lipase, free and immobilized SAL3 were incubated separately at various temperatures during 60 min and the residual activity was determined. As shown in Fig. 1A, in contrast to the free lipase which lost its activity beyond 70 °C, the immobilized lipase displayed a residual activity of about 88%, 81% or 78% after 60 min-incubation at 70, 80 or 90 °C, respectively (Fig. 1A). At 100 °C, the half-life of the immobilized SAL3 was 55 min (data not shown). This is in line with De Oliveira, Alves, and De Castro (2000), who reported that after 1 h of heat treatment at 60 °C, the immobilized *Candida rugosa* lipase retained 50% of its original activity, and that under the same conditions, the free enzyme lost its full activity. The resistance of immobilized SAL3 to temperature is a potential advantage for practical applications of this enzyme.

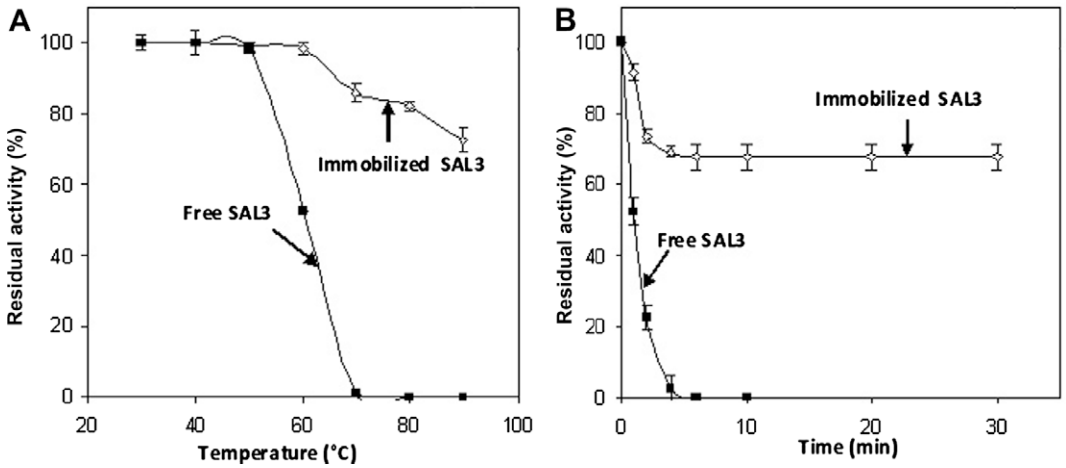

Fig. 1. (A) Thermal stability of free and immobilized *Staphylococcus aureus* lipase. (B) Stability of free and immobilized *Staphylococcus aureus* lipase to microwave radiation. The amount of free or immobilized lipase used was 15 IU.

In addition, when the immobilized SAL3 was incubated in the microwave radiation, it remained stable and exhibited more than 70% of its initial activity during 30 min of incubation (Fig. 1B). These important data confirm previous findings stipulating that the interaction between the support and the enzyme improves the enzyme stability (Mozhaev, Mellik-Nubarov, Sergeeva, Siksnis, & Martinek, 1990; Ogino & Ishikawa, 2001; Penceac'h & Barrati, 1999). Indeed, it was reported that microwave radiation reduced the stability of *Thermomyces lanuginosa* (lipolase) after only 30 s of incubation time. This is probably due to the denaturation caused by in situ generation of heat related to the water molecules vibration, when the exposure exceeds 30 s as was explained by previous studies (Rajan, Prasad, & Abraham, 2006). One can conclude that the immobilization of *Staphylococcus aureus* lipase onto the $CaCO_3$ strongly increased its stability at high temperatures as well as under the microwave vibration. The increase of enzyme stability could be related to the creation of conformational limitation on the enzyme movement as a result of electrostatic interaction and hydrogen bond formation between the enzyme and the support or a low restriction in the diffusion of the substrate at high temperature (Arica, Hasirci, & Alaeddinoglu, 1995; Ye, Xu, Che, Wu, & Seta, 2005).

3.2. Optimization of the starch oleate synthesis

3.2.1. Preliminary study

The ability of immobilized *Staphylococcus aureus* lipase, produced in our laboratory to synthetise starch oleate, was studied and compared to a commercial *Candida antarctica* lipase (Novozyme 435). Esterification was carried out using both a microwave oven and a shaking flask. Several authors have shown that microwave heating accelerates the esterification of starch without any activation of further specific bonds. Consequently this kind of heating will not lead to any kinetic differences compared to uncontrolled forms of heating. The molecular weight distribution and granular structure of modified starches are also not affected as compared to products of traditional heating. As shown previously, the microwave energy enhances the esterfication reaction and decreases the reaction time (Kapusniak & Siemion, 2007; Koroskenyi & McCarthy, 2002). In order to scale up this process, microwave heating (2450 MHz, 2 min) was chosen since it gives fast, uniform heating and a higher conversion percentage for maize starch.

After the microwave radiation, the mixture was carried out in a shaking flask at 200 rpm for 4 h. After a preliminary study, three variables were chosen as the most effective operating variables on the response: the reaction temperature (U1), the amount of immobilized lipase (U2) and the starch/oleic acid molar ratio (U3). Other variables have been checked as reaction time, solvent, stirring speed. Obtained results show that these three last variables have little effect on the esterification yield. Then they have fixed as follow: reaction time: 4 h, solvent: water, stirring speed: 200 rpm.

3.2.2. Experimental design

Experimental conditions: In order to define the experimental domain explored, the level values of variables were chosen in such a way that limits were as wide as possible while all the experiments were feasible. Table 2 shows the selected variable levels. The experiments were carried out in duplicate at each design point. Results are indicated in the last column of the matrix design (Table 1).

Model equation: the coefficients of the postulated model were calculated based on the experimental responses (without including the checked points). The fitted model, expressed in coded variables, was represented by the following equation:

$$\hat{y} = 74.841 - 3.845X1 - 2.711X2 - 3.505X3 - 10.397X1^2 - 5.826X2^2 - 8.633X3^2 - 4.245X1X2 - 4.722X1X3 - 0.840X2X3$$

Analysis of variance and validation of the model: The good quality of the fitted model was attested with analysis of the variance (ANOVA) as shown in Table 3. Indeed, this table shows that the sum of squares related to the regression was statistically significant when using the *F*-test at a 99,9 % probability level, which suggests that the variation accounted for by the model was significantly greater than the residual variation. Likewise, the coefficient of multiple determination of the polynomial model termed R^2, indicated that more than 99% of the variability in the response could be explained by the second-order polynomial predicted equation given above. On the other hand, the validity of the model has been established by comparing the results obtained at the four check points (experiments 21–24) with the predicted values (Table 4). These results seem to confirm the validity of the model.

The response surfaces and the isoresponse curves are plotted in function of two of factors while the third is maintained constant at its mean level (Fig. 2A–C). Examination of these figures suggests the following deductions:

- The coordinates of the stationary point of the isoresponse curves corresponds to a maximum of conversion yield are located near the center of the experimental domain (temperature: 44 °C, immobilized lipase: 386 IU and a starch/oleic acid molar ratio of 0.18).
- The conversion yield dramatically decreased when increasing the temperature at any given enzyme or starch/oleic acid level. This result was confirmed by a kinetic study on the conversion yield as a function of time at different temperatures (Fig. 3).
- The effect of starch/oleic acid molar ratio on the conversion yield is analogous to that of the temperature. The conversion yield decreasing with increasing the molar ratio can be explained by the fact that the viscosity of the resulted product is very high preventing the reaction to easily proceed.

Table 3
Analysis of variance.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Regression	2999.52	9	333.280	70.8159	<0.01***
Residual	227.293	26	8.74204		
Total	3226.81	35			
r-square	0.93				

***Significant at the level 99.9%.

Table 2
Range of variables for the experimental design.

	Level		
Variable	−1	0	+1
U1: temperature (°C)	37	45	53
U2: enzyme amount (IU)	300	400	500
U3: starch/oleic acid molar ratio	0.1	0.2	0.3

Table 4
Validation of the model with check points.

Run	X1	X2	X3	Experimental	Predicted	Difference
21	41.73	376.43	0.18	70.05	74.575	−4.525
22	48.27	376.43	0.18	68.90	72.896	−3.996
23	45.00	447.14	0.18	68.90	72.678	−3.778
24	45.00	400.00	0.25	65.90	70.930	−5.030

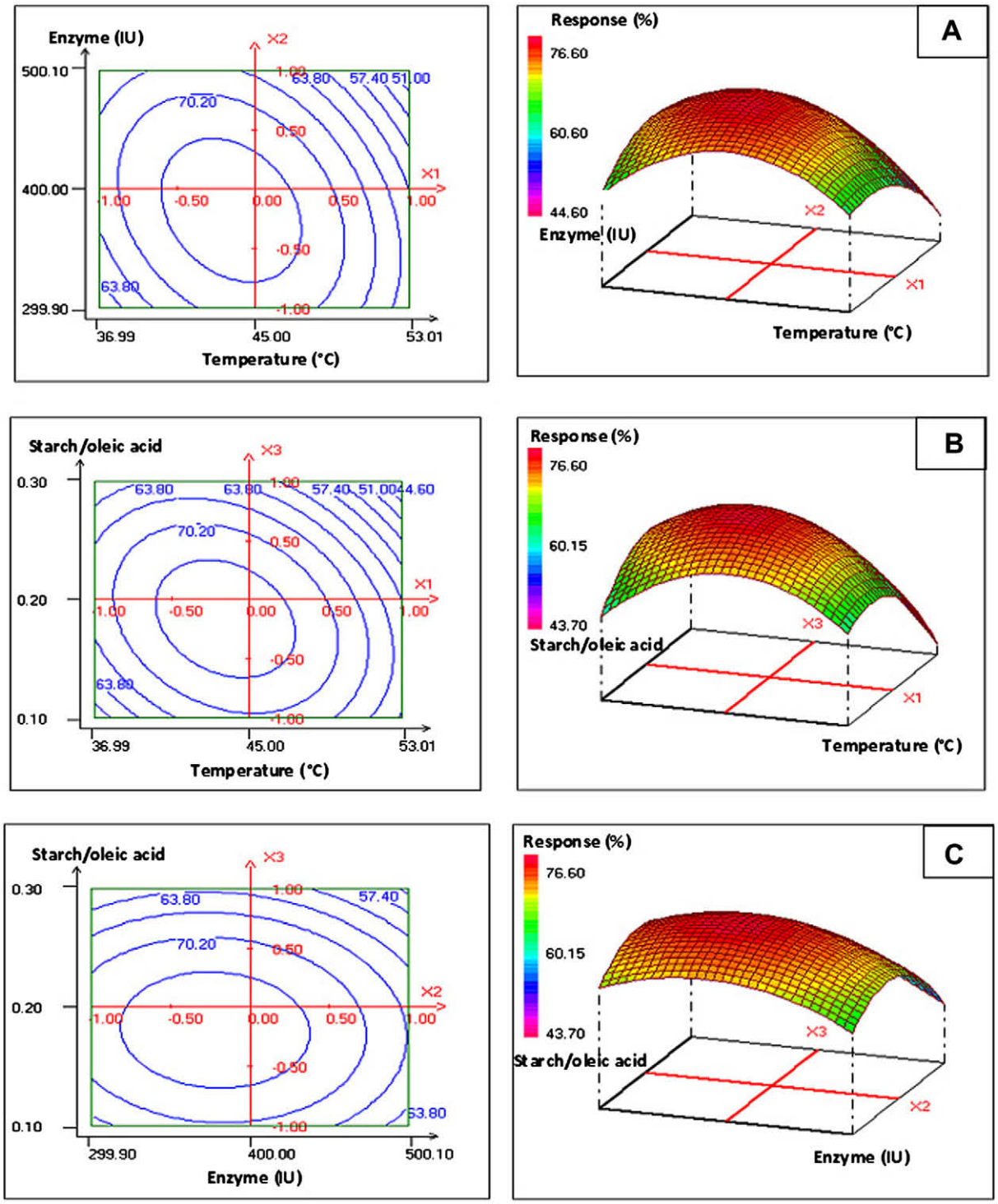

Fig. 2. (A) Contour plots and response surface plot showing the effect of enzyme amount, temperature, and their mutual interaction on starch oleate synthesis at starch/oleic acid molar ratio fixed at 0.18. (B) Contour plots and response surface plot showing the effect of temperature, starch/oleic acid molar ratio, and their mutual interaction on starch oleate synthesis using 386 IU of immobilized lipase. (C) Contour plots and response surface plot showing the effect of enzyme amount, starch/oleic acid molar ratio, and their mutual interaction on starch oleate synthesis at constant temperature equal.

– High level of the enzyme amount has negative effect on the conversion yield.

It is worth noting that, under the same conditions, no conversion was observed when using different amounts of a commercial lipase (Novozyme 435). This is could be attributed to the instability of this enzyme in the esterification conditions.

The optimal conditions leading to the maximum of esterification yield are given by the coordinates of the stationary point which are: temperature: 44 °C, immobilized lipase: 386 IU and a starch/acid molar ratio of 0.18. In order to confirm this result two independent supplementary experiments are carried out under these conditions. The results obtained (77.9%, 76.1%, 76.1%, 75.1% and 74.9%) are close to the predicted value (77.42%).

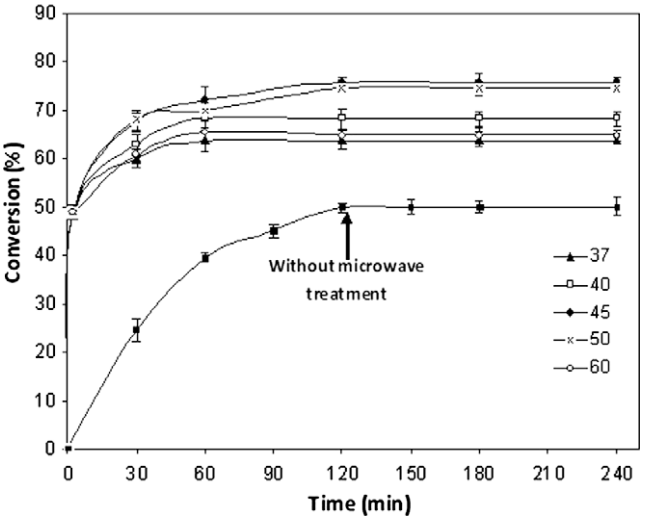

Fig. 3. Influence of temperature on the conversion yield (%). Reaction conditions were 400 IU of immobilized lipase, a starch/oleic acid molar ratio of 0.2 stirred at 200 rpm. A reaction without microwave treatment was carried out under the same conditions at 45 °C.

3.3. Determination of the synthesized starch ester properties

3.3.1. Degree of substitution

The degree of substitution for a modified starch is defined as the moles of substituents of hydroxyl groups per D-glucopyranosyl structural unit of the polymer. The final esterification yield of starch and oleic acid was measured. Therefore, 76% of conversion was obtained after 4 h-esterification under the microwave radiation followed by liquid state esterification with a DS of 2.86. To check the importance of the microwave heating in the esterification yield of starch with oleic acid, a reaction using only shaking flask was carried out under the optimal conditions. 50% of esterification yield with a DS of 1.8 was obtained after 4 h of incubation time. An esterification yield of 45% (DS = 1.6) was obtained when using only microwave heating (Fig. 3). These results confirm that microwave heating and shaking flask were found to be of a great importance to enhance the esterification yield with less time consumption and to reach a high degree of substitution. Rajan et al. (2006) have reported that the highest conversion percentage reached, using only microwave heating and *Candida rugosa* lipase as a biocatalyst to the esterification of cassava starch with recovered coconut oil, was 55% with a DS of 1.1.

3.3.2. FT-IR analysis

Fig. 4 presents the FT-IR spectra of the starch before (Fig. 4A) and after (Fig. 4B) esterification. As shown in this figure, the signals of the starch and its ester indicate that the structure of the original polysaccharide remained intact. The reacted alcoholic groups, shown by a strong peak at 3500 cm^{-1} in the spectrum of unmodified starch, decreased in intensity following esterification which is an indication that the major O–H groups of starch are reacted. One can notes also that the C–H stretching absorbance centered on 2922 cm^{-1} is increased in intensity due to the C–H band of oleic acid upon esterification. In comparison with spectra of the unmodified starch, the major change is the presence of a new peak at 1712 cm^{-1} corresponding to carbonyl group (Fig. 4A). This new absorption peak can be attributed to the characteristic ester group from oleic acid in the starch oleate structure (Fig. 4A). The appearance of the band above 1712 cm^{-1} could be considered as an argument for the esterification of oleic acid with starch because the carbonyl groups from the ester are located in this wavelength region. Chatel, Voirin, and Artaud (1997) differentiated esterified starch from original starch by FT-IR and found that the absorbance around 1724 cm^{-1} correspond to the stretching vibration of the carbonyl group of the ester. Another characteristic band corresponding to the carboxyl group was observed around 1650 cm^{-1} in the reaction mixture before esterfication. The intensity of this band decreased after esterification.

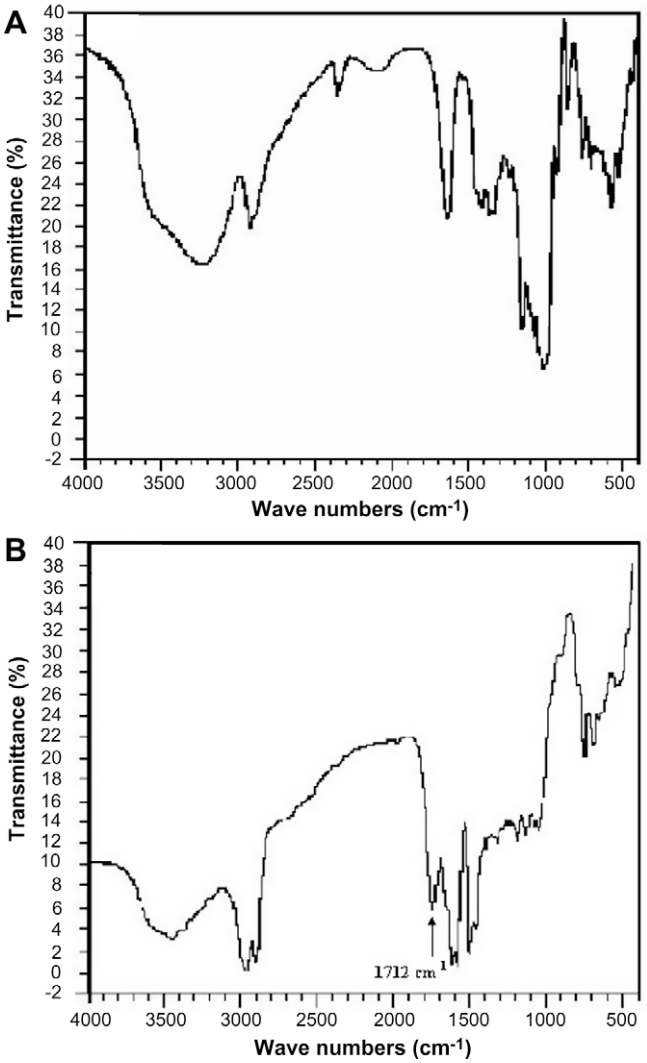

Fig. 4. IR spectrum before esterification (A) and after esterification (B). The Arrow indicates the peak of the new product appeared at 1712 cm^{-1} corresponding to the presence the carbonyl group.

3.3.3. NMR analysis

The chemical structure of starch oleate was confirmed by ^{13}C NMR. Fig. 5 presents the NMR spectra of oleic acid (Fig. 5A), maize starch (Fig. 5B) and starch oleate after 76% of esterification yield with a DS of 2.86 (Fig. 5C). As shown in this figure, new peaks due to oleic acid are all detectable between 50 and 100 ppm, as evidence of the formation of starch oleic acid ester.

Comparing the spectrum of native and esterified starch, a new strong band above 179.7 ppm corresponding to the carbonyl group appeared. The appearance of this band could be considered as an argument for the esterification of oleic acid with starch because the vibrations of the carbonyl group in ester used to reside in this region. All the evidence from the spectra of FT-IR and NMR prove that starch oleic ester was synthesised successfully.

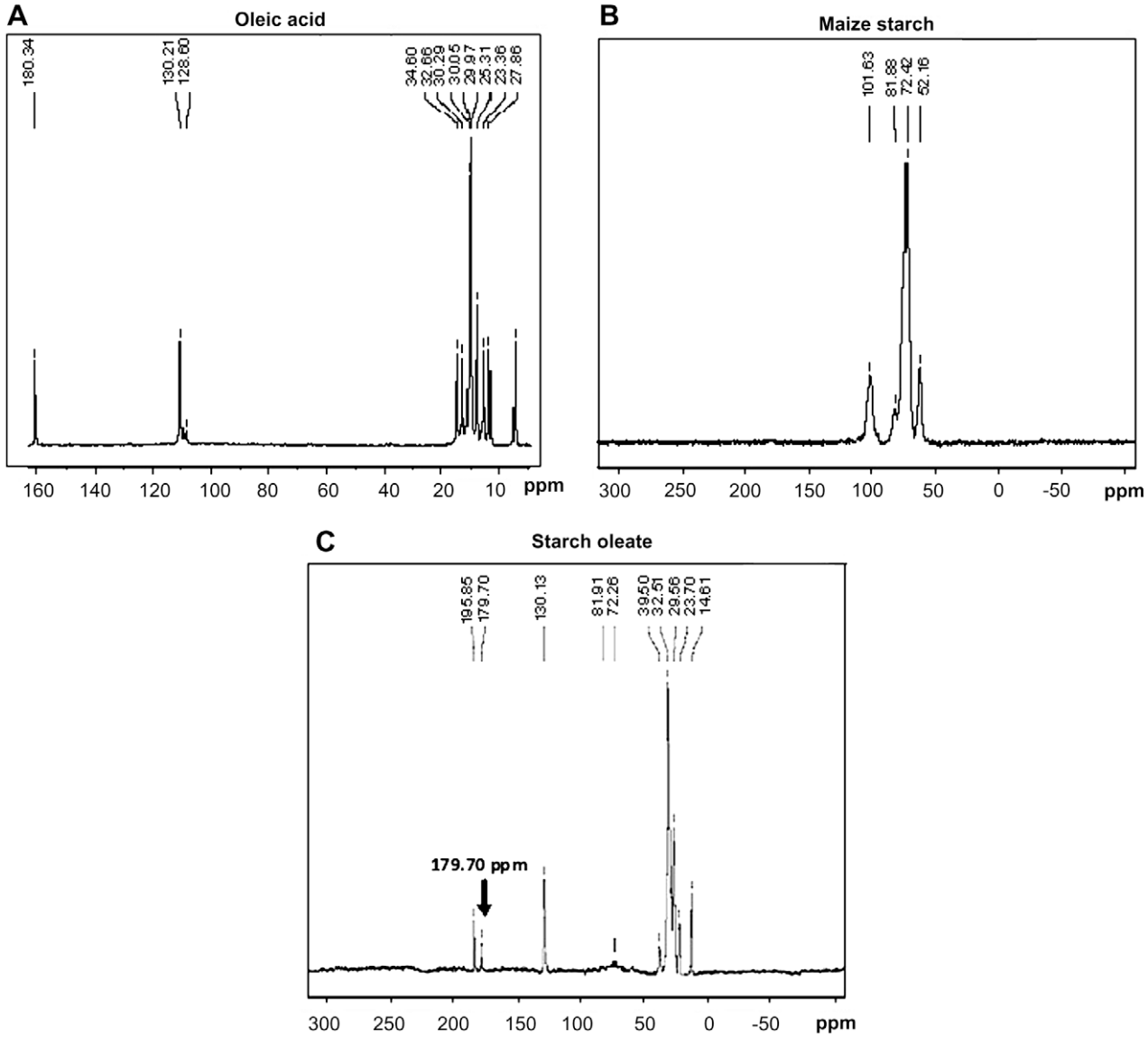

Fig. 5. ^{13}C NMR spectra of oleic acid (A) maize starch (B) and the starch oleic acid ester (C). The presence of the esterified starch was marked by the arrow.

3.3.4. Viscosity measurement

The introduction of an ester group, to polysaccharides constitutes an important synthetic task, as it modifies their hydrophilic nature and enhances their initial properties such as solubility and viscosity. After esterification, the starch exhibited an increase in viscosity due to fatty acid grafted onto starch, rendering the product highly hydrophobic (Rajan et al., 2006). The viscosity profiles of starch before and after esterification are shown in Fig. 6. One can note that the increase of the degree of substitution was accompanied by the increase of the viscosity of the reaction mixture which reaches its maximum value (84.5 mPa S) after 4 h of incubation time.

The increased viscosity of the starch oleate suggests that this material is a good thickener. This is in line with previous findings showing that a starch esterified by alkenyl ketone dimmer has higher Brookfield viscosity compared to the non-esterified starch (Qiao, Gu, & Cheng, 2006).

3.3.5. Digestibility of starch before and after esterification

Usually, the susceptibility to enzymatic hydrolysis has been a critical issue concerning the use of the newly synthesized products.

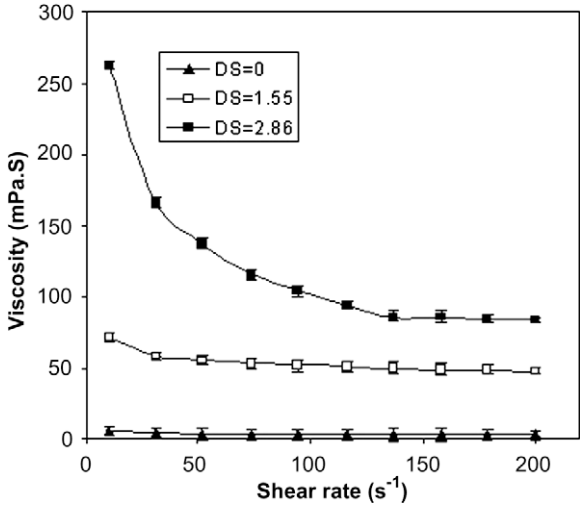

Fig. 6. Viscosity profile of maize starch before and after esterification. DS: degree of substitution.

It has been long known that chemical modifications such as esterification, etherification, and cross-linking of starch can build resistance to α-amylase (Hood & Arneson, 1976; Leegwater & Luten, 1971).

To test the above assumption, native starch or starch oleate, were exposed to enzymatic hydrolysis by porcine pancreatic α-amylase during 60 min (data not shown). The TLC analysis showed that, in contrast to native maize starch, esterified starch (with a DS of 2.86) is not hydrolyzed by porcine pancreatic α-amylase. The lack of the α-amylase digestibility of the esterified starch can be related to the hydrophobicity of the starch esters. On the same way, Rajan et al. (2006) reported that modified starch had lower α-*amylase* digestibility than the native one.

3.3.6. Thermal analysis

Gelatinization enthalpy reflects the energy required for disrupting the granule structure (Liu, Ramsden, & Corke, 1999). DSC results showed that esterification decreases the gelatinization temperature (T_g) and enthalpy (ΔH) (data not shown). In fact the starting starch which is essentially crystalline has a T_g of 86.8 °C. After esterification, the T_g decreased to 61 °C. Obviously, the replacement of hydroxyl groups by long-chain fatty acid has led to a loss of crystallinity of the initially starch. The obtained T_g is in accordance with the T_g values for modified starch reported in the literature (Sagar and Merill, 1995; Aburto et al., 1997). In the above-mentioned studies, it was found that the T_g of esterified starches decrease with increasing chain length of the acid used in esterification.

Our results show also that the enthalpy (ΔH) of native starch (ΔH = 375 J/g) and the esterified one (ΔH = 101 J/g) are different. These changes were explained in previous works by a decrease in inter-molecular hydrogen bonds with replacements of hydroxyl groups (Xu, Dzenis, & Hanna, 2005).

4. Conclusions

The process of biopolymer synthesis presented in this work was conducted in a solvent-free system using a non-commercial immobilized lipase from *Staphylococcus aureus*. For the first time, starch esters with oleic acid were synthesized by combining microwave heating and shaking. The optimized conditions for the synthesis of the biopolymer using response surface methodology were 386 IU of immobilized lipase and an oleic acid/starch molar ratio of 0.18 at 44 °C and 200 rpm. Under these conditions, 76% of conversion with a DS of 2.86 was reached after 4 h of incubation.

Esterification significantly decreased the susceptibility of starch to α-amylolysis due to the increased hydrophobicity of the final product. The hydrophobic starch fatty acid esters produced may have potential industrial applications such as surface coating materials, flavoring agents in food industry and biomedicals for bone fixation and replacements. Furthermore, the process is eco-friendly since no toxic waste products are released. Therefore, enzymatically produced starch esters can be used directly for various food applications.

Acknowledgments

This work is a part of a doctoral thesis by Habib HORCHANI whose research was supported financially by «Ministere de l'enseignement supérieur, de la recherche scientifique et de la technologie-Tunisia» through a grant to «Laboratoire de Biochimie et de Génie Enzymatique des Lipases-ENIS». The authors would like to thank Mr A. Hajji from the Faculty of Letters and Humanities of Kairwan (Tunisia) for his help with English.

References

Aburto, J., Alric, I., Thiebaud, S., Borredon, E., Bikiaris, D., Prinos, J., et al. (1999). Synthesis, characterization, and biodegradability of fatty-acid esters of amylose and starch. *Journal of Applied Polymer Science, 74*, 1440–1451.

Aburto, J., Hamaili, H., Mouysset-baziard, G., Senocq, F., Alric, I., & Borredon, E. (2000). Free-solvent synthesis and properties of higher fatty esters of starch – Part II. *Starch/Stärke, 51*, 302–307.

Aburto, J., Thiebaud, S., Alric, I., Borredon, E., Bikiaris, D., Prinos, J., & Panayiotou, C. (1997). Properties of octanoated starch and its blends with polyethylene. *Carbohydrate Polymers, 34*, 101–122.

Arica, M. Y., Hasirci, V., & Alaeddinoglu, N. G. (1995). Covalent immobilization of α-amylase onto pHEMA microspheres: Preparation and application to fixed bed reactor. *Biomaterials, 16*, 761–768.

Chatel, S., Voirin, A., & Artaud, J. (1997). Starch identification and determination in sweetened fruit preparations. 2. Optimization of dialysis and gelatinization steps, infrared identification of starch chemical modifications. *Journal of Agricultural and Food Chemistry, 45*, 425–430.

De Oliveira, P. C., Alves, G. M., & De Castro, H. F. (2000). Immobilisation studies and catalytic properties of microbial lipase onto styrene-divinylbenzene copolymer. *Biochemical Engineering Journal, 5*, 63–71.

Espigares, I., Elvira, C., Mano, J. F., Vasquez, B., San Yoman, J., & Reis, R. L. (2002). New partially degradable and bioactive acrylic bone cements based on starch blends and ceramic fillers. *Biomaterials, 23*, 1883–1895.

Fang, J. M., Fowler, P. A., Tomkinson, J., & Hill, C. A. S. (2002). The preparation and characterization of a series of chemically modified potato starches. *Carbohydrate Polymers, 4*, 245–252.

Gargouri, Y., Julien, R., Sugihara, A., Sarda, L., & Verger, R. (1984). Inhibition of pancreatic and microbial lipases by proteins. *Biochimica et Biophysica Acta, 795*, 326–331.

Ghamgui, H., Karra-Chaabouni, M., & Gargouri, Y. (2004). 1-Butyl oleate synthesis by immobilized lipase from Rhizopus oryzae: A comparative study between n-hexane and solvent-free system. *Enzyme and Microbial Technology, 35*, 355–363.

Gomes, M. E., Ribeiro, A. S., Malafaya, P. B., Reis, R. L., & Cunha, AM. (2001). A new approach based on injection moulding to produce biodegradable starch based polymeric scaffolds. *Biomaterials, 22*, 883–889.

Heinze, T., Talaba, P., & Heinze, U. (2000). Starch derivatives of high degree of functionalization. 1. Effective, homogeneous synthesis of p-toluenesulfonyl (tosyl) starch with a new functionalization pattern. *Carbohydrate Polymers, 42*, 411–420.

Hood, L. F., & Arneson, V. G. (1976). In vitro digestibility of hydroxypropyl distarch phosphate and unmodified tapioca starch. *Cereal Chemistry, 53*, 282-290.

Horchani, H., Mosbah, H., Ben Salem, N., Gargouri, Y., & Sayari, A. (2009). Biochemical and molecular characterization of a thermoactive, alkaline and detergent-stable lipase from a newly isolated Staphylococcus aureus strain. *Journal of Molecular Catalysis B: Enzymatic, 57*, 237–245.

Kapusniak, J., & Siemion, P. (2007). Thermal reactions of starch with long-chain unsaturated fatty acids. Part 2. Linoleic acid. *Journal of Food Engineering, 78*, 323–332.

Koroskenyi, B., & McCarthy, S. P. (2002). Microwave-assisted solvent-free are aqueous-based synthesis of biodegradable polymers. *Journal of Polymer and Environment, 10*, 93–104.

Leegwater, D. C., & Luten, J. B. (1971). A study on the in vitro digestibility of hydroxypropyl starches by pancreatin. *Starch/Stärke, 23*, 430–432.

Liu, H. J., Ramsden, L., & Corke, H. (1999). Physical properties of cross-linked and acetylated normal and waxy rice starch. *Starch/Stärke, 51*, 249–252.

Malafaya, P. B., Elvira, C., Gallardo, A., San Roman, J., & Reis, R. L. (2001). Porous starch-based drug delivery systems processed by a microwave route. *Journal of Biomaterials Science, Polymer Edition., 12*, 1227–1241.

Miladinov, V. D., & Hanna, M. A. (1999). Physical and molecular properties of starch acetates extruded with water and ethanol. *Industrial and Engineering Chemistry Research, 38*, 3892–3897.

Mozhaev, V. V., Mellik-Nubarov, N. S., Sergeeva, M. V., Siksnis, V., & Martinek, K. (1990). Strategy for stabilizing enzymes. Part 1: Increasing stability of enzymes via their multipoint interaction with a support. *Biocatalysis, 3*, 179–187.

Ogino, H., & Ishikawa, H. (2001). Enzymes which are stable in the presence of organic solvents. *Journal of Bioscience and Bioengineering, 91*, 109–116.

Peltonen, S., & Harju, K. (1996). Application and methods of preparation of fatty acid esters of polysaccharides. US Patent No. 5589577.

Penceac'h, G., & Barrati, J. C. (1999). Properties of free and immobilized lipase from Burkholderia cepacia in organic media. *Applied Microbiology and Biotechnology, 52*, 276–280.

Qiao, L., Gu, Q., & Cheng, H. N. (2006). Enzyme-catalysed synthesis of hydrophobically modified starch. *Carbohydrate Polymers, 66*, 135–140.

Rajan, A., Prasad, V. S., & Abraham, T. E. (2006). Enzymatic esterification of starch using recovered coconut oil. *International Journal of Biological Macromolecules, 39*, 265–272.

Reis, R. L., & Cunha, A. M. (2000). New degradable load-bearing biomaterials composed of reinforced starch based blends. *Journal of Applied Medical Polymers, 4*, 1–5.

Roper, H. (1996). Application of starch and its derivatives. *Carbohydrate Research, 15*, 22–30.

Sagar, A. D., & Merill, E. W. (1995). Properties of fatty acid esters of starch. *Journal of Applied Polymer Science, 85*, 1647–1656.

Sousa, R. A., Mano, J. F., Reis, R. L., Cunha, A. M., & Bevis, M. J. (2002). Mechanical performance of starch based bioactive composite biomaterials molded with preferred orientation for potential medical applications. *Polymer Engineering and Science, 42*, 1032–1045.

Wu, X. Y., Jaasklainen, J., & Linko, Y. (1996). An investigation of crude lipases for hydrolysis, esterification and trans esterification. *Enzyme and Microbial Technology, 19*, 226–231.

Xu, Y. X., Dzenis, Y., & Hanna, M. A. (2005). Water solubility, thermal characteristics and biodegradability of extruded starch acetate foams. *Industrial Crops and Products, 21*, 361–368.

Ye, P., Xu, K., Che, A. F., Wu, J., & Seta, P. (2005). Chitosan-tethered poly(acrylonitrile comaleic acid) hollow fiber membrane for lipase immobilization. *Biomaterials, 32*, 6394–6403.