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ORIGINAL PAPER

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Enzymatic modification of cassava starch by bacterial lipase

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Abstract Enzymatic modification of starch using long chain fatty acid makes it thermoplastic suitable for a myriad of industrial applications. An industrial lipase preparation produced by *Burkholderia cepacia* (lipase PS) was used for modification of cassava starch with two acyl donors, lauric acid and palmitic acid. Reactions performed with palmitic acid by liquid-state and microwave esterification gave a degree of substitution (DS) of 62.08% (DS 1.45) and 42.06% (DS 0.98), respectively. Thermogravimetric analysis showed that onset of decomposition is at a higher temperature (above 600°C) for modified starch than the unmodified starch (280°C). Modified starch showed reduction in α-amylase digestibility compared to native starch (76.5–18%). Swelling power lowered for modified starch as esterification renders starch more hydrophobic, making it suitable for biomedical applications as materials for bone fixation and replacements, carriers for controlled release of drugs and bioactive agents. Thus enzymatic esterification is ecofriendly.

Keywords Cassava starch · Esterification · Degree of substitution · Recovered coconut oil · Lipase · Starch ester

Introduction

Starch is the second most abundant renewable polymer in nature that is inexpensive, fully biodegradable and widely studied for many years in the field of materials [1, 2]. It is a glucose polymer $(C_6H_{10}O_5)_n$ abundant in the seeds of cereal plants and in bulbs and tubers. Starch grains are composed of two kinds of molecules—amylose and amylopectin. Chemical modification of starch is often required to better suit its properties to specific applications. Modification of the starch OH groups to form appropriate degree of substitution (DS) (1.5–3.0 DS) and high % DS (50–95%) imparts thermoplasticity and water resistance to the modified starch over the unmodified one. The main problem here is the incorporation of the fatty acid to the starch, to bring about the esterification. Normally, DMSO and pyridine is used to solubilise the starch to make it more reactive towards esterification. Modified starch-based materials are used in industry as glues, adhesives and auxiliaries of a wide range of rheological and functional properties [3].

A number of authors [4–7] have reported the preparation of esterified starches of higher degrees of substitution, in presence of organic solvents so as to provide suitable reaction conditions. Sophisticated experimental techniques, solvents, or systems of solvents, are used to achieve homogeneous modification of the chosen starch. However, these techniques are expensive, typically use toxic solvents in highly alkaline conditions at high temperatures and are not viable for the large-scale industrial production of modified starches. Downstream processing is expensive in chemical modification whereas use of enzymes is cost effective ($0.35 for 100 mg) and eliminates further purification.

Use of microwave ovens instead of rotating roasters for starch modification is a promising method. Microwave radiation (2,450 MHz) does not activate specific bonds on molecules and consequently this form of heating will not lead to any kinetic differences compared to other form of heating [8]. The rate of temperature rise depends on the moisture content of starches irradiated with microwave [9]. In spite of this there has been a growing interest in the use of microwave heating in organic synthesis, so called "MORE chemistry," microwave oven induced reaction enhancement [10, 11].

The process of starch modification involves the destructurisation of the semi-crystalline starch granules

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and the effective dispersion of the component polymers. In this way, the reactive sites (hydroxyl groups) of the amylopectin polymers become accessible to electrophilic reactants. Starch is effectively destructured by gelatinisation in hot water, the precise temperature (which can vary extensively) depending on the source of starch or, more correctly on the branch chain-length of the amylopectin component.

Chemical esterification of starch is usually done at high pH using NaOH, pyridine and anhydrides of the acids. The aim of this work was to examine the possibility of enzymatic modification of starch by application of microwave instead of rotating roasters or extrusion cookers to obtain starch esters of C-12 to C-18 fatty acids with a structure and functionality of industrial products.

Experimental

Starch

Commercially available cassava starch was procured locally and purified by repeated washing with distilled water and dried in a cross flow drier at 50 ± 2°C.

Enzymes

Lipase PS obtained from *Burkholderia cepacia* (Amano, Nagoya, Japan) had about 6–10% protein. Enzyme activity was 147 U/ml. The extraction of the enzymes from the unwanted filler materials was done with phosphate buffer (0.1 M, pH 7) at 30°C.

Fatty acids

Coconut oil, rich in lauric acid, is used for deep frying banana and jack fruit chips in this part of the country. Once used and recovered coconut oil (C-12 FA, lauric acid 47%, C-14 myristic acid 18%, C18-1 oleic acid 6%, C-16 palmitic acid 9%, C-10 capric 6%, C-18 stearic acid 3%). Coconut oil was hydrolysed with lipase for 8 h in order to release free fatty acids for esterification. The percentage of free fatty acid was estimated by titration against 0.1 N NaOH using phenolphthalein as indicator. Palmitic acid (99.5%, extra pure, SRL, Mumbai, India) was also used. All the other chemicals used are of analytical grade.

Enzyme assay

Lipase assay was done using olive oil as substrate in Tris–HCl buffer pH 8.5 at 30 ± 2°C [12]. One unit (U) of lipase activity is defined as the amount of the enzyme, which liberated 1 μmol of free fatty acid per minute under the assay conditions.

To 10 ml buffer and 10 ml olive oil emulsion, 1 ml enzyme was added. Acetone was added to stop the reaction. It was then titrated against 0.05 M NaOH.

$$\text{Enzyme activity} = \frac{(\text{test titre} - \text{blank titre}) \times \text{normality of NaOH}}{20 \times \text{volume of lipase used}} \times 1{,}000 \qquad (1)$$

Thermo stability and microwave oven heating stability of enzymes

Thermostability of lipase was checked at 30, 40 and 50, 60 and 70°C. Microwave oven stability was checked for different time duration from 15 s to 2 min 15 s under standard conditions.

Esterification

The esterification was done in either liquid state with solvents DMSO/DMF, or with solvents in a domestic microwave oven (BPL Ltd, Bangalore, India).

Liquid state

Cassava starch and hydrolysed coconut oil [1:1 (w/w)] was dissolved in 1 ml solvent (DMF/DMSO) and then 200 mg lipase (143 U) were added and incubated in a water bath at 70 ± 2°C. Under the same conditions hydrolysed coconut oil was replaced by palmitic acid for esterification. The incubation time for esterification with lipase PS is 5 h. The ester formed is then precipitated by adding 10 ml alcohol. The separated ester is washed again and then dried in a hot air oven at 55°C.

Microwave esterification

Esterification was done using a microwave oven having frequency of 2,450 MHz by presetting the power to 80 W with an incubation time of 3 min given intermittently. Starch and fatty acid was taken in the ratio 1:1 and to this 200 mg enzyme was added. The resultant starch esters, was oven dried and were characterised by infrared (IR) spectroscopy.

Fourier transfer infrared measurement

The evidence of esterification was verified by utilising Fourier transfer infrared (FT-IR; Bomem MB Series) showing the shift of the carbonyl of carboxylic acid group to the carbonyl of ester group. For FT-IR spectroscopic analysis, the sample was ground and the fine powder sample was mixed with dry potassium bromide. It was then made into a film that was analysed in the beam of the FT-IR spectrophotometer.

Degree of substitution

The DS of esterified starch was determined using the Miladinov and Hanna [13] method, by hydrolysing and releasing substituted groups (fatty acids) with 1 M NaOH and then titrating back with 0.5 M HCl.

DS was calculated as

$$DS = \frac{(M_{FA} \times MW_{AN})}{(W - M_{FA})(MW_{FA} - MW_{H_2O})} \quad (2)$$

where M_{FA} is the moles of titrated fatty acid, MW_{FA} is the molecular weight of the fatty acid, MW_{H_2O} is the molecular weight of water (18) and MW_{AN} is the molecular weight of anhydrous glucose unit (162).

α-Amylase activity

Gelatinised starch (1%) is hydrolysed with 100 μl of α-amylase (Termamyl 60 l, Novozyme, enzyme activity 3,000 IU/ml) at 60°C for 30 min (pH 6.5) and the reducing sugar equivalents liberated are estimated by DNS method [14]. One unit of α-amylase activity was defined as the release of 1 μmol of reducing sugar from the soluble starch per minute under the experimental conditions. The colour was measured at 540 nm in a spectrophotometer (UV 2100, Shimadzu, Kyoto, Japan) with maltose as standard. The amount of maltose obtained is an index of the amount of starch that got hydrolysed in the samples to give reducing end groups of the dextrins/limit dextrins produced.

Swelling power of modified starch [15]

Starch (0.1 g) is weighed and quantitatively washed with sufficient distilled water to give a total volume equivalent to 9 g. The bottle is kept at 85.0 ± 0.2°C for 30 min with continuous stirring at 200 rpm. The total volume is made up to 10 g and centrifuged at 2,200 rpm for 15 min. The clear supernatant is drawn off to within 0.5 cm of the top surface of the sedimented paste. Five millilitres of this solution is transferred by a pipette to an evaporating dish and evaporated to dryness on steam bath. The dish is dried overnight in an oven at 110°C, cooled in a dessicator and weighed.

To obtain the swelling power, the last 0.5 cm of supernatant above the sedimented starch is drawn off and discarded. The bottle and contents are then weighed to give the weight of the swollen starch granules. % Solubles and swelling power was calculated as

$$\%\text{Solubles (on dry basis)} = \frac{\text{weight of soluble starch} \times 400}{\text{weight of sample on dry basis}}$$
$$\text{Swelling power} = \frac{\text{weight of sedimented paste} \times 100}{\text{weight of sample on dry basis} \times (100 - \%\text{ solubles, drybasis})} \quad (3)$$

Thermal gravimetry analysis

The thermogravimetric analysis (TGA) was carried out in simultaneous differential thermal analyser-thermal gravimetric (DTA–TG) apparatus with a ramping rate of 10 K min^{-1}. Samples of about 3–6 mg were placed in the balance system and heated from ambient temperature to 600°C in a nitrogen atmosphere. Prior to thermal analysis, the samples were lyophilised (Hetosicc).

Results and discussion

Thermostability and microwave oven stability of lipase

The optimum activity was found at 40°C. At 70°C there was a rapid decrease in enzyme activity over time. Half-life of the enzyme was reached in 2 h 30 min at 70°C. Microwave heating reduced the stability of the enzyme as an increase in time. This is due to the denaturation caused by in situ generation of heat due to the vibration of water molecules, if the exposure is above 30 s. Results are given in Figs. 1 and 2.

Thermostability of lipase PS in combination with starch and oil in a microwave oven

Lipase PS activity was checked along with different combinations. Starch and oil were taken in the ratio 1:1. Two hundred milligrams of enzyme was added to it and microwaved at a power rating of 80 W and at a radiation frequency of 2,450 MHz. The results are given in Table 1.

Lipase PS showed better activity in phosphate buffer and it was slightly reduced when in combination with starch and hydrolysed coconut oil. The enzyme activity reduced gradually over the time during microwave heating as the temperature rises to 70–90°C.

Liquid-state esterification

Out of the different combinations of starch with hydrolysed coconut oil and palmitic acid in DMSO and DMF, liquid-state esterification was found to be more effective with palmitic acid as acyl donor. Combination of palmitic acid and DMF showed an ester peak in the region 1,717.55 in IR spectrum (Fig. 4). A DS of 1.45 (62.08 % DS) was obtained in an incubation time of 5 h at 70°C. From a study of the properties of starch esters

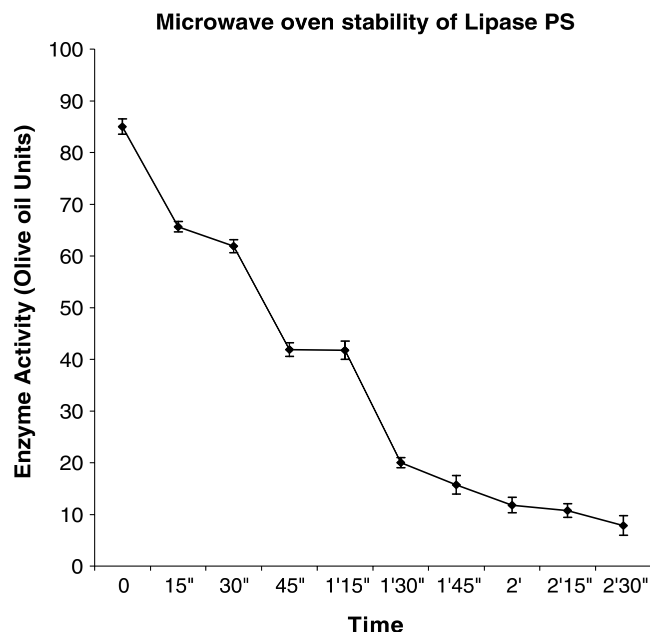

Fig. 1 Microwave oven stability of lipase PS

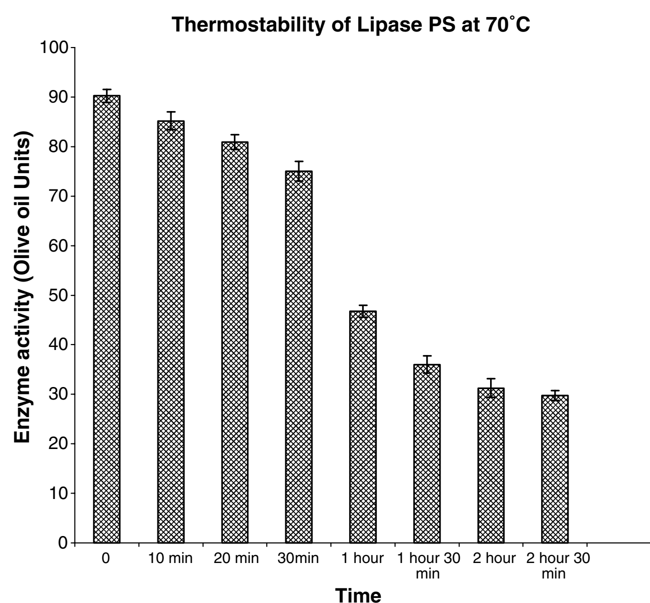

Fig. 2 Thermostability of lipase PS at 70°C

Table 1 Microwave thermostability of lipase PS in combination of starch and oil

Time	Lipase PS	Lipase PS with starch	Lipase PS withstarch and fatty acid
0 s	76.25	65.5	56
30 s	60	55.5	46.75
1 min	54.25	48.75	40.5
1 min 30 s	46.25	36.75	31.75
2 min	38	26.25	25
2 min 15 s	33.25	18	16.25

(butyrate, valerate, hexanoate), Sagar and Merrill [16] showed that the ester groups act like an internal plasticiser, with an increase in the size of fatty acid chain. The resulting starch esters behave like thermostable materials.

Microwave oven esterification

The combination which produced starch modification using microwave energy is cassava starch with palmitic acid and DMF using lipase PS. A shoulder in the ester region was obtained in IR spectrum. The modified starch showed a DS of 0.98 (42.06%).

FT-IR measurement

In the native starch spectrum, the characteristic peaks (958–1,255 cm^{-1}) are attributed to C–O bond stretching [17]. Another strong broad band due to hydroxyl bond stretching appears at 3,000–3,600 cm^{-1} (Fig. 3) which is reduced on esterification (Fig. 4).

A characteristic peak occurred at 1,664.96 cm^{-1}, which is presumably a feature of tightly bound water present in the starch [18, 19]. A strong absorption band at 1,023 cm^{-1}, probably due to the stretching of the C–OH bond, was present in the spectra of the starch consistent with the earlier report by Marcazzan et al. [20]. 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 [6]. The band at 2,919 cm^{-1} is characteristic of C–H stretches. These spectra have similar profiles. In comparison with the spectra of the unmodified starches, the major change is the presence of a carbonyl C–O absorption frequency at 1,717.55 cm^{-1}. The strong O–H stretching band at 3,400 cm^{-1} in the native starches decreased in intensity following esterification of starch with fatty acids. The ester groups impart plasticity, with increase in the size of fatty acid chain used. The resulting starch esters behave like thermoplastic materials.

Degree of substitution

The DS for a starch derivative is defined as moles of substituents of hydroxyl groups per D-glucopyranosyl structural unit of the starch polymer; with three hydroxyl groups per unit, the theoretical maximum of DS is three. The results from the elemental analysis showed that the products of starch modification in aqueous media with solvents DMSO/DMF using lipase as the catalyst had high DS. Comparison of the DS and % DS of different methods are given in Table 2.

The study shows that starch esters with high DS can be prepared in solvents DMSO/DMF using lipase as the

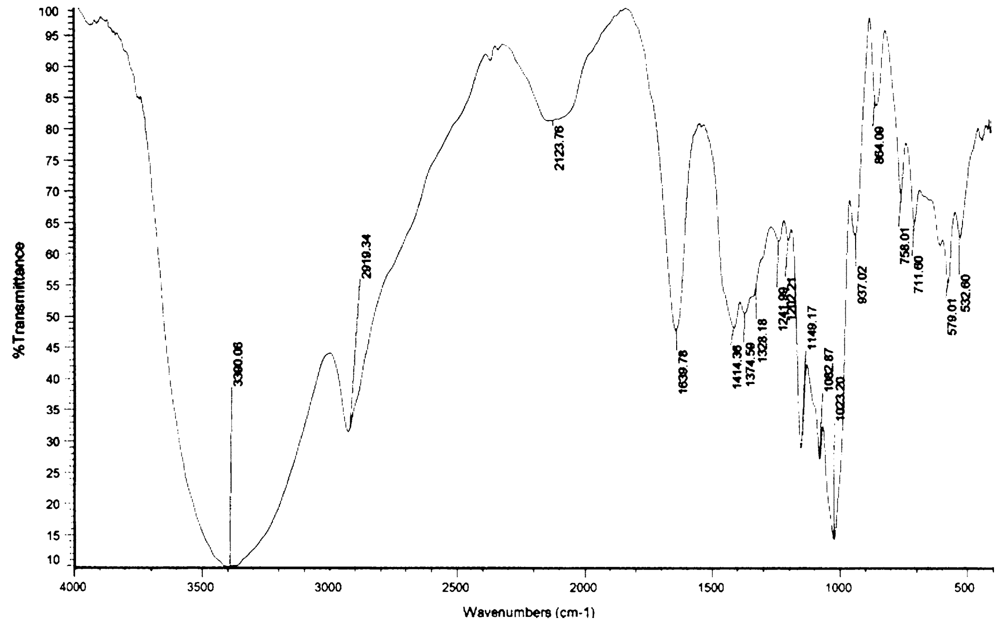

Fig. 3 Infrared spectrum of cassava starch

catalyst. The study proved that microwave radiation can bring about starch modification within seconds. Microwave energy is non-ionising and causes a rise in the temperature within a penetrated medium as a result of rapid changes in the electromagnetic field. The process is ecofriendly, there are no toxic waste products and hence the starch esters can be used directly for various end uses.

Swelling power of modified starch

Swelling behaviour is very important in polymeric systems which will be employed as implants because the capacity to capture water influences parameters such as mechanical properties and surface mobility [21]. The swelling power of modified starch with DS 1.45 is 8.42 (11.2% solubles). DS of starch ester seemed to be a main factor affecting the water uptake [22]. Esterification renders starch more hydrophobic, which would lead to an enhancement of dimensional stability in blends (less swelling) by reducing the possibility of hydrogen bond formation between hydroxyl groups in the starch and water, thereby reducing the water uptake. The higher DS of the starch ester and the more hydrophobic side chains make them less sensitive to water.

Estimation of α-amylase digestibility starch

Unmodified starch control had α-amylase activity of 76.5% which was reduced to 18% by modification (DS

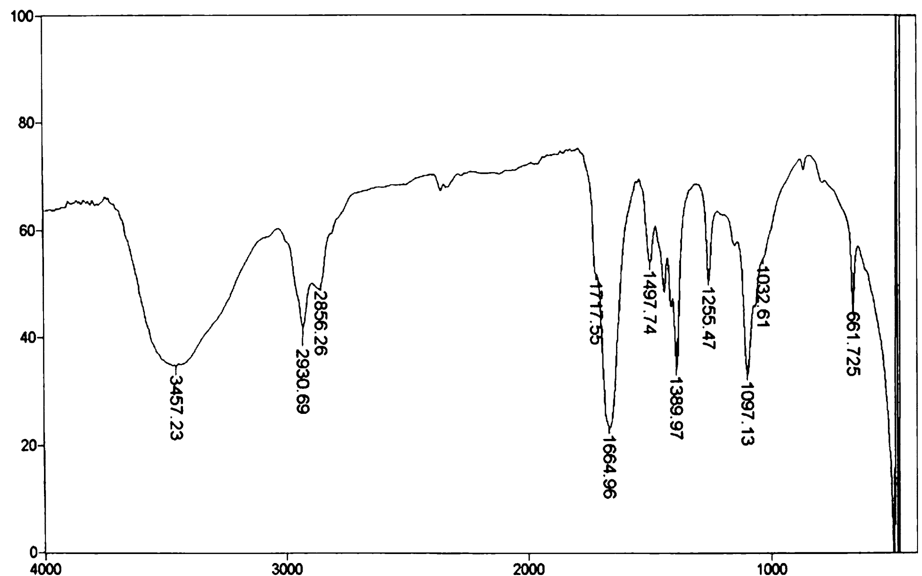

Fig. 4 Infrared spectrum of liquid-state esterification of cassava starch with palmitic acid in DMF with lipase PS

Table 2 Effect of lipase catalysed esterification methods on degree of substitution (DS) and % DS of cassava starch

	DS	% DS
Liquid-state esterification		
Five hours at 70°C, 1 g cassava starch + 0.1 g palmitic acid + 2 g lipase PS enzyme + 10 ml DMF	1.45	62.08
Microwave esterification		
0.1 g cassava starch + 0.1 g palmitic acid + 2 g lipase PS + 10 ml DMF	0.98	42.06

1.45). The high percentage of modification resulted in loss of α-amylase digestibility due to the hydrophobicity of the starch esters and as there is no proper gelatinisation which in turn exposes fewer α 1,4 bonds for the amylase to hydrolyse.

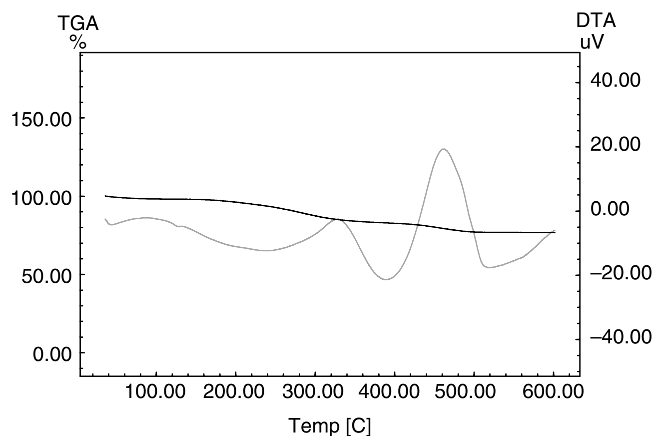

Fig. 6 Thermogravimetric analysis/DTA curve of liquid esterification of cassava starch with palmitic acid and DMF using lipase PS

Thermogravimetric analysis

Thermogravimetric curves were used to examine the changes in thermal stability caused by modification. The TGA thermograms of native starch and starch palmitate with DS 1.45 before and after esterification are shown in Figs. 5 and 6. The initial degradation of native starch began at 280°C with a weight loss of 30%. Further heating, to 600°C, resulted in carbonisation and ash formation as reported [7]. The TGA has shown that the esterification of starch increases its thermal stability. After modification, thermal degradation onset temperature increased from 280°C (unmodified) to above 600°C (for DS 1.45). This increase in thermal stability with increasing DS was attributed to the low amount of remaining hydroxyl groups in starch molecules after modification. The decomposition of starch occurs as a result of the inter- or intramolecular dehydration reactions having water as a main product of decomposition [7]. The opportunity for this condensation reaction was reduced with substitution of hydroxyl group by fatty acid ester group and higher DS contribute to its thermal stability. Hence the resultant weight loss was reduced noticeably.

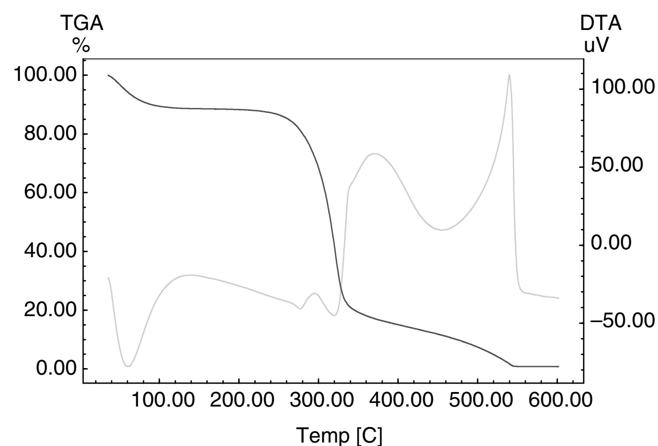

Fig. 5 Thermogravimetric analysis/DTA curve of unmodified cassava starch

Water is the main product of decomposition at temperatures < 300°C formed by intermolecular or intramolecular condensation of starch hydroxyls. There is also a beneficial influence of increasing carbon chain-length of the ester on thermal stability as it acts as a more efficient internal plasticiser. No decomposition was observed at 600°C in TGA. No T_{max} could be obtained as the modified starch showed no degradation even at 600°C.

Modified starch is used in various biodegradable plastic materials, which is mouldable. Starch/synthetic polymer blends have been used for distinct biomedical applications. These include starch-based biomaterials as scaffolds for the tissue engineering of bone and cartilage [23], materials for bone fixation, replacement as well as for filling bone defects [24, 25], carriers for the controlled release of drugs and other bioactive agents [26], new hydrogels and partially degradable bone cements [27]. Starch esters have various industrial applications; for making thermoplastics, glues, chewing gum base, adhesives, etc.

Conclusions

Lipase obtained from *B. cepacia* was found to be a useful biocatalyst in the starch modification. In liquid-state esterification, where cassava and palmitic acid was esterified with lipase for 5 h at 70°C, a DS of 1.45 was obtained. Microwave radiation produced modified starch with a DS of 0.96. Compared to microwave, liquid-state esterification produced an ester carbonyl band which was verified by IR spectrum. Moreover DS was higher in liquid-state esterification. TGA studies showed that no decomposition was observed even at 600°C for starch palmitate. α-Amylase digestibility and swelling power was considerably reduced for modified

starch. These studies showed that esterification renders starch more hydrophobic and thermostable, which would lead to an enhancement of dimensional stability in blends (less swelling) by reducing the possibility of hydrogen bond formation between hydroxyl groups in the starch and water, that is, by reducing the water uptake. Modification of starch with fatty acids makes the blends appropriate for applications, where water absorption must be minimal. The esterification of starch with fatty acids enhances its thermostable character and DS.

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