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Starch Depolymerization with Diluted Phosphoric Acid and Application of the Hydrolysate in Astaxanthin Fermentation

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Summary

An innovative alternative for cassava starch hydrolysis has been developed using diluted (about 0.1 %) phosphoric acid at 160 °C. This technology is advantageous for developing countries where enzyme costs are prohibitive and hydrochloric acid is currently the only catalyst used for starch depolymerization. Lower concentrations of the byproduct hydroxymethyl furfural (HMF) were generated during starch hydrolysis when using phosphoric acid as compared to hydrochloric acid at any given acidic pH. Glucose was the major product from phosphorolysed starch under most reaction conditions, although maltosaccharides with degrees of polymerization from 2 to 7 were also produced, with their relative amounts depending on hydrolysis conditions. Neutralization of the acid with aqueous ammonia produced a hydrolysate with sources of C (free sugars), P (phosphate), and N (ammonium) that could find several applications. We demonstrated one of these, namely the potential for the use of the hydrolysate as a fermentation feedstock, by cultivating the astaxanthin-producing red yeast *Xanthophyllomyces dendrorhous* on it. Cassava wastewater, a polluting byproduct of starch processing, was found to be a convenient source of nitrogen for this fermentation process.

Key words: cassava starch, phosphorolysis, acid hydrolysis, depolymerization, phosphoric acid, astaxanthin

Introduction

Syrups produced from starch by mineral acid or enzymatic hydrolysis represent a potentially important fermentation feedstock. Mineral acids are potentially cheaper than amylases but present problems that still need to be solved. Acid catalysis involves final concentrations in the reactor of 0.01 to 0.1 M, with heating under pressure and short residence times (5 to 10 min) (*1*). Syrups of dextrose equivalents (DE) from 20 to 80 can be produced, although most commonly the DE does not exceed 42. The low acid concentration, lower cost, shorter reaction time and higher starch granule loads (approx. 40 % of solids), all compensate for the fact that enzymes typically operate at lower temperatures. It must also be remembered that the energy costs of the enzymatic hydro-

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lysis are not small since a previous gelatinization step is necessary.

Hydrochloric acid is the only mineral acid still used industrially for starch hydrolysis, but it presents several problems. Firstly, hydrochloric acid produces toxic fumes. Secondly, after neutralization, it is typically necessary to desalinate the syrup using high cost ion exchange resins. Thirdly, undesirable byproducts are produced even when syrups of an average DE are produced, since free glucose is converted to dehydration products such as hydroxymethylfurfural (HMF) and levulinic and formic acids, which can inhibit microbial growth in a subsequent fermentation step (*2*). From the industrial standpoint, the situation is even more complicated since, despite extensive washing, some residual protein and fat remain attached to the granules. These react with HCl to produce coloured byproducts such as bitter Maillard compounds, the removal of which brings additional costs.

Use of phosphoric acid rather than hydrochloric acid presents several possible advantages. Firstly, phosphoric acid is non-volatile and therefore safer to handle. Secondly, preliminary studies have indicated that byproduct formation is reduced (*1*). Thirdly, when concentrated sugar syrups (40 % of solids) are diluted on addition to foods or beverages, the phosphoric acid concentration falls to values as low as 0.01 to 0.05 %, giving a pleasant taste of acidity at pH values around 3.0. Finally, if the hydrolysate is to be used in a subsequent fermentation step, there is no need to eliminate the phosphoric acid. In fact, neutralization with ammonia leads to ammonium phosphate, a convenient supplement for growth. Taken together, these various considerations have the potential to compensate for the fact that phosphoric acid is about twice as expensive as hydrochloric acid.

Development of a starch hydrolysis process based on phosphoric acid and its application in the production of syrups from cassava starch would be of particular importance for developing countries in tropical and subtropical climates, for two reasons: firstly, cassava crops have high yields in these climates and, secondly, starch processing is typically an important economic activity in these countries. For example, in the Brazilian state of Paraná, which contributed around 2 % of the estimated world production of $203 \cdot 10^6$ tonnes of cassava in 2005 (FAOSTAT DataBase of the United Nations Food and Agricultural Organization), the company Indemil produces more than 50 000 tonnes of glucose-enriched syrups annually from cassava starch, in a process that uses hydrochloric acid.

Cassava starch has a high amylopectin content of 83 % and, consequently, debranching enzymes must be added in order to achieve reasonable hydrolysis rates in the enzymatic process. Furthermore, cassava amylopectin has a natural content of phosphate of around 0.5 %, occurring mainly at the C6 primary alcoholic group of the anhydroglucosyl units, and this can interfere with enzymatic hydrolysis. Such problems and the disadvantages of hydrolysis with hydrochloric acid can both be avoided by using phosphoric acid to hydrolyze cassava starch.

Given that the conditions of pH and temperature for cassava starch hydrolysis by phosphoric acid have not been well investigated (*1*), the aim of the current work is to optimize phosphorolysis of cassava starch to produce a syrup rich in glucose, maltose and short maltosaccharides, while minimizing the production of undesirable byproducts. To demonstrate its potential as the basis of a culture medium, the hydrolysate was used for the production of the oxycarotenoid astaxanthin by the basidiomycetous red yeast *Xanthophyllomyces dendrorhous*. This pigment, which has a market of over US$500 million per year, is of interest to the aquaculture and poultry industries since, when incorporated into the diet, it leads to a darker red colouring of the flesh of the animal (*3–6*). It has previously been produced in media based on raw sugar cane juice and urea (*7*). However, sugar cane is not available in all regions, and therefore it is interesting to explore its production on culture media derived from starch.

Materials and Methods

Cassava starch processing

Cassava roots were peeled and finely crushed with excess water. The coarse suspension was filtered through double cheesecloth. The filtrate was left to sediment, forming a thick white starch paste. The supernatant (cassava wastewater, CWW or 'manipueira') was then decanted. Samples of both starch paste and CWW were oven-dried to calculate the dry solids. The final dry solids content of the remaining paste was then adjusted to 30 % (g per 100 mL of solution) and aqueous phosphoric acid was added to give final pH values from 1.15 to 3.75. This suspension was heated and pressurized in a steel reactor at gauge pressures from 1 bar (120 °C) up to 7 bar (175 °C), for the stated holding time.

The saccharide concentrations from duplicate samples were measured by HPLC using an HPX 87H Bio-Rad column eluted with 8 mM sulphuric acid at a flow rate of 0.5 mL/min and at 65 °C, which also allows the detection of 5-hydroxymethylfurufural (HMF) and formic acid using diode array and refractive index detectors. The hydrolysis profile was also checked by thin layer chromatography (TLC) using silica-gel 60 chromatoplates from Merck, with acetone/acetonitrile/ethyl acetate/propanol/water (5:10:5:20:10) as the mobile phase. A second run was made with the same solvent system but using different proportions (5:30:5:30:10). Sugars were visualized with hot 1 % anisaldehyde in methanol/sulphuric acid (95:5). The fact that glucose was the only residual free sugar in the culture medium after 72 h was confirmed by treating the plate with glucose oxidase, peroxidase, phenol and 4-aminophenazone from a commercial glucose kit. HMF was quantified through its absorbance at 283 nm (*8*). The DE value was obtained by the sum of each individual sugar divided by its DP (degree of polymerization) from glucose (G1) to maltohexaose (G6). The standard errors for the replicate determinations were less than ±2 % for reducing sugars, less than ±3 % for yeast biomass, and less than ±5 % for astaxanthin.

Yeast and fermentation conditions for biomass and astaxanthin production

Xanthophyllomyces dendrorhous (formerly *Phaffia rhodozyma;* strain 24202) was obtained from the American Type Culture Collection (ATCC) and maintained on yeast

malt agar. The hot phosphoric acid hydrolysate was filtered through four layers of cheese cloth, diluted to 4.0 or 6.5 % (by mass per volume) dry solids, neutralized with aqueous ammonia to pH=5.5 and supplemented with nitrogen sources: yeast extract, lyophilized cassava wastewater or ammonium nitrate. Each 125-mL Erlenmeyer flask received 25 mL of medium, was autoclaved at 121 °C for 15 min and, after cooling, was inoculated with a fresh culture of *X. dendrorhous* in order to obtain an initial absorbance of 0.25 measured at 650 nm. The flasks were incubated on a rotary shaker at 150 rpm for 6 days at 25 °C. Note that this temperature was chosen on the basis of preliminary experiments that showed slightly higher biomass yields in relation to cultivation at 22 °C, without compromising the astaxanthin content.

Cells were harvested by centrifugation, washed with cold water and lyophilized to obtain the dry mass. The intracellular oxycarotenoids were extracted from dried cells (100 mg) with 24 mL of a mixture of dimethyl sulphoxide/acetone/ethyl ether (2:12:10 by volume). The crude extract was then partitioned against 0.9 % sodium chloride (10 mL). The carotenoids in the upper organosolvent layer were measured by HPLC with a 5 μm, 25 cm×4.6 mm Supelcosil LC-18 (Supelco) column operated isocratically with a mixture of chloroform/acetonitrile/water (6:3.5:0.5).

Results and Discussion

Comparison of phosphoric and hydrochloric acids

We compared cassava starch hydrolysis using phosphoric and hydrochloric acids at temperatures from 140 to 160 °C and pH values below 2.0. The holding time was kept at 5 min in order to minimize the amount of degradation products.

At 140 and 152 °C, the efficiency of hydrolysis with hydrochloric acid was greater than that obtained under corresponding conditions with phosphoric acid; however, for both acids, the DE obtained was lower than the desired value of 85, as demonstrated by the results for 152 °C in Table 1. At 160 °C, the desired DE was obtained with both acids: it was 85 at both pH=1.6 and pH=1.8 with hydrochloric acid and 83 at pH=1.4 with phosphoric acid (Fig. 1). Higher temperatures and lower pH values led to higher concentrations of HMF and formic acid. Their concentrations were almost always higher for hydrolysis with hydrochloric acid than for hydrolysis with phosphoric acid, as shown by the results for 152 °C in Table 1, confirming the advantage of using phosphoric acid. In the case of both hydrochloric and phosphoric acids, significant concentrations of glucose, maltose and maltotriose were produced (Table 1).

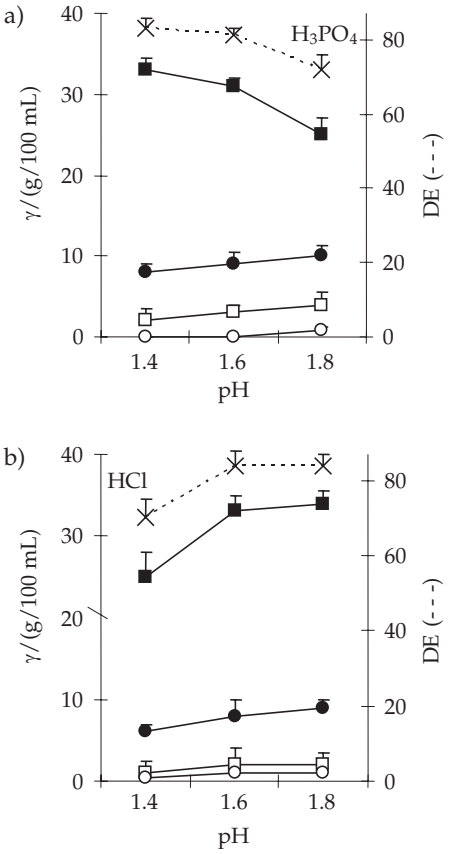

Fig. 1. Effect of pH on the hydrolysis of cassava starch by phosphoric acid (a) and hydrochloric acid (b) at the temperature of 160 °C (5 bar) during 10 min. Final concentrations of hydrolysis products and the final DE are shown. Key: (– x –) dextrose equivalents (DE), (■) glucose, (●) maltose, (□) maltotriose, (○) maltotetraose and higher

Phosphoric acid hydrolysis at higher temperatures

Given the higher DE obtained at 160 °C in the previous experiment, we investigated the possibility of using a longer holding time of 20 min at this temperature, and also at a higher temperature of 176 °C. Milder pH values of 2.0 to 3.2 were used in an attempt to minimize the production of degradation products.

Hydrolysis was best at pH values of 2.0 and 2.25 at 160 °C (Fig. 2). At these pH values the DE values obtained at 176 °C were relatively low, probably due to significant degradation of glucose caused by the combina-

Table 1. Concentrations of hydrolysis products and HMF produced during acid hydrolysis of cassava starch with hydrochloric and phosphoric acid, at pH=1.5 and 152 °C (4 bar) with a holding time of 5 min

Acid	γ(hydrolysis products)/(g/100 mL)					DE	γ(HMF)/(mg/mL)
	G1	G2	G3	G4	G5+G6		
Hydrochloric	10.43	10.02	9.54	5.08	4.02	52	0.185
Phosphoric	7.62	8.56	9.72	5.57	8.40	46	0.075

G1=glucose, G2=maltose, G3=maltotriose, G4=maltotetraose, G5=maltopentaose, and G6=maltohexaose

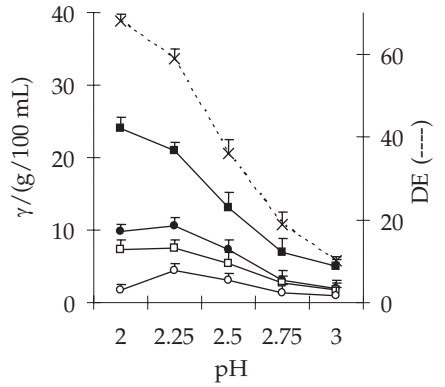

Fig. 2. Effect of milder pH on the hydrolysis of cassava starch with phosphoric acid at 160 °C (5 bar) during 20 min. Key: (– x –) dextrose equivalents (DE), (■) glucose, (●) maltose, (□) maltotriose, (○) maltotetraose and higher

tion of high temperature and low pH, as indicated by the high HMF levels in these hydrolysates (Table 2). Unfortunately, the concentrations of HMF in the hydrolysates produced at the lower pH values at 160 °C were also relatively high (Table 2). The generation of HMF during hydrolysis and its negative impact on microbial growth in a subsequent fermentation of the syrup have already been reported (*9–12*), although the potential still remains to use these syrups generated under milder conditions, as we show in the next section.

Table 2. Hydroxymethyl furfural (HMF) production during phosphoric acid hydrolysis of cassava starch at 160 and 176 °C with a holding time of 20 min

pH	γ(HMF)/(mg/mL)	
	160 °C (5 bar)	176 °C (8 bar)
2.0	1.18	4.34
2.25	0.70	4.02
2.5	0.44	3.10
2.75	0.42	2.84
3.0	0.28	2.04
3.2	–	1.84

Bioconversion of phosphoric acid hydrolysates to astaxanthin

Our previous work (*1*) indicated the feasibility of bioconversion of phosphoric acid hydrolysates to oxycarotenoids but we had used yeast extract as the nitrogen source. We investigated the possibility of using cassava wastewater (CWW), a highly polluting material originating from the processing of cassava roots, to extract starch granules. Yeast extract and ammonium nitrate were also used for the purposes of comparison. Preliminary experiments had indicated the ability of *X. dendrorhous* to use maltosaccharides (results not shown), so we used the hydrolysate produced in the previous section at pH=2.75, with holding at 160 °C for 20 min, since, although this hydrolysate had a relatively high maltosaccharide content, its HMF content was low.

In the control cultures undertaken with yeast extract, higher yeast extract levels led to greater biomass concentrations, although the mass fraction of astaxanthin in the biomass decreased, reaching values as low as 220 μg/g for a yeast extract concentration of 2.0 g/L (Table 3). On the other hand, the biomass concentration also increased with increasing concentrations of CWW, but without any significant decrease in astaxanthin levels. In fact, the astaxanthin level was as high as 380 μg/g for a CWW concentration of 1.5 g/L. Mass fraction of astaxanthin in the biomass was slightly higher (from 390 to 500 μg/g) when various combinations of the supplements (yeast extract, CWW and NH_4NO_3) were used. However, biomass con-

Table 3. Effects of treatments involving various nitrogen sources on growth and astaxanthin production. The hydrolysate for this experiment was produced at pH=2.75 and 160 °C (5 bar), with a 20-minute holding time

Treatment	γ(biomass)/(g/L)	w(astaxanthin, biomass)/(μg/g)	γ(astaxanthin)/(mg/L)
Hydrolysate*	1.35	232	0.31
+1.0 g/L yeast extract	7.33	332	2.43
+1.5 g/L yeast extract	9.10	229	2.09
+2.0 g/L yeast extract	10.45	220	2.30
+1.0 g/L CWW	4.59	307	1.41
+1.5 g/L CWW	6.07	380	2.30
+2.0 g/L CWW	7.55	360	2.72
+1.0 g/L NH_4NO_3	1.47	309	0.46
+1.5 g/L NH_4NO_3	1.14	155	0.18
+2.0 g/L NH_4NO_3	1.02	120	0.12
+0.75 g/L each yeast extract and CWW	8.05	390	3.14
+0.75 g/L each yeast extract and NH_4NO_3	4.89	496	2.42
+0.75 g/L each CWW and NH_4NO_3	4.40	487	2.15
+0.05 g/L each yeast extract, CWW and NH_4NO_3	7.47	447	3.34

*A volume of 4 mL of hydrolysate produced at pH=2.75 and 160 °C, with a holding time of 20 min, was added to 25 mL of water, giving a solution containing 6.5 g of maltooligosaccharides per 100 mL

centrations were not improved and in some cases fell significantly. In conclusion, in terms of overall astaxanthin yield, the culture with 2 g/L of CWW performed competitively, without the need for yeast extract.

Yeast biomass growth and astaxanthin production were followed in more detail. During the period of rapid growth, the mass fraction of astaxanthin in the biomass remained around 100 to 120 μg/g (Fig. 3a). As growth slowed at 72 h, the astaxanthin mass fraction rose rapidly to over 350 μg/g and continued to rise slowly during the stationary phase, reaching almost 500 μg/g by 144 h. This behaviour of the astaxanthin mass fraction in biomass, combined with the growth profile, led to very low concentration of astaxanthin during the rapid growth phase, with a sudden spurt of production between 48 and 72 h, rising from less than 0.5 to around 2.5 mg/L. The concentration of astaxanthin then rose slowly to reach values of over 4 mg/L at 144 h.

During the culture, *X. dendrorhous* consumed all of the maltosugars from G2 to G6, which accounted for about 64 % of the initial reducing sugars (Fig. 3b). In fact, by 72 h, glucose was the only sugar still left in the medium. Its concentration at 144 h was further reduced to only 7 % of the total sugars fed at the beginning of the experiment. The ability of *X. dendrorhous* to use low DP maltosaccharides makes it an excellent candidate for cultivation on phosphorolysed starches. This ability is not common amongst yeasts and has not been noted previously for *X. dendrorhous* (*13*).

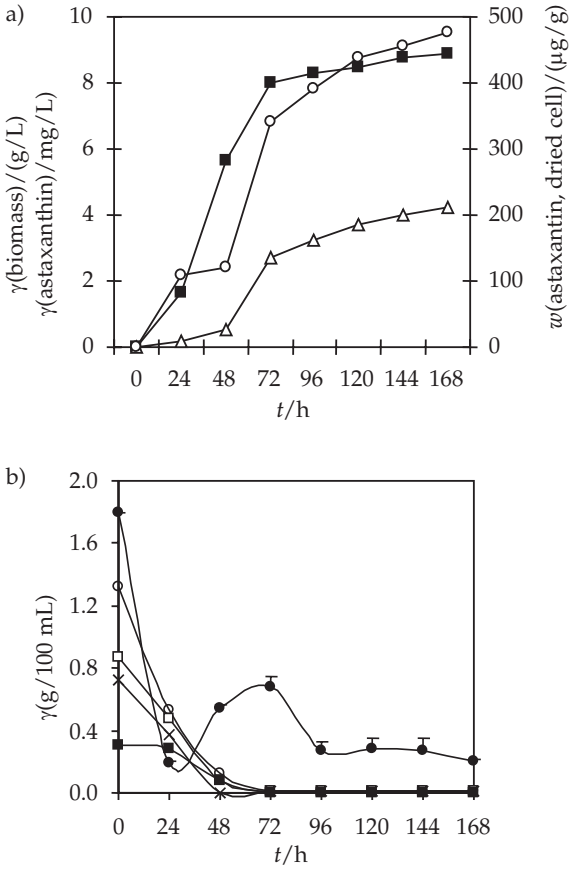

Fig. 3. Kinetics of growth and astaxanthin production from a 4 % (by mass) starch hydrolysate obtained at 160 °C (5 bar) at pH=3 for 20 min supplemented with 2.5 g/L of dried CWW and 0.5 g/L of ammonium nitrate. Key: a) (■) biomass concentration, (○) astaxanthin concentration, (△) mass fraction of astaxanthin in the dry biomass; b) free maltosugars concentration: (●) glucose, (○) maltose, (□) maltotriose, (x) maltotetraose and (■) maltopentaose

Our best production of 3.3 mg/L of astaxanthin (Table 3) is similar to the results of a cultivation done under similar conditions: Tinoi *et al*. (*14*) obtained a yield of 2.34 mg/L with a mass fraction of 497 μg per g of biomass for a cultivation done in shake flasks with a medium containing glucose, malt extract and yeast extract. Since astaxanthin yields can increase significantly, to values around 20 mg/L, when *X. dendrorhous* is cultivated with high aeration (*15*), we can expect to improve astaxanthin yields on cassava starch hydrolysates supplemented with cassava wastewater in future experiments conducted in bioreactors.

Conclusions

Diluted phosphoric acid can be used to produce high DE syrups from cassava starch solutions containing 30 % of total solids. It is probable that this process can also be applied to other starch sources. The syrups may find applications in the confectionery, food, and pharmaceutical industries and this innovation is subject of a patent request (*16*). In some cases the residual phosphate can be left in the final hydrolysate. For example, we have shown the potential for using phosphoric acid hydrolysates as fermentation feedstock by using them to produce oxycarotenoids from the red yeast *Xanthophyllomyces dendrorhous*. For this particular application the use of an inexpensive nitrogen source, cassava wastewater, was useful.

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