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Carbohydrate Polymers 38 (1999) 203–209

Carbohydrate Polymers

Pullulan production by *Aureobasidium pullulans* growing on hydrolysed potato starch waste

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Received 3 March 1998; revised 30 March 1998; accepted 31 March 1998

Abstract

Enzymic hydrolysis of potato starch waste yielded a substrate suitable for growth and exopolysaccharide production by *Aureobasidium pullulans*. Hydrolysates produced by α-amylase alone gave the lowest yields of exopolysaccharide with a low proportion of pullulan. Continued hydrolysis with pullulanase and amyloglucosidase gave higher yields with a better proportion of pullulan, but prolonged hydrolysis did not improve the yield further. The oligosaccharide composition of the hydrolysates was determined by capillary electrophoresis following reductive amination with ethyl *p*-aminobenzoate. Maltose-rich hydrolysates, generated with β-amylase and pullulanase, yielded over twice as much pullulan as the corresponding glucose-rich syrups generated with amyloglucosidase and pullulanase. It is concluded that the composition of the starch hydrolysate is important in determining pullulan yield.

Keywords: Pullulan; Exopolysaccharide; *Aureobasidium pullulans*; Poly(propylene imine)

1. Introduction

Pullulan is an exocellular homopolysaccharide of economic importance produced by the yeast-like fungus *Aureobasidium pullulans*. It is composed of maltotriosyl units linked through $\alpha 1 \rightarrow 6$ glycosidic bonds (Saha and Zeikus, 1989). Its industrial applications have been thoroughly reviewed by Deshpande et al. (1992), while its structure and biosynthesis have been studied extensively by Catley (1971). The use of agro-industrial wastes as substrates for pullulan production has been reported by many researchers (Israilides et al., 1994a; Le Duy et al., 1983; Le Duy and Boa, 1982; Shin et al., 1989; Zajic et al., 1979).

Starch waste has been shown to be a suitable feedstock for industrial fermentations and is comparable to traditional substrates such as molasses and syrups in many cases (Underkofler and Lockwood, 1976). Some biopolymers produced by the industrial fermentation of starch are single cell protein by *Candida utilis* (Cousin, 1980), xanthan gum (Silman and Rogovin, 1972), stereoglucan (Davis et al., 1965) and pullulan (Yuen, 1974). However, many industrially important organisms, including *A. pullulans*, lack the hydrolytic enzymes necessary to liquify the starch and, consequently, the starch must be pre-hydrolysed. Enzymic hydrolysis is carried out in two stages. The first stage involves the use of thermostable α-amylases to liquify the starch at temperatures usually exceeding 100°C. Depending on the desired product, the second stage involves pullulanase and amyloglucosidase (glucoamylase) to produce high glucose syrups (HGS) or a combination of pullulanase and β-amylase to yield high maltose syrups (HMS).

Pullulan is usually recovered from the fermentation broth by ethanol or methanol precipitation after the removal of cells. It has been shown, however, that the purity of pullulan in the crude agglutinating substances may vary according to the substrate used for the fermentation (Israilides et al., 1994b). Furthermore, pullulan produced in such fermentations is often characterised by heterogeneity of composition and molecular weight (Israilides et al., 1994a). While it is not practical to define the chemical composition of the substrate when using waste materials, there is a need to evaluate for each potential substrate the major carbon sources, as these can influence the amount and purity of pullulan produced.

This paper reports the pullulan yields from potato starch waste (PSW), a by-product of potato snack production, hydrolysed to varying extent by α-amylase, pullulanase, amyloglucosidase and β-amylase. A capillary electrophoresis method has been developed to evaluate the composition of the starch hydrolysate. In the study, estimations of crude

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PII: S0144-8617(98)00092-7

pullulan were made by weighing the ethanol precipitate from the cell-free fermentation broth. More accurate estimations of pullulan were made by the coupled enzyme assay (Israilides et al., 1994b).

2. Materials and methods

2.1. Micro-organism

A. pullulans NRRLY-6220 was maintained at 4°C on potato dextrose agar (Oxoid) slopes.

2.2. Media

Potato starch waste (PSW) was donated by PepsiCo. All other chemicals and reagents were GPR grade obtained from BDH Chemicals, unless otherwise stated. The pH of a slurry of PSW at the required concentration was adjusted to 6.5 and heated to 65°C for 30 min in the presence of 3.5 μg ml^{-1} α-amylase (Sigma, *Bacillus licheniformis*, A 3403), and then at 90°C for 2 h. After liquefaction, the temperature was reduced to 55°C and the pH adjusted to 4.5. Pullulanase (Novo Nordisk Bioindustries, *Bacillus pullulyticus*, No. 7307727, 3.5 μg ml^{-1}), and amyloglucosidase (Sigma, *Rhizopus*, A 7255, 290 μg ml^{-1}) were added and left until the desired level of hydrolysis was reached. Alternatively, after liquefaction with α-amylase, pullulanase and β-amylase (Sigma, A7130; 0.05 mg ml^{-1}, approx. 2 μg ml^{-1}) were added. The inoculum medium was that described by Israilides et al. (1994a), and the fermentation media were prepared by adding to the hydrolysate 0.1% (w/v) K_2HPO_4 and 0.1% (w/v) NH_4NO_3 and adjusting the pH to 6.0.

2.3. Fermentation

Inocula were prepared by scraping the culture from a slant into 10 ml medium and inoculating 50 ml medium in a 200 ml Erlenmeyer flask. After three days incubation on a gyrotary shaker (29°C, 200 rpm), 10 ml of this inoculum was used to inoculate 100 ml medium in a 500 ml Erlenmeyer flask. After seven days incubation (29°C, 200 rpm), the cells were removed by centrifugation (3000 *g*; 15 min).

Polysaccharide material in the supernatant was removed by the slow addition of two volumes of 99% ethanol at 4°C whilst stirring. The mixture was stored at 4°C overnight to maximize precipitation. The resultant precipitate was removed by centrifugation (3000 *g*; 15 min). The cell mass and ethanol precipitate were dried to constant weight under vacuum at 50°C. The pullulan content of the ethanol precipitate was determined by the coupled-enzyme assay technique described by Israilides et al. (1994b) with the following modification. A control was run exactly as described in the above method but without the addition of pullulanase. The rate calculated for this control was subtracted from the rate determined in the presence of pullulanase.

Total sugars were estimated by the method of Dubois et al. (1956) and reducing sugars by the method of Somogyi (Nelson, 1944).

2.4. Capillary electrophoresis of sugars

PSW hydrolysates were analysed by capillary electrophoresis using a modification of the method described by Oefner et al. (1992). Samples (2 mg dry wt.) were derivatised by adding 1 ml of a methanolic solution (HPLC grade) of ethyl *p*-aminobenzoate (10% w/v), sodium cyanoborohydride (1% w/v) and acetic acid (10% w/v) and heating for 2 h at 50°C. Samples were cooled to room temperature and diluted 1:10 or 1:100 with methanol before analysis. Analyses were performed on a Beckman P/ACE system 2100 operating at 25°C and with an applied voltage as indicated. Samples were run in a fused silica capillary with an internal diameter of 75 μm, total length (L) and a distance (l) to the detector as indicated. Samples were introduced on to the column by applying pressure (3.5 kPa) for the times shown. Between samples the capillary was flushed with 1.0 M NaOH for 3 min, then 0.001 M NaOH for 2 min and finally equilibrated for 2 min with running buffer (200 mM borate, pH 10.0). Photometric detection of the carbohydrates was carried out on the column at 280 nm.

3. Results and discussion

Potato starch waste (PSW) at concentrations of 10%, 15% and 20% (w/v) was hydrolysed with α-amylase followed by further hydrolysis with pullulanase and amyloglucosidase to yield the dextrose equivalent (DE) values shown in Table 1.

Table 1
Dry cell matter (DCM), exocellular polysaccharide (EPS) and pullulan in seven-day fermentation brews[a]

% w/v PSW	DE[b]	DCM (g l^{-1})	EPS (g l^{-1})	Pullulan (g l^{-1})
10	42	15 (0.8)	23 (0.4)	16 (0.3)
	56	16 (0.4)	29 (2.1)	25 (1.8)
	64	17 (0.8)	29 (0.8)	27 (0.7)
15	29	19*	21*	13*
	52	21 (2.3)	33 (1.0)	21 (0.6)
	64	23 (2.2)	22 (1.9)	ND[c]
20	26	26 (0.3)	46 (0.5)	26 (0.3)
	89	19 (0.2)	34 (0.8)	27 (0.6)
	95	19 (0.8)	36 (1.8)	27 (1.4)

[a]Data are means of two shake flask cultures in each case, except *, which is only one result. Figures in brackets are standard deviation.
[b]DE is the dextrose equivalent of the partially hydrolysed potato starch waste (PSW) used as the substrate.
[c]ND = not determined.

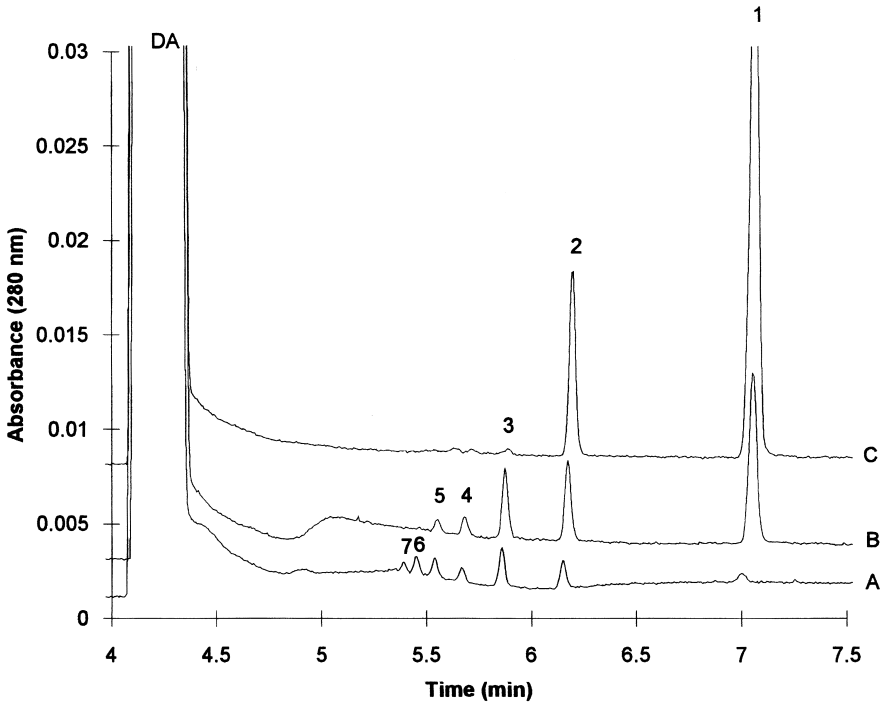

Fig. 1. Capillary zone electropherograms of various stages of PSW hydrolysis. Conditions: electrolyte, 200 mM borate, pH 10.0; capillary, fused silica, $L =$ 67 cm, $l = 60$ cm, $\varnothing = 75$ μm; voltage, 20 kV; temperature, 25°C; injection, 3 s pressure. Peak identification: DA, derivatising agent; 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraose; 5, maltopentaose; 6, maltohexaose; 7, maltoseptaose. Electropherogram: (a) α-amylase hydrolysis; (b) α-amylase, 22 h pullulanase and AMG hydrolysis; (c) α-amylase, 70 h pullulanase and AMG hydrolysis.

The hydrolysates (HPSW) were supplemented with 0.1% (w/v) K_2HPO_4 and 0.1% (w/v) NH_4NO_3 and fermented with *A. pullulans* for seven days. The resulting biomass (DCM) extracellular polysaccharide (EPS) and pullulan were determined (Table 1). There was the possibility, even after seven days fermentation, that there were significant levels of partially hydrolysed starch remaining in the cell free supernatant which would be precipitated on the addition of ethanol. The coupled enzyme assay was modified to correct for this. Pullulan being a linear polymer

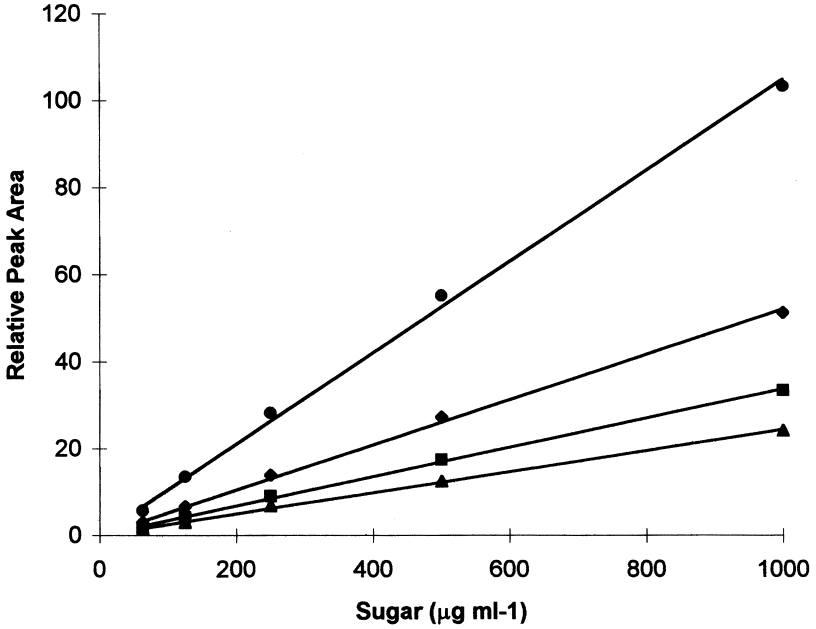

Fig. 2. A plot of relative peak area against sugar concentration (μm ml^{-1}) for glucose ●, maltose ♦, maltotriose ■ and maltotetraose ▲. Data taken from the capillary electropherograms of the ethyl *p*-aminobenzoate derivatisation of the sugars using galactose as the internal standard. The correlation coefficients (R^2) were not less than 99.8% in any of the cases.

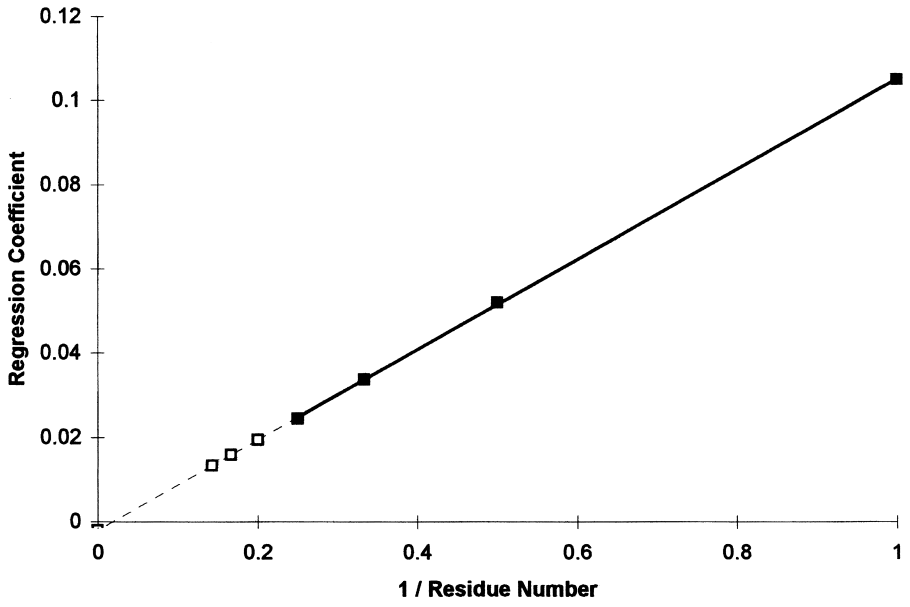

Fig. 3. A plot of the regression coefficients for the slopes of relative peak area against sugar concentration (Fig. 2) against 1/residue number. Filled squares (■) are used for glucose, maltose, maltotriose and maltotetraose. The line is extrapolated and the open squares (□) are for the data for the peaks which are presumed to be maltopentaose, maltohexaose and maltoseptaose.

is only hydrolysed at a very slow rate by amyloglucosidase in the absence of pullulanase. Starch, because of its highly branched structure, and oligosaccharides are good substrates for amyloglucosidase being rich in non-reducing termini, thus giving high rates of hydrolysis. Therefore, by using a pullulanase-free control, it is possible to correct the coupled enzyme assay rate for the level of oligosaccharides and branched chain polyglucans.

A. pullulans NRRLY-6220 does not grow to any significant extent on unhydrolysed starch (data not shown). Under the conditions used in these fermentations, the DCM at day seven of the fermentation showed only a small increase on

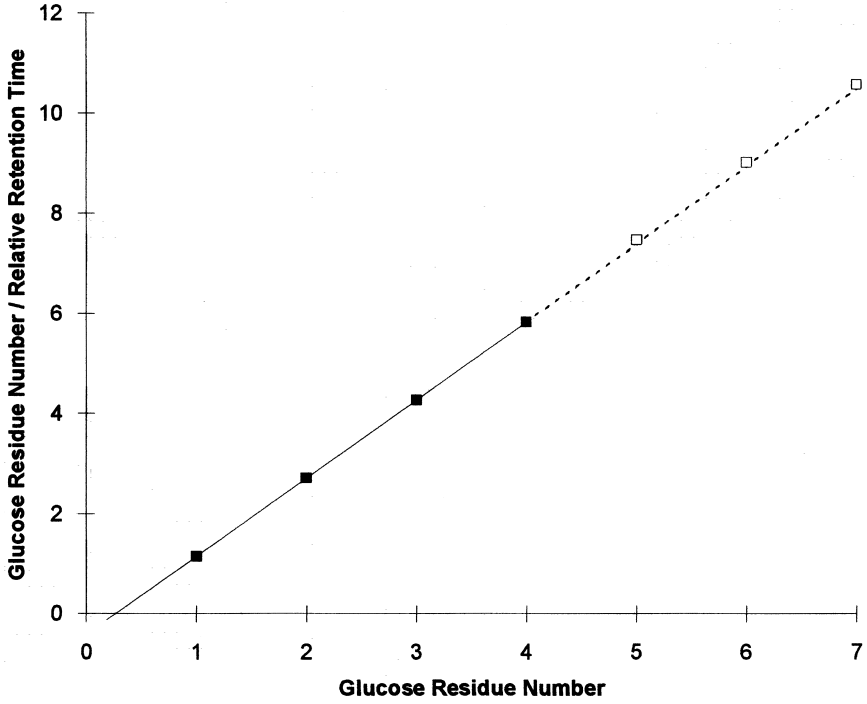

Fig. 4. A plot of number of glucose residues in the oligosaccharide/relative retention time against number of glucose residues in the oligosaccharide. Filled squares (■) are used for glucose, maltose, maltotriose and maltotetraose and the data are extracted from the electropherograms. The open squares (□) are for the data for the peaks which are presumed to be maltopentaose, maltohexaose and maltoseptaose as shown in Tables 3 and 4.

Table 2
Percentage (w/w) of saccharides containing up to seven glucose residues present in hydrolysed potato starch waste[a]

Treatment	Total (%)	Percentage composition (w/w) of hydrolysates						
		Number of glucose residues in saccharide						
		1	2	3	4	5	6	7
A[b]	89.47	1.04	5.39	12.62	6.26	16.49	21.96	25.71
B[c]	84.04	23.07	16.48	24.56	9.89	10.04	0.00	0.00
C[d]	100.1	61.93	36.06	0.81	1.31	0.00	0.00	0.00

[a]Figures are from a single run. On the basis of five successive injections of a glucose standard, the mean concentration derived from the calibration curve was 99% of the true value with a standard deviation of ± 2.8%. Other sugars gave similar values.
[b]Hydrolysis with α-amylase for 2 h.
[c]Hydrolysis with α-amylase for 2 h followed by hydrolysis with pullulanase and amyloglucosidase for 22 h.
[d]Hydrolysis with α-amylase for 2 h followed by hydrolysis with pullulanase and amyloglucosidase for 70 h.

going from a 10% to a 15% (w/v) starch hydrolysate and no significant increase on a further increase to a 20% (w/v) starch hydrolysate. Under these conditions, nitrogen is limiting (Catley, 1971). EPS yields were comparable when either 10%, 15% or 20% (w/v) HPSW was used, but the degree of hydrolysis of PSW (measured by the DE) affected the EPS produced in the fermentation. When the 10% PSW was hydrolysed to a DE of 42, the fermentation yield of EPS was correspondingly low. This is not unexpected as the hydrolysed PSW would contain a large proportion of saccharides of several glucan units. The pullulan content of the EPS was 70% (w/w). Similar results were obtained with the 15% HPSW. The 20% HPSW with a DE value of 26 gave high DCM and EPS values. The EPS figure includes starch oligosaccharides which were precipitated with the pullulan, as indicated in Table 1. Further hydrolysis with pullulanase and amyloglucosidase to give a higher DE (56) gave higher fermentation yields of EPS with a higher proportion of pullulan (87% w/w). Continued hydrolysis with pullulanase and amyloglucosidase to give DE values of 89 and 95, however, did not realise increases in EPS when fermented and the proportion of pullulan in the EPS remained similar (90% w/w). It would appear that hydrolysis to give a high proportion of glucose results in very little, if any, increase in EPS yield, suggesting that glucose is not a good substrate for this fermentation. This is in keeping with the data reviewed by Tsujisaka and Mitsuhashi (1993).

In order to examine the oligosaccharide composition of the HPSW, a capillary electrophoresis method was developed. Underivatised sugars can be separated as their borate complexes by capillary electrophoresis, but detection is by indirect fluorescence which requires laser excitation (Garner and Yeung, 1990), a facility not available in all laboratories. Precolumn derivatisation by reductive amination with cyanoborohydride and ethyl *p*-aminobenzoate, as described by Oefner et al. (1992) allows detection of sugars by UV monitoring at 280 nm. This method was developed to monitor the hydrolysis of starch. In order to separate the oligosaccharides, a high concentration of borate is required (200 mM), otherwise, the peaks are lost in the reagent peak and the optimum pH is 10.0. Peaks in the electropherogram were identified by comparisons with standards for glucose, maltose, maltotriose and maltotetraose (Fig. 1) and calibration curves of relative peak area against concentration constructed using galactose as an internal standard

Table 3
Percentage of saccharides containing up to seven glucose residues present in hydrolysed potato starch waste[a]

Treatment	DE	Total (%)	Percentage composition (w/w) of hydrolysate						
			Number of glucose residues in saccharide						
			1	2	3	4	5	6	7
A[b]	26	67	10.9	14.0	12.4	7.6	13.0	9.2	0.0
B[c]	89	101	98.4	0.4	0.3	2.4	0.0	0.0	0.0
C[d]	95	105	102	0.3	0.8	1.3	0.0	0.0	0.0
D[e]	35	95	10.4	43.2	31.5	9.6	0.0	0.0	0.0
E[f]	42	95	14.0	47.1	33.6	0.0	0.0	0.0	0.0

[a]Figures are from a single run. On the basis of five successive injections of a glucose standard, the mean concentration derived from the calibration curve was 99% of the true value with a standard deviation of ± 2.8%. Other sugars gave similar values.
[b]Hydrolysis with α-amylase for 2 h.
[c]Hydrolysis with α-amylase for 2 h followed by pullulanase and amyloglucosidase for 20 h.
[d]Hydrolysis with α-amylase for 2 h followed by pullulanase and amyloglucosidase for 70 h.
[e]Hydrolysis with α-amylase for 2 h followed by pullulanase and β-amylase for 20 h.
[f]Hydrolysis with α-amylase for 2 h followed by pullulanase and β-amylase for 70 h.

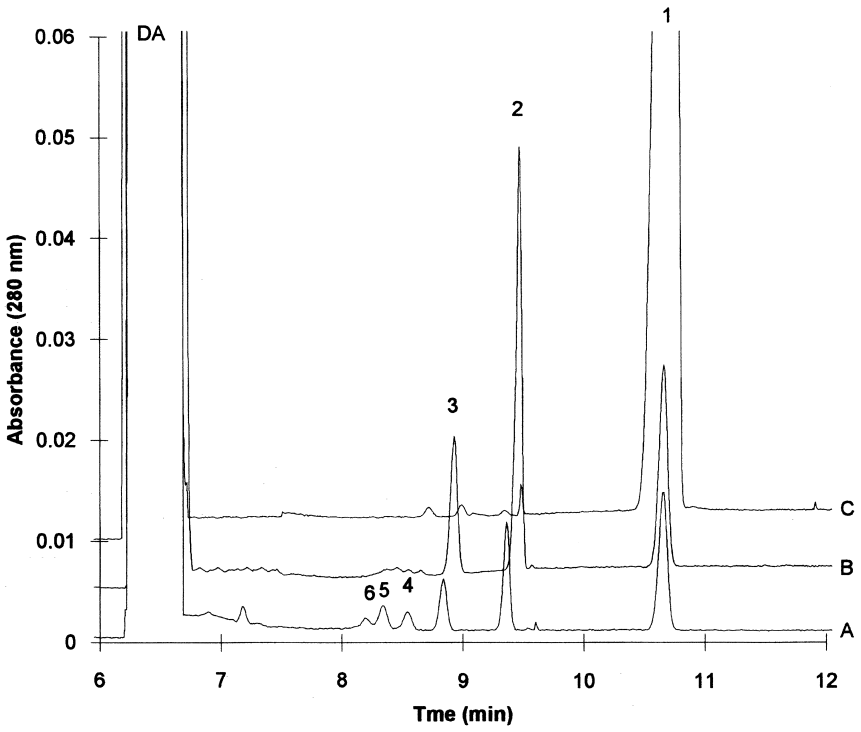

Fig. 5. Capillary zone electropherograms of the hydrolysis of 20% PSW by various enzymes. Conditions: electrolyte, 200 mM borate, pH 10.0; capillary, fused silica, $L = 47$ cm, $l = 40$ cm, $\varnothing = 75$ μm; voltage, 12 kV; temperature, 25°C; injection, 2 s pressure. Peak identification: DA, derivatising agent; 1, glucose; 2, maltose; 3, maltotriose; 4, maltotetraose; 5, maltopentaose; 6, maltohexaose. Electropherogram: (a) α-amylase hydrolysis; (b) α-amylase, 70 h pullulanase and β-amylase hydrolysis; (c) α-amylase, 40 h pullulanase and AMG hydrolysis.

(Fig. 2). Reproducibility was determined to be (99.0 ± 2.8)% from five successive injections. Since only the reducing end of the sugar is derivatised, the response is related to the molar concentration, thus, on a weight per ml basis, the sensitivity decreases as the number of glucose residues in the oligosaccharide increases. As expected, the sensitivity halves as the number of glucose residues in the oligosaccharide doubles (Fig. 3) and this limits the method to the analysis of oligomers with no more than about 10 sugar residues. This relationship is used to estimate the concentration of polyglucans with 5, 6 and 7 glucose residues. The relative retention time is inversely related to the number of residues in the oligosaccharide and there is a straight line relationship between residue number/relative retention time and residue number (Fig. 4). This relationship is used to tentatively identify oligosaccharides with five, six and seven glucose residues as shown in Figs. 1, and 5.

Analysis by capillary electrophoresis of the resulting oligosaccharides following PSW hydrolysis with α-amylase, pullulanase and amyloglucosidase for varying times is shown in Fig. 1 and the data summarised in Table 2. Trace A, the hydrolysate resulting from 2 h treatment with α-amylase, shows a predominance of oligosaccharides from 2 to 7 glucose residues. Also present at 4.4 min is a shoulder (on the peak for the derivatising agent) and at 4.9 min a peak indicating the presence of high and medium molecular weight material. Peaks corresponding to maltoseptaose, maltohexaose, maltopentaose, maltotetraose and maltotriose are all present, as well as peaks for maltose and glucose. Following hydrolysis with pullulanase and amyloglucosidase for 22 h (Trace B) it can be seen that maltoseptaose and maltohexaose have disappeared, there is more maltose and maltotriose and a significant increase in the amount of glucose. The shoulder at 4.4 min has disappeared and there is an increased peak at 4.9 min. After 70 h hydrolysis (Trace C), glucose and maltose predominate with only trace amounts of other oligosaccharides.

Considering that the organism produces low yields of EPS on HPSW with a high DE, 20% w/v PSW was hydrolysed with β-amylase replacing amyloglucosidase to yield a hydrolysate containing, predominantly, maltose rather than glucose. The capillary electropherograms of the hydrolysates are shown in Fig. 5 and the data summarised in

Table 4
Dry cell matter (DCM), exocellular polysaccharide (EPS) and pullulan in seven-day fermentation brews[a]

DE[b]	DCM (g l^{-1})	EPS (g l^{-1})	Pullulan (g l^{-1})
35	27 (0.7)	55 (3.8)	40 (2.8)
42	26 (0.9)	69 (1.6)	58 (1.4)

[a]Data are means of two fermentation flasks. Figures in brackets are standard deviation.
[b]DE is the dextrose equivalent of 20% (w/v) potato starch waste (PSW) hydrolysed with pullulanase and β-amylase after 2 h initial hydrolysis with α-amylase.

Table 3. Fermentation of the hydrolysates containing 0.1% (w/v) K_2HPO_4 and 0.1% (w/v) NH_4NO_3, as before, gave yields of EPS and DCM shown in Table 4. It can be seen that after 20 h hydrolysis the DE was 35, DCM was 27 g l^{-1} and the yield of EPS was 55 g l^{-1}, of which 40 g l^{-1} was pullulan; significantly more than previously obtained when PSW was hydrolysed with amyloglucosidase. Furthermore, continued hydrolysis for 70 h resulted in a DE of 42, and the fermentation yielded similar DCM, but in this case the EPS was higher at 69 g l^{-1}, of which 58 g l^{-1} was pullulan. With amyloglucosidase, there was no increase in EPS or pullulan yield after prolonged hydrolysis. Analysis of the hydrolysate by capillary electrophoresis (Fig. 5) shows that the hydrolysate contains proportionally more maltose than when hydrolysed with amyloglucosidase.

It is known that the growth of the organism must be limited by limiting nitrogen levels in order to achieve pullulan elaboration (Catley, 1971), a factor which, in turn, determines the concentration of carbohydrate substrate in the fermentation. The fact that PSW hydrolysed with β-amylase, rather than amyloglucosidase, yielded 115% more pullulan indicates that maltose is a better substrate than glucose for pullulan production.

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