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Pullulan content of the ethanol precipitate from fermented agro-industrial wastes

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Abstract Ethanol-precipitated substances after fermentation of various agro-industrial wastes by *Aureobasidium pullulans* were examined for their pullulan content. Grape skin pulp extract, starch waste, olive oil waste effluents and molasses served as substrates for the fermentation. A glucose-based defined medium was used for comparison purposes. Samples were analysed by an enzyme-coupled assay method and by high-performance anion-exchange chromatography with pulsed amperometric detection after enzymic hydrolysis with pullulanase. Fermentation of grape skin pulp extract gave 22.3 g l^{-1} ethanol precipitate, which was relatively pure pullulan (97.4% w/w) as assessed by the coupled-enzyme assay. Hydrolysed starch gave only 12.9 g l^{-1} ethanol precipitate, which increased to 30.8 g l^{-1} when the medium was supplemented with NH_4NO_3 and K_2HPO_4; this again was relatively pure pullulan (88.6% w/w). Molasses and olive oil wastes produced heterogeneous ethanol-precipitated substances containing small amounts of pullulan, even when supplemented with nitrogen and phosphate. Overall, grape skin pulp should be considered as the best substrate for pullulan production. Starch waste requires several hydrolyis steps to provide a usable carbon source, which reduces its economic attraction as an industrial process.

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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 structure and biosynthesis have been studied extensively by Catley (1971) and its industrial applications have been thoroughly reviewed by Deshpande et al. (1992). Pullulan is used as a low-calorie ingredient in foods, as a viscosity imparter and binder and, because of its low oxygen permeability, as a packaging agent. It is used in the pharmaceutical industry as a bulking agent and binder and as an oxidation-prevention agent for tablets. Other applications include films and adhesives, microencapsulating agents for flavour and spices and as a water-solubility enhancer in fertilisers. The use of agro-industrial wastes as substrates for pullulan production has been reported by many researchers (Israilides et al. 1994a,b; Le Duy et al. 1983; Le Duy and Boa 1982; Shin et al. 1989; Zajic et al. 1979).

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 ethanol-precipitated 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 mass (Israilides et al. 1994b). While it is not practical to define the composition of the feedstock chemically when using waste materials, there is a need to optimise the levels of additional nutrients, such as phosphate and nitrogen sources, for each agro-industrial waste to maximise production of pullulan.

The estimation of pullulan in mixtures is usually by hydrolysis with pullulanase followed by estimation of the resulting maltotriose by chromatography, radiometry or other methods (Catley 1972; Finkelman and Vardanis 1982). Simon et al. (1993) used the number of

residues in repeating units (ratio of glucose produced by total hydrolysis to the reducing sugar produced by hydrolysis with pullulanase) as a means of differentiating pullulan from other polysaccharides produced in the broth.

In this study, initial estimations of crude pullulan were made by weighing the ethanol-precipitated substances (EPS) from the cell-free fermentation broth. More accurate estimations of pullulan were carried out using the coupled-enzyme assay (Israilides et al. 1994a) and by hydrolysis with pullulanase followed by determination of maltotriose and maltotetraose by high-performance anion-exchange chromatography (HPAEC).

Grape skin pulp extract (GSPE) is an aqueous extract of a residue from the grape juice and wine industries. Starch waste (SW) results from potato processing, olive oil waste (OW) from the production of olive oil and molasses (M) from sugar refining. All are produced in large quantities. This paper reports a study of these raw materials as potential feedstocks for the production of pullulan by fermentation with *A. pullulans*. This would provide an environmentally friendly and economically advantageous bioconversion.

Materials and methods

Micro-organism

Aureobasidium pullulans, NRRLY 6220, was used as the fermenting organism. It was maintained on potato/dextrose/agar (Oxoid Ltd, Basingstoke, UK) slants at 4 °C. The inoculum was grown on the medium used for the fermentation.

Substrates

Total sugars were estimated by the method of Dubois et al. (1956) and reducing sugars by the Somogyi-Nelson method (Nelson 1944). Fermentation media were prepared from agro-industrial wastes as follows. GSPE was prepared by adding one part hot water (65–70 °C) to two parts grape skin pulp. After stirring thoroughly (30 min), the solids were allowed to settle for 30 min and removed. SW, a by-product of the potato crisp industry, was supplied by PepsiCo Ltd., Leicester, UK. A suspension of 10% dry solids was autoclaved at 121 °C for 30 min, then liquefied by incubating for 2 h at 90 °C and pH 6.9 with α-amylase (Sigma, A-3403; 31 units g dry weight starch^{-1}). Pullulanase (Novo, Promozyme) and glucoamylase (Sigma, A-7255) were added at 2.8 and 1.4 units g dry weight^{-1} starch respectively and the incubation was continued at 55 °C and pH 5.5 for 18 h to hydrolyse the starch partially to a dextrose equivalent of about 50%. OW, from the Greek island of Crete was used without dilution (total sugars 1.24% w/v, which is less than the normal value of 2.6% w/v). M, obtained from British Sugar, Newark, UK, was diluted 1 in 10 (w/v) with distilled water (total sugars 5% w/v). Olive oil waste effluents plus molasses (OW/M) were used in the ratio of three parts of olive oil waste to one part of a 20% (w/v) molasses solution. Yeast extract, NH_4NO_3 and K_2HPO_4 were added to these media as indicated.

Defined medium (D) contained (% w/v) yeast extract 0.3, malt extract 0.3, peptone 0.5, dextrose 1.0, NH_4NO_3 0.1 and K_2HPO_4 0.1.

All media were adjusted to pH 5.5 with 1.0 M HCl and were sterilised at 121 °C for 15 min. Unless otherwise stated, all chemicals were supplied by BDH-Merck and were general-purpose reagent grade.

Fermentation and product recovery

Fermentation conditions and precipitation of polysaccharide by the addition of two volumes of ethanol to the cell-free extract have been reported previously (Israilides et al. 1994a). Dry cell weight was determined by separation of the biomass by centrifugation at 2000 *g* for 15 min and drying at 105 °C to constant weight.

Pullulan assays

The pullulan content of the ethanol precipitates was estimated by a coupled-enzyme assay and by determining the amount of maltotriose and maltotraose released on exhaustive hydrolysis of the ethanol precipitate with pullulanase.

For the coupled-enzyme method (Israilides et al. 1994a) calibration standards of pullulan (Sigma P4516) in the range 0.0781–10.0 g l^{-1} were hydrolysed using pullulanase and amyloglucosidase and the glucose produced was oxidised with glucose oxidase using 4-aminophenazone as the terminal electron acceptor (Trinder 1969). The reaction was monitored by measuring the rate of change of absorbance at 515 nm (rate *A*). At each pullulan concentration, the assay was repeated in an identical manner except that no pullulanase was added (rate *B*). The corrected rate ($A-B$) was used to construct a Lineweaver-Burk plot. Known amounts of the ethanol precipitates were assayed by the same method and their pullulan content determined from the Lineweaver-Burk calibration plot. The amount of ethanol precipitate used was adjusted to give rates no greater than 80% of V_{max}.

Pure pullulan, on complete hydrolysis with pullulanase, yields only maltotriose and maltotetraose and therefore the yield of these oligosaccharides from the exhaustive hydrolysis of the EPS can be used to estimate their pullulan content. Pullulan-containing ethanol precipitates were dissolved in distilled water (20 mg l^{-1}) and 0.5-ml aliquots were exhaustively hydrolysed with pullulanase (20 nkat, Sigma P-5420) in a water bath at 37 °C. After 90 min, samples were taken, passed through a 0.45-μm filter and kept frozen at −20 °C for further chromatographic separation.

These pullulan hydrolysates were analysed by HPAEC (Hardy 1990; Lee 1990) with the Dionex anion 450 chromatograph (Dionex Corp., Sunnyvale, Calif.). Samples of 100 μl (10–50 μM oligosaccharide) were injected on to the Carbo Pac PA1 (Dionex Corp) column (4 × 250 mm) and eluted with a linear gradient of sodium acetate from 50 mM to 400 mM in sodium hydroxide at a constant concentration of 100 mM over 25 min at 1 ml min^{-1}. The elution profile was monitored by pulsed amperometric detection (PAD) with applied potentials of 0.05, 0.60 and −0.60 V for 480, 120 and 60 ms respectively. The retention times of glucose, maltose, panose, maltotriose, maltotetraose and maltopentaose were determined by running standards. Quantitative analysis was based on the peak areas obtained by injection of standard maltotriose and maltotetraose solutions in the range 0–40 μM and was carried out automatically by Dionex software.

Results

The total sugars concentration (w/v) of the prepared substrates was: GSPE, 7.4%; hydrolysed SW, 9.5%; OW, 1.24% and M (diluted 1:10) 5%. The dextrose equivalent of the hydrolysed SW was 50%.

Table 1 lists the values for biomass and EPS in relation to fermentation time using GSPE, SW, OW, D, M and a mixture of OW and M (1:1) as substrates. Using GSPE as the substrate, both the biomass and EPS increased over the 7-day period. The biomass also increased with time when hydrolysed SW was used, but in this case the concentration of EPS decreased over the 7-day period. Both OW and M gave roughly the same

Table 1 Biomass and ethanol-precipitated substances (*EPS*) as a function of fermentation time using grape skin pulp extract (*GSPE*), hydrolysed starch waste (*SW*), olive oil waste (*OW*), and molasses (*M*, 1;10 dilution) and defined medium (*D*) as substrates, with and without addition of supplements. The data are the mean of two fermentations and the standard deviation is 5% or less

Substrate	Fermentation time (days)	Biomass ($g\ l^{-1}$)	EPS ($g\ l^{-1}$)
GSPE	1	4.2	6.9
	2	7.0	12.1
	3	8.8	17.0
	7	23.1	22.3
GSPE + yeast extract (0.2%)	1	5.1	5.5
	2	9.0	11.2
	3	11.2	15.6
	7	24.2	21.0
SW	1	7.4	18.8
	4	9.4	15.6
	7	10.8	12.9
SW + yeast extract (0.2%)	1	6.3	24.0
	4	13.1	18.8
	7	15.2	14.9
SW + NH_4NO_3 (0.2%) and K_2HPO_4 (0.2%)	1	7.7	20.2
	4	24.0	30.8
	7	28.1	30.8
OW	7	9.2	7.9
OW + NH_4NO_3 (0.1%) and K_2HPO_4 (0.1%)	7	22.1	10.7
D	7	4.2	1.7
M	7	13.8	6.0
M + NH_4NO_3 (0.1%) and K_2HPO_4 (0.1%)	7	21.3	4.2
(OW + M) + NH_4NO_3(0.1%) and K_2HPO_4 (0.1%)	7	15.7	9.7

amount of biomass as hydrolysed SW after 7 days but, in each case, the yield of EPS was substantially lower than with GSPE and hydrolysed SW. Supplementing the GSPE with yeast extract (0.2% w/v) had very little effect over the 7-day period of fermentation, though hydrolysed SW with added yeast extract gave increased biomass (41%) and slightly increased EPS (15%). Again the concentration of EPS decreased over the 7-day period. Adding NH_4NO_3 and K_2HPO_4 (both 0.2%, w/v) to the hydrolysed starch waste improved the yield of biomass and EPS remarkably (160% and 139% more than unsupplemented hydrolysed SW respectively), though adding the salts at 0.1% (w/v) to OW, M and the mixture of OW plus M increased the biomass but had less effect on the yield of EPS.

Table 2 shows the percentage pullulan present in the EPS, on the basis of both the coupled-enzyme assay and on the maltotriose and maltotetraose produced by pullulanase hydrolysis (HPAEC-PAD). Anion-exchange chromatography gave baseline resolution of glucose, maltose, panose, maltotriose, maltotetraose and maltopentaose. Calibration graphs for maltotriose and maltotetraose were linear in the range used (0–40 μM). The correlation coefficients were 0.994 and 0.991 respectively. The major carbohydrate present in the hydrolysed EPS was maltotriose, while maltotetraose was present in small amounts. The amount of pullulan in the EPS determined by each method is comparable though, apart from the pullulan standard, HPAEC-PAD gave higher amounts in each case. With the exception of GSPE, the EPS were from the substrates supplemented with NH_4NO_3 and K_2HPO_4, as indicated in Table 1. The table also shows the results for the coupled enzyme assay for starch and glycogen. Enzymic hydrolysis rates of the EPS from the fermentations and, for comparison, some reference polysaccharides are shown. The EPS derived from fermented GSPE was the richest in pullulan (97.4% w/w), and that from hydrolysed SW also contained a high proportion of pullulan (88.6% w/w). OW and M, however, gave low yields of EPS that contained a low proportion of pullulan.

Discussion

OW and M are poor substrates for pullulan production. Supplementation with NH_4NO_3 and K_2HPO_4 significantly increases biomass, but has little effect on pullulan production. Measuring EPS alone is a poor indicator of pullulan yield, as in the case of starch fermentations it is evident that much of the precipitated material is simply oligosaccharide derived from the starch hydrolysis. Nevertheless the results in Table 1 do show that, in the case of starch, supplementation with nitrogen and phosphate leads to an increase in yield of pullulan. The fact that there is no significant difference in yield when yeast extract is added to GSPE suggests that this material provides an adequate medium and there is no advantage in supplementing.

The coupled-enzyme assay (Table 2, *A*) is specific for polyglucans with $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glycosidic bonds, but highly branched polyglucans, such as glycogen and starch, also give high rates. The assay can be made more specific by comparing these rates with those where pullulanase is omitted from the assay (Table 2, *B*). The rate is now dependent on the number of non-reducing ends available, which in turn largely depends on the degree of branching, but also, to a lesser extent, on the molecular mass of the polysaccharide. Thus linear polysaccharides such as pullulan give very low rates while highly branched polysaccharides give high rates. The results in Table 2 confirm that the coupled-enzyme assay is capable of differentiating between linear polyglucans, such as pullulan, and branched-chain polyglucans, such as glycogen and starch. High values (above 10) for the ratio of the rate of the coupled-enzyme reaction to the amyloglucosidase reaction rate (*A*/*B*) indicate a linear polyglucan with predominately $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glycosidic bonds. The ratio is still dependent on the molecular mass of the pullulan. In fact, if the pullulan was pure it should be possible to

Table 2 Pullulan content of EPS as estimated by coupled-enzyme assay and by high-pressure anion-exchange chromatography/pulsed amperometric detection (*HPAEC-PAD*) for various agro-industrial wastes supplemented with NH_4NO_3 and K_2HPO_4 after 7 days fermentation. *A* The rate observed for the coupled-enzyme assay; *B* the rate observed as for *A*, less pullulanase. Pullulan is expressed as a percentage of the ethanol-precipitated substances (*EPS*). *GSPE* grape skin pulp extract, *SW* hydrolysed starch waste, *OW* olive oil waste, *M* molasses, 1:10 dilution) and defined medium (*D*) as substrates, with and without addition of supplements. The pullulan content is based on the coupled-enzyme assay corrected for the amyloglucosidase rate (*A*−*B*)

Substrate	Concentration of EPS (% w/v)	Coupled-enzyme assay				Pullulan (% dry weight)	Pullulan by (HPAEC-PAD) (% w/w)
		A ($\Delta A min^{-1}$)	*B* ($\Delta A min^{-1}$)	*A*−*B* ($\Delta A min^{-1}$)	*A*/*B*		
GSPE	0.25	0.1084	0.001	0.1074	108	97.4	125.8
SW	0.25	0.1642	0.0112	0.1530	14.7	86.9	70.1
	0.125	0.0916	0.0065	0.0851	14.1	90.3	
M	0.5	0.0295	0.0024	0.0771	12.3	4.98	7.7
	0.25	0.0150	0.0016	0.0134	9.4	3.96	
OW	1.0	0.0215	0.0054	0.0161	4.0	2.1	2.8
	0.5	0.0068	0.0003	0.0065	22.6	1.3	
OW/M	0.5	0.0021	0.0017	0.0004	1.2	0.05	1.7
D	0.25	0.0635	0.0025	0.0611	25.4	112	16.3
	0.125	0.0418	0.0019	0.0399	22.0	105	
Pullulan (reference)	0.25	0.2224	0.0035	0.2189	63.5	104.6	100
	0.125	0.1165	0.0018	0.1147	64.7	100.7	
Starch (reference)	0.25	0.0229	0.0286	–	0.80	–	–
	0.125	0.0131	0.0159	–	0.82	–	–
Glycogen (reference)	0.125	0.3269	0.3291	–	1.02	–	–
	0.0625	0.1763	0.1667	–	1.05	–	–

standardise the method to give an approximate molecular mass for the pullulan.

The results with the coupled-enzyme assay were confirmed with HPAEC-PAD. HPAEC gives very good resolution, and PAD provides a very sensitive method for estimating carbohydrates that is not influenced by elution gradient (as is the case for the refractive-index detector) and does not require ultrapure solvents (as in the case of short-wave UV). GSPE and SW supplemented with NH_4NO_3 and K_2HPO_4 proved to be good substrates, in that the EPS was essentially pullulan. Even for these substrates, however, the yields were significantly lower than that achieved with a glucose-rich medium (Kondrat'eva and Lobacheva 1990). Molasses and olive oil produced small amounts of EPS that contained negligible quantities of pullulan. These results are in accord with previously published data (Le Duy and Boa 1982).

The purity of the pullulan produced from agro-industrial wastes is an important factor in terms of economic viability, and the assays described provide a reliable estimate of purity. Some waste materials that may not themselves be good substrates for pullulan production may, by the modification of the fermentation parameters and the addition of suitable supplements, become useful sources of pullulan. Thus the viability of the production of pullulan from an agro-industrial waste may be more dependent on the costs of separation and purification than on the yield.

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