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Sweet potato: A novel substrate for pullulan production by *Aureobasidium pullulans*

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ABSTRACT

A strain *Aureobasidium pullulans* AP329, was used for the production of pullulan by employing hydrolysed sweet potato as cultivation media. Hydrolysis with α-amylase alone resulted in the lowest yields of pullulan. In contrast continuous hydrolysis with pullulanase and the β-amylase in sweet potato itself gave higher yields, but prolonged hydrolysis with amyloglucosidase decreased the yield. The maximum pullulan yield (29.43 g/l) was achieved at the dextrose equivalent value of 45 and pH of 5.5 for 96 h. As a substitute of sucrose, hydrolysed sweet potato was found to be hopeful and the yield of pullulan was higher than that of glucose and sucrose. The molecular weight of pullulan obtained from hydrolysed sweet potato media was much higher than that of sucrose and glucose media. Results of this work indicated that sweet potato was a promising substrate for the economical production of pullulan.

1. Introduction

Pullulan is an exocellular homopolysaccharide produced by *Aureobasidium pullulans*. It is a linear mixed linkage α-D-glucan consisting mainly of maltotriose repeating units interconnected by α-(1 → 6) linkages (Saha & Zeikus, 1989). The regular alternation of α-(1 → 4) and α-(1 → 6) bonds results in structural flexibility and enhanced solubility (Leathers, 1993). Pullulan can form thin films that are transparent, oil resistant and impermeable to oxygen. Pullulan can be used as coating and packaging material, sizing agent for paper, starch replacer in low-calorie food formulations, cosmetic emulsions and industrial applications (Deshpande, Rale, & Lynch, 1992). In recent years, many authors have reported that modified pullulan has many pharmaceutical and chemical industry applications (Akiyoshi et al., 1998; Alban, Schauerte, & Franz, 2002; Masci, Bontempo, & Crescenzi, 2002; Shibata, Asahina, Teramoto, & Yosomiya, 2001; Sivakumar & Rao, 2003; Sung, Na, & Bae, 2004).

The cost of pullulan production currently is relatively high and therefore, it is prudent to search for inexpensive carbon and nitrogen sources, which are nutritionally rich enough to support the growth of the microorganism as well as the production of pullulan. Carbon sources that have been investigated are glucose, sucrose, maltose, lactose, peat hydrolysate, Jerusalem artichoke tubers, carob pads, Jaggery, beet molasses and hydrolysed potato starch waste (Barnett, Smith, Scanlon, & Israilides, 1999; Imshenetskii, Kondrat'eva, Dvadtsamova, & Vorontosova, 1985; Lacroix, LeDuy, Noel, & Choplin, 1985; LeDuy & Boa, 1982; Roukas, 1998; Roukas & Biliaderis, 1995; Shin, Kim, Lee, & Cho, 1989; Vijayendra, Bansal, Prasad, & Nand, 2001). Sweet potato, a cheap and available agriculture product, contains a large amount of starch, which is a suitable feedstock for industrial fermentation. It also contains a considerable amount of highly active β-amylase, which is the primary saccharifying enzyme in sweet potato. At the same time, *A. pullulans* lacks the hydrolytic enzymes necessary to hydrolyse the starch. Therefore, there is probably no need to add expensive β-amylase when the starch in sweet potato needs to be saccharified. In this paper, sweet potato was hydrolysed to varying extent by α-amylase, pullulanase, the β-amylase in sweet potato itself and amyloglucosidase. The hydrolysates were used as substrates for pullulan production by *A. pullulans* AP329. The dextrose equivalent values (DE) of the hydrolysed sweet potato (HSP) and initial pH were optimized.

2. Materials and methods

2.1. Microorganism

Aureobasidium pullulans AP329 was kindly supplied by Professor Qunyi Tong in School of Food Science and Technology, Jiangnan University. The microorganism was maintained at 4 °C on potato dextrose agar (PDA) and subcultured every 2 weeks.

Abbreviations: HSP, hydrolysed sweet potato; DE, the dextrose equivalent value; *A. pullulans*, *Aureobasidium pullulans*; PDA, potato dextrose agar; BDW, biomass dry weight; PC, pullulan content; PY, pullulan yield; RSC, the residual sugar content; HPLC, high-performance liquid chromatography; Mw, molecular weight.

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2.2. Preparation of inoculum medium

The inoculum medium contained (g/l), sucrose, 50.0; yeast extract, 2.0; KH_2PO_4, 5.0; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; NaCl, 1.0 and distilled water 1 l. The medium was autoclaved for 15 min at 121 °C after the pH of the medium was adjusted to 6.5.

2.3. Preparation of fermentation media

Sweet potato, containing about 420,000 μ/g β-amylase according to the method described by Teotia, Khare, and Gupta (2001), was purchased from a local agricultural market. The pH of a slurry of sweet potato at the concentration of 15% (w/v) was adjusted to pH 6.5 and instantly heated to 70 °C within 3 min in the presence of 4.0 μg ml^{-1} α-amylase (Sigma–Aldrich Co., *Bacillus licheniformis*, A3403). After liquefaction, the temperature was quickly reduced to 55 °C within 1 min and the pH adjusted to 5.0. Pullulanase (Genencor International, Inc., L-1000, 4.0 μg ml^{-1}) was added to the hydrolysate and allowed to further hydrolyse until the desired DE values were reached. Amyloglucosidase (Sigma–Aldrich Co., *Rhizopus*, A7255, 300 μg ml^{-1}) was then added and incubated for a prolonged hydrolysis. After hydrolysis, the hydrolysates was filtered through a Whatman GF/A filter. The fermentation media were prepared by replacing sucrose in cultivation medium with different quantities (5%, 7.5%, 10%, 12.5% and 15%, w/v) of HSP and autoclaved at 121 °C for 15 min after the pH was adjusted to 6.5.

2.4. Fermentation

Seed cultures were prepared by inoculating cells grown on a PDA agar slant into a 250-ml flask that contained 50 ml of the inoculum medium and subsequently incubated at 28 °C for 48 h with shaking at 200 r/min. About 2.5 ml of the seed culture were transferred into the 250-ml flask containing 50 ml of the fermentation media. The culture was shaken at 28 °C and with 200 r/min for 96 h.

2.5. Isolation and purification of pullulan

The culture was centrifuged to remove the microorganism at 15,000g for 20 min. The biomass (mycelia and yeast-like cells) dry weight (BDW) was determined by washing the sediment with distilled water and drying at 105 °C overnight. Three milliliters of the supernatant were transferred into a test tube, and then 6 ml of cold ethanol was added to the test tube and mixed thoroughly and held at 4 °C for 12 h to precipitate the exocellular polysaccharide. After removal of the residual ethanol, the precipitate was dissolved in 3 ml of deionized water at 80 °C and the solution was dialyzed against deionized water for 48 h to remove small molecules in the solution. The polysaccharide was precipitated again by using 6 ml of the cold ethanol and the precipitate was filtered through a pre-weighted Whatman GF/A filter and dried at 80 °C to a constant weight (Badr-Eldin, El-Tayeb, El-Masry, Mohamad, & El-Rahman, 1994). The reducing sugars were estimated by the method of Somogyi (Nelson, 1944). The residual sugar content (RSC) was determined by the Bernfeld method (Bernfeld, 1995). The pullulan content (PC) of the ethanol precipitate was determined by the modified coupled-enzyme assay technique described by Barnett et al. (1999). The BDW and the PC was expressed as g/l and pullulan yield (PY) was expressed as gram pullulan per 100 g of sugar consumed.

2.6. Analytical methods

The pH of the culture medium was recorded using a digital pH meter (Model: PHS-3C, CD Instruments, China). Ash, moisture, fat and protein content of the samples were determined as per standard methods (Anonymous, 1984). The composition of the sugar in HSP was analysed by Water600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mmid × 300 mm) used pure water as mobile phase at a flow rate of 0.5 ml/min and the column temperature was maintained at 85 °C. The second column (SpherisorbNH$_2$, 4.6 mmid × 250 mm) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 ml/min and the column temperature was 30 °C. The detector sensitivity was four and the inject volume was 10 μl. Pullulan molecular weight (Mw) was determined by HPGFC on a Ultrahydrogel Size Exclusion Column, which is capable to detect Mws in the range of 10^3–10^6. In the size exclusion chromatography studies, 0.1 N $NaNO_3$ was used as an eluent at a flow rate of 0.9 ml/min. The detector used was a High Sensitive Refractive Index Detector, Model ERC-7515A (ERC Inc., Japan). The calibration of the detector was done with known concentrations of commercial available pullulan (Sigma). An aliquot of 20 μl was injected to the column after filtration through 0.45 μm Millipore filter, at ambient temperature and the procedure was repeated three times. The software used was the Multi-channel Chromatography Data Station (version 144A, 1993–1997, Ampersand Ltd.). The characterisation of pullulan was done by Gas Chromatography (Model: GC-15A, Shimadzu, Japan) described by Sawardeker, Sloneker, and Jeanes (1965).

3. Results and discussion

3.1. Effect of DE values of HSP on fermentation

The DE values of HSP after treatment with enzymes are shown in Fig. 1A. *pullulans* AP329 did not grow obviously on unhydrolysed sweet potato (data not shown). Under the conditions used in these fermentation studies, the degree of hydrolysis (measured by the DE) affected the BDW and pullulan produced in the fermentation. When the 5.0% (w/v) sweet potato was liquefied with α-amylase, the fermentation yields of pullulan (g/l) and BDW (g/l) were low (Fig. 1). This is expected as the liquefied sweet potato contained a large proportion of saccharide of several glucan units and dextrin (Table 1). Further hydrolysis with pullulanase and the β-amylase in sweet potato itself to give higher DE gave higher fermentation yields of pullulan and BDW. At the DE of 45 of HSP, containing 54.6% (w/w) maltose (Table 1), the highest yields of pullulan (28.57 g/l) and BDW (10.64 g/l) were achieved (Fig. 1). But continuous hydrolysis with amyloglucosidase, giving very high glucose content (>99%, w/w), decreased the pullulan yields and BDW, indicating that maltose is a better substrate than glucose for pullulan production, which is in accordance with the observation reported

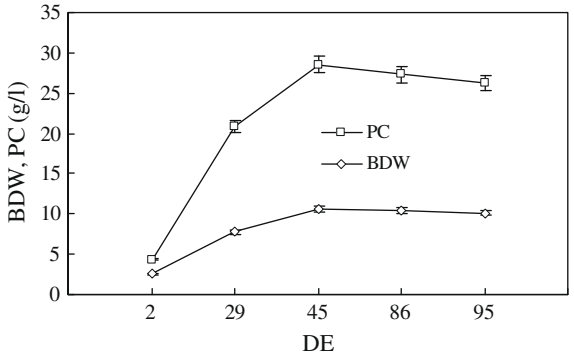

Fig. 1. The effect of DE on biomass dry weight (BDW) and pullulan content (PC) during the fermentation (the results are from three replicate experiments).

Table 1
The sugar composition of HSP.[a]

DE (%)	Total	Percentage composition (w/w) of hydrolysate				
		Glucose	Maltose	Maltotriose	Maltotetraose	Maltopentaose
2[b]	9.6 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	1.6 ± 0.1	2.1 ± 0.2	4.2 ± 0.2
29[c]	87.5 ± 2.1	6.7 ± 0.2	43.6 ± 1.5	27.3 ± 1.2	9.9 ± 0.4	0.00
45[d]	94.7 ± 2.4	7.3 ± 0.2	54.6 ± 1.3	32.8 ± 1.1	0.00	0.00
86[e]	103.9 ± 2.5	99.7 ± 2.2	3.6 ± 0.2	0.6 ± 0.1	0.00	0.00
95[f]	106.9 ± 2.8	106.1 ± 2.4	0.7 ± 0.1	0.1 ± 0.1	0.00	0.00

[a] Mean ± *SD* from three independent experiments.
[b] Instantly liquefied with α-amylase.
[c] Instantly liquefied with α-amylase followed by pullulanase and the β-amylase in sweet potato itself for 24 h.
[d] Instantly liquefied with α-amylase followed by pullulanase and the β-amylase in sweet potato itself for 48 h.
[e] Instantly liquefied with α-amylase followed by pullulanase and the β-amylase in sweet potato itself for 48 h and then hydrolysed with amyloglucosidase for 24 h.
[f] Instantly liquefied with α-amylase followed by pullulanase and the β-amylase in sweet potato itself for 48 h and then hydrolysed with amyloglucosidase for 48 h.

by Barnett et al. (1999). The results in Fig. 1 and Table 1 suggest that the DE of 45 of HSP, liquefied with α-amylase first and then hydrolysed with pullulanase and the β-amylase in sweet potato itself, is the most optimal for pullulan production. Therefore, all subsequent experiments were carried out using this kind of HSP.

3.2. Effect of the concentration of HSP on fermentation

The yield of pullulan mostly depended on the initial concentration of sugar that present in the media. Since the initial concentration of 5% (w/v) sugar was optimal for maximum yield of pullulan (Ueda, Fujita, Komatsu, & Nakashima, 1963), the effect of initial levels of HSP varying from 5% to 15% (w/v) on pullulan yield was investigated. The fermentation was carried out at the pH of 6.5 and temperature of 28 °C for 96 h on a rotary shaker. Under the conditions used in these fermentation experiments, BDW accumulation on the forth day of fermentation showed a gradual and steady increase as the level of HSP in the medium increased. The maximum production of BDW was observed at 15% (w/v) of HSP (Fig. 2). Microscopic examination revealed that the proportion of the yeast like cells increased with the increasing HSP, resulting in higher PC, which was similar to Ronen's observation (Ronen, Guterman, & Shabtai, 2002).

There was a small increase in the pullulan content as the level of HSP was increased. At the same time, the pullulan yield in terms of sugar utilized was comparatively low and most of the sugar (as high as 4–8%, w/v) remained unutilized in the medium at higher concentration of HSP in the media. Although the pullulan content obtained was slightly lower with an initial concentration of 5% (w/v) of HSP, the yield in terms of the sugar consumed was the highest (57.14%). In addition to lower pullulan yields, the high quantity of residual sugar in the fermentation broth is not economical and also can lead to environmental pollution. Taking these into consideration, the optimal concentration of HSP is judged to be 5% (w/v). Therefore, all subsequent experiments were carried out at this concentration of HSP.

3.3. Effect of the initial pH on fermentation

The initial pH of fermentation media can influence the morphology of the *A. pullulans* AP329, which may subsequently influence pullulan production (Cately, 1979). Therefore, it was of interest to investigate the effects of different initial pH values ranging from 2.5 to 7.5 in the media on pullulan production by the microorganism. Yeast-like cell growth was observed at all the initial levels of pH. Interestingly, relatively low pH (about at pH 3.5) was more optimal for biomass growth and this observation was in agreement with those reported by Shingel (2004). Fig. 3 shows that the maximum of 29.43 (g/l) of pullulan in the medium broth was achieved at an initial pH of 5.5. In contrast to the results we observed here, Roukas and Biliaderis (1995) reported that a pH of 6.5 was the optimal condition for pullulan production by *A. pullulans*. Auer and Seviour (1990) observed a maximum polysaccharide content at an initial pH of 7.5 in shake flask or batch culture. Ono et al. noted that an initial pH of 6.0 was optimal for the polysaccharide production (Ono, Yasuda, & Ueda, 1977). Vijayendra et al. (2001) found that an initial pH of 5.0 was optimum for pullulan production. The different optimal pH conditions reported in the literature may be due to the differences in the types of strain, composition of fermentation medium and culture conditions used. From the results in Fig. 3, a pH of 5.5 is the most optimal for pullulan

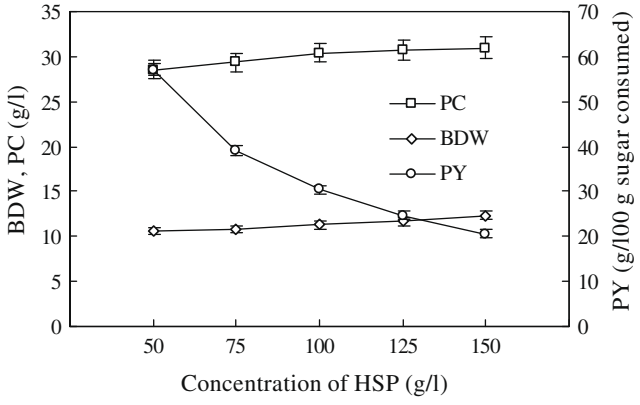

Fig. 2. The effect of the concentration of hydrolysed sweet potato (HSP) on biomass dry weight (BDW), pullulan content (PC) and pullulan yield (PY) during the fermentation (the results are from three replicate experiments).

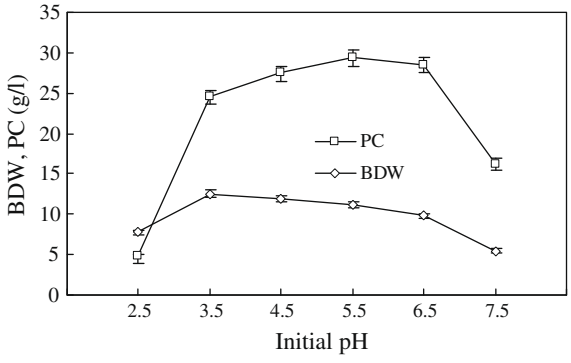

Fig. 3. The effect of initial pH on biomass dry weight (BDW) and pullulan content (PC) during the fermentation (the results are from three replicate experiments).

production using HSP as a substrate under the experimental conditions we employed.

3.4. Use of HSP as a carbon source

In our study, the suitability of HSP for pullulan production by *A. pullulans* AP329 was investigated by replacing HSP in fermentation medium with equal quantity (5%, w/w) of sucrose or glucose. The results are summarized in Table 2. HSP, as a substitute of sucrose, was found to be hopeful and the yield of pullulan was 139.07% more than that of glucose and 39.02% more than that of sucrose. Therefore, it is evident from the results that HSP is a suitable carbon source for the biomass growth as well as pullulan production. In contrast to our findings, LeDuy and Boa (1982) reported maximum polysaccharide levels of 12–14 g/l for various strains of *A. pullulans* grown in peat hydrolysates. Roukas and Biliaderis (1995) reported a 6 g/l PC from carob pad extract in shake flask culture. Vijayendra et al. (2001) achieved a PC of 23.01 g/l using Jaggery as a carbon source. Barnett et al. (1999) observed a 58 g/l PC employing hydrolysed potato starch waste as a carbon source for polysaccharide production at DE of 42 and a concentration of 20% (w/v). These variations in the results may be due to strain variation, composition of medium and fermentation conditions.

3.5. Kinetics of pullulan production

The time course studies on the production of pullulan by *A. pullulans* AP329 were made for a period of 120 h in the fermentation medium. As shown in Fig. 4, there was an obvious increase within 72 h and a slow increase from 72 to 96 h in the production of pullulan. The maximum yield of pullulan was achived after 96 h. The BDW continued to increase till the end of the experimental period. So it is evident from the results that the production of pullulan was not associated with cell growth.

3.6. Characterization of the pullulan

The pullulan obtained from the cell free supernatant of fermentation broth of sucrose, glucose and HSP was characterised. As shown in Table 3, although there was no much difference in pullulan, ash and moisture content of the samples obtained from media, the Mw of pullulan obtained from HSP media was much higher than that of sucrose and glucose media. The pullulan extracted from all media showed the presence of glucose, mannose and traces of rhamnose after acid hydrolysis and GC analysis. Glucose was the major sugar present. These results are similar to the report by Zajic and LeDuy (1973), where the presence of glucose as a major sugar was observed in pullulan. The confirmation of the polysaccharide as pullulan was done in our earlier study (data not shown) by subjecting the polysaccharide to hydrolysis with pullulanase. The hydrolysates are identified as maltotriose units by comparing the Rf value (using paper chromatography) of this product with that of pure pullulan (Sigma–Aldrich Co.). All samples are comparable in color, texture and water soluble ability.

Table 2
Effect of glucose, sucrose and HSP on pullulan fermentation.[a]

Parameter	Carbon source (5%, w/w)		
	Glucose	Sucrose	HSP
PC (g/l)	12.31 ± 1.23	21.17 ± 0.54	29.43 ± 0.78
BDW (g/l)	4.23 ± 0.68	7.92 ± 0.44	11.23 ± 0.55

[a] Mean ± *SD* from three independent experiments.

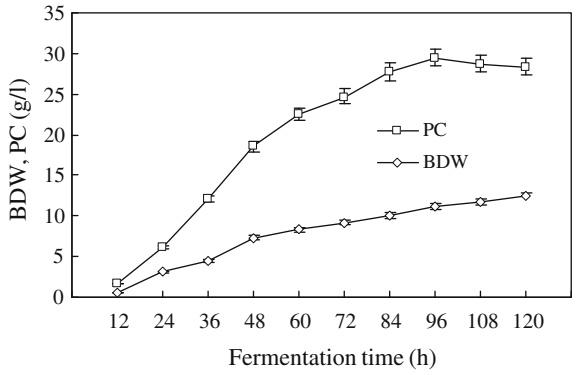

Fig. 4. The effect of time course on biomass dry weight (BDW) and pullulan content (PC) during the fermentation (the results are from three replicate experiments).

Table 3
Comparison of quality of pullulan produced from sucrose, glucose and HSP media.[a]

Parameter	Source of Pullulan		
	Sucrose medium	Glucose medium	HSP medium
Pullulan content (%, w/w)	95.07 ± 0.67	95.01 ± 0.57	95.18 ± 0.62
Mw ($\times 10^5$)	1.7 ± 0.24	1.3 ± 0.13	3.4 ± 0.32
Ash content (%, w/w)	1.7 ± 0.20	1.6 ± 0.21	1.6 ± 0.18
Moisture content (%, w/w)	2.3 ± 0.30	2.4 ± 0.31	2.4 ± 0.27

[a] Mean ± *SD* from three independent experiments.

4. Conclusions

The concentration of the HSP in the fermentation medium affects the biomass accumulation and the yield of pullulan produced by *A. pullulans*. A HSP concentration of 5% (w/v) in the medium, liquefied with α-amylase first and then hydrolysed with pullulanase and the β-amylase in sweet potato itself, resulted in 29.43 g/l of pullulan at the DE of 45 and a pH of 5.5. The Mw of pullulan obtained from HSP media was higher than that of sucrose and glucose media.

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