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Fungal production of the polysaccharide pullulan from a plant hydrolysate

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Abstract: The ability of the fungus *Aureobasidium pullulans* ATCC 42023 to produce pullulan from hydrolysates of the native grass known as prairie cordgrass was investigated and examined relative to polysaccharide and cell biomass production, yield, and pullulan content of the polysaccharide. A pullulan concentration of 9.7 g l⁻¹ and yield of 0.78 g g⁻¹ was produced by ATCC 42023 when grown for 168 h at 30°C on a phosphate-buffered hydrolysate. The highest biomass level of 7.7 g l⁻¹ was produced by ATCC 42023 after 168 h on a hydrolysate-containing complete medium. The pullulan content of the polysaccharide produced by ATCC 42023 after 168 h on the hydrolysate medium alone was 77%. Unlike what has been observed for other biomass substrates, a polysaccharide with a high pullulan content can be produced at a relatively high yield by a fungus grown on a grass hydrolysate indicating that pullulan could be produced using a biomass-based process.

Keywords: biomass; fungus; hydrolysate; polysaccharide; pullulan.

1 Introduction

Pullulan is an exocellular complex polysaccharide that is elaborated by the yeast-like fungus *Aureobasidium pullulans* [1, 2]. In the genus *Aureobasidium*, it seems that the synthesis of polysaccharide with high pullulan content is restricted to certain varieties of *A. pullulans* [1]. This polysaccharide has been shown to consist primarily of cross-linked maltotriose residues [1, 2]. Because this neutral biopolymer is water-soluble, several applications for the commercial use of this fungal gum exist [1, 2]. These applications include its use as a food additive, flocculant,

blood plasma substitute, dielectric material, adhesive, and packaging film [1, 2]. The effect of medium composition such as carbon and nitrogen source, mineral salts, and yeast extract concentrations upon pullulan synthesis by *A. pullulans* has been previously studied. A number of carbon sources have been shown to support pullulan production including glucose, sucrose, maltose, and xylose [3]. Nitrogen availability was found to be an important factor with respect to the onset of polysaccharide biosynthesis by the fungus [3]. The presence of mineral salts and vitamins in the growth medium has been explored and it was found that mineral salt addition affected pullulan production [4].

A biobased approach for fungal pullulan production is to utilize the glucose released during the hydrolysis of cellulose contained within the plant biomass [5]. A plant biomass that could be used for pullulan production is the native grass known as prairie cordgrass (*Spartina pectinata*). The high-yielding prairie cordgrass contains a high cellulose level [5]. A high concentration of cellulose can be released from the plant biomass by treatment at high temperature and pressure [5]. The resultant cellulose can be subjected to enzymatic treatment to produce glucose, which can serve as a source of carbon [5]. In this study, the production of a polysaccharide with a high pullulan content and yield by *A. pullulans* was examined relative to its growth on the prairie cordgrass hydrolysate supplemented with culture medium components.

2 Materials and methods

The primary composition of the prairie cordgrass was 33% cellulose, 13.5% xylose, and 21% lignin [5]. The cordgrass hydrolysate was prepared by mixing the dried grass (milled to a 2 mm particle size) with 0.5% (w/v) potassium phosphate dibasic buffer (pH 5.0) and then autoclaving the mixture (10% solids, w/v) at 121°C for 30 min. After the autoclaved cordgrass suspension was cooled to 40°C, it was treated with 192.3 units of cellulase (g solids)⁻¹ and 184.3 units cellobiase (g solids)⁻¹ for 48 h at 40°C on a rotary shaker (100 rev min⁻¹). After boiling the suspension to halt enzymatic hydrolysis, the suspension was concentrated 10-fold [5]. Glucose was measured

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using a coupled hexokinase and glucose-6-phosphate dehydrogenase spectrophotometric assay, which has been previously described [5]. The glucose concentration present in the prairie cordgrass hydrolysates was determined by monitoring the production of NADH at 340 nm at 25°C [5]. Using this coupled enzymatic assay, it was determined that the glucose concentration in the hydrolysate was 1.1% (w/v). Biochemical reagents used in this study were obtained from the Sigma-Aldrich Corp., St. Louis, MO, USA. To determine the xylose and arabinose concentrations in the hydrolysates, hydrolysate samples (10 µl) were analyzed using a Waters HPLC unit equipped with a Waters 7171 Plus autosampler and a Waters 2410 refractive index detector (Waters Corp., Milford, MA, USA). After sample injection into a heated 300 mm Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) (65°C), it was eluted from the column using a mobile phase of 5 mM sulfuric acid at a flow rate of 0.6 ml min⁻¹ [6]. The concentrations of xylose and arabinose in the hydrolysate were found to be 0.72% (w/v) and 0.21% (w/v), respectively.

The pullulan-producing strain *A. pullulans* ATCC 42023 was used in this study [3]. The strain was grown either in an autoclaved complete culture medium at pH 6.0 [0.5% (w/v) K₂HPO₄, 0.1% (w/v) NaCl, 0.02% (w/v) MgSO₄ · 7H₂O, 0.04% (w/v) yeast extract, and 0.06% (w/v) (NH₄)₂SO₄ with 1.1% (w/v) glucose, 0.72% (w/v) xylose, and 0.21% arabinose as carbon sources] or in a filter sterilized (0.2 µm) 0.5% (w/v) potassium phosphate-buffered, hydrolysate-containing medium (pH 6.0) in which the medium was unsupplemented or supplemented with 0.1% (w/v) NaCl, 0.02% (w/v) MgSO₄ · 7H₂O, 0.04% (w/v) yeast extract or 0.06% (w/v) (NH₄)₂SO₄ or both 0.02% (w/v) MgSO₄ · 7H₂O and 0.1% (w/v) NaCl to learn if their addition affected pullulan production and yield, biomass synthesis, or pullulan content of the polysaccharide elaborated [7].

Batch cultures (50 ml) in 250 ml Erlenmeyer flasks, done in triplicate, were shaken (200 rev min⁻¹) at 30°C for a period of 168 h after inoculation with approximately 10⁸ cells from 24-h cultures of ATCC 42023 grown on the same medium. Samples (5 ml) of each culture were removed at intervals of 24 h and the samples were centrifuged at 14,600 × g for 30 min at 4°C. The supernatant was saved for pullulan determination whereas the cell pellet was retained for dry weight analysis. To quantify the pullulan levels, two volumes of 95% (v/v) ethanol were added for each volume of supernatant containing pullulan [7]. The precipitated pullulan was collected on preweighed Millipore (EMD Millipore Corp., Billerica, MA, USA) 0.45 µm HVLP filters (25 mm diameter) whereas the washed cell

pellet [7] was collected on preweighed 0.45 µm HVLP filters (47 mm diameter). All filters were dried to constant weight at 80°C and reweighed to determine pullulan concentrations and cell dry weights. For statistical analyses, Student's *t*-test was used.

3 Results and discussion

The effect of supplementing components of the phosphate-buffered fungal culture medium was studied. The effect of a single component addition to hydrolysate-containing 0.5% potassium phosphate-buffered medium (pH 6.0) on pullulan production was initially explored. Polysaccharide production on the unsupplemented or supplemented buffered hydrolysate was highest after 168 h of growth compared with 120 h or 144 h of growth (Figure 1A). Production of the polysaccharide pullulan by *A. pullulans* ATCC 42023 on the prairie cordgrass hydrolysate in a phosphate-buffered medium was increased slightly after 168 h of growth by the addition of MgSO₄ · 7H₂O or NaCl compared with unsupplemented medium (Figure 1A) with the difference in production being statistically significant (*P* < 0.05). The increase in pullulan production by ATCC 42023 after the addition of MgSO₄ · 7H₂O or NaCl is likely related to the need for these metal ions during intracellular ATP synthesis, which is required for fungal pullulan production [8]. The addition of yeast extract or (NH₄)₂SO₄ to the hydrolysate-containing medium did not affect pullulan production by ATCC 42023 after 168 h of growth at 30°C compared with the unsupplemented medium (Figure 1A). Because the hydrolysate was found to contain glucose, xylose, and arabinose, the complete culture medium containing these sugars was examined for its ability to support pullulan production by ATCC 42023 (Figure 1A). The supplementation of the two medium components (MgSO₄ · 7H₂O and NaCl) to the phosphate-buffered hydrolysate that individually increased polysaccharide production by ATCC 42023 compared with its production on the phosphate-buffered hydrolysate alone was investigated to learn if pullulan production could be further elevated by the addition of both components. It was found that the addition of both MgSO₄ · 7H₂O and NaCl did increase polysaccharide production (Figure 1A). The addition of both 0.02% MgSO₄ · 7H₂O and 0.1% NaCl to a peat hydrolysate was also observed to be required for optimum pullulan production (12–14 g l⁻¹ at 25°C) by *A. pullulans* strains [9]. Pullulan production by ATCC 42023 on the medium containing glucose, xylose, and arabinose after 168 h of growth was slightly higher than pullulan synthesis by

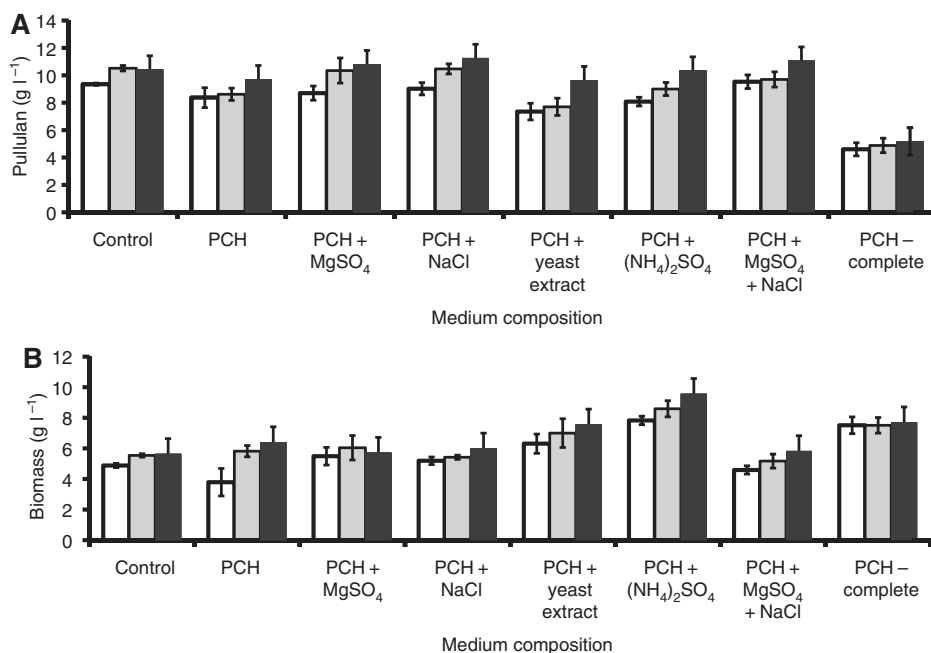


Figure 1: Effect of supplementation to a prairie cordgrass hydrolysate-containing 0.5% potassium phosphate-buffered medium (pH 6.0) on pullulan (A) or biomass (B) production by *A. pullulans* ATCC 42023. Pullulan concentrations (g l⁻¹) or biomass levels (g l⁻¹) produced by ATCC 42023 after growth for 96 h (□), 120 h (▒), or 144 h (■) at 30°C on the control [complete culture medium (pH 6.0) containing 1.1% glucose, 0.72% xylose and 0.21% arabinose]; prairie cordgrass hydrolysate (PCH) in phosphate buffer (pH 6.0); PCH medium (pH 6.0) with 0.02% MgSO₄ · 7H₂O; PCH medium (pH 6.0) with 0.10% NaCl; PCH medium (pH 6.0) with 0.04% yeast extract; PCH medium (pH 6.0) with 0.06% (NH₄)₂SO₄; PCH medium (pH 6.0) with 0.02% MgSO₄ · 7H₂O and 0.10% NaCl; and the complete culture medium (pH 6.0) containing PCH. Error bars indicate the standard deviations of mean data values.

ATCC 42023 grown on the hydrolysate-containing phosphate buffer although no statistical difference in production was noted (Figure 1A). In previous studies, processing coproducts from ethanol production have been studied for their ability to support fungal pullulan production using 2.5% (v/v) corn syrup as a carbon source. When ethanol stillage was utilized as a nitrogen source, it was observed that the highest pullulan level produced by *A. pullulans* ATCC 201253 was approximately 7 g l⁻¹ after 168 h of growth at 30°C if the medium was supplemented with 0.04% yeast extract [10]. Similarly, the utilization of corn steep solids as a nitrogen source in a medium containing 0.04% yeast extract resulted in a pullulan concentration of 7.4 g l⁻¹ being detected after 168 h of ATCC 201253 growth at 30°C [11]. The use of plant biomass hydrolysate in this study as a substrate for *A. pullulans* to produce pullulan seemed to be as effective as the peat hydrolysate and more effective than processing coproducts.

No difference in biomass production by ATCC 42023 could be observed after growth for 120, 144, or 168 h of growth when MgSO₄ · 7H₂O was added to the phosphate-buffered hydrolysate (Figure 1B). The supplementation of yeast extract or (NH₄)₂SO₄ to the buffered hydrolysate increased biomass production by ATCC 42023 after 168 h

of growth compared with biomass production on the buffered hydrolysate alone, with the difference being statistically significant ($P < 0.01$). Also, the addition of (NH₄)₂SO₄ to the buffered hydrolysate elevated biomass production by 1.3-fold relative to yeast extract addition to the buffered hydrolysate (Figure 1B). The supplementation of MgSO₄ · 7H₂O and NaCl slightly reduced biomass production by ATCC 42023 after 168 h of growth compared with the level of biomass produced by ATCC 42023 after 168 h of growth in the phosphate-buffered hydrolysate, with the difference in levels not being statistically significant (Figure 1B). When ATCC 42023 was grown on the complete medium containing glucose, xylose, and arabinose, the strain produced less biomass than it did when grown on the phosphate-buffered hydrolysate (Figure 1B) with the difference in biomass production being statistically significant ($P < 0.05$). Biomass production by ATCC 42023 grown on the complete medium containing glucose, xylose, and arabinose after 168 h was found to be statistically significantly lower ($P < 0.01$) compared with ATCC 42023 biomass production on the complete medium containing the hydrolysate. It has been shown that the addition of 0.06% (NH₄)₂SO₄ to the peat hydrolysate ATCC 201253 stimulated biomass production by *A. pullulans* [9].

The presence of yeast extract in the fungal culture medium has been observed to increase cellular biomass [11]. The effect of using processing products such as ethanol stillage or corn steep solids as a nitrogen source with 2.5% (v/v) corn syrup as a carbon source was also explored. Independent of whether the medium was supplemented with yeast extract, ethanol stillage as a nitrogen source produced higher ATCC 201253 cell dry weights after 168 h of growth at 30°C than did corn steep solids as a nitrogen source [10, 11].

The highest polysaccharide yield was observed when the hydrolysate medium contained $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl (Table 1). The difference in yield between the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl and the pH 6.0 buffered hydrolysate (Table 1) was significantly different ($P < 0.01$). The difference in yield between the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl and the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Table 1) was also significantly different ($P < 0.05$). No statistical difference in yield between the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl and the hydrolysate medium containing yeast extract was noted. The lowest polysaccharide yield was noted when the complete medium contained the hydrolysate (Table 1).

The pullulan content of the polysaccharide was highest when ATCC 42023 was grown on the medium containing glucose, xylose, and arabinose although the pullulan content of the polysaccharide produced by ATCC 42023 was only slightly lower when grown on the hydrolysate-containing medium alone or supplemented with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Table 1). Pullulan content of the polysaccharide produced by ATCC 42023 was highest on the phosphate-buffered hydrolysate containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl (Table 1) compared with the pullulan content of the polysaccharide produced after growth on the

phosphate-buffered hydrolysate alone ($P < 0.05$). The difference in pullulan content of the polysaccharide produced by ATCC 42023 on the phosphate-buffered hydrolysate containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ relative to phosphate-buffered hydrolysate containing NaCl or $(\text{NH}_4)_2\text{SO}_4$ was statistically significant ($P < 0.05$). The difference in pullulan content of the polysaccharide produced by ATCC 42023 on the phosphate-buffered hydrolysate containing yeast extract compared with phosphate-buffered hydrolysate containing $(\text{NH}_4)_2\text{SO}_4$ was also statistically significant ($P < 0.05$). Previous studies have found that authentic pullulan was produced if ATCC 42023 was grown on medium containing 2.5% (v/v) corn syrup as a carbon source and 0.06% (w/v) $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source after 168 h of growth at 30°C [12]. In contrast, the pullulan content of the polysaccharide produced by ATCC 42023 grown on 2.5% (v/v) glucose and 0.06% (w/v) $(\text{NH}_4)_2\text{SO}_4$ for 168 h at 30°C was determined to be 62% [12]. When ethanol stillage or corn steep solids served as the nitrogen source in the presence of 2.5% (v/v) corn syrup in a culture medium lacking yeast extract, the pullulan content of the polysaccharide produced by ATCC 201253 was very low [10, 11]. It is interesting to note that the pullulan content of the polysaccharide produced by ATCC 42023 on the buffered hydrolysate after 168 h of growth was 77% (Table 1). Although the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl produced the highest polysaccharide yield, the pullulan content of the polysaccharide produced was very low (Table 1). Unfortunately, the content of the pullulan synthesized by *A. pullulans* strains on the peat hydrolysate supplemented with 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% NaCl was not determined [9]. In addition, fungal growth on the complete medium containing hydrolysate produced a polysaccharide with low pullulan content (Table 1) compared with the fungal polysaccharide produced on

Table 1: Yield and pullulan content of the polysaccharide produced by *A. pullulans* ATCC 42023 grown on selected medium conditions after 168 h at 30°C.

Medium	Yield ^a	Pullulan content ^a
Complete medium with 1.1% (w/v) glucose, 0.72% (w/v) xylose, and 0.21% (w/v) arabinose	0.51 (0.01)	80 (6)
Complete medium with hydrolysate	0.38 (0.04)	39 (3)
Hydrolysate in pH 6.0 buffer	0.78 (0.06)	77 (7)
Hydrolysate in pH 6.0 buffer with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.84 (0.04)	77 (5)
Hydrolysate in pH 6.0 buffer with NaCl	0.79 (0.06)	67 (8)
Hydrolysate in pH 6.0 buffer with yeast extract	0.86 (0.06)	71 (5)
Hydrolysate in pH 6.0 buffer with $(\text{NH}_4)_2\text{SO}_4$	0.74 (0.07)	71 (4)
Hydrolysate in pH 6.0 buffer with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl	0.94 (0.06)	38 (5)

^aYield is expressed as g pullulan (g reducing sugar utilized)⁻¹ whereas pullulan content is given as % pullulan. Each result indicates the mean of three separate trials with the number in parentheses representing the standard deviation of the mean.

the medium containing glucose, xylose, and arabinose or the hydrolysate medium containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and NaCl ($P < 0.01$). Based on the observed pullulan content of the polysaccharide produced, pullulan production by ATCC 42023 from an unsupplemented or minimally supplemented prairie cordgrass hydrolysate would seem to be a superior option compared with using other biomass substrates.

4 Conclusions

Polysaccharide and biomass production, yield as well as pullulan content by ATCC 42023 grown on the hydrolysate were affected by supplementation. The findings from this study indicated that pullulan can be produced by ATCC 42023 at a high concentration, yield, and pullulan content from prairie cordgrass hydrolysates that are unsupplemented or minimally supplemented. In particular, the fungus grown on the hydrolysate alone produced a polysaccharide with a high pullulan content. This investigation provides evidence that a plant biomass-based substrate can be used to produce authentic pullulan unlike the polysaccharide produced on other biomass substrates.

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