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Polysaccharide production by *Aureobasidium pullulans*: factors affecting polysaccharide formation

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***Aureobasidium pullulans* NRRL 6220 synthesized polysaccharide most actively in media containing sucrose, fructose or maltose with $(NH_4)_2SO_4$ (0.6 g/l) or ammonium acetate giving greatest yields of the polysaccharide. With $(NH_4)_2SO_4$ at ≥ 1.2 g/l, production of polysaccharide was decreased considerably. Polysaccharide production was highest with an initial pH of 6.5 while biomass formation was better below an initial pH of 5.5. Optimum phosphate concentration for polysaccharide production was 0.03 M.**

Key words: Aureobasidium pullulans, polysaccharide, pullulan.

Microbial polysaccharides have diverse application in the food, chemical, energy and pharmaceutical industries (Sutherland & Ellwood 1979). Pullulan, a major polysaccharide produced by strains of *Aureobasidium pullulans*, is a linear polymer of malto-triose units linked through α (1→6) bonds on the terminal glucose units (Wallenfels *et al.* 1965). The potential uses of pullulan in food, drug and other industries (Yuen 1974; Zajic & LeDuy 1977) have led to increased interest in the production of this polysaccharide by *A. pullulans*.

A research programme for developing an improved fermentation process for pullulan production has led to the selection of *A. pullulans* NRRL 6220. The present study was designed to determine the best culture conditions controlling pullulan formation by this strain.

Materials and Methods

Organism and Inoculum Preparation

Aureobasidium pullulans NRRL 6220, selected because it produces a high yield of pigment-free pullulan, was maintained on an agar medium containing (% w/v): sucrose (food grade), 5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 0.06; NaCl, 0.1; $MgSO_4.7H_2O$, 0.2; yeast extract, 0.04; and agar, 2.0. The initial pH was adjusted to 6.5 with 1 M HCl and then the medium was autoclaved at 121°C for 20 min. The organism was grown on slants at 28°C for 3 days and stored at 5°C. Cells from such a 3-day-old stock culture were transferred to 50 ml of maintenance medium in a 250-ml Erlenmeyer flask and incubated at 27°C on a rotary shaker (150 rev/min). A 2% (v/v) inoculum was used for subsequent subcultures.

Polysaccharide Production in Batch Culture

The basal medium used for the physiological studies of polysaccharide production was the maintenance medium supplemented with 10% sucrose (autoclaved separately), unless stated otherwise. The composition of the culture medium varied according to the requirements of the specific experiments. The experiments were conducted in 250-ml Erlenmeyer flasks, each containing 50 ml of medium, incubated with shaking (150 rev/min) at 28°C for 96 h. The experiments were carried out in triplicate and the results given are mean values.

Determination of Biomass

A sample of the culture broth (usually 10 ml), diluted 1- to 3-fold with distilled water if necessary, was centrifuged at 12,000 × *g* for 15 min. The clear supernatant was collected for substrate and product analysis. The sedimented cells were then washed twice with distilled water, placed in a pre-weighed aluminium dish and dried to a constant weight at 105°C.

Determination of Polysaccharide

The polysaccharide (pullulan) was precipitated from the supernatant by addition of 1 to 2 volumes of ethanol and allowed

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Table 1. Effect of carbon source on polysaccharide production.*

Carbon source	Polysaccharide (g/100 ml)	Biomass (g/100 ml)	Polysaccharide (g/g biomass)	Residual sugars (g/100 ml)	Polysaccharide yield† (%)	Final pH
Sucrose	4.19	0.91	4.60	2.93	59.3	4.3
Glucose	2.01	0.70	2.87	4.20	34.7	4.0
Fructose	3.35	0.86	3.90	5.88	81.3	4.1
Mannose	2.76	0.82	3.37	5.33	59.1	4.3
Xylose	2.33	0.77	3.03	6.15	60.5	4.2
Lactose	0.25	0.79	0.32	2.57	3.4	4.1
Maltose	3.25	0.84	3.87	3.71	51.7	4.1
Raffinose	1.77	1.13	1.57	2.70	24.3	4.3

* Basal medium consisted of (% w/v): $(NH_4)_2SO_4$, 0.06; $MgSO_4.7H_2O$, 0.02; NaCl, 0.1; K_2HPO_4, 0.5; and yeast extract, 0.04. Initial pH was adjusted to 6.5 and cultivations were for 96 h.

† Based on sugar consumed.

Table 2. Effect of nitrogen source on polysaccharide production.*

Nitrogen Source	Polysaccharide (g/100 ml)	Biomass (g/100 ml)	Polysaccharide (g/g biomass)	Final pH
$(NH_4)_2SO_4$	3.27	0.64	5.10	3.6
NH_4Cl	2.15	0.52	4.20	4.9
NH_4NO_3	1.93	0.60	3.80	5.0
$NaNO_3$	1.74	0.85	3.30	5.1
Ammonium acetate	3.69	1.19	3.44	4.5
Ammonium citrate	2.71	0.63	4.36	5.1
Ammonium formate	0.41	0.27	1.64	6.9
Ammonium oxalate	2.25	0.59	3.98	5.4
Urea	1.17	0.60	2.00	5.4

*Nitrogen sources equivalent to 0.127 g N/l were added to the basal medium, initial pH was adjusted to 6.5 and cultivations were for 96 h.

Table 3. Effect of $(NH_4)_2SO_4$ concentration on polysaccharide production.*

$(NH_4)_2SO_4$ concentration (g/100 ml)	Polysaccharide (g/100 ml)	Biomass (g/100 ml)	Polysaccharide (g/g biomas)	Final pH
0.03	0.34	0.22	1.55	6.4
0.06	2.60	0.60	4.33	4.8
0.12	1.95	0.87	2.24	3.0
0.24	0.84	0.94	0.89	2.6

*Basal medium consisted of (% w/v): sucrose, 10; $MgSO_4.7H_2O$, 0.02; NaCl, 0.1; K_2HPO_4, 0.5; and yeast extract, 0.04. Initial pH was adjusted to 6.5 and cultivations were for 96 h.

to stand overnight at 4°C. The precipitate was separated by centrifugation, washed first with 66% (v/v) ethanol and then with absolute alcohol and then dried under vacuum at 40°C. The recovered polysaccharide taken at 24-h intervals from typical batch experiments showed an infra-red spectrum similar to that of an authentic pullulan sample (Sigma); there were bands at 925, 768 and 750 cm^{-1} characteristic of pullulan (α-glucan) (Bouveng *et al.* 1963), but the band at 890 cm^{-1} characteristic of the β-glycosidic linkage was absent (Leal-Serrano *et al.* 1980).

Determination of Residual Sugar

The sugar remaining in the culture broth was determined by the phenol-sulphuric acid method (Dubois *et al.* 1956).

Results

Formulating the Medium for Optimum Polysaccharide Formation

Of the sugars tested as carbon sources, highest yields of polysaccharide were produced in media containing sucrose, fructose or maltose (Table 1).

Of the nitrogen sources tested, $(NH_4)_2SO_4$ and ammonium acetate gave greatest amounts of polysaccharide but led to a decrease in the final pH of the culture broth (Table 2).

The concentration of $(NH_4)_2SO_4$ affected both biomass formation and polysaccharide production (Table 3). Poly-

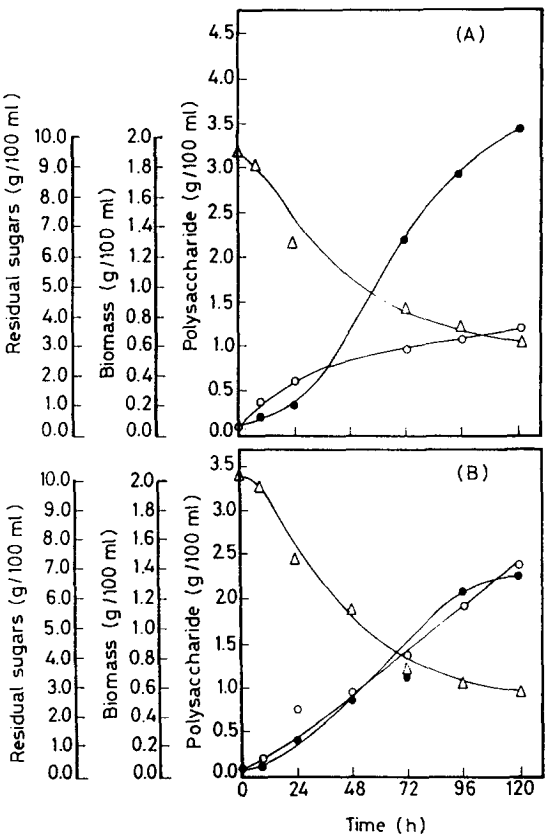

Figure 1. Polysaccharide production in the basal medium with 0.06% (A) or 0.12% (B) $(NH_4)_2SO_4$. ●—Polysaccharide; ○—biomass; △—residual sugars.

saccharide increased progressively during growth in medium containing 0.06% $(NH_4)_2SO_4$ but the final yield was 40% less in medium with 0.12% $(NH_4)_2SO_4$ (Figure 1).

The ability of the organism to synthesize polysaccharide depended upon initial pH: an initial pH 6.5 gave the highest yield (Table 4).

The optimum concentration of K_2HPO_4 for polysaccharide production was found to be 0.03 M (0.5%) (Table 5).

Discussion

The nature of the carbon and nitrogen sources affected both biomass formation and polysaccharide production by *A. pullulans*. Sucrose, fructose or maltose were the better carbon sources. Strains used by Catley (1971) and by West & Reed-Hamer (1991) gave maximum amounts of polysaccharide with sucrose while the lowest was with maltose. Ammonium acetate and $(NH_4)_2SO_4$ stimulated maximal yields of the polysaccharide and biomass whereas urea gave a low yield. In contrast, the strains used by Imshenetskii *et al.* (1981) gave the highest yields of polysaccharide using $NaNO_3$ or urea and the lowest yield with $(NH_4)_2SO_4$. Thus, our results emphasise that there are strain differences in *A. pullulans* in terms of their capacity to assimilate various carbon or nitrogen sources for polysaccharide synthesis.

The observed pattern of growth indicates that increase in NH_4^+ concentration enhances carbon flow into biomass forma-

Table 4. Effect of phosphate concentration on polysaccharide production.*

K_2HPO_4 concentration (M)	Polysaccharide (g/100 ml)	Biomass (g/100 ml)	Polysaccharide (g/g biomass)	Residual sugars (g/100 ml)	Polysaccharide yield† (%)	Final pH
0.01	1.93	0.85	2.27	3.90	31.64	3.3
0.03	2.35	0.62	3.79	4.12	39.97	4.5
0.06	2.05	0.58	3.54	4.68	38.53	4.9
0.12	1.60	0.55	2.91	5.58	36.20	6.4
0.29	0.75	0.47	1.60	7.35	28.30	7.0

*Basal medium consisted of $(NH_4)_2SO_4$, 0.06; $MgSO_4.7H_2O$, 0.02; NaCl, 0.1; and yeast extract, 0.04. Initial pH was adjusted to 6.5 and cultivations were for 96 h.

† Based on sugar consumed.

Table 5. Effect of initial pH on polysaccharide production.*

Initial pH	Polysaccharide (g/100 ml)	Biomass (g/100 ml)	Polysaccharide (g/g biomass)	Residual sugars (g/100 ml)	Polysaccharide yield† (%)	Final pH
2.0	0.12	0.70	0.17	3.9	10.9	2.0
3.5	1.01	0.63	1.60	2.1	34.8	2.5
5.5	1.59	0.65	2.45	1.4	40.6	2.8
6.5	2.28	0.66	3.46	0.9	55.6	4.4
7.5	1.59	0.50	3.18	2.2	56.8	4.9
8.0	1.52	0.48	3.17	2.3	56.3	4.8

*Basal medium consisted of (% w/v): carbon source, 5; $(NH_4)_2SO_4$, 0.06; $MgSO_4.7H_2O$, 0.02; NaCl, 0.1; K_2HPO_4, 0.5; and yeast extract, 0.04. Cultivations were for 96 h.

† Based on sugar consumed.

tion, with a concomitant decrease in polysaccharide level (Figure 1). Thus production of *A. pullulans* polysaccharide is favoured by a high C/N ratio in the culture medium, probably because low nitrogen content restricts biomass formation while the high concentration of carbon source left in the culture can be used for polysaccharide production.

Polysaccharide production depended on initial culture pH: a high initial pH (6.5) favoured polysaccharide production whereas cell growth was better at a much lower pH (2.0). Incorporation of a high concentration of inorganic phosphate in the medium suppressed polysaccharide formation, probably due to the increased buffering capacity preventing the change to a favourable pH for polysaccharide synthesis.

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