Carbohydrate Polymers 86 (2011) 1085–1088

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Effect of different nitrogen sources on activities of UDPG-pyrophosphorylase involved in pullulan synthesis and pullulan production by *Aureobasidium pullulans*

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ARTICLE INFO

Article history:
Received 6 April 2011
Received in revised form 2 May 2011
Accepted 12 May 2011
Available online 20 May 2011

Keywords:
Pullulan
Biomass
Nitrogen source
UDPG-pyrophosphorylase
$NaNO_2$

ABSTRACT

Investigated was the effect of nitrogen source on fermentation time, pullulan production, biomass, and UDPG-pyrophosphorylase activity. Pullulan production fell when excess nitrogen source was present. Biomass accumulation increased as the level of nitrogen source raised. Pullulan production in the medium containing $NaNO_2$ was 58.53% (w/w), greater than that with $(NH_4)_2SO_4$, reaching maximum production one day earlier compared to that with $(NH_4)_2SO_4$. The optimum fermentation time for pullulan production, and UDPG-pyrophosphorylase activity were also affected by the nitrogen source in the medium. UDPG-pyrophosphorylase activity was highly correlated with the increasing production rate of pullulan.

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1. Introduction

Pullulan, an exocellular polysaccharide produced by *Aureobasidium pullulans*, is a linear mixed linkage α-D-glucan consisting mainly of maltotriose repeating units interconnected by α-(1 → 6) linkages. Pullulan may be used as a coating and in packaging materials, as a sizing agent for paper, a starch replacement in low-calorie food formulations, in cosmetic emulsions and in other industrial applications (Singh, Saini, & Kennedy, 2008).

It has been reported that UDP-glucose plays pivotal role in the biosynthesis of pullulan and cannot be replaced by ADP-glucose during pullulan production by *A. pullulans*, indicating that the pullulan precursors originate from UDP-glucose (Shingel, 2004). It has been confirmed that UDP-glucose is formed from UTP and glucose 1-phosphate in reverse reaction catalyzed by UDPG-pyrophosphorylase (Daran, Dallies, Thines-Sempoux, Paquet, & Francois, 1995): UTP + glucose-1-phosphate → UDP-glucose + PPi. This is an essential step for the formation of UDP-glucose, a precursor for pullulan synthesis in *A. Pullulans* (Shingel, 2004). Therefore, the activity of UDPG-pyrophosphorylase, the key enzyme involved in the sugar metabolism and potentially involved in the production of precursors for pullulan biosynthesis in *A. Pullulans* should be one of important aspects in the production of pullulan. It has been reported that the activity of UDPG-pyrophosphorylase was highly correlated with the amount of pullulan produced and the carbon source used (Duan, Chi, Wang, & Wang, 2008). However, the relationship among nitrogen source, UDPG-pyrophosphorylase and pullulan production have not been investigated as yet.

The objectives of this study were to investigate the effect of different types and the concentration of nitrogen source and fermentation time on pullulan production, biomass accumulation, UDPG-pyrophosphorylase and morphology of *A. Pullulans* AP329.

2. Materials and methods

2.1. Microorganism

A. Pullulans AP329 was obtained from Professor Qunyi Tong, School of Food Science and Technology, Jiangnan University. Stock cultures were maintained on potato dextrose agar at 4 °C and subcultured every 2 weeks.

2.2. Preparation of inoculum medium

The inoculum medium contained 50 g glucose, 2.0 g yeast extract, 5.0 g K_2HPO_4, 0.6 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1.0 g NaCl in 1 l distilled water. The pH was adjusted to 6.5, and the medium was autoclaved at 121 °C for 15 min.

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0144-8617/$ – see front matter. Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2011.05.016

Table 1
Effect of nitrogen source (at 0.15 g/l total N) on pullulan production, cell growth and UDP-glucose pyrophosphorylase. Fermentation conditions: fermentation temperature, 28 °C; pH, 6.5; fermentation time, 4 days.

Nitrogen source	Pullulan (g/l)	Biomass (g/l)	Yeast (% of Biomass)	UDP-glucose pyrophosphorylase (mU/mg of protein)
$NaNO_2$	22.46 ± 0.21[a]	10.51 ± 0.15	81 ± 3.2	45.07 ± 2.3
$(NH_4)_2SO_4$	12.57 ± 0.17	4.65 ± 0.16	73 ± 1.4	32.23 ± 1.7
$NaNO_3$	7.04 ± 0.11	3.07 ± 0.14	54 ± 0.9	23.46 ± 0.5
NH_4NO_3	4.97 ± 0.21	3.86 ± 0.11	41 ± 1.1	12.51 ± 0.6
KNO_3	4.34 ± 0.13	3.21 ± 0.06	32 ± 0.5	7.59 ± 0.3
$(NH_4)_2CO_3$	2.15 ± 0.23	2.43 ± 0.07	17 ± 0.8	3.83 ± 0.1

[a] Mean ± S.D. from three independent experiments.

2.3. Preparation of fermentation medium

The fermentation medium was prepared by replacing $(NH_4)_2SO_4$ in the inoculum medium with other types of nitrogen sources as indicated in Table 1.

2.4. Fermentation

Seed culture was prepared by inoculating cells grown on a potato dextrose agar slant into a 250-ml flask that contained 50 ml of the inoculum medium and subsequently incubated at 28 °C for 2 days with shaking at 200 rpm. A 5-l stirred tank fermentor (5M-2002, Shanghai Baoxing Bio-engineering Equipment Co., China) with a working volume of 3 l was used for the production of pullulan in batch culture. Fermentor was consisted of a glass vessel with stainless-steel endplates and three equally spaced vertical baffles. Agitation was provided by a six-flat-blade impeller (diameter 4 cm) located 3 cm above the bottom of the vessel. The fermentor was sterilized at 121 °C for 15 min. After cooling, 3 l of production medium was added into the fermentor. The medium was inoculated with 150 ml inoculum. The fermentor was incubated at 28 °C in a thermostated chamber. The impeller speed was 800 rpm and the sterile air flow 4 l/min. The pH was controlled at 6.5 by feeding with either 2 M NaOH or 2 M HCl.

2.5. Preparation of cell-free extract

The cells in 5.0 ml of the culture were collected by centrifugation at 8000 × *g* for 5 min at 4 °C, washed 3 times with ice-chilled distilled water and suspended in 1.0 ml ice-chilled 1.0 M Tris–HCl (pH 7.6) to make a thick paste. The product was homogenized in a Homogenizer (DY89-I, Xinzhi, Zhejiang, China) for 1 h on the ice bath. The cell debris was removed by centrifugation at 10,000 × *g* at 4 °C for 30 min. The supernatant was the cell-free extract (also the enzyme preparation). Protein concentration in the cell-free extract was determined by the method of Bradford with bovine serum albumin as standard (Duan et al., 2008).

2.6. Enzymes assays

The reaction mixture contained 1.4 mM $NADP^+$, 4.0 mM $MgCl_2$, 1.0 mM UDP-glucose, 10 μM glucose 1,6-diphosphate, 66 mU of a-phosphoglucomutase, 220 mU of glucose 6-phosphate dehydrogenase, 75 mM Tris–HCl (pH 7.0) and 10 μM of a diluted enzyme preparation in a final volume of 0.5 ml. The absorbance was measured at 340 nm on a spectrophotometer using a 1 cm path cuvette at 30 °C. 1 unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1.0 μM of substrate in 1 min. The mixture with the diluted enzyme preparation that had been heated at 100 °C for 5 min was used as the control (Duan et al., 2008).

2.7. Isolation and purification of pullulan

The culture was centrifuged at 15,000 × *g* for 20 min to remove the microorganisms. After the supernatant was decanted, cells were weighed and then separated into unicellular and mycelial fractions using a 45 μm nylon mesh (Heald & Kristiansen, 1985). Weights of the unicellular and mycelial fractions were determined. An aliquot (3 ml) of the supernatant was transferred into a test tube, and then mixed thoroughly with 6 ml cold ethanol. The prepared mixture was left in a refrigerator (4 °C) for 12 h to precipitate the exocellular polysaccharide. Residual ethanol was removed carefully, then 3 ml deionized water was added and the mixture was heated to 80 °C in a water bath to dissolve the precipitate. The solution was dialyzed against deionized water for 48 h to remove small molecules. The polysaccharide was reprecipitated by adding 6 ml cold ethanol, and was recovered by filtering the mixture through pre-weighed Whatman GF/A filter paper. The filter paper with the recovered precipitate was dried at 80 °C to a constant weight (Badr-Eldin, El-Tayeb, El-Masry, Mohamad, & El-Rahman, 1994). The pullulan content of the ethanol precipitate was determined using the coupled-enzyme assay technique described by Israilides, Bocking, Smith, and Scanlon (1994). Cell biomass and pullulan content were expressed as g/l.

2.8. Analytical methods

Ash, moisture, fat and protein content of the samples were determined as per standard methods (Anon, 1984). Pullulan molecular weight (Mw) was determined by High Performance Gel Filtration Chromatography (HPGFC) on a Ultrahydrogel Size Exclusion Column, which is capable to detect Mw 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-7515 A (ERC Inc., Japan). The calibration of the detector was done with known concentrations of commercially 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., Alphabet City, NY, USA).

3. Results and discussion

3.1. Effect of changing nitrogen source on pullulan production

Effect of different nitrogen source on cell growth and pullulan production by *A. Pullulans* AP329 are shown in Table 1. The highest biomass accumulation (10.51 g/l), pullulan production (22.46 g/l) and the activity of UDPG-pyrophosphorylase (45.07 mU/mg of protein) were observed in the media containing $NaNO_2$ as a nitrogen source, and the lowest all three parameters were observed in the

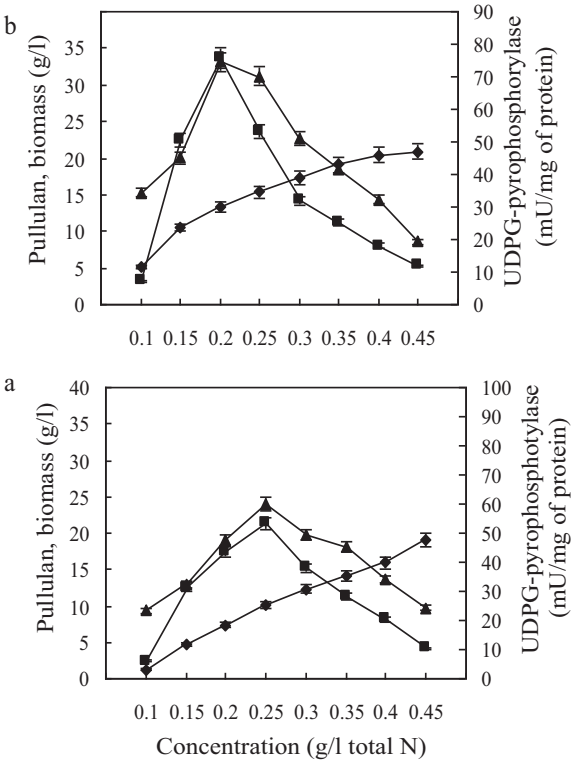

Fig. 1. Effect of level of N source on pullulan production (■), cell growth (♦) and UDPG-pyrophosphorylase (▲). (a) Nitrogen source, $(NH_4)_2SO_4$; (b) nitrogen source, $NaNO_2$. Fermentation conditions: fermentation temperature, 28 °C; initial pH, 6.5; fermentation time, 4 days. Data are shown as mean ± S.D. ($n = 3$).

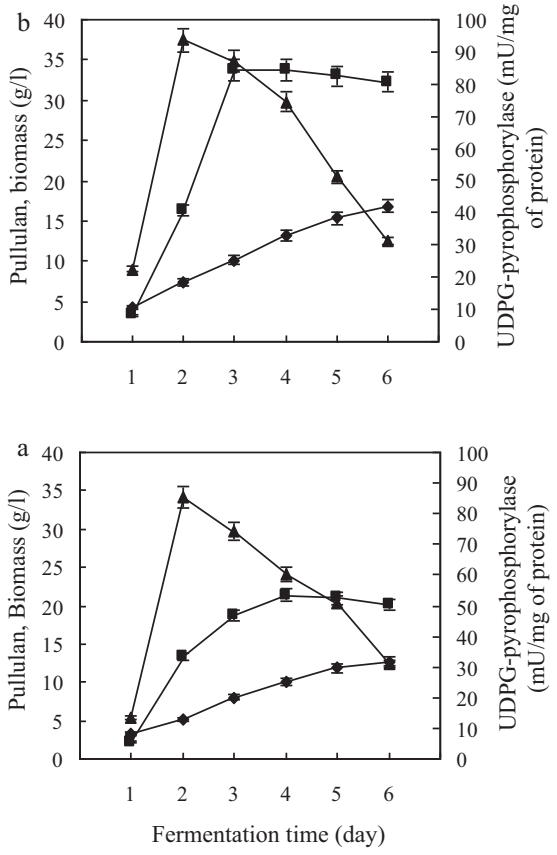

Fig. 2. Effect of time course on pullulan production (■), cell growth (♦) and UDPG-pyrophosphorylase (▲). Fermentation conditions: (a) nitrogen source, 0.25 g/l $(NH_4)_2SO_4$; pH, 4.5; fermentation temperature, 28 °C; (b) nitrogen source, 0.2 g/l $NaNO_2$; pH, 5.5; fermentation temperature, 28 °C. Data are shown as mean ± S.D. ($n = 3$).

medium containing $(NH_4)_2CO_3$. $(NH_4)_2SO_4$, although used as a universal nitrogen source for pullulan production, was proved not the best nitrogen source. Biomass accumulation (4.65 g/l), pullulan production (12.57 g/l) and the activity of UDPG-pyrophosphorylase (32.23 mU/mg of protein) of $(NH_4)_2SO_4$ containing medium were lower than those of $NaNO_2$ containing medium, but higher than those of other nitrogen source containing media. These results clearly showed that the type of nitrogen source in the culture medium affected biomass accumulation, pullulan production and the activity of UDPG-pyrophosphorylase. Therefore, $(NH_4)_2SO_4$ and $NaNO_2$ were selected for further study. Inaddition, the activity of UDPG-pyrophosphorylase was highly correlated with the amount of pullulan produced. This result was agreed with the previous reports (Duan et al., 2008). When $(NH_4)_2SO_4$ and NH_4NO_3 were used as the nitrogen sources, the biomass was almost entirely unicellular (81–83%, w/w). With $NaNO_2$, the biomass was predominantly unicellular (61%, w/w), lower than that of $(NH_4)_2SO_4$ and NH_4NO_3. These results indicate that nitrogen source also affect the morphology of the fungal population.

3.2. Effect of the concentration of nitrogen source on fermentation.

When $(NH_4)_2SO_4$ was used as the nitrogen source, maximum pullulan production (21.34 g/l) and the highest activity of UDPG-pyrophosphorylase (60.15 mU/mg of protein) were observed in medium containing 0.25 g/l N (Fig. 1a). However, pullulan production and the highest activity of UDPG-pyrophosphorylase decreased as the concentration of the nitrogen level increased above 0.25 g/l, even though biomass continued to increase. The biomass was almost exclusively unicellular cell biomass that continued to increase as the nitrogen level increased. With $NaNO_2$, pullulan yields and the highest activity of UDPG-pyrophosphorylase reached a maximum (33.68 g/l and 74.48 mU/mg of protein, respectively) in medium containing 0.2 g/l N, and decreased with nitrogen concentrations above 0.2 g/l (Fig. 2b). Results of these experiments confirmed the previous findings, namely, the activity of UDPG-pyrophosphorylase was highly correlated with the amount of pullulan produced. The unicellular cell biomass increased as the nitrogen level increased and finally was predominantly unicellular (71%, w/w). The biomass was almost exclusively unicellular cell biomass that continued to increase as the nitrogen level increased. These results indicated that increasing nitrogen levels can shift the pattern of carbon efflux within the cells of this fungus that agreed with the general view and affect the morphology of the fungal population.

3.3. Kinetics of pullulan production

Time-course studies on the production of pullulan by *A. Pullulans* AP329 were carried out for a period of 6 days in the fermentation media (Fig. 2). With $(NH_4)_2SO_4$, pullulan yield increased to reach a maximum within 4 days (21.34 g/l), and thereafter production leveled off (Fig. 2a). In the case of $NaNO_2$, the maximum pullulan production (33.83 g/l) occurred at day 3 (Fig. 2b), 58.53% greater production than $(NH_4)_2SO_4$ reaching maximum production one day earlier. In other reports, maximum pullulan production were 25.95 g/l (Vijayendra, Bansal, Prasad, & Nand, 2001), 25.1 g/l (Prasongsuk et al., 2007) and 30.28 g/l (Jiang, 2010), respectively, when $(NH_4)_2SO_4$ was used as nitrogen source. All these values were lower than those we obtained in this study $NaNO_2$. The biomass continued to increase until the end of the

Table 2
Comparison of quality of pullulan produced from $(NH_4)_2SO_4$ and $NaNO_2$.

Parameter	Source of pullulan	
	$(NH_4)_2SO_4$	$NaNO_2$
Pullulan content (%, w/w)	96.03 ± 0.67[a]	95.89 ± 0.57
Mw ($\times 10^5$)	2.7 ± 0.24	1.2 ± 0.13
Ash content (%, w/w)	1.5 ± 0.20	1.4 ± 0.21
Moisture content (%, w/w)	2.5 ± 0.30	2.6 ± 0.31

[a]Mean ± S.D. from three independent experiments.

experimental period with both nitrogen sources. This is probably due to the fact that pullulan production is not closely associated with cell growth. With both nitrogen sources, the biomass was predominantly unicellular within 1 day, mainly mycelial after day 2, and almost entirely unicellular after day 4. This indicates that mycelial is responsible for pullulan production. This result is consistent with the previous reports (Heald & Kristiansen, 1985; Cately, 1979). However, increase in pullulan production and the activity of UDPG-pyrophosphorylase reached maximum at day 2 (Fig. 2). Therefore, the activity of UDPG-pyrophosphorylase was highly correlated with the increasing rate of pullulan, rather than the amount of pullulan produced.

3.4. Characterization of the pullulan

The pullulan obtained from the cell free supernatant of fermentation broth of $(NH_4)_2SO_4$ and $NaNO_2$ was characterised. As shown in Table 2, although there was no much difference in pullulan, ash and moisture content of the samples obtained from media, the Mw of pullulan obtained from $NaNO_2$ media was higher than that of $(NH_4)_2SO_4$ media. 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.). Interestingly, the pullulan produced from $(NH_4)_2SO_4$ and $NaNO_2$ was pigment free. All samples are comparable in color, texture and water soluble ability.

4. Conclusions

The influence of nitrogen source on the pullulan production and cell growth in the 10 culture of *A. Pullulans* AP329 was explored. The optimal nitrogen sources for both cell growth and pullulan production were $NaNO_2$ and $(NH_4)_2SO_4$. With $(NH_4)_2SO_4$, the optimal concentration for pullulan production was 0.25 g/l N, and with $NaNO_2$, the optimal concentration was 0.20 g/l N. The maximum pullulan production was obtained on day 4 in medium containing $(NH_4)_2SO_4$. In medium containing $NaNO_2$, the optimal pullulan production occurred at day 3. Biomass continued to increase until the end of the experimental period (6 days) in both media. It is not unicellular, but mycelial that was responsible for pullulan production, and was not disagree with the previous reports (Heald & Kristiansen, 1985; Cately, 1979). The activity of UDPG-pyrophosphorylase was highly correlated with the increasing rate of pullulan. The type and concentration of nitrogen source, and fermentation time all affected the morphology of the fungal population, activity of UDPG-pyrophosphorylase and pullulan production.

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