BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Continuous pullulan fermentation in a biofilm reactor

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Abstract Biofilm is a natural form of cell immobilization in which microorganisms attach onto solid support. In this study, a pigment-reduced pullulan-producing strain, *Aureobasidium pullulans* (ATCC 201253), was used for continuous pullulan fermentation in a plastic composite support (PCS) biofilm reactor. Optimal conditions for the continuous pullulan production were determined by evaluating the effects of the feeding medium with various concentrations of ammonium sulfate and sucrose and dilution rate. Pullulan concentration and production rate reached maximum (8.3 g/l and 1.33 g/l/h) when 15 g/l of sucrose, 0.9 g/l of ammonium sulfate, and 0.4 g/l of yeast extract were applied in the medium, and the dilution rate was at 0.16 h^{-1}. The purity of produced pullulan was 93.0%. The ratio of hyphal cells of *A. pullulans* increased when it was grown on the PCS shaft. Overall, the increased pullulan productivity can be achieved through biomass retention by using PCS biofilm reactor.

Keywords Pullulan production · PCS biofilm reactor · Nitrogen limitation · Continuous fermentation · Cell morphology

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Introduction

Pullulan, a water-soluble exopolysaccharide synthesized by a yeast-like fungus, *Aureobasidium pullulans*, is often described as α-1,6-linked maltotriose (Ueda et al. 1963; Catley et al. 1986). With this unique linkage pattern, pullulan demonstrates distinctive physical properties, such as the adhesive ability, the capacity to form fibers, and the capacity to form thin and biodegradable film, which is transparent and highly impermeable to oxygen (Yuen 1974). Accordingly, pullulan has been used for various applications, such as blood plasma substitutes, food and cosmetic additives, adhesive additives, flocculants, resins, and environmental remediation agents (Leathers 2003; Rekha and Sharma 2007; Iyer et al. 2005).

For pullulan production, the effect of nitrogen source and its concentration has drawn much attention, and it is generally accepted that the exopolysaccharide is only produced under nitrogen-limiting conditions (Seviour et al. 1992). The depletion of nitrogen is regarded as a signal for exopolysaccharide formation of *A. pullulans* fermentation (Gibbs and Seviour 1996). Catley et al. (1986) examined the nitrogen limitation effect on *A. pullulans* for pullulan production. Their results indicated that, with similar rates of carbon utilization, the diversion of glucose from incorporation into cellular material to the production of polysaccharide relied on ammonium ion concentration. Campbell et al. (2003) also reported that NH_4^+ might impact the production of pullulan-degrading enzymes controlling carbon flow within the cell. Excess nitrogen supply could contribute to the accumulation of biomass, but not polysaccharide (Orr et al. 2009; Cheng et al. 2011).

Batch pullulan fermentation has been studied thoroughly in the past decade, and the highest pullulan concentration reached 60.7 g/L (Cheng et al. 2010c). Continuous fermentation, compared with batch fermentation, provides several advantages, such as constant environmental conditions for biological systems, elimination of substrate inhibition, and reduction of toxicity due to metabolite accumulation (Calcott 1981). In fact, several attempts have been made to enhance pullulan production by using chemostat fermentations. However, the results were not significant when compared with those in the batch fermentation. It was found that either the proposed dilution rate was extremely low (0.015 h^{-1}) with a productivity of 1.17 g/L/h (Seo et al. 2006) or the production rate was not significantly improved (0.16 and 0.73 g/L/h, respectively) (Schuster et al. 1993; Reeslev et al. 1997).

Other pullulan fermentation methods using cell immobilization have been investigated, including agar, calcium alginate, carrageenan, and polyurethane (Mulchandani and Luong 1988; Lebrun et al. 1994; West 2000; West and Strohfus 2001; Ürküt et al. 2007). However, the main concern of these immobilization materials is their durability. Calcium alginate beads, for example, only worked for four 120-h repeated batch fermentations. Pullulan production decreased from 25 to 15 g/L, and the beads lost their hardness and were disintegrated at the end of the seventh run (Ürküt et al. 2007).

Biofilm, on the other hand, grows on the solid support, which is a natural form of cell immobilization (Characklis and Marshall 1990). Plastic composite support (PCS) provides an ideal surface for biofilm formation and is an extrusion product of a mixture of polyprolylene and nutritious compounds (Pometto III et al. 1997). Polypropylene acts as a matrix and integrates agricultural mixtures, including ground soybean hulls, soybean flour, and microbial nutrients (such as bovine albumin, red blood cells, yeast extract, peptone, and mineral salts). Therefore, PCS not only provides an ideal physical structure for biofilm formation but also slowly releases nutrients during fermentation (Ho et al. 1997a). Detailed summary of advantages of biofilm reactors over suspended cell reactors, including higher biomass density and operation stability, reducing the risk of washing out when operating at high dilution rates during continuous fermentation, and eliminating need for reinoculation during repeated batch fermentation, can be found in the review articles (Demirci et al. 2007; Cheng et al. 2010a).

With these advantages of biofilm reactor mentioned previously, the scope of the present work was to investigate the effectiveness of continuous pullulan production by using a PCS biofilm reactor. Variation of initial ammonium sulfate and sucrose concentration on pullulan production was also investigated.

Materials and methods

Microorganism culture preparation

A. pullulans (ATCC 201253) was obtained from the American Type Culture Collection (Rockville, MD, USA) and was a reduced pigmentation mutant of *A. pullulans* NYS-1 (West and Strohfus 2001). The culture was grown at 30 °C for 24 h in a medium containing 50 g of glucose, 5 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 1 g of NaCl, 5 g of KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water at an initial pH 5.0 with agitation at 150 rpm. The working culture was stored at 4 °C and subcultured bi-weekly in order to maintain its viability. For long-term storage, stock cultures were maintained at −80 °C in a 20% glycerol solution.

Plastic composite support

PCS tubes were manufactured using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, South Hackensack, NJ, USA) as described by Pometto III et al. (1997). Polypropylene (50% (*w/w*)) and other ingredients (35% (*w/w*) of soybean hulls, 5% (*w/w*) of soybean flour, 5% (*w/w*) of yeast extract, 5% (*w/w*) of dried bovine red blood cell, and 0.272% (*w/w*) of sodium acetate, 0.0004% (*w/w*) of $MgCl_2 \cdot 6H_2O$, and 0.002% (*w/w*) of NaCl) were mixed together and extruded at 13 rpm through a medium pipe die with barrel temperatures of 200 °C, 220 °C, and 200 °C and a die temperature of 165 °C. The nutrition composition of PCS (soybean hulls, defatted soy bean flour, yeast extract, dried bovine red blood cell, and mineral salts) was selected as described in our previous study based on the amount of biofilm formation on the PCS (CFU per gram PCS) and pullulan production (grams per liter; Cheng et al. 2009). The extruded tubes with a wall thickness of 2.5 mm and an outer diameter of 10.5 mm were cut into 6.5-cm length tubes with both ends cut at a 45° angle (Cheng et al. 2009).

Continuous fermentation system

A continuous stir tank Sartorious Biostat B Plus bioreactor (Allentown, PA, USA) equipped with a 2-L vessel (1.5 L working volume) was implemented for studies. Twelve PCS tubes were bound to the agitator shaft in a grid-like fashion, with six rows of two parallel tubes. The reactor vessel with PCS was autoclaved with water at 121 °C for 45 min. Sucrose and nitrogenous components with mineral salts were autoclaved separately and added to the reactor aseptically after draining the water from the reactor as recommended by Pometto III et al. (1997). In order to establish biofilm on the PCS, after inoculating with a

24-h grown culture of *A. pullulans* (1%, *v/v*), five repeated batch fermentations were carried out with original medium obtained from our previous study (Cheng et al. 2010b), including 75 g of sucrose, 3 g of yeast extract, 5 g of ammonium sulfate, 1 g of NaCl, 5 g KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water, at a constant pH 5.0 with 200 rpm agitation at 30 °C for 120 h for each run.

Continuous fermentation was carried out initially in a optimized basal medium, including 100 g of sucrose, 1 g of NaCl, 5 g of KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter of deionized water, at a constant pH 5.0 with 200 rpm agitation at 30 °C (Cheng et al. 2010c). Suspension cultures without PCS were also carried out as control. Different ammonium sulfate concentrations (0.3 to 1.2 g/L) and sucrose concentration (10 to 100 g/L) on pullulan production were evaluated. Dilution rate was set from 0.05 to 0.2 h^{-1}. The results of each condition were the average of duplicates.

Sample analysis

For each change of fermentation condition, at least three volume exchanges were allowed before sampling was initiated. Samples were then collected every 12 h until consistent data were obtained.

Biomass Biomass in suspension medium was measured to evaluate the detachment of microorganism and suspension growth. Samples (1 ml) from culture broth were centrifuged at 12,000×*g*, 4 °C for 20 min. The precipitated pellets were washed twice with distilled water and centrifuged again to remove impurities. Finally, the pellet was dried at 80 °C overnight to determine biomass dry weight.

Crude pullulan After culture broth (5 ml) was centrifuged at 3,300×*g*, 4 °C for 20 min, 4 ml of supernatant was mixed with 8 ml of 95% ethanol and incubated at 4 °C for 12 h to precipitate the crude polysaccharides, which were then separated by centrifuging at 3,300×*g* for 30 min. Finally, the pellet was dried at 80 °C overnight to determine the weight of crude pullulan.

Sugar analysis For sucrose concentration, fermentation broth (3 ml) was centrifuged at 3,300×*g* for 10 min and hydrolyzed by hydrochloric acid (6 N) before analyzing with the dinitrosalicylic (DNS) method (Miller 1959).

Ammonium sulfate concentration For residual ammonium sulfate concentration, a modified indophenol method was adopted from Scheiner (1976). Briefly, 2 ml of fermentation broth samples was centrifuged at 3,000×*g* to remove biomass and insoluble debris. The supernatants were then diluted with 100-fold DI H_2O and neutralized to pH 7 with NaOH (4 N). Twenty-five milliliters of each sample was added with 10 ml phenol-nitroprusside buffer and mixed. Hypochlorite reagent (15 ml) was then added to the test and placed in room temperature, 45 min for color development. The absorbance of the sample was red under 630 nm using a spectrophotometer (DU series 500, Beckman, Fullerton, CA, USA). Reagent blank was used as control.

Cell morphology analysis At the end of fermentation, the PCS tubes in the biofilm reactor were dissembled and used for cell morphology analysis. *A. pullulans* cells on the PCS tube were detached by using a sand strip method described earlier (Ho et al. 1997b). The cell suspension was then centrifuged at 6,000×*g*, 4 °C for 20 min. After decanting the supernatant, the cells were washed three times with potassium phosphate buffer (50 mM, pH 6.8). Images were taken, including about 250 cells per sample, by using an image system consisting of an Olympus digital microscope (Model: CX41; Olympus Co., Center Valley, PA, USA) and processed by an Infinity Capture software (version 4.4.0; Lumenera Co., Lawrenceville, GA, USA).

Results

Effect of PCS biofilm reactor on continuous pullulan fermentation

Results of continuous pullulan fermentation in the PCS biofilm reactor using optimized base medium (Cheng et al. 2010c) were summarized in Table 1. *A. pullulans* produced 3.5 g/l of pullulan with a 0.17-g/l/h production rate when dilution rate was at 0.05 h^{-1} and approached zero when dilution rate was at 0.15 h^{-1}. The suspension control (Suspension 1, i.e., without PCS) also demonstrated that, without nitrogen supply, the amount of biomass was low when dilution rate was at 0.05 h^{-1} and has been completely washed out at 0.1 h^{-1}, which resulted in extremely low pullulan production. Therefore, various ammonium sulfate concentrations and 0.4 g/l of yeast extract were added as new nitrogen sources and evaluated for their effects on pullulan production.

Effects of ammonium sulfate concentration on continuous pullulan fermentation

Results of continuous pullulan fermentation with various ammonium sulfate concentrations in the PCS biofilm reactor were summarized in Table 2. With the addition of 0.9 g/l of ammonium sulfate at 0.17 h^{-1} dilution rate, *A. pullulans* produced 7.5 g/l of pullulan with a 1.28-g/l/h production

Table 1 Results of continuous pullulan fermentation using optimal medium from batch culture and controls

	Basal medium[a]			Suspension 1[b]			Suspension 2[c]		
Dilution rate (h^{-1})	0.05	0.1	0.15	0.05	0.1	0.15	0.05	0.1	0.15
Pullulan (g/l)	3.5±0.2	1.8±0.2	0.3±0.0	0.7±0.1	ND	ND	1.1±0.2	ND	ND
Pullulan production rate (g/l/h)	0.17	0.18	0.06	0.04	0.00	0.00	0.06	0.00	0.00
Residue sucrose (g/l)	94.5±3.1	97.7±3.5	98.8±2.7	99.2±1.9	100.2±2.1	100.3±2.9	98.50±3.8	100.2±4.1	100.4±3.4
$Y_{p/s}$ (%)[d]	63.6	72.2	23.0	77.7	0.0	0.0	68.7	0.0	0.0
Biomass in suspension (g/l)	0.5±0.01	0.3±0.02	0.2±0.01	0.5±0.03	ND	ND	1.0±0.01	ND	ND
Ammonium sulfate (g/l)	ND	ND	ND	ND	ND	ND	0.8±0.03	0.9±0.05	0.9±0.02

ND not detected

[a] Optimal medium obtained from batch pullulan fermentation in the PCS biofilm reactor (Cheng et al. 2010c)

[b] Continuous fermentation without PCS using base medium

[c] Continuous fermentation without PCS using optimal medium

[d] Pullulan production yield ($Y_{p/s}$)=(total pullulan produced/total sugar consumed)×%

rate, which was the highest value among all tested conditions. The pullulan production yield was 82.4%. The biomass in suspension was only 0.3 g/l, indicating that the biofilm was still firmly attached on the PCS tubes. At the mean time, ammonium sulfate was completely consumed.

The result of pullulan production with the presence of ammonium sulfate was higher than that without ammonium sulfate. A predicted optimal dilution rate, 0.16 h^{-1}, was obtained when plotting the results of pullulan production rate vs. dilution rate. The maximum pullulan production rate

Table 2 Summary of continuous pullulan fermentation in biofilm reactors with different ammonium sulfate concentrations

Ammonium sulfate (g/l)	Dilution rate (h^{-1})	Pullulan (g/l)	Pullulan production rate (g/l/h)	Residue sucrose (g/l)	$Y_{p/s}$ (%)	Biomass in suspension (g/l)	Ammonium sulfate (g/l)
0.3	0.05	8.0±0.3	0.40	88.3±2.3	67.2	0.3±0.03	ND
	0.08	7.5±0.2	0.60	89.2±3.7	68.2	0.5±0.05	ND
	0.10	6.2±0.3	0.62	91.5±2.8	71.3	0.4±0.01	ND
	0.15	3.2±0.2	0.48	94.2±3.1	53.3	0.3±0.02	ND
	0.20	1.0±0.1	0.20	97.5±3.5	37.0	0.0±0.00	0.3±0.06
0.6	0.05	9.2±0.2	0.46	87.8±3.2	74.2	0.7±0.03	ND
	0.08	8.8±0.4	0.70	90.1±4.0	87.1	0.2±0.02	ND
	0.10	8.4±0.1	0.84	89.9±2.1	81.6	0.5±0.06	ND
	0.15	4.5±0.2	0.68	93.5±3.7	67.2	0.4±0.01	0.4±0.02
	0.20	1.0±0.1	0.20	98.4±1.9	55.6	0.2±0.01	0.6±0.05
0.9	0.05	9.8±0.2	0.49	88.8±1.6	86.0	0.4±0.02	ND
	0.08	9.2±0.3	0.74	87.6±2.8	73.0	0.6±0.02	ND
	0.10	8.4±0.3	0.84	89.7±2.7	80.0	0.3±0.01	ND
	0.13	8.1±0.2	1.05	89.2±3.4	73.6	0.4±0.02	ND
	0.15	7.8±0.1	1.17	90.2±1.7	78.0	0.5±0.05	ND
	0.17	7.5±0.1	1.28	91.1±2.5	82.4	0.3±0.03	0.1±0.02
	0.20	1.3±0.0	0.26	98.1±2.2	61.9	0.1±0.00	0.8±0.05
1.2	0.05	9.3±0.4	0.47	89.7±4.1	88.6	0.7±0.06	0.3±0.01
	0.08	9.0±0.1	0.72	90.2±3.2	90.0	0.8±0.03	0.3±0.01
	0.10	7.7±0.1	0.77	91.7±2.9	90.6	0.3±0.01	0.4±0.03
	0.15	5.9±0.2	0.89	90.4±3.1	60.2	0.6±0.03	0.5±0.01
	0.20	0.8±0.0	0.16	97.3±1.9	27.6	0.1±0.00	1.2±0.03

The results were the average of two duplicates

ND not detected

reached 1.33 g/l/h at the dilution rate of 0.16 h^{-1} when ammonium sulfate was at 0.9 g/l (Table 3).

Effects of sucrose concentration on continuous pullulan fermentation

Due to the economic concern, the reduced amount of sucrose was applied to evaluate pullulan production with the optimal dilution rate and ammonium sulfate concentration obtained in the previous section. Significant decrease of pullulan concentration and production rate were observed when 10 g/l or lower sucrose concentration was used (Table 3). As a result, 15 g/l of sucrose was selected as the optimal concentration for continuous fermentation.

Morphology of *A. pullulans*

The microscopic results (Fig. 1) clearly showed that the cell type of *A. pullulans* switched from yeast-like form to hyphal form when performing pullulan fermentation in PCS biofilm reactor. The ratio of hyphal cells detached from PCS tubes was higher than that obtained in the suspension culture.

Discussion

It was reported that pullulan was a secondary metabolite and began to be synthesized by *A. pullulans* in the late exponential phase, which can be better produced when the growth becomes limited or as the cells approach stationary phase (Pouliot et al. 2005). Nevertheless, in a continuous fermentation system, cells were maintained in a growth phase, and hence the pullulan production was limited. Accumulation of less productive variants during fermentation could be another reason (Seo et al. 2006).

In this study, a PCS biofilm reactor, which can grow biomass on the solid support, was implemented for pullulan fermentation. Reasonable biomass retention on the PCS
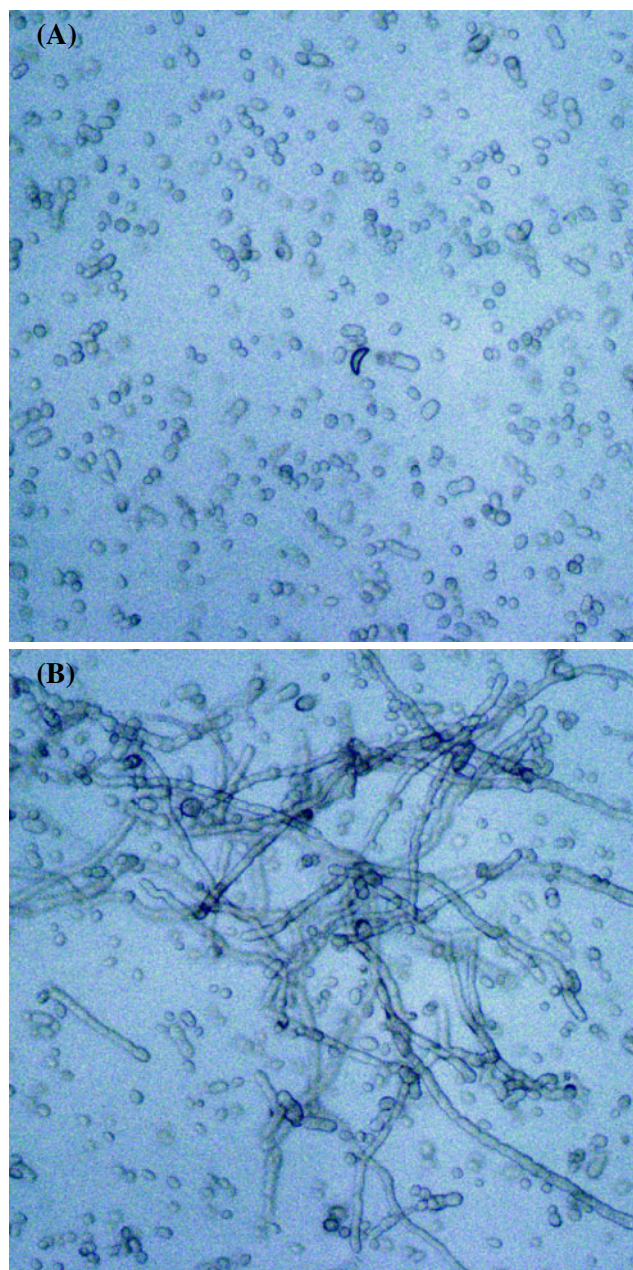

Fig. 1 Morphology of *A. pullulans* cells in the suspension culture (**a**) and detached from PCS (**b**)

Table 3 Summary of continuous pullulan fermentation in biofilm reactors with reduced sucrose concentrations

Sucrose (g/l)	Pullulan (g/l)	Pullulan production rate (g/l/h)	Residue sucrose (g/l)	$Y_{p/s}$ (%)
100	8.3±0.2	1.33	90.2±3.7	84.2
50	8.3±0.3	1.32	40.7±3.1	89.0
20	8.2±0.3	1.31	10.3±0.6	84.6
15	8.3±0.1	1.33	5.8±0.3	90.3
10	6.2±0.1	0.99	2.1±0.5	78.5

Dilution rate was set at 0.16 h^{-1}. Ammonium sulfate was at 0.9 g/l

improved both dilution rate and pullulan production and resulted in the increase of production rate. Moreover, the biomass (biofilms) grown on the solid support is considered as a pseudo-steady state, which was reported as a favored stage for pullulan production (Pouliot et al. 2005).

The PCS was noted for slowly releasing nitrogen source to the broth during fermentation (Ho et al. 1997a); however, both low pullulan concentration and production rate indicated that the slow release of nitrogen ingredients from PCS tubes can not compensate the absence of nitrogen sources in the medium during continuous fermentation. The

possible reason is that *A. pullulans* in a chemostat system may need more nitrogen to maintain its physical functions. In this study, additional 0.9 g/l of ammonium sulfate yielded the highest pullulan production rate (1.28 g/l/h; Table 2). Meanwhile, the extremely low residual ammonium sulfate also indicated that the high utilization of nitrogen source may prevent *A. pullulans* from growing excess biomass and/or producing pullulan-degrading enzymes.

Although the major type of cell which is responsible for pullulan production is still under debate, yeast-like cells and chlamydospores are considered as the main polysaccharide producers in the culture (Ronen et al. 2002; Campbell et al. 2004; Li et al. 2009). The hyphal forms of *A. pullulans* may produce exopolysaccharide which is different from pullulan (Simon et al. 1995). In this study, the ratio of hypha cells of the biomass detached from the PCS was higher than that obtained from the suspension culture. The possible reason is that the solid support is more suitable for the attachment of filamentous cells. *A. pullulans* may adjust themselves during fermentation. However, an adequate amount of pullulan-producing cells is still on the PCS, which prevent *A. pullulans* from producing other exopolysaccharides. The purity of produced pullulan maintains around 93% after 60 volume exchanges were performed at optimized condition (data not shown).

In summary, the use of PCS biofilm reactors demonstrated a positive effect on biomass density, pullulan production rate, and dilution rate. A dilution rate at 0.16 h^{-1} with 15 g/l of sucrose and 0.9 g/l of ammonium sulfate addition resulted in the highest production rate (1.33 g/l/h), which is the highest number reported to date. Utilization of low-nutrient medium is an important factor to reduce fermentation cost. Further study is needed to improve the bioreactor design for industrial purposes. First, the optimal type and quantity of nitrogen ingredients on the PCS and their leaching rate can be further investigated; second, the shape of PCS can be adjusted to accumulate more biomass (e.g., discs). The use of agricultural waste as substitute carbon source may also reduce the cost for industrial production.

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