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ORIGINAL PAPER

Pullulan production by tropical isolates of *Aureobasidium pullulans*

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Abstract Tropical isolates of *Aureobasidium pullulans* previously isolated from distinct habitats in Thailand were characterized for their capacities to produce the valuable polysaccharide, pullulan. *A. pullulans* strain NRM2, the so-called ''color variant'' strain, was the best producer, yielding 25.1 g pullulan l^{-1} after 7 days in sucrose medium with peptone as the nitrogen source. Pullulan from strain NRM2 was less pigmented than those from the other strains and was remarkably pure after a simple ethanol precipitation. The molecular weight of pullulan from all cultures dramatically decreased after 3 days growth, as analyzed by high performance size exclusion chromatography. Alpha-amylase with apparent activity against pullulan was expressed constitutively in sucrose-grown cultures and induced in starch-grown cultures. When the alpha-amylase inhibitor acarbose was added to the culture medium, pullulan of slightly higher molecular weight was obtained from late cultures, supporting the notion that alpha-amylase plays a role in the reduction of the molecular weight of pullulan during the production phase.

Keywords Amylase · *Aureobasidium pullulans* · Pullulan

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

Pullulan, an exopolysaccharide (EPS), is a linear homopolymer composed of maltotriose subunits interconnected with α-1,6 glucosidic linkages [9, 10]. This polymer is exploited in the food, cosmetic, and manufacturing industries [4]. Pullulan is produced by the yeast-like fungus *Aureobasidium pullulans* cultured on certain carbon sources, such as glucose, sucrose, and starch [9, 10].

A. pullulans, an Ascomycete in the order Dothideales, is widespread worldwide and colonizes a range of habitats, especially surfaces, such as the phyllosphere, leaf litter, and painted and plastic surfaces [3, 4, 16]. This ''black yeast'' typically produces a black melanin pigment, but brightly pigmented, the so-called ''color variant'' strains have been reported only from tropical latitudes [11, 20]. Most reported strains of *A. pullulans* are from temperate zones in the Americas and Europe and studies from tropical spheres such as Thailand are sparse, but the isolation of distinctive color variants has prompted further study of tropical strains [16]. Prasongsuk et al. [15] reported the isolation of *A. pullulans* from a variety of habitats in Thailand.

These isolates included both typical black pigmented and color variant strains. Color variant strains produce pullulan with less contaminating pigment and thus are of interest for industrial production of pullulan. In this study, the production of pullulan by these new isolates is assessed.

Alpha-amylase is produced when growing *A. pullulans* on various carbon sources [8]. Although pullulan is generally resistant to amylases, Catley [2] suggested that alpha-amylases attack rare maltotetraose subunits of pullulan, resulting in the decreased molecular weight and viscosity of pullulan often observed especially in older cultures. In this regard, the quality of pullulan (high molecular weight) potentially could be improved by inhibition of alpha-amylase activity. In this study, a decrease in the molecular weight of pullulan was observed in late cultures, and alpha-amylase activity was detected in cultures grown on starch and sucrose. The use of an amylase-inhibitor, acarbose, was evaluated for its effect on pullulan molecular weight.

Materials and methods

Microorganisms

Tropical isolates of *A. pullulans* were obtained from diverse habitats in Thailand, from leaves to indoor surfaces [15]. The higher-yielding pullulan isolates BK4, BK6, LB3, NRM2, and SK3, were identified as *A. pullulans* var. *pullulans* based on morphological characteristics [15].

Exopolysaccharide production

EPS was prepared by growing cultures in 100 ml of production medium (PM) containing glucose (5%), $(NH_4)_2SO_4$ (0.06%), K_2HPO_4 (0.5%), $MgSO_4 \cdot 7H_2O$ (0.04%), NaCl (0.1%), and yeast extract (0.04%), in 250-ml Erlenmeyer flasks. The cultures were incubated at 150 rpm and 30 ± 2°C. EPS was recovered by first removing the cells by centrifugation (10,000 × *g*, 15 min), followed by the precipitation of the EPS from the culture supernatant with two volumes of 95% ethanol. EPS was dried at 60°C.

In order to optimize EPS production, a range of nutritional parameters were evaluated, including carbon (glucose and sucrose) and nitrogen [$NaNO_3$, $(NH_4)_2SO_4$, and peptone] sources.

Analysis of EPS

Hexose content analysis

The total hexose content of EPS was determined by using the anthrone assay [7] using glucose (Sigma, St. Louis, MO) as the standard.

^{13}C-nuclear magnetic resonance spectroscopy

^{13}C-nuclear magnetic resonance (NMR) spectroscopy was carried out using a 500 MHz Bruker Avance Spectrometer (Bruker Biospin Corp., Billerica, MA) equipped with a 5 mm inverse broadband probe operating at 125.7 MHz and 70°C. The EPS sample was dissolved in 50% (v/v) D6-dimethylsulfoxide (D6-DMSO, Cambridge Isotope Laboratories Inc., Andover, MA) and H_2O at a final concentration of 2% (w/v). Samples, 0.7 ml each, in NMR tubes (Wilmad, Buena, NJ) were subjected to NMR analysis. Chemical shifts were expressed as parts per million downfield from an internal DMSO standard.

Molecular weight determination

The molecular weight of EPS was determined using high performance size-exclusion chromatography (HPSEC) and multi-angle light scattering. Samples (15 mg ml^{-1}) were prepared by dissolving dry EPS in 50 mM $NaNO_3$ containing 0.02% sodium azide. These preparations (10 µl) were applied to a Shodex KB-806 M HPSEC column (8 × 330 mm^2, Showa Denko, Tokyo, Japan) equilibrated with $NaNO_3$ (50 mM) containing sodium azide (0.02%) using a flow rate of 0.5 ml min^{-1}. Eluate from the column was analyzed on-line by multi-angle light scattering at 690 nm (Dawn EOS; Wyatt Technology Corporation, Santa Barbara, CA) with refractive index (RI) detection (Optilap DSP; Wyatt Technology Corporation, Santa Barbara, CA). A value of 0.147 g ml^{-1} was used for the dn/dc of the EPS. Molar mass and size were calculated from the light scattering and RI signals using Astra for Windows (version 4.73, Wyatt Technology Corporation, Santa Barbara, CA). Molecular weight estimates based on elution positions were made using pullulan molecular weight standards (Shodex 5,800 to 1.66 million).

Viscosity measurement

EPS samples were dissolved in distilled water (10 mg ml^{-1}). Viscosity was measured using a Brookfield Digital Rheometer model DV-III+ (Brookfield,

Middleboro, MA) at 25°C with a rotation of 125 rpm and a shear rate of 264 s^{-1}.

Enzyme assays

Alpha-amylase (E.C. 3.2.1.1) screening

Cultures of *A. pullulans* isolates were screened for their abilities to hydrolyze starch using solid culture medium containing soluble starch (1% w/v), $NaNO_3$ (0.2%), $MgSO_4 \cdot 7H_2O$ (0.05%), NaCl (0.05%), $FeSO_4$ (0.001%), and yeast extract (0.04%). After 5 days of growth, the plates were flooded with iodine solution. Haloes or pale yellow zones around the colony indicated the presence of alpha-amylase activity.

Alpha-amylase (E.C. 3.2.1.1) assay

Cultures were grown in 1% starch medium [18] and amylase activity in the culture medium assessed by measurement of the production of reducing sugars using borohydride-reduced starch as substrate [19]. The reaction mixture contained 0.5 ml of 1% borohydride-reduced starch in 50 mM sodium acetate buffer, pH 5.0 and 0.5 ml of appropriately diluted enzyme. After 30 min incubation at 50°C, reducing sugars liberated in the reaction mixture were measured by the Somogyi-Nelson method [14]. One unit (U) of alpha-amylase activity is defined as the amount of enzyme that produces 1 μmol reducing sugar per minute at 50°C.

Pullulan-degrading activity assay

Pullulan-degrading activity [18] was assayed in the supernatant of starch-grown cultures using borohydride-reduced pullulan as substrate [19]. The reaction mixture contained 0.5 ml of 1% borohydride-reduced pullulan in 50 mM sodium acetate buffer, pH 5.0 and 0.5 ml of appropriately diluted enzyme. After 30 min incubation at 50°C, the reducing sugar liberated was measured using the Somogyi-Nelson method [14]. One unit (U) of pullulan-degrading activity was defined as the amount of enzyme that produced 1 μmol reducing sugar per minute under these conditions.

Inhibition of amylase activity

Filter-sterilized amylase inhibitor, acarbose (50 mg ml^{-1}, Bayer, Leverkusen, Germany) was added to the EPS sucrose production culture medium at 0.05% (w/v). The EPSs were ethanol recovered as described above. The molecular weight of the EPSs from the 3-, 5- and 7-day cultures were determined and compared to those from cultures lacking acarbose.

Results and discussion

Exopolysaccharide production

EPS production of all 15 *A. pullulans* isolates was compared using a glucose-based medium (Table 1). Five higher-yielding strains were selected for optimization of pullulan production: BK4 (7.9 g l^{-1}), BK6 (6.1 g l^{-1}), LB3 (4.6 g l^{-1}), NRM2 (7.7 g l^{-1}), and SK3 (5.8 g l^{-1}), the latter three being apparent ''color variant'' strains.

Optimal production periods (5–7 days) were determined for these strains grown on either sucrose or glucose and using one of three nitrogen sources (Table 2). In all cases, strains gave greater yields on the disaccharide (Table 2), consistent with a number of previous studies [9, 10].

Among nitrogen sources, $(NH_4)_2SO_4$ was optimal for BK4 (23.1 g l^{-1}), BK6 (17.0 g l^{-1}) and SK3 (10.4 g l^{-1}) while peptone was better for NRM2 (25.2 g l^{-1}) and LB3 (15.2 g l^{-1}) (Table 2). In previous studies, *A. pullulans* strain NRRL 6220 gave a higher EPS yield (32.7 g l^{-1}) on $(NH_4)_2SO_4$ than on other nitrogen substrates including ammonium citrate, $NaNO_3$, and urea [1]. On the other hand, *A. pullulans* strain ATCC 42023 has given greater pullulan yields on complex nitrogen sources including peptone than that on ammonium sulfate [17]. In commercial production, peptone has been used as nitrogen source [9, 10]. Overall, these yields compare favorably with those from a number of previously described strains [9, 10].

Table 1 Extracellular polysaccharide (EPS) production from tropical isolates of *Aureobasidium pullulans*

A. pullulans strain	Colony color	EPS (g l^{-1})	Production period (days)
BK1	Black	3.7	6
BK2	Black	3.7	7
BK3	Black	4.1	7
BK4	Black	7.9	6
BK5	Black	4.6	7
BK6	Black	6.1	7
BK7	Black	4.8	7
LB1	Pink	4.0	6
LB2	Black	3.8	7
LB3	Red	4.6	6
NRM1	Pink	5.5	7
NRM2	Pink	7.7	7
SK1	Black	4.4	7
SK2	Red	4.4	7
SK3	Yellow	5.8	7

Table 2 Extracellular polysaccharide (EPS) yields of higher-yielding isolates on various carbon and nitrogen sources

A. pullulans strain	Carbon/nitrogen sources	Highest EPS yield (g l^{-1})	Production period (days)
BK4	Glucose/$(NH_4)_2SO_4$	8.0	6
	Sucrose/$(NH_4)_2SO_4$	23.1	6
	Sucrose/$NaNO_3$	14.5	6
	Sucrose/peptone	18.0	6
BK6	Glucose/$(NH_4)_2SO_4$	6.1	7
	Sucrose/$(NH_4)_2SO_4$	17.0	7
	Sucrose/$NaNO_3$	14.5	6
	Sucrose/peptone	15.9	7
LB3	Glucose/$(NH_4)_2SO_4$	5.1	6
	Sucrose/$(NH_4)_2SO_4$	13.8	6
	Sucrose/$NaNO_3$	12.4	7
	Sucrose/peptone	15.2	7
NRM2	Glucose/$(NH_4)_2SO_4$	7.7	7
	Sucrose/$(NH_4)_2SO_4$	19.6	7
	Sucrose/$NaNO_3$	17.4	6
	Sucrose/peptone	25.2	7
SK3	Glucose/$(NH_4)_2SO_4$	6.4	5
	Sucrose/$(NH_4)_2SO_4$	10.4	6
	Sucrose/$NaNO_3$	6.3	7
	Sucrose/peptone	9.2	7

During the production period, cell morphology and pH also were examined. In all cases, the pH sharply decreased within 24 h from an initial value of pH 6.5 to around pH 3, where it remained throughout EPS production. The morphology of all isolates during production was predominantly yeast blastospores, which began to germinate at day 7. Isolate SK3 also produced hyphae. Black pigmented isolates (BK4 and BK6) produced a large amount of melanin after 3 days of cultivation. Color variant isolates (LB3, NRM2, and SK3) remained cream colored throughout the 7-day fermentation.

EPS characterization

The purity of the EPSs was high, with the hexose content ranging from 95 to 99%. The EPS from *Aureobasidium* SK3 had the highest hexose content (99%) followed by that of BK4 and LB3, both at 98%, and NRM2 and BK6 at 97%. Relatively high purity (90–93%) has been obtained using *A. pullulans* strain 105-22 [7].

Structural characterization of the EPSs by ^{13}C-NMR spectroscopy yielded spectra similar to that of the pullulan standard (Showa Denko, Tokyo, Japan) (Fig. 1). The NMR spectra confirmed the homogeneity of pullulan and the absence of other glucans, such as aubasidan. Two anomeric carbon signals at 100.8 and 100.4 ppm are indicative of the C-1/C-4 α-1,4 glucosidic linkages. The signal at 98.4 ppm corresponds to the α-1,6 glucosidic bond (C-1/C-6). Overall, the spectra indicate that the basic glucosidic components are linked with a ratio of α-1,4 to α-1,6 bonds of 2:1. These NMR spectra correlate well with the structure of pullulan [6]. High purity of pullulan obtained by simple ethanol precipitation is a useful attribute for commercial production.

EPSs were recovered at days 3, 5 and 7 and their molecular weights assessed using HPSEC with detection by light scattering and RI. The molecular weights of the EPSs decreased late in culture, from a range of 547,000–2,450,000 Da at day 3 to 10,200–167,000 Da by day 7 (Table 3). The highest EPS molecular weights were from isolates BK6 and NRM2 on day 3, at 2,450,000 and 1,770,000 Da, respectively. The smallest EPS was 10,200 Da from LB3 at day 7 (Table 3). Two molecular weight species were recovered from *A. pullulans* BK4 at day 3, and both were smaller by day 5 (Table 3). On day 7, only a single EPS species was apparent. This trend also was found with the EPSs from isolates LB3 and SK3, with only a single low-molecular weight EPS polymer present by day 5. For *A. pullulans* BK6, three EPS species were recovered on day 3, which were reduced to two lower-molecular weight species on day 5 and a single species by day 7. For isolate NRM2, two high-molecular weight species and one of lower molecular weight similarly were progressively reduced to a single low-molecular weight polymer (Table 3). Thus, the trend of reduction in size of initially higher molecular weight EPS was common to all cultures studied.

The viscosities of the EPSs generally correlated with the observed molecular weight data (Table 3). Viscosities decreased from 17.41–3.49 cP on day 3 to 2.63–1.53 cP on day 7, with the highest viscosity from isolate BK6 (17.41 cP) on day 3 and the lowest from isolate SK3 on day 7. The overall viscosity of the pullulan product is a key commercial parameter.

Enzyme production

All of the five selected *Aureobasidium* cultures produced alpha-amylase based on the starch agar plate culture assay (Fig. 2). The slower growing isolate SK3 formed well-defined haloes, perhaps a result of clearer visualization as the culture did not overgrow the potential zones of clearing. Several yeasts are known to produce amylases, including *Candida*, *Cryptococcus*, *Debaryomyces*, *Rhodotorula*, and *Trichosporon* [12]. *A. pullulans* strains in particular can produce both alpha-amylase and glucoamylase [12]. In a major comparative study, all of 198 strains of *A. pullulans* showed amylase activities on starch (0.2%) agar plates [5].

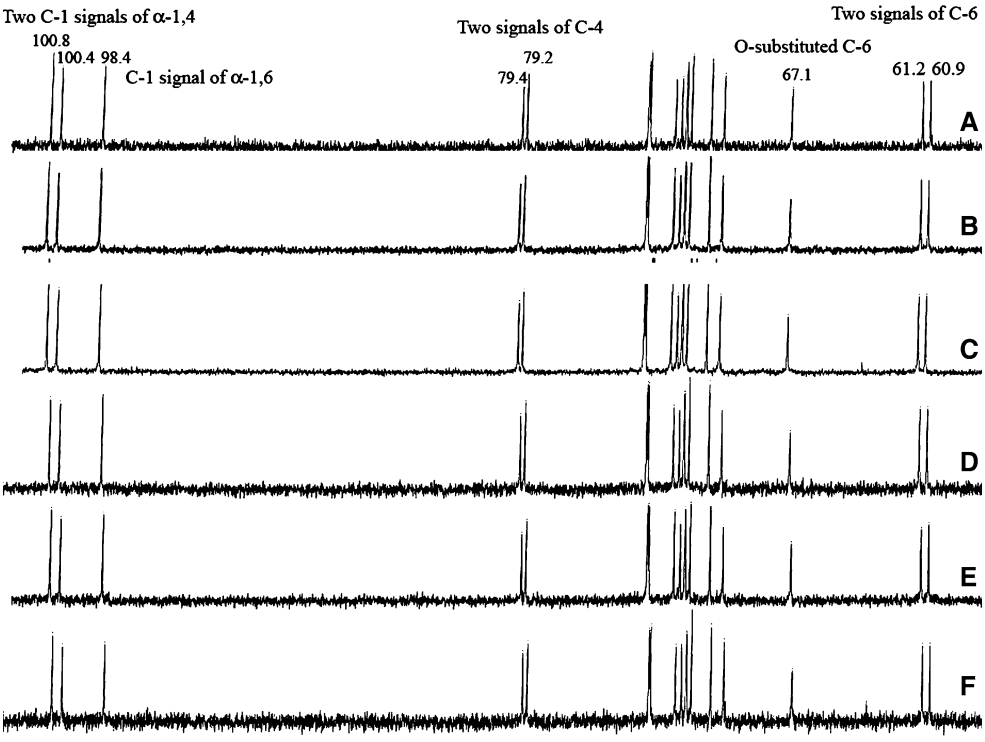

Fig. 1 ^{13}C-NMR spectrum of (*A*) pullulan, (*B*) EPS from *A. pullulans* BK4, (*C*) EPS from *A. pullulans* BK6, (*D*) EPS from *A. pullulans* LB3, (*E*) EPS from *A. pullulans* NRM2 and (*F*) EPS from *A. pullulans* SK3

All five cultures exhibited greater alpha-amylase activity than pullulan-degrading activity (Table 4). The maximal alpha-amylase activity was from *Aureobasidium* NRM2 grown on starch on day 7 (0.738 U ml^{-1}) while the maximal pullulan-degrading activity was produced by strain BK6 at day 5 (0.040 U ml^{-1}). The lowest alpha-amylase and pullulanase levels were from *Aureobasidium* SK3 on day 7 (Table 4). On sucrose medium, *A. pullulans* isolates produced alpha-amylase ranging from 0.024 up to 0.408 U ml^{-1}, and pullulan-degrading activity from 0.002 to 0.013 U ml^{-1} (Table 4). The highest alpha-amylase yield from sucrose-grown cells was from *A. pullulans* NRM2 on day 7 (0.408 U ml^{-1}) while the highest pullulan-degrading activity was from *A. pullulans* BK4 (0.013 U ml^{-1}). Overall, strains appeared to produce constitutive levels of enzymes when cultured on sucrose, and induced enzyme levels on starch. These results are consistent with previous studies of *Aureobasidium* strain NRRL Y-12974 [8].

Table 3 Reduction of molecular weight and viscosity of EPS from *A. pullulans* during fermentation

EPS of *A. pullulans* strain	Production period (days)	Number of polymer	Molecular weight (Da)	Viscosity (cP)
BK4	3	2	580,000/335,000	15.26
	5	2	151,000/86,700	4.50
	7	1	13,300	1.53
BK6	3	3	2,450,000/582,000/27,000	17.41
	5	2	372,000/19,400	2.84
	7	1	22,400	2.15
LB3	3	2	385,000/162,000	6.05
	5	1	27,500	2.63
	7	1	10,200	2.60
NRM2	3	3	1,770,000/1,325,000/30,500	15.25
	5	1	315,000	3.18
	7	1	167,000	2.15
SK3	3	2	470,000/113,000	3.49
	5	1	149,000	2.56
	7	1	147,000	1.53

Inhibition of amylase activity

As discussed above, *Aureobasidium* NRM2 produced a high yield of pullulan (25.1 g l^{-1}) by day 7 but the molecular weight was reduced with time (Table 3). We further established that this strain produces alpha-amylase and pullulan-degrading activities (Table 4). Catley [2] proposed that amylase activity in culture supernatants could reduce the molecular weight of pullulan in the later stages of the fermentation. In order to produce a higher molecular weight pullulan, the addition of an amylase inhibitor to the culture medium was considered. Thus, the molecular weights of the EPSs produced in the presence and absence of the alpha-amylase inhibitor acarbose were compared. EPS produced by strain NRM2 at day 3 was of high molecular weight. At days 3 and 5, pullulan showed

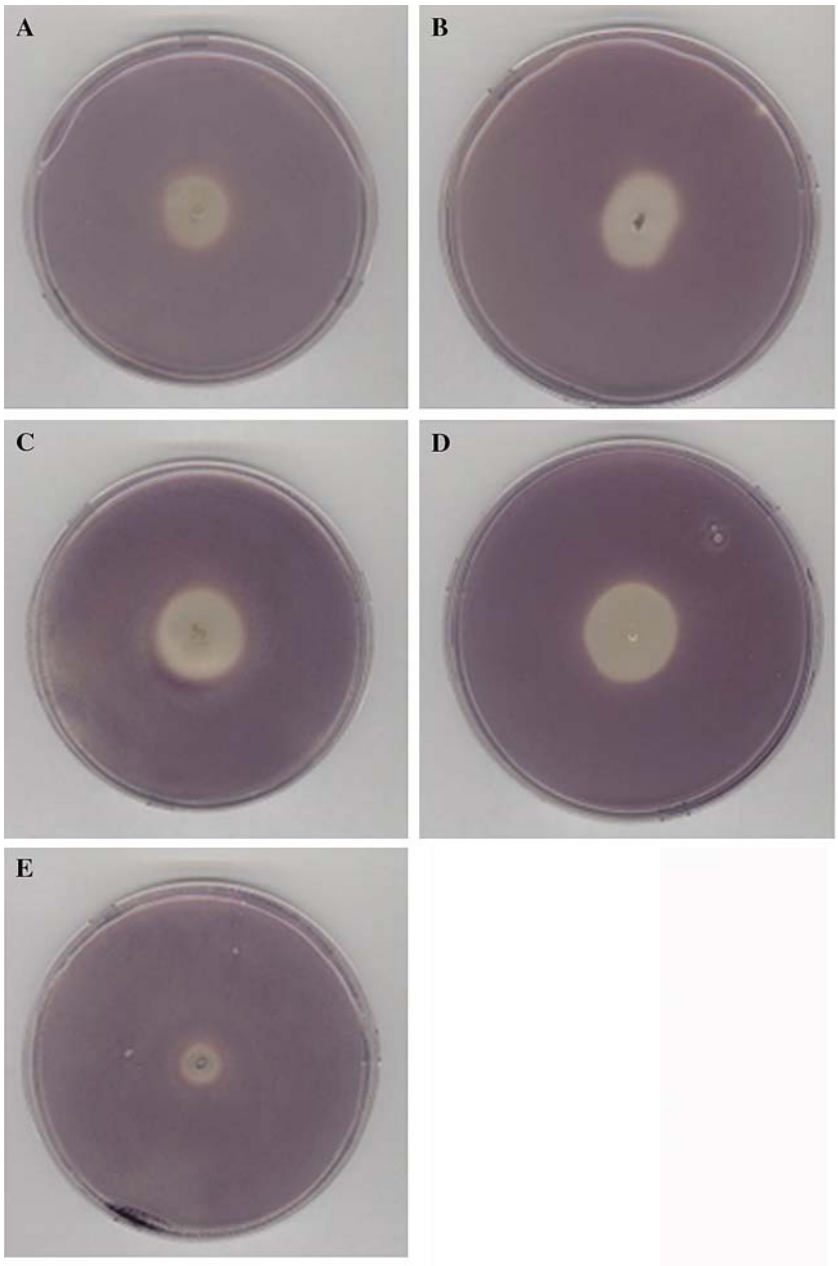

Fig. 2 Alpha-amylase/iodine screening of *A. pullulans* strain. (*A*) *A. pullulans* BK4, (*B*) *A. pullulans* BK6, (*C*) *A. pullulans* LB3, (*D*) *A. pullulans* NRM2 and (*E*) *A. pullulans* SK3

Table 4 Alpha-amylase and pullulan-degrading activities in culture supernatant of *A. pullulans* grown on sucrose and starch media

A. pullulans	Starch				Sucrose			
	Alpha-amylase activity (U ml^{-1})		Pullulan-degrading activity (U ml^{-1})		Alpha-amylase activity (U ml^{-1})		Pullulan-degrading activity (U ml^{-1})	
	Day 5	Day 7	Day 5	Day 7	Day 5	Day 7	Day 5	Day 7
BK4	0.250	0.402	0.036	0.020	0.070	0.186	0.007	0.013
BK6	0.153	0.230	0.040	0.024	0.053	0.129	0.005	0.009
LB3	0.247	0.176	0.029	0.010	0.065	0.134	0.004	0.002
NRM2	0.695	0.738	0.016	0.017	0.170	0.408	0.004	0.005
SK3	0.135	0.130	0.033	0.009	0.024	0.100	0.004	0.004

considerable degradation to lower molecular weight species (Table 3). In the presence of acarbose, the ratio of high molecular weight to lower molecular weight products was reduced, although considerable degradation still was apparent (data not shown). These results are consistent with the findings of Miura et al. [13] and suggest that alpha-amylase does play a role in the reduction of pullulan molecular weight in strain NRM2, although other enzymes such as pullulanase may also be involved. Although it may be possible to enhance the molecular weight of pullulan by the judicious use of inhibitors, a more practical approach might be to use alpha-amylase negative mutants. The full potential role of enzymatic reduction of pullulan molecular weight remains to be resolved.

In summary, tropical isolates of *A. pullulans* from Thailand produced EPSs, giving better yields when cultured on sucrose than on glucose. The production of the EPSs was dependent on the nitrogen source and differed among strains. $(NH_4)_2SO_4$ resulted in higher yields from strains BK4, BK6, and SK3, while peptone was the better nitrogen source for strains NRM2 and LB3. Under optimal nutrient status (sucrose and peptone), a maximal EPS yield of 25.1 g l^{-1} was obtained from strain NRM2 after 7 days. The culture pH decreased from pH 6.5 to around pH 3 within 24 hr and remained acidic through 7 days. Only blastospores and hyphae appear responsible for EPS production. All EPSs were homogeneous pullulans as determined by anthrone carbohydrate analysis and ^{13}C-NMR spectroscopy. The higher-molecular weight EPSs were from strain BK6 (2,450,000 Da) and NRM2 (1,770,000 Da) after 3 days of culture. The molecular weight of EPSs from all cultures decreased in late cultures, presumably as a result of extracellular hydrolytic enzymes such as alpha-amylase. In assays for these enzymes, all strains of *A. pullulans* were positive for alpha-amylase and pullulan-degrading activity in liquid starch-based medium. Cultures grown on sucrose produced lower, constitutive enzyme activities. In an attempt to retain high-molecular weight pullulan in late cultures, an amylase inhibitor (acarbose) was incorporated into the culture medium. EPS from late cultures of strain NRM2 was somewhat less degraded in the presence of acarbose. This suggests that alpha-amylase is at least partly responsible for the reduction in the molecular weight of pullulan from strain NRM2, and efforts to reduce this activity would yield a higher molecular weight product.

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