Carbohydrate Polymers 87 (2012) 1696–1700

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Optimization of fermentation conditions for the production of pullulan by a new strain of *Aureobasidium pullulans* isolated from sea mud and its characterization

Shengjun Wu*, Jing Chen, Saikun Pan

School of Marine Science and Technology, HuaiHai Institute of Technology, 59 Cangwu Road, Lianyungang 222005, China

ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form
13 September 2011
Accepted 26 September 2011
Available online 1 October 2011

Keywords:
Pullulan
DNA characterization
Sea mud
Fermentation conditions optimization

ABSTRACT

A yeast-like fungal strain was isolated from sea mud samples from the beach of the yellow sea near Lianyungang, Jiangsu, China. The characteristics of this strain, i.e., colony, morphology, DNA molecular, and product indicated that it is related to *Aureobasidium pullulans* family and named *A. pullulans* CJ001. The fermentation conditions were optimized: fermentation time 4 d, initial pH 6.0, and temperature 22 °C. Under these optimized conditions, this strain produced 31.25 g/L of melanin-free exopolysaccharide (EPS). Fourier-transform infrared (FTIR) spectroscopy of this EPS is almost identical to that of standard pullulan, and High Performance Liquid Chromatography (HPLC) of the hydrolysates of the EPS with pullulanase, which is capable of cutting off α-(1 → 6) linkages, showed that the main composition is maltotriose, and thus confirming the pullulan structure of this EPS.

1. Introduction

Pullulan is an exocellular polysaccharide produced by yeast-like fungus *Aureobasidium pullulans*. It is mainly a linear maltotriose repeating units interconnected by α-(1 → 6) glycosidic bonds. Pullulan can form thin films that are transparent, oil resistant and impermeable to oxygen, and therefore it can be used as coating and packaging material, sizing agent for paper, starch replacer in low-calorie food formulations, cosmetic emulsions and industrial applications (Deshpande, Rale, & Lynch, 1992). In recent years, modified pullulan has been frequently reported for its applications in pharmaceutical and chemical industry (Alban, Schauerte, & Franz, 2002; Masci, Bontempo, & Crescenzi, 2002; Sivakumar & Rao, 2003).

A. pullulans is a black yeast or yeast-like fungus widely spread in all ecological niches, e.g., forest soils, fresh and sea water, plant, and animal tissues, etc. (Leathers, 2003). However, there are very few reports of isolation of *A. pullulans* from sea mud. A number of sea mud samples were collected from the Eastern of China, and 113 strains were isolated from these sea mud samples in our laboratory. Among the strains, an isolate was suspected to be *A. pullulans* according to the phenotypic characteristics that were identical to those of *A. pullulans* and named *A. pullulans* CJ001. Therefore, in this study, it was further characterized by sequencing the 26 S DNA, and the fermentation conditions were optimized, and the EPS molecular structure was characterized.

2. Materials and methods

2.1. Isolation and phenotypic characterization of the LDT-1

The fungal strain LDT-1 used was isolated from sea mud samples from the beach of the yellow sea near Lianyungang, Jiangsu, China. The sea mud samples were homogenized in 5.0 mL sterile water and filtered. 100 μL of filtrates were serially diluted from 10^{-1} to 10^{-6} with sterile water and spread on YPD plates containing (g/L) 10 yeast extract, 20 peptone, 20 dextrose, 15 agar supplemented with chloramphenicol (50 mg/l) and streptomycin (30 mg/L) to suppress bacterial growth. The plates were incubated at 25, 30 and 37 °C and were observed daily for the presence of yeast colonies. The colonies were isolated at different time intervals, purified, and stored in 10% glycerol at −70 °C and liquid nitrogen for long-term maintenance (Choudhurya, Salujab, & Prasadb, 2011). The morphological, physiological and biochemical properties of LDT-1are determined according to the methods described by Yarrow (1998).

2.2. DNA molecular characterization of LDT-1

DNA isolation was performed according to the methods described by Ausubel et al. (2011). Amplification and sequencing of the 26 S DNA were done also as described by Ausubel et al. (2011). The similarity of gene sequence was analyzed using GenBank BLASTN (Altschul et al., 1997), and the gene sequences were

* Corresponding author. Tel.: +86 0518 85895427; fax: +86 0518 85895428.
E-mail addresses: wushengjun008@sina.com, chenjwxy1973@163.com (S. Wu).

0144-8617/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2011.09.078

aligned employing the CLUSTAL X program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). In the case of the neighbor-joining analysis, distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980).

2.3. Microorganism

LDT-1, isolated from the sea mud in the east of china, was maintained at 4 °C on potato dextrose agar (PDA) and subcultured every 2 weeks.

2.4. Preparation of medium

The medium contained: 50 g sucrose, 2.0 g yeast extract, 0.77 g K_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, and 1.98 g NaCl in 1 L distilled water (Chen, Wu, & Pan, 2011). The pH was adjusted to 5.5, and the medium was autoclaved at 121 °C for 15 min.

2.5. Fermentation

Seed cultures were prepared by inoculating cells grown on a PDA agar slant into a 250-mL flask that contained 50 mL of the inoculum medium and subsequently incubated at 22 °C for 48 h with shaking at 200 rpm. 2.5 mL of the seed culture were transferred into the 250-mL flask containing 50 mL of the fermentation media. The culture was shaken at 22 °C and with 200 rpm for 6 d (Wu, Jin, Tong, & Chen, 2009).

2.6. Isolation and purification of pullulan

The culture was centrifuged at 15,000 × *g* for 20 min to remove the microorganisms. The biomass (mycelia and yeast-like cells) dry weight was determined by washing the sediment with distilled water and drying at 105 °C overnight. 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). Pullulan content and biomass were expressed as g/L.

2.7. Analytical methods

Pullulan molecular weight (M_W) was determined by High Performance Gel Filtration Chromatography (HPGFC) (LC-10A, Shimadzu, Japan) on a Ultrahydrogel Size Exclusion Column, which is capable of determining M_W 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.) (Jiang, Wu, & Kim, 2011). FT-IR spectra of representative pullulan samples were collected in KBr pellets on a Nicolet Nexus FTIR 470 spectrophotometer over a wavelength range of 400–4000 cm^{-1}. The representative pullulan solutions (3%, w/w) were hydrolyzed at pH 5.0 and temperature 45 °C for 6 h by 10 ASPU/g pullulanase (Wu, Chen, Tong, Xu, & Jin, 2009). The composition of the sugar in the hydrolysates was analyzed by Water600 HPLC equipped with a double column system. The first column (Sugarpark 1, 6.5 mm × 300 mm i.d.) used pure

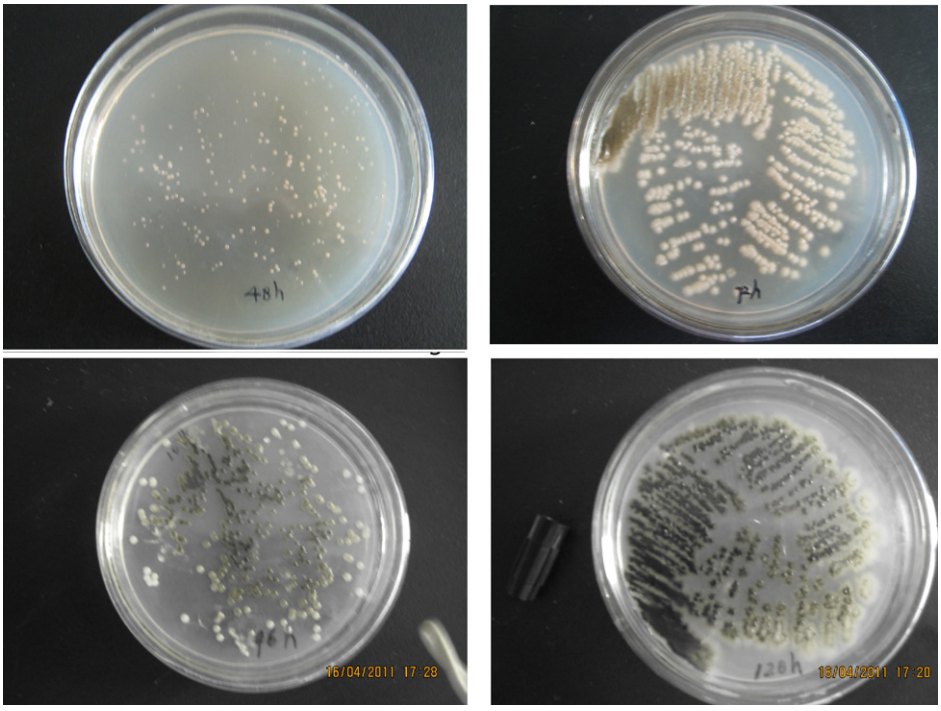

Fig. 1. The photos of colonies of LDT-1.

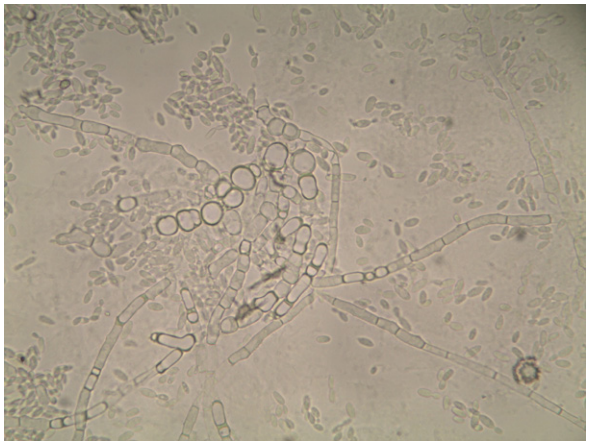

Fig. 2. The photos of morphology of LDT-1.

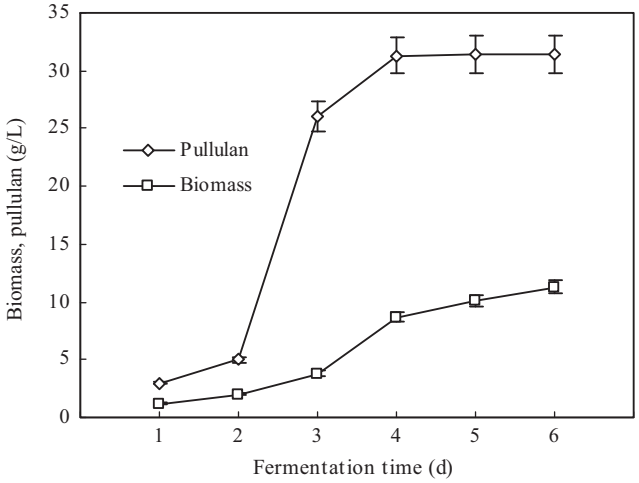

Fig. 4. Effect of fermentation time on pullulan production and cell growth. Data are shown as mean ± SD ($n = 3$).

water as mobile phase at a flow rate of 0.5 mL/min and the column temperature was maintained at 85 °C. The second column (SpherisorbNH$_2$, 4.6 mm × 250 mm i.d.) used acetonitrile/water (70/30, v/v) as mobile phase at a flow rate of 1 mL/min and the column temperature was 30 °C. The detector sensitivity was 4 and the injection volume was 10 μL (Wu, Jin, et al., 2009).

3. Results and discussion

3.1. Phenotypic characteristics of LDT-1

The early colonies of LDT-1 showed dirty white and slimy, and then gradually transformed into black leather-like wrinkles (Fig. 1). LDT-1 has five different cell morphologies: yeast-like cells, young blastospores, swollen blastospores, chlamidospores and mycelia (Fig. 2). All these phenotypic characteristics of the isolate are identical to those of *A. pullulans*, strongly indicating that LDT-1 belongs to *A. pullulans* family (Slepecky & Starmer, 2009).

3.2. DNA molecular characterization of LDT-1

Phylogenetic tree constructed with 26 S DNA sequences of LDT-1 with type strains of accepted varieties of *A. pullulans* and related species showed that LDT-1 locates between *A. pullulans* (EU304243) and *A. pullulans* (HQ629552) (Fig. 3), confirming that LDT-1 belongs to *A. pullulans* family. Therefore, LDT-1 was named *A. pullulans* CJ001.

3.3. Time course of fermentation

Kinetics studies on the production of pullulan by *A. pullulans* CJ001 were made for a period of 6 d. There was a sharp increase over 2–3 d and a slight increase from 3 d to 4 d in pullulan production. Maximum pullulan yield was observed after 4 d. However, the biomass increased during the whole experimental period (Fig. 4). So it is evident from the results that the production of pullulan is not associated with cell growth.

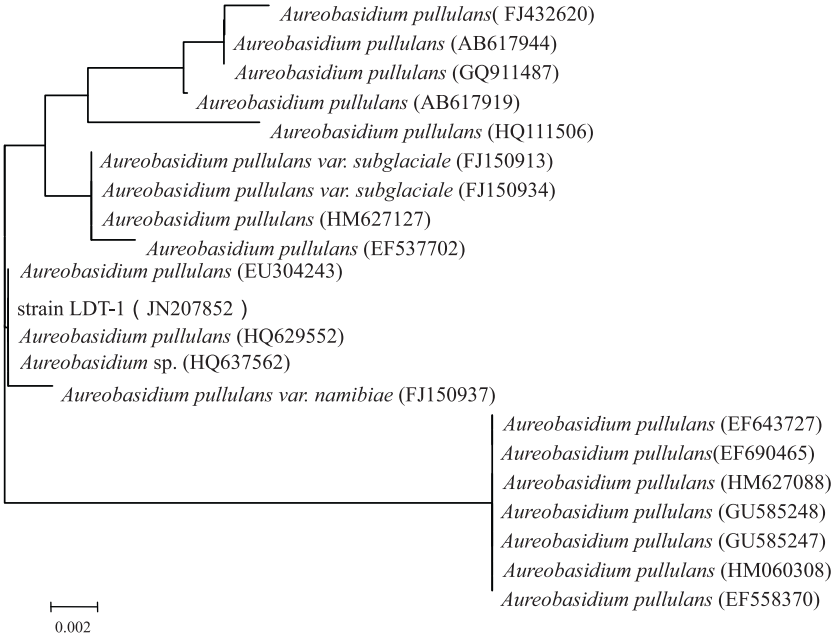

Fig. 3. Neighbor-joining tree of LDT-1.

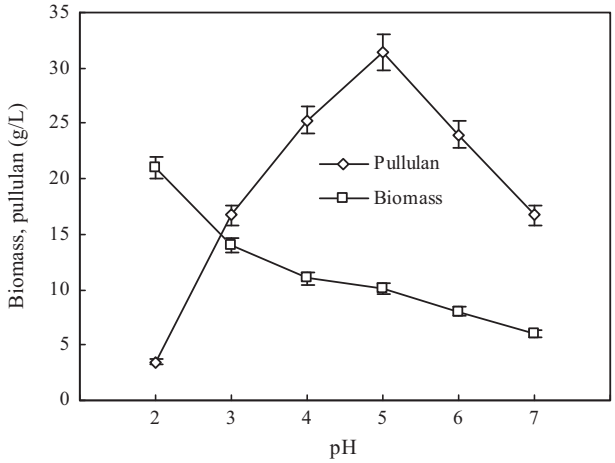

Fig. 5. Effect of pH on pullulan production and cell growth. Data are shown as mean ± SD ($n = 3$).

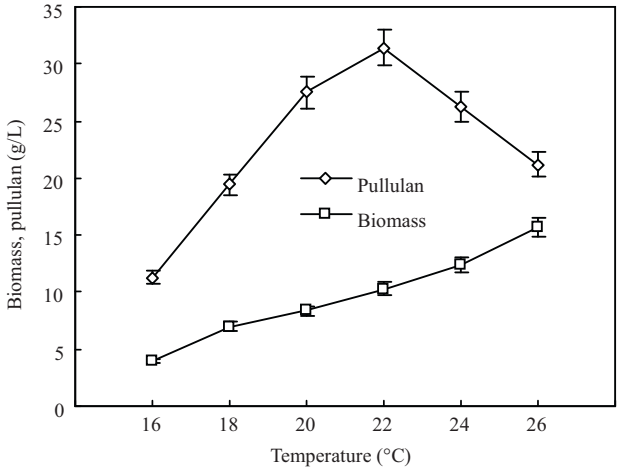

Fig. 6. Effect of temperature on pullulan production and cell growth. Data are shown as mean ± SD ($n = 3$).

3.4. Effect of initial pH on pullulan production

Effect of initial pH values ranging from 2 to 7 in the media on pullulan production by the microorganism was investigated and the results were shown in Fig. 5. Yeast-like cell was observed at all the initial levels of pH, and relatively low pH is suitable for biomass growth and this observation is in agreement with those reported by Wu, Jin, et al. (2009). Maximum pullulan production (31.4 g/L) in the medium broth was observed at an initial pH of 5. In contrast to our results, several previous reports indicated that the optimal pH values for pullulan production were obtained at initial pH of 5.0 (Vijayendra, Bansal, Prasad, & Nand, 2001), 5.5 (Wu, Jin, et al., 2009), 6.0 (Ono, Yasuda, & Ueda, 1977), 6.5 (Roukas & Biliaderis, 1995), and 7.5 (Auer & Seviour, 1990). The different optimum initial pH conditions reported may be related to function of the strain type, medium composition, and fermentation conditions used. From the results in Fig. 4, an initial pH of 5.5 is optimum for pullulan production.

3.5. Effect of temperature on pullulan production

A study was conducted to investigate the effect of temperature varying from 16 °C to 26 °C. Maximum pullulan production was achieved at a temperature of 22 °C (Fig. 6). In contrast, other reports have described optimal conditions for pullulan production as temperature 20 °C (Roukas & Biliaderis, 1995), 24 °C (McNeil & Kristiansen, 1990), and 26 °C (Wu, Chen, Jin, & Tong, 2010). The different optimal temperature conditions reported in the literature may be due to the differences in the types of strain, composition of fermentation medium and culture conditions used. However, cell growth of *A. pullulans* CJ001 increased with temperature in the range of 16–26 °C. Therefore, the optimal temperature of cell growth is also not in accordance with that of pullulan production.

3.6. Characterization of the exopolysaccharide

The M_W of the exopolysaccharide prepared in this study is 2.6×10^5 Da. FT-IR spectra of the exopolysaccharide prepared in this experiment and the pullulan obtained from Japan Pharmacopoeia are almost identical, indicating the major composition of this exopolysaccharide is pullulan (Fig. 7) (Chen et al., 2011). The confirmation of the polysaccharide as pullulan was also done by subjecting the polysaccharide to hydrolysis with pullulanase, which is capable of cutting off α-(1 → 6) linkages. The main sugar form of the hydrolysates of the exopolysaccharide by pullulanase

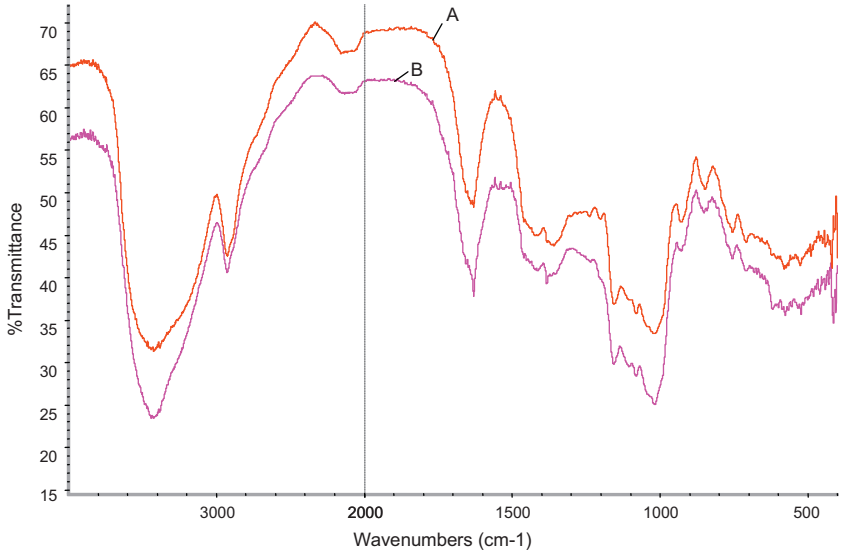

Fig. 7. FT-IR spectra of the pullulan prepared in this experiment (A) and obtained from Japan Pharmacopoeia (B).

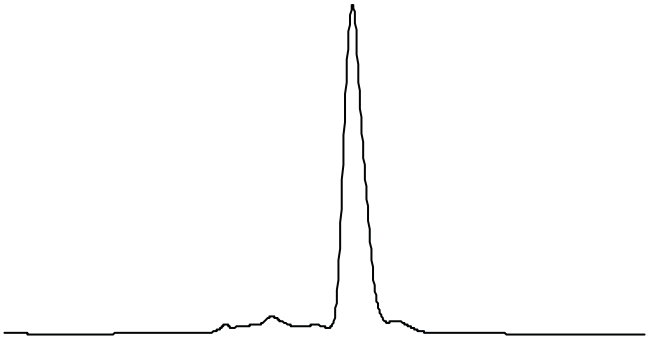

Fig. 8. HPLC profile of the hydrolysates.

was judged to be maltotriose by HPLC method, indicating pullulan structure of this exopolysaccharide (Fig. 8) (Chen et al., 2011).

4. Conclusions

An isolate from sea mud was judged to be *A. pullulans* by phenotypic and DNA molecular characterization. The optimized fermentation conditions for the pullulan production by *A. pullulans* CJ001 were found to be time 4 d, pH 5.0, and temperature 22 °C. Under the optimized conditions, the maximum pullulan production of 31.40 (g/L) was reached in the fermentation culture of *A. pullulans* CJ001. The molecular structure of pullulan prepared in this study was conformed by FT-IR and HPLC method.

References

Alban, S., Schauerte, A. & Franz, G. (2002). Anticoagulant sulfated polysaccharides: Part I. Synthesis and structure–activity relationships of new pullulan sulfates. *Carbohydrate Polymers*, *47*, 267–276.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, *25*, 3389–3402.

Auer, D. P. F. & Seviour, R. J. (1990). Influence of varying nitrogen sources on polysaccharide production by *Aureobasidium pullulans* in batch culture. *Applied Microbiology and Biotechnology*, *32*, 637–644.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. M., Smith, J. A. & Struhl, K. (2011). *Current protocols in molecular biology*. New York, USA: Greene Publishing Associates.

Badr-Eldin, S. M., El-Tayeb, O. M., El-Masry, E. G., Mohamad, O. A. & El-Rahman, O. A. A. (1994). Polysaccharide production by *Aureobasidium pullulans*: Factors affecting polysaccharide formation. *World Journal of Microbiology and Biotechnology*, *10*, 423–426.

Chen, J., Wu, S. J. & Pan, S. K. (2011). Optimization of medium for pullulan production using a novel strain of *Aureobasidium pullulans* isolated from sea mud through response surface methodology. *Carbohydrate Polymers*, doi:10.1016/j.carbpol.2011.08.062

Choudhurya, A. R., Salujab, P. & Prasadb, G. S. (2011). Pullulan production by an osmotolerant *Aureobasidium pullulans* RBF-4A3 isolated from flowers of *Caesulia axillaris*. *Carbohydrate Polymers*, *83*, 1547–1552.

Deshpande, M. S., Rale, V. B. & Lynch, J. M. (1992). *Aureobasidium pullulans* in applied microbiology: A status report. *Enzyme and Microbial Technology*, *14*, 514–527.

Israilides, C., Bocking, M., Smith, A. & Scanlon, B. (1994). A novel rapid coupled enzyme assay for the estimation of pullulan. *Biotechnology and Applied Biochemistry*, *19*, 285–291.

Jiang, L. F., Wu, S. J. & Kim, J. M. (2011). Influence of varying nitrogen sources on activities of UDPG-pyrophosphorylase involved in pullulan synthesis and pullulan production by *Aureobasidium pullulans*. *Carbohydrate Polymers*, *86*, 1085–1088.

Kimura, M. (1980). A simple method for estimation evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111–120.

Leathers, T. D. (2003). Biotechnological production and applications of pullulan. *Applied Microbiology and Biotechnology*, *62*, 468–473.

Masci, G., Bontempo, D. & Crescenzi, V. (2002). Synthesis and characterization of thermoresponsive N-isopropylacrylamide/methacrylated pullulan hydrogels. *Polymer*, *43*, 5587–5593.

McNeil, B. & Kristiansen, B. (1990). Temperature effects on polysaccharide formation by *Aureobasidium pullulans* in stirred tanks. *Enzyme and Microbial Technology*, *12*, 521–526.

Ono, K., Yasuda, N. & Ueda, S. (1977). Effect of pH on pullulan elaboration by *Aureobasidium pullulans* S.1. *Agricultural and Biological Chemistry*, *44*, 2113–2118.

Roukas, T. & Biliaderis, C. G. (1995). Evaluation of carob pod as a substrate for pullulan production by *Aureobasidium pullulans*. *Applied Biochemistry and Biotechnology*, *55*, 27–44.

Sivakumar, P. A. & Rao, K. P. (2003). The use of cholesteryl pullulan for the preparation of stable vincristine liposomes. *Carbohydrate Polymers*, *51*, 327–332.

Slepecky, R. A. & Starmer, W. T. (2009). Phenotypic plasticity in fungi: A review with observations on *Aureobasidium pullulans*. *Mycologia*, *101*(6), 823–832.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The Clustal × windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *24*, 4876–4882.

Vijayendra, S. V. N., Bansal, D., Prasad, M. S. & Nand, K. (2001). Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77. *Process Biochemistry*, *37*, 359–364.

Wu, S. J., Chen, H., Jin, Q. & Tong, Z. Y. Q. Y. (2010). Effect of two-stage temperature on pullulan production by *Aureobasidium Pullulans*. *World Journal of Microbiology and Biotechnology*, *26*, 737–741.

Wu, S. J., Chen, H. Q., Tong, Q. Y., Xu, X. M. & Jin, Z. Y. (2009). Preparation of maltotriose by hydrolyzing of pullulan with pullulanase. *European Food Research and Technology*, *229*, 821–824.

Wu, S. J., Jin, Z. Y., Tong, Q. Y. & Chen, H. Q. (2009). Sweet potato: A novel substrate for pullulan production by *Aureobasidium pullulans*. *Carbohydrate Polymers*, *76*, 645–649.

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In C. P. Kurtzman, & J. W. Fell (Eds.), *The yeasts, a taxonomic study* (4th ed., pp. 77–100). Amsterdam, The Netherlands: Elsevier.