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Pullulan production by an osmotolerant *Aureobasidium pullulans* RBF-4A3 isolated from flowers of *Caesulia axillaris*

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ABSTRACT

Phenotypic and molecular characterization of five yeast-like fungal isolates from flowers of wild plants showed that they are related to *Aureobasidium pullulans*. Compared to other isolates, an osmotolerant and non-pigmented isolate *A. pullulans* RBF-4A3 produced 26.35 g l^{-1} of melanin-free exopolysaccharide (EPS) in 96 h at 30 °C in 5% glucose containing medium. At higher concentrations of glucose (7.5–25% (w/v)), the EPS produced by this organism increased from 34.68 to 66.79 g l^{-1} up to 15% (w/v) glucose, with a productivity of 16.69 g l^{-1} per day. Beyond 15% (w/v) glucose concentration, the EPS production decreased gradually to 43.29 g l^{-1} at 25% (w/v) glucose. Fourier-transform infrared (FTIR) spectroscopy confirmed that chemical structures of the exopolysaccharide produced by *A. pullulans* RBF-4A3 and standard pullulan were identical. This is the first report of pullulan production at 15% (w/v) concentration of glucose by an osmotolerant strain of *A. pullulans*.

1. Introduction

Microbially produced polysaccharides have properties that are very useful in various industrial applications. Pullulan a biopolymer synthesized by yeast-like fungal species *Aureobasidium pullulans*, is a linear α-D-glucan built of maltotriose subunits, connected by α-1,6-D-glucosidic and α-1,4-D-glucosidic linkages in 2:1 ratio to produce a linear glucan (Shingel, 2004). This typical feature is responsible for structural flexibility and superior solubility of pullulan (Leathers, 1993), and confers it with certain unique physical and chemical properties such as film and fibre-forming capability, impermeability to oxygen, non-reducing and biodegradable nature (Leathers, 2003). The film- and fibre-forming characteristics of pullulan make it an ideal material for compression mouldings, in packing industries for coating and packing material, as a sizing agent for paper and in plywood manufacturing (Leathers, 2003; Singh, Saini, & Kennedy, 2008). Pullulan is non-mutagenic, non-toxic, tasteless, odourless and edible (Kimoto et al., 1997), and is being used as a starch replacer in low-calorie food formulations, in cosmetics, lotions and shampoos (Leathers, 2003).

Pullulan is being used extensively in the food industry as a food ingredient for over 20 years in Japan, and has Generally Regarded As Safe (GRAS) status in the USA (US FDA, 2002). Pullulan which was earlier considered as an indigestible polymer was shown to be slowly digestible and found application as a low-calorie food additive providing bulk and texture (Wolf, 2005). Recently pullulan is also being investigated for its biomedical applications in various aspects like targeted drug and gene delivery, tissue engineering, wound healing and in diagnostic imaging using quantum dots (Rekha & Sharma, 2007). Despite the large number of uses, some of the problems associated with fermentative production of pullulan are (i) the formation of a melanin pigment; (ii) the inhibitory effects caused by high sugar concentrations in the medium; and (iii) the high cost associated with pullulan precipitation and recovery (Youssef, Roukas, & Biliaderis, 1999).

The producer of pullulan, *A. pullulans,* is a black yeast or yeast-like fungus widely spread in all ecological niches including forest soils, fresh and sea water, plant and animal tissues, etc. (Leathers, 2003). It is also found on the phylloplane along with other yeast-like fungi of the genera *Taphrina* and *Lalaria* (Inácio et al., 2004). Interestingly, most of the recent reports of pullulan production by *A. pullulans* are from plant leaves (Chi & Zhao, 2003; Manitchotpisit et al., 2009; Prasongsuk, Sullivan, Kuhirun, Eveleigh, & Punnapayak, 2005; Singh & Saini, 2008). Although it has been long known that flowers in general and floral nectar in particular often contains dense yeast populations (Brysch-Herzberg, 2004; Herrera, de Vega, Canto, & Pozo, 2009), there are very few reports of isolation *A. pul-*

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Table 1
Details of *Aureobasidium pullulans* isolates used in this study and their nucleotide sequence accession numbers.

S. no.	Strain designation, and EMBL accession number of nucleotide sequence	Colony colour	Source of isolation	Place of isolation
1.	RBF-3B2 FN665417	Cream	Flowers of *Andropogonis echioides*	Kota, Rajasthan, India
2.	RBF-4A3 FN665413	Cream	Flowers of *Caesulia axillaris*	Rawatbhata, Rajasthan, India
3.	RBF-8B1 FN665419	Cream to pink	Flowers of wild plant	Rawatbhata, Rajasthan, India
4.	RBF-17A2 FN665420	Cream to pink	Flowers of a Fabaceae family plant	Near Chambal River, Kota, Rajasthan, India
5.	RBF-17BR13 FN665421	Cream to pink	Flowers of a Malavaceae family plant	Rawatbhata, Rajasthan, India

lulans from flowers (Lachance et al., 2001; Loncaric, Oberlerchner, Heissenberger, & Moosbeckhofer, 2009). In a recent study *A. pullulans* was found in 7% of nectar samples from different flower samples, and a significant correlation was found between incidence of yeast species in nectar and their reported ability to grow in a medium containing 50% glucose (Pozo et al., 2010). Literature survey has shown that there are no reports of exopolysaccharide production by *A. pullulans* strains isolated from flowers.

During the survey of yeast species associated with flowers, about 150 yeast strains were isolated from Rawatbhata town (25: 10: 34N, 15: 49: 51E), Rajasthan state of India. The isolates were divided into different groups based on microsatellite fingerprinting patterns. A few isolates from each group were characterized by sequencing the D1/D2 region of the large-subunit rRNA gene (LSUrRNA gene). These identifications revealed the presence of some species belonging to the genera *Aureobasidium, Candida*, *Cryptococcus*, *Debaryomyces*, *Lodderomyces*, *Metschinikowia*, *Pichia*, *Pseudozyma*, *Rhodotorula*, *Sympodiomycopsis*, *Trichosporonoides*, and *Wickerhamiella* (Saluja, 2010; Saluja & Prasad, 2007, 2008). Among these isolates, five yeast-like fungi showing *Aureobasidium*-like morphology were observed. In the present paper we describe the pullulan production by a non-pigmented, osmotolerant and high pullulan producing isolate RBF-4A3.

2. Materials and methods

2.1. Isolation and phenotypic characterization of isolates

The fungal strains used in this study were isolated from flower samples from places nearby Rawatbhata town (25: 10: 34N, 15: 49: 51E), Rajasthan state in India, details of the place and source of isolation are given in Table 1. After removing of the petals the flowers were homogenized in 2.0 ml sterile water. One hundred microliters of serial dilutions from 10^{-1} to 10^{-6} were spread on YPD plates containing ($g\,l^{-1}$) 10 yeast extract, 20 peptone, 20 dextrose, 15 agar) supplemented with chloramphenicol ($50\,mg\,l^{-1}$) and streptomycin ($30\,mg\,l^{-1}$) 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. The methods used to determine the morphological, physiological and biochemical properties are as described by Yarrow (1998). Ability of the cultures to grow at different concentrations of glucose was examined by supplementing the yeast nitrogen base medium with different concentration of glucose.

2.2. Molecular characterization of isolates

DNA isolation was done with the MasterPure Yeast DNA purification kit (Epicentre Technologies) according to the manufacturer's instructions. Amplification and sequencing of the Internal Transcribed Spacer region (ITS1, 5.8S rDNA and ITS2 regions) of rRNA gene cluster and the D1/D2 domain of LSU rRNA gene were done as described earlier (Saluja & Prasad, 2008). Processing of the samples for loading onto an ABI 3130 *xl* Genetic Analyzer sequencer was performed according to the instructions of the manufacturer (Applied Biosystems). A sequence-similarity search was done using GenBank BLASTN (Altschul et al., 1997). Sequences were aligned using the CLUSTAL X program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). For the neighbour-joining analysis (Saitou & Nei, 1987), distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

2.3. Screening for exopolysaccharide (EPS) production

Inoculum was prepared inoculating yeast colonies grown on a YPD agar plates into a 25-ml flask that contained 5 ml of the medium (pH 6.0) with subsequent incubation at 30 °C for 24 h with shaking at 200 rpm. 2.5 ml of the cultures was transferred into the 250-ml flask containing 50 ml of the production medium (pH 6.0) containing 5% (w/v) carbon source. The culture flasks were incubated at 30 °C and 200 rpm for 144 h, samples were taken for analysis at 24 h intervals up to 144 h.

2.4. Effect of different carbon sources on EPS production

The production medium for EPS production contained 5% (w/v) of carbon source viz. glucose, sucrose, maltose or lactose. Other constituents of the media and fermentation conditions were kept unchanged. EPS and biomass were measured at 24 h intervals up to 144 h.

2.5. Effect of different concentrations of glucose on EPS production

The media for production of EPS with various concentrations of glucose ranging from 5% to 25% (w/v) was same as the above except for the concentration of glucose. Biomass and residual glucose concentration were also measured for each sample. Residual sugar concentration was measured as per the method of Miller (1959) using Perkin Elmer spectrophotometer (Lambda 35 UV/VIS). All experiments were done in triplicate and average values are presented in results.

2.6. Isolation, purification and characterization of EPS

The methods for isolation and purification of EPS were adopted from earlier studies (Chi & Zhao, 2003; Leathers, Nofsinger, Kurtzman, & Bothast, 1988) with minor modifications. Samples were withdrawn at 24 h intervals the culture broth was centrifuged (Sigma 6K 15) at 12,000 rpm and 4 °C for 10 min to remove cells. For precipitation of exopolysaccharide (EPS), two volumes of ethanol were added to 5 ml of cell free culture broth in a test tube, and kept at 4 °C for 12 h. The precipitate was separated using centrifugation at 12,000 rpm at 4 °C for 10 min (Sigma 6K 15). Small molecules precipitated along with the EPS were separated by dissolving the precipitate in 5 ml of deionised water at 80 °C, followed by dialysis against deionised water for 48 h. The EPS was re-precipitated

using two volumes of cold ethanol as mentioned earlier, and the precipitate was dried at 80 °C to a constant weight.

Polysaccharide (%) was estimated as grams of pullulan (dry weight) produced per 100 ml of fermented broth. Polysaccharide yield was expressed as polysaccharide per 100 g of sugar consumed, whereas sugar utilization was taken as a ratio of sugar consumed over the total amount of added sugar multiplied by 100.

The characterization of EPS was carried out using IR spectroscopy. Pullulan samples (2 mg) were manually blended with 60 mg of 95% potassium bromide powder. These mixtures were then desiccated overnight at 50 °C under reduced pressure prior to FTIR measurement. The FT-IR spectra were measured over potassium bromide pellets and pullulan from Sigma, USA was used as a standard. Fourier transform infrared (FTIR) spectra were recorded with a Perkin Elmer spectrophotometer over a range of 4000–400 cm^{-1}, 16 scans with a resolution of 2 cm^{-1} were acquired and averaged.

3. Results and discussion

3.1. Phenotypic and molecular characterization of A. pullulans isolates

Five yeast-like fungal isolates used in this study showed morphological similarity with *A. pullulans*. Variations in colony morphologies were observed among these isolates. Colonies of two isolates (3B2 and 8B1) were cream to pink coloured for 2 days on the plates, then turned to black. Three other isolates (RBF-17A2, RBF-17Br13 and RBF-4A3) remained cream coloured at 25 °C for 2 weeks. On detailed characterization, isolate RBF-4A3 was found to be osmotolerant and was able to grow in presence of 50% glucose concentration. This isolate could assimilate glucose, fructose, D-galactose, L-sorbose, D-ribose, D-xylose, L-arabinose, L-rhamnose, sucrose, maltose, melizitose, α,α-trehalose, methyl-α-D-glucoside, melibiose, lactose, inulin, starch, erythritol, myo-inositol, salicin, arbutin, glycerol, ribitol, xylitol, L-arabinitol, D-glucono-1,5-lactone, D-gluconic acid sodium salt and 2-keto-D-gluconate. These observations are similar to the characteristics of the type strain of *A. pullulans* (Kurtzman & Fell, 1998). It could not assimilate D-glucitol, D-mannitol, galactitol, ribitol, D-glucuronate, DL-lactate, succinate, citrate, where as the type strain of *A. pullulans* was reported to assimilate D-glucitol, D-mannitol and ribitol. Variations in utilization of different carbon and nitrogen compounds by different *A. pullulans* isolates were reported earlier (Singh & Saini, 2008). It is unable to grow in the presence of 0.01% cycloheximide, and is able to grow in 50%, but not at 60% glucose. Acetic acid production is absent, production of starch like compounds is negative.

Sequence analysis of ITS-D1/D2 domains confirmed that these five isolates are related to *A. pullulans*. All the sequences are submitted in the nucleotide sequence database and the isolates along with their EMBL accession numbers are given in Table 1. Molecular characterization of isolates is very important for correct identification of the producing strains, in some of the earlier reports a strain of *Rhodotorula bacarum* Y68 was reported to produce pullulan (Chi & Zhao, 2003). The authors claimed that it was identified using BIOLOG system and routine phenotypic methods used for yeast identification (Chi & Zhao, 2003; Zhao & Chi, 2003). In subsequent papers the same strain Y68 was reported as *A. pullulans* (Duan, Chi, Li, & Gao, 2007; Duan, Chi, Wang, & Wang, 2008) leading to confusion for several years.

Phylogenetic tree constructed with ITS and D1/D2 domain sequences of our isolates with type strains of accepted varieties of *A. pullulans* and related species showed that the two pigment forming isolates (RDF-3B2 and RBF-8C1) are related to *A. pullulans* var. *melanigenum* that is known to produce melanin (Fig. 1). Three non-pigmented isolates RBF-17A2, RBF-17BR13, and 4A3 are closely related to each other and formed a separate cluster supported by 100% bootstrap values, and they formed a loose cluster and appear to be sister-group of *Kabatiella microstricta* CBS 114.64 and *Kabatiella caulivora* CBS 242.64.

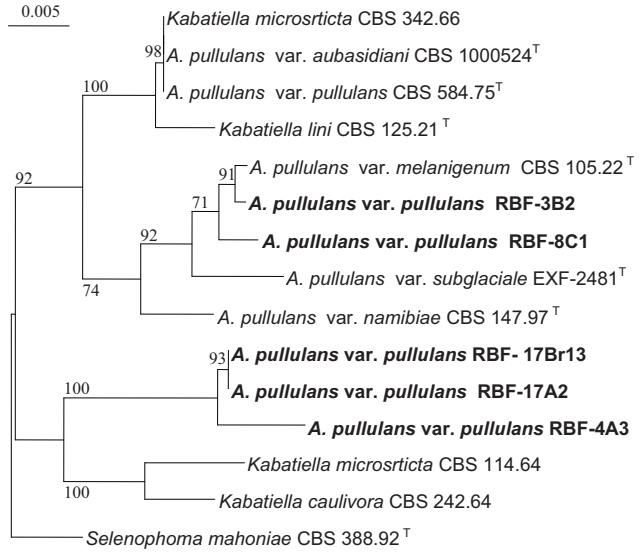

Fig. 1. Neighbor-joining tree of *Aureobasidium* and related species based on combined sequences Internal Transcribed Spacer (ITS) region and of the D1/D2 domain of LSU rRNA gene. Evolutionary distances were calculated according to Kimura (1980). Numbers at the node represent 100 replicate bootstrap samplings. Bootstrap values less than 70% are not shown. CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; EXF = Culture Collection of Extremophilic Fungi, Ljubljana, Slovenia.

3.2. Exopolysaccharide production by A. pullulans isolates

The exopolysaccharide (EPS) production by different isolates in 5% glucose medium ranged from 9.5 to 26.35 g l^{-1} EPS in 120 h (Fig. 2) isolate RBF-4A3 being the highest EPS producer, followed by RBF-3B2 (g l^{-1}) (Fig. 2). With similar glucose concentration, Lee et al. (2001) obtained 15 g l^{-1} pullulan with *A. pullulans* ATCC 42023. Tropical isolates of *A. pullulans* from Thailand produced

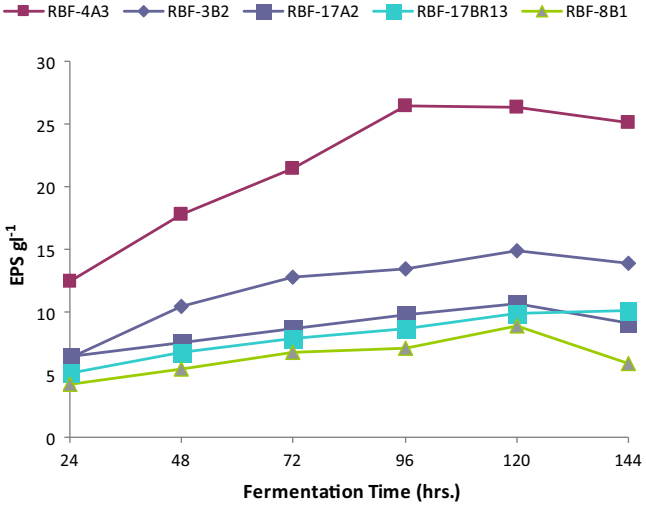

Fig. 2. Exopolysaccharide production by five flower isolates of *A. Pullulans* in 5% (w/v) glucose containing medium 30 °C. *X*-axis = fermentation time (h); *Y*-axis = g l^{-1} exopolysaccharide produced (results are average of three experiments).

Table 2
Comparative study of EPS production and yield by five *A. pullulans* flower isolates in 5% glucose containing medium.

Strain designation	EPS ($g l^{-1}$)	EPS yield ($g l^{-1}$ sugar)	Biomass ($g l^{-1}$)	EPS yield ($g l^{-1}$ biomass)	Residual sugar ($g l^{-1}$)	Utilization of carbon source (%)
RBF-3B2	14.9	0.298	9.24	1.61	5.1	89.9
RBF-4A3	26.35	0.525	9.97	2.63	4.5	91.0
RBF-8B1	8.85	0.177	7.32	1.20	5.6	88.8
RBF-17A2	10.62	0.212	6.51	1.63	6.3	87.4
RBF-17BR13	9.91	0.198	6.47	1.53	6.5	87.0

3.7–7.9 $g l^{-1}$ EPS in 6–7 days (Prasongsuk et al., 2007). In a recent study 52 isolates (43 isolates collected from leaves) of *A. pullulans* from Thailand were examined for pullulan production and xylanase activity (Manitchotpisit et al., 2009). Most of these isolates were found to be poor or moderate producers of EPS on 5% sucrose containing medium, only 2 isolates could produce more than 25 $g l^{-1}$ EPS. Seo et al. (2001) showed that with optimization of culture conditions the EPS production by some *A. pullulans* strains can be increased from 10.4 to 25.2 $g l^{-1}$ for different strains. Compared to the above studies, one of our flower isolate RBF-4A3 produced 26.35 $g l^{-1}$ of EPS in 5 days with 5% glucose medium without any optimization of fermentation parameters. The culture was found to maintain its cream colour throughout the fermentation period and produced exopolysaccharide free of melanin pigment.

The effect of 5% glucose concentration on EPS production, biomass production, EPS yield and sugar utilization pattern of five flower isolates is shown in Table 2. Even though the sugar consumption pattern of all the five isolates is almost similar, the EPS production and yield by the isolate RBF-4A3 are markedly different from all other isolates. The results show that the EPS yield in terms of per gram sugar consumed (0.525), and yield in terms of EPS/g biomass (2.63) is highest for flower isolate RBF-4A3. These results are better as compared with earlier results (Lee et al., 2001; Singh & Saini, 2008).

3.3. Effect of different carbon sources on EPS production

Most of the reports on pullulan productions have used either glucose (Chi & Zhao, 2003; Prasongsuk et al., 2005, 2007; Punnapayak, Sudhadham, Prasongsuk, & Pichayangkura, 2003) or sucrose as the substrate (Gibson & Coughlin, 2002; Singh & Saini, 2008; Youssef et al., 1999) for production of pullulan. Based on the preliminary experiments using 5% glucose as carbon source, isolate RBF-4A3 that produced higher exopolysaccharide with high yield and sugar consumption patterns was also examined for its ability to produce EPS in three other different carbon sources (sucrose, maltose and lactose) at 5% (w/v) concentration. From Table 3 it is evident that glucose is a better carbon source for production of EPS by RBF-4A3, lactose supported least EPS production. Most of the earlier reports suggest that sucrose is a better substrate for EPS production compared to glucose. Gibson and Coughlin (2002) examined effect of glucose and sucrose on EPS production by *A. pullulans* NRRLY-2311-1 in 5% glucose and sucrose medium and found that sucrose was better substrate for EPS yield and conversion efficiency by this isolate. Prasongsuk et al. (2007) compared EPS production by 5 different isolates of *A. pullulans* using sucrose or glucose as carbon source and found that sucrose supported 2–4 times more EPS production. In a recent study Ravella et al. (2010) examined effect of five different carbon sources at 5% concentration on pullulan production and found that sucrose is best carbon source, followed by fructose. Interestingly glucose supported very little EPS production with their isolate. Contrary to the above reports, Duan et al. (2007) found that glucose was better substrate for pullulan production compared to other carbons sources. Our results confirm the earlier observations that the preference of substrate for production EPS is strain specific.

Table 3
Comparative study of biomass and EPS production by *A. pullulans* RBF-4A3 in media containing different carbon sources (5% (w/v)).

Age (h)	Biomass ($g l^{-1}$)				EPS ($g l^{-1}$)			
	Glucose	Sucrose	Maltose	Lactose	Glucose	Sucrose	Maltose	Lactose
24	12.7	11.2	12.97	4.2	18.2	14.3	7.8	2.2
48	15.8	14.6	14.2	4.9	20.1	15.7	9.4	4.7
72	16.6	17.9	12.6	4.6	22.3	18.3	9.2	4.3
96	17.3	17.5	10.5	3.5	26.5	20.5	8.1	4.2
120	17.6	15.2	8.6	2.3	25.5	19.5	7.1	3.1

3.4. Effect of different concentrations of glucose on exopolysaccharide and biomass production by A. pullulans 4A3

Despite the large number of fermentation studies of *A. pullulans* reported in the literature, there are very few reports of isolates using beyond 5% sugar-containing carbon source for pullulan production. It is believed that inhibitory effects caused by high sugar concentration (more than 5%), is one of the reasons for not using more than 5% sugar concentration for production of pullulan. Shin, Kim, Lee, Kim, and Byun (1987) reported that the inhibitory effect of high sugar concentration could be overcome and a higher pullulan level (58 $g l^{-1}$) may be achieved using a fed-batch culture system. EPS production by *A. pullulans* Y68 (reported as *R. bacarum* in the paper, and later as *A. pullulans*) was examined at 4–10% (w/v) glucose concentration, and 8% glucose was found to be optimum for production of EPS by this culture, there after it declined (Chi & Zhao, 2003). Gibson and Coughlin (2002) examined pullulan production by *A. pullulans* NRRLY-2311-1 in three different concentrations (2.5%, 5%, and 10%) of sucrose, and found there was very marginal increase in pullulan production at 10% (w/v), compared to 5% (w/v) sucrose concentration, and pullulan yield was better at 5% (w/v).

As the physiological characterization of isolate *A. pullulans* RBF-4A3 showed that it is able to grow at 50% glucose concentration, the ability of this isolate to produce EPS at higher concentrations of glucose was examined. Glucose concentrations ranging from 5% to 25% (w/v) were used for examining the EPS production, biomass, change in pH, consumption of glucose. Results presented in Fig. 3 show that the organism is able produce EPS at all the concentrations examined, optimum being 15% (w/v) glucose, at which it produced 66.79 $g l^{-1}$ EPS in 96 h, the biomass was also found to be highest (34.94 $g l^{-1}$) at this concentration. Sugar utilization pattern, production of biomass and EPS, and change in the pH of the optimum glucose concentration (15% (w/v)) medium are given in Fig. 4. The pH of the medium dropped down from initial 6.5 to 5.2 till 72 h, when the consumption of sugar was vigorous. From 96 h onwards it started increasing and reached 6.8 at 144 h. Our observation with isolate RBF-4A3 are different from earlier studies that showed generally there is drop in pH of the medium from pH 6.5 to pH 4.5 after 24 h. Chi and Zhao (2003) reported pH 7.0 is better for pullulan production by *A. pullulans* Y68, and found that the pH drops down to 6.0 after 60 h. In another study pH of the medium dropped 6.5 to around 3.0 pH within 24 h and continued to be acidic for 7 days

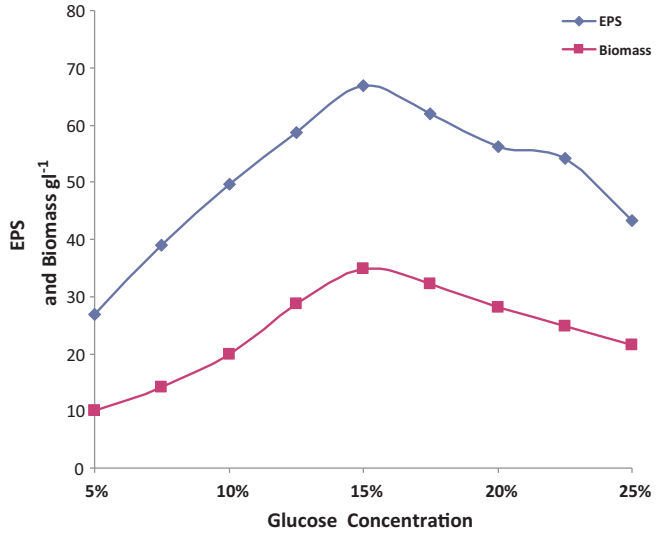

Fig. 3. Effect of different concentrations of glucose on exopolysaccharide and biomass production by *A. pullulans* 4A3 at 96 h time at 30 °C (results are average of three experiments).

(Prasongsuk et al., 2007). The consumption of glucose also coincided with the production of EPS and biomass, at 24 h the organism could consume 56 g l^{-1} of glucose and produced 28.23 g l^{-1} of EPS (Fig. 4). More than 95% of glucose was consumed by 96 h and at this point of time the EPS production was 66.79 g l^{-1}. Recently Ravella et al. (2010) reported 40.1 g l^{-1} EPS with 12.5 g l^{-1} productivity per day by *A. pullulans* strain isolated from biogas reactor. Our culture could produce 66.79 g l^{-1} EPS with 16.69 g l^{-1} productivity per day. This is also the first report of exopolysaccharide production by *A. pullulans* in a media containing more than 10% (w/v) glucose.

As observed in 5% glucose concentration, the EPS produced by RBF-4A3 at higher concentrations was also found to be free of melanin pigment. This is significant as any strain that produce high amounts of pullulan along with melanin may not be useful for industrial production of pullulan, because demelanization of pullulan by adsorption on activated charcoal or by use of solvent/salt combinations increases the cost of pullulan production (Kachhawa, Bhattacharjee, & Singhal, 2003).

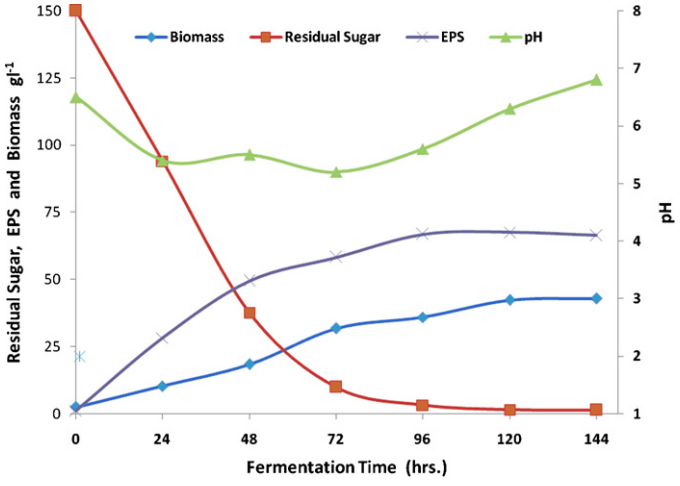

Fig. 4. Time course of exopolysaccharide and biomass production, utilization of glucose and changes in pH by *A. pullulans* 4A3 in 15% (w/v) glucose medium at 30 °C (results are average of three experiments).

Table 4
Comparison of FTIR data from Sigma pullulan and EPS produced by *A. pullulans* RBF-4A3.

Assignment	Sigma pullulan wave number (cm^{-1})	EPS of RBF-4A3 wave number (cm^{-1})
O–H str	3399.4	3397.9
C–H str	2924.7	2927.9
O–C–O str	1651.9	1653.1
C–O–H bend	1418.6	1418.4
C–O–C str	1156.0	1154.7
α-1,6-Glucosidic bonds	1022.1	1018.7
α-D-Glucopyranoside	852.8	847.5
α-1,4-D-Glucosidic bonds	756.0	755.3

3.5. FTIR analysis of the exopolysaccharide

FT-IR spectra for standard pullulan (Sigma) used as a reference and purified exopolysaccharide EPS obtained from the *A. pullulans* 4A3 given in Table 4 are almost identical. FTIR spectrograms of our isolate and Sigma pullulan standard are given in Appendix BSupplementary Fig. 1. The strong absorption at 3397.9 cm^{-1} indicated that both the pullulans have some repeating units of –OH as in sugars. The other strong absorption at 2927.9 cm^{-1} indicated a sp3 C–H bond of alkane compounds existed in the sample. In the specific area (1500–650 cm^{-1}) which is characteristic for the pullulan molecule as a whole, the spectra for standard pullulan as well as that produced by RBF-4A3 exhibited similar features. Absorption in 847.5 cm^{-1} is characteristic of the α-D-glucopiranoside units. Absorption in 755.3 cm^{-1} indicates the presence of α-(1-4)-D-glucosidic bonds, and spectra in 1018 cm^{-1} proved the presence of α-(1-6)-D-glucosidic bonds. These results confirmed that the chemical structure of the EPS of isolate RBF-4A3 is pullulan.

4. Conclusions

Efficient strains of *A. pullulans* normally convert 50–65% of carbon source into exopolysaccharide. Therefore one way of increasing the exopolysaccharide production is screening osmotolerant strains that can tolerate higher concentrations of carbon source and convert it efficiently to exopolysaccharide. In the present study we have shown that a non-pigmented, osmotolerant yeast strain isolated from flowers could produce 66.79 g l^{-1} in 15% (w/v) glucose, with a productivity of 16.69 g l^{-1} per day. Optimization of fermentation conditions for further higher production and yield of exopolysaccharide in fermentor by this isolate are being examined in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.10.003.

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