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Downstream processing and characterization of pullulan from a novel colour variant strain of *Aureobasidium pullulans* FB-1

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ABSTRACT

Exopolysaccharide produced by a new novel colour variant strain of *Aureobasidium pullulans* FB-1 was purified by cell harvesting and precipitation of the polymer. Various organic solvents were screened for pullulan precipitation. Isolation and purification of pullulan from fermentation broth was carried out using single-step purification strategy by isopropyl alcohol precipitation. Ratio of culture supernatant to isopropyl alcohol and time of precipitation were optimized for pullulan precipitation. Maximum yield (4.47%, w/v) of polysaccharide was obtained when two volumes of ice-cold isopropyl alcohol were added to one volume of supernatant with precipitation time of 12 h. IR spectra as well as carbon-13 and proton NMR spectra in aqueous solution of intact polysaccharide obtained from *A. pullulans* FB-1 and commercially available pullulan (Sigma, USA) revealed solely α-(1 → 6) linked maltosyl units, in accord with the generally accepted structure of pullulan. Maximum hydrolysis (94.25%) of purified pullulan at 50 °C by pullulanase was achieved under agitation (150 rpm) after 360 min.

1. Introduction

Microbial polysaccharides are water-soluble polymers and may be ionic or non-ionic in nature. The repeating units of these exopolysaccharides are very regular, branched or unbranched, and interconnected by glycosidic linkages. In the recent years, a major emphasis has been laid on the search of novel microbial exopolysaccharides and a wide variety of microbial strains are reported to produce exopolysaccharides (EPS) of varied compositions (Yalpani & Sandford, 1987). Their interesting physico-chemical and rheological properties with novel functionality has lead them to act as new biomaterials and find wide range of applications in many industrial sectors like textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, cosmetology, pharmacology, and as food additive.

Pullulan which is a linear α-D-glucan with 'maltosyl units' i.e. α-(1 → 4)Glu*p*-α-(1 → 4)Glu*p*-α-(1 → 6)Glu*p*-, as a regularly repeating structural unit, produced extracellularly by *Aureobasidium pullulans*. Thus, the polysaccharide is viewed as a succession of α-(1 → 6)-linked (1 → 4)-α-D-triglucosides i.e. maltotriose (G_3). Due to its strictly linear structure, pullulan is very valuable in basic research as well as a well-defined model substance (Leathers, 2002). Pullulan can form thin films which are transparent, colourless, tasteless, odourless, tenacious, resistant to oil and grease and unaffected by small thermal variations. Besides, the films are also impermeable to oxygen, non-toxic, biodegradable and edible (Leathers, 2003). It is insoluble in many solvents including methanol, ethanol and acetone, but soluble in water to form a transparent, colourless, viscous adhesive solution (Shingel, 2004). Now-a-days, pullulan has been gaining attention as an excellent material for food, pharmaceutical and biomedical applications (Singh, Saini, & Kennedy, 2008).

Pullulan is produced by yeast-like cells of *A. pullulans* in late exponential and early stationary phase of cultures. An undesirable characteristic feature of most of the strains of *A. pullulans* is the production of dark pigment, which is a melanin-like compound and appears dark green to black in colour (Singh et al., 2008). Melanin is one of the major obstacle in pullulan production and is responsible for dark green to black colour of the broth. Thus, an appropriate downstream processing of the fermentation broth is required to alleviate the pigmentation problem in case of melanin producing strains. The colour-variant strains are differentiated from typically pigmented (off-white to black in appearance) strains of *A. pullulans* by their brilliant pigments of red, yellow, pink or purple colours and produce melanin-free pullulan (Singh et al., 2008). Generally from strains producing melanin-free pullulan, recovery and purification is accomplished with one precipitation step using a suitable organic solvent. The solvents of relatively higher molecular weight

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and slightly lower hydrophilicity are more suitable for such processes in comparison with those having lower molecular weight and higher hydrophilicity as methanol, ethanol and acetone (Kato & Nomura, 1977). The solvents having relatively low hydrophilicity such as propyl alcohol, isopropyl alcohol, tetrahydrofuran, dioxane, etc. are capable of effecting complete precipitation of pullulan with an addition of less volume of the solvent. These solvents also display sufficient impurity removing efficacy. Further purification of pullulan can be achieved by ultrafiltration and ion-exchange resins (Kachhawa, Bhattacharjee, & Singhal, 2003).

Both upstream and downstream processing contribute to the cost of process to obtain a pure biopolymer from the fermentation broth. A simple downstream processing is more cost-effective. In the present investigation, an attempt has been made to systematically investigate recovery and purification of pullulan and to develop a simple one-step downstream processing. The structural characteristics of purified pullulan by the IR and NMR spectroscopy have been done. Hydrolysis of purified pullulan by pullulanase has also been investigated.

2. Materials and methods

2.1. Organism

Aureobasidium pullulans FB-1, used for the present work has been isolated, identified and maintained as reported earlier (Singh & Saini, 2008). The culture has been deposited in Microbial Type Culture Collection, Chandigarh, India and assigned Accession No. MTCC 6994.

2.2. Exopolysaccharide production

The production of pullulan was carried out in shake-flasks as described earlier (Singh, Singh, & Saini, 2009). Briefly, inoculum was prepared in a medium containing (g/L) sucrose, 50.0; K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 1.0; NaCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.05; $FeSO_4$, 0.01; $MnSO_4$, 0.01; $ZnSO_4$, 0.01 and adjusted to pH 7.0, at 30 °C on a refrigerated rotary shaker (Innova 4335, New Brunswick Scientific, USA) under agitation (150 rpm) for 4 days. Production medium (50 mL) containing (g/L) sucrose, 50.0; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; yeast extract, 2.0 and adjusted to pH 6.5 was inoculated with 5% (v/v) of starter culture, and incubated at 30 °C on a rotary shaker at 150 rpm for 7 days.

2.3. Recovery and purification of pullulan

The fermented broth was clarified using a refrigerated centrifuge (Beckmann Avanti™ 30) and the cell-free supernatant was used as the source of polysaccharide. Yeast cells were separated from the broth by centrifugation at 2500*g* for 10 min at 4 °C and pullulan precipitation was carried out from the culture supernatant with two volumes of ice-chilled isopropyl alcohol. The precipitates obtained were centrifuged (2500*g*, 20 min, 4 °C). After the removal of residual solvent, the precipitates were dissolved in deionized water and again precipitated with the same solvent. The precipitates thus, obtained were washed with acetone and deionized water and dried at 80 °C to a constant weight.

2.3.1. Screening of organic solvents

Various organic solvents namely ethanol, tetrahydrofuran and isopropyl alcohol (one volume of supernatant and two volumes of ice-chilled organic solvent) were screened to maximize the pullulan precipitation.

2.3.2. Ratio of culture supernatant to isopropyl alcohol

Various ratios of culture supernatant to isopropyl alcohol (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, and 1:3) were used to optimize the volume of organic solvent required for maximum pullulan precipitation.

2.3.3. Time of precipitation

To optimize the time required for maximum pullulan precipitation by isopropyl alcohol, precipitation was carried up to 16 h at 4 °C and samples were analyzed at 2 h intervals.

2.3.4. Fractionation of the exopolysaccharide

Crude polysaccharide was purified by dissolving the precipitates and reprecipitating them with ice-chilled isopropyl alcohol up to three fractions. Each fraction obtained was washed with acetone before reprecipitation. Exopolysaccharide (EPS) obtained in third fraction was further subjected to IR and NMR spectroscopy for structural characterization.

2.4. Characterization of purified pullulan

2.4.1. Infra-red spectroscopy

The characterization of purified EPS was carried out using IR spectroscopy. Dry precipitates were ground prior to the addition of 95% potassium bromide. IR spectra were determined on an FTIR spectrophotometer (Perkin-Elmer, Norwalk, CT). Pullulan procured from Sigma, USA was used as a standard for characterization.

2.4.2. Nuclear magnetic resonance spectroscopy

NMR spectroscopy of pullulan was carried out at NMR Spectroscopy Facility, Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, USA and compared with commercially available pullulan (Sigma, USA). Pulsed field NMR experiments were performed on a Bruker AVANCE 500 MHz Spectrometer (1996) with an Oxford Narrow Bore Magnet (1989). A 5-mm i.d. 1H/BB (^{109}Ag–^{31}P) triple-axis gradient probe (ID500-5 EB, Nalorac Cryogenic Corp.) was used for all diffusion measurements. The NMR probe was turned to C-13 frequency which is 125.75 MHz in the 500 MHz Spectrophotometer (^{1}H Frequency-500.128 MHZ).

NMR samples were prepared by dissolving each of samples twice (once for proton and once for carbon) in approximately 0.6 mL of deionized water and the solution was transferred to a 5 mm NMR tube for analysis. These dilutions were performed in glass. Approximately 1% solution for ^{1}H NMR and 25% solution for ^{13}C NMR and 2D analysis were used. NMR data were obtained using a 500 MHz Bruker DRX NMR spectrophotometer. All spectra were acquired at 298 K. DSS (2,2-dimethylsilapentane-5-sulfonic acid, $\delta = 0.00$) was used as internal standard. The standard instrument parameters for acquisition of the gradient 2D COSY, 2D HMQC and 2D HMBC were used. Data were processed with Bruker software XWINMR 2.5 and standard processing parameters.

2.5. Hydrolysis of pullulan using pullulanase

Purified pullulan from *A. pullulans* FB-1 was hydrolysed using pullulanase (Source: *Bacillus acidopullulyticus*, Sigma, USA). Pullulan (0.4%, w/v) was incubated with pullulanase (4 IU) at 50 °C under agitation at 150 rpm for different time intervals (0.5–6.0 h). The reducing sugars released were quantified and the extent of hydrolysis (%) was calculated as under:

$$\text{Hydrolysis}(\%) = \frac{\text{Amount of reducing sugars released}}{\text{Amount of pullulan}} \times 100$$

2.6. Analytical techniques

2.6.1. Determination of exopolysaccharide

Exopolysaccharide (pullulan) was precipitated from the supernatant by addition of two volumes of ice-chilled isopropyl alcohol and allowed to stand overnight at 4 °C. The precipitates were separated by centrifugation (2500*g*, 10 min), washed with alcohol, and then dried to a constant weight at 80 °C. Exopolysaccharide (%) was estimated as grams of pullulan (dry weight) produced per 100 mL of fermented broth.

2.6.2. Estimation of reducing sugars

The reducing sugars were estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959).

2.7. Statistical analysis

Experiments were carried out in triplicates and the mean values were calculated. One-way analysis of variance (ANOVA) and pair wise multiple comparison procedures (Tukey's test) were carried out using the statistical software SigmaStat, version 2.0 (Jandel Corp., San Rafael, CA, USA). Values are expressed as means ± SEM. The level of significance was set at $P < 0.001$.

3. Results and discussion

3.1. Recovery and purification of pullulan

Downstream processing was kept simple and inexpensive to facilitate the scaling-up. The conventional single-step method of solvent precipitation was used for the purification of crude pullulan from *A. pullulans* FB-1. To obtain the pure biopolymer from the fermentation broth, cell harvesting, and precipitation of the polymer are essential. Fermented broth after 7 days of cultivation was subjected to centrifugation. Pullulan precipitation was carried out from the culture supernatant with two volumes of ice-chilled isopropyl alcohol. After fermentation, biomass separation is usually the first step in the downstream processing of biological products.

3.1.1. Screening of organic solvents

Different organic solvents namely ethanol, tetrahydrofuran and isopropyl alcohol (one volume of supernatant and two volumes of ice-chilled organic solvent) were used for the precipitation of crude pullulan (100 mL). A comparison of different solvents on the efficiency of recovery of pullulan is depicted in Fig. 1 and Tukey's test has confirmed that the difference in polysaccharide yield was statistically significant ($P < 0.001$). Isopropyl alcohol gave maximum pullulan yield (4.47%, w/v) followed by ethanol. Isopropyl alcohol having relatively low hydrophilicity, is capable of affecting complete precipitation of pullulan (Kato & Nomura, 1977).

Pullulan purification by precipitation of centrifuged broth with tetrahydrofuran (THF) has been carried out by Leathers, Nofsinger, Kurtzman, and Bothast (1988). Pullulan precipitation from the supernatant with an equal volume of ethanol has also been reported (Mulchandani, Luong, & LeDuy, 1989). Pollock and coworkers have recovered polysaccharide from the clarified broth by precipitation with isopropyl alcohol (Pollock, Thorne, & Armentrout, 1992). Roukas and Biliaderis (1995) initially precipitated crude polysaccharide with two volumes of acetone, dissolved in distilled water and reprecipitated with absolute ethanol. Pullulan precipitation by addition of ethanol at 4 °C has been reported by a number of workers (Chi & Zhao, 2003; Forabosco et al., 2006; Göksungur, Dağbağli, Uçan, & Güvenç, 2005). Kachhawa et al. (2003) compared different solvents including methanol, ethanol, isopropyl alcohol, ethyl methyl ketone, tetrahydrofuran and acetone for efficiency of pullulan recovery and reported ethanol to be most efficient.

3.1.2. Ratio of culture supernatant to isopropyl alcohol

Various ratios of culture supernatant to isopropyl alcohol (1:0.5, 1:1, 1:1.5, 1:2, 1:2.5, and 1:3) were used to optimize the volume of organic solvent required for maximum pullulan precipitation. The use of two volumes of ice-cold isopropyl alcohol to one volume of supernatant showed a better precipitation and recovery (4.47%, w/v) of the biopolymer (Fig. 2). Tukey's test confirmed that the difference in polysaccharide recovery with respect to ratio of culture supernatant to isopropyl alcohol was statistically significant ($P < 0.001$).

Total pullulan precipitation is possible only when 2–3 volumes of organic solvent are added per volume of the fermentation broth (Youssef, Roukas, & Biliaderis, 1999). The pullulan concentration in the solution also influences the volume of the precipitating agent added. Pollock et al. (1992) recovered polysaccharide from the clarified broth by precipitation with one volume of isopropyl alcohol. Vijayendra and coworkers purified pullulan by precipitation using two volumes of isopropyl alcohol to one volume of cell-free fermentation broth followed by washing of precipitates with acetone (Vijayendra, Bansal, Prasad, & Nand, 2001). Pullulan precipitation from the supernatant with an equal volume of ethanol has also been reported (Mulchandani et al., 1989). A number of investigations have reported pullulan precipitation using two volumes of ethanol (Kachhawa et al., 2003; Lazaridou, Roukas, Biliaderis, & Vaikousi, 2002). Sena, Costelli, Gibson, and Coughlin (2006) reported pullulan precipitation from the supernatant using one volume of 2-propanol per volume of supernatant.

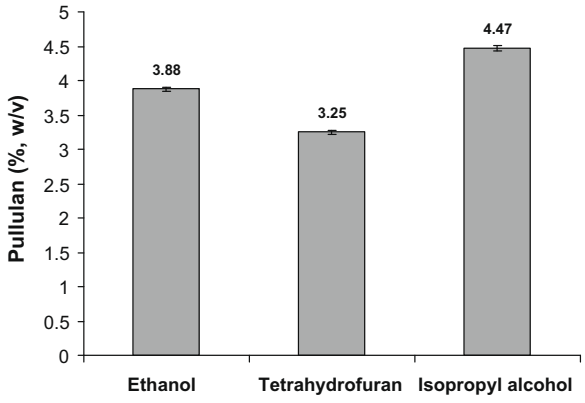

Fig. 1. Effect of different organic solvents on the precipitation of pullulan.

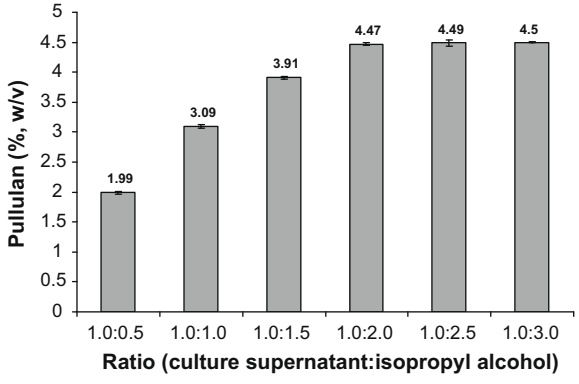

Fig. 2. Effect of ratio of culture supernatant to isopropyl alcohol on pullulan precipitation.

3.1.3. Time of precipitation

An increase in pullulan yield by using two volumes of isopropyl alcohol at 4 °C was obtained up to precipitation time of 12 h and thereafter polysaccharide yield was constant (Fig. 3) and results were found to be statistically significant by Tukey's test ($P < 0.001$).

Chi and Zhao (2003) reported 12 h precipitation time at 4 °C to precipitate extracellular pullulan produced by *Rhodotorula bacarum*. The polysaccharide produced by *A. pullulans* has been precipitated with two volumes of ethanol at 4 °C for 6 h (Youssef et al., 1999). Pullulan precipitation using two volumes of ethanol at 4 °C for 1 h has been reported (Göksungur et al., 2005; Lazaridou et al., 2002). Forabosco et al. (2006) treated culture filtrates with three volumes of cold ethanol for pullulan precipitation at −20 °C for 24 h.

3.2. Characterization of purified pullulan

3.2.1. Infra-red spectroscopy

The summary of characterization of all pullulan samples is summarized in Table 1. The investigations into the conformation of pullulan chains by means of IR spectroscopy are rare in literature. The structural characterization of the crude EPS and purified fractions by IR spectroscopy yielded spectra similar to that of the pullulan standard from Sigma. Information on the conformation of glucopyranosyl units in the polysaccharide can be acquired in the 1000–700 cm^{-1} region. For pullulans, the band at around 900 cm^{-1} describes α-(1 → 6) linkages. α-(1 → 4) linkages were observed at around 925 cm^{-1}. The absorptions at around 850 and 765 cm^{-1} showed that pullulan had a $^4C_1$ chair conformation. The broad band at approximately 3424.4 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The band at about 1639 cm^{-1} has been previously assigned to valent vibrations of the C–O–C bond and glycosidic bridge (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The band at around 2927.3 cm^{-1} was due to C–H stretching vibration. In the pullulan macromolecule, there is a large proportion of the primary C–OH groups at C-6 position, and an intense bend at about 1366 cm^{-1} was observed. The broad peak at around 1021 cm^{-1} should be most likely ascribed to the vibration of the C–O bond at C-4 position of a glucose residue (Kačuráková & Mathlouthi, 1996). A pullulan-like peak at around $\lambda = 850$ cm^{-1} indicating the α-configuration within the EPS has been reported (Prasongsuk, Sullivan, Kuhirun, Eveleigh, & Punnapayak, 2005). Madi, Harvey, Mehlert, and McNeil (1997) also reported a peak at $\lambda = 859.6$ cm^{-1} of EPS from *A. pullulans* (de Bary) Arnaud (IMI145194), which they interpreted as α-configuration. IR spectra confirmed the absence of the other glucans as aubasidan which have a significant peak at $\lambda = 890$ cm^{-1}.

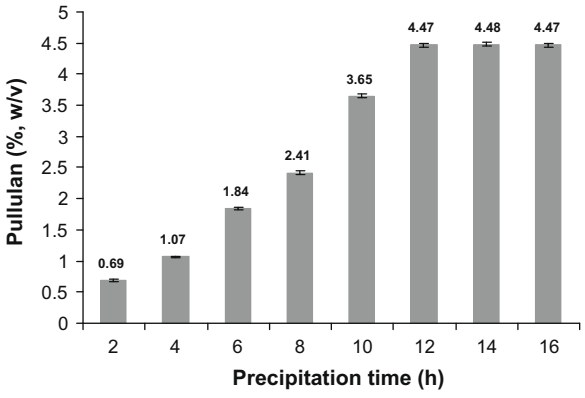

Fig. 3. Effect of time of precipitation on pullulan yield.

Table 1
IR spectroscopy of purified fractions of pullulan from *A. pullulans* FB-1 in comparison to standard pullulan (Sigma, USA).

Assignment	Wave number (cm^{-1})			
	Sigma	FB-1 1st fraction[a]	FB-1 2nd fraction[b]	FB-1 3rd fraction[c]
O–H str.	3424.4	3379.5	3338.5	3391.7
C–H str.	2929.3	2928.2	2929.5	2926.0
O–C–O str.	1639.7	1647.9	1653.6	1648.1
C–O–H bend	1366.4	1370.2	1369.5	1368.6
C–O–C str.	1154.9	1156.7	1158.0	1156.7
C–O str.	1021.4	1021.7	1020.6	1024.8
α-configuration	851.0	858.2	856.8	851.4

[a] 1st fraction obtained after one time purification step from crude exopolysaccharide.
[b] 2nd fraction obtained after repeating purification step for 1st fraction.
[c] 3rd fraction obtained after repeating purification step for 2nd fraction.

3.2.2. Nuclear magnetic resonance spectroscopy

Structural characterization of the purified fraction of pullulan from *A. pullulans* FB-1 in comparison with pullulan (Sigma, USA) was carried out by NMR spectroscopy. NMR spectra were acquired for the pullulan samples in order to examine the detailed structural features of the polysaccharide. Pullulan is usually thought to consist of a series of maltosyl units connected by α-(1 → 6) bonds. It contains both α-(1 → 4) and α-(1 → 6) linkages. One-dimensional proton and carbon-13 NMR spectra of both samples were obtained under complete relaxed conditions so as to quantify the proportion of α-(1 → 6) linkages and to reveal any difference in the spectra of the samples. The spectra obtained from both the samples indicated the presence of α-(1 → 4) and α-(1 → 6) linkages in the ratio of 2:1 as expected for α-(1 → 6) maltosyl repeating unit. In addition, the spectra of both the samples showed the occurrence of α-(1 → 6) linkage between every third glucose ring. Integration of the spectra from Sigma pullulan as well as purified fractions of FB-1 pullulan sample indicated no minor peaks due to terminal glucose rings or homogeneity of the repeating maltosyl unit. Proton spectrum gave the proportion of α-(1 → 6) linkages of around 0.34 and 0.32 from the ^{13}C spectrum. These values signify the presence of only maltosyl units in the polysaccharide samples.

The one-dimensional ^1H NMR spectrum permits the assignment of the signal at 5.02 ppm for Sigma sample and 5.04 ppm for *A. pullulans* FB-1 sample due to the anomeric proton at the site of the α-(1 → 6) linkage on the basis of their chemical shifts. The signals at 5.46 and 5.42 ppm (Sigma pullulan) and 5.44 and 5.48 ppm (FB-1 pullulan) are due to the α-(1 → 4) linkage based on their chemical shifts. Very weak signals at 5.42 and 4.72 ppm indicates α and β anomers of the terminal glucose residue. In ^{13}C spectrum, the anomeric α-(1 → 6) carbon signal was found at 98.76 ppm for Sigma pullulan and 99.00 ppm for FB-1 pullulan. Depending upon the linkage of the preceding residue, the shifts of the anomeric (1 → 4) linked carbons were 100.64 or 101.10 ppm and 100.60 or 101.10 ppm for Sigma and FB-1 pullulan samples, respectively. The ^{13}C NMR data further confirmed the homogeneity of both the pullulan samples as no other resonance lines in the anomeric carbon region were found in the spectrum.

In addition to one dimensional spectra, conventional double-quantum-filtered COrrelated Spectroscopy (COSY), TOtal Correlation Spectroscopy (TOCSY) spectra were also recorded. Two dimensional gradient enhanced Heteronuclear Single-Quantum Coherence (HSQC) and high resolution Heteronuclear Multiple Bond Correlation (HMBC) spectra were recorded at 298 K with carbon decoupling during acquisition time. The above cited results were confirmed by a series of two-dimensional ^1H–^1H homonuclear correlation spectra i.e. COSY and TOCSY and ^1H–^{13}C heteronuclear correlated NMR spectra i.e. HSQC and HMBC. The two

Table 2
Chemical shifts (ppm) of pullulan protons at 298 K (DSS reference).

Pullulan	H-1	H-2	H-3	H-4	H-5	H-6
Sigma, USA						
(1 → 4)-(1 → 6)-(1 → 4)Glc	5.02	3.94	4.08	3.76	3.88	3.92, 4.01
(1 → 4)-(1 → 4)-(1 → 6)Glc	5.46	3.72	4.04	3.68	4.01	3.88, 3.96
(1 → 6) (1 → 4)-(1 → 4)Glc	5.42	3.70	3.79	3.55	4.01	3.86, 4.00
A. pullulans FB-1						
(1 → 4)-(1 → 6)-(1 → 4)Glc	5.04	4.00	4.09	3.75	3.72	3.99, 3.85
(1 → 4)-(1 → 4)-(1 → 6)Glc	5.48	3.71	4.05	3.69	4.01	3.74, 3.68
(1 → 6) (1 → 4)-(1 → 4)Glc	5.44	4.00	3.78	3.54	4.01	3.87, 4.02
α Reducing Glc	5.42	3.73	4.08	3.68	n.d.	n.d.
β Reducing Glc	4.72	3.68	3.94	3.68	n.d.	n.d.

n.d., not determined.

Table 3
Chemical shifts (ppm) of pullulan carbons at 298 K (DSS reference).

Pullulan	C-1	C-2	C-3	C-4	C-5	C-6
Sigma, USA						
(1 → 4)-(1 → 6)-(1 → 4)Glc	98.76	72.48	74.27	78.78	72.38	61.61
(1 → 4)-(1 → 4)-(1 → 6)Glc	100.64	72.59	74.18	78.41	72.23	61.32
(1 → 6) (1 → 4)-(1 → 4)Glc	101.10	72.48	73.92	70.38	72.02	67.42
A. pullulans FB-1						
(1 → 4)-(1 → 6)-(1 → 4)Glc	99.00	72.49	74.24	79.00	72.34	61.80
(1 → 4)-(1 → 4)-(1 → 6)Glc	100.60	72.60	74.18	78.80	72.22	61.50
(1 → 6) (1 → 4)-(1 → 4)Glc	101.10	72.49	73.88	70.35	72.02	67.80
α Reducing Glc	92.62	n.d.	n.d.	n.d.	n.d.	n.d.
β Reducing Glc	96.53	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not determined.

dimensional spectra were used to obtain complete assignment of the proton as well as carbon-13 spectra (Tables 2 and 3).

Absolute chemical shifts were slightly different from those reported by McIntyre and Vogel (1991, 1993). This may be probably due to differences in the sample temperature, concentration and reference standards. McIntyre and Vogel (1991, 1993) reported the use of NMR spectroscopy to probe the primary structure of the glucan pullulan. Confirmation of structural characteristics of pullulan samples by ^{13}C NMR spectroscopy has been reported by Youssef et al. (1999). The structure of pullulan-like polysaccharides produced as exocellular material by different strains of *Cryphonectria parasitica*, the fungus responsible for chestnut cankers, has been investigated with nuclear magnetic resonance techniques (Delben, Forabosco, Guerrini, Liut, & Torri, 2006).

The presence of well defined and sharp peaks in the spectrum testified the high purity of the samples as well as the absence of appreciable extent of oligomers. The spectrum recorded was compatible with the expected structure for the pullulan, i.e., the polymaltosyl structure. All the spectral properties of pullulan produced by *A. pullulans* FB-1 agree with the presence of maltosyl sequences in the polysaccharide backbone.

3.3. Hydrolysis of pullulan by pullulanase

Purified pullulan produced by *A. pullulans* FB-1 was used as a substrate for hydrolysis by pullulanase. Pullulan hydrolysis was carried out using free pullulanase at 50 °C under agitation (150 rpm) with respect to time. A comparative analysis of the percent hydrolysis at different time intervals at 150 rpm reveal that maximum pullulan hydrolysis (94.25%) was achieved after 360 min yielding 3.77 ± 0.07 mg/mL of reducing sugars (Fig. 4). Complete conversion of pullulan into maltosyl by pullulanase from *Fervidobacterium pennavorans* Ven5 has been reported at 85 °C (Koch, Canganella, Hippe, Jahnke, & Antranikian, 1997). Bertoldo, Duffner, Jorgensen, and Antranikian (1999) reported more than

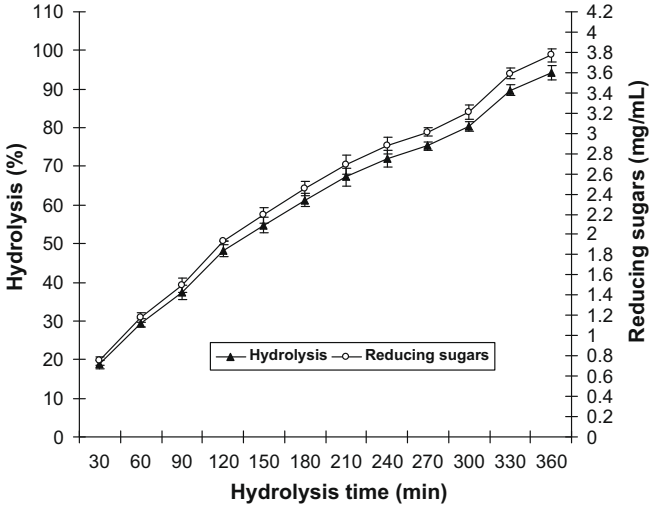

Fig. 4. Hydrolysis of purified pullulan by pullulanase at 50 °C under agitation (150 rpm) with respect to time.

98% pullulan hydrolysis after 1 h incubation at 80 °C by thermostable pullulanase from *Fervidobacterium pennavorans* Ven5. Similar hydrolysis rate of pullulan by pullulanase aqueous solution at 25 °C for each molecular weight of pullulan has been reported (Ohtani, Ishidao, Iwai, & Arai, 1999). Random decomposition of pullulan has been suggested by aqueous solution of pullulanase with the proceeding time.

4. Conclusions

Simple and inexpensive downstream processing of EPS being a significant part of the total production cost is considerable to facilitate the scaling-up. The spectral features of isopropyl alcohol precipitated polymers were typical of pullulan revealing the effectiveness of single-step precipitation for purification of pullulan. The structural characterization of the EPS by IR spectroscopy yielded spectra similar to that of the pullulan standard from Sigma. A pullulan-like peak at around $\lambda = 850\ cm^{-1}$ indicating the α-configuration within the EPS has been found. Further, the molecular homogeneity of the alcohol-precipitated polysaccharides from the fermentation broth as well as the structural features of pullulan was confirmed by NMR spectra. Spectra recorded indicated the presence of high purity and ordered configuration. More than 94% hydrolysis of purified pullulan by pullulanse also confirms its high purity.

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