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Original Research

Pullulan gum production from low-quality fig syrup using *Aureobasidium pullulans*

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Abstract: Pullulan is an important polysaccharide with several potential applications in food science, pharmaceutical and cosmetic industries, but high costs of pullulan production are the main limitation for commercial utilization. Therefore, a cost-effective process for pullulan production was developed using fig syrup as an exclusive nutrient source. In particular, the feasibility of using low quality fig syrup as a supplemental substrate for pullulan gum production by *Aureobasidium pullulans* was investigated. Fermentation was carried out over a range of fig syrup and sucrose degrees Brix (5-15%). Maximum pullulan gum production was observed after 96h using 12.5% fig syrup, yielding approximately14.06 g/L. This value of pullulan production (14.06 g/L) was higher than the amount of pullulan produced using sucrose as substrate (5.01 g/L). In conclusion, fig syrup was an effective substrate for pullulan production by *Aureobasidium pullulans*, and, therefore, this byproduct deserves attention for the cost-effective and environmentally friendly pullulan production.

Key words: Polysaccharides; Fermentation; FTIR; Byproducts.

Introduction

Pullulan is a water-soluble polysaccharide produced from starch by the yeast-like fungus *Aureobasidium pullulans* under aerobic conditions (1). It is a linear α-D-glucan consisting of maltotriose units connected by an α-1,6 glycosidic bond, whereas three glucose units in maltotriose are connected by an α-1,4 glycosidic bond (2). Because of its non-toxic and non-immunogenic properties, pullulan possesses a wide range of commercial and industrial applications in many fields like food science, pharmacy, cosmetics and even in lithography (3). In particular, it can be used to produce edible, transparent, biodegradable, oil resistant and high gas barrier films. Although pullulan is commercially produced since 25 years, however, due to the high costs of production, only few potential uses of this polymer have been widely developed. Nevertheless, the last few years have seen resurgence of interest in pullulan, particularly for high-value health and pharmaceutical applications (3). Currently, the costs of production of this polysaccharide are comparatively high. Therefore, it is pivotal to search for inexpensive carbon and nitrogen sources to support both fungal growth and pullulan biosynthesis (4).

The plants have always been remarkable in all cultures of throughout the world, being used not only for food but also for medicinal uses and many other purposes (5-30). Figs (*Ficus carica* L., Moraceae) are highly nutrient and healthy food produced worldwide, rich in carbohydrates (almost 65-70%) and vitamins (31). Fig tree originated from Western Asia and, then, spread to the Mediterranean area. In 2003, an estimated annual fig production of 1,077,211 tons has been recorded according to Food and Agriculture Organization (FAO), with Turkey and Iran ranking 1st and 2nd, respectively, among all countries producing figs (32). Various data have been reported on pullulan gum production by different sources of carbon such as jiggery (4), hydrolyzed potato starch waste (33), sweet potato (34), coconut by-products (35) and beet molasses (36, 37), and their yield were 51.9, 19, 29.43, 58.0 and 49 g/L, respectively.

Pullulan is a natural exopolysaccharide with many useful characteristics. However, pullulan is more costly than other exopolysaccharides, which limits its effective application. Currently, the costs of production of this polysaccharide are comparatively high. With exponential growth of science and technology across throughout the world (38-40), finding low-cost sources for its production can be beneficial. The purpose of this study was to adapt a novel strategy for maximizing pullulan production, mainly using low-quality fig syrup as a low-cost substrate for fermentation by *Aureobasidium pullulans*. Therefore, a model of pullulan fermentation was carried out by using different carbon sources, namely sucrose and fig syrup, at different concentrations, focu-

sing on the effect of temperature on fungal cell growth and pullulan production. Therefore, the main aim of this study was to optimize the fermentation conditions for pullulan production from fig syrup by *Aureobasidium pullulans*.

Materials and Methods

Fig syrup preparation

The initial extract was prepared by soaking 100 g of low quality dried fig fruits in 500 mL distilled water, and mixed for 1 min at low speed, and 3 min at high speed. The homogenized extract was maintained in hot water for 20 min, and then centrifuged at 10000 g for 30 min at room temperature to remove solids. Produced fig syrup was diluted to 5%, 7.5%, 10%, 12.5%, and 15% (w/v) by distilled water. All prepared solutions were kept at 4 °C.

Microorganism and inoculum preparation

Aureobasidium pullulans 51 was kindly supplied by Department of Biology, Isfahan University. The microorganism was maintained on potato dextrose agar (PDA) plates at 4 °C and subcultured each 2 weeks. Two loops of *A. pullulans* colonies grown on PDA at 28 °C were transferred to 250 mL conical flasks containing 100 mL of activation medium. The activation medium contained 5.0 g/L K_2HPO_4, 1.0 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L $(NH_4)_2SO_4$, 2 g/L yeast extract and 1 L distilled water. The flask was incubated at 28 °C for 48h in a rotary shaker incubator at 160 rpm. This culture was used to inoculate the production medium at 5% (v/v). The production medium contained 3% w/v sucrose, 5.0 g K_2HPO_4, 0.4 g yeast extract, 0.6 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 1 L distilled water and 50-150 g sucrose. The initial pH of the medium was adjusted to 6.5. For experiments with fig syrup, the cultivation medium was prepared replacing sucrose with fig syrup. This media was sterilized at 121 °C for 20 min. Sucrose and fig syrup were autoclaved separately and then mixed with other substrates. Seed cultures were prepared by inoculating cells grown on a PDA slant into 250 mL flask containing 50 mL of inoculum medium and were subsequently incubated at 28 °C for 48 h while being shaken at 160 rpm. Then, 2.5 mL of seed culture was inoculated in a 250 mL flask containing 50 mL of fermentation medium. Batch pullulan fermentation was carried out in a shaker autoclave at 160 rpm and 28 °C.

Effect of temperature on pullulan production

The temperature of incubation ranged from 20 to 30 °C. The production media was fig syrup at 12.5% Brix. All other process conditions were constant.

Isolation of biomass

Every 24 h, fermentation broth was collected and centrifuged at 10,000 g for 20 min to remove cells. The biomass dry weight was determined by washing the sediment with distilled water and drying at 80 °C overnight. The biomass dry weight was expressed as g/L.

Isolation and purification of pullulan

Fifty milliliters of the supernatant were transferred into a 250 mL conical flask and, then, 100 mL of cold ethanol were added and mixed thoroughly. The flasks were held at 4 °C for 12 h to precipitate the extracellular polysaccharides. Then, the residual ethanol was removed, the precipitate was dissolved in 50 mL of deionized water at 80 °C and the solution was dialyzed against deionized water for 48 h to remove small molecules. The polysaccharides were precipitated again by using 100 mL of the cold ethanol; the precipitate was filtered through a pre-weighted Whatman GF/A filter and dried at 80 °C to a constant weight (41). The pullulan content was expressed as g/L. All tests were performed in triplicate and the results are expressed as mean and standard deviation.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was applied using a Shimadzu 8300 at 400 to 4000 (cm^{-1}) over KBr pellet. Pullulan sample (2 mg) was manually well blended with 100 mg of KBr powder. The mixture was desiccated overnight at 50 °C under reduced pressure prior to FTIR measurement (42).

Statistical analysis

The results represent the mean of three independent replicates, and the error bars indicate the standard deviation (SD) of the mean. Statistical significance was determined by ANOVA at $P < 0.05$.

Results and Discussion

Effect of initial substrate concentration on pullulan production

The production of exopolysaccharide in fermentation media containing sucrose and fig syrup (5, 7.5, 10, 12.5, and 15 degrees Brix, pH=6.0) was investigated up to 144 h of incubation at 28 °C. As shown in Tables 1 and 2, initial concentrations of carbon sources present in the media mostly affected the yield of pullulan production.

Table 1. Production of pullulan under different concentrations of fig syrup.

	Time (h)					
	24	48	72	96	120	144
Brix (TSS)	g/L					
5	3.84 ± 0.52^{Bc}	4.28 ± 0.48^{ABdc}	5.3 ± 0.41^{Ac}	5.4 ± 0.53^{Ac}	4.87 ± 0.13^{ABbc}	4.7 ± 0.19^{ABbc}
7.5	3.89 ± 0.18^{Ac}	4.01 ± 0.33^{Ad}	5.2 ± 0.21^{Ac}	5 ± 0.22^{Ac}	4.7 ± 0.26^{Ac}	4.2 ± 0.34^{Ac}
10	4.8 ± 0.11^{ABc}	5.6 ± 0.28^{ABbc}	4.98 ± 0.18^{ABc}	5.3 ± 0.28^{ABc}	6.18 ± 0.91^{Aab}	4.52 ± 0.22^{Bc}
12.5	9.1 ± 0.09^{Ba}	9.4 ± 0.29^{Ba}	13.2 ± 0.71^{Aa}	14.06 ± 0.18^{Aa}	6.66 ± 0.72^{Ca}	6.4 ± 0.09^{Ca}
15	6.3 ± 0.32^{Bb}	6.38 ± 0.22^{Bb}	7.1 ± 0.19^{Bb}	9.18 ± 0.19^{Ab}	6.04 ± 0.83^{Babc}	5.99 ± 0.28^{Bab}

Results represent the mean ± standard error; n = 3. Means with different superscript lower case letters in the columns and capital letters in the rows are significantly different ($P < 0.05$).

Table 2. Production of pullulan under different concentrations of sucrose.

	Time (h)					
	24	48	72	96	120	144
Brix (TSS)			g/L			
5	1.56±0.32^{Bb}	2.44±0.92^{ABb}	2.96±0.28^{ABb}	3.11±0.28^{Ac}	3.16±0.77^{Aa}	2.16±1.02^{ABb}
7.5	2.42±0.81^{Bab}	3.08±0.69^{ABab}	3.47±0.52^{ABab}	4.12±0.81^{Abc}	3.45±0.12^{ABa}	3.11±1.13^{ABab}
10	3.62±0.38^{Aa}	4.28±0.77^{Aa}	4.91±0.39^{Aa}	5.01±0.16^{Aa}	4.42±0.52^{Aa}	4.14±0.88^{Aa}
12.5	3.22±0.37^{Ba}	4.00±0.82^{Aa}	4.34±0.11^{Aab}	4.2±0.19^{Ab}	3.66±0.73^{Ba}	3.21±0.61^{Bab}
15	3.72±0.61^{Aa}	4.35±0.18^{Aa}	3.64±0.19^{Aab}	3.12±0.18^{Ac}	3.36±0.79^{Aa}	3.04±0.71^{Aab}

Results represent the mean ± standard error; n = 3. Means with different superscript lower case letters in the columns and capital letters in the rows are significantly different ($P < 0.05$).

Under the conditions used in these fermentation media, concentration of produced polysaccharide increased significantly with the increase of initial fig syrup concentration from 5 to 12.5 g/L, while, in higher concentration (15 g/L), polysaccharide production slightly decreased. This might be due to the fact that higher degrees Brix of fig syrup can have a negative impact on the metabolic activities of microbial cells, ultimately affecting the production of the exopolysaccharide. The maximum production of pullulan occurred in fermentation medium containing fig syrup at 12.5 degrees Brix at 96 h and continuous agitation, while, for the fermentation medium containing sucrose, the maximum pullulan production was reached at 10 degrees Brix within 96 h. The highest yields of pullulan were 14.06 and 5.01 g/L for fig syrup and sucrose, respectively.

In general, for both carbon sources, production of pullulan showed an increasing trend along the fermentation time, up to 96 h. Conversely, after 96 h, the concentration of this exopolysaccharide decreased. The reduction in pullulan production with the increase in fermentation time might be due to the pH decrease in the medium as well as to the aging of *A. pullulans* cells. Overall, our results demonstrated that the fig syrup is a better carbon source than sucrose for pullulan production by *A. pullulans*.

In comparisons to sucrose, fig syrup supported a higher pullulan production. This may be attributed to the presence of suitable ratio of carbohydrates and proteins in fig syrup. The amount of pullulan obtained in the present study was higher than that reported by Seo et al. (2004) for glucose (7.6 g/L) (42). The results reported by Göksungur et al. (2004), Vijayendra et al (2001) and Göksungur (2011) indicate that beet molasses, jaggery and hydrolyzed potato starch waste supported higher pullulan production too (4, 33, 43).

Effect of incubation temperature on pullulan production

Incubation temperatures significantly affect fungal growth and pullulan production (44). Therefore, determination of optimum temperature for maximizing pullulan production is pivotal. To examine the effect of temperature on pullulan production, the incubation temperature was varied from 20 to 30 °C and fig syrup used as carbon source. As reported in Figure 1, maximum pullulan production was obtained at 28 °C (14.91 g/L). It also can be noticed that the amount of produced pullulan significantly decreased when the fermentation temperature was higher than 28 ◦C and this observation was in agreement with previous (34, 37, 45). This means that

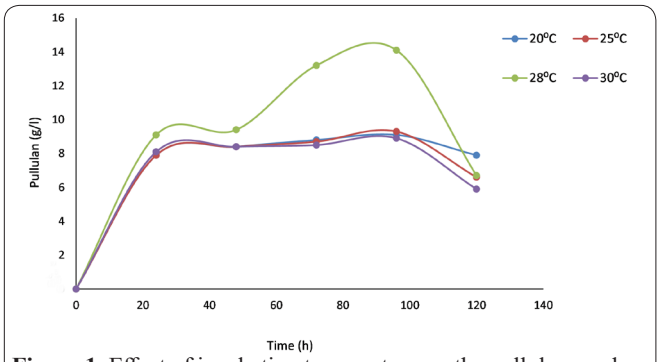

Figure 1. Effect of incubation temperature on the pullulan production by *Aureobasidium pullulans*.

production of pullulan by *A. pullulans* 51 was sensitive to higher temperature than 28 °C. Sharma et al. (2013) also reported that by increasing the incubation temperature, the pullulan production raised (46). Contrary to our findings, other reports have described optimal conditions for pullulan production at temperatures of 20 ºC (47), 25 ºC (45) and 26 ºC (48). This result could be related to the differences in the types of strain, composition of fermentation medium, and culture conditions used.

Changes of pH during fermentation

Under the conditions used in this study, the pH of fermentation medium changed in the range 6.5-3.85, as shown in Figure 2. Since the fermentation process is acidogenic, the pH of medium decreased slightly during the fermentation process. Changing pH could affect pullulan production. The initial pH of medium was adjusted to 6.5 and, with pH decreasing from 6.5 to 4.26 in 96 h, pullulan production reached the maximum, while a further decrease in pH significantly reduced pullulan yield.

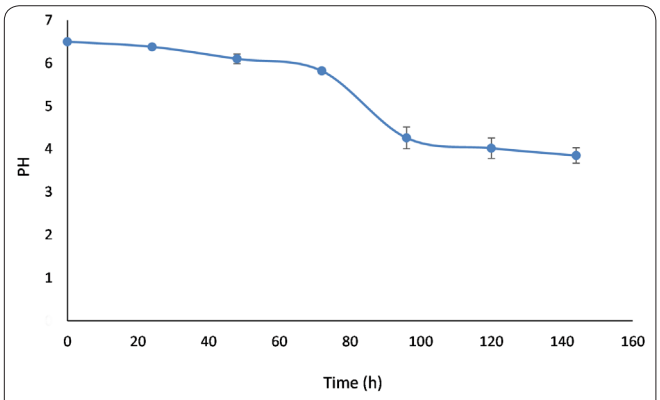

Figure 2. pH changes of the medium during fermentation. Results represent the mean ± standard error (n = 3). Means with different letters are significantly different ($P < 0.05$).

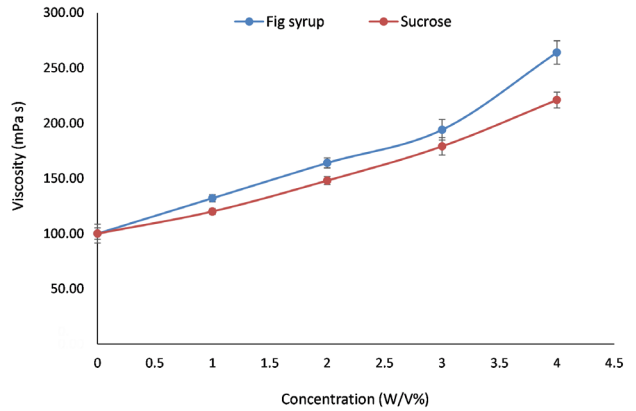

Figure 3. Effect of pullulan concentration on medium viscosity. Results represent the mean ± standard error (n = 3). Different letters indicate differences within groups ($P < 0.05$).

Effect of pullulan concentration on viscosity

The effect of pullulan concentration on viscosity is shown in Figure 3. Viscosity increased when pullulan concentration increased. The viscosity of the biopolymer extracted from the medium with fig syrup as carbon source was higher compared with those obtained from the medium with sucrose as carbon source, at identical biopolymer concentrations.

Fourier transform-infrared (FTIR) spectra of pullulan

The FTIR spectra of pullulan were obtained between 400 and 4000 wave number (cm^{-1}) (Figure 4). This indicated the presence of carbonyl groups, a tertiary amine salt and phenyl nucleus skeletal stretching. The broad band at approximately at 3322 cm^{-1} indicated that pullulan has repeating -OH units, as in polysaccharides. Absorptions at about 832 cm^{-1} and 720 cm^{-1} indicated the presence of α-D-glucopyranoside units and α-(1- 4)-D-glucosidic linkages, respectively. The peaks at 1615 cm^{-1} has been previously corresponded to vibrations of the C–O–C bond and glycosidic bridge (49). The peaks in around 2927.3 cm^{-1} governed by stretching vibration of C–H.

In conclusions, this study reported the production of pullulan by a strain of *A. pullulans*. It was shown that fig syrup is better than sucrose as carbon source during fermentation. Therefore, the use of fig syrup as nutrient in the fermentation medium might represent a potential application for this agriculture waste, with a significant decrease of costs of pullulan production. Noteworthy, increasing the degrees Brix of syrup up to 12.5% significantly raised the level of pullulan production. In conclusion, the present study developed a novel low cost process for pullulan production, by using the fig syrup, an agricultural waste, as nutrient medium. Further studies are in process for optimization and scaling up of the industrial process.

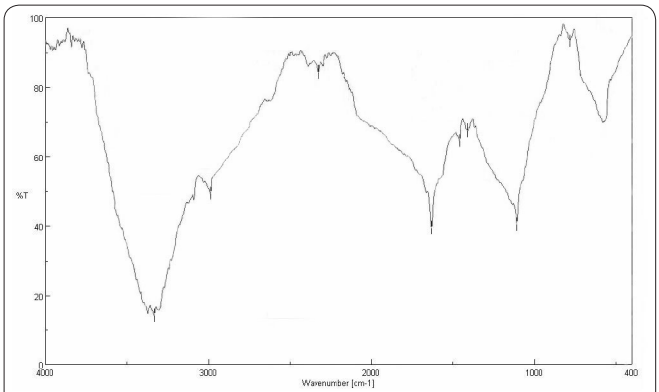

Figure 4. Fourier transform infrared spectroscopy (FTIR) spectra of pullulan.

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Conflicts of Interest

The authors declare no conflict of interest.

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