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Production of Pullulan From Beet Molasses and Synthetic Medium by *Aureobasidium pullulans*

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Abstract: The production of pullulan from molasses and synthetic medium by *Aureobasidium pullulans* P56 in batch culture was investigated. Among the pretreatments of molasses used to increase polysaccharide production, sulfuric acid + activated carbon treatment gave better results with regard to polysaccharide concentration, polysaccharide yield and sugar utilization. A maximum polysaccharide concentration of 21.4 g l^{-1} and a pullulan concentration of 16.7 g l^{-1} were obtained in synthetic medium whereas 35.0 g l^{-1} of polysaccharide and 16.9 g l^{-1} of pullulan were obtained in molasses medium containing 50 g l^{-1} of initial sugar at pH 7.5. The highest pullulan contents of the crude polysaccharide were 85.3 and 48.3% for synthetic medium and molasses medium, respectively and this showed that *A. pullulans* produced a mixture of pullulan and other polysaccharides in molasses medium. The experiments performed with synthetic medium showed that higher initial sugar concentrations led to a significant decrease in pullulan yield. A polysaccharide concentration of 20.4 g l^{-1} and a pullulan concentration of 6.6 g l^{-1} were obtained in a stirred tank bioreactor with synthetic medium. These concentrations were lower than those in shake flask culture.

Key Words: pullulan, beet molasses, synthetic medium, *Aureobasidium pullulans*

Pancar Melası ve Sentetik Ortamdan *Aureobasidium pullulans* ile Pullulan Üretimi

Özet: Bu çalışmada pancar melası ve sentetik ortamdan *Aureobasidium pullulans* P56 ile pullulan üretimi araştırılmıştır. Polisakkarit üretimini artırmak için melasa uygulanan ön işlemlerden sülfürik asit + aktif kömür ile daha yüksek polisakkarit, polisakkarit verimi ve şeker tüketim değerleri elde edilmiştir. Sentetik ortam ile 21.4 g l^{-1} polisakkarit ve 16.7 g l^{-1} pullulan, melas ile ise 35.0 g l^{-1} polisakkarit ve 16.9 g l^{-1} pullulan üretimi, 50 g l^{-1} başlangıç şeker konsantrasyonunda (pH 7.5) elde edilmiştir. Sentetik ortamdan elde edilen toplam polisakkaritin % 85.3'ünün pullulan olduğu belirlenirken, bu oran melas içeren ortamda % 48.3'tür. Bu sonuç sentetik ortamdan yüksek saflıkta pullulan elde edilirken melastan elde edilen polisakkaritin yaklaşık yarısının pullulan dışı diğer polisakkaritlerden oluştuğunu göstermektedir. Sentetik ortam ile gerçekleştirilen denemelerde, yüksek başlangıç şeker konsantrasyonlarının pullulan veriminde azalmaya neden olduğu belirlenmiştir. Karıştırmalı tank tipi biyoreaktörde yapılan denemede, 20.4 g l^{-1} polisakkarit ve çalkalamalı kültüre göre çok daha düşük bir değer olan 6.6 g l^{-1} pullulan konsantrasyonu elde edilmiştir.

Anahtar Sözcükler: pullulan, pancar melası, sentetik ortam, *Aureobasidium pullulans*

Introduction

Pullulan is an extracellular water-soluble microbial polysaccharide produced by strains of *Aureobasidium pullulans*. It is a linear mixed linkage α-D-glucan consisting mainly of maltotriose units interconnected via α-(1→6) linkages (1). Typical industrial uses of pullulan are as food coatings and packaging material due to its good film-forming properties, as an ingredient of low calorie foods and as a starch substitute; as films, with properties similar to those of polyvinyl alcohol, but superior in many ways as well as being biodegradable; as an adhesive in the form of pastes with water, as a construction material (after esterification) with fibers similar in strength and elasticity to those in nylon; and as a bulking agent and stabilizer for tablets in the pharmaceutical industry (2).

A. pullulans is a polymorphic fungus that synthesizes several polysaccharides, including pullulan (3). The production of pullulan from a synthetic medium by different strains of *A. pullulans* has been described (4-7). The use of agro-industrial wastes like potato starch waste, peat hydrolyzate, whey, molasses, brewery wastes and olive oil waste effluents as substrates for pullulan production has also been reported by many researchers

(1,8-13). Utilization of these substrates would seem to be ecologically sound and economically advantageous as they have low or even negative costs (2). However, the pullulan produced from different substrates may vary in purity and this is more pronounced when agro-industrial wastes are used as a carbon source for fermentation. *A. pullulans* produces various polysaccharides other than pullulan and it has been shown that the purity of the pullulan may vary according to the substrate used (2,8,10).

In this study, beet molasses, a by-product of the sugar industry, pretreated with different techniques were compared to synthetic medium for polysaccharide and pullulan production by *A. pullulans*. Estimations of crude polysaccharide were made by weighing the ethanol precipitate from the cell-free fermentation broth and estimations of pullulan were made by coupled enzyme assay (14). In addition, the effect of various fermentation parameters such as pH and initial sugar concentration on kinetic parameters of pullulan fermentation and also on the pullulan content of crude polysaccharide in synthetic medium were studied.

Materials and Methods

Microorganism and culture conditions

A. pullulans P56, a strain deficient in melanin production, was kindly supplied by Prof. Dr. T. Roukas of the Aristotle University of Thessaloniki. The microorganism was maintained on potato dextrose agar slants at 4 °C and subcultured every 3 weeks. Cells for inoculation of the culture medium were obtained from cultures grown on potato dextrose agar slants at 28 °C for 48 h. Two loops of *A. pullulans* cells were transferred to 250 ml conical flasks containing 50 ml of culture medium (pH 5.5) of the following composition (g l^{-1}): sucrose 30.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4.7H_2O$ 0.2 and NaCl 1.0. The flasks were incubated at 28 °C for 48 h in a rotary shaker incubator (B. Braun Certomat) at 200 rpm. These cultures were used to inoculate the production medium at a level of 5% (v/v).

Pretreatment of molasses

Beet molasses (containing 54% total sugar) used throughout the study were supplied by the Pakmaya Yeast Co., İzmir. Molasses consist of water, sucrose, proteins, vitamins, amino acids, organic acids and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium etc., which cause a critical problem during fermentation, together with some colored substances. Three methods were used to remove these substances from beet molasses (1). For *sulfuric acid treatment*, the molasses (pH 7.4) were diluted to 35 ° Brix, acidified to pH 4.0 with 2 N H_2SO_4 and boiled for 5 min. The liquid obtained after boiling was centrifuged at 4000 x g for 10 min and filtered through filter paper. For *potassium ferrocyanide treatment*, the pH of the molasses solution containing 100 g l^{-1} of total sugars was adjusted to pH 5.5 with 5 N HCl and the solution heated at 100 °C for 15 min. The hot liquid was treated with 100 ppm potassium ferrocyanide to encourage the precipitation of heavy metals, allowed to stand for 24 h at room temperature and then centrifuged at 4000 x g for 10 min. The supernatant was used for fermentation. For *sulfuric acid and activated carbon treatment*, following the sulfuric acid treatment, the supernatant was treated with activated carbon at a ratio of 5% (w/v). The mixture was stirred and heated at 60 °C for 1 h. The solution was filtered through coarse filter paper. The filtrate was refiltered and the solution was again treated with activated carbon as described above.

Fermentation conditions

The production medium used for shake flask experiments and stirred tank bioreactor experiments had the following composition (g l^{-1}): sucrose 50.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4.7H_2O$ 0.2 and NaCl 1.0 (pH 7.5). The substrate was sterilized at 121 °C for 15 min. When molasses were used for fermentation experiments, the molasses solution after the above treatments was diluted to contain 50 g l^{-1} of total sugars and pH was adjusted to 7.5 with 10 N NaOH. Following sterilization at 121 °C for 15 min, the molasses were used for the production of pullulan. The shake flask experiments were conducted in a temperature controlled shaker (B. Braun Certomat) operated at 200 rpm, 28 °C. The shake flasks were 250 ml Erlenmayer flasks containing 50 ml of either molasses or synthetic medium as the production medium. The bioreactor experiment with synthetic medium was performed in a 7 l Chemap AG fermentor with a working volume of 5 l. The bioreactor consisted of a glass vessel with stainless-steel endplates and 4 equally spaced vertical baffles. Agitation

was provided by 2 four-flat-blade impellers. After sterilization and cooling, 5 l of production medium was added to the bioreactor. The medium was inoculated with 250 ml of inoculum. The agitation speed and aeration rate used were 400 rpm and 2 vvm (vol air/vol medium/min), respectively.

To determine the effect of pH of the production medium on pullulan production, a series of conical flasks containing 50 ml of synthetic medium were prepared. The pH of the medium was adjusted to 4.5, 5.5, 6.5, 7.5 and 8.5 with 10 N NaOH and the substrate was inoculated with 5% (v/v) of the inoculum. To determine the effect of initial sugar concentration on pullulan production, 50 ml of synthetic medium containing 30, 50 and 70 g l^{-1} of sucrose in 250 ml flasks was prepared and inoculated with 5% (v/v) of the inoculum. The flasks were incubated under the same conditions as described earlier.

Analytical techniques

Biomass dry weight was determined by centrifuging 50 ml of fermentation broth at 4000 x g for 10 min, and washing with distilled water followed by drying at 103 °C. Polysaccharide content was determined by adding the first supernatant from biomass dry weight determination to the washings, and the polysaccharide was precipitated with 2 volumes of ethanol at 4 °C for 1 h. The precipitate was centrifuged at 4000 x g for 10 min followed by drying at 80 °C overnight and was then weighed. To determine the pullulan content of the polysaccharide, the precipitate was resuspended in 0.05 M sodium acetate (pH 5.0) at a concentration of 10 mg ml^{-1} and 10 µl of pullulanase (Promozyme D2, Novozymes A/S, Denmark) was added to 1 ml of sample. The mixture was incubated at 25 °C for 21 h according to the procedure of Leathers et al. (14) The enzyme was also added to a pure sample of pullulan (Hayashibara Biochemical Co., Japan) of the same concentration as described earlier. Using a reducing sugar assay (15), the glucose reducing equivalents were determined and the actual pullulan content was derived. Total sugar was determined according to the phenol sulfuric acid method using sucrose as the standard (16). All experiments were done in triplicate samples and mean values were calculated. The polysaccharide yield was expressed as grams of polysaccharide per 100 g of sugar consumed. The sugar utilization was calculated by dividing the sugar consumed during fermentation by the initial sugar quantity and multiplying the result by 100.

Results and Discussion

Effect of treatment of beet molasses on polysaccharide production

Fermentation media containing 50 g l^{-1} of initial sugar were prepared from pretreated molasses and synthetic medium and the kinetics of biomass growth and polysaccharide production in shake flasks at 28 °C were determined. The kinetics of the production of polysaccharide by *A. pullulans* are given in Figure 1. The highest polysaccharide concentration (35.0 g l^{-1}) was obtained in molasses treated with sulfuric acid + activated carbon, followed by synthetic medium (21.6 g l^{-1}).

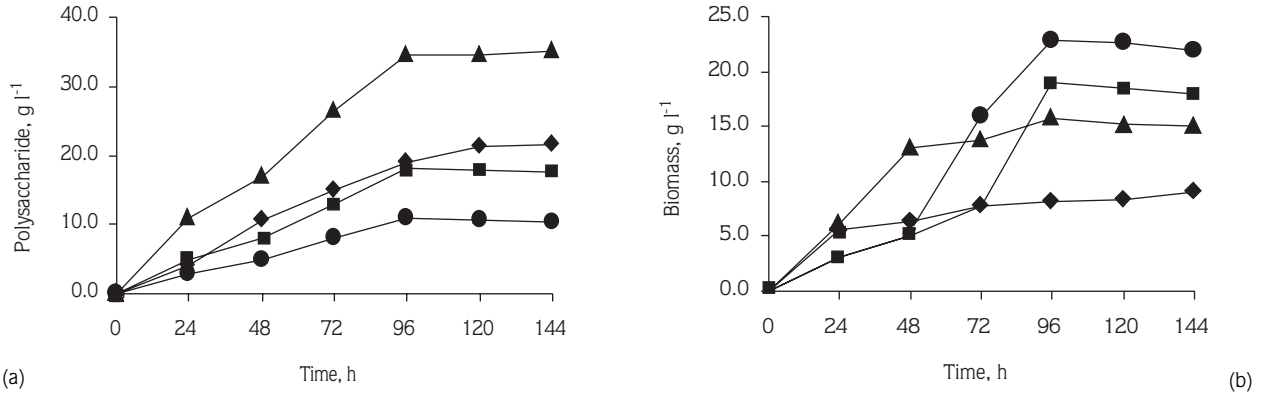

Figure 1. Polysaccharide (a) and biomass (b) production of *A. pullulans* in synthetic medium (◆), sulfuric acid + activated carbon treated molasses (▲), sulfuric acid treated molasses (■), potassium ferrocyanide treated molasses (●) in shake flasks at 28 °C, 200 rpm (pH 7.5, initial sugar concentration = 50 g l^{-1}).

Molasses treated with sulfuric acid and potassium ferrocyanide gave polysaccharide yields of 18.0 and 11.0 g l^{-1}, respectively. As seen in Figure 1b, the highest biomass (22.8 g l^{-1}) was obtained in potassium ferrocyanide treated molasses medium at the 96th h of fermentation. Molasses treated with sulfuric acid, sulfuric acid + activated carbon and synthetic medium gave biomass yields of 19.0, 15.8 and 9.0 g l^{-1}, respectively. The biomass dry weight followed a pattern similar to that of polysaccharide production during fermentation, which indicates that pullulan production takes place during the growth phase of the culture. In molasses medium, the pH of the fermentation medium decreased during the first 72 h of fermentation from an initial value of 7.5 to 5.5 and then increased to 8.1 at the end of fermentation. In synthetic medium pH decreased from 7.5 to 3.0 during the fermentation. Synthesis of organic acid could be attributed to the increase in acidity of the fermentation broth, whereas the increase in the pH of molasses medium could be due to the deamination of amino-acids in molasses by *A. pullulans* and the production of ammonia, which increased the pH of the fermentation medium.

The residual sugar concentrations in molasses treated with sulfuric acid, sulfuric acid + activated carbon and potassium ferrocyanide were 6.0, 2.0 and 12.3 g l^{-1}, respectively. The residual sugar concentration in the synthetic medium was 9.0 g l^{-1} . The corresponding yield values were 54.0, 40.9, 74.5 and 29.2% and sugar utilization values were 81.6, 88.0, 95.9 and 75.4% for synthetic medium, molasses treated with sulfuric acid, sulfuric acid + activated carbon and potassium ferrocyanide, respectively.

These results showed that the complex ingredients and composition of molasses enhanced the growth of *A. pullulans* since higher biomass yields were observed in molasses medium than in synthetic medium. In addition, an inverse relation between polysaccharide production and cell weight seemed to exist. Other researchers (17,18) also stated that good biomass production does not always give high polysaccharide yields.

The superiority of the combined treatment of sulfuric acid and activated carbon over other treatments of molasses regarding polysaccharide production may be due to the significant removal of heavy metals as well as color substances from molasses. These compounds found in high concentrations in crude molasses are generally considered fermentation inhibitors, limiting the utilization of molasses as substrate in industrial fermentations (19). The effect of sulfuric acid treatment on the mineral content of molasses in fermentation processes has been stated elsewhere (20).

Israilides et al. (2) produced pullulan from the fermentation of various agro-industrial wastes and reported a relatively low yield of polysaccharides from untreated molasses by *A. pullulans*. Roukas (1) studied the effect of different pretreatments of molasses supplemented with K_2HPO_4, L-glutamic acid, olive oil and Tween 80 on the production of pullulan. That study obtained the maximum polysaccharide concentration (32.0 g l^{-1}), biomass dry weight (33.8 g l^{-1}), polysaccharide yield (63.5%) and sugar utilization (97.5%) in sulfuric acid treated molasses by *A. pullulans*. Lazaridou et al. (19) found the highest pullulan concentration (24 g l^{-1}) and biomass dry weight (14 g l^{-1}) by *A. pullulans* in molasses pretreated with sulfuric acid and activated carbon. LeDuy and Boa (9) used peat hydrolyzate as the substrate for the production of pullulan and obtained ≈ 14 g l^{-1} of maximum polysaccharide with various strains of *A. pullulans*. Shin et al. (13) obtained pullulan concentrations of 17.5 and 15.5 g l^{-1} using inulin and Jerusalem artichoke extracts, respectively. The reasons for such variability in the literature are the strain of microorganism, the chemical composition of the substrate, the pretreatment method and the conditions employed during fermentation.

In general, the results of this study showed that the pretreatment of molasses with sulfuric acid in combination with activated carbon significantly improved the pullulan production. Hence molasses medium pretreated with sulfuric acid + activated carbon and synthetic medium, which gave the highest yields of polysaccharide, were used for further experiments.

Pullulan content of polysaccharide produced by *A. pullulans* in molasses and synthetic medium

Since *A. pullulans* produces various polysaccharides other than pullulan, the pullulan content of the polysaccharide produced by *A. pullulans* was determined in molasses medium and synthetic medium. The kinetics of polysaccharide and pullulan production by *A. pullulans* in molasses medium treated with sulfuric acid + activated carbon and synthetic medium is given in Figure 2. The

maximum pullulan concentration (16.9 g l^{-1}) was obtained at the 144th h of fermentation in molasses medium whereas 16.7 g l^{-1} of pullulan was obtained at the 120th h of fermentation in synthetic medium. The highest pullulan proportions were 85.3 and 48.3% of the total polysaccharide in synthetic medium and molasses medium, respectively. These results showed that pullulan produced from molasses medium contained a high percentage of impurities and other polysaccharides while pullulan produced from chemically defined synthetic medium was of much higher purity. Israilides et al. (10) also stated that molasses resulted in the formation of heterogeneous ethanol-precipitated substances containing small amounts of pullulan and found that 4.98% of the polysaccharides was pullulan in the molasses medium. Israilides et al. (2) used different substrates for pullulan production and found that 40% of the total mass of polysaccharide produced from molasses was pullulan. Roukas and Liakopoulou-Kyriakides (21) found that the pullulan content of crude polysaccharide was 35% when molasses treated with sulfuric acid were used as substrate. Roukas (1) found that the pullulan content of the crude polysaccharide was 30-35% and concluded that *A. pullulans* grown on molasses medium produced other polysaccharides. The results of this study are in agreement with the literature. However, the pullulan content of the polysaccharide proportion obtained from molasses medium was higher compared to those of the other studies.

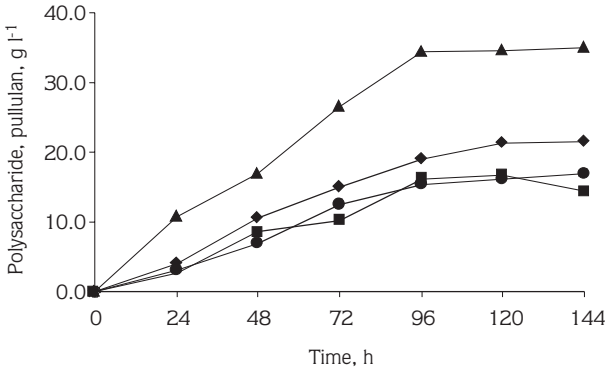

Figure 2. Polysaccharide and pullulan production by *A. pullulans* in molasses medium treated with sulfuric acid + activated carbon and synthetic medium in shake flasks at 28 °C, 200 rpm (polysaccharide in synthetic medium, ◆; pullulan in synthetic medium, ■; polysaccharide in molasses medium, ▲; pullulan in molasses medium, ●)

Effect of initial sugar concentration

To determine the effect of sugar concentration on the final concentration of polysaccharide and pullulan, synthetic meda containing 30, 50 and 70 g l^{-1} of sugar were used. Fermentation was performed in shake flasks at 28 °C, pH 7.5. When the sugar concentration was increased from 30 to 50 g l^{-1}, both the polysaccharide and the pullulan concentrations increased (Figure 3).Beyond a 50 g l^{-1} sugar concentration, the polysaccharide concentration remained relatively constant, but there was a significant decrease in the pullulan concentration due to inhibition produced by the high sugar concentration, which is a characteristic of a batch culture. Chul Shin et al. (22) also found that pullulan production was inhibited at high initial sucrose concentrations and stated that the yields could be increased by using a fed-batch fermentation. The maximum polysaccharide concentrations obtained from synthetic medium containing 30, 50 and 70 g l^{-1} of sugar concentration were 13.6, 21.6 and 20.8 g l^{-1}, respectively. The corresponding pullulan concentrations were 9.2, 16.7 and 5.6 g l^{-1}, respectively.

The concentration of sugars in the fermentation medium declined during fermentation (data not shown), following an inverse trend to polysaccharide and pullulan production. In the cultures with 30 and 50 g l^{-1} initial sugar concentrations, there was almost complete depletion of sugars after 72 h and 144 h of incubation,

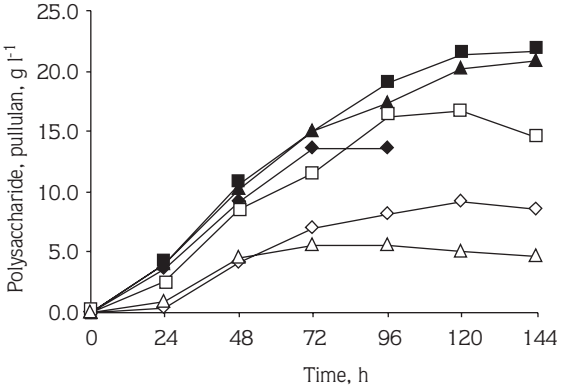

Figure 3. Polysaccharide and pullulan production with *A. pullulans* from synthetic medium containing different initial sugar concentrations in shake flasks at 28 °C, 200 rpm (polysaccharide from 30 g $^{-1}$ initial sugar, ◆; polysaccharide from 50 g $^{-1}$ initial sugar, ■; polysaccharide from 70 g $^{-1}$ initial sugar, ▲; pullulan from 30 g $^{-1}$ initial sugar, ◊; pullulan from 50 g $^{-1}$ initial sugar, ❑; pullulan from 70 g $^{-1}$ initial sugar, Δ).

respectively. When the maximum concentration of polysaccharides was obtained in the fermentation medium, the residual sugar concentrations were 0.2, 3.8 and 30.0 g l^{-1} in media containing 30, 50 and 70 g l^{-1} of initial sugar, respectively. The corresponding polysaccharide yield values were 50.2, 47.8 and 55.3% and sugar utilization values were 97.8, 91.6 and 56.2%, respectively. In all fermentations (Figure 3) there was a slight decline in the pullulan yield at later stages of culture growth. This decline was most probably due to the hydrolysis of pullulan by an endogenous glucoamylase A released by the microorganism during the later stages of fermentation (19).

Effect of pH

The pH of the medium is important in pullulan production by *A. pullulans* since it affects the morphology of the organism, which in turn influences pullulan production. The effect of initial pH (4.5-8.5) on the kinetics of polysaccharide and pullulan production from synthetic medium by *A. pullulans* is given in Figure 4. The polysaccharide and pullulan concentrations increased when the initial pH of the culture medium increased from 4.5 to 7.5 and decreased thereafter. However, the biomass dry weight decreased with each unit increase of pH. The highest polysaccharide (21.4 g l^{-1}) and pullulan concentrations (16.7 g l^{-1}) were obtained in cultures grown at an initial pH of 7.5. The highest biomass dry weight (14.1 g l^{-1}) was obtained at an initial pH of 4.5. Lacroix et al. (23) studied pullulan production in sucrose based synthetic medium using 2 strains of *A. pullulans* and found that at very low initial pH values (pH 2.0) the polysaccharide production was very low. They stated that at higher initial pH values (pH 5.5) the maximum polysaccharide concentrations were obtained, and that contrary to polysaccharide production *A. pullulans* grew best at a very low initial pH of 2.0. Similar results were obtained by Heald and Kristiansen (6) who showed that the more acidic the environment, the lower the accumulation of pullulan. Auer and Seviour (24) observed maximum polysacharide concentration at an initial pH of 7.5. Roukas (11) studied pullulan production from deproteinized whey and determined the maximum polysaccharide concentration at an initial pH of 6.5. Ono et al. (25) used synthetic medium and determined optimum polysaccharide production at a pH of 6.0. Lacroix et al. (23) reported an optimal pH of 5.5 for pullulan production by *A. pullulans* grown in a chemically defined medium. The observed variation in optimum pH is possibly due to the different strains of the microorganism employed as well as the substrate composition (e.g., amount and type of nitrogen source) and the various fermentation systems used in these studies. The results in Figure 4 clearly indicate the importance of initial pH of the medium to microbial growth and pullulan production.

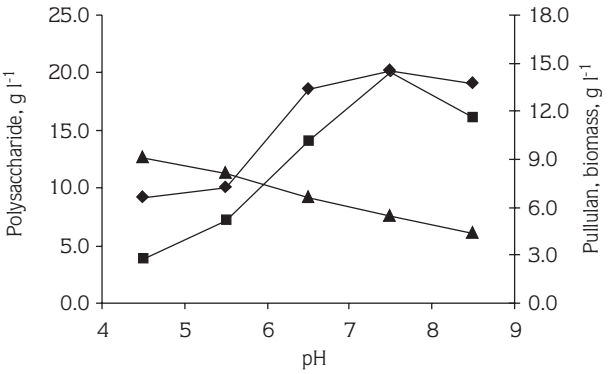

Figure 4. The effect of pH on pullulan, polysaccharide and biomass production by *A. pullulans* in shake flasks at 28 °C, 200 rpm (polysaccharide, ◆; pullulan, ■; biomass, ▲).

Production of pullulan in a stirred tank bioreactor

A. pullulans was grown at 28 °C in a stirred tank bioreactor using synthetic medium containing 50 g l^{-1} of initial sugar (pH 7.5). The aeration rate and agitation speed were 2 vvm and 400 rpm, respectively. As shown in Figure 5, polysaccharide concentration increased steadily during the entire period of fermentation up to a level of 20.4 g l^{-1} at 168 h of incubation. Maximum pullulan concentration (6.6 g l^{-1}) was obtained at the 96th h of fermentation and decreased afterwards, possibly due to the hydrolysis of pullulan by an endogenous glucoamylase A released by *A. pullulans* (19). The biomass concentration increased during the fermentation and remained virtually constant after the 144th h of fermentation, and a maximum biomass of 9.4 g l^{-1} was obtained. As expected, the concentration of residual sugars decreased to a minimum of 3 g l^{-1} during the course of fermentation, coinciding with the increase in biomass and pullulan production (Figure 5). Polysaccharide yield and sugar utilization values were 43.4 and 94.0, respectively. While the polysaccharide concentration obtained in the bioreactor was almost the

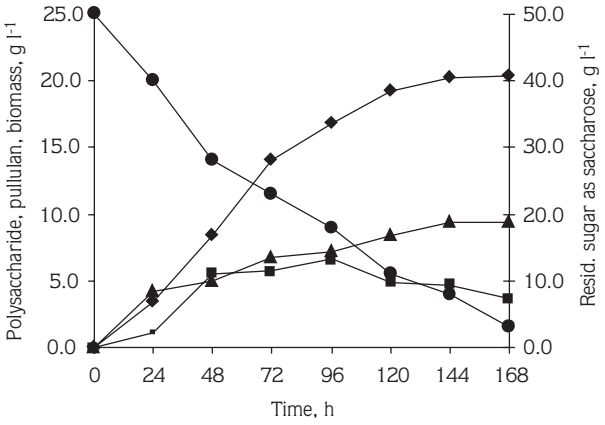

Figure 5. Polysaccharide and pullulan production by *A. pullulans* in a stirred tank bioreactor using synthetic medium (polysaccharide, ◆; pullulan, ■; biomass, ▲; saccharose, ●).

same as the polysaccharide concentration in the shake flask experiments (21.6 and 20.4 g l^{-1}, respectively), the pullulan concentration (6.6 g l^{-1}) obtained in the stirred tank biorector was significantly lower than that in the shake flasks (16.7 g l^{-1}). Thus, it was concluded that the scale up studies in the stirred tank bioreactor resulted in higher production of polysaccharides other than pullulan by *A. pullulans*. Gibbs and Seviour (3) found maximum polysaccharide levels of 6 g l^{-1} when *A. pullulans* was grown in synthetic medium in a stirred tank bioreactor. Using a fast-producing strain of *A. pullulans* and a concentrated synthetic medium (80 g glucose l^{-1}), Moscovici et al. (7) observed final polysaccharide concentrations as high as 50 g l^{-1} in a stirred tank bioreactor. Roukas and Liakopolou-Kyriakides (21) obtained a maximum polysaccharide concentration of 23 g l^{-1} from beet molasses medium in a stirred tank bioreactor and the highest value of pullulan proportion was 35% of the total polysaccharide.

Conclusions

The results of this study show some important aspects of pullulan production from beet molasses and synthetic medium. In molasses medium, the highest polysaccharide was obtained from molasses pretreated with sulfuric acid and activated carbon. *A. pullulans* grown on beet molasses produced a mixture of pullulan and other polysaccharides since the highest values for pullulan were 85.3 and 48.3% of the total polysaccharide for synthetic medium and molasses medium, respectively. The maximum polysaccharide and pullulan concentration was obtained at an initial sugar concentration of 50 g l^{-1} and pH 7.5 from synthetic medium. In a stirred tank bioreactor with synthetic medium, the pullulan yield decreased (39.3% of crude polysaccharide) compared to that from the shake flasks, which showed that the pullulan content of the fungal polysaccharide was strongly influenced by the substrate used and the fermentation technique. Further work is required for increasing the pullulan content of the total polysaccharide in the molasses medium and for optimizing the process on a pilot and industrial scale.

Acknowledgments

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