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Evaluation of Carob Pod as a Substrate for Pullulan Production by *Aureobasidium pullulans*

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ABSTRACT

The production of extracellular polysaccharides from carob pod extract by *Aureobasidium pullulans* in batch fermentation was investigated. Optimum conditions for polysaccharide productivity, polysaccharide yield, and fermentation efficiency were: initial sugar concentration of 25 g/L, initial pH 6.5, and temperature 25–30°C. A maximum polysaccharide concentration (6.5 g/L), polysaccharide productivity (2.16 g/L/d), total biomass concentration (6.3 g/L), and polysaccharide yield (30%) were obtained with inoculum at 10% (v/v), initial sugars in carob pod extract of 25 g/L, pH 6.5, and 25°C. The highest values of pullulan proportion (70% of total polysaccharides) and fermentation efficiency (89%) were assumed at initial sugar concentration of 25 g/L, pH 6.5 and 30°C. Structural characterization of purified pullulan samples by monosaccharide and methylation analyses, ^{13}C-NMR, and pullulanase digestion combined with size-exclusion chromatography revealed the presence of mainly α-(1 → 4) (68%) and α-(1 → 6) (31%) glucosidic linkages; however, small amounts (< 1%) of triply linked (1, 3, 4- and 1, 4, 6-Glc) glucose residues were detected. The molecular-size distribution and intrinsic viscosity of pullulan derived from culture grown at pH 4.5 (30°C) showed a higher molecular weight than its counterpart obtained at pH 6.5 (30°C).

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Index Entries: Pullulan; fermentation; carob pod; polysaccharide; structure.

INTRODUCTION

The carob pod is the fruit of carob tree (*Ceratonia siliqua*), which is mainly cultivated in the Mediterranean countries and in some semiarid regions of North America. The annual world production is about 340–400 × 10^3 metric tons (*1*). Greece is a main producer with an annual harvest of 21 × 10^3 tons (*2*). Carob pod consists of the kibble and the seeds, which contain a storage polysaccharide (galactomannan, also known as locust bean gum) that is highly valued in the food, textile, and cosmetic industries (*3*). A typical carob kibble composition (expressed as g/100 g of kibble) is: moisture 10–15, total sugars (glucose, fructose, sucrose, and maltose) 40–50, protein 3–4, pectin 1–2, cellulose 7, hemicelluloses 5, phenolic compounds 20, fat 0.5–1.0, and ash 2–3 (*2*). The carob pod is used as animal feed, in the preparation of antiarrheic and antiemetic products, baking pastries, and as a cocoa substitute (*4*). Because of its high sugar content, the carob kibble may find new and more attractive uses, e.g., as a fermentation substrate. Recently, the production of ethanol from carob pod extract by free and immobilized *Saccharomyces serevisiae* cells has been described (*2,5,6*).

Pullulan is an extracellular water-soluble microbial polysaccharide produced by several microorganisms. It is a linear mixed-linkage polysaccharide of mainly maltotriose units interconnected via α-(1 → 6) linkages and is commercially produced by fermentation using *Aureobasidium pullulans*. The most interesting applications of this biopolymer are related to its film-making properties; pullulan films are impermeable to gasses (particularly 0_2) and exhibit good flavor retention, oil resistance, and antistatic properties. Pullulan has been tested as coating and packaging material, as a sizing agent for paper, in cosmetic emulsions, as a starch replacer in foods, and in other industrial and medicinal applications (*7,8*).

The commercial production of pullulan from synthetic media by *A. pullulans* has been reported (*9–12*). However, utilization of sucrose or glucose as carbon source is not economical, and a less expensive carbohydrate source would be beneficial. LeDuy and coworkers (*13*) and Boa and LeDuy (*14*) employed lactose and peat hydrolyzates, respectively, as fermentation substrates for pullulan production. Recently, attention has been given to agricultural crops as potential substrates, e.g., Jerusalem artichoke tubers (*15*). Considering the low price of kibbles (US $50/ton), the aim of the present investigation was to examine the carob pod as a substrate for pullulan production. The effect of various parameters, such as inoculum level, initial sugar concentration, pH, and temperature, on the kinetic aspects of fermentation were studied. Structural characterization of the pullulan was also carried out.

MATERIALS AND METHODS

Microorganism and Culture Conditions

A. pullulans SU No. M18, kindly supplied by L. Harvey of the University of Strathclyde, was employed throughout these studies. The microorganism was maintained on potato dextrose agar plates at 4°C and subcultured every 2 wk. Cell suspension for inoculation of culture medium was obtained from culture grown on a potato dextrose agar plate at 28°C for 48 h. The cells were suspended in 10 mL of sterile distilled water and transferred to a 500-mL conical flask containing 100 mL of culture medium (pH 5.5) of the following composition (g/L): sucrose 50, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5, $MgSO_4 \cdot 7H_2O$ 0.2, and NaCl 1.0. The flask was incubated at 28°C for 48 h in a rotary shaker/incubator (Lab-Line Orbit-Environ shaker, Lab-Line Instr., Inc., Melrose Park, IL) at 200 rpm. This culture broth was used to inoculate the production medium at a ratio of 10% (v/v) or at some other specified level.

Preparation of Fermentation Medium

Carob pods (cultivar *Tylliria*) were obtained from the local market. After removing the seeds, the kibbles were comminuted into small particles (0.3–0.4 cm diameter). Comminuted kibbles (45 g) were mixed with 180 mL distilled water (solid/liquid 1:4), and the mixture was shaken in a rotary shaker/incubator at 250 rpm for 2 h at 70°C to extract sugars from the kibble. The suspension was centrifuged at 4000*g* for 15 min, and the supernatant was diluted with distilled water to obtain 2.5 and 5% (w/v) initial sugar concentration. The pH of these extracts was adjusted to 6.5 with 1*N* NaOH and sterilized (121°C for 15 min). Carob pod extracts so prepared (production medium) were used for production of pullulan by *A. pullulans*. The fermentation was carried out in 500-mL conical flasks, containing 100 mL of extract inoculated with 10 mL of inoculum, and incubated at 30°C using a rotary shaker/incubator at 200 rpm.

Effect of Fermentation Parameters

Initial Sugar Concentration

Carob pod extracts containing either 2.5 or 5% (w/v) initial sugars were used to examine the effect of sugar concentration on the kinetic aspects of fermentation.

Nutrients

A set of conical flask experiments were performed at different levels of nutrients to examine their effect on pullulan production. These flasks, containing 100 mL of production medium (initial sugars 25 g/L, pH 6.5), were further supplied with either the same amounts of nutrients present in the culture medium (except sucrose) or with different amounts of

$(NH_4)_2SO_4$ (0.3, 0.6, and 1.2 g/L). The flasks were inoculated with 10 mL of inoculum and incubated at 30°C for 72 h.

Amount of Inoculum

A series of conical flasks containing 100 mL of production medium were inoculated with 2.5, 5.0, 7.5, and 10 mL inoculum, and incubated at 30°C for 72 h.

Initial pH and Bistaged pH

Conical flasks (100 mL production medium) were adjusted at different initial pH, (3.5, 4.5, 5.5, and 6.5), inoculated with 10 mL of inoculum, and incubated at 30°C for 72 h. The production of polysaccharide was also examined in a bistaged pH process. In this case, the initial pH was adjusted to 6.5 or 3.5, and after 48 h of incubation (first phase), the pH was adjusted to 3.5 or 5.0, respectively. The fermentation was then allowed to proceed for an additional 72 h (second phase).

Temperature

Portions (100 mL) of the production medium were inoculated with 10 mL of inoculum and incubated at different temperatures (25, 30, 35, and 40°C) for 72 h.

Separation and Purification of Pullulan

The fermentation broth (after appropriate dilution by a factor up to 1:5) was centrifuged at 10,000*g* for 20 min to remove the cells of the microorganism. The crude polysaccharide was initially precipitated with 2 vol of acetone, dissolved in distilled water, and reprecipitated with absolute ethanol. The polysaccharides were then dissolved in water and fractionated into soluble and insoluble fractions by adding an equal volume of cetyltrimethylammonium bromide (CTAB) solution at pH 6.0 (*16*); the amount of detergent present was equal by weight with the crude polysaccharide. The CTAB-soluble fraction, corresponding to pullulan, was precipitated with 2 vol ethanol and refluxed (3x) with 100 mL ethanol to remove residual detergent. The pullulan preparation was then dissolved (70°C) in distilled water and treated with 10% (v/v) toluene to remove contaminating proteins; denatured proteins partition at interface. The aqueous phase was treated with 2 vol of ethanol to precipitate the polysaccharide. This procedure was repeated, and the polysaccharide was finally washed with ether and dried under vacuum (40°C) overnight. This preparation was designated purified pullulan.

Chemical Analyses

Fermentation Aspects

At specific time intervals, the flasks were removed and the fermentation broth was analyzed. Total biomass (mycelial and yeast cells) dry weight was determined by centrifugation of the broth (after appropriate

dilution) at 10,000*g* for 20 min, washing (2x) the sediment with distilled water, and drying at 105°C overnight. The first supernatant was combined with the washings, and the crude polysaccharide was precipitated with 2 vol ethanol. The precipitate was filtered through a preweighed Whatman GF/A filter and dried at 105°C for 6 h. Residual sugars as glucose were determined in the filtrate according to ref. (*17*). Mycelial and yeast forms were separated by filtration using nylon mesh with pore size of 45 μm (*18*). Mycelium dry weight was estimated by filtration of 10 mL of fermentation broth through preweighed nylon mesh, washing with 20 mL of distilled water, and drying (105°C, 6 h). The yeast cells dry weight was calculated from the difference between total biomass and mycelium. Polysaccharide yield was expressed as crude polysaccharide/100 g of sugars consumed, whereas as fermentation efficiency was taken as the ratio of sugars consumed over initial sugars multiplied by 100.

All experiments were repeated three times, and the reported data are averages. Variability is expressed by coefficient of variation (cv) values.

Structural Characterization of Pullulan

Monosaccharide analysis of pullulan was carried out by GC using an SP-2330 glass capillary column, 30 m × 0.75 mm id, according to Englyst and coworkers (*19*). The proton-decoupled ^{13}C-NMR spectra (300 Hz) were recorded on a Bruker AM 300 FT spectrometer at 65°C, polymer concentration 2.0% (w/v) in D_2O, 30,000 pulses, pulse repetition time 1.245 s, and r.f. pulse angle 80.0°. Chemical shifts (δ) are expressed in ppm downfield from external Me_4Si, but were actually measured by reference to internal 1,4-dioxane (Aldrich Chemical Co. Inc., Milwaukee, WI) (δ = 67.4 ppm). Methylation analysis was carried out according to ref. (*20*). GC-MS was performed on an SP-2330 capillary column (60 m × 0.25 mm), whereas quantitative measurements of partially permethylated acetyl alditols were made on an SP-2330 column (30 m × 0.75 mm), programmed from 160 to 210°C at 2°C/min; helium was used as carrier gas (15 psi). Limiting viscosity measurements of aqueous solutions of pullulan (in deionized water) were made using Ubbelohde capillary viscometers at 20°C. The intrinsic viscosity was calculated from the Huggins equation (*21*) and the weight-average molecular weight from the equation $[\eta] = (0.000258)\ Mw^{0.646}$ (*22*). The molecular-size distribution of pullulan was performed on a Sepharose 2B column (2.5 × 98.5 cm). Aliquots (3 mL) of pullulan (8–10 mg) in 0.1*M* NaCl applied on the column were eluted with 0.1*M* NaCl containing 0.05% NaN_3 at a flow rate of 50 mL/h at 25°C. Effluent fractions (5.25 mL) were monitored for total carbohydrates by the phenol-sulfuric method (*17*). Debranching of pullulan (30 mg) in 10 mL 0.1*M* acetate buffer (pH 4.7) was carried out with 32 IU of crystalline pullulanase (Hayashibara Biochem. Lab. Inc., Okayama, Japan) at 37°C for 12 h (*23*). Following debranching, the enzyme was inactivated in a boiling water bath (20 min), the digest was filtered, and 4 mL were applied on a Biogel P-2 column (2.5 × 94 cm) eluted with 0.1*M* sodium acetate (pH 4.7)

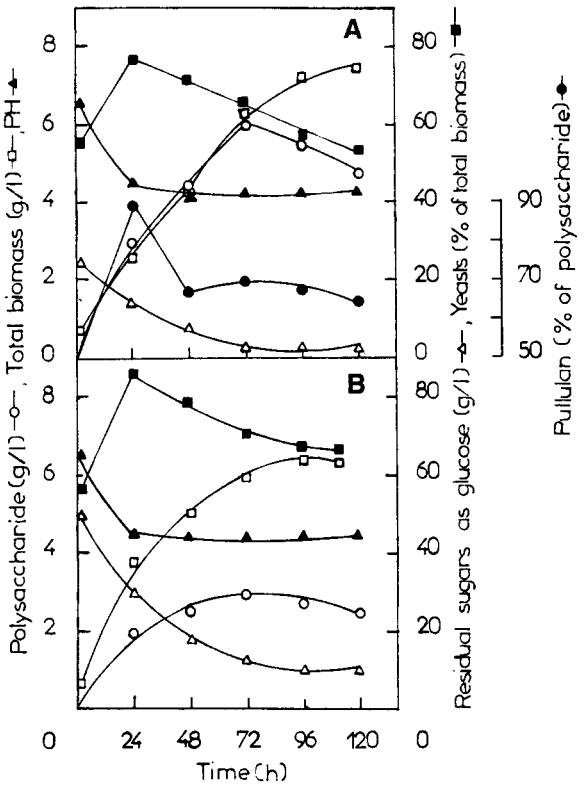

Fig. 1. Fermentation kinetics of carob pod extract by *A. pullulans* in shake-flask culture at two initial levels of sugar concentration: (**A**) total sugars 25 g/L; (**B**) total sugars 50 g/L. Data are means of triplicate experiments.

containing 0.02% NaCl at 32 mL/h (25°C). Fractions of 3.125 mL were analyzed for total carbohydrates (*17*).

RESULTS AND DISCUSSION

Effect of Initial Sugar Concentration

The effect of initial sugar concentration on the kinetic aspects of carob pod fermentation by *A. pullulans* is shown in Fig. 1. The crude polysaccharide concentration increased as fermentation progressed up to 72 h and then decreased. This decline may reflect hydrolysis of polysaccharides by endogenous hydrolases released by the microorganism at later stages of fermentation (*24*). Moreover, total polysaccharides decreased with an increase of the initial sugar concentration (Fig. 1A vs B). The highest concentration of polysaccharide (6.0 g/L) was obtained for culture grown in medium of 25 g/L sugar concentration after 72 h of incubation; the medium containing 50 g/L sugars gave almost half the amount of polysaccharide at

72 h. This observation may imply the presence of inhibitory factors in the carob pod extract that exert a detrimental effect on growth and biosynthesis of extracellular glucans when present at high concentration. Gibbs and Seviour (*10*) reported maximum levels for polysaccharides of 3 and 6 g/L when *A. pullulans* (ATCC 3092) was grown in synthetic medium in airlift vessels and shirred-tank fermentors, respectively. Using a fast-producing strain of *A. pullulans* (ICCF-68), Moscovici and coworkers (*11*) have recently reported final polysaccharide concentrations as high as 50 g/L in synthetic medium (80 g glucose/L) and a stirred-tank fermenter. LeDuy and Boa (*8*) found maximum levels of polysaccharide at 12–14 g/L for various strains of *A. pullulans* grown in peat hydrolyzate in shake-flask culture. Finally, Shin and coworkers (*15*) found that a coculture of *Kluyveromyces fragilis* and *A. pullulans* (SH 8646) produced 15.5 g/L exopolysaccharides when Jerusalem artichoke extract containing 50 g/L total carbohydrates was fermented in submerged culture. Several factors could account for the variation in yield reported in these studies; these include the strain of microorganism, the chemical composition and any impurities present in the medium (inhibitors, promoters, and so forth), the fermentation system, and the conditions employed during fermentation.

The proportion of pullulan (as percent of total polysaccharides) increased rapidly during the first 24 h, and then decreased and remained practically constant between 48 and 120 h (Fig. 1A); the maximum level of pullulan produced (4.2 g/L) was obtained at 72 h. Schuster and coworkers (*12*), using the mutant strain *A. pullulans* p56 in batchwise fermentation with synthetic media of various sugars, found pullulan concentrations of about 4.5 g/L at the time of carbon exhaustion.

Total biomass concentration increased during fermentation. In cultures grown at initial sugar concentration of 25 and 50 g/L, maximum biomass was 7.5 and 6.5 g/L, respectively. The percent biomass represented by yeast-like cells reached a maximum at 24 h of incubation and then decreased substantially (Fig. 1) owing to better growth of the mycelium form at lower pH. However, when expressed as yeast cell concentration, this variable increased steadily during the first 72 h and then remained constant (data not shown in Fig. 1). This trend coincided with that of pullulan production, supporting the notion that pullulan is primarily synthesized by the yeast-like cells of the microorganism. The same observations were made in stirred-tank fermentation studies with *A. pullulans* grown in synthetic media (*25,26*).

Throughout the fermentation, the pH decreased (Fig. 1). In addition to metabolic products, synthesis of acidic polysaccharides could contribute to increased acidity of the fermentation broth. The concentration of residual sugars decreased during fermentation, particularly during the first 72 h. An almost complete sugar depletion was observed for the culture grown in extract containing 25 g/L sugars initially (Fig. 1A), whereas 10–12% residual sugars remained in the fermentation broth of the more

concentrated carob pod extract (Fig. 1B). Moreover, at 72 h of fermentation, higher yields of crude polysaccharide (27 vs 8%) and greater fermentation efficiency (89 vs 75%) were obtained with the more dilute medium.

During fermentation of carob pod extract (usually after 48 h), formation of a melanin-like pigment was observed. This pigment is known to be synthesized via the pentaketide pathway with 1,8-dihydroxynapthalene as a precursor (*27*). The appearance of melanin is probably associated with the morphological change of *A. pullulans*; this microorganism exists in two distinct morphologies, nonpigmented blastospores, and pigmented chlamydospores (*27*). The formation of melanin makes the recovery and purification of pullulan more difficult, since it necessitates discoloration with activated carbon. Heald and Kristiansen (*25*) observed minimal melanin formation in experiments with controlled pH at 5.0–6.3. Recently, a new strain of *A. pullulans* that produces high-mol-wt pullulan with reduced pigmentation has been reported (*28*).

Effect of Added Nutrients

The concentration of crude polysaccharide decreased significantly from 6.0 to 3.0 g/L when carob pod extract was supplemented with nutrient ingredients (except sucrose), whereas the total biomass increased from 6.2 to 10.2 g/L (data not shown). Moreover, addition of $(NH_4)_2SO_4$ in the medium (concentrations up to 1.2 g/L) led to a drastic reduction in polysaccharide concentration from 6.0 to 2.2 g/L and an increase in total biomass from 6.2 to 9.0 g/L. Clearly, $(NH_4)_2SO_4$ affects biomass and polysaccharide production in different ways. These findings are consistent with the data of Seviour and Kristiansen (*29*), Bulmer and coworkers (*30*), and Auer and Seviour (*31*), who studied the effect of different nitrogen sources on polysaccharide production by *A. pullulans* in batch culture. There are several possible explanations for the action of the ammonium ion. According to Seviour and Kristiansen (*29*), the cells may become irreversibly committed, in response to high initial NH_4^+ levels, to a continued carbon flow into biomass rather than polysaccharide synthesis. Auer and Seviour (*31*) further suggested that both type and concentration of the nitrogen source may be important in regulating key enzymic systems involved in nitrogen assimilation, thereby affecting the pool of certain intracellular metabolites. The latter, acting as allosteric effectors of carbon flow in the cells, could divert cell metabolism toward growth or polysaccharide synthesis. The physiological changes brought about by NH_4^+ may resemble the modification of citric acid production by *Aspergillus niger* where both phosphofructokinase and pyruvate kinase are activated by this cation leading to enhanced biomass production (*29*). The results of these studies clearly showed that maximal polysaccharide concentration (6.0 g/L) can be obtained from carob pod extract fermentation without any addition of nutrients. Carob pod seems to contain the necessary nutrients for the controlled growth of the microorganism and the production of extracellular polysaccharides.

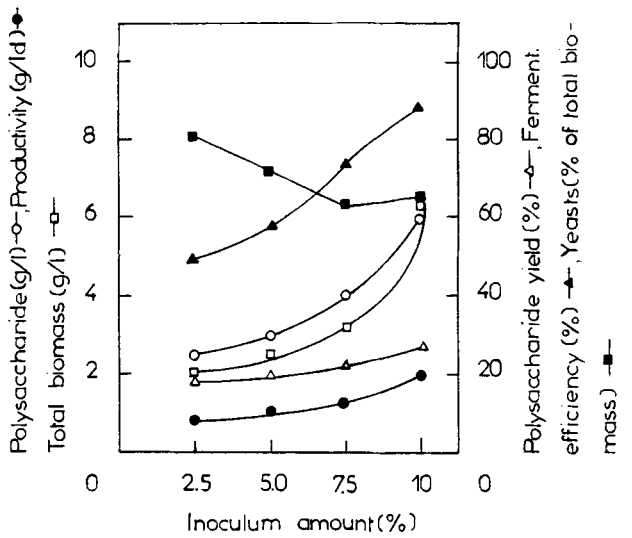

Fig. 2. Effect of inoculum amount on carob pod extract fermentation (sugar concentration 25 g/L, pH 6.5, 30°C, fermentation time 72 h). Data are means of triplicate experiments; cv for all measured parameters did not exceed 8%.

Effect of Inoculum

The effect of inoculum level (2.5–10.0%, v/v) on fermentation of carob pod extract is shown in Fig. 2. Maximum polysaccharide concentration (6.0 g/L), polysaccharide productivity (2.0 g/L), polysaccharide yield (27%), fermentation efficiency (89%), and total biomass concentration (6.2 g/L) were obtained with inoculum of 10% (v/v). At this level of inoculum, only the proportion of yeast cells was reduced (as percent of total biomass). Nevertheless, the 10% inoculum was used in all subsequent experiments, since maximum polysaccharide concentration was obtained under these conditions.

Effect of Initial pH

The effect of initial pH (3.5–6.5) on carob pod fermentation is shown in Fig. 3. Except for the proportion of yeast cells and pullulan, all other fermentation parameters slightly decreased with increasing pH up to 4.5 and then increased. The proportion of yeast cells followed the reverse trend, whereas pullulan as percent of crude polysaccharide increased continuously with the pH. Maximum polysaccharide concentration (6.0 g/L), polysaccharide productivity (2 g/L/d), polysaccharide yield (27%), pullulan concentration (4.2 g/L), fermentation efficiency (89%), and total biomass concentration (6.2 g/L) were obtained in cultures grown at pH 6.5. Using a sucrose synthetic medium, Lacroix and coworkers (32) found maximum polysaccharide production at pH 5.5, whereas maximum biomass concentration was at a very low initial pH of 2.0 for two strains (2552

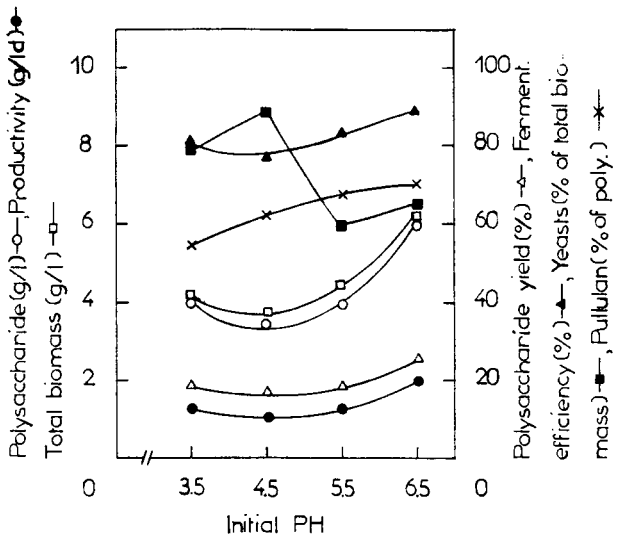

Fig. 3. Effect of initial pH on carob pod extract fermentation (sugar concentration 25 g/L, 30°C, fermentation time 72 h). Data are means of triplicate experiments; cv for all measured parameters did not exceed 6%.

and 140 B) of *A. pullulans*. Ono and coworkers (*24*) also reported maximum production of pullulan with initial pH 6.0 in synthetic medium. In their studies as well as in those of Auer and Seviour (*31*), optimum pH for yeast-like cells and mycelium forms was 6.5 and 2.5–3.5, respectively. In the present work, maximum concentrations for mycelium (2.2 g/L) and yeast cells (4.0 g/L) were obtained at an initial pH of 6.5. The results of Fig. 3 clearly indicate the importance of initial pH of the culture medium on microorganism growth and pullulan production.

Bistaged pH Fermentation

The effect of a two-stage pH adjustment during carob pod extract fermentation is shown in Fig. 4. Cultures grown at initial pH of 6.5 (top) and 3.5 (bottom) and subsequently changed (at 48 h) to 3.5 and 5.0, gave maximum polysaccharide concentrations of 5.5 and 4.5 g/L, respectively. These values are lower than the yield obtained with conventional uncontrolled pH fermentation (6.0 g/L, Fig. 1). With synthetic media, Heald and Kristiansen (*25*) found very little effect on pullulan production by changing the pH of the fermentation broth, whereas Lacroix and coworkers (*32*) reported an increase in polysaccharide concentration by 44–48% when a two-stage pH fermentation procedure (initial pH 2.0, then stepchanged to pH 4–5) was employed. The lower polysaccharide concentration observed on fermentation of carob pod extract (Fig. 4) using a two-step pH process could be related to the relatively poor growth of the mycelial form of the microorganism (1.0 and 0.5 g/L) for the two experimental protocols of Fig. 4.

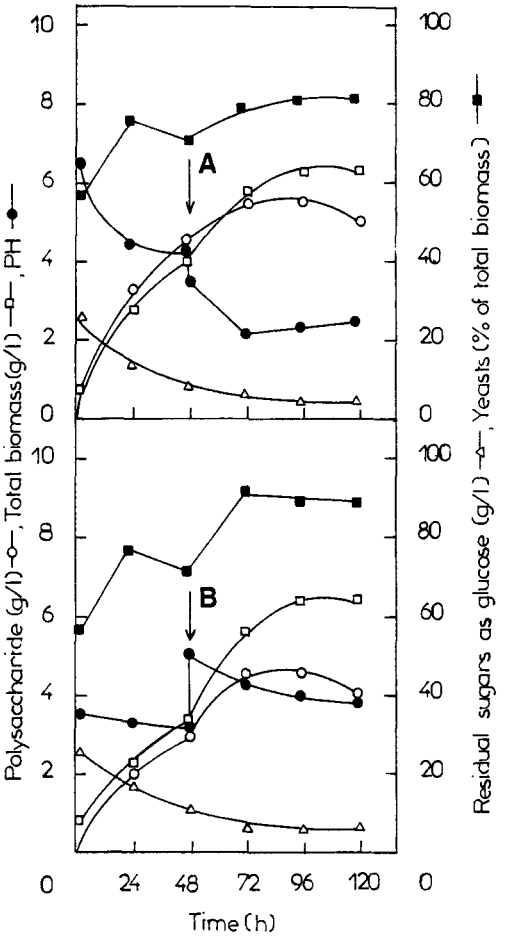

Fig. 4. Fermentation kinetics of a bistaged pH process: (**A**) initial pH 6.5, which shifted to 3.5 after 48 h; (**B**) initial pH 3.5, which shifted to 5.0 after 48 h. Data are means of triplicate experiments.

Effect of Temperature

As shown in Fig. 5, increasing the fermentation temperatures from 25 to 40°C significantly affected the fermentation characteristics. Polysaccharide concentration and productivity as well as fermentation efficiency remained constant between 25 and 30°C and decreased at higher temperatures. This certainly reflects the pronounced reduction in total biomass concentration at temperatures above 30°C. High temperatures may cause denaturation of key enzyme systems in yeast, reducing their capacity to grow and produce secondary metabolites (33). Maximum polysaccharide concentration (6.5 g/L), polysaccharide productivity (2.1 g/L/d), polysaccharide yield (30%), and total biomass concentration (6.3 g/L) were obtained at 25°C. With a sucrose synthetic medium, McNeil

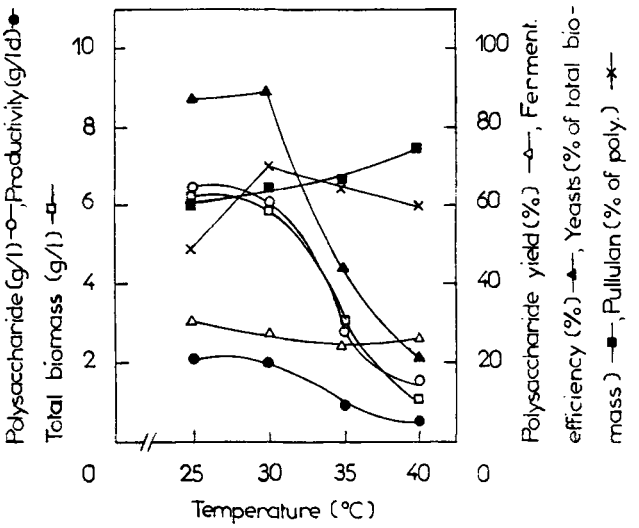

Fig. 5. Effect of temperature on carob pod extract fermentation (sugar concentration 25 g/L, pH 6.5, fermentation time 72 h). Data are means of triplicate experiments; cv for all measured parameters did not exceed 5%.

and Kristiansen (34) reported an optimum temperature of 24°C for polysaccharide production. The results of Fig. 5 indicate that the largest proportion of pullulan (70%) and maximum fermentation efficiency (89%) occur at 30°C. Although the proportion of yeast cells increased continuously with the temperature, the total biomass concentration decreased from 25 to 40°C. As a result, pullulan production decreased with increasing fermentation temperature.

Structural Features of Pullulan

Pullulan samples isolated after 72 h of fermentation (cultures grown in carob pod extract at initial sugar concentration of 25 g/L) of initial pHs 4.5 and 6.5 were subjected to structural analysis using different approaches. The molecular-size distribution profiles of these samples on Sepharose 2B showed single peaks, indicative of rather uniform polysaccharide populations (Fig. 6). The first observation made was the difference in peak elution volumes; pullulan isolated from the culture grown at pH 6.5 eluted in a lower-molecular-size region than that at pH 4.5. The intrinsic viscosity numbers confirmed the molecular-size difference; 0.488 dL/g (pH 6.5) vs 0.749 dL/g (pH 4.5) (Table 1). The corresponding average molecular weights, mol wt, calculated from the Mark-Houwink-Sakurada equation, were 118,000 (pH 6.5) and 229,000 (pH 4.5). Molecular-weight values for pullulan reported in the literature generally vary between 10^5 and 10^6 (35,36), although molecular weights as high as 10^7 have been reported for samples produced from some new strains of *A. pullulans* (28). Although

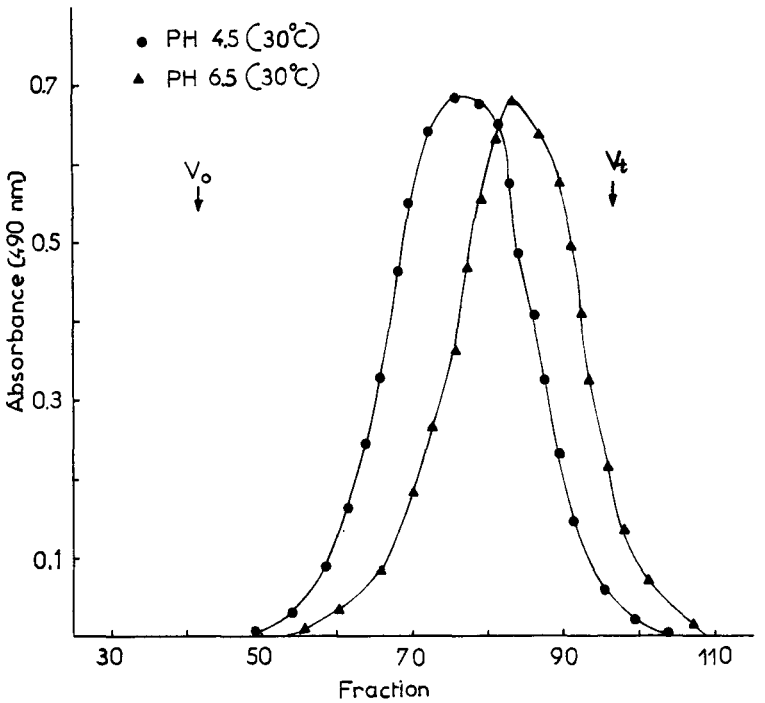

Fig. 6. Elution profiles (Sepharose 2B, 2.5 × 98.5, eluted with 0.1*M* NaCl at 50 mL/h, 25°C) of pullulans isolated from fermentation broths of *A. pullulans* grown in carob pod extract (pH 4.5, 6.5 at 30°C). V_o and V_t denote peak elution volumes of Blue Dextran and glucose, respectively.

Table 1
Relative Molar Ratio of Partially Permethylated Acetyl Alditols of Pullulan, Limiting Viscosity and Estimates of Molecular Weight

Sample	Alditol acetate	Model of linkage	Molar composition[a]	$[\eta]$, dL/g	Mol wt[b]
pH 4.5/30°C	2,3,4,6 Me_4Glc	Terminal Glc	0.37	0.749	2.29×10^5
	2,3,4 Me_3Glc	→1)-Glc-(6 →	32.0		
	2,3,6 Me_3Glc	→1)-Glc-(4 →	67.3		
	Other Glc derivatives[c]		0.33		
pH 6.5/30°C	2,3,4,6 Me_4Glc	Terminal Glc	0.57	0.488	1.18×10^5
	2,3,4 Me_3Glc	→1)-Glc-(6 →	30.8		
	2,3,6 Me_3Glc	→1)-Glc-(4 →	68.2		
	Other Glc derivatives[c]		0.43		

[a]Molar ratio of partially permethylated glucoses as percent of total glucose present in the sample.

[b]The weight average molecular weight was calculated from equation: $[\eta] = (0.000258)\ Mw^{0.646}$ (22).

[c]Partially permethylated acetyl alditols of 1,3,4- and 1,4,6-linked glucose residues.

differences in the reported estimates of molecular weights could be, at least in part, attributed to the method used for determining molecular weight, it is known that several fermentation parameters affect the size of the polysaccharide accumulated in the culture medium. These include pH, substrate composition, time of culture harvest, strain of microorganism, and so forth (*28,35,36*). The influence of pH and sucrose concentration on the molecular size of pullulan was recently studied by Lee and Yoo (*36*). Higher-molecular-size pullulan was obtained at a low initial pH of 3.0 compared to pH 4.5–7.5; our findings (Table 1) concur with their observations. There appears to be a general inverse relation between pullulan yield and molecular size of the polysaccharide (*28*). In this respect, the much higher yield of pullulan obtained from the culture grown at pH 6.5 than at 4.5 (Fig. 3) is not surprising. The underlying physiological basis for the inverse relationship between yield and molecular size is not known. The possibility of interference by the more viscous pullulan solution around the cell with nutrient uptake cannot be excluded (*28*); i.e., the mechanical barrier of a hydrated high-molecular-size polysaccharide network around the cell would make it less efficient in uptaking nutrients from the medium, thus reducing the overall yield.

Methylation analysis of pullulan gave mainly 2,3,6-tri-*O*-methyl- and 2,3,4-tri-*O*-methyl-glucopyranose derivatives at a ratio close to 2:1, consistent with linear chain segments of 4-*O*- and 6-*O*-substituted glucose residues. The ratio of α-(1 → 4) to α-(1 → 6) linkages in pullulans may vary between 2.2:1 and 3:2 (*37*). The 2, 3, 4, 6-tetra-*O*-methyl-glucopyranose originates from the nonreducing glucosyl termini. The fine structure of pullulan from *A. pullulans* cannot be described by a simple polymaltotriosyl polymeric structure (*38–40*). The occurrence of maltotetraose units has been verified and may reach up to 6% of the trisaccharide units depending on the strain (*39*). The presence of other glucosidic linkages, e.g., α-(1 → 3), has been severally described, as reviewed by Catley and coworkers (*40*). It is not certain, however, whether such minor linkages arise from other contaminating glucan impurities or they are present in the native pullulan structure. Methylation of pullulan samples from cultures grown at initial pHs 6.5 and 4.5 (30°C) also revealed minor amounts of triply linked glucose residues (Table 1). As suggested by Catley and coworkers (*40*), pullulan produced by some strains may have tightly packed branched points where additional polymaltotriosyl chains are linked to the central or terminal residues of a maltotriosyl residue. Monosaccharide analysis of both pullulan samples, pHs 6.5 and 4.5 (Table 1), did not show the presence of any other monosaccharide residue except glucose.

Pullulanase treatment and Biogel P-2 chromatography of the debranched pullulan digest revealed mainly maltotriose oligomers (Fig. 7). A small amount of a high-molecular-size gel-excluded fraction may indicate the presence of a contaminating glucan species and/or a resistant oligosac-

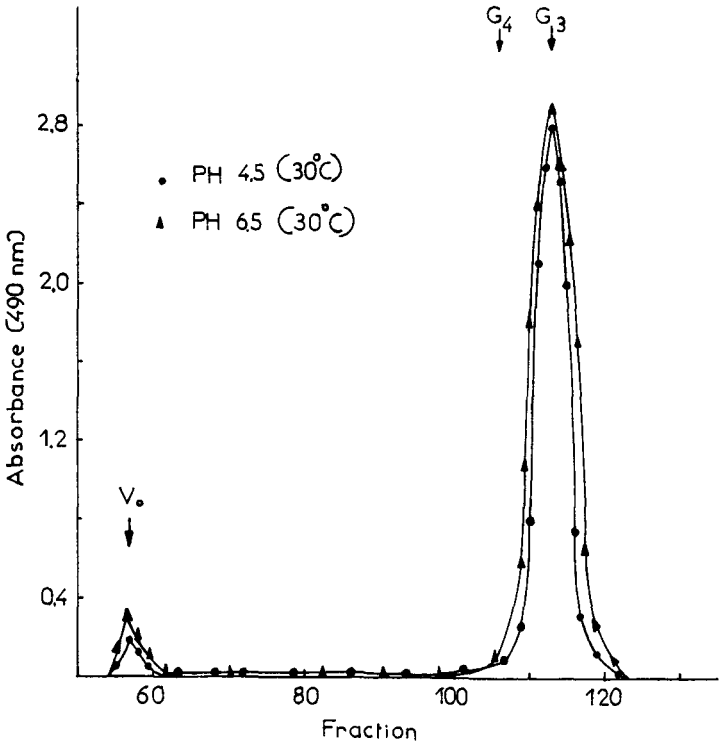

Fig. 7. Elution profiles (Biogel P-2, 2.5 × 94 cm, eluted with 0.1*M* sodium acetate, pH 4.7, at 32 mL/h, 25°C) of pullulanase treated pullulans. G_4 and G_3 denote the peak elution volume of maltotetraose and maltotriose chromatographic standards.

charide fragment to pullulanase, e.g., owing to branching with linkage other and α-(1 → 6). This is consistent with the methylation data where minor linkages, in addition to α-(1 → 4) and α-(1 → 6), were detected (Table 1). Structural characterization of the native pullulan samples was also carried out by ^{13}C-NMR spectroscopy. The spectral features of both pullulan samples were typical of this α-D-glucan based on previous work; the pullulan spectrum published by Gorin (*41*) is included in Fig. 8 (inset) for comparison. The anomeric carbon region shows three signals corresponding to α-(1 → 4) (101.5, 100.9 ppm) and α-(1 → 6) (99.0 ppm) linkages, whose relative area (sum of 101.5, 100.9 resonances over that of 99.0 ppm) was approx 2:1, in accordance with the methylation data. The splitting of C-4 (79.0, 79.3 ppm) and C-6 (61.8, 62.0 ppm) resonances of the (1 → 4) linked glucose units is the result of the sensitivity of these carbon positions to the nature of the linkage at C-1, i.e., the C-6 signals at 61.8 and 62.0 ppm are those of the two types of 1,4-linked α-D-glucose, whereas the single resonance at 68.5 ppm corresponds to C-6 of the 1,6-linked α-D-glucose (*41,42*).

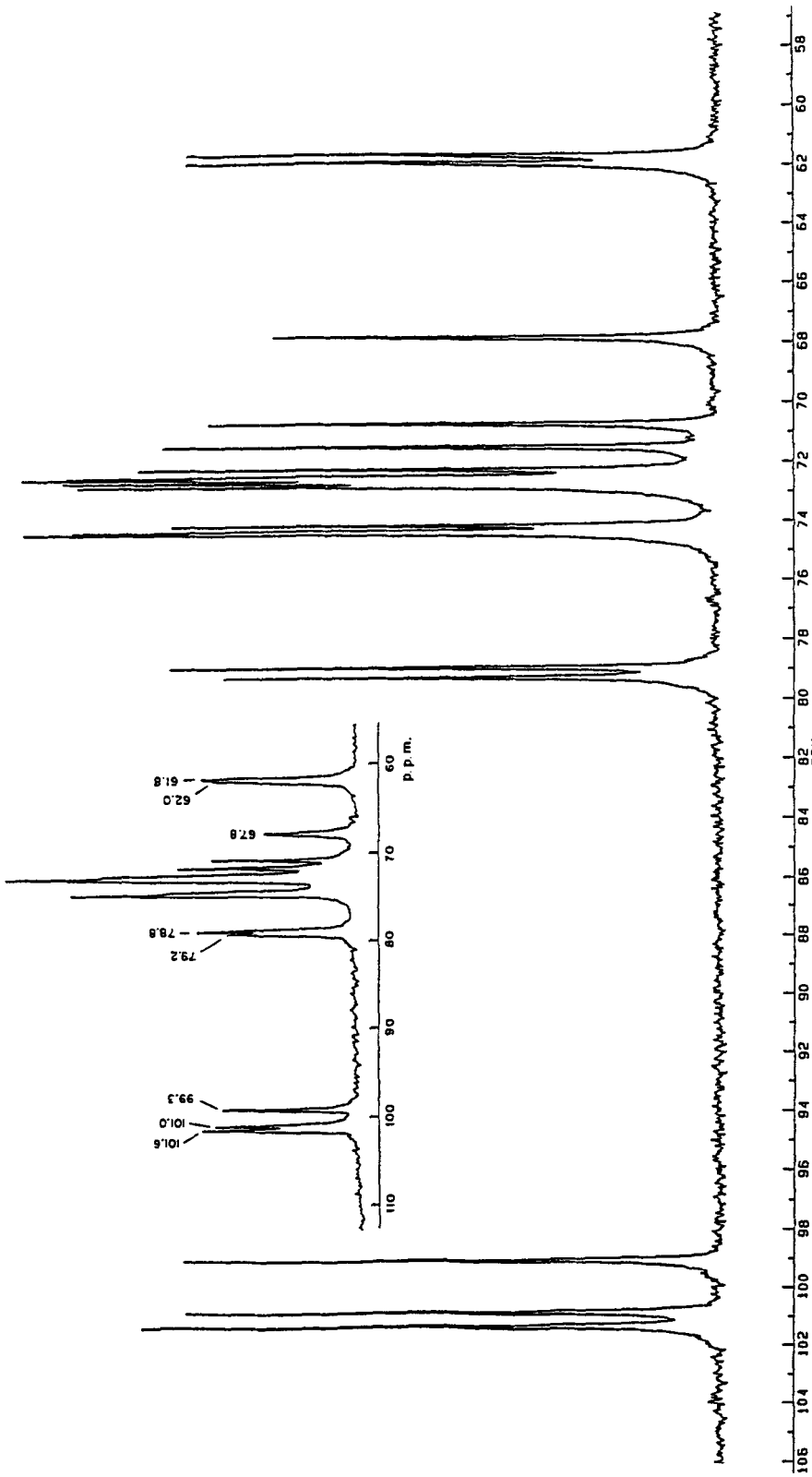

Fig. 8. ^{13}C-NMR spectrum of pullulan isolated from the fermentation broth of *A. pullulans* grown in carob pod extract (pH 6.5, 30°C). Inset shows a corresponding pullulan spectrum from the literature (*41*).

CONCLUSION

The results of the present work revealed some interesting aspects of carob pod extract fermentation and production of extracellular polysaccharide by *A. pullulans*. Optimum conditions for fermentation and polysaccharide production were at an initial sugar concentration of the extract at 25 g/L, initial pH 6.5, and temperature of 25–30°C. External addition of nutrients in the extract or pH adjustment in a two-step procedure during fermentation did not improve the fermentation characteristics. Overall, carob pod extract is an attractive medium for pullulan production by this organism. Further screening of a number of strains and optimization of the respective fermentation conditions would be desirable to identify higher yielding strains that could efficiently grow on this substrate.

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