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Production and Characterization of Pullulan from Beet Molasses Using a Nonpigmented Strain of *Aureobasidium pullulans* in Batch Culture

Athina Lazaridou,[1] Costas G. Biliaderis,*[,1] Triantafyllos Roukas,[1] and Marta Izydorczyk[2]

[1]Department of Food Science and Technology, Aristotle University of Thessaloniki, Box 256, Thessaloniki 540 06, Greece, E-mail: biliader@agro.autho.gr; and [2]Grain Research Laboratory, 1404-303 Main Street, Winnipeg, Manitoba, R3C 3G8, Canada

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Abstract

The production of pullulan from beet molasses by a pigment-free strain of *Aureobasidium pullulans* on shake-flask culture was investigated. Combined pretreatment of molasses with sulfuric acid and activated carbon to remove potential fermentation inhibitors present in molasses resulted in a maximum pullulan concentration of 24 g/L, a biomass dry wt of 14 g/L, a pullulan yield of 52.5%, and a sugar utilization of 92% with optimum fermentation conditions (initial sugar concentration of 50 g/L and initial pH of 7.0). The addition of other nutrients as carbon and nitrogen supplements (olive oil, ammonium sulfate, yeast extract) did not further improve the production of the exopolysaccharides. Structural characterization of the isolated polysaccharides from the fermentation broths by ^{13}C-nuclear magnetic resonance spectroscopy and pullulanase digestion combined with size-exclusion chromatography confirmed the identity of pullulan and the homogeneity (>93% dry basis) of the elaborated polysaccharides by the microorganism. Using multiangle laser light scattering and refractive index detectors in conjunction with high-performance size-exclusion chromatography molecular size distributions and estimates of the molecular weight (M_w = 2.1–4.1 × 10^5), root mean square of the radius of gyration (R_g = 30–38 nm), and polydispersity index (M_w/M_n = 1.4–2.4) were obtained. The fermentation products of molasses pretreated with sulfuric acid and/or activated carbon were more homogeneous and free of contaminating proteins. In the concentration range

*Author to whom all correspondence and reprint requests should be addressed.

of 2.8–10.0 (w/v), the solution's rheologic behavior of the isolated pullulans was almost Newtonian (within 1 and 1200 s^{-1} at 20°C); a slight shear thinning was observed at 10.0 (w/v) for the high molecular weight samples. Overall, beet molasses pretreated with sulfuric acid and activated carbon appears as an attractive fermentation medium for the production of pullulan by *A. pullulans*.

Index Entries: Pullulan; fermentation; *Aureobasidium pullulans*; molecular weight; beet molasses; light scattering; ^{13}C-nuclear magnetic resonance; pullulanase.

Introduction

Pullulan is an extracellular water-soluble microbial polysaccharide produced by different strains of *Aureobasidium pullulans*. It is a linear mixed linkage α-D-glucan consisting mainly of maltotriose units interconnected via α-(1→6) linkages. While not extensively exploited by the industry, the physical properties of this biopolymer make it suitable for a multitude of applications in the food, cosmetic, and pharmaceutical industries. Purified pullulan can form thin films or molded products by solution casting or thermoplastic extrusion, which are biodegradable, transparent, oil resistant, and impermeable to oxygen. Pullulan and its derivatives may thus be used as coating and packaging materials, as sizing agents for paper, and as fat replacers in low-calorie food formulations *(1,2)*.

The production of pullulan from defined chemical media by different strains of *A. pullulans* has been described *(3–6)*. However, utilization of glucose or sucrose as a sole carbon source is not economical, and a less expensive carbohydrate source would be beneficial. Furthermore, there is some evidence to suggest that high sucrose concentrations can inhibit pullulan synthesis *(7)*. Recently, considerable interest has been shown in using inexpensive substrates such as peat hydrolysates, spent sulfite liquor, Jerusalem artichoke tubers (inulin hydrolysates), carob pods, fuel ethanol byproducts, various crude agroindustrial wastes, deproteinized whey, and brewery wastes *(8–15)*.

Molasses is a byproduct of the sugar industry readily available at relatively low cost. It consists of water, sucrose (47–50% [w/w]), proteins, vitamins, amino acids and other nitrogen-containing compounds, organic acids, and heavy metals such as iron, zinc, copper, manganese, magnesium, and calcium. Israilides et al. *(15)*, Roukas *(16)*, and Roukas and Liakopoulou-Kyriakides *(17)* examined the production of pullulan from molasses by *A. pullulans*. They have reported that *A. pullulans* grown on crude beet molasses produced a mixture of pullulan and other polysaccharides; the pullulan content of the crude polysaccharides produced was very low.

The aim of the present investigation was to examine the production of pullulan from pretreated molasses with sulfuric acid and activated carbon to remove heavy metals and other microbial growth inhibitors in order to increase the concentration of polysaccharide and the pullulan proportion

of the crude polysaccharide. The effects of pH and other nutrients/substrates on the fermentation parameters were also studied. Structural characterization of the pullulan samples was carried out by enzymic means and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy. Moreover, the molecular size of the polysaccharides and the rheologic behavior of their aqueous solutions were determined.

Materials and Methods

Microorganism and Culture Conditions

A. pullulans P 56, a strain deficient in melanin production, was used throughout this investigation *(6)*. The microorganism was maintained on potato dextrose agar plates at 4°C and subcultured every 3 wk. Cells for inoculation of the culture medium were obtained from cultures grown on potato dextrose agar plates at 28°C for 48 h. From the Petri dish, two loops of *A. pullulans* cells were transferred to 500-mL conical flasks containing 100 mL of culture medium (pH 5.5) of the following composition: 30 g/L of sucrose, 0.6 g/L of $(NH_4)_2SO_4$, 0.4 g/L of yeast extract, 5.0 g/L of K_2HPO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, and 1.0 g/L of NaCl. The flasks were incubated at 28°C for 48 h in a rotary shaker incubator (Lab Line Orbit-Environ Shaker; Lab-Line, Melrose Park, IL) at 200 rpm. These cultures were used to inoculate the production medium at a level of 5% (v/v).

Pretreatment of Molasses

Beet molasses was obtained from a local sugar refinery (Platy, Thesalloniki, Greece). Two methods were used to pretreat molasses and reduce potentially harmful compounds to the microorganism (e.g., coloring substances, amino acids, heavy metals) that may act as growth inhibitors during fermentation.

Activated Carbon Treatment

Molasses solution containing 5% (w/v) total sugars (as sucrose, pH 7.0) was treated with activated carbon at a ratio of 3% (w/v). The mixture was heated at 60°C for 1 h under stirring. The solution was filtered through a Whatman no. 1 filter. This pretreatment was repeated three to four times until the solution was almost colorless (absorbance readings at 490 nm of <0.02).

Sulfuric Acid Treatment and Activated Carbon Treatment

Molasses solution (5% [w/v] sugars) was adjusted to pH 3.0 with 0.5 *M* H_2SO_4. The liquid was allowed to stand for 24 h and then centrifuged at 5000*g* for 15 min. Following the sulfuric acid treatment, the supernatant was also treated with activated carbon (one to two times) as already described but without heating to avoid hydrolysis of sucrose. The pH of the solution was then adjusted to 7.0 with 10 *M* NaOH and used for the production of the polysaccharide. Following the pretreatments, the molasses solu-

tion was concentrated in a rotary evaporator and adjusted to a desired level of sugar concentration, as specified in the fermentation protocol; the initial sugar concentration in the fermentation medium was measured according to the method of Dubois et al. *(18)*.

Fermentation Conditions

All fermentation experiments were carried out in 500-mL conical flasks containing 100 mL of untreated or treated molasses solution (50 g/L of initial sugar, pH 7.0). The flasks were inoculated with 5 mL of the inoculum at 28°C in a rotary shaker incubator at 200 rpm.

Fermentation Variables

Initial Sugar Concentration

An initial molasses solution of 100 g/L sugar content (treated with sulfuric acid and activated carbon, pH 7.0) was diluted with distilled water in order to obtain solutions of 35, 50, 70, and 100 g/L sugars content. Samples of molasses so prepared (production medium) were used to investigate the effect of initial sugar concentration on polysaccharide production by *A. pullulans*. A series of conical flasks containing 100 mL of production medium was inoculated with 5 mL of the inoculum and incubated at 28°C in a rotary shaker incubator at 200 rpm.

Initial pH

The pH of a set of conical flasks containing 100 mL of molasses solution (50 g/L of initial sugars, treated with sulfuric acid and activated carbon) was adjusted to 4.0, 5.0, 6.0, 7.0, and 8.0 with dilute HCl or NaOH solutions, and the substrate was inoculated with 5 mL of the inoculum. The flasks were incubated under the conditions specified earlier.

Nutrients

A series of conical flask experiments was performed at different concentrations of nutrient ingredients to investigate the effect of supplements on polysaccharide production. Three different experiments were carried out on conical flasks containing 100 mL of production medium (treated with sulfuric acid and activated carbon, 50 g/L of initial sugar). The flasks were supplied with (1) 0.03 and 0.02 g of $(NH_4)_2SO_4$ and yeast extract, respectively; (2) 0.06 and 0.04 g $(NH_4)_2SO_4$ and yeast extract, respectively; and (3) 2.5 mL of olive oil and 0.5 mL of Tween-80, respectively. The pH of the substrate was adjusted to 7.0 with 10 *N* NaOH, and the medium was inoculated with 5 mL of the inoculum. The flasks were incubated under the same conditions as described earlier.

Chemical Analyses, Isolation, and Characterization of Pullulan

Fermentation Aspects

At specific time intervals, the flasks were removed and the fermentation broth was analyzed. Total biomass (mycelial and yeast cells) dry wt

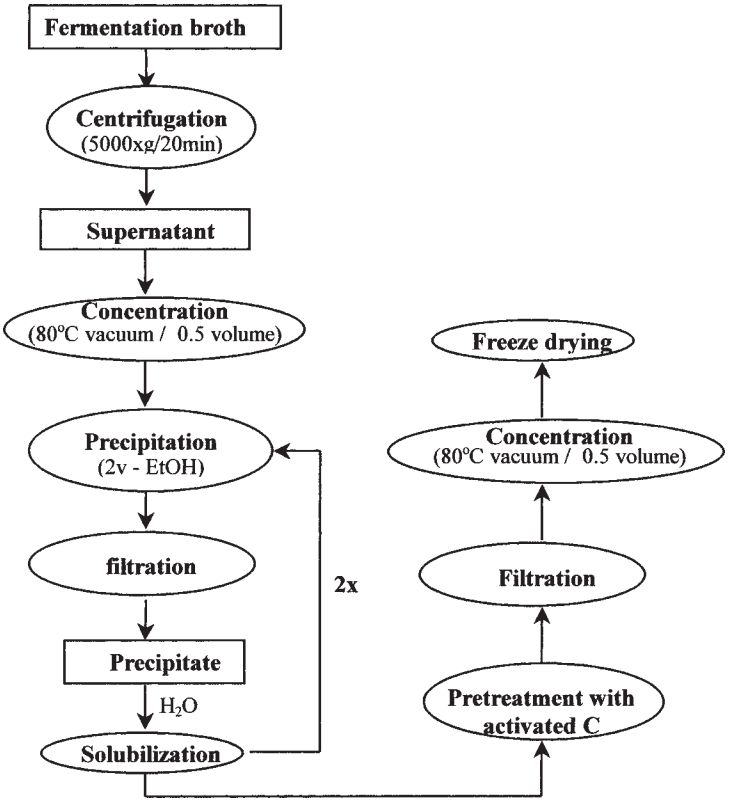

Fig. 1. Isolation scheme of pullulan from beet molasses fermentation broth of *A. pullulans*.

was determined by centrifuging the fermentation broth (after appropriate dilution at 10,000*g* for 20 min), washing the sediment with distilled water, and drying at 105°C overnight. The first supernatant was combined with the washings, and the pullulan was precipitated with 2 vol of ethanol at 4°C for 1 h. The precipitate was filtered through the preweighed Whatman GF/A filter and dried at 105°C overnight. The pH was measured using a Knick 646 pH meter equipped with a glass electrode. Residual sugars were determined as sucrose by the method of Dubois et al. *(18)*. Pullulan yield was expressed as grams of polysaccharide/100 g of sugar consumed. Sugar utilization was calculated as a ratio of the sugars consumed during fermentation over the initial sugars and multiplying the result by 100. Each fermentation experiment was repeated at least two times, and the results reported herein are averages of two replications. The differences between replicates did not exceed 9% for all measured parameters.

Isolation of Pullulan

Figure 1 illustrates the sequence of steps for isolation and partial purification of the polysaccharide from the fermentation broths. The pro-

tein content of the freeze-dried polysaccharides was determined by the Lowry method *(19)*.

Structural and Physicochemical Characterization of Pullulan

The structure and purity of the elaborated polysaccharides from *A. pullulans* were assessed by ^{13}C-NMR and enzymic methods. The proton-decoupled ^{13}C-NMR spectra (300 MHz) were recorded on a Bruker AM 300 FT spectrometer at 70°C, a polysaccharide concentration of 2% (w/v) in 50% (v/v) d6-dimethylsulfoxide/H_2O, 30,000 pulses with a pulse repetition time of 1.245 s, and an radio frequency pulse angle of 80.0°. Chemical shifts are expressed in parts per million downfield from external Me_4Si but were actually measured by reference to internal 1,4-dioxane ($\delta = 67.4$ ppm). Structural analysis of the pullulan samples was also carried out by debranching (30 mg of polysaccharide in 5 mL of 0.1 *M* acetate buffer, pH 5.5) with 32 IU of crystalline pullulanase (Hyashibara Biochem., Okayama, Japan) at 37°C for 6 h, according to the method of Biliaderis et al. *(20)*. Following debranching, the enzyme was inactivated in a boiling water bath (20 min), and the digest was filtered and applied on a BioGel P-2 (Bio-Rad, Richmond, CA) column (2.5 × 95 cm). Elution of the carbohydrates was carried out with 0.1 *M* sodium acetate buffer (pH 4.7) containing 0.02% NaN_3 at 35 mL/h (25°C). Fractions of 5 mL were analyzed for total carbohydrates *(18)*.

Size-exclusion chromatography of the pullulan samples was carried out with a high-performance size-exclusion chromatography (HPSEC) system consisting of a pump (Waters 510), an injection valve (Model 7010; Rheodyne) with a 200-µL sample loop, a guard column (TSK PWH; TosoHaas GmbH, Stuttgart, Germany), an SEC column (TSK G5000 PW column, 7.8 × 600 mm) (TSK PW; TosoHaas GmbH), a multiangle laser light scattering (MALLS) detector (Dawn DSP; Wyatt, Santa Barbara, CA), and a refractive index (RI) detector (Waters 410). Chromatography was performed at room temperature. The flow rate of the mobile phase (aqueous 0.15 *M* $NaNO_3$ containing 0.02% NaN_3), which was filtered through 0.2-µm and then 0.1-µm cellulose acetate membranes, was 0.4 mL/min. M_w and R_g were calculated using the Astra 4.72 software (Wyatt). Calculations were carried out with a $(dn/dc) = 0.147$ mL/g. Pullulan standards with known M_w values (P-50, $M_w = 47{,}300$; P-400, $M_w = 404{,}000$; P-800, $M_w = 788{,}000$) were used to determine the proper experimental setup and calculations. The M_w of macromolecules from light scattering (LS) measurements can be calculated by the following equation:

$$Kc/R_\theta = [M_w P(\theta)]^{-1} + 2A_2 c + \ldots$$

where K = a polymer constant for a particular scattering system: $K = 4[\pi n_0(dn/dc)]^2/\lambda^4 N_A$; dn/dc = the change in RI with polymer concentration at the wavelength (λ) of the scattered light; N_A = the Avogadro number; c = polymer concentration; R_θ = excess Rayleigh factor or excess scattering of the polymer, $R_\theta = R_{\theta,\text{solution}} - R_{\theta,\text{solvent}}$; M_w = weight average molecular weight;

$P(\theta)$ = particle scattering function, related to the radius of gyration (R_g); and A_2 = second virial coefficient, a measure of the solvent-polymer interactions R_g that can be obtained from the initial slope of a plot of Kc/R_θ vs $\sin^2\theta/2$ and $1/M_w$ from the intercept.

Steady shear viscosity was measured on a rotational Physica MCR 300 rheometer (Physica Messtechnic GmbH, Stuttgart, Germany) using a concentric cylinder (diameter of cup and bob = 28.92 and 26.66, respectively) and a double-gap cylindrical geometry. Polysaccharide solutions (2.8–10.0% [w/v] in distilled water) were subjected to shear sweeps between 1 and 1200 s^{-1} at 20 ± 0.1°C.

Results and Discussion

Effect of Molasses Treatment on Pullulan Production

Figure 2 presents the results of the production of pullulan from untreated and pretreated molasses using different chemical methods to remove the color substances and the heavy metals. Polysaccharide production from untreated molasses was very low (~5 g/L) in comparison with the treated molasses media. Molasses treated with sulfuric acid and activated carbon gave a marked increase in pullulan concentration (24 g/L). This implies that beet molasses contains undesirable substances, which affect polysaccharide elaboration by the microorganism. In relevant work from our laboratory *(16)*, it was also found that *A. pullulans* P 56 grown on molasses treated with sulfuric acid alone produced higher amounts of polysaccharide (2.5x) than from a medium based on crude molasses. The superiority of the combined treatment (H_2SO_4-activated C, Fig. 2D) over other treatments of molasses on pullulan production may be owing to the significant removal of heavy metals as well as amino acids and 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. Moreover, the polysaccharide produced from fermentation of crude molasses was contaminated with a substantial amount of proteins, as shown in Table 1; by contrast, all polysaccharide samples produced from pretreated molasses contained <1.3% (w/w) proteins. The biomass dry wt followed a pattern similar to that of polysaccharide production during fermentation (Fig. 2); maximum levels of biomass solids were between 14 and 16 g/L. These findings indicate that pullulan production is taking place during the growth phase of the culture.

The yield and purity of pullulan produced from fermentation of different substrates seem to depend on the chemical composition of the medium, the strain of the microorganism, the fermentation system, and the conditions employed during fermentation. Israilides et al. *(15,21)* have reported a relatively low yield of polysaccharides (~6 g/L), which consisted only of 5–8% (w/w) of pullulan and had about 8% (w/w) contaminating proteinaceous material, from fermentations of untreated molasses

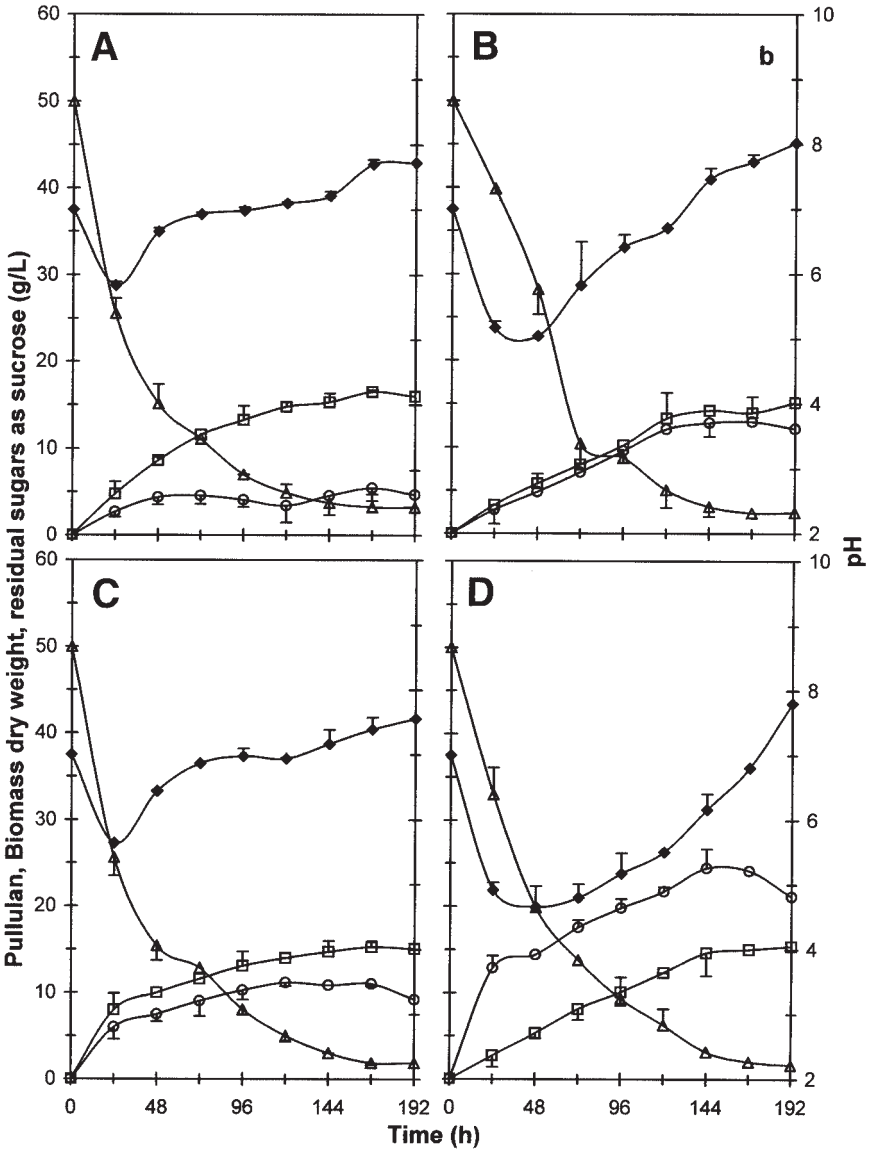

Fig. 2. Fermentation kinetics of *A. pullulans* P 56 during pullulan production from different media made of beet molasses (5% [w/v] sugars, initial pH 7.0): **(A)** crude molasses (control); **(B)** molasses pretreated with activated carbon; **(C)** molasses pretreated with H_2SO_4; **(D)** molasses pretreated with H_2SO_4 and activated carbon. (—○—), Pullulan concentration; (—□—), biomass dry weight; (—△—), residual sugars as sucrose; (—◆—), pH.

with *A. pullulans* NRRLY-6220 in shake-flask culture. LeDuy and Boa *(22)* have found maximum polysaccharide levels of ~12–14 g/L for various strains of *A. pullulans* grown on peat hydrolysates in shake-flask cultures. Shin et al. *(10)* found that a mixed culture of *A. pullulans* and *Kluyveromyces*

Table 1
Characteristics of Exopolysaccharides (pullulan) Isolated from Fermentation Broths (at 192 h) of *A. pullulans* Grown in Media Made from Beet Molasses with Different Treatments

Sample no.	Treatment/nutrient concentration	Maximum pullulan concentration (g/L)	Protein (% d.b.)	Molecular characteristics		
				$M_w \times 10^5$ (M_n)	R_g (nm)	Polydispersity index (M_w/M_n)
1	Crude molasses (control)	5.5	15.5	2.40 ± 0.2 (0.96 ± 0.2)	37 ± 1.7	2.4 ± 0.1
2	Activated C/3.5% (w/v) sugars	8.2	1.1	3.57 ± 0.0 (2.10 ± 0.0)	30 ± 0.0	1.7 ± 0.0
3	Activated C/5.0% (w/v) sugars	12.9	0.8	2.78 ± 0.1 (1.72 ± 0.0)	35 ± 0.1	1.6 ± 0.0
4	Activated C, H_2SO_4/5.0% (w/v) sugars	24.1	0.3	2.07 ± 0.0 (1.32 ± 0.0)	38 ± 1.0	1.6 ± 0.0
5	Activated C/5.0% (w/v) sugars, 2.5% (v/v) olive oil	9.4	1.2	3.72 ± 0.4 (2.62 ± 0.4)	36 ± 2.5	1.4 ± 0.1
6	Activated C, H_2SO_4/5.0% (w/v) sugars, 2.5% (v/v) olive oil	23.6	0.6	4.06 ± 0.1 (2.71 ± 0.1)	30 ± 1.2	1.5 ± 0.2

fragilis (SH 8646) produced ~15.5 g/L of exopolysaccharides when Jerusalem artichoke extracts (containing 50 g/L of total carbohydrates, mainly inulin hydrolysate) were fermented in a submerged culture system. According to Barnett et al. *(23)*, hydrolysates of potato starch wastes rich in maltose rather than glucose were more effective substrates for pullulan production (higher yields and a better proportion of pullulan in the isolated exopolysaccharide material). West and Reed-Hamer *(7)* reported that the pullulan content of the crude polysaccharide was 54 and 100% when *A. pullulans* ATCC 42023 was grown on sucrose or corn syrup as a carbon source, respectively. In similar experiments carried out in our laboratory *(14)*, it was found that *A. pullulans* P 56 produced ~6.0 g/L of crude polysaccharide from brewery wastes. Finally, Roukas and Biliaderis *(11)* reported a maximum polysaccharide concentration of ~6.5 g/L (with a highest pullulan proportion value of ~70%) and a polysaccharide yield of ~30% on fermentation of carob pod extract by another pigmented strain of *A. pullulans* (SU-M18).

Throughout the fermentation, the pH of the cultures decreased during the first 48 h of fermentation and then increased at late stages of the process (Fig. 2). Synthesis of organic acids could contribute to the increased acidity of the fermentation broth up to 48 h of growth, whereas deamination of some amino acids initially present in molasses could account for the subsequent rise in pH as fermentation progressed.

In general, the results of these studies have shown that the pretreatment of molasses with activated carbon in combination with sulfuric acid significantly improved the pullulan production by this nonpigmented strain (P 56) of *A. pullulans*. This pretreatment of molasses was therefore selected over other methods for all subsequent fermentation experiments.

Effect of Initial Sugar Concentration

The polysaccharide concentration increased with increasing levels of sugars, up to 50 g/L, present initially in the medium, and remained relatively constant (~23–25 g of polysaccharide/L) beyond this value (Fig. 3). The highest concentration of pullulan (24.7 g/L) was obtained with an initial sugar concentration of 50 g/L after 144 h of incubation. In previous studies in our laboratory on the production of pullulan from chemically defined medium (strain P 56), carob pod extracts (strain SU-M18), and molasses treated with sulfuric acid (strain P 56), the maximum polysaccharide concentration was observed at an initial sugar concentration of 60, 25, and 70 g/L, respectively *(5,11,16)*. The concentration of sugars in the medium continuously declined during fermentation, following an inverse trend to those of biomass and polysaccharide production. In the cultures with 35 and 50 g/L initial sugar concentration, there was almost a complete depletion of sugars after 120 h of incubation. When the maximum concentration of polysaccharides was achieved, the residual sugars in the fermentation medium were 5.4, 3.6, 19.7, and 27.1% of the initial sugars for media

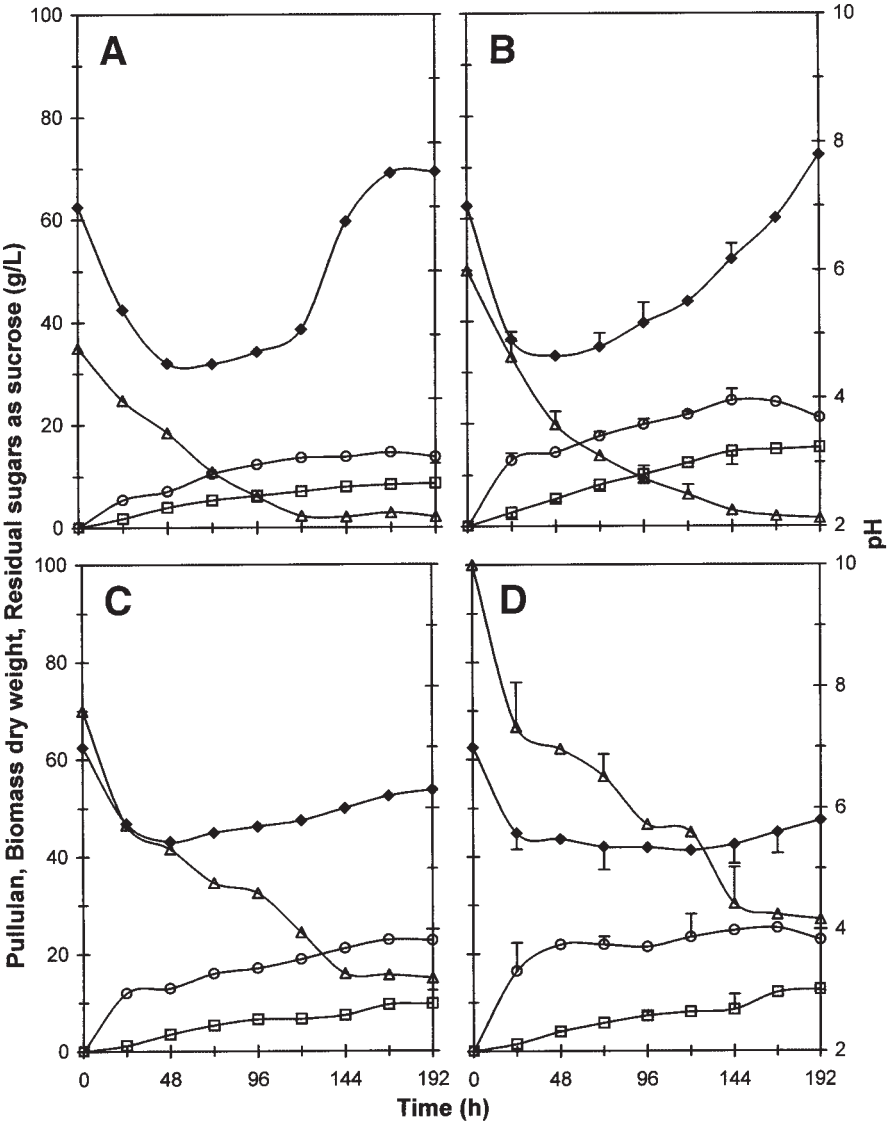

Fig. 3. Fermentation kinetics of *A. pullulans* P 56 during pullulan production from beet molasses at different initial sugar concentrations: **(A)** 35 g/L; **(B)** 50 g/L; **(C)** 70 g/L; **(D)** 100 g/L. Symbols are as in Fig. 2.

adjusted at initial sugar concentrations of 35, 50, 70, and 100 g/L, respectively. Moreover, the respective maximum biomass dry wt was 8, 14, 9, and 10 g/L for these fermentation systems. In some fermentations (Figs. 1 and 2), there was even evidence for a slight decline in the pullulan yield after 144 h of incubation. Although until recently there was no convincing proof that *A. pullulans* itself produces hydrolytic enzymes capable of degrading pullulan, West and Strohfus *(24)* have reported on an endogenous glucoamylase A released by the microorganism at late stages of fermenta-

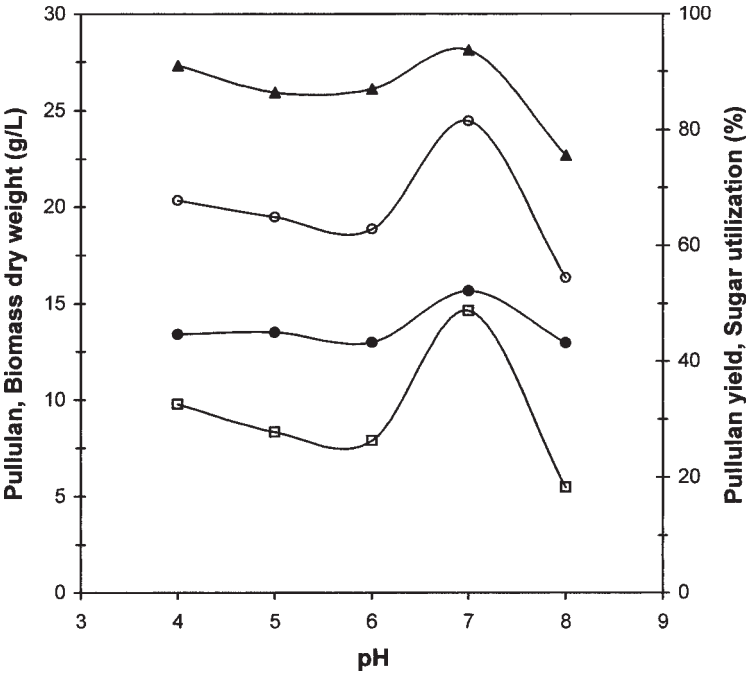

Fig. 4. Kinetic parameters of beet molasses fermentation by *A. pullulans* at different initial pH values. (—○—), Pullulan concentration; (—□—), biomass dry weight; (—●—), pullulan yield; (—▲—), sugar utilization.

tion. Such a hydrolytic activity could account for the reduction in pullulan yield at late stages of culture growth.

Effect of Initial pH

The pH of the medium is probably a very influential factor in pullulan production by *A. pullulans* because it affects the morphologic features of this microorganism, which may itself be related to polysaccharide production *(25)*. LeDuy et al. *(26)* as well as Lacroix et al. *(27)* found little or no polysaccharide production at an initial pH of 2.0, although high biomass concentrations were achievable under these conditions. Auer and Seviour *(28)*, however, showed that the pH response depends greatly on the nitrogen source added to the medium; thus, if glutamate were used instead of $(NH_4)_2SO_4$, polysaccharide synthesis would occur to a similar extent at all controlled pH values of 2.5, 4.5, and 6.5 tested. For the present study, Fig. 4 illustrates the influence of initial pH (4.0–8.0) on pullulan production from molasses by *A. pullulans* P 56. Pullulan concentration and biomass dry wt solids slightly decreased when the initial pH of the culture medium increased from 4.0 to 6.0. All fermentation parameters subsequently increased by a further rise of pH from 6.0 to 7.0 and decreased thereafter. The highest pullulan concentration (24 g/L), biomass dry wt (14 g/L),

pullulan yield (52.5%), and sugar utilization (92.0%) were obtained in cultures grown at an initial pH of 7.0. With this initial pH, the fermentation broth seemed to be maintained between 5.0 and 6.0 throughout a long period of the fermentation (Fig. 3B). This range of pH seems to favor mostly the growth of yeastlike cells as well as some mycelial production.

In view of the complex life exhibited by *A. pullulans* (a polymorphic microfungus with a life cycle involving hyphae, blastospores, and resting intermediate forms, such as swollen cells and chlamydospores), the hypothesis that exopolysaccharide and, more specifically, pullulan production is associated with certain form(s) of the microorganism has received considerable attention. According to the early work of Catley *(29,30)*, blastospores might be the major and possibly the sole morphologic form responsible for pullulan synthesis. Heald and Kristiansen *(31)* have also suggested that pullulan is elaborated primarily by the yeastlike forms of *A. pullulans*. In recent studies, Simon et al. *(32,33)* have reported that *A. pullulans* chlamydospores are responsible for the synthesis of pullulan and that swollen cells may in fact be responsible for the synthesis of other polysaccharides that could be subsequently transformed into pullulan when these cells develop into chlamydospores, depending on the culture conditions. Their findings have indicated that hyphae do not play a role in the synthesis of extracellular polysaccharide. According to these researchers, polysaccharide production in batch culture could be the result of a morphologic and physiologic response of the blastospores when the environmental conditions in the medium become unfavorable (e.g., glucose and nitrogen depletion, low pH); more specifically, pullulan production coincided with the highest amount of chlamydospores in the culture medium. Shabtai and Mukmenev *(3)* also reached the same conclusion in two-stage fermentation experiments with two substrates as carbon sources; the morphogenic shift toward swollen blastospores or germinating blastospores seemed to trigger the elaboration of pullulan. In this context, the influence of medium pH on the morphologic differentiation of the microorganism is an important parameter for exopolysaccharide production in cultures of *A. pullulans*. Shabtai and Mukmenev *(3)* and Lacroix et al. *(27)* reported an optimal initial pH of 5.5 for pullulan elaboration by *A. pullulans* grown in chemically defined media. Roukas and Biliaderis *(11)* have found that an initial pH of 6.5 supported the highest pullulan concentration when *A. pullulans* was grown in carob pod extracts in shake-flask cultures. Auer and Seviour *(28)* and Papon et al. *(34)* observed a maximum polysaccharide concentration at a slightly higher initial pH of ~7.0–7.5.

It would appear that the optimal initial pH for pullulan production depends on several fermentation parameters. These include the composition of the substrate, the type of nitrogen source added to the medium, and the strain of the microorganism. Overall, the results in Fig. 3 clearly indicate that synthesis of pullulan by *A. pullulans* P 56 is largely influenced by the initial pH of the medium.

Effect of Added Nutrients

In numerous studies, the type and concentration of carbon and nitrogen sources were shown to affect exopolysaccharide production by *A. pullulans (25)*. In most studies, NH_4^+ as the nitrogen source, often in combination with yeast extract, has been employed. The general consensus from these studies is that pullulan formation occurs only after NH_4^+ is exhausted; it has been suggested that NH_4^+ may regulate the activities of key enzymes, causing a shift in carbon flow to biomass production at the expense of polysaccharide synthesis, when its concentration in the medium exceeds a certain level. Figure 5 illustrates the effect of other nutrients (olive oil, Tween-80, ammonium sulfate, and yeast extract) on the kinetic aspects of fermentation of molasses by *A. pullulans*. As shown in Fig. 5A,B, the pullulan concentration and the biomass dry wt did not increase substantially when the medium was supplemented with olive oil and Tween-80; note, however, that pullulan level rose faster in the medium supplemented with olive oil and Tween-80. On the other hand, the addition of $(NH_4)_2SO_4$ and yeast extract at different concentrations led to a slight reduction in pullulan concentration from 24 to ~20 g/L, while the biomass concentration was maintained almost constant (15 g/L) (Fig. 5A,C,D). These results agree with those of Roukas and Biliaderis *(11)*, who studied the effect of substrate composition on pullulan production from carob pod extract by *A. pullulans*. The enhancement of pullulan production from sucrose-based media in the presence of a second carbon source added as a supplement in shake-flask culture (e.g., soybean oil or olive oil), previously observed by Shabtai and Mukmenev *(3)* and Youssef et al. *(5)* was not noticed in the present work. These findings suggest that the chemical nature of the main substrate used in fermentations of *A. pullulans* controls the impact of the addition of a second carbon source as far as polysaccharide yield is concerned. Thus, the addition of other supplements to beet molasses does not seem to largely improve the growth of the microorganism and the production of extracellular polysaccharide.

Structural Characterization of Polysaccharides

Polysaccharide samples were isolated at 144 h of fermentation (maximum levels of exopolysaccharides in culture broth) following the scheme described in Fig. 1 and subjected to structural analysis using destructive and nondestructive analytical approaches. Enzymic digestion of the polysaccharide samples with a highly purified preparation of pullulanase and chromatography of the debranched digests on a BioGel P-2 column mainly showed maltotriose oligomers. A representative chromatograph of a pullulanase digest of the exopolysaccharide produced by *A. pullulans* P 56 (sample 2, as specified in Table 1) is shown in Fig. 6, and all the results of the debranching experiments are summarized in the inset. Enzyme digestion and chromatography of all the samples revealed a very small proportion of gel-excluded carbohydrate material (eluted at V_0),

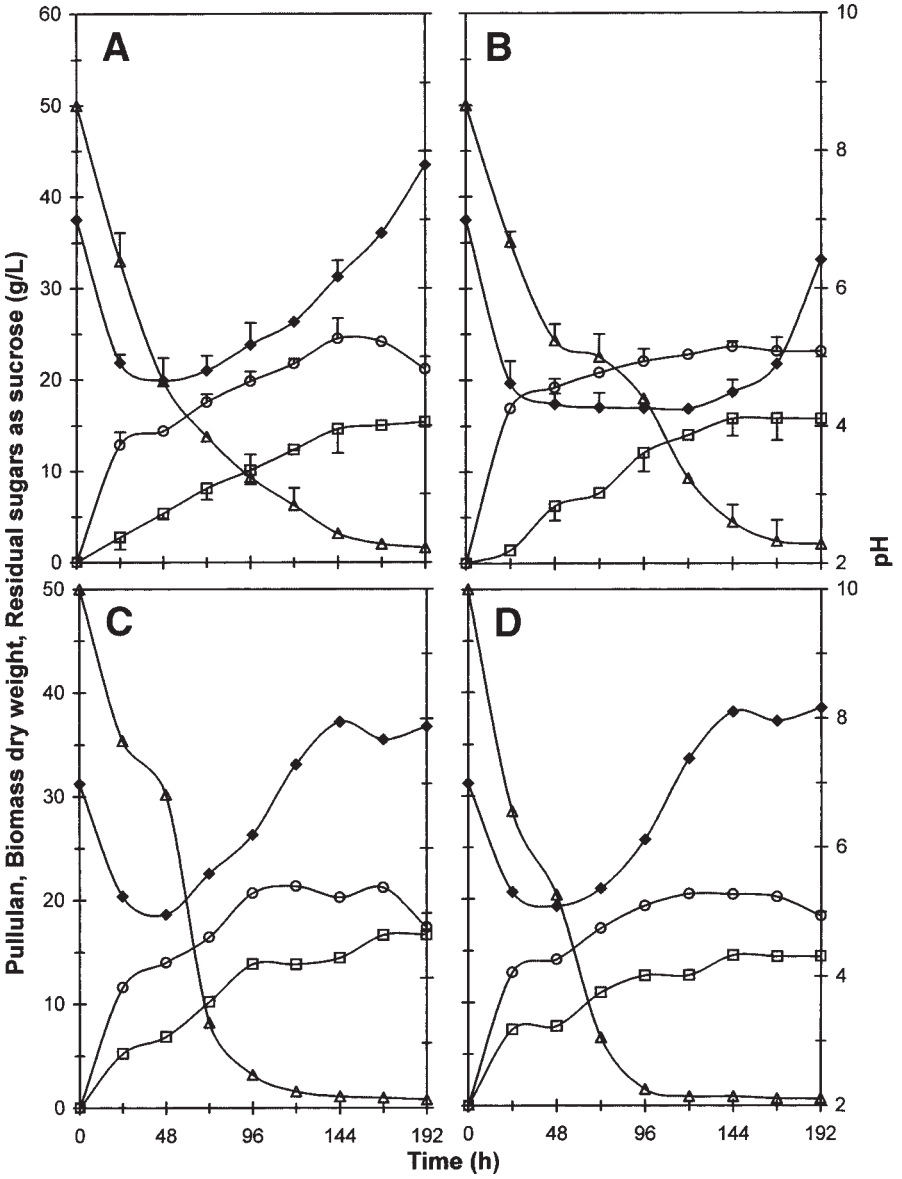

Fig. 5. Effect of other nutrients added in medium on kinetic parameters during fermentation of beet molasses by *A. pullulans* in shake-flask cultures (5% [w/v] sugars in pretreated molasses with H_2SO_4 and activated carbon): **(A)** control (basic medium with no added supplements); **(B)** as in (A) supplemented with 2.5% (v/v) olive oil and 0.5% (v/v) Tween-80; **(C)** as in (A) supplemented with 0.3 g/L of $(NH_4)_2SO_4$ and 0.2 g/L of yeast extract; **(D)** as in (A) supplemented with 0.6 g/L of $(NH_4)_2SO_4$ and 0.4 g/L of yeast extract. Symbols are as in Fig. 2.

indicative of a contaminating glycan (other than pullulan) and/or resistant oligosaccharide fragments to further pullulanase hydrolysis, such as with branching linkages other than α-(1→6). However, the gel-excluded mate-

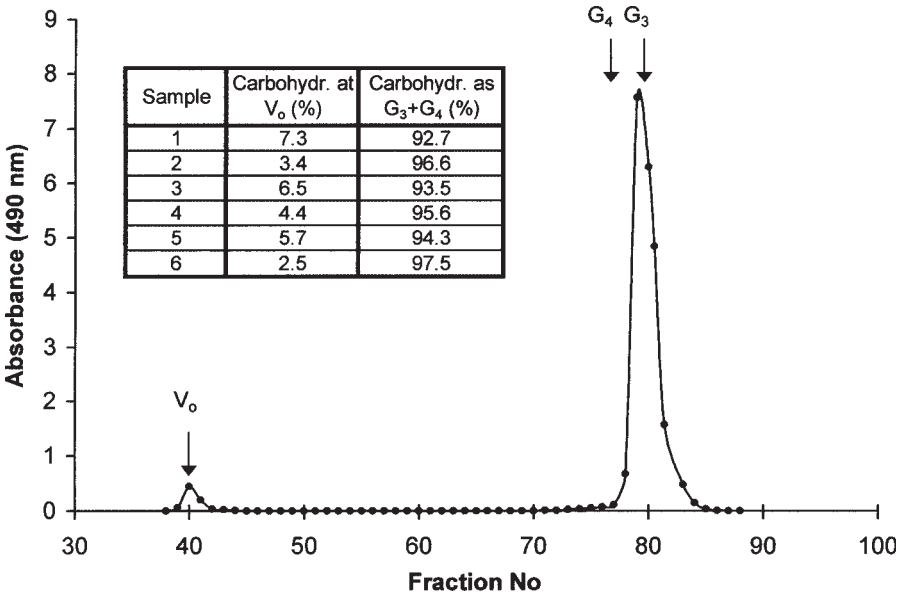

Fig. 6. Elution profile (BioGel P-2 column, 2.5 × 95 cm, eluted with 0.1 *M* sodium acetate buffer, pH 4.7, containing 0.02% NaN_3 at 35 mL/h and 25°C) of pullulanase digest of exopolysaccharides of sample 2 (see Table 1). G_4 and G_3 denote the peak elution volumes of maltotetraose and maltotriose standards, whereas V_0 denotes the gel-excluded carbohydrate fraction. The inset shows the proportion of carbohydrates eluted at V_0 and in the G_4 and G_3 region of the chromatogram for the pullulanase digests of different samples (as specified in Table 1).

rial was <7.5% in all cases, even for the sample derived from fermentation of crude molasses. This finding is in direct contrast with the results of Israilides et al. *(15)*, who reported ~5–7% pullulan of the total exopolysaccharides isolated from fermentation broths of crude molasses using another strain of *A. pullulans* (NRRLY 6220). Obviously, the purity of pullulan produced is dependent on the strain and fermentation conditions employed. The purity of the pullulan produced in the present work was also verified by ^{13}C-NMR spectroscopy. The spectral features of the samples were all typical of pullulan, based on the assignments of Gorin *(35)*, as shown in Fig. 7 for a series of samples. Thus, the anomeric carbon region showed only three resonances, corresponding to α-(1→6) (~99 ppm) and α-(1→4) (~100.8 and 101.3 ppm) linkages. The splitting of the C-4 (79.4 and 79.6 ppm) and C-6 (61.8 and 62.1 ppm) resonances of the (1→4) linked glucose units is the result of the sensitivity of these carbons to the nature of the linkage at C-1; for example, the C-6 signals at 61.8 and 62.1 ppm are those of the two types of 1,4-linked α-D-glucose, whereas the signal at 68.0 ppm corresponds to C-6 of the 1,6-linked α-D-glucose *(35)*. Although some minor differences were shown in the relative intensities of some resonance lines (e.g., at 100.8 vs 101.3 ppm of sample 5, compared with other samples), the ^{13}C-NMR spectra confirmed the homogeneity of the pullulan

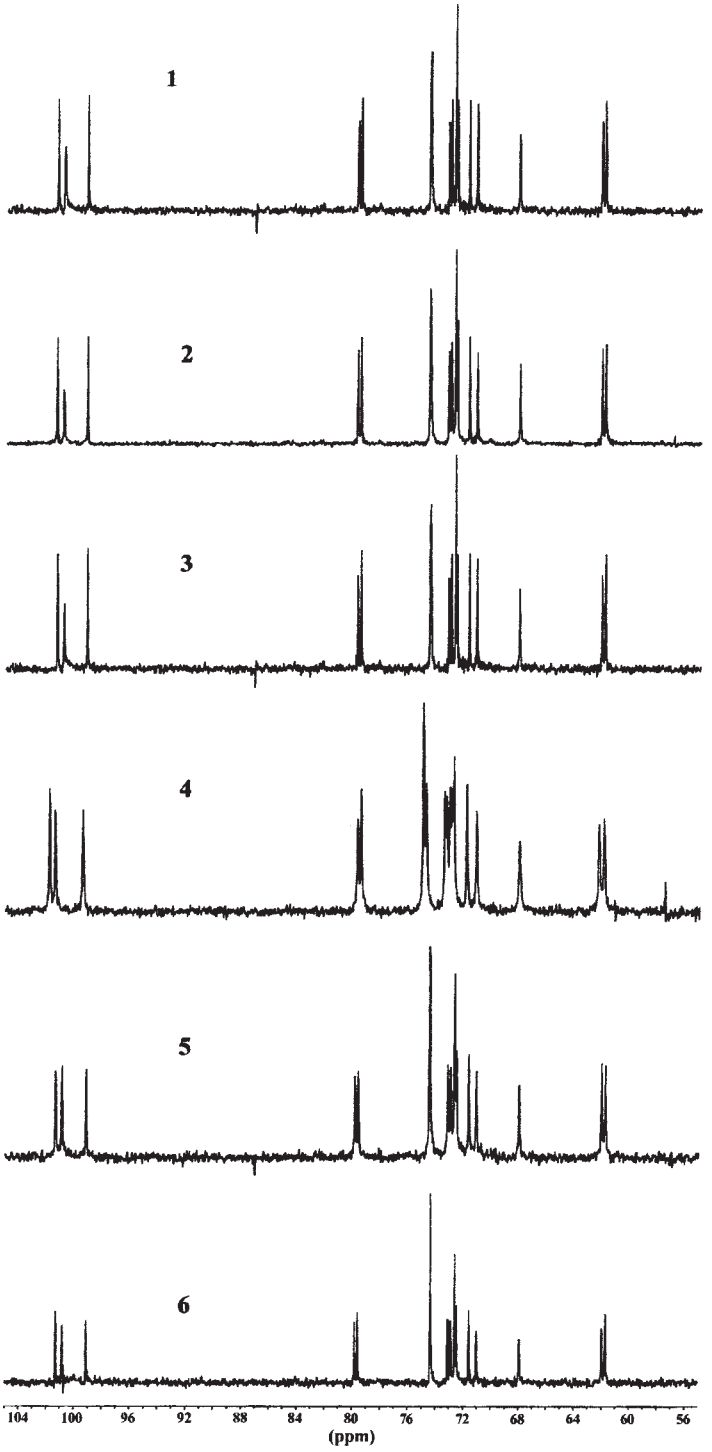

Fig. 7. ^{13}C-NMR spectra of exopolysaccharides isolated from fermentation broth of *A. pullulans* grown in media made from beet molasses with different treatments (as specified in Table 1).

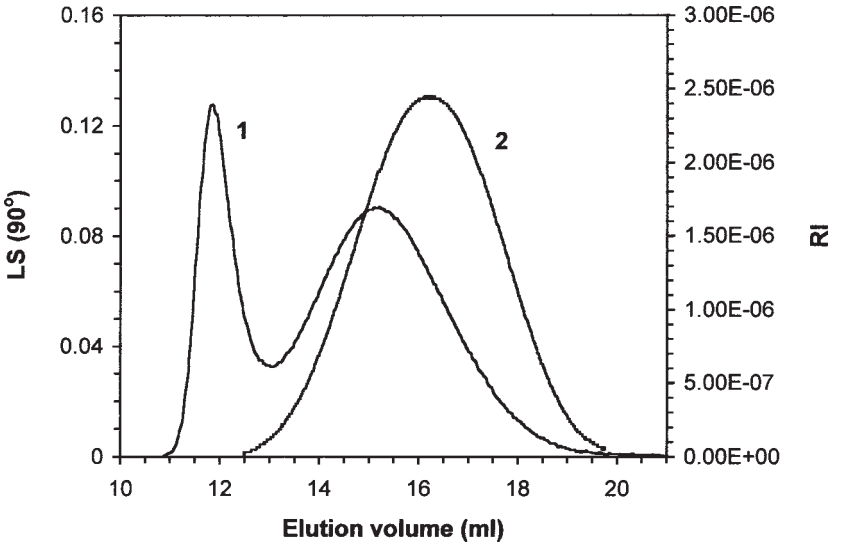

Fig. 8. Superimposed chromatographic traces for exopolysaccharides of sample 2 (as specified in Table 1) detected by LS (at an angle of 90°) (1) and RI (2) measurements.

derived from all fermentation broths of *A. pullulans* P 56; that is, no other major resonances were shown that would advocate the presence of other contaminating glucans.

Weight average molecular weight (M_w), radius of gyration (R_g), molecular size distribution, and polydispersity index were measured by HPSEC coupled with MALLS and differential RI detectors. Typical chromatograms of superimposed traces of LS (at 90°) and RI obtained for a pullulan sample are shown in Fig. 8, whereas several elution RI profiles of different samples are shown for comparison in Fig. 9. In addition, Table 1 summarizes the estimates of molecular characteristics (M_w, R_g, M_w/M_n) of the samples. A very small fraction of pullulans (<1.0% of the total mass) gave a sharp, very intense scattering peak from the LS 90° chromatogram (Fig. 8, line 1), before the main fraction of the polymer was eluted. This peak could indicate the presence of minute amounts of very high molecular weight species, and in most cases, the corresponding RI response was negligible. The RI curve was mainly dominated by species eluting between 12 and 20 mL (Figs. 8 and 9). The molecular characteristics were therefore calculated from this region of the chromatograms. For most samples, the main fraction of the eluting species gave a normal distribution, except for the sample isolated from the fermentation broth of crude molasses. For the latter sample, there was also a small molecular size component (Fig. 9, line 1, elution volume of 21–23 mL); this material was found to strongly absorb in the ultraviolet region and most likely represents the contaminating proteins of this sample (Table 1). The light-scattering responses of this region were not taken into account in calculating the average molecular features of the sample. The M_w and R_g values of the pullulans varied between 2.07–

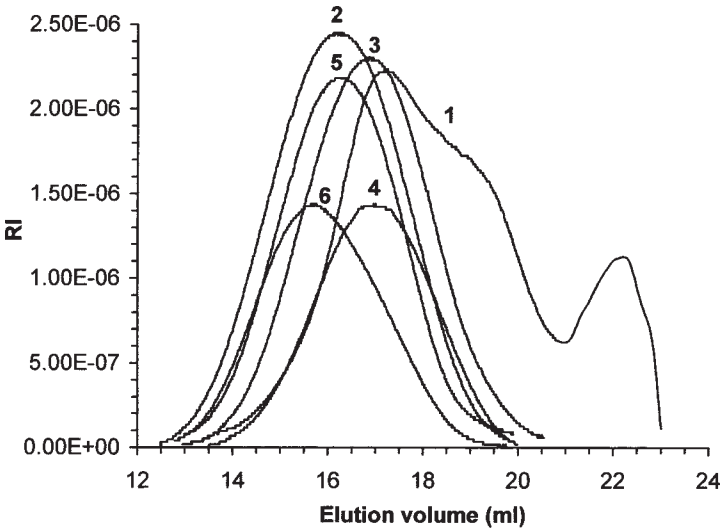

Fig. 9. Chromatographic traces (RI detector) of exopolysaccharides isolated from fermentation broths of *A. pullulans* grown in media made from beet molasses with different treatments (trace numbers as specified in Table 1).

4.06×10^5 and 30–38 nm, respectively. Surprisingly, the R_g values did not correlate with the molecular size, implying conformational differences among samples. The M_w/M_n ratios did not vary among the samples except for the sample obtained from fermentation of untreated molasses that exhibited the highest polydispersity index.

Israilides et al. *(21)* have reported similar values for M_w/M_n and slightly higher M_w for pullulans produced by fermentation of various agroindustrial wastes or a defined synthetic medium. Furthermore, with a sucrose-based medium, Catley *(36)* has found a decrease in molecular weight of pullulan from about $3–6 \times 10^6$ to 1 to 2×10^5 after 5 d of culture. Lee and Yoo *(37)* have also reported a molecular weight range for pullulans between 2.0 and 6.0 $\times 10^5$, depending on the culture conditions (pH, sucrose concentration of medium). A general inverse relation between pullulan yield and molecular weight has often been shown in cultures of *A. pullulans (6,37,38)*. This relationship was not established with the isolated pullulan samples of the present study, as shown by the data in Table 1. The molecular size of pullulan is important for its end-use properties, e.g., as a food coating or packaging or encapsulating wall material (mechanical and gas barrier properties), as an adhesive (viscosity, adhesiveness, moisture retention), and as a fat mimetic in low-calorie food formulations (viscosity, dispersibility).

Pullulan solutions are known to exhibit a Newtonian flow behavior over a relatively broad range of concentrations compared with other plant or microbial polysaccharides. Solutions of pullulan samples isolated from various fermentation protocols started to become more viscous and showed

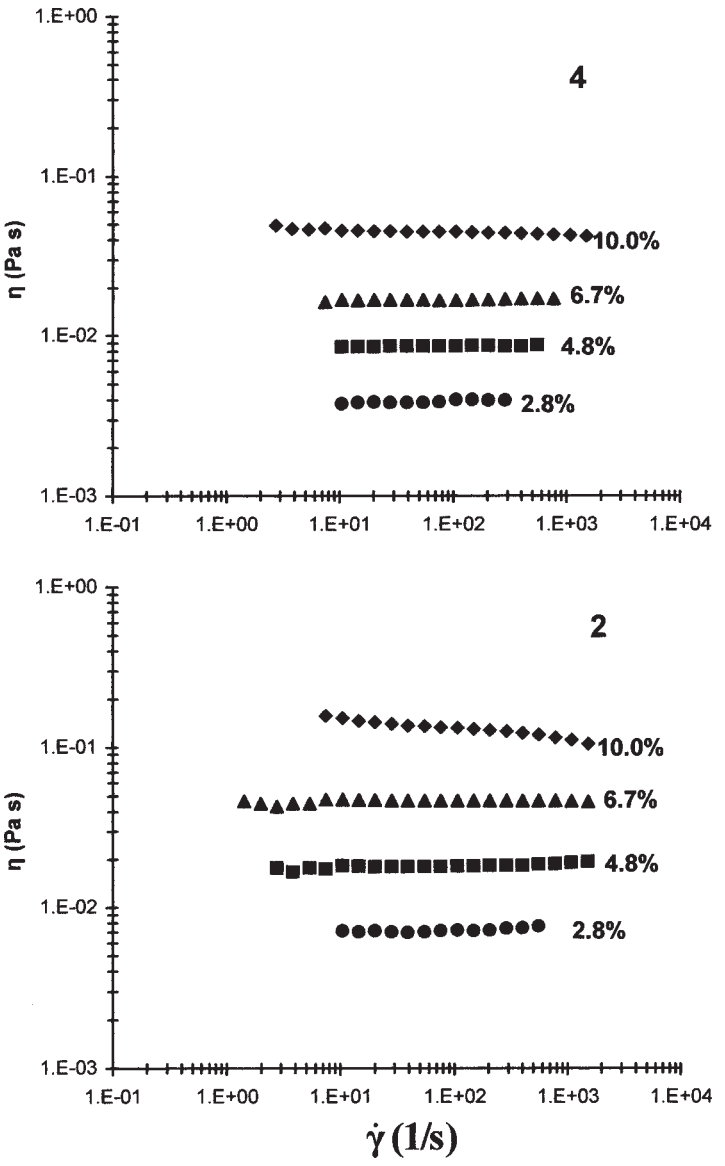

Fig. 10. Flow curves of exopolysaccharides (samples 2 and 4, as specified in Table 1) at different polymer concentrations (% [w/v] at 20°C).

slight deviations from Newtonian flow at concentrations above 8–10% (w/v) (Fig. 10). The solution behavior of pullulan, typical of a flexible random coil, is believed to originate at the α-(1→6) linkage, which has much greater rotational freedom than other types of linkages on a glucan chain *(39)*. Overall, the relatively low viscosity of pullulan solutions and their pH and salt tolerance compared to other hydrocolloids make this biopolymer unique as a coating and encapsulating carrier *(40)*.

Conclusion

The results of our work have revealed some interesting aspects of pullulan production from beet molasses using a pigment-deficient strain of *A. pullulans*. The maximum concentration of polysaccharides (24 g/L) could be achieved by pretreatment of molasses with sulfuric acid and activated carbon. Optimum fermentation conditions for polysaccharide production were an initial sugar concentration of 50 g/L and pH 7.0. The addition of other nutrients (olive oil, ammonium sulfate, and yeast extract) as supplements did not seem to improve further the fermentation efficiency. The exopolysaccharides isolated from the fermentation broths consisted mainly of pullulan (>93% dry basis) as detected by enzymic methods and ^{13}C-NMR spectroscopy. Light-scattering measurements in combination with HPSEC revealed normal distributions of the polysaccharide components of all samples isolated from the fermentation broths of treated molasses. Estimates of weight average molecular weights ranged between 2.1 and 4.1 × 10^5 and root mean square of the radius of gyration between 30 and 38 nm.

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