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Enhancement of Pullulan Production by *Aureobasidium pullulans* in Batch Culture Using Olive Oil and Sucrose as Carbon Sources

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ABSTRACT

The production of pigment-free pullulan by *Aureobasidium pullulans*, using olive oil and sucrose as carbon (C) sources, in shake flasks, was investigated. Optimum medium composition for pullulan elaboration was 80 g/L sucrose, 25 mL/L olive oil, 5 mL/L Tween-80, 10 g/L glutamic acid, and an initial pH of 5.5. Maximum pullulan concentration (51.5 g/L), productivity (8.6 g/L·d), and yield (80.3%) were achieved under these conditions after 120 h of fermentation. The principal advantage of using olive oil and sucrose simultaneously as C sources was the elimination of the inhibitory effect of high sucrose concentrations (>60 g/L) on pullulan production by the microorganism. Structural characterization by ^{13}C-NMR, monosaccharide, and methylation analyses, and pullulanase digestion, combined with size-exclusion chromatography, confirmed the identity of pullulan and the homogeneity of the released polysaccharide in the fermentation broths. There were no significant differences in structure between pullulan samples isolated from either olive oil-supplemented media or olive oil-free media. The molecular size of pullulan from the combined olive oil–sucrose fermentation was slightly lower (1.1×10^6) than that of conventional fermentation with sucrose as a single C source (1.4×10^6). Lowering the initial pH of the medium resulted in increased molecular size for the released polymer, but a lower pullulan yield.

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Index Entries: Pullulan; fermentation; *Aureobasidium pullulans;* olive oil; batch culture; polysaccharide structure.

INTRODUCTION

Pullulan, the chief extracellular homopolysaccharide produced by strains of *Aureobasidium pullulans,* is a predominantly linear a-D-glucan, consisting of a-(1→6)-linked maltotriose units. The physical properties of this biopolymer make it suitable for a wide range of applications in the food, cosmetic, and pharmaceutical industries *(1–4).* A. pullulans is a polymorphic fungus that undergoes a series of morphological changes, depending on culture conditions: hyphae, blastospores (conidial form), swollen cells, and chlamydospores. Although, in some reports, no casual relationship between polysaccharide-producing capacity and culture morphology was shown *(5,6),* in other studies it was found that pullulan production is closely associated with the morphology of the producer microorganism *(7–9).* According to the recent work of Simon and coworkers *(10,11),* the resting forms (swollen cells and chlamydospores) of the microorganism are primarily involved in polysaccharide elaboration; the filamentous forms are less productive than the yeast-like forms. In addition to a wide diversity in pullulan production observed among different strains of *A. pullulans,* important environmental parameters for polysaccharide synthesis are temperature, *(12)* pH of the medium, *(13–16)* oxygen supply *(17,18),* nitrogen (N) source and concentration *(19–22),* and C source *(1,19,23,24).* The microorganism uses sugar substrates for cell growth and biosynthesis of pullulan, the latter being produced mostly when growth slows down, and after cessation of cell growth, e.g., when N supply becomes a limiting factor *(13,18,23).* This suggests that fermentation of a shorter duration may be established if the biomass is rapidly built up at the beginning of the fermentation.

Several undesirable features of fermentation with *A. pullulans* cultures have been observed. First, there is a decrease in the molecular size of pullulan as fermentation progresses *(25).* Adjustments of fermentation conditions (e.g., initial pH, inoculum size, time of harvest) to vanquish this shortcoming, or the development of new strains capable of producing high mol wt pullulan *(26),* generally lead to reduced polysaccharide yields. The second problem is related to the simultaneous synthesis of a dark melanin-like pigment, which contaminates the product and impedes the recovery steps for isolation and purification of the polysaccharide from the fermentation broth. The development of melanin-deficient strains *(27)* and/or the restraining of morphogenesis of the microorganism from the yeast-like forms (swollen cells) to the filamentous hyphae, or to the heavily pigmented chlamydospores during the production stage *(28),* have been recently proposed to overcome this drawback. Moreover, there is a need

for developing fermentation systems in which media with higher concentrations of sugar and/or other C substrates can be efficiently utilized to enhance pullulan yield, and reduce the cost for the downstream recovery of the polysaccharide. In this respect, a two-stage fermentation process was described in which a cell-growth stage and a pullulan-production stage have been separated using two entirely different C sources: cell biomass was rapidly built up in the presence of soy bean oil; pullulan accumulation in the broth occurred by shifting the culture to a sucrose-containing and N-restricted medium *(28)*. A high conversion rate of sugar to pullulan was attained (0.65 g/g), and no pigment was formed in the culture during the production stage. For the latter, the authors claimed that the culture is morphogenetically arrested in a yeast-like form during the growth phase, using soy bean oil as the sole C source, and therefore it produces a pigment-free polysaccharide. The utilization of high concentrations of sugar in *A. pullulans* fermentation has been also explored with other fermentation systems, including fed-batch culture *(29)*. Shin and coworkers *(29)* have overcome the inhibitory effect of high sucrose concentration (10%) in batch culture by using a fed-batch culture and an intermittent feeding mode; a high polysaccharide concentration (58 g/L from a total 10% sucrose concentration) was observed. However, in this work, as well as in most fermentation studies involving *A. pullulans,* the reported high theoretical yields are based on total extracellular polysaccharides secreted by the microorganism, and not on true pullulan levels.

In the present study, the production of pullulan by a nonpigmented strain of *A. pullulans,* using olive oil and sucrose as C sources, in shake flasks, was explored in an attempt to increase the polysaccharide-producing capacity of the culture. The effects of glutamic acid and emulsifier on the fermentation parameters were also examined. Moreover, structural characterization and molecular size determination of the recovered polymers from the culture broths were carried out.

MATERIALS AND METHODS

Microorganism and Culture Conditions

A. pullulans P 56, a strain deficient in melanin production, was kindly supplied by Prof. Mersmann of the Technical University of Munich. The microorganism was maintained on potato dextrose agar plates at 4°C, and subcultured every 2 wk. Cell suspension for inoculation of the culture medium was obtained from cultures grown on potato dextrose agar plates at 28°C for 48 h. The cells were suspended in 10 mL sterile water and transfered to 500-mL conical flasks containing 150 mL culture medium (pH 5.5) with the following composition (g/L): 30.0 sucrose, 0.6 $(NH_4)_2SO_4$, 0.4 yeast

extract, 5.0 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, and 1.0 NaCl. The flasks were incubated at 28°C for 48 h in a rotary shaker incubator (Lab Line Orbit-Environ Shaker, Lab-Line Instruments, Melrose Park, IL) at 200 rpm. These cultures were used to inoculate the production medium at a ratio of 5% (v/v).

Fermentation Conditions

A series of fermentation experiments were performed in conical flasks to examine the effect of olive oil on pullulan production. The fermentation media had the following composition (g/L): 0.2 yeast N base, 5.0 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 1.0 NaCl, 0.01 $FeSO_4 \cdot 7H_2O$, and sucrose (60, 80, and 100), olive oil (0.0, 10.0, 20.0, and 25.0 mL/L), Tween-80 (0.0 and 5.0 mL/L), and glutamic acid (5.0 and 10.0 g/L). Control experiments were also carried out using a medium of the following composition (g/L): sucrose (60.0 or 80.0), 0.6, $(NH_4)_2SO_4$, 0.4 yeast extract, 5.0 K_2HPO_4, 0.2 $MgSO_4 \cdot 7H_2O$, and 1.0 NaCl. The pH of the medium was adjusted with either 1 *N*NaOH or 1 *N*HCI, and sterilized at 121°C for 15 min.

All of the above experiments were carried out in 500 mL conical flasks containing 100 mL of fermentation medium. The flasks were incubated at 28°C for 120 h in a rotary shaker/incubator (Lab Line Orbit-Environ Shaker, Lab-Line) at 200 rpm.

Isolation of Pullulan

The fermentation broth (after appropriate dilution by a factor of 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 acetone, dissolved in distilled water, and finally precipitated with absolute ethanol. Preliminary experiments indicated that it was not necessary to further purify the secreted polysaccharides in the fermentation broths. This strain, under the conditions employed in this work, was not found to produce acidic extracellular polysaccharides which can be precipitated by the cationic detergent cetyltrimethylammonium bromide *(30,31)*.

Chemical Analyses

Fermentation Aspects

At specific time intervals, total biomass dry wt was determined by centrifugation of the broth (after appropriate dilution) at 10,000*g* for 20 min, washing the sediment with distilled water (twice), and drying at 105°C overnight. The first supernatant was combined with the washings, and the 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 were determined in the filtrate, according to ref. *(32)*. Pullulan yield was expressed as polysaccharide/100 g of sucrose

consumed; fermentation efficiency was taken as a ratio of sugar consumed over the total amount of added sugar, multiplied by 100.

Structural Characterization of Polysaccharide

Monosaccharide analysis of pullulan was carried out by GC, using a SP-2330 glass capillary column, 30 m × 0.75 mm id, according to ref. *(33)*. The proton-decoupled ^{13}C-NMR spectra (300 Hz) were recorded on a Bruker AM 300 FT spectrometer at 65°C, polymer concentration 2% (w/v) in D_2O, 30,000 pulses, with a pulse repetition time 1.245 s, and r.f. pulse angle 80.0 degrees *(31)*. Methylation analysis was carried out according to ref. *(34)*. GC-MS was performed on an SP-2330 capillary column (60 m × 0.25 mm); qualitative measurements of partially permethylated acetyl alditols were made on an SP-2330 column (30 m × 0.75 mm), programmed from 160 to 210°C/min; helium was used as carrier gas (15 psi). The molecular size distribution of the polysaccharides was determined on a Sephacryl S-500 column (2.5 × 95 cm). Aliquots of polysaccharides (10 mg) in 5 mL of 0.1 *M* NaCl were applied on the column, and eluted with 0.1 *M* NaCl containing 0.05% NaN_3, at a flow rate of 45 mL/h at 25°C. Effluent fractions (4 mL) were monitored for total carbohydrates by the phenol–sulfuric method *(32)*. Estimation of V_o and V_t for the column were obtained by chromatography of blue dextran and glucose, respectively. The first fraction detected with blue dextran by means of absorption at 280 nm was taken as V_o; the glucose peak determined by the phenol–sulfuric method was taken as V_t. Calibration of the column was carried out using a series of pullulan standards with known mol wt (0.58, 1.22, 2.37, 4.8, 10.0, 18.6, 38.0, and 166.0 × 10^4) obtained from Showa Denko K.K. (Okayama, Japan). The mol wt of the unknown samples was determined from the calibration curve (log mol wt vs K_{av} for the series of standard pullulan samples), using the peak fraction of the eluted polysaccharides (V_e = the number of peak fraction × vol of effluent fraction). The K_{av} is defined as $(V_e - V_o)/(V_t - V_o)$, when V_e is the elution volume, V_o the exclusion volume, and V_t the total volume. Debranching of pullulan (30 mg) in 5 mL of 0.1 *M* acetate buffer (pH 5.5) was carried out with 32 IU of crystalline pullulanase (Hayashibara Biochem., Okayama, Japan) at 37°C for 12 h, according to ref. *(35)*. 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 column (2.5 × 95 cm). Elution was carried out with 0.1 *M* sodium acetate (pH 4.7) containing 0.02% NaN_3 at a flow rate of 35 mL/h at 25°C. Fractions of 3 mL were analyzed for total carbohydrates *(32)*.

Statistical Analysis

Analysis of variance was carried out on the fermentation data, and differences among various fermentation regimes were determined by

Table 1
Influence of Sucrose Concentration on Fermentation Parameters by *A. pullulans* Grown in Olive Oil-Free Medium[1]

Sucrose (g/L)	Pullulan (g/L)	Productivity (g/L.d)	Biomass dry wt (g/L)	Pullulan Yield (%)	Fermentation efficiency (%)
60	$33.1^b \pm 1.9$	$6.6^b \pm 0.4$	$9.4^a \pm 0.6$	$58.9^b \pm 2.6$	$93.8^b \pm 1.4$
80	$21.2^a \pm 0.4$	$4.3^a \pm 0.1$	$10.1^a \pm 0.2$	$40.1^a \pm 1.1$	$66.1^a \pm 0.7$

[1]pH 7.5, 120 h fermentation. Data are means of triplicate experiments; values with different letters in each column are significantly different ($p < 0.05$).

Duncan's multiple range test, using the SPSS statistical software (vers. 5.0, Kaysville, UT). Data presented are means of triplicate runs. Variability was also expressed by coefficient of variation (cv) values.

RESULTS AND DISCUSSION

Effects of Medium Composition and Fermentation Conditions

Shake-flask fermentations were performed for the production of pigment-free pullulan using the *A. pullulans* P 56. Control experiments were carried out to examine the effect of sucrose concentration (60 and 80 g/L) on pullulan production by *A. pullulans* grown in an olive oil-free medium, for comparative purposes (Table 1). In these experiments, the use of $(NH_4)_2SO_4$ as a N source necessitated adjustment of initial pH of the medium to 7.5 *(36)*. Apart from biomass concentration, all other fermentation parameters significantly decreased when sucrose concentration increased to 80 g/L. Biomass concentration was found to remain practically constant. It appears that a sugar concentration higher than 60 g/L largely inhibits pullulan synthesis; pullulan concentration decreased from 33.1 to 21.2 g/L when sucrose concentration increased from 60 to 80 g/L. Similarly, Shin and coworkers *(29)* reported that pullulan synthesis by *A. pullulans* is inhibited when sucrose concentration exceeds 50 g/L.

The effect of varying sucrose concentrations (60, 80, and 100 g/L) on pullulan production by *A. pullulans*, grown in culture media containing 25 mL/L olive oil, was subsequently studied. The culture medium in these experiments was supplemented with glutamic acid (10 g/L), to suppress the morphogenetic shift from yeast to filamentous growth, and with Tween-80 (5 mL/L) as an emulsifier. It has been previously reported that certain N sources (e.g., amino acids) suppress the morphogenetic shift from yeast to filamentous growth of *A. pullulans (28,37)*. When sucrose concentration increased from 60 to 80 g/L, the pullulan level increased

Table 2
Effects of Sucrose and Olive Oil Concentration on Fermentation Parameters by *A. pullulans*[1]

Sucrose (g/L)	Olive oil (mL/L)	Pullulan (g/L)	Productivity (g/L.d)	Biomass dry wt (g/L)	Pullulan Yield (%)	Fermentation efficiency (%)
60	25	$43.2^{b} \pm 3.2$	$8.6^{b} \pm 0.7$	$11.9^{a} \pm 0.5$	$82.8^{b} \pm 3.0$	$94.0^{c} \pm 7.8$
80	25	$51.5^{c} \pm 1.7$	$10.3^{c} \pm 0.4$	$14.0^{b} \pm 0.6$	$80.3^{b} \pm 0.7$	$88.6^{b} \pm 0.7$
100	25	$33.9^{a} \pm 4.0$	$6.8^{a} \pm 0.8$	$22.4^{c} \pm 0.1$	$43.2^{a} \pm 4.0$	$78.5^{a} \pm 2.1$
80	20	$34.8^{b} \pm 0.5$	$7.0^{b} \pm 0.1$	$15.0^{b} \pm 0.4$	$52.8^{b} \pm 0.2$	$73.4^{b} \pm 0.7$
80	10	$34.1^{ab} \pm 0.9$	$6.8^{a} \pm 0.1$	$16.0^{c} \pm 0.1$	$49.9^{a} \pm 0.2$	$72.4^{a} \pm 0.3$

[1]10 g/L glutamic acid, 5 mL/L Tween 80, pH 5.5, 120 h fermentation. Data are means of triplicate experiments; values with different letters in each column are significantly different ($p < 0.05$).

Table 3
Effect of Tween 80 on Fermentation Parameters[1]

Tween 80 (g/L)	Pullulan (g/L)	Productivity (g/L.d)	Biomass dry wt (g/L)	Pullulan Yield (%)	Fermentation efficiency (%)
0	$25.3^{a} \pm 0.3$	$5.1^{a} \pm 0.1$	$22.1^{b} \pm 0.7$	$35.7^{a} \pm 0.2$	$85.7^{a} \pm 0.7$
5	$51.1^{b} \pm 1.7$	$10.3^{b} \pm 0.4$	$14.0^{a} \pm 0.9$	$80.3^{b} \pm 0.7$	$88.6^{b} \pm 0.7$

[1]8% w/v sucrose, 2.5% v/v olive oil, 10 g/L glutamic acid, 120 h fermentation. Data are means of triplicate experiments; values with different letters in each column are significantly different ($p < 0.05$).

from 43.2 to 51.5 g/L (Table 2). With further increase in sucrose concentration to 100 g/L, the level of pullulan decreased to 33.9 g/L. In contrast, the biomass constantly increased with sucrose concentration, but the fermentation efficiency decreased. Pullulan yield remained almost constant, up to a sucrose concentration of 80 g/L. A drastic drop in pullulan yield was observed with further increase in sucrose concentration to 100 g/L, or with a lower concentration of olive oil. The results of Table 2 indicate that the optimal sucrose and olive oil concentration for pullulan production by *A. pullulans* P 56 is 80 g/L and 25 mL/L, respectively.

To examine the influence of emulsification of olive oil, media supplemented with and without Tween-80 (sorbitan monooleate) were tested for pullulan production. The fermentation data are summarized in Table 3. In the absence of emulsifier, there was a reduction in all fermentation parameters, except for biomass concentration. Although the concentration of pul-

Table 4
Effect of Glutamic Acid on Fermentation Parameters[1]

Glutamic acid (g/L)	Pullulan (g/L)	Productivity (g/L.d)	Biomass (g/L)	Pullulan Yield (%)	Fermentation efficiency (%)
5	$36.4^a \pm 3.3$	$7.3^a \pm 0.7$	$14.0^a \pm 1.0$	$64.6^a \pm 6.1$	$94.0^a \pm 0.3$
10	$43.2^b \pm 3.2$	$8.6^b \pm 0.7$	$14.2^a \pm 0.5$	$72.8^b \pm 5.4$	$98.8^b \pm 0.4$

[1]2.5% v/v olive oil, 6% w/v sucrose, pH 5.5, 120 h fermentation. Data are means of triplicate experiments; values with different letters in each column are significantly different ($p < 0.05$).

lulan fell by 50% in a Tween-80-free medium, the cell-mass concentration increased from 14 to 21.2 g/L. This may indicate that, in the presence of Tween-80, olive oil suppresses the morphogenetic shift from yeast to filamentous growth. It is well known that yeast-like forms are more productive than filamentous forms of the microorganism *(8,10,11,18,38)*. The effect of glutamic acid concentration (5 and 10 g/L) on the kinetic aspects of fermentation by *A. pullulans*, grown in olive oil-supplemented medium, is shown in Table 4. Apart from biomass concentration, all other fermentation parameters were found to significantly decrease when the concentration of glutamic acid decreased from 10 to 5 g/L.

The influence of initial pH on pullulan production by *A. pullulans* P 56, grown on olive oil and sucrose as C sources, was also studied. At the end of the fermentation period (120 h), for the medium containing 2.5% (v/v) olive oil and 6% (w/v) sucrose (10 g/L glutamic acid, 5 mL/L Tween-80), the highest biomass concentration (14.2 g/L), pullulan concentration (42.3 g/L), and pullulan yield (72.8%) were obtained with an initial pH of 5.5 (Fig. 1). Apart from biomass concentration, all other fermentation parameters decreased when the initial pH of the culture medium increased from 5.5 to 7.5. The optimal pH of 5.5 found for the olive oil-containing medium is much lower than that (pH 7.5) for olive oil- and glutamic acid-free medium (i.e., medium containing $(NH_4)_2SO_4$ as N source *(36)*; and it might be a reflection of differences in medium composition between the two regimes. It is well known that culture pH is affected by the N source. With 0.05–0.15 g/L N in the form of $(NH_4)_2SO_4$, Auer and Seviour *(22)* found a large drop in the pH of fermentation broth, compared to other N sources, such as glutamate. The low optimal pH of 5.5 concurs with the data of Shabtai and Mukmenev *(28)*, who reported an optimal initial pH of 5.5 for pullulan elaboration by *A. pullulans* (ATCC 42023) grown in culture medium containing 3% (v/v) soybean oil. In another study, Lacroix et al. *(16)* found that an initial pH of 5.5 supports the highest pullulan concentration in a culture medium containing yeast extract. It was also shown

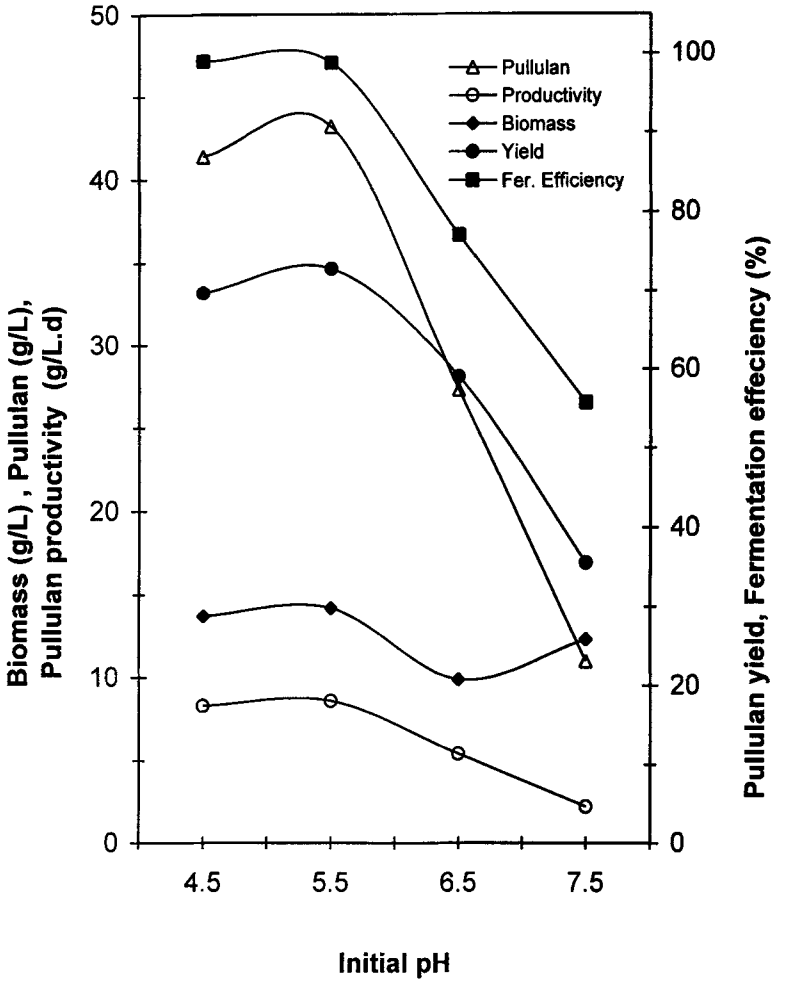


Fig. 1. Effect of initial pH on batch-fermentation parameters (*A. pullulans* P 56) at 120 h, using olive oil (25 mL/L) and sucrose (60 g/L) as C sources. Data are means of triplicate experiments; cv values for all measured parameters did not exceed 5.1% in all cases.

that a glucose-based synthetic medium with an initial pH of 4.5 stimulates more polysaccharide synthesis than when the initial pH is 7.0 *(21)*. Roukas and Biliaderis *(31)* found that an initial pH of 6.5 is optimal for pullulan production by *A. pullulans* (SU No. M18) grown on carob pod extracts. In other studies, an initial pH of 7.5 was reported as the optimal pH value for pullulan synthesis, using a synthetic medium with $(NH_4)_2SO_4$ as the N source *(22,29)*. It would appear that the optimal initial pH for pullulan synthesis depends on several fermentation parameters. These include the strain of the microorganism, the composition of the culture medium, and other fermentation conditions *(26)*. The results of Fig. 1 clearly indicate

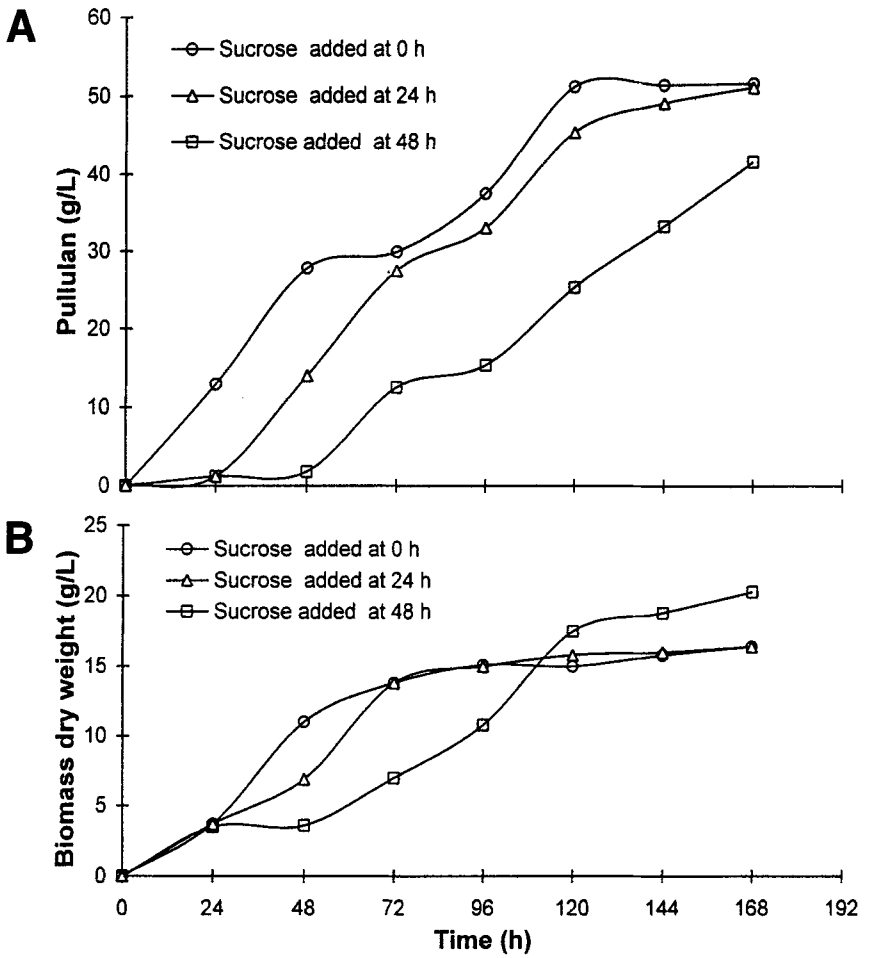


Fig. 2. Pullulan **(A)** and biomass **(B)** levels during growth of *A. pullulans* P 56 in media containing olive oil (25 mL/L), and sucrose (80 g/L) added at various fermentation times. Data are means of triplicate experiments; cv values did not exceed 5.6% in all cases.

that synthesis of pullulan by *A. pullulans* P 56, with olive oil and sucrose as C substrates, is largely influenced by the initial pH of the medium.

According to the work of Shabtai and Mukmenev *(28)*, the growth of the microorganism and the elaboration of the polysaccharide is favored with different medium conditions; i.e., biomass buildup took place initially (72 h) in the presence of triglycerides, but polysaccharide synthesis was stimulated after the C source for the culture was shifted to sucrose (production stage). Thus, the effect of sucrose addition at various fermentation times (0, 24, and 48 h) on the growth of *A. pullulans* P 56, and the synthesis of pullulan in culture media supplemented with olive oil at 0 h,

was examined. The levels of pullulan elaborated in the fermentation broth were found to be influenced by delaying the addition of sucrose (Fig. 2A). With a medium containing sucrose at the beginning of fermentation, synthesis of pullulan commenced immediately, showing no lag period for its appearance. In this culture, pullulan concentration increased as fermentation progressed, until 120 h. In contrast, cultures fed with sucrose at 24 or 48 h exhibited very small amounts of pullulan (less than 2 g/L), before sucrose was introduced into the media. In these cultures, pullulan synthesis started immediately after the addition of the sugar. In the culture medium containing sucrose and olive oil at 0 h, a maximum pullulan concentration of 51.5 g/L was achieved after 120 h of fermentation. The same maximum pullulan concentration was also obtained from the culture that was fed with sucrose at 24 h, but after 168 h of fermentation. For the culture fed with sucrose at 48 h, a lower pullulan concentration (41.7 g/L) was obtained at 168 h. These findings clearly indicate that the duration of fermentation is prolonged by delaying sucrose addition (e.g., at 24 or 48 h). Moreover, pullulan productivity, calculated on the basis of a 120-h fermentation period, was highest (10.3 g/L·d) when both sucrose and olive oil were present in the culture medium at 0 h: the lowest productivity (5.1 g/L·d) was observed when sucrose was fed at 48 h. Delaying the addition of sucrose also led to decreased pullulan yield (from 80.3% at 0 h to 54.2% at 48 h delaying time) and fermentation efficiency (from 85.7% at 0 h to 58.3% at 48 h delaying time). These results suggest that addition of sucrose at the beginning of fermentation is optimal for pullulan production by *A. pullulans* P 56 grown in an olive oil-supplemented medium.

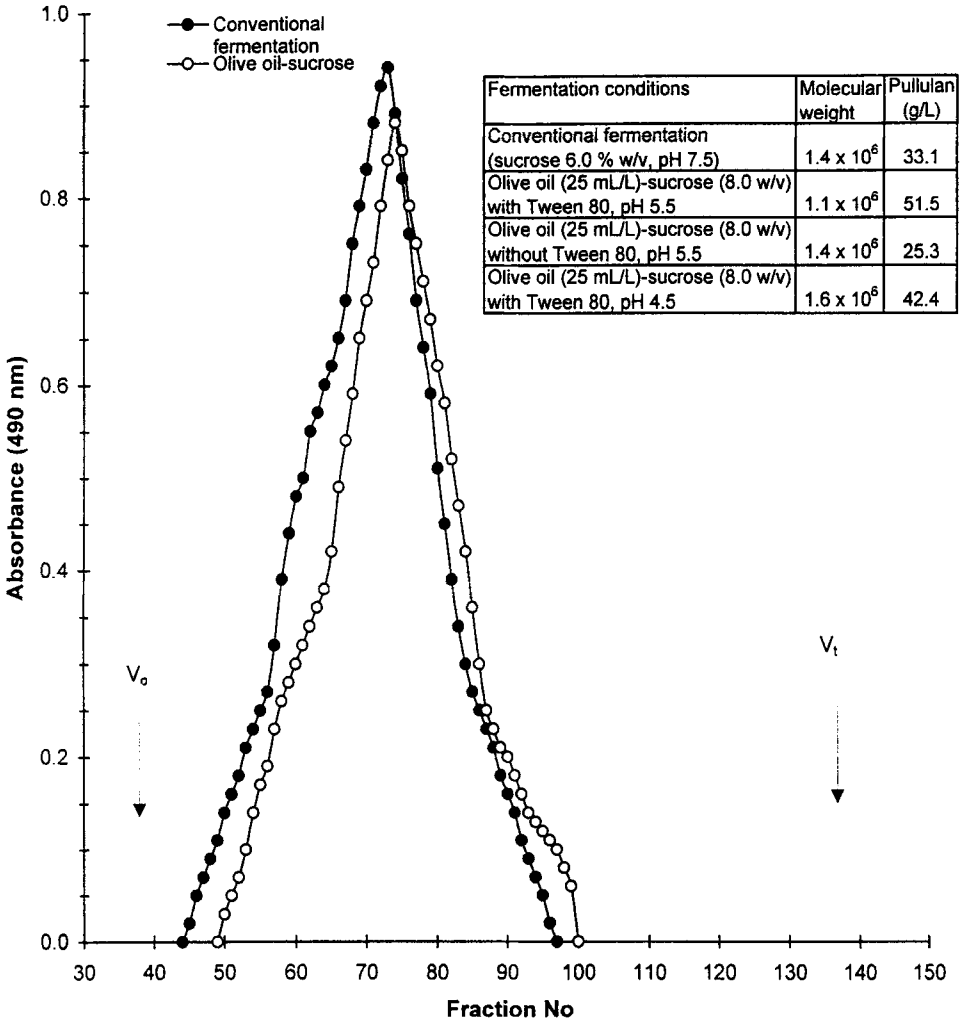
The biomass concentration of *A. pullulans* in culture media containing olive oil, and fed with sucrose at various fermentation times (0, 24, 48 h), is presented in Fig. 2B. During the first 24 h of fermentation, all cultures exhibited similar growth responses (about 3.6 g/L). After this period, the growth of the microorganism followed the order of sucrose addition, i.e., the culture with sucrose added at 0 h gave the fastest growth rate. These observations imply that olive oil does not effectively support cell growth or pullulan synthesis by *A. pullulans* P 56, unless the culture medium is simultaneously supplemented with sucrose. These results are not consistent with the data of Shabtai and Mukmenev *(28)*, who found that soybean oil as a sole C source can give high cell-mass concentration (about 15 g/L) after 20 h of fermentation; however, a different strain (ATCC 42023) was employed in their studies. As shown in Fig. 2, the culture fed with sucrose at 48 h gave the highest cell-mass concentration (about 22 g/L), but the lowest pullulan concentration (41.7 g/L) after 168 h of fermentation. It would appear that delaying the addition of sucrose to an olive oil-supplemented medium leads to late microbial growth at the expense of pullulan synthesis.

The growth of *A. pullulans* in a synthetic medium containing olive oil (2.5% v/v), and lacking sucrose, was finally examined. The microorganism was found to grow on olive oil, yielding a biomass concentration of 3.6 g/L at 24 h. Following this period, the fungal cell levels appeared to remain constant at about 3.7 g/L. This biomass level is much lower than that of 6.0 g/L, achieved if sucrose at 5% w/v was used as the sole C source. Shabtai and Mukmenev *(28)* have noted that another strain of *A. pullulans* (ATCC 42023) grows nearly twice as fast on soybean oil as on sucrose.

Overall, the above findings indicate that the simultaneous addition of sucrose and olive oil in the culture medium (at 0 h) is pivotal for shortening the duration of fermentation and achieving high pullulan concentration in the fermentation broth. Moreover, including Tween-80 in the olive oil-, glutamic acid-, and sucrose-supplemented media positively influences the level of pullulan elaborated by the *A. pullulans* P 56.

Characterization of Polysaccharides

Polysaccharide samples isolated from different culture media after 120 h of fermentation were subjected to structural analysis using different approaches. The molecular size distribution profiles of these materials were obtained using a Sephacryl S-500 column. The elution profiles of polysaccharides showed single peaks (Fig. 3), indicative of rather single polymer populations. From the estimates of mol wt of the polysaccharide samples, included in the inset of Fig. 3, it appears that the molecular size of pullulan produced in an olive oil-supplemented medium was lower (1.1×10^6) than that of pullulan produced with conventional fermentation using sucrose as the sole C source (1.4×10^6). This is not surprising, since a much higher pullulan concentration was found in the culture medium of olive oil. An inverse relationship has been shown between pullulan yield and molecular size of this polysaccharide. *(26)* The underlying physiological basis for this relationship is not known *(31)*, although the possibility for less efficient nutrient uptake from the medium by cultures producing high mol-wt pullulan cannot be excluded *(26)*. The molecular size of pullulan produced in a culture medium containing olive oil and lacking Tween-80 was slightly higher (1.4×10^6) than that of pullulan produced in culture medium containing olive oil and Tween-80 (1.1×10^6). This finding may be also a reflection of the lower level of pullulan produced in the absence of Tween-80. With respect to the initial pH of the medium, the molecular size of pullulan isolated from the culture grown at pH 4.5 was slightly higher (1.6×10^6) than that of pullulan obtained from the culture grown at pH 5.5. These results confirm the observations of Lee and Yoo *(39)*, who reported higher mol-wt pullulan from cultures grown at a low initial pH of 3.0,



Fermentation conditions	Molecular weight	Pullulan (g/L)
Conventional fermentation (sucrose 6.0 % w/v, pH 7.5)	1.4×10^6	33.1
Olive oil (25 mL/L)-sucrose (8.0 w/v) with Tween 80, pH 5.5	1.1×10^6	51.5
Olive oil (25 mL/L)-sucrose (8.0 w/v) without Tween 80, pH 5.5	1.4×10^6	25.3
Olive oil (25 mL/L)-sucrose (8.0 w/v) with Tween 80, pH 4.5	1.6×10^6	42.4

Fig. 3. Elution profiles (Sephacryl S-500, 2.5 × 95 cm, eluted with 0.1 M NaCl at a flow rate of 45 mL/L, 25°C) of polysaccharides from batch fermentation with olive oil (25 mL/L)–sucrose (80 g/L), or conventional fermentation with sucrose (60 g/L).

compared to pH 4.5 and 7.5. Pullulanase treatment and chromatography of the polysaccharide digest on a Biogel P-2 column revealed mostly maltotriose oligomers (Fig. 4). All pullulan samples from different Biogel P-2 fermentation conditions gave almost the same elution profile, and the debranching results are summarized in the inset of Fig. 4. A small amount (<3.0%) of a high molecular-size gel-excluded fraction (at V_o) may orginate from the presence of a contaminating glucan species or resistant oligosaccharide fragments to pullulanase action, i.e., because of branching with linkages other than α-(1→6). The high concentration of (maltotriose+mal-

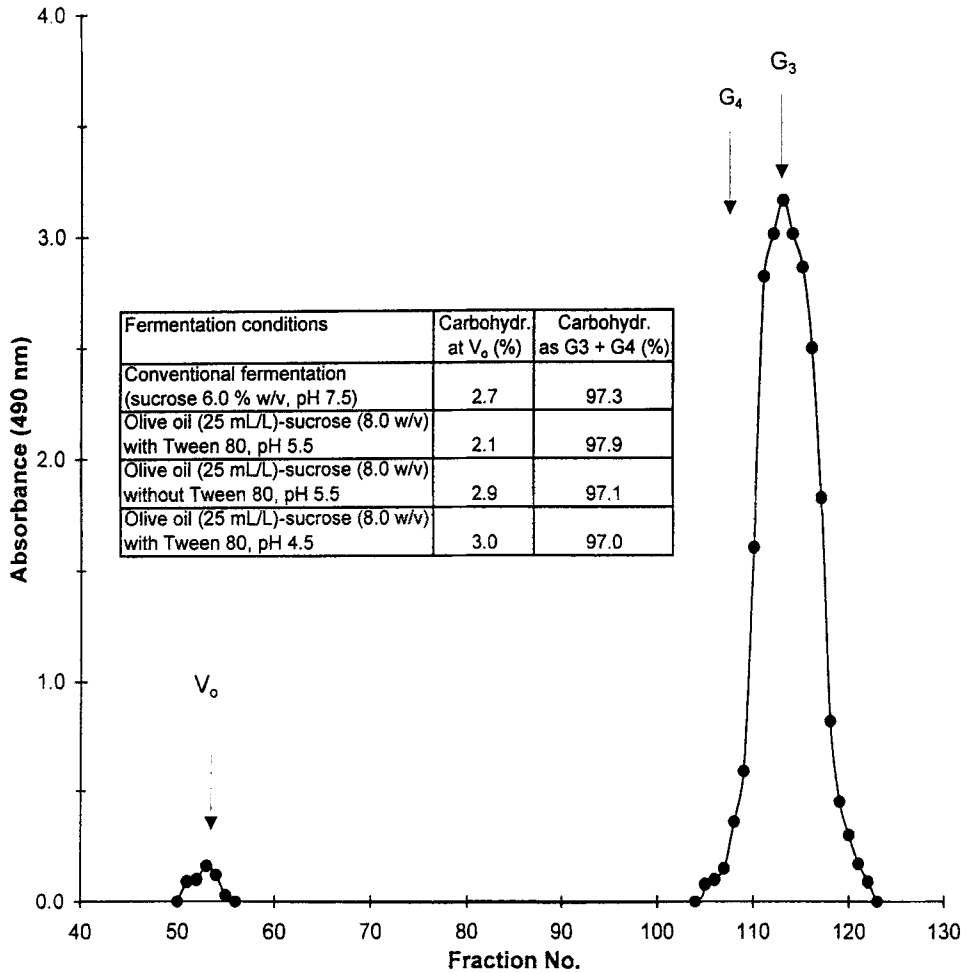


Fig. 4. Elution profile (BioGel P-2, 2.5 × 95 cm, eluted with 0.1 M sodium acetate buffer, pH 4.7 at 35 mL/h, 25°C) of pullulanase-debranched polysaccharide isolated from the fermentation broth of *A. pullulans* P 56 grown on olive oil (25 mL/L)–sucrose (80 g/L) medium at 120 h. G_3 and G_4 denote the peak elution volumes of pure maltotriose and maltotetraose oligosaccharides. The inset shows the relative amounts of carbohydrates eluted at V_o and (G_3 + G_4) for samples obtained under various fermentation conditions.

totetraose) oligomers following pullulanase treatment confirmed the purity of the acetone–ethanol-precipitated polysaccharides from the fermentation broths.

The methylation data of pullulan obtained from the culture grown on olive oil–sucrose are given in Table 5, along with the results of a sample obtained from conventional fermentation using sucrose as the sole C source. Methylation analysis of all samples gave mostly 2,3,6-tri-*O*-methyl- and 2,3,4-tri-*O*-methyl-glucopyranose derivatives at a ratio close to 2:1.

Table 5
Relative Molar Ratio of Partially Permethylated Acetyl Alditols of Polysaccharides from *A. pullulans* Fermentation Broths With Olive Oil- and Sucrose-Supplemented Medium[a] and Conventional Fermentation With Sucrose[b]

Alditol acetate	Linkage type	Conventional fermentation[b]	Fermentation with sucrose-olive oil[a]
2,3,4,6-Me_4-Glc	(Glc*p*) 1→	2.3	1.7
2,3,4-Me_3-Glc	→6 (Glc*p*) 1→	29.8	30.1
2,3,6-Me_3-Glc	→4 (Glc*p*) 1→	65.0	66.0
2,6-Me_2-Glc	→3,4 (Glc*p*) 1→	0.5	0.4
3,6-Me_2-Glc	→2,4 (Glc*p*) 1→	0.6	0.4
2,4-Me_2-Glc	→3,6 (Glc*p*) 1→	0.1	0.3
2,3-Me_2-Glc	→4,6 (Glc*p*) 1→	1.7	1.1

[a] Olive oil 2.5% (v/v)-sucrose 8.0% (w/v), Tween 80 5 mL/L, glutamic acid 10 g/L, initial pH 5.5.
[b] Sucrose 6.0% (w/v), $(NH_4)_2SO_4$ 0.06% (w/v), initial pH 7.5.

This is in agreement with a linear chain of 4-*O*-and 6-*O*-substituted glucose residues. The ratio of α-(1→4) to α(1→6) linkages in pullulan may vary between 1:1 and 2.7:1. *(40)* The 2,3,4,6-tetra-*O*-methyl-glucopyranose originates from the nonreducing glucosyl termini of the polymer structure. According to Catley and coworkers *(41,42)*, the fine structure of pullulan from *A. pullulans* cannot be described by a simple polymaltotriosyl polymeric structure. The occurrence of maltotetraose units has been verified, and may reach up to 6% of the trisaccharide units, depending on the strain of microorganism *(43)*. The data of Table 5 also indicate the presence of other than α-(1→4) and α-(1→6) glucosidic linkages in minor quantities, as has often been reported *(42)*. It is not certain, however, whether such minor linkages arise from other contaminating glucan impurities, or if they are present in the native pullulan structure. As shown in Table 5, there seemed to be no major differences in the molecular ratio of permethylated alditol derivatives among the two samples.

Structural characterization of the native polysaccharides precipitated by acetone–alcohol from the fermentation broths was also carried out by ^{13}C-NMR spectroscopy. The spectral features of all samples analyzed were typical of pullulan, based on previous work published by Gorin *(44)*, confirming the homogeneity of the elaborated polysaccharide by the *A. pullulans* P 56. Some typical ^{13}C-NMR spectra of these polysaccharides isolated from cultures grown on olive oil-supplemented and olive oil-free media

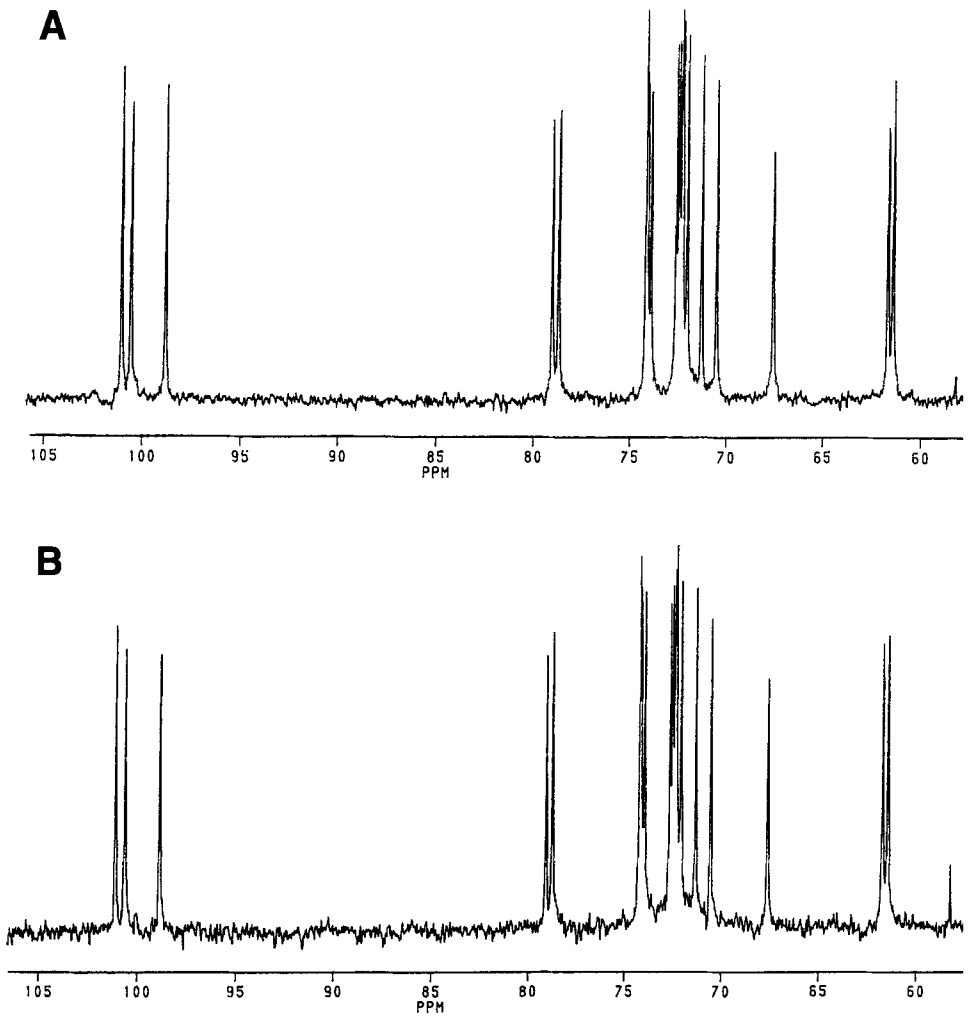


Fig. 5. ^{13}C-NMR spectra of polysaccharides isolated from batch cultures of *A. pullulans* P 56 using media with olive oil (25 mL/L)–sucrose (80 g/L) (**A**), or only sucrose (60 g/L), in conventional fermentation (**B**).

after 120 h fermentation are shown in Fig. 5. The anomeric C region shows three signals corresponding to α-(1→4) (101.1, 100.6 ppm) and α-(1→6) (98.8 ppm) linkages, whose relative area (sum of the 101.1, 100.6 resonances over that of 98.8 ppm) was approx 2:1, in accordance with the methylation data. The splitting of the C-4 (79.0, 78.7 ppm) and C-6 (61.7, 61.4 ppm) resonances of the 1,4-linked glucose units is the result of the sensitivity of these C positions to the nature of the linkage at C-1; e.g., the C-6 signals at 61.4 and 61.7 ppm are those of the two types of 1,4-linked α-D-glucose residues, but the single resonance at 67.6 ppm corresponds to C-6 of the 1,6-linked α-D-glucose *(44,45)*.

CONCLUSIONS

The results of the present study indicate that pullulan production with olive oil and sucrose as C sources in batch culture has several advantages, compared with conventional fermentation using sugar as a single C substrate. The addition of olive oil to the culture medium of *A. pullulans* P 56 resulted in overcoming the inhibitory effect caused by a high sucrose concentration (>6%), and a large amount of biomass (14 g/L) was rapidly built up (during the first 48 h of fermentation). A high conversion (80%) of sucrose into pullulan was also attained. This conversion ratio is much higher than the maximum value (70%) reported previously by Yuen *(1)*. The simultaneous presence of olive oil and sucrose in the culture medium led, within 120 h of fermentation, to a higher pullulan concentration (51.5 g/L) than with any medium containing only sucrose; this would have a direct bearing on lowering the cost for precipitation and recovery of pullulan. Overall, these findings provide the basis for using cheap vegetable oil byproducts, in conjunction with sugars, to enhance the elaboration of pullulan by *A. pullulans.* This may also result in a fermentation process with greater productivity and lower costs, because the utilization of agroindustrial wastes seems to be a very promising ecological and economical route of bioconversion. Further investigations are needed to optimize the fermentation conditions, in order to carry out the process on an industrial scale.

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