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PRODUCTION OF PULLULAN POLYMER BY *Aureobasidium pullulans*

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ABSTRACT

Three strains of *A. pullulans* showed different ability to produce pullulan in static and shaked cultures. Shaked cultures produced high amounts (2->5 folds) than static cultures. *A. pullulans* ATCC 42023 recorded the highest pullulan accumulation, followed by *A. pullulans* ATCC 12535. *A. pullulans* ATCC 16628 showed feeble pullulan production in spite of its good characteristics. Production of pullulan was characterized by appearance of sharp peak coinciding with the 3 days' interval of incubation for *A. pullulans* ATCC 16628 shaked cultures. For other shaked cultures, the peaks coincided with 6 days. Pullulan production was highly dependent on the carbon source on which they were cultivated. Some carbon sources were able to induce pullulan production (2.05–24.18 g/l). The pH of pullulan producing cultures decreased to a pH range of 2.69-3.00. A pH range of 5.46–6.29 were recorded in cultures containing sugar cane and sugar beet molasses as carbon and/or energy sources. Sugar cane molasses or sugar beet molasses, and their treatment methods affected pullulan production. EDTA treated sugar beet molasses promoted the higher pullulan production (14.83 g/l) for *A. pullulans* ATCC 12535. Growing of *A. pullulans* ATCC 42023 on treated molasses resulted in pullulan production smaller than those produced during growth on glucose syrup. Growing on molasses as the sole carbon source led to little change in culture pH. Molasses promoted remarkable growth of pullulan producing strains.Urea+ yeast extract promoted the highest production of pullulan with *A. pullulans* ATCC 12535, while yeast extract alone appeared as being the most favorable nitrogen source for *A. pullulans* ATCC 42023. $Na_3PO_4.12H_2O$ allowed the highest production of the polymer pullulan with *A. pullulans* ATCC 12535, while Na_2HPO_4 appeared as being the most favorable phosphorus source for *A. pullulans* ATCC 42023. The majority of phosphorus sources were not favorable for the test strains. pH 5.5 seemed to be optimal for pullulan accumulations by the two test strains. Outside pH 5.5, the pullulan formation was repressed. Extremes of both acidity and alkalinity adversely affected both the pullulan and biomass production. 5% and 10% inoculum size allowed the highest production of pullulan with *A. pullulans* ATCC 12535 and *A. pullulans* ATCC 42023, respectively. Conversion coefficient (%) at 5% inoculum size reached 59.48% for *A. pullulans* ATCC 12535 and at 10% inoculum size reached 73.67% for *A. pullulans* ATCC 42023. Pullulan yield % at (5%) inoculum size reached 48.60% for *A. pullulans* ATCC 12535 and at 10% inoculum size reached 66.70% for *A. pullulans* ATCC 42023. Pullulan produced has greenish colour, soluble in water at 25°C, the pH for solution soluble in water (from 5-7), maximum ash 2% and melting point over 300°C. IR was studied for pullulan.

Keywords: production, *Aureobasidium pullulans*, pullulan, molasses, biopolymer.

INTRODUCTION

Pullulan is the generic name given to water-soluble homopolysaccharide that is produced extracellularly by the polymorphic micromycete *A. pullulans*. It is a predominantly linear α-D-glucan, made mainly of maltotriose repeating units interconnected by α-1,6 linkages. The

regular alternation of α-1,4 and α-1,6 bonds results in two distinctive properties of structural flexibility and enhanced solubility (Singh *et al.*, 2008).

The physical properties of this biopolymer make it suitable for a wide range of applications in food, agricultural, pharmaceutical and chemical industries. Pullulan produces a high viscosity solution at a relatively low concentration and can be used for oxygen-impermeable films and fibers, thickening or extending agents or adhesives or encapsulating agents. Despite being a α-D-glucan, pullulan is resistant to α-D-amylolysis and may be used in low-calorie food formulations (Leathers (2003) and Singh *et al.* (2008)).

In addition to a wide diversity in pullulan production observed among different strains of *A. pullulans*, important environmental parameters for polysaccharide synthesis are temperature, culture pH, oxygen supply, nitrogen and carbon source. 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 nitrogen supply becomes a limiting factor. This suggests that fermentation of a shorter duration may be established if the biomass is rapidly built up at the beginning of the fermentation (Roukas and Liakopoulou-Kyriakides, 1999, Lee *et al.*, 2001, Chi and Zhao, 2003 and El-Tayeb *et al.*, 2005).

Considerable attention has been paid to the microbiology of the pullulan-producing organisms; production of pullulan with respect to the nutrient sources, fermentation conditions and process technology for commercial manufacture of pullulan. The reason for this investigation was the paucity of knowledge available concerning the producer fungus, factors governing pullulan production and the properties of pullulan.

MATERIALS AND METHODS

Microorganism

A. pullulans ATCC 16628, *A. pullulans* ATCC 12535 and *A. pullulans* ATCC 42023 were obtained from Microbiol. Resources Center (Cairo MIRCEN), Fac. of Agric., Ain Shams Univ. Cairo, Egypt. The used culture strains were subcultured on agar slants at 28°C, maintained at 4°C and transferred 3 weeks.

Morphological observation

Slide cultures were made using PDA, which were stained with lactophenol-cotton blue and observed by wet mounting using bright field microscopy. The *Aureobasidium* strains were compared with the descriptions of *Aureobasidium* by Hermanides-Nijhof (1977), Domsch *et al.* (1993) and Barnett and Barry (1998).

Media

A. pullulans ATCC 16628 and *A. pullulans* ATCC 12535 were maintained on Emmons' modification of Sabouraud's agar (ATCC medium: 28 Emmons' modification of Sabouraud's agar Sabouraud Dextrose Broth (BD 238220), 30.0 g, agar, 20.0 g, distilled water, 1.0 L, adjust pH to 6.8-7.0. Autoclave at 121°C for 15 min) and *A. pullulans* ATCC 42023 was maintained on malt extract agar (Blakeslee's formula) (ATCC medium: 325 malt extract agar (Blakeslee's formula), malt extract, 20.0 g, glucose, 20.0 g, peptone, 1.0

g, agar, 20.0 g, distilled water, 1.0 L, add glucose prior to sterilization. Autoclave at 121°C for 15 min.). The microorganism was maintained at 4°C and subcultured every 3 weeks.

Göksungur *et al.* (2004) medium was used for inoculum preparation. The medium has the following composition (g/l): sucrose 30.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4.7H_2O$ 0.2 and NaCl 1.0. The pH of the medium was adjusted at pH 5.5. Göksungur *et al.* (2004) medium was used for pullulan production after replacement of its carbon or nitrogen source with a certain sources. The medium has the following composition (g/l): sucrose 50.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4.7H_2O$ 0.2 and NaCl 1.0. The pH of the medium was adjusted at pH 5.5.

Preparation of Standard Inoculum

Cells for inoculation of the culture medium were obtained from cultures grown on agar slants at 28°C for 48 h. Two loops of *A. pullulans* cells were transferred to 250 ml conical flasks containing 50 ml of culture medium (pH 5.5). The flasks were incubated at 28°C for 48 h in a rotary shaker incubator (Lab-Line Incubator-Shaker) at 200 rpm. These cultures were used to inoculate the production medium at a level of 5% (v/v), (Göksungur *et al.*, 2004).

Fermentation

The production medium used for shake flask experiments had the following composition (g/l): sucrose 50.0, $(NH_4)_2SO_4$ 0.6, yeast extract 0.4, K_2HPO_4 5.0, $MgSO_4.7H_2O$ 0.2 and NaCl 1.0 (pH 5.5). The medium was sterilized at 121°C for 15 min. When molasses were used for fermentation experiments, the molasses solution was diluted to contain 50 g/l of total sugars and pH was adjusted to 5.5 with 10 N NaOH. Following sterilization at 121°C for 15 min, the molasses were used for the production of pullulan. The shake flask experiments were conducted in a temperature controlled shaker (Lab-Line Incubator-Shaker) operated at 200 rpm, 28°C. The shake flasks were 250 ml Erlenmayer flasks containing 50 ml of either molasses or synthetic medium as the production medium.

Pretreatment of molasses

Sugar beet molasses (65% total sugars) was obtained from the Sugar beet Factory (Kalabsho, Belkas, Dakhlia Governorate). Sugar cane molasses (50% total sugars) was obtained from the Upper Egypt Sugar Cane Factory (Naga Hammadi, Qena Governorate). Molasses was diluted with distilled water to obtain 7% (w/v) total sugar. The molasses solution was used for H_2SO_4, K ferrocyanide, EDTA and ammonium oxalate pretreatment methods (Mashhoor *et al.*, 1987 and Roukas, 1998).

Separation and Purification of Pullulan

The fermentation broth was centrifuged at 4000 xg for 20 min to remove the cells of the microorganism. The crude polysaccharide was initially precipitated in the culture supernatant with two volumes 95% ethanol at 4°C for 1 h (Göksungur *et al.*, 2004).

Pullulan concentration

The first supernatant from biomass dry weight determination was combined with the washings, and the crude polysaccharide was precipitated

with 2 volumes ethanol at 4°C for 1h. The precipitate was filtered through a preweighed Whatman No 1 filter and dried at 80°C overnight and was then weighd. Pullulan yield (%) = pullulan (g)/original sugar (g) x 100. Conversion coefficient (%) = pullulan (g)/consumed sugar (g) x 100.

Total biomass

Total biomass (mycelial and yeast cells) dry weights were determined by centrifugation of the broth at 4000xg for 20 min, washing (2x) the sediment with distilled water, and drying at 105°C overnight.

Total sugars

Total sugars were determined in the fermented liquor according to the phenol sulfuric acid method using glucose as the standard (Dubois *et al.*, 1956).

pH

The final pH of culture media employed for pullulan production was measured with Jenway 3020 pH digital meter.

Ash content:

Ash content was determined using the method of Herbert *et al.* (1971).

Melting point

Melting point was measured using Fisher-Johns melting point apparatus.

Infra-red (IR) spectroscopic analysis

The polymer was confirmed by infra-red (IR) (MATTISON 5000 FTIR spectrometer) in Mansoura. Univ., Fac. of Sci., Chem. Dept., Spectral Analysis Unit. The IR spectra of the pullulan were measured using KBr disk on a MATTISON 5000 FTIR spectrometer. Calibration of the frequency reading was made with a polystyrene film at Mansoura Uni., Fac. of Sci., Chem. Dept., Spectral Analysis Unit.

RESULTS AND DISCUSSION

Time course of pullulan production

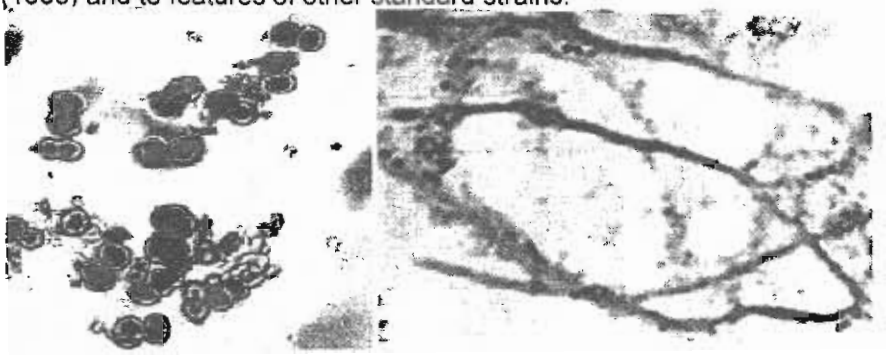
The time course of a typical fermentation of *A. pullulans* ATCC 16628 shows the relationships among biomass production, pullulan production and pH change (Table 1). Throughout the fermentation, the pH decreased. In addition to metabolic products, synthesis of acidic polysaccharides could contribute to increased acidity of the fermentation broth. Maximum depletion of the sugar substrate occurs after about 3-4 days and a maximum pullulan concentration, at about the same period. The decreasing pullulan concentration appears to be associated with increasing biomass concentration. This suggests the pullulan is being hydrolyzed and utilized by the fungus after the substrate is consumed. For strain *A. pullulans* ATCC 12535, maximum pullulan production occurred at day 6. The pH of the fermentation broth fell from 5.50 to only 2.88 within the first 24 hours and remained near 2.8 through day 7. Cell morphology was essentially mycelial from day 1 onwards. Pigment formation began on day 3 and turned the fermentation broth completely dark by day 5. Strain *A. pullulans* ATCC 42023 was the strain with the higher yield, as well as the one that produced the less biomass and had pigment formation.

Table 1: Effect of time course on pullulan production by *A. pullulans*.

Strain	Culture	Time (day)	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Con-version coefficient%	Pullulan yield%
A. pullulans ATCC16628	Static culture	1	4.73	0.31	0.71	3.43	1.42
		2	4.21	1.05	1.33	6.19	2.66
		3	3.96	1.95	1.41	6.40	2.82
		4	3.81	2.01	1.51	6.83	3.02
		5	3.80	2.08	1.25	5.68	2.50
		6	3.85	2.10	0.99	4.51	1.97
		7	3.83	1.85	0.87	4.02	1.74
	Shaked culture	1	3.36	0.51	0.81	3.86	1.61
		2	3.00	1.59	2.28	10.19	4.55
		3	2.87	1.60	2.98	13.12	5.96
		4	2.92	2.04	2.73	11.98	5.46
		5	2.93	2.15	1.86	8.30	3.72
		6	2.85	1.96	1.65	7.47	3.30
		7	2.88	1.94	0.74	3.40	1.47
A. pullulans ATCC 12535	Static culture	1	4.50	0.57	0.66	3.15	1.31
		2	4.38	0.63	0.93	4.40	1.85
		3	4.35	0.65	1.40	6.59	2.80
		4	4.03	1.09	1.47	6.80	2.93
		5	3.62	1.50	1.60	7.32	3.20
		6	3.54	2.21	1.03	4.67	2.05
		7	2.19	1.05	0.53	2.52	1.06
	Shaked culture	1	2.88	2.78	1.56	6.89	3.11
		2	2.86	4.99	6.78	25.26	13.55
		3	2.89	6.88	6.83	24.41	13.66
		4	2.84	7.23	8.76	29.90	17.52
		5	2.82	7.82	9.33	31.16	18.65
		6	2.80	8.82	10.40	33.37	20.80
		7	2.79	8.99	10.24	32.78	20.48
A. pullulans ATCC 42023	Static culture	1	4.11	1.27	1.32	6.10	2.63
		2	3.69	1.54	1.81	8.20	3.61
		3	3.44	1.98	1.90	8.49	3.79
		4	3.22	2.02	3.45	14.84	6.90
		5	3.09	2.07	3.83	16.28	7.65
		6	2.77	2.96	3.63	15.18	7.25
		7	2.94	2.19	2.73	11.92	5.45
	Shaked culture	1	2.82	3.16.	6.95	26.84	13.90
		2	2.81	4.63	14.81	47.41	29.62
		3	2.81	4.98	17.09	52.10	34.17
		4	2.77	5.06	17.93	53.79	35.86
		5	2.76	5.27	18.22	54.28	36.44
		6	2.69	5.74	20.44	58.20	40.88
		7	2.76	4.82	16.13	50.24	32.25

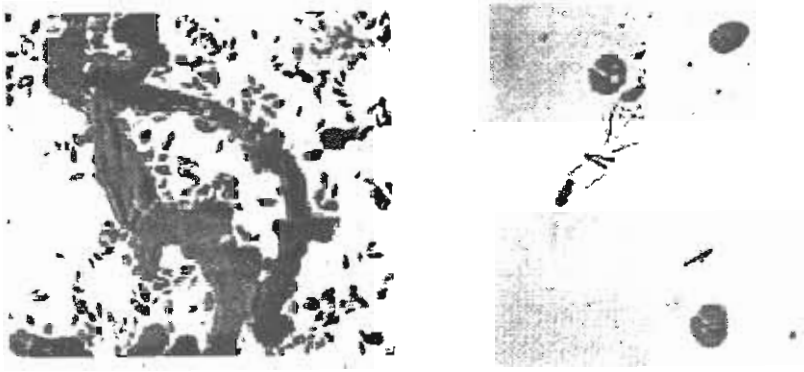
As can be seen, maximum pullulan production occurred also at 6 days. The pH of the fermentation broth fell from 5.50 to 2.82 within the first 24 hours and stayed near 2.75 for the remainder of the fermentation. Yeast-like cells were the dominant morphology up to day 3, when mycelial growth and chlamydospores began to appear. The obtained results are in agreement with those obtained by Kaplan *et al.* (1987), Leathers *et al.* (1988) and Simon *et. al.* (1995). Examination of the cell morphology of the most active strain (ATCC 42023) by bright field light microscopy showed the

classic *A. pullulans* polymorphology with blastospores, yeast like cells, hyphae, and chlamydospores (Fig. 1). Both morphological and colony characteristics corresponded well with the *A. pullulans* descriptions by Hermanides-Nijhof (1977), Domsch *et al.* (1993) and Barnett and Barry (1998) and to features of other standard strains.



Yeast like cells hyphae

Conidial apparatus



Mycelium chlamydospores

Fig. 1. Morphology of *Aureobasidium pullulans* ATCC 42023

Carbon sources

The kind of carbon sources affected greatly the amount of pullulan produced by the used strains (Table 2). Sucrose promoted the highest amount of pullulan from *A. pullulans* ATCC 12535. However, sucrose have been reported to be the better carbon sources for pullulan production

(Schuster *et al.*, 1993 and Seo *et al.*, 2006). Also, it could be noticed that the high pullulan production was accompanied with a low pH value being 2.80. Using glucose syrup (5% sugars) as a sole carbon source promoted the highest pulluluan production from *A. pullulans* ATCC 42023. Such increase in pullulan formation may be attributed to the nature of glucose syrup. Glucose syrup is a refined, concentrated aqueous solution of D(+)–glucose, maltose and other sugars of D–glucose obtained by the controlled partial hydrolysis of edible starch. Glucose syrup has unique sugar content, that it contains monosaccharides, disaccharides, trisaccharides, tetrasaccharides, pentasaccharides, hexasaccharides and higher sugars (Jackson, 1995). Finally, the results in Table 2 led us to suggest that molasses treatment may enhance pullulan production. Thus, the subsequent experiment will deal with using pretreated molasses as carbon source.

Treatment of molasses

In this experiment the carbon source was replaced by molasses, for comparing each of them with other and with previously investigated carbon sources. Fermentation media containing 50 g/l of initial sugar were prepared from pretreated molasses and synthetic medium and the kinetics of biomass growth and pullulan production in shake flasks at 28°C were determined. The kinetics of the production of pullulan by *A. pullulans* are given in Table 3. The highest pullulan concentration (14.83 g/l) produced by *A. pullulans* ATCC 12535 was obtained in beet molasses treated with EDTA, followed by sugar cane molasses (14.40 g/l) treated with the same substance. Molasses treated with potassium ferrocyanide gave also high concentrations of pullulan. The highest biomass was obtained in potassium ferrocyanide treated molasses medium at the 6 days of fermentation. In sugar cane molasses medium, the pH of the fermentation medium decreased during the 6 days of fermentation from an initial value of 5.5 to 5.0 in ammonium oxalate treatment and increased to 6.31-6.38 in other treatments.

Table 2: Effect of carbon sources on pullulan production by *A. pullulans*.

Strain	Carbon source	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Con-version coefficient %	Pullulan yield %
A. pullulans ATCC 12535	Sucrose	2.80	2.82	10.40	33.37	20.80
	Starch	2.75	13.99	4.20	13.74	8.40
	Lactose	3.00	5.05	2.05	8.47	4.10
	Fructose	2.85	8.66	6.04	21.16	12.08
	Glucose	2.80	8.89	3.75	13.71	7.51
	Sugar cane molasses	6.24	12.69	7.32	23.21	14.65
	Sugar beet molasses	5.56	12.89	9.15	27.90	18.30
	Glucose syrup	2.81	12.37	2.88	9.97	5.75
A. pullulans ATCC 42023	Sucrose	2.69	5.74	20.44	58.20	40.88
	Starch	2.78	10.69	15.61	44.56	31.23
	Lactose	2.81	6.54	3.10	12.13	6.21
	Fructose	2.97	5.96	16.91	50.84	33.82
	Glucose	2.72	7.67	11.37	36.68	22.75
	Sugar cane molasses	6.29	9.78	12.95	39.02	25.90
	Sugar beet molasses	5.46	9.33	15.04	44.09	30.08
	Glucose syrup	2.72	7.10	24.18	63.51	48.35

Table 3: Effect of treated molasses on pullulan production by *A. pullulans*.

Strain	Molasses	Inorganic nitrogen source	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Conversion coeffic-ient%	Pullulan yield%
A. pullulans ATCC 12535	Sugar cane molasses	Untreated	6.24	12.69	7.32	23.21	14.65
		H_2SO_4	6.31	12.85	12.03	34.94	24.05
		K ferrocyanide	6.38	15.46	13.39	36.50	26.77
		EDTA	6.35	15.12	14.40	38.86	28.80
		Amm. Oxalate	5.00	12.13	3.47	11.94	6.94
	Sugar beet molasses	Untreated	5.56	12.89	9.15	27.90	18.30
		H_2SO_4	5.68	15.52	7.58	22.73	15.15
		K ferrocyanide	5.70	16.99	12.68	34.14	25.35
		EDTA	5.68	15.06	14.83	39.76	29.65
		Amm. Oxalate	3.88	16.45	2.89	9.08	5.77
A. pullulans ATCC 42023	Sugar beet molasses	Untreated	6.29	9.78	12.95	39.02	25.90
		H_2SO_4	6.54	10.92	14.34	41.46	28.67
		K ferrocyanide	6.61	13.38	15.88	43.04	31.76
		EDTA	6.52	13.01	14.84	41.07	29.67
		Amm. Oxalate	5.78	9.22	6.82	23.27	13.64
	Sugar cane molasses	Untreated	5.46	9.33	15.04	44.09	30.08
		H_2SO_4	5.97	10.93	14.90	43.19	29.80
		K ferrocyanide	5.97	11.71	18.65	49.71	37.30
		EDTA	6.00	12.57	17.84	47.45	35.68
		Amm. Oxalate	5.00	11.92	10.28	31.26	20.55

In sugar beet molasses medium pH decreased from 5.5 to 3.88 during the fermentation in ammonium oxalate treated molasses and increased from 5.5 to 5.68-5.70 in other treatments. Synthesis of organic acid could be attributed to the increase in acidity of the fermentation broth, whereas the increase in the pH of molasses medium could be due to the deamination of amino-acids in molasses by *A. pullulans* and the production of ammonia, which increased the pH of the fermentation medium. The complex ingredients and composition of molasses enhanced the growth of *A. pullulans* since higher biomass yields were observed in molasses medium than in synthetic medium. Seviour *et al.* 1992 and West and Reed-Hammer 1993 also stated that good biomass production does not always give high polysaccharide yields.

The superiority of the treatment of EDTA over other treatments of molasses regarding pullulan production may be due to the significant removal of heavy metals as well as color substances from molasses. These compounds found in high concentrations in crude molasses are generally considered fermentation inhibitors, limiting the utilization of molasses as substrate in industrial fermentations (Lazaridou *et al.*, 2002). Roukas (1998) studied the effect of different pretreatments of molasses supplemented with K_2HPO_4, L-glutamic acid, olive oil and Tween 80 on the production of pullulan. That study obtained the maximum polysaccharide concentration (32.0 g/l), biomass dry weight (33.8 g/l), polysaccharide yield (63.5%) and sugar utilization (97.5%) in sulfuric acid treated molasses by *A. pullulans*. Lazaridou *et al.* (2002) found the highest pullulan concentration (24 g/l) and biomass dry weight (14 g/l) by *A. pullulans* in molasses pretreated with

sulfuric acid and activated carbon. LeDuy and Boa (1983) used peat hydrolyzate as the substrate for the production of pullulan and obtained 14 g/l of maximum polysaccharide with various strains of *A. pullulans*. The reasons for such variability in the literature are the strain of microorganism, the chemical composition of the substrate, the pretreatment method and the conditions employed during fermentation. In general, the results of this study showed that the pretreatment of molasses with EDTA significantly improved the pullulan production. Hence sugar beet molasses medium pretreated with EDTA and synthetic medium, which gave the highest amount of pullulan, were used for further experiments.

Nitrogen sources

The $(NH_4)_2SO_4$ and yeast extract was replaced by other nitrogen sources, for comparing each of them with other sources. These nitrogen sources were added to the basal medium, in amounts calculated to give equal amounts of final nitrogen (on "N" basis) irrespective of the chemical concentration. Relying upon the conclusion yielded from testing carbon compounds and molasses, the carbon source applied was EDTA treated sugar beet molasses for *A. pullulans* ATTC 12535 and glucose surup for *A. pullulans* ATTC 42023. Accordingly different nutritive carbon and nitrogen sources and experimental conditions were undertaken. The nitrogen sources greatly affected the production of pullulan (Table 4).

Urea + yeast extract allowed the highest productivity of pullulan polymer with *A. pullulans* ATTC 12535. On the other hand yeast extract alone allowed the highest production of pullulan with the strain *A. pullulans* ATTC 42023. This strain proved by far, as being the most active pullulan producer. $(NH_4)_2HPO_4$ + yeast extract and $(NH_4)_2SO_4$ + malt extract and $(NH_4)_2SO_4$ + proteose peptone appeared to be not favorable nitrogen source for elaboration of the extracellular polysaccharide pullulan. Conversion coefficient% and yield of pullulan% reached to 58.24% and 40.72% in *A. pullulans* ATCC 12535 media containing urea and 72.91% and 62.85%, in *A. pullulans* ATCC 42023 media containing yeast extract alone as nitrogen source, respectively. Park (1982) characterized a different strain in which mycelial germination is supported by a number of amino acids. The bioreactor design and nitrogen source in fermentation medium affect pullulan formation by *A. pullulans* strain QM 3092, but not its morphology (Gibbs and Seviour, 1992). *A. pullulans* strain ATCC 42023 (ppKM-3) revealed that ammonium and complex nitrogen sources are superior to nitrate for pullulan production (West and Reed-Hamer, 1991 and Reed-Hamer and West, 1994). The production of pullulan by *A. pullulans* HP-2001 was enhanced by yeast extract as a nitrogen source (Seo *et al.*, 2004).

Phosphate requirement

The addition of $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$, $NaH_2PO_4.2H_2O$ and Na_2HPO_4 to the production medium did not improve polysaccharide production for *A. pullulans* ATCC 12535 (Table 5).

However, such phosphorus sources improved some growth in comparison with control. The strain grew well (11.14 g biomass dry weight/l), but produced 17.36 g pullulan /l of production medium supplemented with $NaH_2PO_4.2H_2O$. The addition of KH_2PO_4 pronounced with 21.94 g pullulan/L

and 8.13 g biomass dry weight/l. The maximum pullulan concentration, conversion coefficient and pullulan yield was obtained in medium containing $Na_3PO_4.12H_2O$. The change in pH value ranged between 4.20 to 5.78.

The addition of KH_2PO_4, $(NH_4)H_2PO_4$, $(NH_4)_2HPO_4$, $NaH_2PO_4.2H_2O$ and Na_3PO_4 $12H_2O$ to the culture medium employed for pullulan production did not improve pullulan formation by *A. pullulans* ATCC 42023 (Table 5). However, the effect of such phosphorus sources on growth varied to some extent in comparison with control.

Table 4: Effect of nitrogen sources on pullulan production by *A. pullulans*.

Strain	Nitrogen source	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Con-version coefficient %	Pullulan yield%
	Inorganic source + yeast extract					
A. pullulans ATCC 12535	$(NH_4)_2SO_4$	5.68	15.06	14.83	39.76	29.65
	NH_4NO_3	6.53	16.29	16.00	41.27	31.99
	$NaNO_3$	6.61	15.11	15.35	40.83	30.70
	$Ca(NO_3)_2$	6.65	15.74	13.67	36.96	27.33
	KNO_3	6.77	14.87	14.80	39.86	29.60
	$C_6H_{14}N_2O_7$	6.40	14.98	17.20	44.19	34.39
	$(NH_4)_2HPO_4$	5.05	14.95	15.45	41.08	30.89
	$Co(NH_2)_2$	5.78	6.95	20.36	58.24	40.72
	$(NH_4)_2SO_4$ alone	4.54	17.83	15.07	38.57	30.14
A. pullulans ATCC 42023	$(NH_4)_2SO_4$	2.72	7.10	24.18	6.51	48.35
	NH_4NO_3	5.67	5.76	25.63	67.19	51.25
	$NaNO_3$	6.24	5.40	15.96	49.36	31.91
	$Ca(NO_3)_2$	5.30	9.96	22.43	57.51	44.85
	KNO_3	6.25	8.87	20.08	54.63	40.15
	$C_6H_{14}N_2O_7$	4.37	22.87	27.65	56.26	55.30
	$(NH_4)_2HPO_4$	2.78	7.79	8.68	29.37	17.35
	$Co(NH_2)_2$	6.42	9.23	15.49	45.19	30.97
	$(NH_4)_2SO_4$ alone	2.58	10.54	19.72	52.55	39.43
	$(NH_4)_2SO_4$ + Organic source					
A. pullulans ATCC 12535	Meat extract	5.11	16.00	14.60	38.75	29.20
	Malt extract	4.99	17.55	15.88	40.48	31.76
	Peptone	5.21	16.30	15.57	40.49	31.14
	Proteose peptone	5.03	15.98	14.20	37.91	28.39
	Tryptone	5.10	16.48	15.49	40.27	30.97
	Corn steep liquor	5.29	16.31	15.85	41.06	31.70
	Yeast extract	5.68	15.06	14.83	39.76	29.65
	Yeast extract alone	6.24	15.11	15.96	42.02	31.92
A. pullulans ATCC 42023	Meat extract	2.74	11.34	21.90	55.82	43.79
	Malt extract	2.77	15.15	4.10	13.16	8.20
	Peptone	2.77	10.11	15.83	45.16	31.65
	Proteose peptone	2.74	11.71	9.27	28.80	18.53
	Tryptone	2.76	10.80	17.25	47.65	34.20
	Corn steep liquor	2.77	8.54	13.71	41.71	27.42
	Yeast extract	2.72	7.10	24.18	63.51	48.35
	Yeast extract alone	4.27	8.54	31.43	72.91	62.85

Table 5: Effect of phosphorus sources on pullulan production by *A. pullulans*.

Strain	Phosphorus source	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Conversion coefficient %	Pullulan yield%
A. pullulans ATCC 12535	K_2HPO_4	5.78	6.95	20.36	58.24	40.72
	KH_2PO_4	4.76	8.13	21.94	58.71	43.87
	$(NH_4)H_2PO_4$	4.77	7.78	17.69	50.92	35.37
	$(NH_4)_2HPO_4$	4.20	7.87	14.79	44.67	29.57
	$NaH_2PO_4.2H_2O$	4.76	11.14	17.36	47.55	34.72
	Na_2HPO_4	4.93	9.01	16.01	46.41	32.02
	$Na_3PO_4.12H_2O$	5.74	11.84	24.30	59.48	48.60
A. pullulans ATCC 42023	K_2HPO_4	4.27	8.54	31.43	72.91	62.85
	KH_2PO_4	4.25	7.24	31.21	73.87	62.42
	$(NH_4)H_2PO_4$	2.22	11.77	20.39	52.80	40.77
	$(NH_4)_2HPO_4$	2.01	10.64	21.63	55.90	43.25
	$NaH_2PO_4.2H_2O$	4.25	7.60	26.19	66.23	52.37
	Na_2HPO_4	4.28	9.98	32.84	73.41	65.67
	$Na_3PO_4.12H_2O$	4.25	5.83	28.16	71.09	56.32

The strain grew well (11.77 g biomass dry weight/l), but produced 20.39 g of pullulan/l in production medium supplemented with $(NH_4)H_2PO_4$. The addition of Na_2HPO_4 led to the maximum pullulan concentration (32.84 g pullulan/l) and yield (65.67%). The change in pH ranged between 2.01 to 4.28. Sugimoto (1978) and Tsujisaka and Mitsuhashi (1993) reported that low initial phosphate and pH levels have been reported to favor the formation of high-molecular-weight pullulan. $Na_3PO_4.12H_2O$ and Na_2HPO_4 as the best phosphorus sources may confirm their previous findings.

Initial culture pH

The pullulan concentrations increased when the initial pH of the culture medium of *A. pullulans* ATCC 12535 increased from 2.5 to 5.5 and decreased thereafter (Table 6). However, the biomass dry weight increased with each unit increase of pH till pH 6.5 then decreased. The highest pullulan concentration (24.3 g/l) was obtained in cultures grown at an initial pH of 5.5. The highest biomass dry weight (12.97 g/l) was obtained at an initial pH of 6.5. Conversion coefficient (%) and pullulan yield % at pH 5.5 reached 59.48% and 48.60%, respectively.

The pullulan concentrations also increased when the initial pH of the culture medium of *A. pullulans* ATCC 42023 increased from 2.5 to 5.5 and decreased thereafter. However, the biomass dry weight increased with each unit increase of pH till pH 4.5. The highest pullulan concentration (32.84 g/l) were obtained in cultures grown at an initial pH of 5.5. The highest biomass dry weight (11.17 g/l) was obtained at an initial pH of 4.5. Conversion coefficient (%) and pullulan yield % at pH 5.5 reached 73.41% and 65.67%, respectively. Thus, the pH of the growth medium employed for pullulan production is an important factor in pullulan accumulation by the fungus *A. pullulans*, since it affects the pullulan production due to its influence in turn on morphology of this microfungus. Ono *et al.* (1977) used synthetic medium and determined optimum pullulan production at a pH of 6.0. Lacroix *et al.* (1985) reported an optimal pH of 5.5 for pullulan production by *A. pullulans*

grown in a chemically defined medium. The variation in optimum pH is possibly due to the different strains of *A. pullulans* employed as well as the substrate composition (*e.g.*, amount and type of nitrogen source) and the various fermentation systems used. At very low initial pH values (pH 2.0) in sucrose based synthetic medium the pullulan production by 2 strains of *A. pullulans* was very low. At higher initial pH values (pH 5.5) the maximum pullulan concentrations were obtained, and that contrary to polysaccharide production *A. pullulans* grew best at a very low initial pH of 2.0. Outside pH 5.5, the pullulan formation as regards the two test strains, was repressed. Extremes of both acidity and alkalinity adversely affected both the pullulan and biomass production. Heald and Kristiansen (1985) showed that the more acidic the environment, the lower the accumulation of pullulan. Auer and Seviour (1990) observed maximum pullulan concentration at an initial pH of 7.5. Roukas (1999) studied pullulan production from deproteinized whey and determined the maximum polysaccharide concentration at an initial pH of 6.5.

Table 6: Effect of initial culture .pH on pullulan production by *A. pullulans*.

Strain	Initial pH	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Con-version coefficient %	Pullulan yield%
A. pullulans ATCC 12535	2.5	2.47	3.27	20.06	59.37	40.11
	3.5	3.82	8.61	21.29	57.08	42.57
	4.5	5.72	11.81	22.57	56.63	45.13
	5.5	5.74	11.84	24.30	59.48	48.60
	6.5	6.34	12.97	23.07	55.93	46.13
	7.5	6.90	10.89	22.98	58.11	45.95
	8.5	7.10	8.26	20.46	55.90	40.91
	9.5	7.12	6.44	19.81	58.12	39.62
A. pullulans ATCC 42023	2.5	2.31	7.65	22.80	60.63	45.59
	3.5	3.04	10.87	30.00	68.85	60.00
	4.5	3.74	11.17	30.86	69.72	61.72
	5.5	4.28	9.98	32.84	73.41	65.67
	6.5	5.11	7.58	27.07	67.66	54.13
	7.5	6.17	6.96	23.73	62.86	47.46
	8.5	6.49	6.86	23.19	62.06	46.38
	9.5	6.68	6.48	21.38	59.18	42.76

Inoculum size

In *A. pullulans* ATCC 12535, maximum pullulan concentration (24.65 g/l), and total biomass dry weight (11.84 g/l) were obtained with inoculum of 5.0% (v/v) (Table 7). At this level of inoculum, the pH of the culture medium was 5.74. Conversion coefficient (%) and pullulan yield % at 5% inoculum size reached 61.39% and 49.30%, respectively, for *A. pullulans* ATCC 12535. The 5.0% inoculum was used in subsequent experiment, since maximum polysaccharide concentration was obtained under these conditions. Low inoculum densities appear to favor mycelial development in some strains of *A. pullulans* and hence pullulan production (Ramos and Garcia Acha, 1975

and Park, 1984). In *A. pullulans* ATCC 42023, maximum pullulan concentration (33.35 g/l), and total biomass dry weight (10.48 g/l) were obtained with inoculum of 10.0% (v/v), Table 7. At this level of inoculum, the pH of the culture medium was 3.89. Conversion coefficient% and pullulan yield % at 10% inoculum size reached 73.67% and 66.70%, respectively, for *A. pullulans* ATCC 42023.The 10.0% inoculum was used in subsequent experiment, since maximum polysaccharide concentration was obtained under these conditions. High inoculum densities seem to favor mycelial growth in other strains of *A. pullulans* and hence pullulan production (Catley, 1980; Vinroot and Torzilli, 1988).

Table 7: Effect of inoculum size on pullulan production by *A. pullulans*.

Strain	Inoculum Size %	Final culture pH	Mycelium DW (g/l)	Pullulan Conc. (g/l)	Con-version coefficient %	Pullulan yield%
A. pullulans ATCC 12535	2.5	5.67	10.27	24.01	58.51	48.02
	5.0	5.74	11.84	24.30	59.48	48.60
	7.5	4.97	12.45	23.29	57.32	46.57
	10.0	4.92	12.55	23.16	57.02	46.32
A. pullulans ATCC 42023	2.5	4.26	7.96	31.68	73.90	63.36
	5.0	4.28	9.98	32.84	73.41	65.67
	7.5	3.91	10.08	23.86	73.34	65.72
	10.0	3.89	10.48	33.35	73.67	66.70

Pullulan Properties

Fig. 2 shows the IR spectrum of the pullulan produced by *A. pullulans* ATCC 42023. The spectrum demonstrated that at 2927, 1636, 1380, 1730, 1154 and 1022 δ, respectively, for methylene, alkin, methyl, aldehide, ethers and alcohol. The chains have the CH_3 groups responsible for the good mechanical properties of the pullulan. The presence of ether-linkage gives the polymer elasticity and flexibility. Some properties of pullulan produced by *A. pullulans* ATCC 42023 are presented in Table 8. The results are in line with (Leathers, 2002, 2003; Shingel, 2004 and Singh *et al.* 2008).

Table 8: Some properties of pullulan produced by *A. pullulans* ATCC 42023

Appearance	Greenish powder
Water solubility (25C)	easy soluble
pH Sol. 1%	5-7
Mineral residue ash (sulphated), %	max. 2
Melting point	Over 300

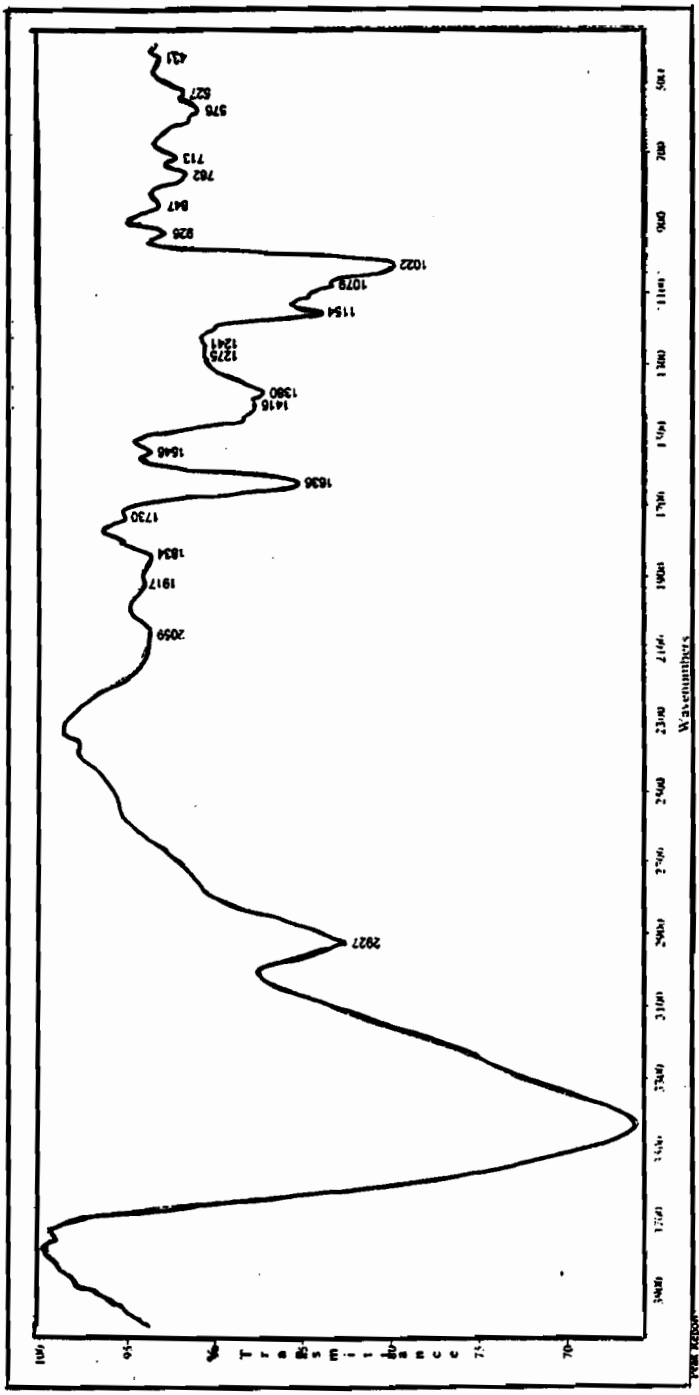


Fig. 2. IR spectra of the polymer pullulan produced from *A. pullulans* ATCC 42023.

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إنتاج بوليمر البوليولان بواسطة فطر ايروبازيديم بوليولانس

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استهدف البحث مقارنة ثلاثة سلالات فطرية هى *A. pullulans* ATCC 16628 و *A. pullulans* ATCC 12535 و *A. pullulans* ATCC 42023 لإنتاج البوليولان فى مزارع ساكنة وأخرى مهتزة . وقد أظهرت النتائج أن السلالات الثلاث تفاوتت فى إنتاج البوليمر الحيوى ، وقد أعطت المزارع المهتزة إنتاج أعلى عن مثيلتها الساكنة بمقدار من ٢-> ٥ أضعاف . وقد سجلت السلالة *A. pullulans* ATCC 42023 أعلى إنتاج ، يليها *A. pullulans* ATCC 12535 ، فى حين سجلت السلالة *A. pullulans* ATCC 16628 أقل إنتاج على الرغم من تميزها بخلو البوليولان المنتج من صبغة الميلانين .

تميز إنتاج البوليولان بظهور ذروة واحدة تطابقت مع فترة الثلاثة أيام حضانة للمزرعة المهتزة *A. pullulans* ATCC 16628 ، فى حين تطابقت مع فترة الستة أيام للسلالتين *A. pullulans* ATCC 12535 و *A. pullulans* ATCC 42023 .

لوحظ عدم وجود ارتباط بين إنتاج البوليمر وتكوين الميسيليوم فى السلالات المختبرة ، وقد تميزت مزارع إنتاج البوليولان بمدى من التركيز الأيونى الأيدروجينى فى بيئات الزرع من ٢,٦٩- ٤,٧٣ .

تأثر إنتاج البوليولان بنوع مصدر الكربون فى بيئة الإنتاج ، ولقد أدت بعض المصادر إلى حث إنتاج البوليولان بصورة واضحة (من ٢,٠٥ إلى ٢٤,١٨ جم / لتر) .

لوحظ انخفاض pH مزارع إنتاج البوليولان إلى المدى ٢,٦٩ – ٣,٠٠ ، وذلك باستثناء المزارع المحتوية على المولاس والتى كان مدى التركيز الأيونى للأيدروجين بين درجتى ٥,٤٦ – ٦,٢٩ . كما لوحظ عدم وجود علاقة بين زيادة النمو وإنتاج بوليمر البوليولان .

تأثر إنتاج البوليولان فى بيئة الإنتاج بنوع المولاس المستخدم وطريقة معاملته ، وقد أعطى الزرع على مولاس بنجر السكر المعامل بمركب الـ EDTA أعلى إنتاج من البوليولان (١٤,٨٣ جم / لتر) من السلالة *A. pullulans* ATCC 12535 ، فى حين لم تؤدى معاملات الزرع على المولاس المعامل الأخرى إلى زيادة كبيرة ، أما بالنسبة للسلالة *A. pullulans* ATCC 42023 فلم تصل الإنتاجية على المولاس فى جميع الأحوال إلى مثيلتها على شراب الجلوكوز . ولم يؤدى زرع السلالات المستخدمة على أنواع المولاس إلى تغير ملحوظ فى التركيز الأيونى للأيدروجين . وبصفة عامة أدى الزرع على المولاس إلى زيادة ملحوظة فى نمو سلالات الفطر المنتجة لبوليمر البوليولان .

أعطت اليوريا+ مستخلص الخميرة أعلى إنتاج من البوليولان مع السلالة *A. pullulans* ATCC 12535 ، بينما أعطى مستخلص الخميرة أعلى إنتاج للبوليولان فى السلالة *A. pullulans* ATCC 42023 .

أعطت الفوسفات ثلاثية الصوديوم أعلى إنتاج للبوليولان مع السلالة *A. pullulans* ATCC 12535 ، بينما أعطت فوسفات الصوديوم ثنائية القاعدية أعلى إنتاج للبوليولان فى السلالة *A. pullulans* ATCC 42023 ، وقد كانت غالبية مصادر الفوسفور المستخدمة غير ملائمة لإنتاج البوليون بكمية كبيرة .

تطابق أمثل إنتاج للبوليولان فى كلا السلالتين عند درجة pH ٥,٥ ، وقد تناقص إنتاج البوليولان خارج هذه الدرجة من التركيز الأيونى للأيدروجين بالنسبة للسلالتين موضع الاختبار ، وقد أثرت التركيزات المتطرفة (حموضة أو قلوية) لأيون الأيدروجين تأثيرا عكسيا على إنتاج البوليولان والبيوماس .

تطابق أمثل إنتاج للبوليولان عند مستوى اللقاح ٥% ، ١٠% للسلالة *A. pullulans* ATCC 12535 والسلالة *A. pullulans* ATCC 42023 ، على الترتيب ، وقد تناقص الإنتاج خارج هذا المستوى ، وقد بلغ بلغ معدل التحويل عند مستوى اللقاح ٥% (59.48%) فى حالة السلالة *A. pullulans* ATCC 12535 وعند مستوى اللقاح ٥% (٧٣,٦٧%) فى حالة السلالة *A. pullulans* ATCC 42023 ، بينما بلغ محصول البوليولان عند مستوى اللقاح ٥% (48.60%) فى حالة السلالة *A. pullulans* ATCC 12535 و عند مستوى اللقاح ١٠% (٦٦,٧٠%) فى حالة السلالة *A. pullulans* ATCC 42023 .

وجد أن البوليولان المنتج من السلالة *A. pullulans* ATCC 42023 ذات مظهر زيتونى ، ويذوب فى الماء على ٢٥°م ، وتركيز أيون الأيدروجين للمحلول الذائب فى الماء (من ٥–٧) ، وأقصى نسبة للرماد به ٢% ، ونقطة الانصهار أعلى من ٣٠٠°م ، وقد تم التأكد من البوليمر بدراسة الـ IR .