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Comparative studies on pullulan synthesis, melanin synthesis and morphology of white mutant *Aureobasidium pullulans* B-1 and parent strain A.p.-3

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Abstract

A mutant with reduced pigmentation was isolated from *Aureobasidium pullulans* A.p.-3 by physical mutagenesis. The ability to synthesize pullulan and melanin was compared between the mutant and the parent strain. Morphological differences between the strains were observed under scanning and transmission microscope (SEM and TEM). The chemical structure of the pullulan synthesized by the strains evaluated was compared using FT-IR spectroscopy with that of pullulan available commercially.

The amount of pullulan synthesized by the mutant and parent strains was similar whereas the melanin content was significantly reduced. The chemical structure of the pullulan was the same as that of the pullulan from commercial sources. The changes in the appearance of mutant capsules were observed under SEM. Smaller amount, lower size of the melanin molecules and their dislocation influenced the brightness of mutant chlamydospores.

Keywords: *Aureobasidium*; Pullulan structure; Melanin; Mutant strain

1. Introduction

Recently, the polymers synthesized by microorganisms have received increasing interest, mainly because of their useful physicochemical features and uncomplicated biodegradability in the natural environment. Pullulan, being one such polymer is synthesized by the fungi belonging to the *Aureobasidium pullulans* species (Bender, Lehmann, & Wallenfels, 1959; Leathers, 2003; Yuen, 1974). Pullulan is a linear α-D-glucan built of maltotriose subunits, connected by (1 → 6)-α-D-glucosidic linkages (Catley, 1970; Jakovljevic, Vrvic, Radulovic, & Hranissavljevic-Jakovljevic, 2001; McIntyre & Vogel, 1993; Taguchi, Kikuchi, Sakano, Sakuma, & Kobayashi, 1973; Wallenfels, Bender, Keilich, & Bechtler, 1961). The regular alternation of (1 → 4)-α and (1 → 6)-α bonds influences two distinctive properties: structural flexibility and enhanced solubility (Leathers, 1993). Such properties suggest that pullulan might be useful in different technological processes, e.g. food production and packaging (Leathers, 2003; Yuen, 1974). Synthetic media containing various mono- or disaccharides as the carbon sources, ammonium sulfate or peptone as the nitrogen source and basic salts are used for cultivation (Catley, 1971; Dufresne, Thibault, LeDuy, & Lencki, 1990; Gniewosz & Sobczak, 1992; Shabtai & Amukmenev, 1995; Silman, Bryan, & Leathers, 1990; Yurlova, 1994). Aerobic conditions are required for pullulan biosynthesis and the process efficiency increased during intensive aeration (Moscovici et al., 1996; Rho, Mulchandani, Loung, & LeDuy, 1988; Wecker & Onken, 1991). Biosynthesis of pullulan begins in the late logarithmic growth phase (~25 h of cultivation), when there is lack of nutrient compounds in the media (Catley & McDowell, 1982; Dominguez, Goni, & Uruburu, 1978; Finkelman & Vardanis, 1982). Under such conditions fungus blastospores undergo

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changes in their resting forms (Simon, Bouchet, Caye-Vaugien, & Gallant, 1995). The highest pullulan synthesis is observed in the stationary growth phase (between 70 and 90 h of cultivation) when the resting forms account for the 40% of the population (Guterman & Shabtai, 1996).

During cultivation of *A. pullulans* pullulan, as well as other metabolites produced by the fungi, are secreted to the environment. One among them is melanin pigment, which transforms the color of the culture medium from dark green to black (Gadd, 1980; Siehr, 1981; Simon et al., 1995). Demelanisation of pullulan by adsorption on activated charcoal or by use of solvent/salt combinations increased the costs of pullulan production (Kachhawa, Bhattacharjee, & Singhal, 2003). For this reason the improvement of *A. pullulans* strains toward limited melanin synthesis is essential. Majority of such strains were obtained as the result of inductive selection where different mutagenic sources, e.g. UV radiation, nitrous acid, MMS (Pollock, Thorne, & Armentrout, 1992; Tarabasz-Szymańska & Galas, 1993; West & Reed-Hamer, 1993; Seo et al., 2004).

In this study comparison between the synthesis of pullulan and melanin compounds by white mutant *A. pullulans* B-1 obtained by associated mutagenisation with parent strain *A. pullulans* A.p.-3 was evaluated. The changes in morphology of mutant B-1 were also observed.

2. Materials and methods

2.1. Organisms and growth conditions

Aureobasidium pullulans, A.p.-3 strain and its mutant B-1 obtained from the Collection of Pure Cultures, Food Biotechnology and Microbiology Division of Warsaw University of Life Sciences, Warsaw, Poland, were used in the studies.

White B-1 mutant was selected after associated mutagenisation of the natural *A. pullulans* A.p.-3 black strain using of ethylenoimine (0.3% EI for 90 min) and was then subjected to UV (1.5 min.) radiation. Mutant screening was conducted in order to obtain the most desirable white colonies. The selected white B-1 mutant was investigated and compared with the parent *A. pullulans* A.p.-3 strain.

Cultures were inoculated on the slants, stored at 4 °C and then transferred to the liquid media containing (g/l): sucrose 60, K_2HPO_4 7.5, NaCl 1.5, $(NH_4)_2SO_4$ 0.7, $MgSO_4{\cdot}7\ H_2O$ 0.4, yeast extract 0.4. The medium pH was 6.0. The cultures were shaken (150 rpm) for 24 h on ROSI 1000 (Thermolyne, USA) at 28 °C. A total of 1 ml of preinoculum was then transferred to the fresh medium and cultivated for 24 h under the same conditions described above. After cultivation was complete the number of cells was $\sim 1 \times 10^7$ cfu/ml. A total of 1 ml of inoculum was transferred to the fresh liquid medium, and the cultures were cultivated under the same conditions as described above (28 °C, 150 rpm, 96 h). The samples were collected at 96 h.

2.2. Isolation of exocellular polysaccharide

After 96 h the culture broth was centrifuged at 19,000*g* for 20 min. to remove cells. The supernatant was mixed with two volumes of 96% ethanol and incubated at 4 °C for 24 h to precipitate the crude pullulan (polysaccharide). The precipitate was separated by centrifugation at 19,000*g* for 20 min, rewashed with ethanol, and vacuum-dried for 3 days.

2.3. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (Nicolet, Magma-IR 550, USA) was used to test the chemical structure of pullulans.

2.4. Cellular and exocellular melanins

Melanin compounds produced by the parent strain and the mutant were determined after 192 h of cultivation by the methods of Gadd (1980) and West and Reed-Hamer (1993). The absorbance was measured at $\lambda = 540$ nm (Genesis 20 Spectrophotometer, Thermolyne, USA). The standard curve was elaborated with the use of the synthetic melanin (Sigma, Poznan, Poland).

2.5. Cellular observations and cells measurement

The morphology of the cells was observed using a light microscope (Olympus AX 70 Provis) with the photographic cap (Olympus SC 35) and the camera (Sony DXC 950 P Power HAD). The pictures were saved in AnaliSIS-Pro Program, which also have been used for measurement of 100 randomly selected cells chosen from each strain evaluated. Data from this part of the study were analyzed by Tukey's Multiple Range Test and Analysis of Variance (Statgraphic 4.0).

2.6. Ultrastructural observation

2.6.1. Scanning electron microscope (SEM)

Centrifuged (1400*g*) cells of the fungus were fixed in 2% (v/v) glutaraldehyde (pH 7.4) prepared on a phosphate buffer for 2 h. Cell fixation and initial contrasting was performed with 2% water solution of OsO_4 for 2 h. After washing in distilled water the dehydration was carried out in a series of ethanol solutions and twice in acetone. The samples were dried at critical point (CPD 7501 Critical Point Drier, Polaron, Great Britain). The cells were observed under a scanning electron microscope (JSM-35, Jeol, Japan).

2.6.2. Transmission electron microscope (TEM)

Centrifuged cells (1400*g*) were fixed in 2.5% (v/v) glutaraldehyde prepared on a phosphate buffer for 2 h and then in a phosphate buffer alone (pH 7.2) for another 2 h. The cells were then fixed with 1% water solution of OsO_4 (osmium tetroxide) for 2 h. Dehydration was carried out in a series of ethanol solutions and twice in acetone for

30 min. The samples were embedded in an epon resin acetone mixture (1:1) for 12 h at room temperature and then in epon resin alone for 2 h. Containers with the biological material were kept in an incubator at a temperature of 32 °C for 24 h and then at a temperature of 60 °C for 48 h. The samples were cut with ultramicrotome (LKB) into 60–70-nm thick sections. The preparation was stained first with uracyl acetate and then with lead citrate (Reynolds, 1963). The cells were observed under a transmission electron microscope (JEM-1220, Joel, Japan). About 100 randomly selected cells were photographed and their photos were scanned. The thickness of the whole walls and melanin was measured using Zeiss LSM Image Browser software for Digital Image Analysis.

3. Results and discussion

3.1. Synthesis of crude pullulan and cellular and exocellular melanin by white mutant and parent strains

The evaluated strains were studied on a comparative basis. The results indicated that the selected mutant B-1 synthesized the same level of pullulan as the parents strain A.p.-3 and that the differences were not significant (Table 1). Low melanin content was observed in the biomass of white mutant B-1, whereas much higher melanin content was observed in the biomass of the parent strain A.p.-3 (Table 1). As compared with the parent strain A.p.-3., mutant B-1 synthesized 13 times less melanin compounds. The exocellular melanin was present in crude pullulan preparations obtained from parent strain (Table 1). Crude pullulan produced by A.p.-3 parent strain contained 32 mg melanin/g, whereas these compounds were not present in crude pullulan produced by B-1 mutant strain. The results indicated that melanin compounds were not present in the slimy capsule of chlamydospores and did not pollute the preparations obtained from the mutant. West and Reed-Hamer (1993) reported similar results for mutant obtained by chemical mutagenesis of *A. pullulans* ATCC 42023 strain. Mutant with reduced pigmentation produced 11 times less melanin in the cellular biomass and 18 times less stains in the crude pullulan preparation as compared with the parent strain.

Table 1
Crude pullulan, cellular and exocellular melanin production by mutant B-1 and parent strain *A. pullulans* A.p.-3

Strain	Crude pullulan (g/l ± SD)	Cellular melanin (mg/g ± SD)	Exocellular melanin (mg/g ± SD)
B-1	27.6 (± 0.8) a[a]	7.4(± 0.3) b	nd[b]
A.p.-3	28.2 (± 0.5) a	95.1(± 0.5) c	3.3 (± 0.2)

The culture samples collected at 96 h.
[a] Each value represents a mean of six replicates with standard deviations in bracket; a and b – means with the same letters are not significantly different (according to Tukey's test).
[b] not detected.

3.2. Structural characterisation of pullulans

FT-IR spectra for commercial pullulan (Sigma) used as a reference and those for crude pullulans obtained from the strains evaluated are compared in Fig. 1. In the specific area (1500–650 cm^{-1}) which is characteristic for the pullulan molecule as a whole, the spectra for commercial pullulan as well as those for evaluated samples exhibited similar features (Fig. 1). Such results confirmed the identical chemical structure of the samples. Strong absorption in 850 cm^{-1} is characteristic of the α-D-glucopiranosid units. Absorption in 755 cm^{-1} indicates the presence of α-(1 → 4)-D-glucosidic bonds, and spectra in 932 cm^{-1} proved the presence of α-(1 → 6)-D-glucosidic bonds. Besides, in the areas for reference and evaluated samples the frequencies are the same (Seo et al., 2004).

3.3. Morphology of cells and colonies of mutant B-1 and parents strain

The colonies of B-1 mutant on the slants and those on the liquid medium were visually evaluated after 72 and 96 h of incubation and were found to be white in color. The colonies of the wildtype A.p.-3 strain became dark and changed their color from olive-green after 48 h to black after several days of incubation.

The same morphological forms – blastospores – for B-1 mutant (Fig. 2a) as for parent strain A.p.-3 (Fig. 2b) were observed in light micrographs. Blastospores are produced in the logarithmic growth phase, when the hyphae and resting forms (swollen cells and chlamydospores) appeared during later stages of growth. The appearance of blastospores in both strains evaluated did not differ (Fig. 2a and b). The highest numbers of these thin-walled, elongated, hyaline forms were observed during first 48 h of cultivation. After a certain period of time when the amount of nutrients in the medium was reduced, a part of the blastospores were transformed into swollen forms. The largest differences between the two strains were observed in the morphology of chlamydospores (Fig. 2a and b). Chlamydospores of

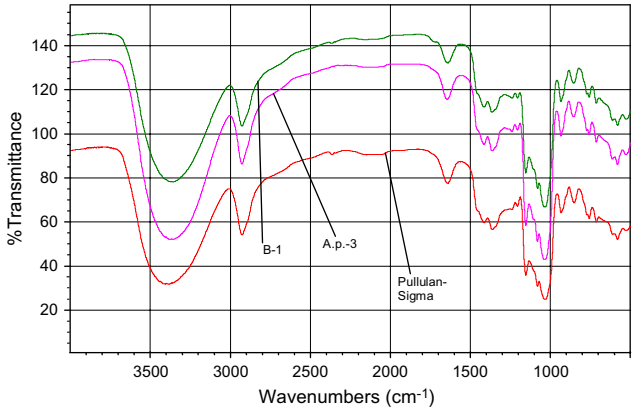

Fig. 1. FT-IR spectra of pullulans elaborated by: mutant B-1, parent strain A.p.-3 and standard Sigma. The culture samples collected at 96 h.

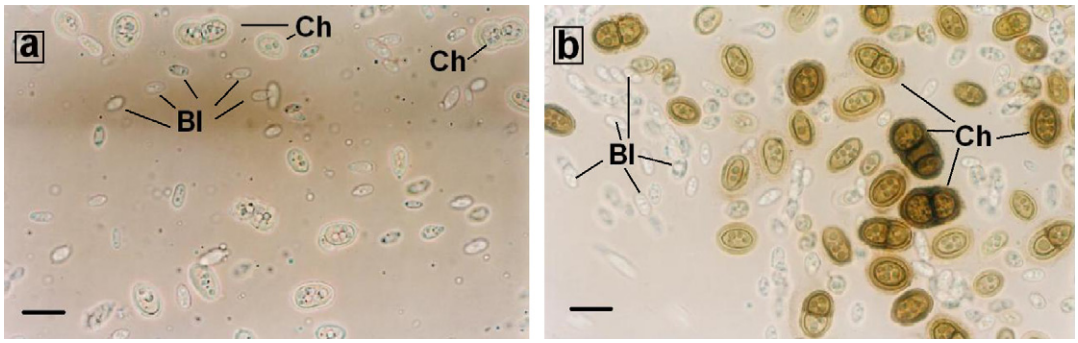

Fig. 2. Light micrographs of various morphological forms of *A. pullulans*: mutant B-1 (a), parent strain A.p.-3 (b). Blastospore (Bl), chlamydospore (Ch). Scale bar = 10 μm. The culture samples collected at 96 h.

parent strain A.p.-3 were surrounded by thick, dark cell walls with characteristic olive-green non-regular outer edge. Dark color of the cell walls and presence of the outer edge is attributed to the presence of the melanin compounds (Simon et al., 1995). The chlamydospores of the B-1 mutant also had thick cell walls, but they were made of hyaline and were bright as well as the outer edge. Similar differences were observed in the morphology of mycelium. During the initial stages of cultivation the strain A.p.-3 mycelium was thin and made of hyaline, but after few days it became dark and had thick walls in contrast to the colorless and thin mycelium of the B-1 mutant.

The sizes of blastospores and chlamydospores of the two strains evaluated are presented in Table 2. Chlamydospores of the strains were few times larger than blastospores. There were no significant differences between the mean sizes of blastospores of parent (A.p.-3) and mutant (B-1) strains. It was observed that the number of mutant B-1 chlamydospores was significantly lower than the number of parents A.p.-3. The shape factors for both strains were not significantly different (Table 2).

3.4. Observations of the cells morphology of mutant B-1 and parent strain under the scanning microscope

Morphological forms observed for mutant and parent strains are presented in Fig. 3. The morphology of blastospores for the two strains did not differ significantly (Figs. 3a and e). The forms were elliptical with very smooth surface, often formed diametrically on two ends of the blastospores. The blastospores originated from polyblastic budding of chlamydospores, swollen cells, or hyphae. Chlamydospores differ significantly from the blastopores, mainly with respect to mean size and the presence of slimy capsule surrounding thick cells. The capsule surface was unequal and folded (Fig. 3b, c, f and g). In the regions where the chlamydospores divided into two cavities, the hollows in the capsules were observed. There were significant differences in the structure of the capsules between the two strains. The capsule of the mutant B-1 chlamydospores seems to be slightly granular and lumpy, whereas the surface of the chlamydospores capsule of the parent strain was granular and lengthwise folded. Chlamydospores were connected by long filaments that emerged from capsules. It was observed that the filaments emerged from the capsules and are integral part of them. However, the arrangement and the structure of the filaments differed for each strain evaluated. The filaments connecting chlamydospores of A.p.-3 parent strain were often irregular, curved, not uniform in thickness and spliced in thicker ropes (Figs. 3d and h). Part of the filaments was adherent to the capsule along the surface. This observation suggests that the whole capsule is built from filaments. After a certain period of time, one end of the filament gets separated, resulting in the formation of splicing network. The filaments observed in the chlamydospores of B-1 mutant are regular in thickness as well as in their network.

3.5. Observations of the cell ultrastuctures in mutant B-1 and parents strain under the transmission microscope

The blastospores of the mutant and parent strains were uniform with thin and bright cell walls. The thickness of the cell walls was 60–150 nm (Figs. 4a and d). Many light

Table 2
Mean sizes of blastospores and chlamydospores of mutant B-1 and parent strain A. pullulans A.p.-3

Strain	Blastospores				Chlamydospores			
	Lenght (μm ± SD)	Circle (μm ± SD)	Size (μm^2 ± SD)	Shape factor (±SD)	Lenght (μm ± SD)	Circle (μm ± SD)	Size (μm^2 ± SD)	Shape factor (±SD)
B-1	6.4 ± 0.8[a] g	20.5 ± 2.7 f	23.6 ± 5.7 e	0.70 ± 0.07 h	9.3 ± 0.6 c	29.6 ± 1.5 b	52.5 ± 7.7 a	0.77 ± 0.11 d
A.p.-3	6.3 ± 0.7 g	20.2 ± 3.1 f	22.8 ± 5.4 e	0.69 ± 0.07 h	11.2 ± 1.8 c	31.3 ± 3.9 b	61.3 ± 8.6 a	0.78 ± 0.05 d

The culture samples collected at 96 h.

[a] Each value represents a mean of one hundred replicates with standard deviations; a and b – means with the same letters are not significantly different (according to Tukey's test).

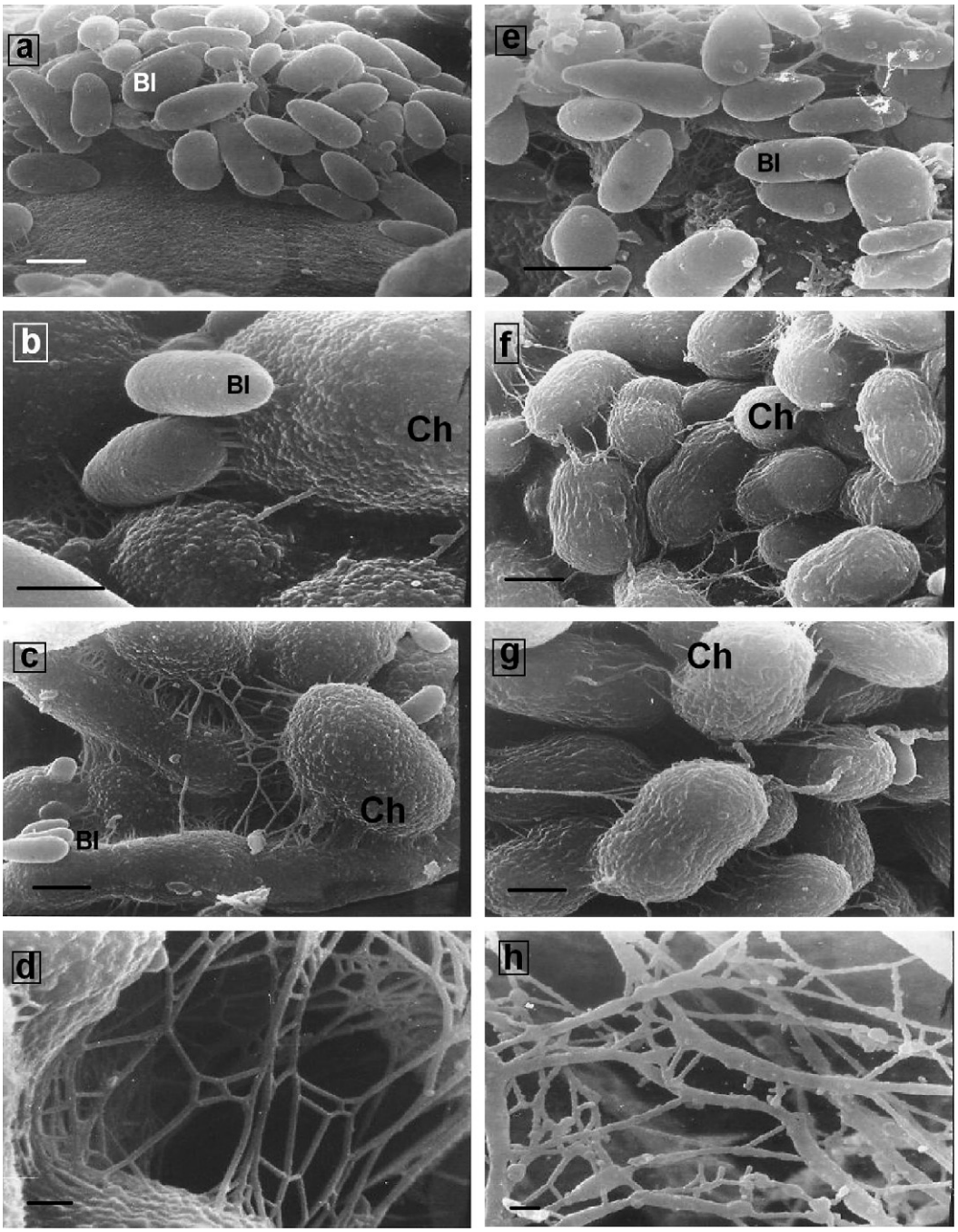

Fig. 3. Scanning electron (SEM) micrographs of various morphological forms of *A. pullulans*: mutant B-1 (a–c), parent strain A.p.-3 (e–g); scale bar = 5 μm. Extracellular polysaccharide of parent strain (d) and mutant B-1 (h); scale bar = 1 μm. Blastospore (Bl), chlamydospore (Ch). The culture samples collected at 96 h.

granules with glycogen and lipids were observed inside the cells. Our results are in agreement with those of Simon et al. (1995).

During transformation of the swollen cells to chlamydospores, the synthesis of slimy capsules is observed. The cell wall enlarges and the size can reached 500–1200 nm and many melanosomes are seen inside. In the young chlamydospores melanosomes were present only in the cell walls and were localized mainly close to the inner layer (Fig. 4b and c). In the mature chlamydospores of A.p.-3 strain the melanosomes were present in the whole capsules area and their number was very high (Fig. 4e and f). The size of the melanosomes produced by the parent A.p.-3 strain was 30–100 nm. During the biosynthesis of melanin in the chlamydospores, the culture on the medium led to change in color from dark green to black after longer cultivation times (144 h).

The chlamydospore of B-1 mutant with two cavities is presented in Fig. 4b. The thickness of the cell wall was 700–1000 nm, and the width of the septum in the narrow

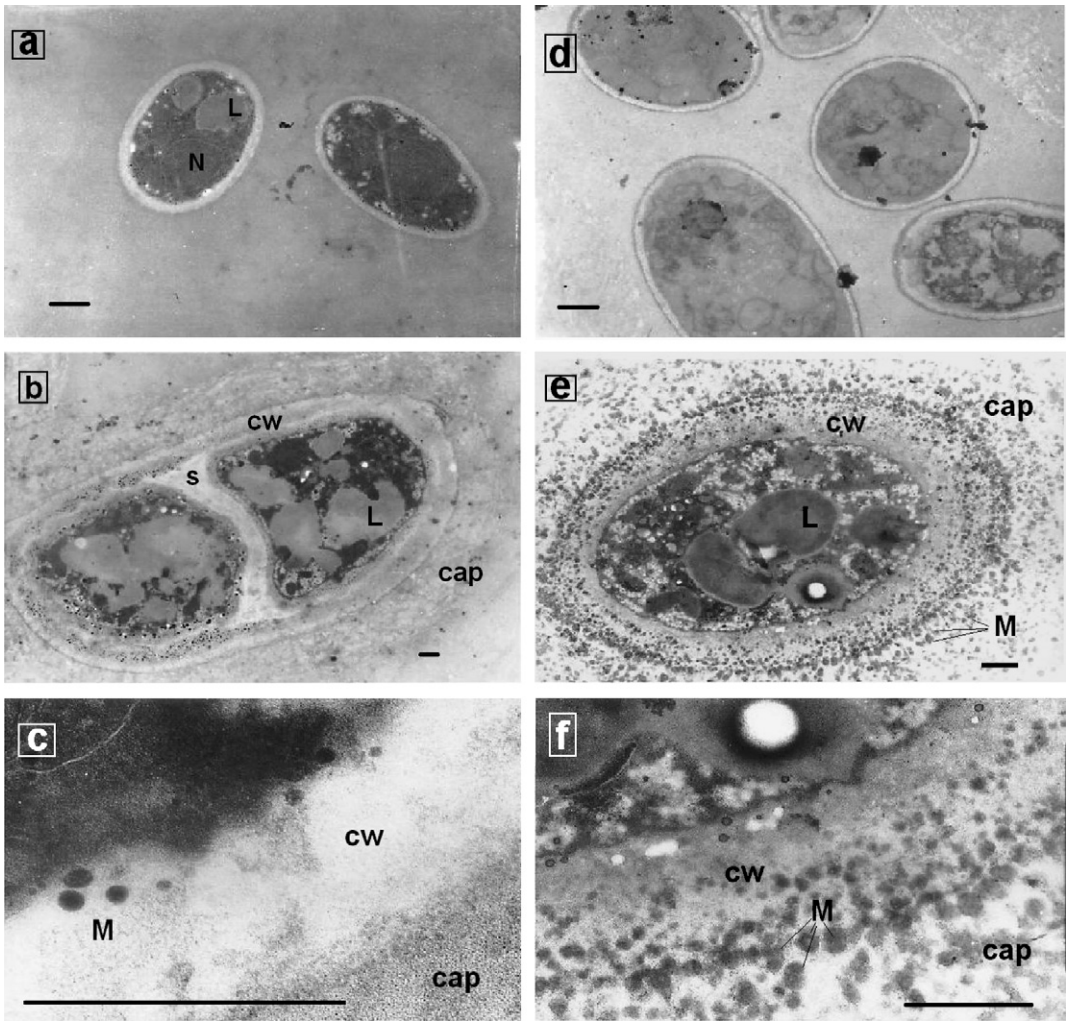

Fig. 4. Transmission electron micrographs of various morphological forms of *A. pullulans*: mutant B-1 (a–c); parent strain A.p.-3 (d–f). Blastospores (a and d), chlamydospores (b, c, e, and f); cell wall (c and f). Melanosome (M), cell wall (CW), capsule (cap), septum (S), lipid (L), nucleus (N). Scale bar = 1 μm. The culture samples collected at 96 h.

region was 500 nm. The size of few melanosomes was much lower (10–30 nm) than that observed in the chlamydospores of the parent strains (Fig. 4b and e). The slimy capsules surrounding chlamydospore cells did not have the dark pigments inside which is responsible for their bright, hyaline appearance in the passing light. It can be concluded that due to the lack of these pigments the batch cultures of the mutants were also white even after long time (more than 192 h) of storage.

Pullulan is synthesized in unfavourable environmental conditions, i.e. characterised by a lack of nitrogen and carbon sources in the medium and/or because of low pH (Dominguez et al., 1978; Finkelman & Vardanis, 1982; Simon, Caye-Vaugien, & Bouchonneau, 1993). Parallel to pullulan are produced also other fungal metabolites, e.g. cellular melanins (Gadd, 1980; Siehr, 1981). Large amounts of both substances are accumulated in the cell wall of chlamydospores and in the capsule formed during the maturation of these morphological forms (Simon et al., 1995). The capsule occurs in the form of a heterogenous fibrous layer. The fibers are made up of pullulan, melanins and also proteins (Simon et al., 1995; Takeo, Nishimura, & Miyaji, 1993). The role of the thin protein structure may consist of binding fibers first to the cell wall region. Subsequent fibers build upon the previous fibers to effectively thicken the fiber layer (Pechak & Crang, 1977; Simon et al., 1995; Takeo et al., 1993). If the cultivation of this fungus is carried out on a liquid medium, the fibers become solubilized. The solubilized pullulan along with soluble melanins are able to penetrate into the cultivation media, which was observed in cultures of a wild strain.

Data from literature indicate that the use of mutant *A. pullulans* strains led to desired changes in the genotype of these fungi, for example, an increase in the capacity for pullulan production and at the same time a decrease in the secretion of other metabolites. Kelly and Catley (1977) used ethidine bromide to mutate a strain of *A. pullulans*. Mutants with normal melanin synthesis but an increased capacity for pullulan synthesis were selected. Elinov, Kossior, Pronina, Tiunova, and Kobzeva (1988)

performed a mutagenisation of *A. pullulans* protoplasts with *N*-nitroso-*N*-methylbiuret. This procedure, however, did not give the expected effect, since mainly negative mitochondrial mutants characterised by a low capacity for pullulan biosynthesis have been obtained. It was only when UV irradiation was used that the morphological mutations decreased and mutations that influenced higher pullulan synthesis increased significantly. In successive studies Tarabasz-Szymańska and Galas (1993) exposed cells of *A. pullulans* to a two-step UV irradiation. After the first irradiation for further investigations a ''negative'' mutant characterised by a very much reduced capacity for pullulan biosynthesis (3 g/l) was selected. The end effect was a mutant with an inhibited synthesis of melanin pigments and an increased production of pullulan (up to 10 g/l). The production of pullulan by this strain, however, was still very small. The results of our investigations are most of all similar to the results obtained by West and Reed-Hamer (1993) and West and Reed-Hamer (1994). These authors used nitric acid (III) and MMS for a mutagenisation of the ATCC 42023 strain of *A. pullulans*. has been isolated The RP-1 mutant of *A. pullulans* prepared by means of the nitric acid (III) mutagenesis had much lower melanin production but pullulan production was not increased. However, after using the MMS an opposite result was obtained. Two mutants which secreted a greater amount of pullulan than the parental strain were selected, but the biosynthesis of melanin compounds in these mutants did not decrease.

4. Conclusions

The results of the studies showed that the amount of the polysaccharide synthesized by the mutant B-1 was similar to that of the parent strain. FT-IR spectra indicated the structure of pullulan synthesized by the strains evaluated was the same as that of pullulan available commercially, which let us to the conclusion that the pullulan synthesis in the mutant B-1 cells were not inhibited by mutation.

The cellular and exocellular melanin production by white mutant was significantly reduced. It was also confirmed by microscopic observations. Small size and low number of melanosomes as well as their dislocation had a considerable influence on the brightness of mutant B-1 chlamydospores. It seems that the production of pullulan with the use of B-1 mutant may reduce the costs of production of this polysaccharide. Future studies should focus on an optimisation of fermentation conditions and the usefulness of this strain in the pullulan synthesis.

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