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ORIGINAL PAPER

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Characterization of *Aureobasidium pullulans* isolated from airborne spores in Thailand

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Abstract Isolates from air in several locations in Thailand were identified as *Aureobasidium pullulans* PR with dark pigmentation (Loei province), *A. pullulans* SU with an unusual conidial apparatus (Chiangmai province), and *A. pullulans* CU with burgundy-red pigmentation (from a shady area in Bangkok). The internal transcribed spacer sequences of the rDNA of *A. pullulans* SU and *A. pullulans* CU confirmed that they were *A. pullulans*. Both *A. pullulans* CU and *A. pullulans* PR preferred 30 °C and pH 7.5 for exopolysaccharide (EPS) production, while *A. pullulans* SU preferred 25 °C and pH 6.5. All three isolates preferred glucose over sucrose and $(NH_4)_2SO4$ over peptone for EPS production. Under optimal conditions, *A. pullulans* PR produced EPS yields of up to 0.225 g g^{-1}, followed by *A. pullulans* CU (0.185 g g^{-1}) and *A. pullulans* SU (0.158 g g^{-1}). Amylase activities were detected during the course of EPS production but gradually decreased as the EPS yields increased. IR spectra suggest that the EPS from these isolates was pullulan. EPS from the three isolates were partially sensitive to pullulanase.

Keywords *Aureobasidium pullulans* · Exopolysaccharide · Pullulan

Introduction

Aureobasidium pullulans is classified as a black yeast, in the Ascomycetes, order Dothideales. It is recognized as an important yeast in industry. The pullulan exopolysaccharide (EPS) produced by *A. pullulans* has applications in the food and plywood industries and in medicine [3]. *A. pullulans* is a saprophytic phylloplane fungus that occurs commonly in temperate zones. There are reports of the isolation of the fungus from tropical regions, such as Brazil, India, Malaysia, and Jamaica [3]. Tokumasu et al. [9] reported *A. pullulans* from a pine forest in Thailand. Microbiologists in Thailand occasionally find the yeast as a contaminant. However, attempts to isolate the culture intentionally are difficult, because secondary saprophytic fungi often predominate in a tropical climate. Therefore, this primary phylloplane colonist is not easily isolated from a plant specimen. In order to find this important yeast in Thailand, we attempted to collect airborne spores and isolate *A. pullulans* from several locations around the country. EPS production by these strains was also investigated.

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Materials and methods

Sample collection

Samples were collected at several locations around Thailand, using corn meal agar (CMA; Difco, Detroit, Mich., USA) plates exposed for 5, 10, 15, 20, 25, or 30 min. The sampling locations included the Phurua mountain area in Loei province, the Doi Suthep pine forest (Chiangmai province), the Narm Now pine forest (Petchabun province), the Tung Salang Laung pine forest (Phitsanulok province), the Khao Yai forest (Nakornratchasima province), and a shady area of Chulalongkorn University (Bangkok).

Cultivation and identification

Exposed CMA plates were incubated at room temperature (30 ± 2 °C) for 7 days. Experiments were performed in triplicate. Yeast-like colonies were picked and were transferred to potato dextrose agar (PDA; Scharlau Chemie, Barcelona, Spain) medium. Identification began with cultivation of the yeasts on malt extract agar (Scharlau Chemie) medium at 20 °C and at room temperature. Cells were collected for microscopic observation. *A. pullulans* ATCC 42023 and *A. pullulans* NRRL 6992 were used for comparison. In addition, identification of the yeast was made by using the characteristics described by Barnett and Barry [1], De Hoog and Guarro [2],

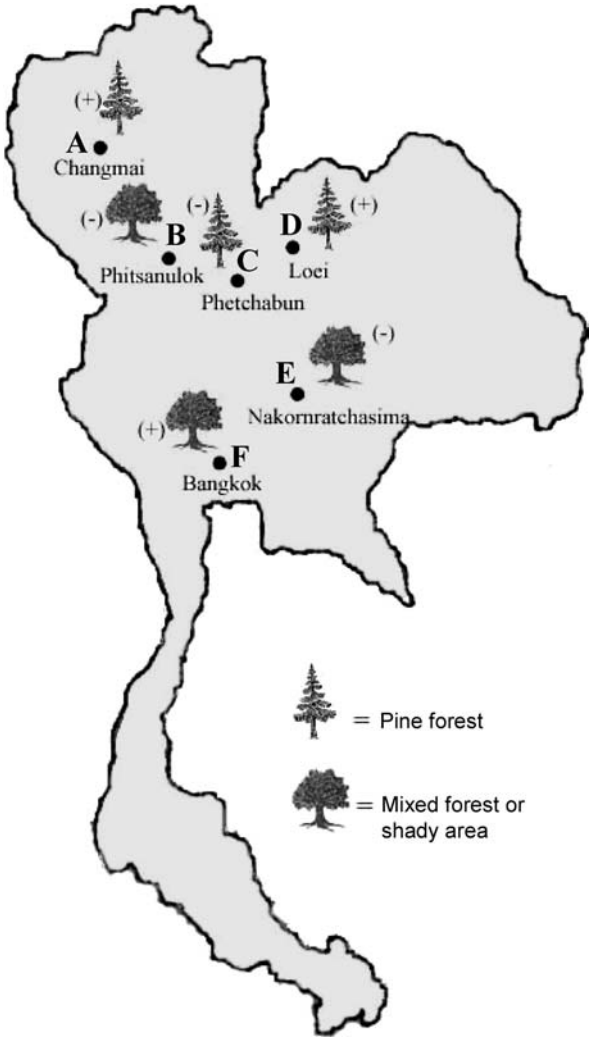

Fig. 1 Collection sites in Thailand where *Aureobasidium pullulans* was *(+)* or was not *(−)* found. The elevation and mean temperature at collection sites were: *A* 900 m, 26.1 °C in March 1999 and 25.4 °C in August 1999, *B* 900 m, 25.8 °C in August 1999, *C* 800 m, 26.7 °C in August 1999, *D* 1,000 m, 26.7 °C in August 1999, *E* 900 m, 28 °C in May 1998, and *F* 2 m, 27.7 °C in November 1999

and Hermanides-Nijhof [5]. Confirmation of the identification was done by sequencing the rDNA internal transcribed spacer (ITS) domains of each isolate, using primers ITS1 and ITS4. The ITS sequencing was performed at the Centraalbureau voor Schimmelcultures (CBS), Utretch, The Netherlands. The techniques described by Gerrits van den Esde and De Hoog [4] were used. Basic local alignment search tool (BLAST) searches (http://www.ncbi.nlm.nhi.gov.) were done in GenBank in order to look for similarity with the neotype strain of *A. pullulans,* CBS 584.75.

EPS production

EPS was produced from the three isolates of *A. pullulans*. The yeast was maintained on a PDA slant at 4 °C. EPS production started with the preparation of 5% (v/v) inoculum (10^7 cells ml^{-1}) in potato dextrose broth (Scharlau Chemie). The optimal parameters for EPS production were determined, including temperature, carbon source, and nitrogen source. The pH optimization began with inoculation of *A. pullulans* into 95 ml of a production medium containing 5% glucose (Sigma Chemical, St. Louis, MO, USA) and 0.06% $(NH_4)_2SO4$ (Carlo Erba, Milan, Italy), with pH adjusted to 4.5, 5.5, 6.5, or 7.5 in individual 250-ml Erlenmeyer flasks. The flasks were incubated at 25 °C, 150 rpm for 5 days. The temperature optimization study was performed using the production medium at the optimal pH for each isolate and incubation temperatures of 25, 30, or 35 °C at 150 rpm for 5 days. Two carbon sources were tested, glucose and sucrose, and each sugar, at 5% (w/v), was used as the sole carbon source in the production medium at the optimal pH and temperature. EPS production was carried out at 150 rpm for 5 days. Two nitrogen sources, $(NH_4)_2SO4$ and bactopeptone (Difco), were tested at 0.06% (w/v) under the optimal conditions for pH, temperature, and carbon source. Again, EPS production was carried out at 150 rpm for 5 days.

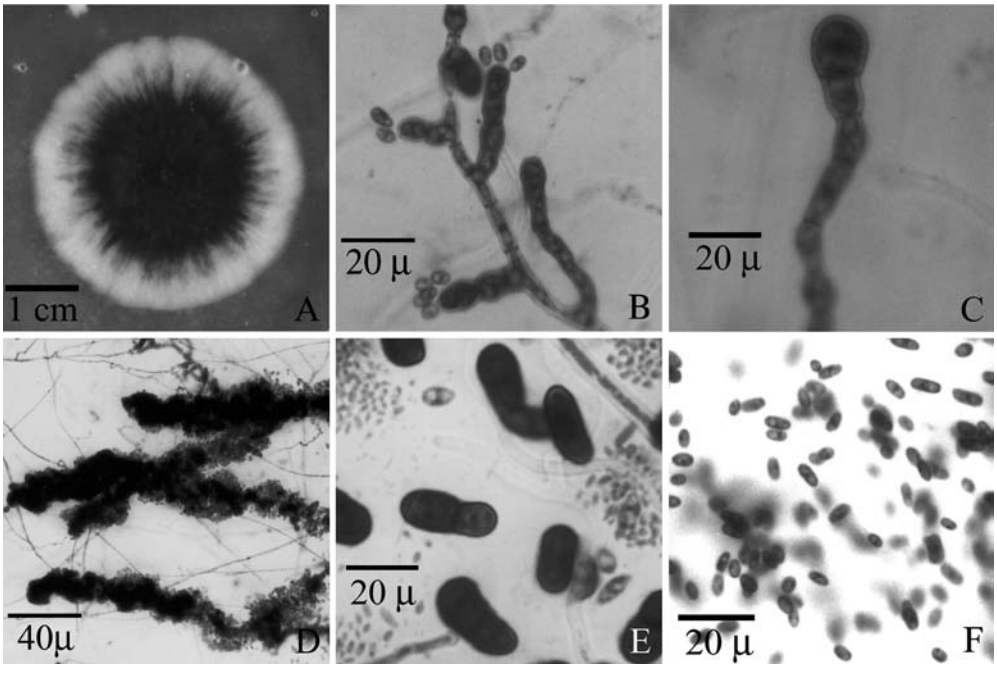

Fig. 2A–F *A. pullulans* PR from Loei province. **A** Colony, **B** conidial apparatus, **C** dark hypha with a chlamydospore at the hyphal tip, **D** dark hyphae with chlamydospores, **E** dark arthroconidia, and **F** conidia

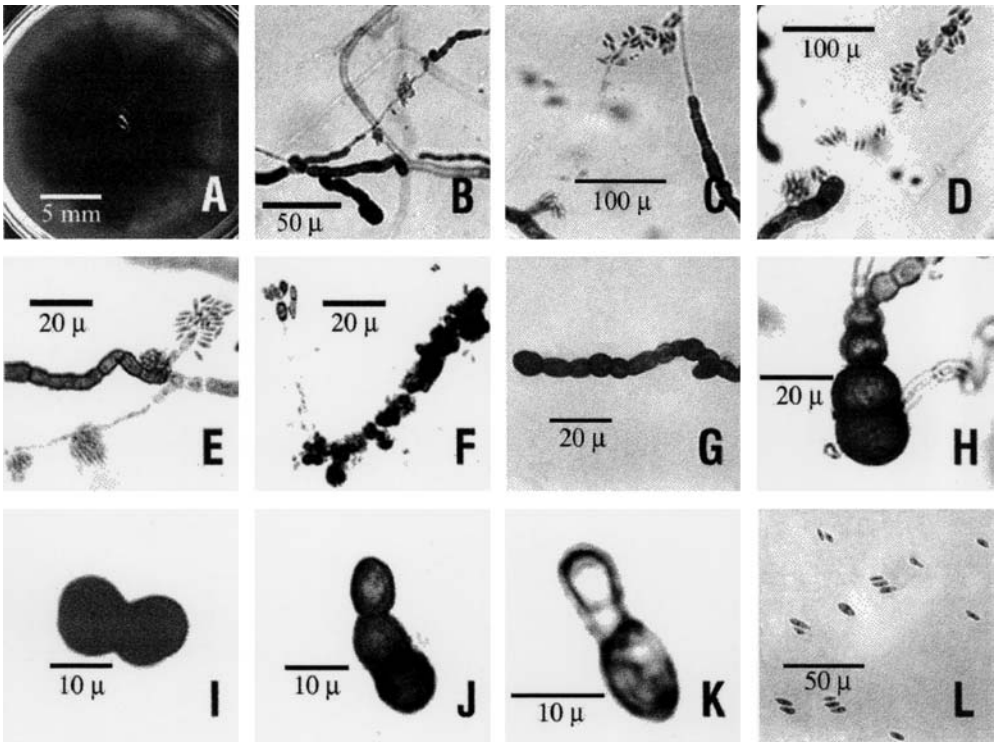

Fig. 3A–L *A. pullulans* SU from Doi Suthep pine forest. **A** Colony, **B–E** conidial apparatus, **F, G** dark hypha, **H–K** dark chlamydospores, and **L** conidia

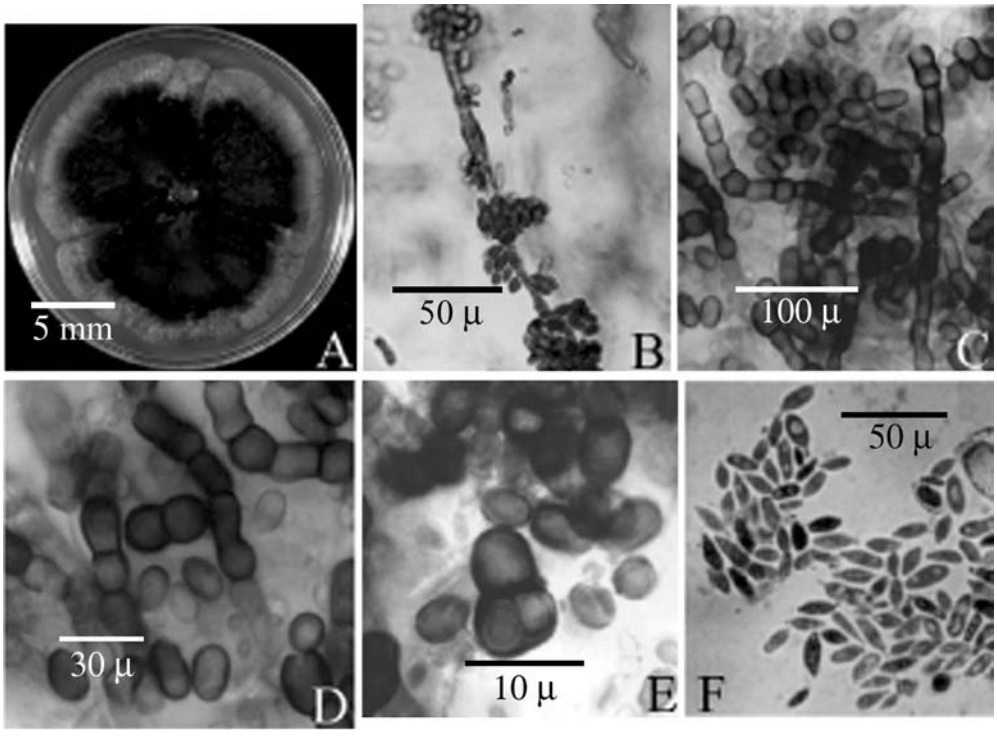

Fig. 4A–F *A. pullulans* CU from Bangkok. **A** Colony, **B** conidial apparatus, **C** dark hyphae, **D, E** intercalary and discrete chlamydospores, and **F** conidia

Enzyme assay

Total amylase activity was assayed in the production medium. The reaction mixture (0.5 ml) contained 1% (w/v) boiled soluble starch (Carlo Erba) solution, 50 mM acetate buffer, pH 5.0, and the enzyme solution. The incubation period was 30 min at 50 °C. The dinitrosalicylic acid method was used to measure the liberated reducing sugar [7]. One unit of amylase activity was the amount of enzyme that produced 1 μmol glucose min^{-1} in the reaction mixture.

Analysis of EPS

EPS was precipitated from spent medium using ethanol. Dry precipitate was ground prior to the addition of 95% KBr. An infrared (IR) spectrophotometer (Perkin-Elmer, Norwalk, Conn., USA) was used for the IR analysis. Pullulanase sensitivity was determined by the method of Leathers et al. [6]. Dry EPS was suspended at 0.1% (w/v) in 50 mM sodium acetate, pH 5.0. Pullulanase from *Klebsiella pneumoniae* (Sigma) was added at 0.1 unit ml^{-1} and incubated for 21 h at 25 °C. Glucose-reducing sugar equivalents were determined by the dinitrosalicylic acid method [7]. Levels of pigmentation were judged by visual observation.

Data on EPS production were averaged from quadruplicate samples. All experiments had a completely randomized design. Statistical analyses included Duncan's multiple range test.

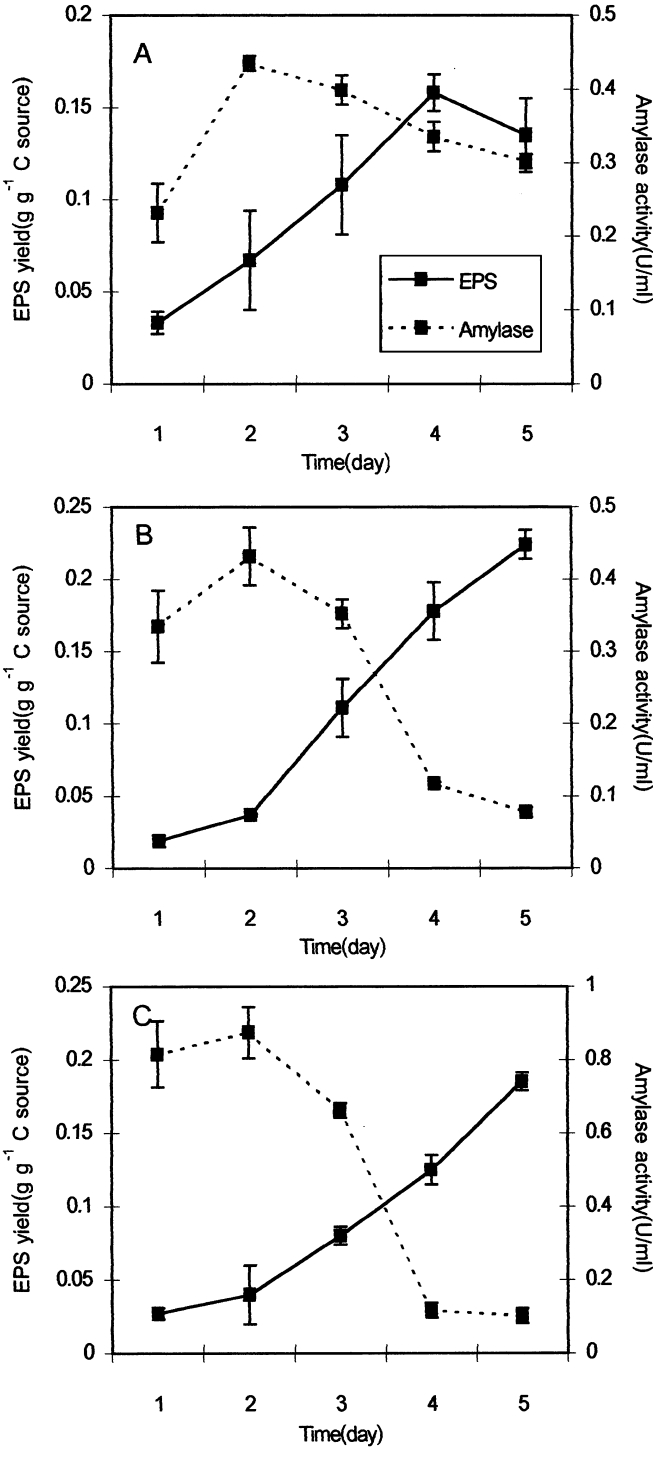

Fig. 5A–C Exopolysaccharide (EPS) yields and amylase activities (*U/ml* units of enzyme activity per milliliter of medium) during the cultivation of *A. pullulans* SU in production medium. **A** SU isolate at pH 6.5 and 25 °C, **B** PR isolate at pH 7.5 and 30 °C, and **C** CU isolate at pH 7.5 and 30 °C

Results and discussion

Sampling sites which showed the presence of *A. pullulans* are indicated in Fig. 1. There was no obvious correlation between recovery of *Aureobasidium* spp and environmental factors in Thailand. The organism was found at both low (2 m) and high (1,000 m) altitudes, at ranges from 25.4 °C to 28.0 °C.

Although sample collections were not done throughout the year, *A. pullulans* was detected in both the rainy season (August) and the dry seasons (March, November).

The microbial flora that emerged from exposure of CMA plates included yeast, bacteria, fungi, and actinomycetes. When the exposure exceeded 15 min, only filamentous fungi were detected. Rapid proliferation of filamentous fungi probably hindered yeast growth. These fungi, common to the tropics, were identified as *Aspergillus* sp., *Cladosporium* sp., *Curvularia* sp., *Neurospora* sp., *Nigrospora* sp., *Penicillium* sp., *Trichoderma* sp., and *Xylaria* sp.

Identification of *Aureobasidium pullulans* was accomplished initially through microscopic examination of morphological characteristics. *A. pullulans* is polymorphic, consisting of blastospores, swollen cells, chlamydospores, hyphae, and pseudohyphae. The conidia are hyaline, smooth, and ellipsoidal. Endoconidia are present. Melanin pigmentation is common. Using the classic guidelines for identification, *A. pullulans* PR, isolated from Phurua, resembled *A. pullulans* var. *melanigenum,* as indicated by the culture rapidly becoming black or dark olivaceous-green [5]. The culture also contained dark arthroconidia (Fig. 2). *A. pullulans* SU, isolated from the Doi Suthep pine forest, also resembled *A. pullulans* var. *melanigenum* but the conidial apparatus was elongated, as in the variety *pullulans*. Therefore, this *A. pullulans* isolate appeared to be a hybrid between var. *pullulans* and var. *melanigenum* (Fig. 3). *A. pullulans* CU, isolated from Bangkok, also resembled *A. pullulans* var. *melanigenum* (Fig. 4). However, its burgundy-red pigment is not typical of *A. pullulans* var. *melanigenum*.

Sequencing of the rDNA ITS domains suggested that *A. pullulans* SU (GenBank accession number AY 139393, CBS 110376) and *A. pullulans* CU (GenBank AY 139391, CBS 110377) were similar to the neotype strain of *A. pullulans*. BLAST searches in GenBank also revealed the highest similarity with *A. pullulans*. The ITS sequencing of *A. pullulans* PR was not conclusive (data not shown). However, this isolate

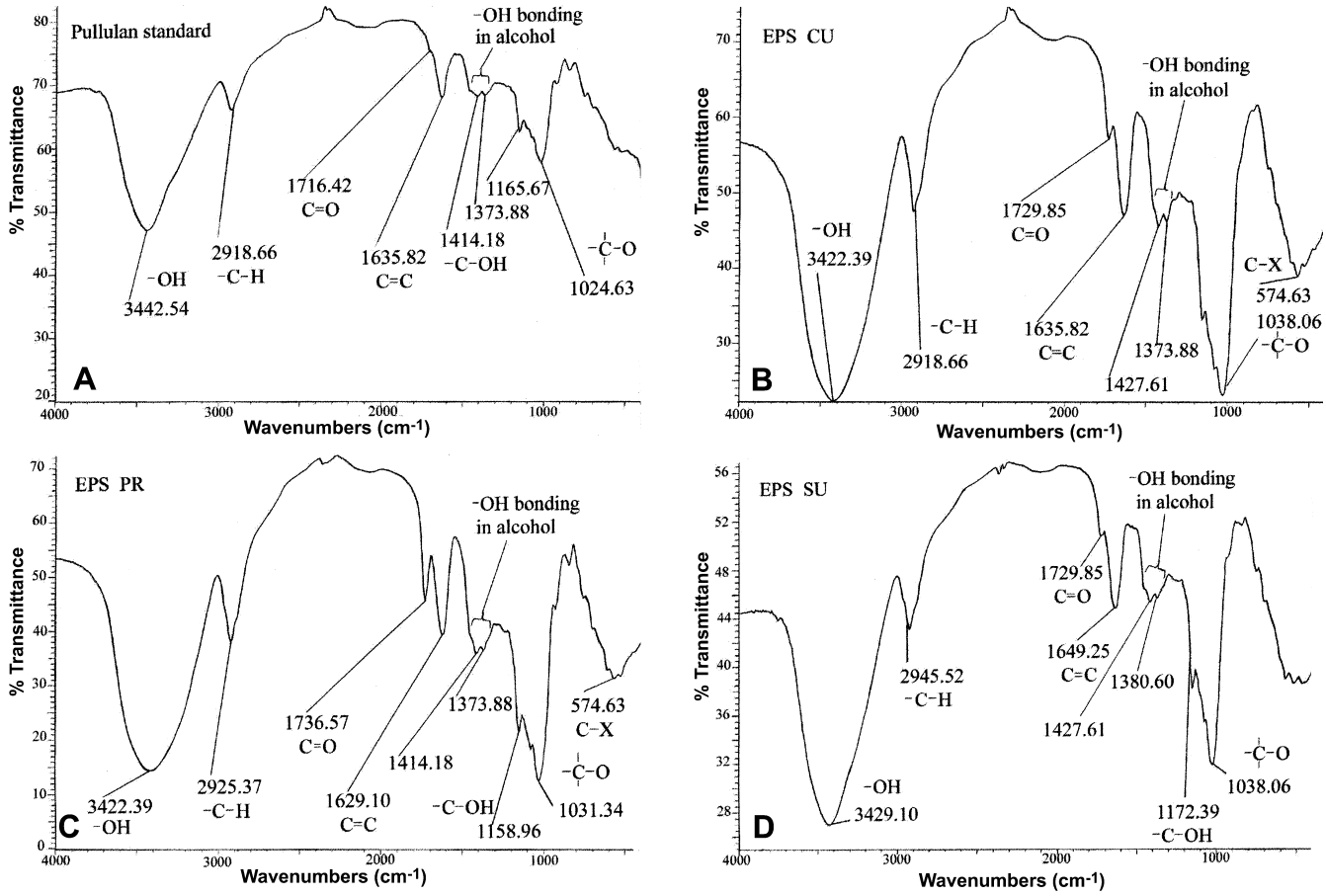

Fig. 6A–D Infrared (IR) spectra. **A** Pullulan standard (Sigma), **B** EPS from *A. pullulans* CU, **C** EPS from *A. pullulans* PR, **D** EPS from *A. pullulans* SU

was also likely to be *A. pullulans* (G.S. De Hoog, personal communication).

In modern yeast taxonomy, the classification of *A. pullulans* into *A. pullulans* var. *melanigenum* based on morphology and melanin pigmentation should be considered inadequate (G.S. De Hoog, personal communication).

Recently Yurlova and De Hoog [12] reported a new variety of *A. pullulans,* var. *aubasidani*, characterized by its EPS structure. By determining the EPS structure, nutritional requirement and molecular features, *A. pullulans* was classified into *A. pullulans* var. *pullulans* and *A. pullulans* var. *aubasidani*. To identify the true variety of these isolates, further investigations will be required.

A pH of 7.5, 25 °C yielded the greatest dry weight of isolates *A. pullulans* CU (0.167 g g^{-1} of carbon source) and *A. pullulans* PR (0.052 g g^{-1}) after 5 days, while isolate *A. pullulans* SU yielded the most EPS at pH 6.5 (0.238 g g^{-1}) after 5 days.

At pH 7.5, 30 °C yielded the maximum EPS dry weight of isolates *A. pullulans* CU (0.186 g g^{-1}) and *A. pullulans* PR (0.225 g g^{-1}) after 5 days. *A. pullulans* SU produced the most EPS (0.158 g g^{-1}) at 25 °C and pH 6.5 after 4 days, while 30 °C yielded significantly less EPS (0.076 g g^{-1}).

As carbon source, Glucose was preferred over sucrose for EPS production by all three isolates. Glucose yielded the greatest dry weight of *A. pullulans* PR (0.225 g g^{-1}) [followed by *A. pullulans* CU (0.185 g g^{-1})] after 5 days and the greatest dry weight of *A. pullulans* SU (0.158 g g^{-1}) after 4 days, while sucrose yielded only 0.020 g g^{-1}(*A. pullulans* PR), 0.122 g g^{-1} (*A. pullulans* CU), and 0.007 g g^{-1} (*A. pullulans* SU) during the same period.

$(NH_4)_2SO4$ was more suitable than peptone for EPS production by all three isolates. $(NH_4)_2SO4$ yielded the greatest dry weight of *A. pullulans* PR (0.225 g g^{-1}) [followed by *A. pullulans* CU (0.185 g g^{-1})] after 5 days and the greatest dry weight of *A. pullulans* SU (0.158 g g^{-1}) after 4 days, while peptone yielded only 0.168 g g^{-1} (*A. pullulans* PR), 0.131 g g^{-1} (*A. pullulans* CU), and 0.030 g g^{-1} (*A. pullulans* SU) during the same period.

During the course of EPS production, amylase activity was detected in the extracellular medium of all three isolates. The highest amylase activity (0.875 units ml^{-1}) was from *A. pullulans* CU on day 2, followed by 0.432 units ml^{-1} from *A. pullulans* PR and 0.435 units ml^{-1} from *A. pullulans* SU (Fig. 5). After reaching maxima on day 2, amylase activities gradually decreased, while EPS yields increased. Since endogenous glucoamylases may attack alternan, it is possible that

accumulation of pullulan in late cultures is related to this decrease in amylase activities [8, 11].

Analysis of the precipitated EPS by IR suggested that the precipitated polymer was pullulan (Fig. 6). These EPS were partially sensitive to pullulanase, ranging from 46.8% sensitivity for the EPS from *A. pullulans* SU to 20.0% (*A. pullulans* CU) and 2.6% for the EPS from *A. pullulans* PR. While the degree of pullulanase sensitivity could be related to the pullulan content and purity, it was observed that highly pigmented EPS (*A. pullulans* PR) was less sensitive to pullulanase. Leathers et al. [6] noted that the presence of melanin could be inhibitory to pullulanase. West and Reed-Hamer [10] suggested the same possibility. However, it is equally possible that these new isolates produce novel polysaccharides analogous to the EPS produced by the recently described variety *aubasidani*. Further studies are planned to resolve this question.

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