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EXPERIMENTAL ARTICLES

Isolation of *Aureobasidium pullulans* and the Effect of Different Conditions for Pullulanase and Pullulan Production[1]

Hani Moubasher[a, *], Salwa S. Wahsh[b, *], and Nabil Abo El-Kassem[b]

[a] *Faculty of Science, Cairo University, Egypt*

[b] *Faculty of Science, Fayoum University, Egypt*

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Abstract—Isolation and production of pullulahase by a new *Aureobasidium pullulans* isolate from the Fayoum Governorate (AUMC 2997) which was identified by the Assiut University Mycological Center was investigated. Another isolate from the Aswan Governorate (AUMC 1695) was kindly provided by the Assiut University Mycological Center. Acetone 2× gave better results for the precipitation of protein than 80% ammonium sulfate in the case of the media containing yeast extract. Very low protein production occurred in media without yeast extract. No enzyme production occurred in the first two days and the production of the enzyme started on the third day. Statistical analysis determined that the optimum conditions for the production of pullulanase were: incubation at 25°C for 5 days, pH 5.5, with sucrose as carbon source at 100 g/L and sodium nitrate as nitrogen source at 2 g/L. Addition of manganese chloride to the medium (1, 2 and 3 g/L) caused inhibition of pullulanase. Also, while the lowest pullulan + pigment concentrations were attained at the fifth day, pH 5.5, at 15°C, 100 g/L sucrose, 2 g/L nitrogen sources, the pullulan + pigment production increased with increasing the concentrations of manganese chloride.

Keywords: pullulanase, pullulan, *Aureobasidium pullulans*

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Aureobasidium pullulans (De Bary) Arnaud, 1918 is widely distributed on dead plants and has been isolated from air, food stuffs, soil, textile, and wood [1]. Pullulan is a linear polymer of α-1–6 linked maltose units. It is produced by *Aureobasidium pullulans*, a yeast-like fungus, which is also known as black yeast because it also produces melanin [2]. Melanin pigment was detemined in the culture supernatant of *Torula corallina* [3]. Amylolytic enzyme, such as α-amylase (EC 3.2.1.1) hydrolyzes soluble starch at α-1,4 glucosidic bonds, producing maltose and maltotriose, while pullulanase type I (EC 3.2.1.41) hydrolyzes soluble starch at α-1,6 glucosidic bonds revealing maltotriose only [4]. The α-1,6 bonds in amylopectin and pullulan are hydrolyzed by pullulanases which are enzymes belonging to the glycosyl hydrolase family 57 [5]. The amylase-pullulanase enzyme from *Bacillus circulans* F-2 displayed dual activity with respect to glycosidic bond cleavage. Pullulanases (pullulan 6-glucanohydrase, EC 3.2.1.41) can hydrolyze α-1,6 glycosidic bonds in pullulan, starch, amylopectin, and related oligosaccharides, while α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) hydrolyze the α-1,4-bonds. Pullulanases do not show activity against linear α-1,4-linked oligosaccharides, and α-amylases show no activity against pullulan or branched substrates. Pullulan-degrading enzymes can be divided into five groups based on substrate specificity and product formation. Pullulan hydrolase type I (EC 3.2.1.135) is an enzyme which attacks α-1,4 glycosidic linkages in pullulan, forming panose. Pullulan hydrolase type II (EC 3.2.1.57) attacks α-1,4 glycosidic linkages in pullulan, forming isopanose which was previously classified as iso-pullulanase. Pullulanase type I (EC 3.2.1.41) specifically hydrolyzes the α-1,6-linkage in pullulan, forming maltotriose which is capable of hydrolyzing α-1,6 glycosidic linkages. Pullulanase type II (EC 3.2.1.3) attacks, in addition to α-1,6-linkages in pullulan, α-1,4-linkages in other polysaccharides; and glucoamylases (EC 3.2.1.3) which sequentially hydrolyze pullulan from the non-reducing terminal to produce glucose [6].

Initial NO_3^- concentration has great effect on the synthesis of the exopolysaccharide and the polysaccharide-cleaving enzyme. When the fungus *A. pullulans* ATCC 9348 was grown in a continuously stirred tank reactor with varying concentrations of NO_3^- (as $NaNO_3$), the final yields of exopolysaccharide obtained fell as the initial NO_3^- concentration was

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* Corresponding author; e-mail: Moubasher54@hotmail.com, Saly4ever2002@yahoo.com

increased from 0.0325 to 0.78 g/L. Polysaccharide-degrading enzyme/s were synthesized by *A. pullulans* during fermentation, and the pullulan content fell to zero after 84 h incubation [7]. pH and temperature has an effect on the production of enzyme; extracellular constitutive pullulan-hydrolysis activity was detected in *Sclerotium rolfsii* when cultivated in a synthetic medium containing starch. The enzyme was most active at pH 4.2 and was stable in the pH range of 3.5–5.5. The optimum temperature for pullulan hydrolysis was 50°C and the activity was stable in the temperature range of 25–60°C at pH 4.2 for 30 min. The enzyme did not produce glucose or maltose from pullulan [8].

Yeast extract and pullulan biosynthesis by *Aureobasidium pullulans* ATCC 42023 was studied in relation to pH (2.0–7.5) during fermentation of phosphate-buffered minimal media based on 2.5% sucrose for 5 days at 30°C. Optimum initial pH for sucrose medium was 6.5 with 0.04% yeast extract but 5.5 with none; pullulan concentration with and without yeast extract was 8.37 and 2.85 g/L, respectively [9].

Pullulan concentration with the effect of different carbon source; cells of *A. pullulans* ATCC 42023, grown at pH 6.0 on minimal medium containing either corn syrup, sucrose, or glucose (25 g/L) were immobilized in sponge cubes, at 30°C. In each cycle the highest pullulan concentration was reached after 5–7 days, being 4.5–5.6 g/L on corn syrup, 5.4–6.2 on sucrose and 5.4–7.1 on glucose [10]. Pullulan biosynthesis was studied in relation to temperature (23–33°C) during batch cultivation of *A. pullulans* ATCC 42023 for 5 days on media containing 2.5% sucrose or corn syrup. For both substrates, the optimum temperature was 26°C. The highest pullulan concentration, reached after 4 days on sucrose, was 17.08 g/L, representing 54% of the total polysaccharide at that time [11]. A new Thermophilic *Bacillus* strain 3183 (ATCC 49341) was isolated from hot-spring sediments. The organism grew on pullulan as a carbon source and had pH and temperature growth optima at pH 5.5 and 62°C, respectively. The optimum pH and temperature for pullulanase activity were pH 6.0 and 75°C, respectively. The enzyme was stable at pH 5.5–7.0 and the temperature up to 70°C in the absence of substrate [12]. Cultivation of the fungus *A. pullulans* ATCC 42023 for 7 days at 30°C on sucrose (25 g/L), supplemented with 5–50 mM $MnCl_2$ resulted in higher polysaccharide outputs. The presence of Mn^{2+} decreased cellular pigmentation (mg melanin/g cell dry weight) from 16.45 to 4.28–1.90 mg/g with sucrose; the melanin content of the polysaccharide was also greatly decreased. On sucrose medium with increasing Mn^{2+} concentration, the cell weight increased within the range of 0–10 mM, and the pullulan content increased at >10 mM [13].

The neutral and water-soluble polysaccharide pullulan is the predominant exopolysaccharide (EPS) produced by the polymorphic fungus *Aureobasidium pullulans*, and this polysaccharide has great potential [14] in food [15] and pharmaceutical industries [16]. Considerable interest has been focused on the factors affecting its production [17].

MATERIALS AND METHODS

Isolates of *Aureobasidium pullulans*. New isolate (AUMC 2997) was obtained from Fayoum soil on Czapek media and another isolate was obtained from Aswan soil (AUMC 1695). Two isolates of *A. pullulans* were identified in Assiut University Mycological Center (AUMC), Egypt.

Effect of yeast extract in the media. Czapek's media, with or without yeast extract (10 g/L) and with sucrose (100 g/L), unless otherwise mentioned, were used for growing the test fungi. Fifty mL of the medium were dispensed into each of 100 mL flasks. Stationary and shaken cultures were used.

Protein precipitation with ammonium sulfate. The fungal isolates were grown on Czapek's 16 medium with 10% sucrose with or without 1% yeast extract for 5 days at 25°C. Precipitation of protein from mycelia and spores was carried out by adding different concentrations of ammonium sulfate (40, 50, 60, and 80%) [18]. Samples were kept overnight, centrifuged at 10.000 rpm for 10 min at 4°C and the protein was redissolved in 20 mM acetate buffer. Samples were dialyzed in a dialysis bag immersed in 50 mM acetate buffer, pH 5.0, overnight with gentle stirring at 4°C. Thereafter, ammonium sulfate was washed out, arid the sample of protein was concentrated using polyethylene glycol (6000 Mwt.)

Protein precipitation with acetone. Ammonium sulfate was replaced by 2× acetone. Acetone was removed by air-drying the protein sample.

Determination of the best conditions for the pullulanase production. Incubation periods: the test organisms were grown for 2, 3, 5 and 7 days in Czapek's medium, with 1% yeast extract and 10% sucrose at pH 5.5 at 25°C.

The initial pH values of 4.0, 5.5, 6.0, 7.0 and 9.0 were used: Czapek's medium, with 1% yeast extract and 10% sucrose was incubated for 5 days at 25°C, at different pH values.

Temperatures (15, 25, 30, 37 and 50°C) Czapek's medium, with 1% yeast extract and 10% sucrose was incubated for 5 days at pH 5.5.

Carbon sources. Sucrose, glucose, maltose, each at 100, 75, and 50 g/L, and starch at 1, 2, and 3 g/L were used in Czapek's medium, with 1% yeast extract was incubated for 5 days at pH 5.5 at 25°C.

Nitrogen sources. Equivalent weight weights of nitrogen of 2, 5 and 10 g/L of $NaNO_3$ and $(NH_4)_2SO_4$ and NH_4Cl were employed in Czapek's medium, with 1% yeast extract and 10% sucrose was incubated for 5 days at pH 5.5 at 25°C.

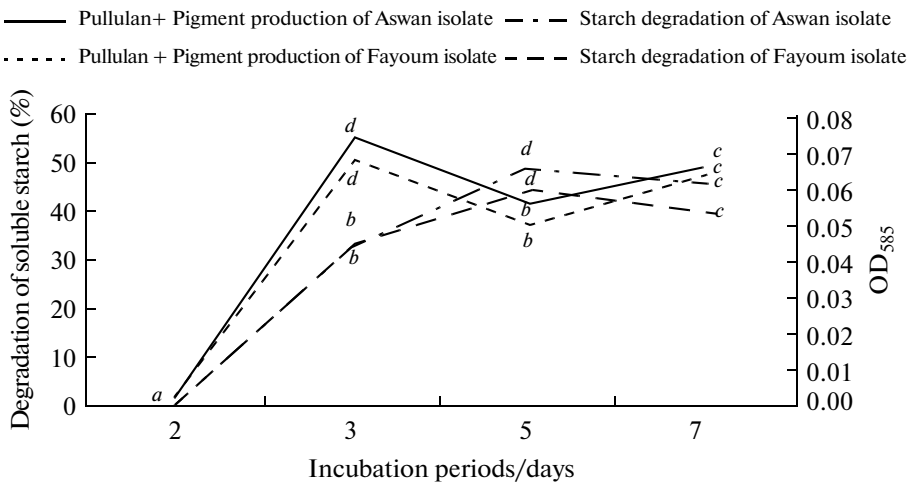

Fig. 1. Effect of different incubation periods on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

The effect of addition $MnCl_2$. 1, 2 and 3 g/L were used in Czapek's medium, with 1% yeast extract and 10% sucrose was incubated for 5 days at pH 5.5 at 25°C.

Statistical analysis. The experimental design was fully randomized. The results are averages of 3–4 biological replicates per treatment or sample. The data presented are the mean ± standard deviations. Data were analyzed using independent samples T-test or analysis of variance (ANOVA) from SPSS-12 statistics program. Multiple comparisons of means were made with Duncan's multiple range tests at 95%.

Determination of pigment associated with pullulan. Pullulan extract of *A. pullulans* is homogenously associated with the dark melanin pigment. The density of the dark melanin pigment is an indicator of the concentration of pullulan in the extract. Melanin pigment was determined in the supernatant using a spectrophotometer at $\lambda = 585$ nm [3].

Enzyme activity assay. Pullulanase activity was determined using starch (0.6 mg/mL) as a substrate and iodine as an indicator (0.1% I_2; 0.5% KI). The control sample was 1 mL 50 mM acetate buffer pH 5.0 + 0.5 mL starch, but the crude sample was 1 mL enzyme-containing solution (3.5 mg/mL) in 50 mM acetate buffer pH 5.0 + 0.5 mL starch and both were incubated overnight at 37°C. The color absorbance was measured using a spectrophotometer (Jenway 6305) at 660 nm using the iodine test [19]. By comparing the difference between zero time (A_0) and time-incubated sample (A_t).

RESULTS AND DISCUSSION

For the precipitation of protein 2× acetone gave better results than all ammonium sulfate concentrations under study in the case of the media containing yeast extract (data not shown) which was similar to the results reported in [20].

Traces of protein were present in the media without yeast extract, while a high amount was detected with yeast extract (data not shown). Two isolates required yeast extract to be added to Czapek's liquid medium for pullulanase production, which means that these isolates may be deficient in one or more of the nitrogenous compounds of yeast extract. In the case of pullulan biosynthesis by *A. pullulans* ATCC 42023 pullulan concentration reached 8.37 g/L with yeast extract and 2.85 g/L without it [9]. The molecular weight of pullulan from all cultures dramatically decreased after 3 days of growth [21]. Shaken cultures were superior to stationary cultures for pullulan + pigment production.

Optimization of enzyme production conditions. Pullulanase production was first detected after two days and reached its maximum value on the fifth day (Fig. 1), a significant difference at $P = 0.05$ according to Duncan's multiple range test. The enzyme activity appeared on the fourth day of fungal growth, and pullulan concentration decreased after 4–7 days [10]. Slight changes in the culture pH occurred during the growth period of *A. pullulans* up to the seventh day, but a sharp decrease in pH value was recorded on the tenth day. Also, the lowest pullulan + pigment concentration was attained at the fifth day, while the highest concentration occurred at the third day (Fig. 1), significant difference at $P = 0.05$ according to Duncan's multiple range tests. The exopolysaccharide pullulan increased on the third day then decreased on the fifth day suggesting that a pullulan-degrading enzyme was synthesized by *A. pullulans* [7].

The optimum for the maximal production of enzyme was pH 5.5. The lowest activity of pullulanase was determined at pH 4.0 after 5 days at 25°C (Fig. 2), significant difference at $P = 0.05$ according to Duncan's multiple range test.

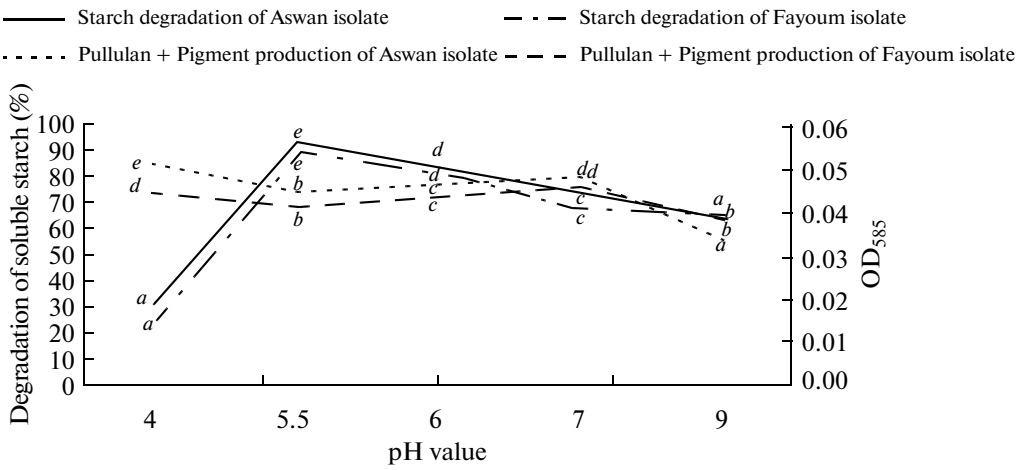

Fig. 2. Effect of different pH values on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

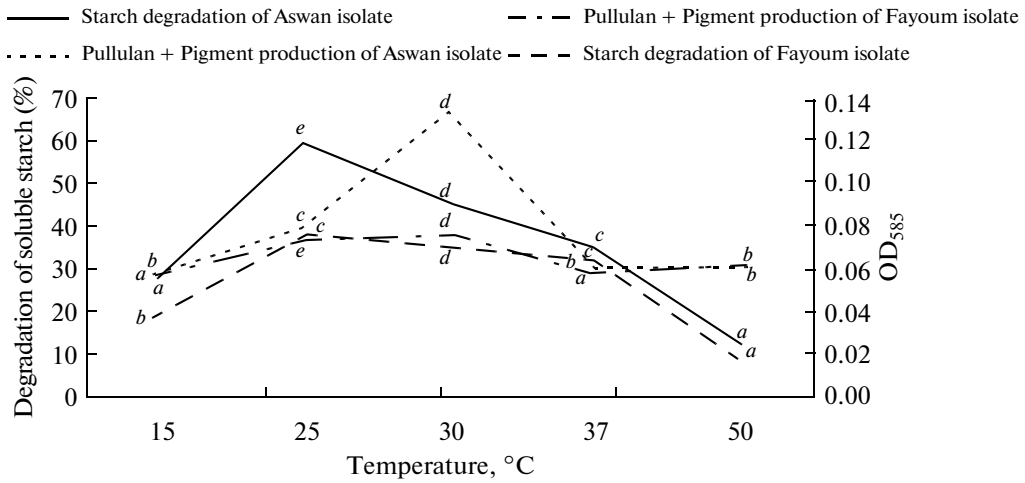

Fig. 3. Effect of different incubation temperatures on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

After 5 days, at 25°C the pH values for all culture media changed to about pH 5.5, except for initial pH 9.0 which decreased to about 6.7–7.0 for both isolates. For *A. pullulans* was grown on sucrose, the pH of the media became acidic probably due to the production of pullulan-acidic glucan with uroric acid components which is in agreement with [22]. The lowest production for pullulan + pigment occurred at pH 9.0 while the highest production occurred at pH 4.0 and 7.0. The optimum pH for pullulanase production and minimum pullulan + pigment production occurred at pH 5.5 for both isolates studied (Fig. 2), significant difference at $P = 0.05$ according to Duncan's multiple range test. Pullulan biosynthesis by *A. pullulans* ATCC 42023 was studied in relation to pH (2.0–7.5), optimum initial pH for sucrose medium was 6.5 with 0.04% yeast extract but 5.5 with none [9]. Extracellular pullulan-hydrolysis activity of *Sclerotium rolfsii* was detected at pH range from 3.5–5.5 and the largest amount of the enzyme was detected at pH 5.5. After 5 days of incubation at 25°C, the pH of the media was almost regularly decreased and that was more pronounced in the alkaline side [8]. It was noticed that pH 9.0 changed to pH 7.0 after 5 days incubation, and the pullulanase activity was lowered so as the production of pullulan + pigment was promoted. The lowering of pH can be attributed to high production of uronic acid components of pullulan [22].

The highest enzyme yield was attained at 25°C, comparable with 15, 30, 37°C, while 50°C resulted in the lowest yield (Fig. 3), significant difference at $P = 0.05$ according to Duncan's multiple range test. The highest yield of pullulan + pigment production was at 30°C, the Aswan isolate showed much higher concentration than the Fayoum isolate, followed by 25, 50 and 37°C; the lowest yield was at 15°C (Fig. 3), signif-

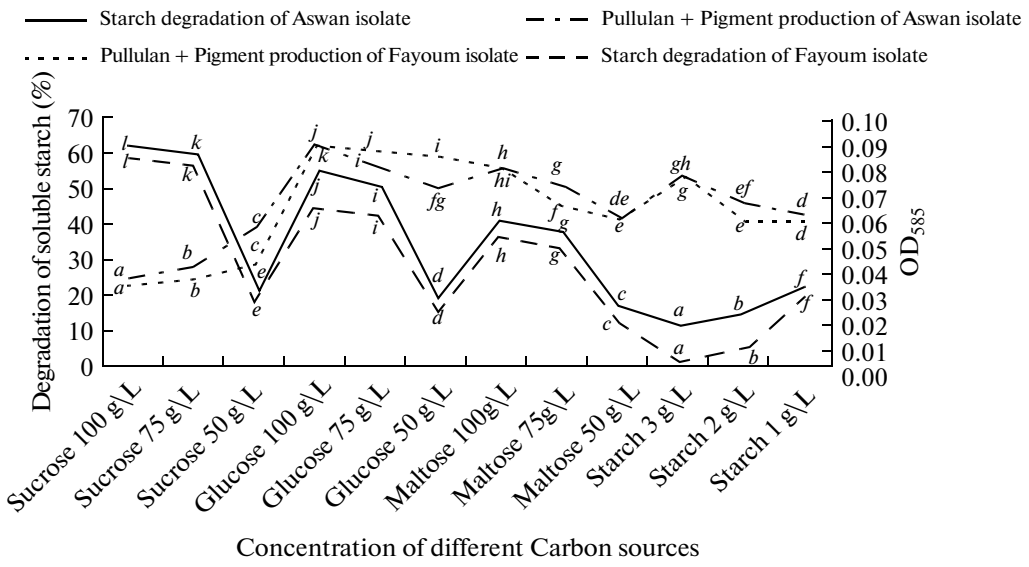

Fig. 4. Effect of different concentrations of carbon sources on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

icant difference at $P = 0.05$ according to Duncan's multiple range test. The pH values of the culture media changed were also affected by the temperature during the growth periods, so that its lowest value was recorded at 30°C. In the case of *A. pullulans* ATCC 42023 grown for 5 days in media containing 2.5% sucrose or corn syrup, for both substrates the optimum temperature was 26°C [11]. *A. pullulans* (ATCC 42023) was grown at pH 6.0 on minimal medium containing either corn syrup, sucrose, or glucose (25 g/L), and its ability to produce pullulan from these media was studied at 30°C [10]. Recording at different temperatures after incubation for 5 days, pH of the medium was regularly decreased and the lowest pH was recorded at 30°C, consequently associated with the highest production of pullulan + pigment. *A. pullulans* CU and PR preferred 30°C and pH 7.5 for exopolysaccharide production, while *A. pullulans* SU preferred 25°C and pH 6.5 [23].

Sucrose, of the three carbon sources under study, was the best for the enzyme production. The optimum concentration of sucrose was 100 g/L (Fig. 4). With respect to soluble starch, it was observed that 1 g/L was the best concentration for pullulanase production, rather than 2 and 3 g/L (Fig. 4). In media containing 100 g/L sucrose, the lowest concentration of pullulan + pigment was attained. As the concentration of the carbon source decreased, the concentration of pullulan + pigment increased. In the presence of glucose and maltose (100 g/L), the highest concentration of pullulan + pigment was attained. As the concentration of glucose and maltose decreased, also the concentration of pullulan + pigment decreased (Fig. 4). With respect to starch, the lowest concentration of pullulan + pigment was observed at 1 g/L, compared to 2 and 3 g/L (Fig. 4), significant difference at $P = 0.05$ according to Duncan's multiple range test. No change in pH was noticed after 5 days of incubation in all carbon sources. The highest pullulan concentrations reached by *A. pullulans* ATCC 42023 after 5–7 days were 4.5–5.6 g/L on corn syrup, 5.4–6.2 g/L on sucrose, and 5.4–7.1 g/L on glucose [11].

Sodium nitrate at all concentrations used regularly induced the highest production of pullulanase, followed by ammonium chloride and ammonium sulfate (Fig. 5); it also stimulated the highest production of pullulan + pigment, followed by ammonium sulfate and ammonium chloride which may indicate that acidity favors pullulan + pigment production (Fig. 5), significant difference at $P = 0.05$ according to Duncan's multiple range test. All nitrogen sources and concentrations after 5 days induced a decrease of medium pH. *A. pullulan* that sodium nitrate was the best source for the production of pullulan [24]. Also, it was found that *A. pullulans* ATCC 11254 produced more pullulan on nitrate than on ammonium as a nitrogen source [19]. When the fungus *A. pullulans* ATCC 9348 was grown in a continuously stirred tank reactor with varying concentrations of NO_3^- (as $NaNO_3$), the final yields of exopolysaccharide obtained fell as the initial nitrate concentration was increased from 0.0325 to 0.78 g/L. In both isolates, the pH value after 5 days exhibited a slight decrease in the case of sodium nitrate compared to ammonium chloride and ammonium sulfate, which caused a considerable pH decrease. This can be explained on the basis of the fact that the fungus utilizes ammonium in both ammonium chloride and ammonium sulfate and

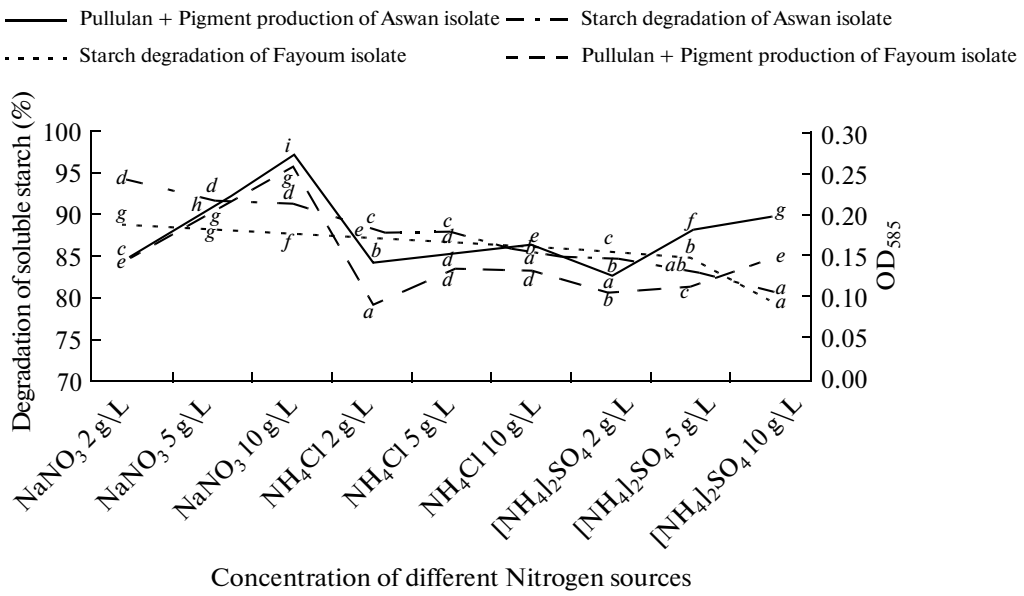

Fig. 5. Effect of different concentrations of nitrogen sources on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

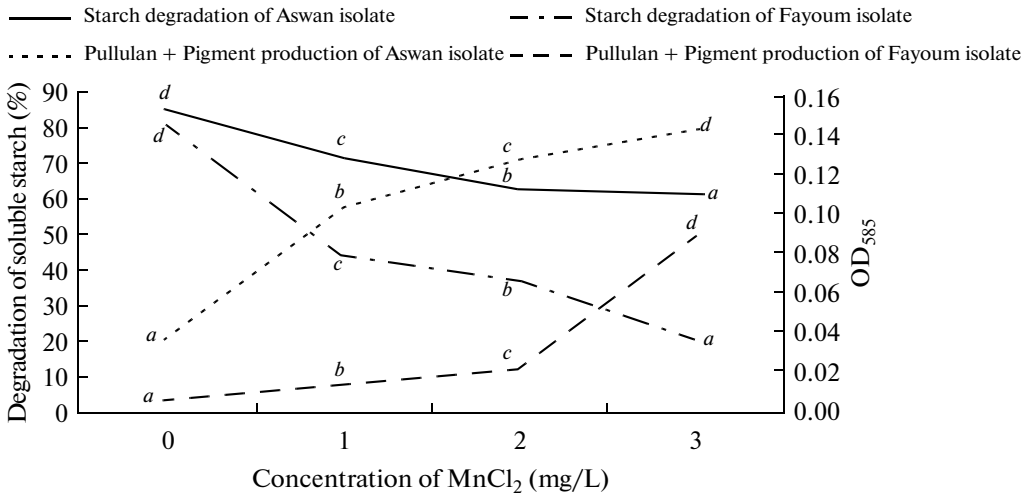

Fig. 6. Effect of different concentrations of manganese chloride on pullulanase and pullulan + pigment production. Standard deviation were indicated by vertical bars and different letters indicate significant difference at $P = 0.05$ according to Duncan's multiple range test.

Cl^- and SO_4^{2-} are released into the medium causing a considerable lowering of pH. On the other hand, in sodium nitrate, the fungus utilizes NO_3^- and releases Na^+ into the medium causing the elevation of pH so that the final pH is slightly lowered compared to the ammonium nitrogen sources [7].

Manganese chloride at 1, 2 and 3 g/L regularly lowered the activity of pullulanase with the increase of dose and a sharper decrease was observed in the Fayoum isolate than in the Aswan one (Fig. 6), while it regularly increased the production of pullulan + pigment (Fig. 6), significant difference at $P = 0.05$ according to Duncan's multiple range test. Increasing concentrations of manganese chloride were associated with a decrease in pH value. Manganese chloride at the dose of 1 mM activated amylase and inhibited pullulanase activity in *Bacillus circulans* F-2 [6]. The presence of manganese chloride (5–50 mM) resulted in higher polysaccharide output using sucrose medium with increasing Mn^{2+} concentration. The mycelia weight also increased with the increase of manganese chloride [13].

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