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ISOLATION AND CHARACTERIZATION OF POLLULAN PRODUCED BY A LOCAL ISOLATE OF Aureobasidium pullulans

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عزل وتشخيص البوليلان المنتج بواسطة عزلة محلية من الفطر

Aureobasidium pullulans

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تم عزل عزلة محلية من الفطر *Aureobasidium pullulans* من على سطح أوراق نبات التين، وأظهرت هذه العزلة تعديدية الأشكال في المستعمرات وإنتاج صبغة الميلانين وإنتاج البوليلان في مزارع الدوارق المهزوزة، لوحظ أن إنتاج العزلة المحلية للبوليلان قارب ثلثي إنتاج السلالة **ATCC 42023** وبينت دراسات الصفات الفيزيائية والكيميائية والتحليل الكيميائي والتركيب السكري والأكسدة بواسطة البيرايوديت وتكسير سمث للبوليلان المنتج بواسطة العزلة المحلية أنه مشابه بصورة مطلقة للبوليلان المنتج من عزلات أخرى للفطر A. pullulans.

Key words : Aureobasidium pullulans, Pullulan Extracellular polysacchairdes.

Running Title: The Production of Fungal Exatracellular polysaccharides (a – glucans), Pullulan by Different strains of Aureobasidium pullulans.

ABSTRACT

A local isolate of *Aureobasidium pullulans* was obtained from fig leaves surface. The isolate was identified as *A. pullulans* on the basis of appearance of polymorphism in the colonies, melanin pigment and pullulan production in shake flask cultures. Pullulan production by the local isolate of *A. pullulans* was two thirds of that produced by ATCC 42023. The studies of physico — chemical properties, chemical analysis, sugar composition periodate oxidation and smith degradation showed that the pullulan produced by the local isolate is nearly similar to the pullulan produced by other strains of *A. pullulans*.

INTRODUCTION

Biopolymers produced by microbes are rapidly emerging as important source of novel and unique plymeric materials. One of biopolymers of great potential for industrial applications is the pullulan produced by the yeast — like fungus Aureobasidium pullulans (pullularia pullulans). Many uses for pullulan have been developed. The films produced from pullulan are water soluble and impervious to oxygen and fibers from pullulan appear to have properties similar to rayon on nylon. Water resistant textile can be synthesized from this polysaccharide (1). It can also serve as a colourles adhesive, moreover a variety of medical applications for pullulan exist (2,3). Pullulan can also be utilized as a food and beverage additive (4).

The chemical structure of pullulan had been elucidated by many investigators (5,6,7). The pullulan consists of linear polymer of maltotriose units connected by α - (1 – 6) linkage.

Aureobasidium Pullulans is a ubiquitous organism. Successful isolations have been achieved from arid and tropical zones. Some of the literature regarding variations in the yield and structure of pullulan can probably be explained in terms of the different origins of strains of A. pullulans. With such a widly distributed fungus it is hardly surprising that polysaccharide yield and properties should vary in different parts of the world (8,9,10). In this article, production of pullulan by a new isolate of A. pullulans and characterization of the pullulan produced are reported.

MATERIALS AND METHODS

Fungal strains and culture media. A. pullulans ATCC 42023 and a local isolate of this fungus, LS1 was obtained according to pollock et al. (11) were used throughout the work. One attached healthy leaf was removed from a fig plant *Ficus carica* and sliced into small pieces. The leaf slices were soaked in sterile distilled water (25ml) for 3 days at 25°C and then 0.1ml was transferred into 10ml of minimal salts medium which included the following (per liter of distilled water): 1g of $(NH_4)_2$; HPO_4; 0.5g of NaCl; 0.05g of $MgSO_4$. $7H_2O$; 2g of K_2HPO_4; 0.01g each of $FeSO_4$, $MnSO_4$ and $ZnSO_4$; 10g of sucrose and 10mg of chloramphenicol. The pH has adjusted to 4 with HC1. After 2 days of shaking at 25°C the turbid culture was allowed to sit undisturbed for 20 min. to allow filaments and aggregates to settle to the bottom. About 20 μl from the upper partially clarified phase that was enriched for yeast – like cells were spread on to agar plates containing the above medium (with 2% agar and pH adjusted to 5). After 4 days independent colonies were purified by replating. An isolate of A. pullulans was obtained and tested in shake flasks for secretion of ethanol precipitable polysaccharide (pullulan), polymorphism of the fungus and culture pigmentation with melanin. The fungi were maintained on PDA slants at 4°C and was subcultured every two weeks.

The incoula were grown in 250ml Erlenmeyer flasks containing 50ml of a standard glucose medium (12). The standard glucose medium included the following (per liter of distilled water); 50g of glucose; 5g of K_2HPO_4; 2g of $MgSO_4$. $_7H_2O$; 0.6g of $(NH_4)_2SO_4$; 1g of NaCl and 0.4g of yeast extract. The medium was adjustged to pH 6.5. five – day – old inocula grown on shaker at 150 rpm and at 28°C were used to seed the culture media at ratio of 2% (v/). The same standard glucose medium and cultural conditions mentioned above were used for the production of pullulan by the two strains of A. pullulans.

Sampling. After the pH measurement, the fermentation broth was centrifuged at 6000 rpm for 20 min. The cells were then dried at 80°C for 24hr. The crude pullulan was precipitated from the supernatant by the addition of 2 volumes of ethanol, then the mixture was centrifuged at 9000 rpm for 30 min. the precipitate was collected and dried at 70°C for 24 hr.

Physico – chemical properties. Before physico – chemical and structural analysis the crude pullulan was purified by dissolving it in distilled water and reprecipitated with ethanol for at least three times. Specific optical rotation was measured using Perkin – Elmer 141 polarimeter. Infrared spectrum of pullulan dispersed in KBr discs was obtained using Pye Unicam SP 1100 infrared spectrophotometer. Elementary analyses were performed in the Department of Chemistry, College of Science, University of Mosul. Protein content was measured using bovine albumin as a standard (13). Residual sugar was determined by the phenol sulphuric acid method (14) using glucose as a standard.

Sugar composition. pullulan (20mg) was hydrolysed with 5ml H_2SO_4 at 100°C for 24 hr and then neutralized with barium carbonate and filtered. The filtrate was concentrated to 1ml at 50°C. The individual sugar constituents of the acid hydrolysate were identified by descending paper chromatography on Whatman no. 1 paper. The solvent system was n – butanol : acetone : water 4:5:1 (v/v) and elution time was 48

hr (15). The spots on the chromatograms were developed with silver nitrate in acetone followed by ethanolic sodium hydroxide (16).

Periodate oxidation. periodate oxidation was performed according to Hay et al. (17). 50 mg of pullulan were dissolved or suspended in 100ml of 0.03 M sodium periodate at 4°C in the dark for 10 days. A blank solution containing periodate at 4°C in the dark for 10 days. A blank solution containing no pullulan was processed similarly. The consumption of periodate was determined by iodometric method in which a 5ml – aliquot of the periodate oxidation solution was added rapidly to a solution containing water (40ml), 20% potassium iodate (2ml), and 0.5N H_2SO_4 (3ml) and the liberated iodine was immediately titrated with 0.1 N sodium thiosulphate solution using starch as the indicator. In the same time the reagent blank was processed in the same way. The difference between the blank and the sample represented the periodate consumed. The formation of formic acid was determined by titration with 0.01 N sodium hydroxide after excess sodium periodate was decomposed by ethylene glycol.

Smith – type degradation of periodate – oxidized pullulan. Smith – degradation was performed according to Frazer and jennings (18). 0.8g of pullulan was dissolved in 250ml of distilled water and the added to 250 ml of 0.5 M sodium periodate. The mixture was kept in dark at 4°C for 14 days. After that 5ml of ethylene glycol was added to the solution, shaken well and left for half hr at room temperature. The solution was dialyzed against tap water for 24 hr. Then 2.5g of sodium borohydride was added as reducing agent. The solution was kept for 24 hr at room temperature. After that drops of acetic acid were added to remove the exces sodium borohydride until pH5. The solution was dialyzed again and evaporated to dryness at 50°C. 50mg of the polyalcohols were hydrolysed with 5ml of 1.75M H_2SO_4 at 110°C for 5 hr for complete Smith degradation (19). The hydrolysate was neutralized with barium carbonate and filtered. The filtrate was concentrated to 1ml at 50°C. The products were identified by descending paper chromatography on Whatman no. 1 paper. The solvent system was pyridine: ethyl acetate: water 23:72:20 (v/v). The elution time was 12hr. The spots on the chromatograms were developed as mentioned previously.

RESULTS AND DISCUSSION

Identification of *A. Pullulans* LS1. Black coloured colonies formed on agar were isolated, as this fungus is characterized by the production of the black pigment, melanin (20). The isolate was examined microscopically. The isolate showed polymorphism in which yeast phase, filament phase and chlamydospores are characteristic features of *A. Pullulans* (21). The isolate was also tested for the production of pullulan in shake flask culture.

Production of pullulan. The results of pullulan production, growth, changes in sugar uptake and final pH of culture of LSI and ATCC 42023 are presented in Fig. 1. As can be seen the production of pullulan highly exceeded that produced by LS1. 21.3g/l of pullulan produced by the foreign strain after 5 days of fermentation compared to only 13.7g/l produced after the same period of fermentation by the local isolate. In contrast to pullulan, growth of LS1 as expressed in biomass production was greater than that obtained with ATCC 42023. The suger consumption reflected very well cell growth and polysaccharide synthesis by the two strains. The decrease in pH after fermentation was exhibited by the two strains as usually observed in this fungus when simple carbohydrate substrates are used as carbon source (22). The differences in pullulan and biomass production by LS1 and ATCC 42023 could be attributed to strain variation as numerous strains of *A. pullulans* were reported to vary in growth and pullulan production (11).

Physico – chemical properties. The specific optical rotation of pullulan produced by LS1 $[\alpha]D^{25}$ was + 183 (cl% in water). The rported specific rotation of pullulan was $[\alpha]D^{23}$ + 189 (6).

The infrared spectrum of pullulan produced by LSI (Fig. 2) showed a absorption band at 850cm^{-1}. The absorption band at 850cm^{-1} is characteristic of α – linked polysaccharide (23). Additional bands at and α – (1-6) glucosidic linkages (24). These linkages are normally the main linkages present in pullulan molecule (6,25).

The aqueous solution of pullulan produced by LS1 did not give any colour with iodine. This result is in agreement with that obtained by Bouveng et al (25).

Chemical analysis. Chemical analysis of LS1 pullulan revealed 89% carbohydrate and protein 0.8%. Ash content was 3%. Elementary analysis gave (%) C,39.98, H, 6.36, N,0.12, O (by difference 54.54). The proportion of C,H and O are close to the theoretical values for a glucan.

Sugar composition. Paper chromatography of the acid hydralysate of LS1 pullulan revealed only glucose as the predominant monosaccharide. It is noteworthy to mention here that paper chromatography of the acid hydrolysate of pullulan produced by ATCC 42023 showed two spots coincided with standard glucose and fructose. It can be said that pullulan produced by LS1 was more pure than that produced by ATCC 42023.

Periodate – oxidation and Smith degradation. The results obtained by periodate oxidation were calculated with reference to the molar concentration of glucose in the pullulan. 1.32 mmol periodate were reduced per mmol anhydroglucose unit and 0.31 mmol formic acid were produced per mmol anhydroglucose unit. This indicate that percentages of α (1-3), α (1-4) and α (1-6) linkages were O, 70 and 31 respectively (26). These results attest to the presence of 1-4 and 1-6 α – D – glucopyranose linkages in the ratio of 2.24:1 which is fairly good agreement with that obtained by Bouveng et al. (25).

Smith degradation products of the reduced periodate – oxidized pullulan were analyzed by descending paper chromatography. Only glycerol and erythritol were detected. The glycerol must arise from the non – reducing terminals and (1-6) linked sugar residues. The detection of erythritol should be due to the presence of (1-4) linked sugar residues. The absence of glucose indicates the absence of oxidation resistant glucose residues such as (1-3) linked paranose units. These rsults confirmed that existence of (1-4) and (1-6) linkages in the molecule of pullulan produced by *A. Pullulans* LS1 (27).

The overall results indicate that the pullulan produced by LSI is absolutely similar to the pullulan produced by other strains of *A. Pullulans.* The objective of this research was to have our own strains of the yeast – like fungus *A. pullulans* and also to investigate the nature of pullulan produced. Attempts to improve the yield of pullulan produced by LS1 either by changing the cultural conditions or by ultraviolet mutagenesis are in progress in our laboratory.

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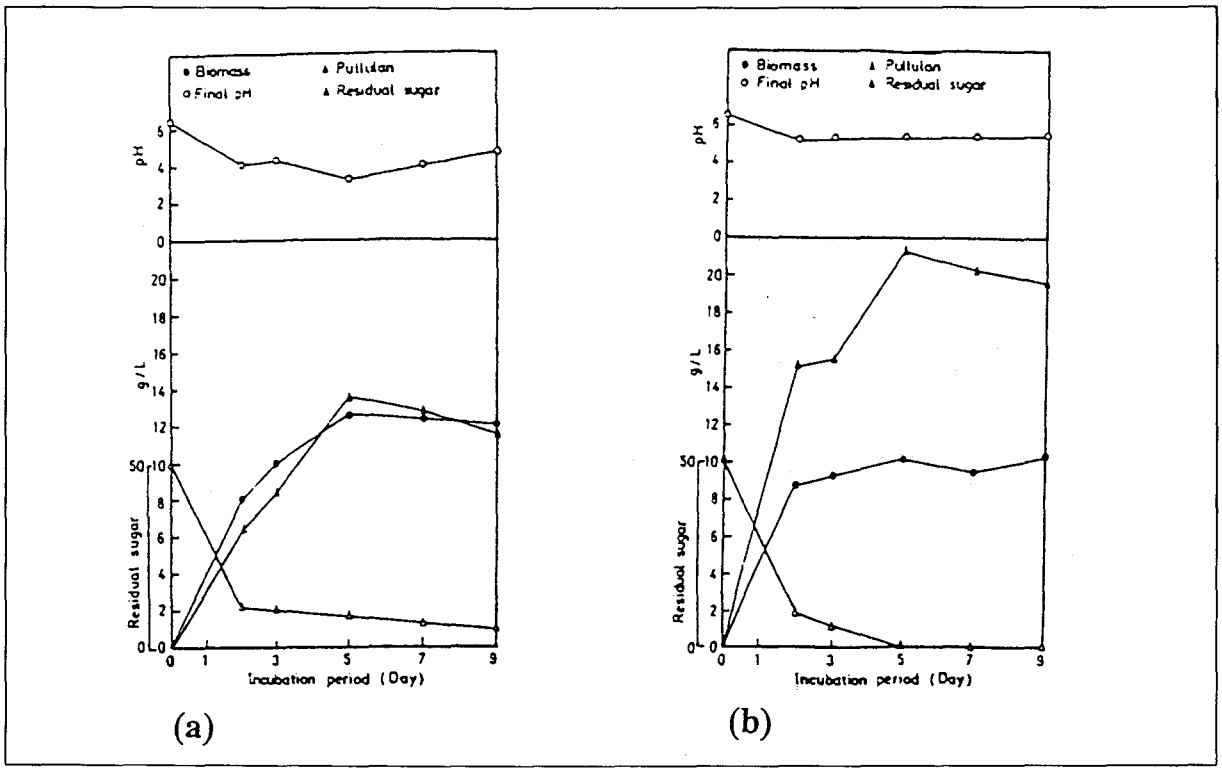

Fig. 1 : Pullulan productin, growth, changes in pH of the medium and residual glucose for A. pullulans strains (a) LS and (b) ATCC 42023 grown in glucose medium.

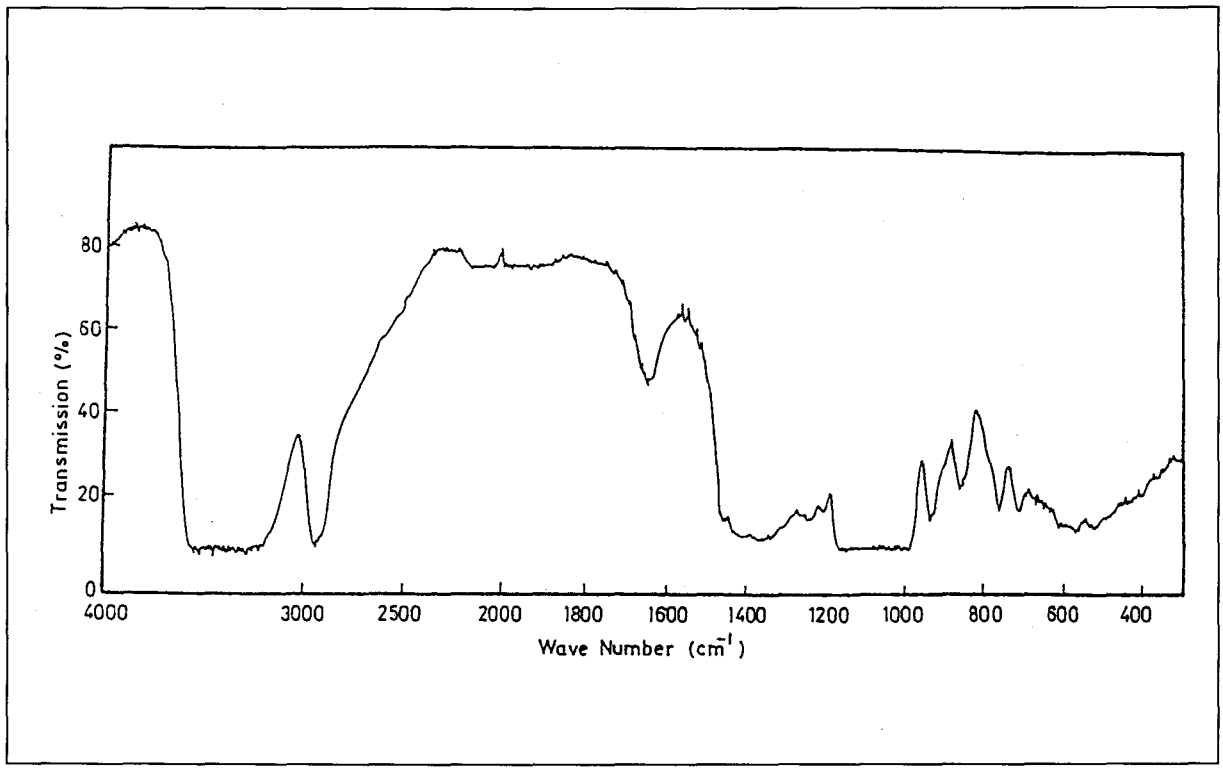

Fig. 2 : Infrared spectrum of pullulan produced by A. pullulans LS1.