

## EFFECT OF SOME CHEMICAL FACTORS ON PULLULAN PRODUCTION FROM DATE SYRUP BY ALOCAL ISOLATE OF THE FUNGUS AUROBISEDIIUM PULLULANS

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### ABSTRACT:

This study was developed to use Iraqi date syrup as a carbon source and basal substrate for the production of the exopolysaccharide “Pullulan” by the yeast like fungus *A. pullulans*. It has been revealed that the best incubation period for high accumulation of pullulan was three days of incubation with initial pH value of 6.5. Also, the fermentation medium of 12% sugar was found to be superior to other sugar concentrations with respect to pullulan production. Moreover, the results of the addition of different nitrogen source containing same amounts of nitrogen present in 0.2% ammonium sulphate show that the urea stimulated pullulan biosynthesis by the fungus more than other tested nitrogen sources. Additionally, highest production of pullulan was obtained when a concentration of 0.2% sodium chloride and was added to the fermentation medium.

**KEYWORDS:** Date syrup; *Aureobasidium*; Exopolysaccharide “pullulan”.

### 1. INTRODUCTION

Pullulan is an  $\alpha$ -glucan polymer of simple chains of three glucopyranosides linked by (1-4)- $\alpha$ -D-glycosidic bonds (Taguchi *et al.*, 1973). These  $\alpha$ -trisaccharide sub-units are multiple-polymerized by (1-6)- $\alpha$ -D-glycosidic bonds occurring at the ends of glucoside residues. The degree of polymerization ranges from 100 to 5000  $\alpha$ -glucopyranoside units and the molecular mass of pullulan may range between 15 - 4 000 kDa depending on the applied strain and selected culture parameters. Parameters influencing pullulan biosynthesis are: temperature, initial pH of culture medium, type and concentration of carbon and nitrogen source in the medium, and culture saturation with oxygen (Kim *et al.*, 2000; McNeil and Kristiancene, 1987; Rho, *et al.*, 1988; Rouks, 1999). Over the last four decades, numerous studies have demonstrated the application of pullulan in the biomedical and pharmaceutical industries as well as in the production of foods and cosmetics (Cheng *et al.*, 2011). Due to its non-toxic, non-mutagenic and non-immunogenic properties, pullulan may be used as biomaterial in tissue engineering and as a carrier of controlled drugs release in a human body (Mishra, 2011). Furthermore, its excellent transparent film forming ability, its colorless, odorless, water soluble characteristics and its thickness are properties making pullulan suitable for use in the production of oral protective capsules for drug and dietary supplements (NPCaps capsules) and rapidly-soluble oral films for use in therapeutic preparations (Cheng *et al.*, 2011). Pullulan has also been demonstrated to be a polysaccharide poorly and slowly degradable by human digestive enzymes and, additionally, one that reduces appetite sensation in men (Peters *et al.*, 2011; Wolf *et al.*, 2003). For these reasons, it may be directly added to foods in order to decrease their calorific value. Raw materials employed in preparation of nutritional media, have been used as useful carbon source for growth of microorganisms such as fungi. Hamer and West, (1994)

revealed that sucrose and yeast extracts can be utilized for production of pullulan by *Aureobasidium pullulans*, as well as Roukas, (1999) showed that barely extract can be used for production of the exopolysaccharide “pullulan” by the fungus *A. pullulans*.

On the other hand, Timothy *et al.*, (1994) explained the use of fuel ethanol byproducts as primary ingredient for production of pullulan sugar, Lazaridou *et al.*, (2002) also demonstrated that cane molasses can be utilized by the fungus *A. pullulans* for production of pullulan sugar. Goksunger and Guvenc, (2004) found he possibility of pullulan production from beets molasses by the fungus *A. pullulans*. Abdel Hafez *et al.*, (2007) reported the possibility of bio-conversion of several industrial and agriculture waste products to pullulan by the fungus *A. pullulans*. Pullulan sugar can be produced by *A. pullulans* from other carbon sources such as date extract, as it is considered as an important source for sugars, it also contains high percentage of amino acids, vitamins and minerals (Najum, 2008). Urkut, (2007) stated that carbon source, nitrogen concentration, initial pH value, oxygen concentration and temperature are represented the main parameters for the biosynthesis of pullulan by the fungus *A. pullulans*. Sharma, *et al.*, (2013) reported that it may also be possible to develop a low cost effective process for production of pullulan by using agro-industrial residues. Cheng, *et al.*, (2011) reported that ammonium ion ( $\text{NH}_4^+$ ), as a nitrogen plays a important role in pullulan production. The depletion of nitrogen is regarded as a signal for the formation of exopolysaccharide “pullulan” by *A. pullulans*. West, (2011) reported that the amount production of the polysaccharide “pullulan” by *A. pullulans* ATCC 42023 was related to carbon source. Ma, *et al.*, (2012) referred that the sodium nitrate can be used as a nitrogen source to produce polysaccharide “pullulan” by *A. pullulans*. But Singh, *et al.*, (2012) were used ammonium sulphate as a nitrogen source for production of pullulan by different thermotolerant *A. pullulans* strains. Moreover, Oliveira, *et al.*, (2015) tested five different nitrogen sources, namely,  $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_4\text{NO}_3$ , urea and residual brewery yeast for pullulan production by two different strains of *A. pullulans*.

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The objective of this study is to study the effect of some chemical factors on the production of pullulan by the local isolate of the yeast like fungus *Aureobasidium pullulans*.

## 2. MATERIALS AND METHODS

### 2.1 The microorganism

The locally isolated strain of the yeast like *Aureobasidium pullulans* was obtained from the culture collection of Mycology Research Laboratory, Department of Biology, College of Science, University of Duhok, which have been previously identified by Asst. Prof. Dr. Asia Saadullah used in the present study. It was maintained at 4°C and activated on potato dextrose agar slants at intervals from 4-5 weeks.

### 2.2 Growth media

**2.2.1 Date extract medium:** Date extract medium was used as a carbon source for the growth of the yeast like fungus *A. pullulans* and production of the exopolysaccharide "pullulan". It was prepared by washing 2 kg of the cheaper Iraqi date fruit, (Zuhdi) then added to 4L of tap water and boiled at 100°C for about 2-3 hours. After cooling the debris was separated from date syrup by filtration using muslin. The sugar concentration of date extract was 64.8 % using the phenol sulphuric acid method (Dubois, *et al.*, 1955).

**2.2.2 Standard medium:** This medium was used to prepare the inocula of the following chemical composition: glucose, 5%; K<sub>2</sub>HPO<sub>4</sub>, 0.1%; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.06%; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.02%; yeast extract, 0.025% and pH was adjusted at 6.5.

### 2.3 Cultural conditions

Date extract media was prepared at concentration of 8% by dissolving 61.72g of date extract into 250 ml distilled water and nitrogen source in the form of ammonium sulphate (0.06) was added, the pH of the media were adjusted to 6.5 using pH meter. Finally the volume completed to 500 ml by sterilized distilled water. The growth and pullulan production media were distributed into 250 ml Erlenmeyer flask in triplicate samples receiving each 50ml. Thereafter, Plugged before autoclaving at 121°C for 15 min. After cooling culture flasks were inoculated with 2% of inocula, (fungal cell suspension), incubated in rotary incubator (150 RPM) for sufficient time at 28±1°C and at specifically interval, three replicate of each treatment were withdrawn randomly for further analysis.

### 2.4 Analytical methods

**2.4.1. Determination of biomass dry weight:** The fungal biomass was separated from the fermentation media by centrifugation at 6000 RPM using. The supernatant was collected and 50 ml of distilled water was added to pellet and centrifuged again at 6000 RPM. After centrifugation, the supernatant was discarded, the pellet was transferred into known weight glass plate and dried at 65-70°C for 24hrs using oven. Finally, the biomass dry weight was measured using a sensitive balance.

**2.4.2. Estimation of sugar concentration:** Initial and residual sugar determination were carried out according to (Dubois *et al.*, 1955).

**2.4.3. Measurement of initial and final pH value:** Initial and final pH measurement was measured by using pH meter (pH HANN instrument, Taiwan). The pH was calibrated by

provided buffers from manufactures and 0.1M HCL and 0.1M NaOH were used to adjusted pH for required solutions.

**2.4.4. Determination of total pullulan:** The amount of pullulan in the supernatant of each treatment was determined by adding 20ml of cold acetone to 10ml of the supernatant, mixing gently followed by centrifugation at 6000 RPM for 15 minutes, the supernatant was discarded and the precipitated pullulan was dried at 50 °C until constant weight was achieved.

### 2.5 Experiments

**2.5.1.** The effect of different incubation periods (2, 3, 4, 5, 6 and 7) days on pullulan production.

**2.5.2.** The effect of different sugar concentrations (4, 6, 8, 12 and 14)% on pullulan production

**2.5.3.** The effect of different nitrogen sources containing same amount of nitrogen{(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2%; NaNO<sub>3</sub>, 0.26%; NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.2%, urea, 0.09%; peptone, 0.25%; Asparagine, 0.22% and (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.35% } on pullulan production

**2.5.4.** The effect of different sodium chloride concentrations (0.05, 0.10, 0.15, 0.2, 0.25 and 0.3)% on pullulan production

## 3. RESULTS AND DISCUSSION

### 3.1 The effect of different incubation periods on pullulan production

The effect of different incubation periods on pullulan production by the yeast like fungus *A. pullulans* is presented in table (3-1). It is clear that high production of pullulan was achieved after 3 days of incubation, 6.67 g/l, (66.43 % of the biomass dry weight), and started to decrease gradually reaching 4.87 g/l at the end of experiment (after 7 days). The decline in the accumulation of the pullulan after three days of incubation could be attributed to the utilization of the carbon source, low oxygen due to high assimilation during the first three days of incubation, or decline in the final pH value due to the metabolic activity of the fungus. The results are in an agreement with previous results obtained by several researchers (Rouks, 1999; West and hamer, 1995; Sena, *et al.*, 2006). It has been stated that the highest production of pullulan by different strains of *A. pullulans* was achieved after three days of incubation. On the other hand, other investigators (Hamer and West, 1993; Sobczake and Gniewosz, 1996; Gniewosz, *et al.*, 1997) found that pullulan production was stimulated after four days of incubation, while Abdel-hafez, *et al.*, (2007) demonstrated that the best accumulation of pullulan in the fermentation media was occurred following five days of incubation. Moreover, Leather and Timotly, (2004), found that seven days of the fungal incubation was the best for high production of pullulan.

### 3.2 The effect of different sugar concentrations on pullulan production

This experiment has been carried out to study the effect of different sugar concentrations in the form of date extract on the production of pullulan by *A. pullulans*. Table (3-2) shows that the fermentation medium containing 12 % sugar was superior to other media containing different sugar concentration with respect to pullulan production (9.98 g/l). This amount is equivalent to 76.34 % of the biomass dry weight, followed by the medium containing 10 % sugar in which the produced amount of pullulan reached 9.22 g/l, (67.1 %). The reported data implied increasing of sugar concentration more than 12% has shown to have suppressing effect pullulan production but stimulated fungal growth. Similar observations were discussed by (Yousif, *et al.*, 1999; Choudhury, *et al.*, 2012).

Table 1. Effect of different incubation periods on pullulan production

| Incubation periods (day) | Biomass dry weight g/l | Pullulan g/l   | Pullulan % | Residual sugar % | Final pH |
|--------------------------|------------------------|----------------|------------|------------------|----------|
| 2                        | 8.46<br>±0.012         | 4.40<br>±0.110 | 52.00      | 0.430            | 5.0      |
| 3                        | 10.04<br>±0.146        | 6.67<br>±0.009 | 66.43      | 0.148            | 5.2      |
| 4                        | 12.18<br>±0.144        | 6.03<br>±0.105 | 49.50      | 0.049            | 4.8      |
| 5                        | 12.86<br>±0.00         | 5.44<br>±0.046 | 42.30      | 0.041            | 4.1      |
| 6                        | 11.94<br>±0.052        | 5.04<br>±0.008 | 42.21      | 0.032            | 4.0      |
| 7                        | 11.15<br>±0.039        | 4.87<br>±0.008 | 43.67      | 0.019            | 3.9      |

The first number represent the mean of three replicate and the second number represent the standard deviation ( $\pm$ S.D.).

Table 2. Effect of different sugar concentrations on pullulan production

| Sugar contain % | Biomass dry weight g/l | Pullulan g/l   | Pullulan % | Residual sugar % | Final pH |
|-----------------|------------------------|----------------|------------|------------------|----------|
| 4               | 6.88<br>±0.140         | 3.21<br>±0.007 | 46.65      | 0.51             | 4.9      |
| 6               | 8.70<br>±0.108         | 4.33<br>±0.001 | 49.77      | 0.49             | 4.7      |
| 8               | 10.01<br>±0.009        | 6.57<br>±0.103 | 65.63      | 0.45             | 4.7      |
| 10              | 12.25<br>±0.116        | 8.22<br>±0.052 | 67.10      | 0.41             | 4.3      |
| 12              | 12.68<br>±0.187        | 9.98<br>±0.105 | 76.43      | 0.43             | 4.0      |
| 14              | 13.17<br>±0.125        | 6.86<br>±0.125 | 52.08      | 0.73             | 4.2      |

The first number represent the mean of three replicate and the second number represent the standard deviation ( $\pm$ S.D.).

### 3.3 The effect of different nitrogen sources on pullulan production

The aim of the experiment was to determine the best nitrogen source added to the fermentation media that supports high accumulation of pullulan by the yeast like fungus *A. pullulans*. Different sources of nitrogen containing equivalent amounts of nitrogen presented in 0.2 % of ammonium sulphate were utilized. The amount of the

produced pullulan was weighed at the end of the experiment. Table (3) shows that the organic nitrogen source in the form of urea was more superior than other nitrogen sources used in the experiment with respect to pullulan production and biomass dry weight. The produced amount of pullulan was 17.60 g/l which is equivalent to (101.28 %) of the biomass dry weight. The culture medium containing ammonium sulphate was second best nitrogen source with respect to pullulan production. The produced amount of pullulan was 13.42 g/l, which is equivalent to 94.0% of the biomass dry weight. Moreover, media containing sodium nitrate or asparagine supports growth of the fungus rather than pullulan production in comparison with the medium containing ammonium sulphate. According to these findings and previously postulated results (Smith and Pace, 1982; Hamer and West, 1994; West and Reed, (1999) the suitability of nitrogen sources for high pullulan production definitely depends on either fungal strain or nature of the fermentation media or both factors.

### 3.4 The effect of different sodium chloride concentrations on pullulan production

The goal of the experiment is to find out the effect of addition of sodium chloride to the fermentation media on pullulan production by the yeast like fungus *A. pullulans*. Addition of sodium chloride (0.05 %) highly stimulated the accumulation of pullulan in the fermentation medium to reach 22.89 g/l (135.66%) comparing to other added concentration of sodium chloride. Moreover, increasing the addition of sodium chloride above 0.2% have suppression effects on pullulan accumulation comparing to the control sample (table 4). The accumulated pullulan is inversely proportional to the concentration of the sodium chloride presented in the media more than 0.2%. This supports the results obtained by Imshenetskii, *et. al.*, (1981) and Najum, (2008). On the other hand, Singh, *et. al.*, (2012) found that the suitable concentration of sodium chloride for high production of pullulan using synthetic media was 0.5%. Therefore, it can be deduced that the best concentration of sodium chloride to be added to the fermentation media for high production of pullulan by the fungus *A. pullulans* is dependant of the nature of the used media (synthetic or natural sources).

Table 3. Effect of different nitrogen sources on pullulan production

| Nitrogen source %                                    | Biomass dry weight g/l | Pullulan g/l    | Pullulan % | Residual sugar % | Final pH |
|--|------------------------|-----------------|------------|------------------|----------|
| (NH <sub>4</sub> ) <sub>2</sub> S<br>O <sub>4</sub>  | 15.34<br>±0.008        | 13.42<br>±0.027 | 94.00      | 0.071            | 4.3      |
| NaNO <sub>3</sub>                                    | 16.30<br>±0.182        | 9.79<br>±0.007  | 60.06      | 0.153            | 5.0      |
| (NH <sub>4</sub> ) <sub>2</sub> H<br>PO <sub>4</sub> | 15.61<br>±0.180        | 10.60<br>±0.003 | 67.90      | 0.095            | 5.3      |
| Asparagi<br>ne                                       | 16.22<br>±0.137        | 12.24<br>±0.102 | 75.46      | 0.102            | 4.7      |
| Peptone  | 12.89<br>±0.183        | 12.28<br>±0.004 | 95.26      | 0.095            | 4.7      |
| Urea   | 16.39<br>±0.005        | 17.60<br>±0.008 | 107.38     | 0.031            | 4.4      |

The first numbers represent the mean of three replicate and the second number represent the standard deviation ( $\pm$ S.D.).

Table 4. The effect of different sodium chloride concentrations on pullulan production

| Na Cl % | Biomass dry weight g/l | Pullulan g/l    | Pullulan % | Residual sugar % | Final pH |
|---------|------------------------|-----------------|------------|------------------|----------|
| 0.00    | 16.23<br>±0.140        | 18.23<br>±0.008 | 136.96     | 0.08             | 5.5      |
| 0.05    | 16.87<br>±0.094        | 22.89<br>±0.017 | 135.66     | 0.07             | 5.5      |
| 0.10    | 17.08<br>±0.107        | 21.87<br>±0.003 | 128.04     | 0.09             | 5.8      |
| 0.15    | 17.73<br>±0.213        | 20.34<br>±0.18  | 114.72     | 0.09             | 6.1      |
| 0.20    | 18.10<br>±0.093        | 19.08<br>±0.134 | 101.41     | 0.11             | 6.4      |
| 0.      | 18.46                  | 17.35           | 93.99      | 0.15             | 6.6      |
| 25      | ±0.192                 | ±0.027          |            |                  |          |
| 0.30    | 18.95<br>±0.008        | 16.77<br>±0.007 | 88.49      | 0.15             | 6.7      |
| 0.35    | 18.98<br>±0.201        | 15.04<br>±0.010 | 79.24      | 0.15             | 6.7      |

The first number represents the mean of three replicate and the second number represent the standard deviation ( $\pm$  S.D.).

#### 4. CONCLUSION

We concluded that date extract can be utilized as a nutritional medium for the growth of *A. pullulan* and production of polysaccharide “pullulan” and at concentrations noticeably lower than sugar. Moreover, determination of period of incubation and nitrogen source and its concentration has shown to enhance the production of pullulan. Finally, the addition of diluted concentrations of sodium chloride has been found to highly increase the rate of pullulan production by locally isolated fungus.

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### گاریگه ریا هندهك ژنده رین کیمیایی لسه ر به رهه م ئینانا بولیولان ل گیراوا خورمى ب ریکا توخمى نافخویى ژ کورویى *Aurobasidium pullulns*

کورتیا لیکولینى:

ئهف لیکولینه هاتیه ریکخستن بؤ بکارئینانا گیراوا خورما ئیراقى وهك ژنده رهكى کاربونی و که رهسته یه کى سه ره کی بؤ به رهه م ئینانا شهکرا جوراوجور (بولیولان) ب ریکا که روى *A. pullulans*. د ئه نجامادا دیاربوو کو باشتیرین دهم بو به رهه م ئینانا و کومکرنا زورتیرین ریژه یا بولیولان برتیه ل سن روژا ل پله یا ترشاتی 6.5. ههروه سا دیاربوو کو چریا شهکرا ناهه ندا ترشینه ری 12% باشتیرین ریژه یه دناقهه را ئه و چریین هاتینه بکارئینان بؤ به رهه م ئینانا بولیولان د لیکولینى دا. لیه ر قى چه ندئ، د ئه نجامادا دیاربوو کو که رهستى یوریا باشتیرین ژنده ره و هانده ره بؤ به رهه م ئینانا بولیولان ل دهمى یوریا دهیته تیگهل کرن دگهل ناهه ندا ترشاندى کو دبیته هه مان ئه و ریژه یا نیتروجینا هه ی دناف 2% ل گوگردئ ئه مونىوم. سه ره رای قى چه ندئ، دیاربوو کو که روو بلندترین ریژا بولیولان ی به رهه م ئینیت ل دهمى کولوریدئ سودیوم دهیته زیده کرن ب چریا 0.2% بؤ ناهه ندا ترشاندى.

### تأثیر بعض العوامل الكیمیائية على إنتاج البولیولان من مسیخلص التمر بواسطة سلالة محلیه من الفطر *Aurobasidium pullulns*

خلاصة البحث:

صممت هذه الدراسة لاستخدام مستخلص التمر العراقى كمصدر كاربونى ومادة اساس لانتاج السكر المتعدد "البولیولان" بواسطة الفطر الشبيه بالخميره *A. pullulans*. واطهرت النتائج ان افضل فتره زمیه للتخصین لتجميع اعلى كميته من البولیولان هي 3 ايام وعند الرقم الهيدروجيني الاولي 6.5. وكذلك تبين ان تركيز السكر لوسط التخمر 12% هو افضل من بين التراكيز المختبره في التجربه من حيث انتاج البولیولان. فضلا عن ذلك اضهرت النتائج ان افضل مصدر نيتروجيني محفز لانتاج البولیولان هو اليوريا عند اضافته الى وسط التخمر بحيث يحتوي على نفس الكميته من النيتروجين الموجود في 2% كبريتات الامونىوم. بالاضافة الى ذلك تبين بان الفطر ينتج اعلى مقدار من البولیولان عند اضافة كلوريد الصوديوم بتركيز 0.2% الى وسط التخمر.