

PULLULAN PRODUCTION FROM SUGAR BEET MOLASSES

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أنتاج البلولان من مولا س بنجر السكر

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البلولان إسم يطلق على المادة الجلوكوزية التي تستخرج من الفطر شبيه الخميرة. في هذه التجربة أستخدم مولا س بنجر السكر بدلاً من الجلوكوز نسبة لرخصه وتوفره وقد تم قياس المادة الحية والسكر المتبقي والرقم الهيدروجيني للوسطين وقد وجد أن إنتاج الجلوكوز يفوق نظيره مولا س السكر من البلولان عند معالته بسلفات الصوديوم أو عند تركيز سكر ٥٪ أو عند حفظ الوسط في حاضنة لمدة خمسة أيام أو عند استخدام سلفات الأمونيوم كمصدر للنيتروجين وبالذات عند تركيز ٠,٤ ٪. وعند دراسة أثر الرقم الهيدروجيني وجد أن التركيز الابتدائي ٣,٥ يساعد علي زيادة إنتاج البلولان (٢٠ جم/ل) وهو ما يقارب إنتاجه عند استعمال الجلوكوز كوسط (٢٢,٢ جم/ل).

Key words : Aureobasidium pullulans, Pullulan

ABSTRACT

Sugar beet molasses was used as the substrate for the production of pullulan with *Aureobasidium pullulans* ATCC 42023. In addition biomass, residual sugar and final pH of the culture medium were investigated. In comparing sugar beet molasses and glucose media, it was found that production of pullulan from glucose medium highly exceeded that produced from molasses medium. Treatment of molasses with sodium sulphate slightly enhanced pullulan production. Highest pullulan production from molasses occurred at 5% sugar concentration. A five-day incubation period was optimum for the highest production of pullulan. Ammonium sulphate as nitrogen source gave the highest production of pullulan particularly when it was used at the concentration of 0.04 % as nitrogen. During the investigation of the effect of initial pH of the molasses medium on the production of pullulan, it was found that at the initial pH 3.5 there was a jump in pullulan production reaching 20.9 g/l, which was nearly equal to that obtained in glucose medium (22.26 g/l).

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INTRODUCTION

The elaboration of microbial polysaccharides has been the subject of increasingly over the last two decades.

Pullulan is the generic name given to any α -glucan elaborated by the yeast-like fungus Aureobasidium pullulans formerly called pullularia pullulans (1). Pullulan is a water soluble neutral glucan and it is a linear homopolymer of glucose composed mainly of repeating maltotriosyl groups linked by α - (1-6) glucosidic linkage (2). A small percentage of the polymer (6.6%) consists of randomly distributed maltotetraosyl groups (3). with increased applications to food, drug and other industries, investigations of the production of pullulan with A. pullulans have been emphasized.

A. Pullulans can grow and synthesize the extracellular polysaccharide (pullulan) from a wide range of carbohydrate substrates including monosaccharides, disaccharides and polysaccharides (4, 5, 6, 7) Pullulan is commercially produced from starch hydrolysate by Hyashibara Biochemical Laboratories Inc., Okayama, Japan (8). Pullulan was also produced from other carbon substrates such as peat hydrolysate (9, 10) . We looked for a cheap carbon substrate on which fermentation production of pullulan could be performed. Sugar beet molasses the by-product of the sucrose industry as it is readily available and relatively low priced could be a suitable substitute. Pullulan production from beet molasses by A. pullulans has not been reported elsewhere in the literature. The objective of our investigation was to produce pullulan from sugar beet molasses as the sole carbon substrate by A. pullulans ATCC 42023. This also included measuring fungal cell dry weight , residual sugar as well as the final pH of the culture medium. In addition the influence of different molasses concentrations, different nitrogen sources and concentrations and other cultural conditions upon polysaccharide synthesis from sugar beet molasses were investigated.

MATERIALS AND METHODS

Microorganism. Aureobasidium pullulans ATCC 42023 was used throughout the work. The fungus was maintained on PDA slants at 4°C and was subcultured every two weeks.

Media and cultural conditions. The crude beet molasses CBM was kindly supplied by the Sugar Factory, Mosul, Iraq. The molasses, was diluted with distilled water to either 5 % sugar and used as untreated molasses medium or to 25 % sugar to which 2.5 % sodium sulphate was added. The mixture was heated at 80°C for 1hr. The muddy precipitate was then removed by centrifugation at 6000 rpm for 20 min. The resultant solution was then diluted with distilled water to the required sugar concentration. Unless otherwise stated 0.06 % ammonium sulphate was added to the medium as a nitrogen source. The medium was adjusted to pH 6.5 and was dispensed in 50 ml portion into 250 ml Erlenmeyer flasks, autoclaved at 1 atm at 121°C for 20 min.

The inoculum was grown in 250 ml Erlenmeyer flasks containing 50 ml of a standard glucose medium (13). The glucose medium has the following composition: 5 % glucose, 0.5 % K₂HPO₄, 0.2 % MgSO₄ · 7H₂O, 0.06 % (NH₄)₂ SO₄, 0.1 % NaCl and 0.04 % yeast extract. The medium also served as a basis of comparison with, molasses medium. The medium was adjusted to pH 6.5 and autoclaved as above. Five-day-old inoculum grown on a shaker at 140 rpm and at 28°C was used to seed the culture media for all experiments at a ratio of 2 % (v/v). A. pullulans was inoculated aseptically in glucose or molasses media and grown at 28°C with shaking at 140 rpm for the required time period. The results are presented as average valued from three different flasks.

Analytical methods. After the pH measurement the fermentation broth was centrifuged at 6000 rpm for 20 min. The cells were then dried at 80°C for 24 hr. The crude pullulan was precipitated from the supernatant by the addition of 2 volumes of either ethanol or acetone, then the mixture was centrifuged at 9000 for 30 min. The precipitate was collected and dried at 70°C for 24 hr. The values of the crude pullulan reported were also corrected for the solvent precipitate from the molasses alone at the specific sugar concentration used. The centrifugate was used for the determination of the residual sugar after the evaporation of the solvent. Initial and residual sugar were determined by the Phenol sulphuric acid method (14) using glucose as a standard.

RESULTS

Chemical composition of the crude beet molasses CBM.

The chemical composition of CBM is shown in Table 1. Water contributed about 51.72 % of its fresh weight. CBM was found to be rich in total sugar (42 %), sucrose proved to be the principal constituent with traces of glucose and xylose. On the other hand CBM was poor in nitrogen and lipids. An appreciable amount of ash also was present.

Table 1. Approximate chemical composition of the crude beet molasses

Constituents	Fresh weight (%)
Total sugar	42.00
Moisture	51.72
Total nitrogen	0.33 ^a
Total lipids	0.22 ^b
Ash content	5.62

a. Determined by kjeldahl method.

b. Determined gravimetrically by extraction of dry beet molasses with petroleum ether.

Comparison of different media for growth and pullulan production : Changes in dry cell weight, pullulan, residual sugar and pH in glucose, treated and untreated molasses media are shown in Fig. 1. It can be readily seen that production of pullulan from glucose medium greatly exceeded that produced from molasses. At the 6th day of fermentation the maximum crude pullulan in glucose, treated and untreated molasses media was 22.26, 9.12 and 6.9 g/l respectively. The amount of pullulan harvested from the growth medium decreased after 6 days. Pullulan production was not proportional to biomass production, but that highest pullulan levels were achieved in glucose medium, where biomass levels were lowest. The total sugar consumption reflected very well cell growth and pullulan production. The pH increased in the molasses media instead of decreasing as observed with glucose medium. The medium of molasses treated with sodium sulphate was used in the subsequent experiments.

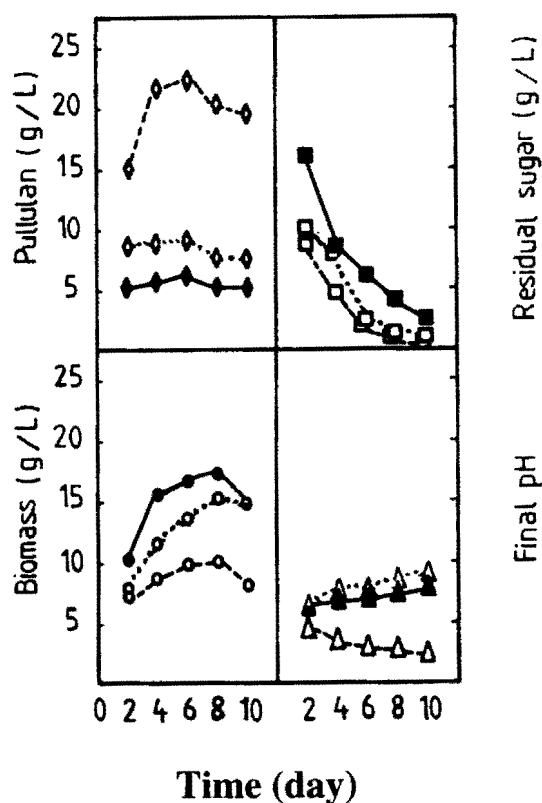
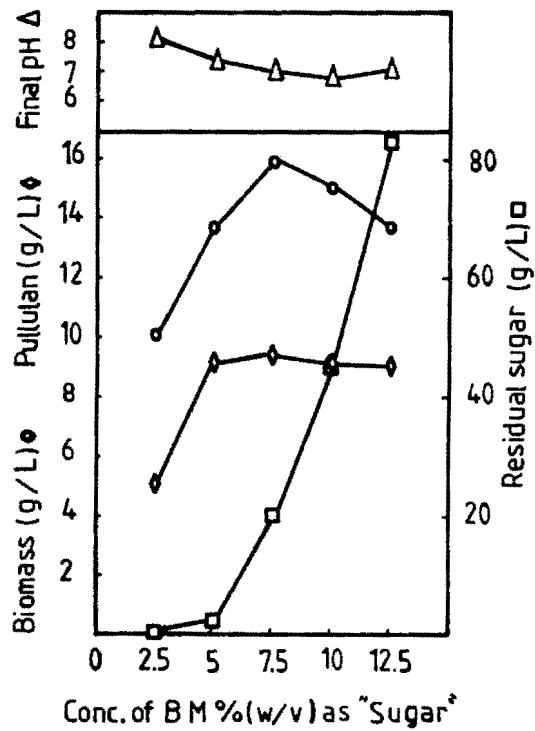


Fig. -1-

Comparison of different media for pullulan production by *A. Pullulus*

- Glucose medium.
 - Untreated molasses medium
 - Molasses medium treated with sodium sulphate.
- For all other details, see text.

The effect of molasses concentration: *A. pullulans* was grown with different concentrations of molasses namely 2.5, 5.0, 7.5, 10.0 and 12.5 % as sugar. The fermentation results after 5 days of incubation are presented in Fig. 2. It was found that pullulan production increased as molasses concentration increased to 5 % as sugar and after this concentration pullulan production remained steadily. On the other hand maximum cell dry weight was obtained in molasses medium with 7.5 % as sugar. Residual sugar in the broth after fermentation was very high especially in media with higher molasses concentration than 5 % as sugar. The pH was higher than its initial value, pH 6.5 The medium contained 5 % molasses as sugar was selected to be the most suitable for the subsequent experiments.

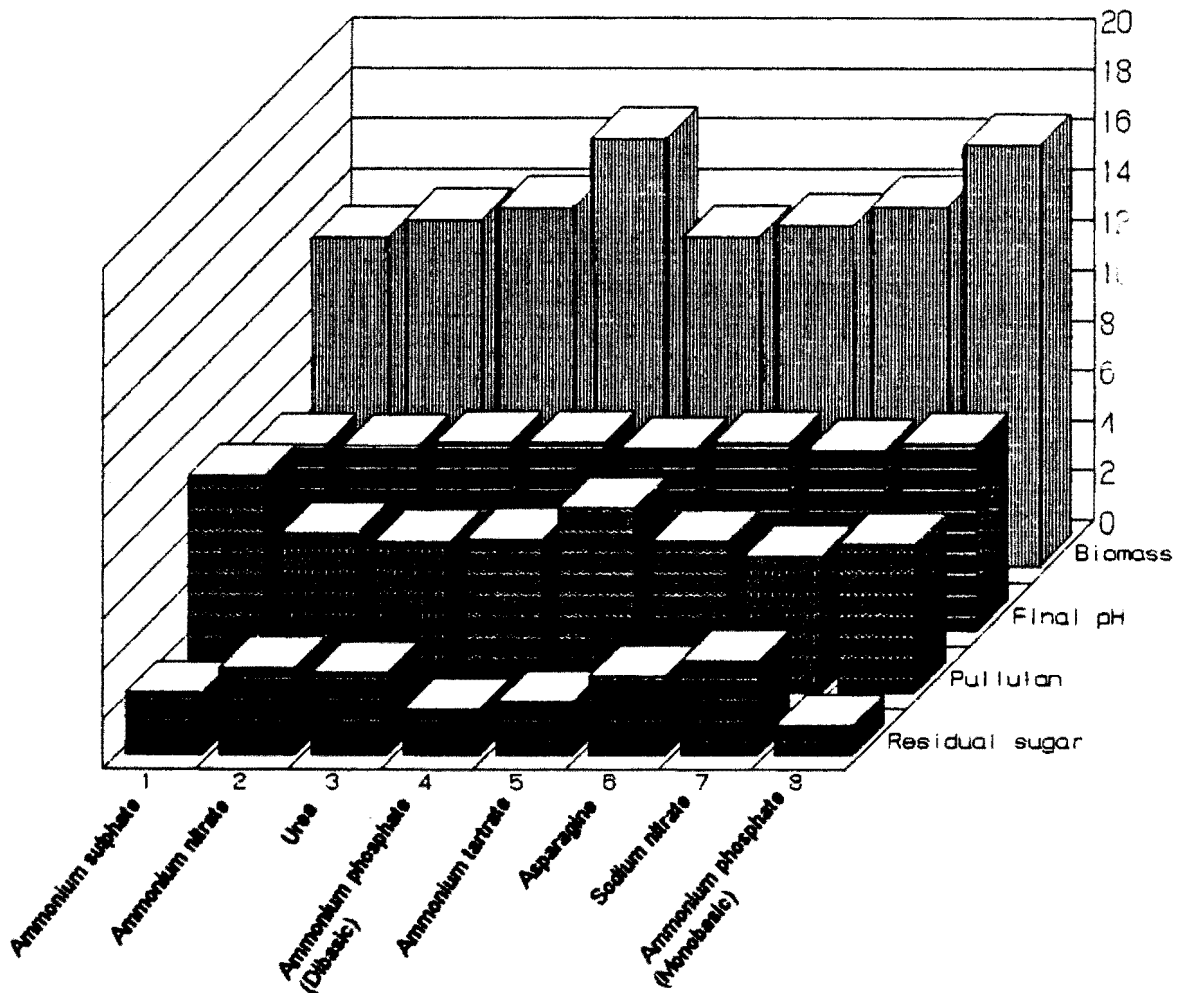


Residual Sugar

Fig. -2-

production of pullulan by *A. pullulans* with different molasses concentrations, on the basis of sugar level. For all other details, see text.

The effect of nitrogen sources: to examine the effect of nitrogen source on pullulan produced, strain ATCC 42023 was cultured in molasses medium at the 5% sugar level supplemented with various nitrogen sources added at a nitrogen level equivalent to 0.6 g ammonium sulphate per liter. The results of pullulan production, fungal growth, residual sugar and pH are shown in Fig.3. It appeared that ammonium sulphate was the best nitrogen source for stimulating pullulan production from molasses. Maximum growth of the fungus was observed (as biomass) and the lowest residual sugar was present when ammonium phosphate was used as nitrogen source. Residual sugar was a reflection of both pullulan production and growth. In all cases, the final pH was greater than the initial pH, that lays between 7.14 and 7.47.



Nitrogen Sources 0.012% (w/v) as "N"

Fig. -3-

Production of pullulan by *A. pullulans* grown in molasses medium supplemented with different nitrogen sources. For all other details, see text.

The effect of ammonium sulphate concentration: The fermentation results of *A. pullulans* at different concentrations of ammonium sulphate are shown in Fig.4. It was found that in medium without ammonium sulphate the production of pullulan was least. However, with increasing ammonium sulphate concentration the amount of pullulan harvested increased roughly linearly up to the equivalent of 0.04 % nitrogen (1.88 g of the salt per liter). Addition of ammonium sulphate had to significant effect on the growth of the fungus when grown in molasses medium. The sugar consumption reflected both cell growth and pullulan production. The pH of the media usually increased as in the previous experiments but nitrogen concentration had little effect on the final pH.

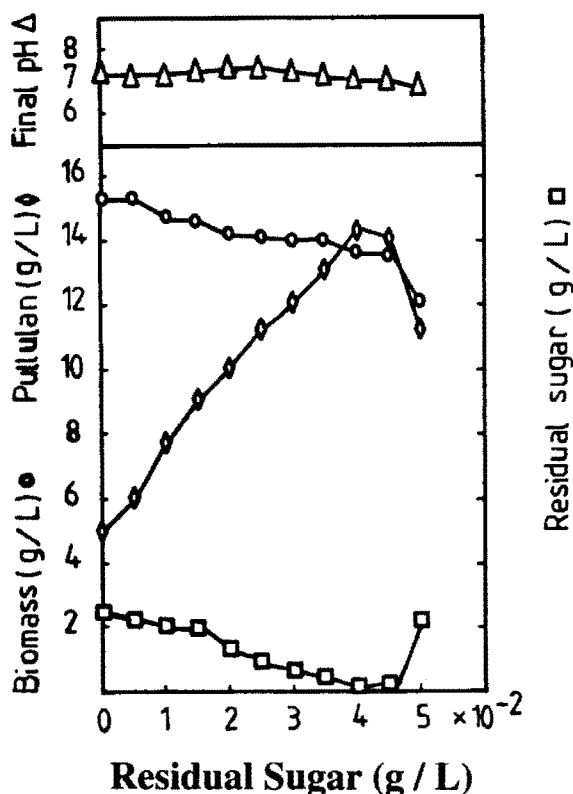


Fig. -4-

Production of pullulan by *A. pullulans* grown in molasses medium supplemented with different ammonium sulphate concentrations. For all other details, see text.

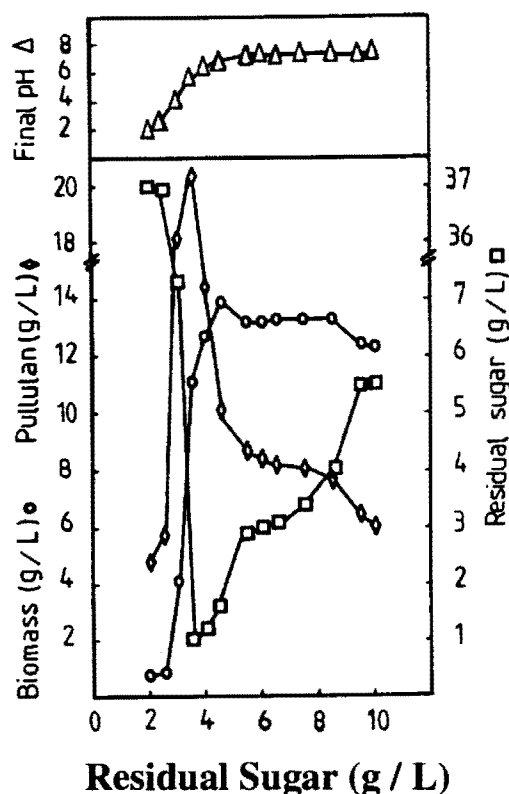


Fig. -5-

Production of pullulan by *A. pullulans* grown in molasses medium at different initial pH values. For all other details, see text.

The effect of initial pH: The results of experiments on the effect of different initial pH values on the fermentation of *A. pullulans* ATCC 24023 on molasses are presented in Fig. 5. Pullulan production increased greatly between pH 2.5 and pH 3.5, achieving a maximum production of 20.9 g/l at pH 3.5, which was more than double that obtained with an initial pH 6.5. At initial pH values greater than 3.5 there was steady decrease in pullulan production. The fungal cell growth in molasses was very poor at a very low initial pH values (2-3, but between pH 3.5 and 4.5 the cell growth increased to a maximum (13.94 g/l). At high pH values (5.5-10) there was no significant change in the cell growth. The sugar consumption was greatest (as reflected in the low amount of residual sugar) when pullulan production was highest (pH 3.5) and at higher initial pH values declined in parallel with the amount of pullulan recovered from the medium. The final pH in the medium was higher than the initial value over the initial range pH 3.0-6.5, but in medium with a higher initial pH values (7.5-10.0) the pH fell to around 6.5.

DISCUSSION

It was intended in the present investigation to try to make use of the large amount of sugar beet molasses produced locally as a by-product of sucrose manufacture. Results presented here indicate that synthesis pullulan by *A. pullulans* from molasses as a sole carbon source is substantially affected by cultural conditions. After comparing the glucose medium with molasses medium for their ability to stimulate pullulan synthesis under given conditions, it was concluded that pullulan level was greatest after growth in glucose medium. Treatment of molasses with sodium sulphate to reduce its heavy elements content and to remove the muddy residue slightly enhanced pullulan production. The biomass concentrations in molasses medium were double those obtained in the glucose medium. This may be mainly due to the presence of growth promoting factors in the molasses. The pH increased in molasses medium instead of decreasing as observed with glucose medium. This in agreement with previous investigation where pH increased in peat hydrolysate medium (9), and in acetate medium (16), and

the pH decreased in glucose and other simple carbohydrate substrates (13, 15, 12). Increasing molasses concentration did not proportionately increase pullulan levels and fungal growth. Similarly it has been found that pullulan levels became static and fungal growth diminished slightly as the concentration of corn syrup was raised (12). The screening of potential nitrogen source in relation to pullulan formation from molasses indicated that ammonium sulphate was the best nitrogen source. Previous studies have indicated that ammonium sulphate was better nitrogen source in promoting pullulan synthesis than other nitrogen sources tested (17,18). Increasing the initial ammonium sulphate concentration in the molasses medium to 0.4 g/l as nitrogen the pullulan production was maximal. It has been reported that in glucose medium the optimal concentration of ammonium sulphate for pullulan production by *A. pullulan* was 0.15 g/l as nitrogen (18) which was much lower than that required by the fungus in molasses medium. In optimizing the sugar beet molasses for maximum production of pullulan, it was found that addition of different concentrations of either dipotassium orthophosphate, sodium chloride, magnesium sulphate, yeast extract of biotin (data not presented) to the molasses medium no significant differences in pullulan production

were observed. Elevation of pH during fermentation with molasses medium was in contrast to the glucose medium where pH decreased encouraged us to study the effect of initial pH of molasses medium on the production of pullulan. As shown in the result (Fig.5), maximum pullulan Production (20.90 g/l) was obtained with initial pH 3.5 which was more than double that obtained with initial pH 6.5 and nearly equal to that obtained in glucose medium (22.26 g/l). From these results it is obvious that initial pH 3.5 highly stimulated the elaboration of pullulan. This means probably that the key enzyme systems which participate in the biosynthesis of pullulan from molasses are stimulated during growth with an initial pH of 3.5 and then the intermediate involved in the biosynthesis may be accumulated and then elaboration of pullulan occurred during the latest days of fermentation. In conclusion it can be said that sugar beet molasses could replace other expensive substrate for the production of pullulan. Also it was determined that firstly the value of initial pH and secondly the concentration of ammonium sulphate do influence the production of pullulan from molasses as a sole carbon source. More work is required to investigate the correlation between initial pH and ammonium sulphate concentration in promoting pullulan production.

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