Optimaization for *β*-glucan production from *Aureobasidium pullulans* NRRL 58543

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Abstract

A. pullulans, a black yeast, is of biotechnological interest since it produces a biodegradable poly-*α*-1,6-maltotriose-based exopolysaccharide (EPS) called pullulan. In addition to pullulan, an EPS with a different structure called aubasidan, a *β*-1,3-D-glucan with *β*-1,6 or *α*-1,4 side chains was reported to be produced from *A. pullulans*. In this research, production of *β*-glucan from *A. pullulans* NRRL 58543 was optimized by growing *A. pullulans* NRRL 58543 in 100 ml of production medium by varying the important factors including carbon source (sucrose or glucose at 5 or 6 or 7 % w/v), nitrogen source (peptone or potassium nitrate or sodium nitrate at 0.04 or 0.06 or 0.08 % w/v) and olive oil as a nutrient supplement (0, 3, 5 and 7% v/v). The effects of initial pH (5.0, 5.5, 6.0, 6.5 and 7.0) and the incubation temperature (25, 28 and 30 ^{o}C) on *β*-glucan production were also investigated. The *β*-glucan was separated from the culture supernatant every 3 days. The maximum *β*-glucan yield (21.04±0.10 g/l) was obtained when the yeast was grown in the production medium containing 6% (w/v) sucrose and 0.06% (w/v) $NaNO_3$, and 5% (v/v) of olive oil as a nutrient supplement with initial pH 6.5 and at 25 °C. The structural analyses using FT-IR and NMR (^{1}H and ^{13}C) suggested that this *β*-glucan was aubasidan-like *β*-glucan.

Keywords: *Aureobasidium pullulans*, *β*-glucan, olive oil

Introduction

Aureobasidium pullulans is a yeast-like fungus well-known for the production of biodegradable exopolysaccharide (EPS) called pullulan. The major structure of pullulan is *α*-1,6- linked polymaltotriose, or a linear glucan containing *α*-1,4 and *α*-1,6 linkages in a ratio of 2:1 (Sowa *et al*. 1963). Pullulan is colorless, tasteless, odorless, non-toxic and highly water-soluble (Leathers, 2003). It has been used as biofilms and adhesives in food, drug, and cosmetic industries. In addition to pullulan, certain isolates of *A. pullulans* have been reported to produce other structurally different EPSs. Kikuchi *et al*. (1973) described an insoluble heteropolysaccharide from *A. pullulans* that contained glucose, mannose and galactose in a ratio similar to that of the polysaccharide from cell wall extracts. Elinov *et al*. (1987) reported that *A. pullulans* produced a glucan with *β*-1,3 linked backbone and *α*-1,4 linked side chains attached by *β*-1,6 linkages which was later named aubasidan. The *β*-glucan from *A. pullulans* was recently reported to exhibit some interesting biological properties such as antitumor and

antiosteoporotic activities and also food allergy prevention (Tada *et al*. 2008). In Thailand, a number of *A. pullulans* strains have been isolated from air-borne spores (Punnapayak *et al*. 2003), foliar samples (Prasongsuk *et al*. 2005; Manitchotpisit *et al.* 2009), bathroom cement walls, and latex-painted surfaces (Prasongsuk *et al*. 2005; Lotrakul *et al*. 2009). In 2009, Manitchotpisit *et al*. reported two non-pullulan (NP) producing strains of *A. pullulans* NRRL 58539 and NRRL 58543, from 2 geographically isolated areas in Thailand and the multilocus phylogenetic analysis placed them on a separated clade from the other *A. pullulans*. Recently, Lotrakul *et al*. (2013) reported the production of *β*-glucan from these *A. pullulans* NRRL 58543 and NRRL 58539 for the first time in Thailand in which *A. pullulans* NRRL 58543 was found to produce higher *β*-glucan yield than that from NRRL 58539. Thus, *A. pullulans* 58543 should be further subjected for optimization of *β*-glucan production.

In this research, the optimization of *β*-glucan production from *A. pullulans* NRRL 58543 was carried out by varying some important factors in the production medium including carbon, nitrogen and nutrient supplement. Moreover, the effects of initial pH and incubation temperature on *β*-glucan production were also investigated. The structural analyses of produced *β*-glucan were performed using FTIR and NMR techniques.

Methodology

1. Microorganisms

Aureobasidium pullulans NRRL 58543 and *A. pullulans* var. *aubasidani* NRRL 58013 were obtained from yeast culture collection of Plant Biomass Utilization Research Unit, Department of Botany, Faculty of Science, Chulalongkorn University and were maintained on yeast malt agar (YMA) at 4 ^{o}C.

2. Optimization of *β*-glucan production

Medium composition and culture conditions

The *β*-glucan was produced by growing *A. pullulans* NRRL 58543 in 100 ml of production medium (PM) (Prasongsuk *et al*. 2007) in a 250-ml flask at room temperature (30±2°C) with initial pH at 6.5 under shaking condition (150 rpm). The *β*-glucan was collected from culture supernatant every 3 days for 12 days by centrifugation at 6,000 g for 15 min to remove yeast cells and the supernatant was precipitated using two volumes of 95% ethanol (Prasongsuk *et al*. 2005). The *β*-glucan was then oven dried at 60°C and its dry weight was recorded. The experiments were performed in triplicate.

Effect of carbon and nitrogen sources on the β-glucan production

The optimization of carbon and nitrogen sources for *β*-glucan production by *A. pullulans* NRRL 58543 was carried out in two stages. Firstly, the factorial experimental design was carried out with two factors of carbon (glucose and sucrose) and nitrogen sources (peptone, sodium nitrate and potassium nitrate) in two and three levels, respectively. After that, a central composite design (CCD) and response surface methodology (RSM) in three levels for each independent variable

was adopted to find the optimum concentration. The best carbon resulted from the above studies was varied at the final concentration of 5, 6 and 7% (w/v) and the best nitrogen was varied at 0.04, 0.06 and 0.08 % (w/v). The obtained responses, *β*-glucan yields, were fitted to a second-order polynomial model and were statistically analyzed using the Desing-Expert software versions 6 for regression coefficients, variable of the model (ANOVA) and the three-dimensional response surface plot (RSM).

Effect of nutrient supplement on the β-glucan production

The effect of additional nutrient in the PM containing the optimal carbon and nitrogen sources from above experiment on *β*-glucan production was examined using olive oil in the concentration of 0, 3, 5 and 7% (v/v). The *β*-glucan yield was assessed as described above. The experiment was performed in triplicale.

Effects of pH and temperature on the β-glucan production

The effects of physical factors including initial pH and incubation temperature on β-glucan production was determined by modification the initial pH at 5.0, 5.5, 6.0, 6.5 and 7.0. The incubation temperature for *β*-glucan production was varied at 25, 28 and 30 ^{o}C. The *β*-glucan yield was assessed as described above. The experiment was performed in triplicate.

Statistical analysis

All the obtained data were subjected to statistical calculate using one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) or Student's t-test (2 tailed) with the SPSS 17.0 software package (SPSS Inc., Chicago, U.S.A.). Differences at $P < 0.05$ were considered significant.

3. Structure analysis of *β*-glucan

The IR spectra of *β*-glucan were determined using potassium bromide (KBr) technique on a Fourier Transform Infrared Spectrometer (Perkin-Elmer, Spectrum One, USA). The average of 16 scans at a resolution of 4 cm^{-1} was performed per sample (Prasongsuk *et al*. 2007; Manitchotpisit *et al*. 2009). The *β*-glucan was dissolved in D_2O to the final concentration of 0.05 mg/ml and was subjected to NMR analyses. The NMR (^{1}H and ^{13}C) spectra were obtained at 50 °C using a Varian Inova-500 NMR System equipped with a CP/MAS solid state probe and nano probe, operating at 500 MHz (Prasongsuk *et al*. 2007; Manitchotpisit *et al*. 2009).

Results

1. Optimization of the *β*-glucan production

Effect of carbon and nitrogen sources on the β-glucan production

For the effect of carbon sources and nitrogen sources on *β*-glucan production, *A. pullulans* NRRL 58543 gave the highest *β*-glucan yield at 8.75±0.03 g/L from the production medium containing sucrose and NaNO3. The concentrations of sucrose and NaNO3 in the production medium were then optimized using response surface methodology (Table1).Using the Desing-Expert program version 6, a second-order polynomial equation for the experimental data was deduced as follows:

Equation# 1: $Z = 8.950-0.340x_1+0.550y_2 -1.570x_1^2-1.230y_2^2+0.760x_1y_2$

Table1: Central Composite Design (CCD) and the observed responses for optimization of the *β*-glucan production by *A. pullulans* NRRL 58543

Natural variables		Coded variables		Response
Sucrose (%) (w/v)	$NaNO_3$ (%) (w/v)	X1	X2	yield (g/l)*
5.0	0.35	-1	-1	5.55±0.02
5.0	0.50	-1	0	4.64±0.05
5.0	0.65	-1	1	6.73±0.02
6.0	0.35	0	-1	7.21±0.03
6.0	0.50	0	0	8.75±0.03
6.0	0.65	0	1	6.49±0.03
7.0	0.35	1	-1	3.32±0.02
7.0	0.50	1	0	5.72±0.01
7.0	0.65	1	1	7.54±0.02

*Mean values ± one standard deviation derived from three replications

According to the CCD model, the actual relationship between the response and significant variables was analyzed. It indicated the variability in the response that could be explained by the 2nd-order polynomial predictive equation given above with a satisfactory coefficient of determination (R^2=0.82). In addition, the P-value was 0.03 that confirmed the fit of polynomial model. The 3D profile of response surface and its contour of the optimal production were shown in Fig. 1. The values in the figure were transformed back to the uncoded (real) values. The *β*-glucan yield at approximately 8.75 g/l (or within the range of 6.29-8.17%) was predicted to be the maximal value and the optimal concentrations of sucrose and NaNO3 for the *β*-glucan production, as predicted by the RSM, were at 6% and 0.06% (w/v), respectively.

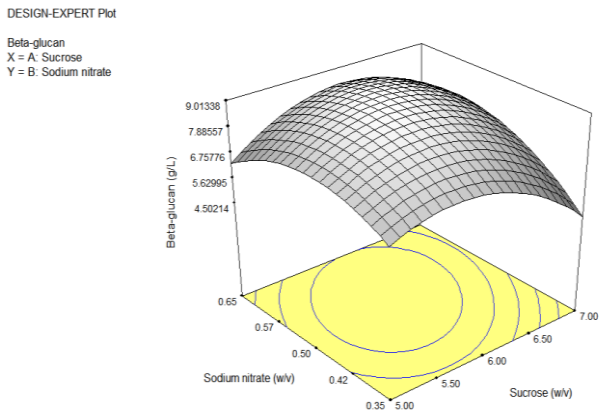

Figure 1: Response surface as a function of sucrose and NaNO3 for the *β*-glucan yield.

To confirm this prediction, the *β*-glucan production in the medium containing sucrose and NaNO3 at the suggested concentrations was repeated. The highest *β*-glucan yield was obtained at 7.21±0.03 g/l which fell within the estimated range, albeit on the lower side, thus confirming the calculation.

Effect of nutrient supplement on β-glucan production

In order to enhance *β*-glucan production from *A. pullulans* NRRL 58543, the effect of olive oil supplement in PM with optimal concentration of sucrose and NaNO3 as described above was investigated. The highest production of *β*-glucan was found in PM supplemented with 5 % (v/v) olive oil (17.57±0.01 g/L) which was significantly higher than those in PM supplemented with 0 % (7.06±0.03 g/L), 3% (16.21±0.18 g/L) and 7% (17.22 ± 0.35 g/L) of olive oil.

Effect of pH on β-glucan production

The effect of pH on *β*-glucan production of *A. pullulans* NRRL 58543 was also investigate by adjusting the initial pH in the range of 5.0, 5.5, 6.0, 6.5 and 7.0 in PM supplemented with olive oil at 5 % (v/v). It was found that *A. pullulans* NRRL 58543 could produce the highest *β*-glucan yield at pH 6.5 (19.52±0.05 g/L) followed by pH 5.5 (18.88±0.30 g/L) and pH of 6.0 (16.21±0.19 g/L).

Effect of temperature on β-glucan production

The effect of temperature on *β*-glucan production was investigated in different temperature at 25, 28 and 30 ^{0}C. The maximum yielded of *β*-glucan production from selected strain was found to be (21.04±0.19 g/L) after incubation at 25^{o}C that followed by (19.36±0.41 g/L) at 28 °C and (19.04 ±0.22 g/L) at 30°C.

2. Structural analyses of *β*-glucan

The structural analysis of *β*-glucan by FT-IR spectra was compared with aubasidan from *A. pullulans* NRRL 58013 (Table 5). The structure of aubasidan and *β*-glucan contained various

functional groups including alkane, carbonyl, ether, hydroxyl, hydroxyl bonding in alcohol and primary alcohol. Functional group indicated *α*-configuration was not found in *β*-glucan produced from *A. pullulans* NRRL 58543 while that indicated *β*-configuration was presented. This structure was as same as aubasidan produced from *A. pullulans* var. *aubasidani* NRRL 58013 which the peak at $\lambda = 891\ cm^{-1}$ was found. Structural analysis of the *β*-glucan from *A. pullulans* NRRL 58543 using ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) revealed the absence the peaks representing *α*-1,6- and *α*-1,4-configuration, which similar to the aubasidan produced by *A. pullulans* NRRL 58013 (Fig. 2). Multiple peaks representing the *β*-1,3 and *β*-1,6 configurations were detected in both the *β*-glucan and aubasidan (chemical shifts for *β*-1,3 /*β*-1,6 at 4.362, 4.363, and 4.408 ppm/4.211, 4.221, 4.246 and 4.270 ppm and aubasidan at 4.513 and 4.497 ppm /4.265, 4.257, 4.249, and 4.241 ppm). For ^{13}C-NMR analysis, peaks of *β*-glucan at around C-1 of *α*-1, 4 (about 100.870 ppm), C-1 of *α*-1, 6 (about 98.530 ppm), C-3 of *β*-1, 3 (about 80.770 ppm) O-substituted C-6 (about 67.350 ppm) and C-6 (about 62.208 ppm) similar to those of aubasidan. However, the peak C-4 (about 79.115 ppm) and peak C-1 of *β*-1,6 (about 104.200 ppm) of the produced *β*-glucan were not detected while they were found in the structure of aubasidan (Fig. 3).

Table 5: Functional groups and the major peaks from FT-IR of *β*-glucan produced by *A. pullulans* NRRL 58543 compared with aubasidan from *A. pullulans* var. *aubasidani* NRRL 58013

Functional groups	FT-IR (wavenumber, cm^{-1})	
	β-glucan (NRRL 58543)	aubasidan (NRRL 58013)
-OH	3411.53	3428.40
-C-H	2922.04	2928.09
C=O	2134.91	2131.33
-C-OH	1426.02	1425.05
-OH bonding in alcohol	1374.85	1373.92
C-O	1078.78	1078.76
α-configuration	-	-
β-configuration	890.58	891.88

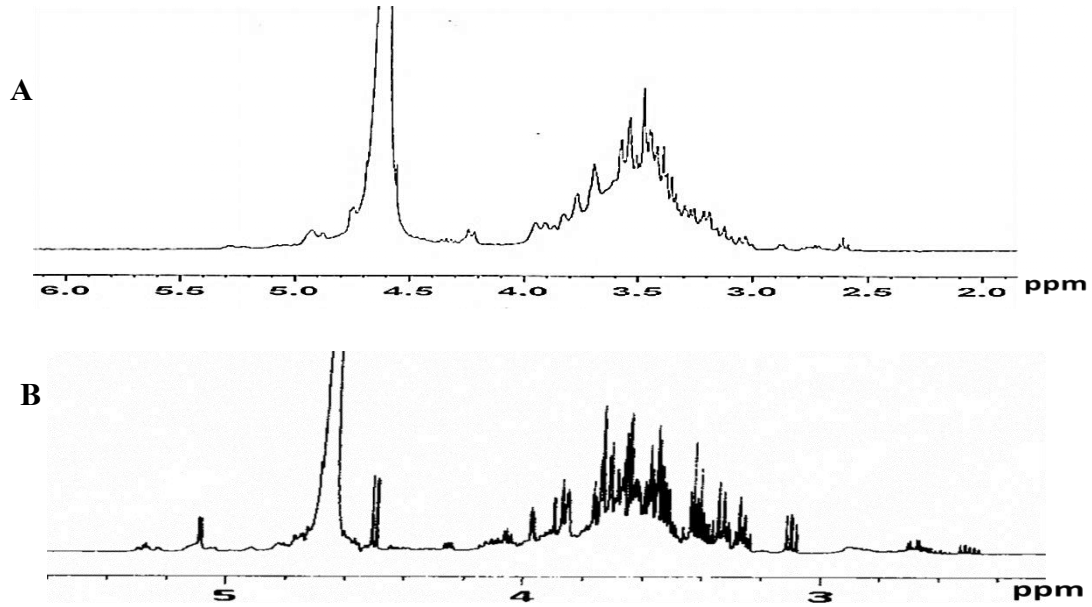

Figure 2: ^{1}H-NMR spectra (A) *β*-glucan from *A. pullulans* NRRL 58543 compared with (B) aubasidan from *A. pullulans* var. *aubasidani* NRRL 58013

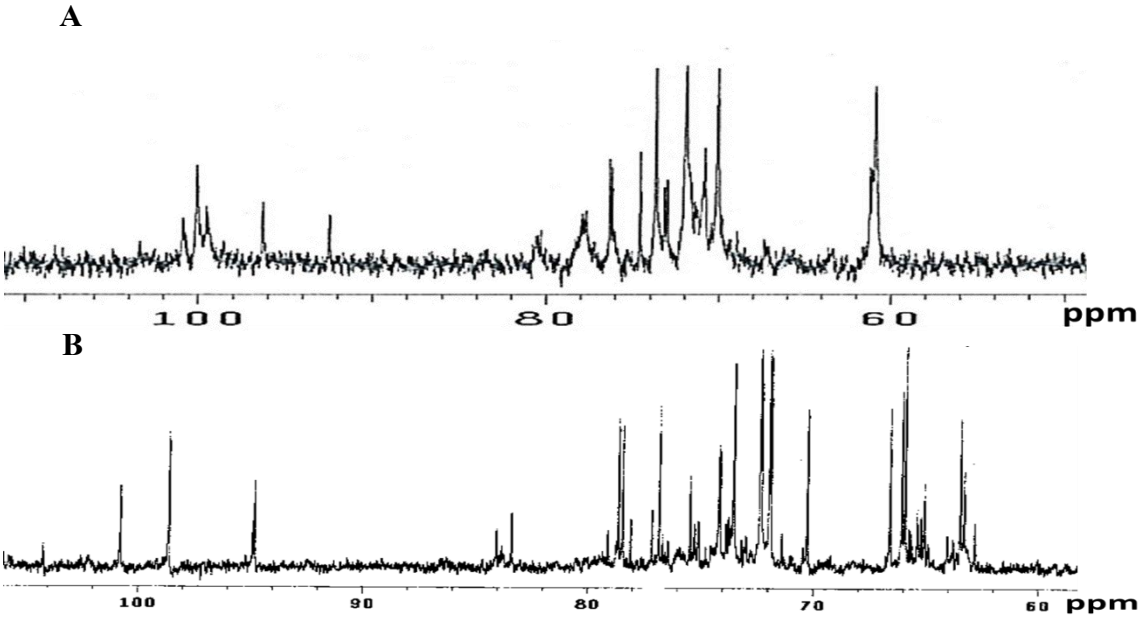

Figure 3: ^{13}C-NMR spectra (A) *β*-glucan from *A. pullulans* NRRL 58543 compared with (B) aubasidan from *A. pullulans* var. *aubasidani* NRRL 58013.

Discussion

β-glucan is a biopolymer that has been gaining interest due to its multiple functional and bioactive properties to against insulin resistance, dyslipidemia, hypertension, and obesity (Khoury *et al*. 2012). *β*-glucan can be produced from yeasts including *A. pullulans*. The *β*-glucan production in different conditions resulted in different production profiles which were independently related with the cell growth in some case. Sucrose and glucose were tested to

find a suitable carbon source for the production of *β*-glucan. The significantly high yield of *β*-glucan was obtained with sucrose for *A. pullulans* NRRL 58543 that was in accordance with the previous report of glucan production from *A. pullulans* (Reese and Maguire, 1971). Among nitrogen sources, NaNO3 resulted in increasing of *β*-glucan production with corresponded well with Yurlova *et al.* (1995) which found that the highest yield of aubasidan produced from *A. pullulans* var. *aubasidani* was obtained when $NaNO_3$ was used as the sole nitrogen source. The concentration of carbon and nitrogen or C/N ratio was also the important factor for the production of metabolites from microorganism. This study predicted the optimal concentration and the yield of β-glucan by RSM using CCD (Ghadge and Raheman, 2006). It was found that 6% (w/v) sucrose and 0.06 % (w/v) $NaNO_3$ gave the highest *β*-glucan yield (8.75±0.01 g/L). Prasongsuk *et al.* (2007) reported that the similar C/N ratio provided the highest yield of pullulan produced from a tropical *A. pullulans* NRM2. Vegetable oil is the rich mineral source of vitamins, fatty acids and amino acid that can act as coenzyme in high-energy required process of normal cell function. Therefore, the cultivation medium supplemented with vegetable oil has been reported to enhance the growth rate and enzyme production in several microorganisms including *A. pullulans*. There was a report concerning the effect of vegetable oil on the enhancement of pullulan production from *A. pullulans* MTCC 2195 (Thirumavalavan *et al.* 2009). To our knowledge, this is the first study of the influence of olive oil on the *β*-glucan production in this yeast. The similar result was also reported in which the *β*-glucan production was increased in higher concentration of olive oil in culture medium which increase *β*-glucan due to vitamins, containing fatty acid, in olive oil. The vitamins are involved in the catalyst. The highest *β*-glucan yield was found to be 17.57±0.01 g/L at 5% (v/v) of olive oil which the consistent with Roukas (1999) concerning the production of pullulan from the waste of beer factory by *A. pullulans* P56. It was found that 6.0±0.3 g/L of pullulan was obtained from the base medium without supplements and it was increased to 8.5±0.3 g/L when 2.5 % (v/v) of olive oil was supplemented into the production medium. The initial pH and incubation temperature are important factors for production of metabolites from microbes. So far, there was no report concerning the effects of pH or temperature on β-glucan production from this yeast. *A. pullulans* NRRL 58543 preferred mild acidic pH at 6.5 for β-glucan production. Several researches reported the maximum pullulan production at pH range of 3.0–7.0 (Prasongsuk *et al.* 2007; Thirumavalavan *et al.* 2009).The temperature is one of important factors that affected with the growth of *A. pullulans* and *β*-glucan production. This fungus has been reported to produce yeast-like cells at 25ºC to 28ºC that mainly responsible for high yield of EPS production but the production and growth decreased when the temperature increased. Ueda *et al.* (1963) found that productivity of pullulan at 25 ºC was higher than that at 30 ºC and tended to decrease when the temperature was higher. West and Hamer (1993) studied the effect of temperature on the production of pullulan at 23 – 33 ºC and found that the optimal temperature was 24–26ºC. However, Singh *et al.* (2012) reported that *A. pullulans* RG-5 yeast like cells could be survived and able to produce in high amount of pullulan after cultivation at 42°C. Lotrakul *et al.* (2013) reported that the exoposaccharide produced from *A. pullulans* NRRL 58539 and NRRL 58543 was similar to the aubasidan produced by *A. pullulans* CBS 100524 deduced from NMR (^{13}C and

^{1}H) and FT-IR. At the optimal condition, the β-glucan from *A. pullulans* NRRL 58543 was analyzed for its structure. The NMR and FT-IR results of produced β-glucan from this strain were mostly similar to that of aubasidan produced by *A. pullulans* var. *aubasidani* NRRL 58013 (Yurlova and De Hoog, 1997). From FT-IR spectra, the b-configuration (λ = 890 cm^{-1}) was found in both β-glucan from *A. pullulans* NRRL 58543 and aubasidan from *A. pullulans* NRRL 58013 indicated that *A. pullulans* 58543 produced glucan with β-configuration under optimal condition. This β-configuration was also found in the structure of glucan produced by *A. pullulans* 105-22 (Leal and Serrano, 1980). The ^{1}H-NMR peaks of *β*-1,3 *β*-1,6 *α*-1,4 and *α*-1,6 in the structure of the *β*-glucan produced from NRRL 58543 were similar to those of aubasidan. However, The ^{13}C-NMR peaks of aubasidan indicating C-4 and C-1 of *β*-1,6 were not found in those of *β*-glucan produced by *A. pulluans* NRRL 58543. The results suggested that *A. pullulans* NRRL 58543 produced aubasidan-like β-glucan polysaccharide.

Conclusion

The highest yield of *β*-glucan at (21.04±0.19 g/l) was achieved when *A. pullulans* NRRL 58543 was grown in the production medium containing 6% (w/v) sucrose, 0.06% (w/v) $NaNO_3$, and 5% (v/v) of olive oil as a nutrient supplement, initial pH 6.5 and at temperature 25°C. The structural analyses using FT-IR and NMR (^{1}H and^{13}C) also suggested that this *β*-glucan was aubasidan-like *β*-glucan.

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