ORIGINAL ARTICLE

Pullulan Production Utilizing Various Carbon Sources by *Aureobasidium melanogenum* DSM 2404 as Biotherapeutic Tool in Biomedical Applications

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ABSTRACT

Introduction: Pullulan is a biodegradable biopolymer made up of maltotriose subunits that are water-soluble. It has a direct glucan structure comprising α-1,4 and α-1,6 linkages in a 2:1 ratio, which gives it exceptional physical and structural properties, making it a suitable choice for application in biomedical applications. Despite its potential benefits, the price of production is expansive, and productivity is low, which are significant drawbacks. Therefore, the objective of this work is to identify the ideal carbon source for *Aureobasidium melanogenum* DSM 2404 to produce high pullulans. **Methods:** In shake flask studies, the effects of different carbon sources, such as maltose, glucose, sucrose, lactose, and xylose, on cell growth and pullulan generation by Aureobasidium melanogenum DSM 2404 were examined. **Results:** The highest pullulan production was obtained from sucrose (13.38 g L^{-1}) compared to other types of carbon sources used in the fermentation medium. The maximal pullulan production rate of 0.112 [g^{-1} L^{-1} h^{-1}] was obtained in sucrose culture. This suggests that sucrose provides favorable conditions for the microorganisms to produce pullulan at a faster rate compared to other sugars. **Conclusion:** Sucrose was found to be the most efficient carbon source for the synthesis of pullulan using *Aureobasidium melanogenum* DSM 2404 generating 13.38 g L^{-1} of pullulan.
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INTRODUCTION

Polysaccharides, often known as a type of natural macromolecular polymer typically consist of chains with either straight or branched glycosidic linkages to interconnect more than ten monosaccharides. Its molecular weight (Mw) can be hundreds or a billion times higher [1]. There are three types of polysaccharides such as capsular polysaccharides (CPSs), lipopolysaccharides (LPSs) and xopolysaccharides (EPSs) [2]. The majority of CPSs are linked to bacteria's pathogenicity and virulence enhancing factors [3]. While for LPSs, most Gram negative bacteria's outer membrane contains LPSs, and some bacteria create LPSs as a virulence factor [4,5]. Lastly, EPSs are produced outside of a cell (extracellular) and this polymer is mostly high Mw [6]. Currently, there are many important EPSs for industrial applications such as kefiran [7], xanthan [8] and pleuran [9]. Microbial polysaccharides have gained significant attention due to their unique properties and wide range of applications in various industries.

One of the most studied and industrialized EPSs named pullulan. It has garnered significant attention

from researchers and industries alike due to its unique properties. During the fermentation of Aureobasidium pullulans, Bauer [10] first examined pullulan. Later, Bernier [11] successfully extract and identify pullulan from the culture solutions of A. pullulans. According to Bender's investigations [12], which showed that it was exclusively composed of -D-glucans with a preponderance of -(1(4) linkages and had a positive optical rotation, the term "pullulan" was given. It is highly soluble in water, forming clear solutions, and can form films with excellent oxygen and moisture barrier properties. Pullulan films are also flexible, transparent, and have good mechanical strength.

The pullulan market has experienced significant growth in recent years due to its versatile properties and wide range of applications. Pullulan's unique characteristics, such as film-forming ability, solubility, and biocompatibility, have made it a sought-after ingredient in various industries. According to market analysts, from 2017 the pullulan market is anticipated to develop at a compound annual growth rate (CAGR) of about 2.2 percent, beginning with a value of US$129 million and reaching US$130 million by 2023[13]. Additionally, according to Adroit Research Market [14] the COVID-19 pandemic has caused the pullulan market size to generally be evaluated to be worth US$ 68 million in 2022 and to increase to US$ 89 million by 2029 with a CAGR of 4.6% during the operational time window 2022-2028. Pharmaceutical firms currently control the largest industries, accounting for about 40.74 percent of the global market share in 2015. The largest market was Japan, with a market share of 667 million tonnes, followed by China (20.65%) and the USA (29.65%) [13]. Overall, the pullulan market is expected to continue growing as industries recognize its potential in various applications. The demand for natural and sustainable ingredients, coupled with the expanding food, pharmaceutical, and packaging sectors, is likely to drive the market's future growth.

The applications of pullulan in biomedical industries are extensive [15]. Pullulan is inert and has non-toxic, non-immunogenic, and biocompatible characteristics that make it broadly used in the biomedical field [16]. Pullulan has a high concentration of hydroxyl groups which could be used as a bioactive polymer and dextran-based blood plasma substitute [17]. Moreover, it can also be shaped into pills or tablets size for the use of pharmaceutical and nutraceutical purposes, such as NPcaps capsules from Capsugel® company, US [18]. Due to pullulan wide applications, it is required to identify the best production strategy for the high production of pullulan. It is worth noting that pullulan production strategies may vary depending on the specific application and industry requirements. Therefore, a thorough understanding of the production process, continuous monitoring, and optimization efforts are necessary to develop the most efficient strategy for high pullulan production. Identifying the best production strategy for high-yield pullulan production is crucial to meet the increasing demand for this versatile polysaccharide. Several factors need to be considered when developing an optimal production strategy, including the choice of microbial strain, fermentation conditions, and downstream processing. By comparing various types of carbon sources, this study sought to identify the best carbon source for Aureobasidium melanogenum DSM 2404 to produce pullulan. The study would typically involve selecting a range of carbon sources to evaluate their impact on pullulan production. Common carbon sources tested for pullulan production include maltose, glucose, sucrose, lactose, and xylose. Each carbon source has a different composition and influence pullulan production differently.

MATERIALS AND METHODS

Microorganism

The research employed Aureobasidium melanogenum DSM 2404 (formerly Aureobasidium pullulans var. melanogenum), which was obtained in freeze-dried form from the Leibniz Institute DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures GmbH).

Preparation of Inoculum and production medium

One mL of a functioning cell bank containing the fungal culture was added to a 250 mL Erlenmeyer flask that contained 50 mL of sterile seed media (SM) to create the inoculum[19]. The SM was made up of the following components per liter of distilled water: sucrose (30.0 g), $(NH_4)2SO_4$ (0.6 g), yeast extract (0.4 g), K_2HPO_4 (5.0 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (1.0 g) and $MnCl_2$ (0.01 g), with the pH adjusted to 5.5 before sterilization for 20 minutes at 121°C. Separately, sucrose was sterilized for 20 minutes at 110°C and added to the Erlenmeyer flask containing the 50 mL sterile SM after cooling. The inoculated flask was then incubated for 48 hours at 200 rpm and 28°C in a rotary shaker incubator. After 48 hours of incubation, 5% of the inoculum was transferred into the production medium, which contained the following components per liter of distilled water: maltose (50.00 g), yeast extract (10.00 g), peptone (20.00 g), $(NH_4)_2SO_4$ (0.60 g), K_2HPO_4 (5.00 g), $MgSO_4.7H_2O$ (0.60 g), and NaCl (1.00 g).

Effect of Different Types of Carbon Sources

In order to find the optimal carbon source for Aureobasidium melanogenum DSM 2404 to produce high pullulans, various carbon sources including maltose, glucose, sucrose, lactose, and xylose were

examined. The production medium composed of (in g L^{-1}): yeast extract, 10.00; peptone, 20.00; (NH4)2SO4, 0.60; K_2HPO_4, 5.00; $MgSO_4$.7H2O, 0.60; NaCl, 1.00; was used in this study. Separately, each carbon source was sterilized for 20 minutes at 110°C, which later was transferred into the production medium. After inoculating the production medium with 5% of the inoculum, for 48 hours at 200 rpm and 28°C, the flask was incubated in a rotating shaker incubator.

Cell Dry Weight Determination

Samples in all flasks containing 50 ml each were withdrawn after 72 hours of cultivation in a centrifugation tube. The culture broth was then centrifuged in 50 ml falcon tubes at 7000 rpm for 15 minutes to settle the cells. Gravimetric methods were used to determine the cell dry weight.

Crude EPS Determination

Crude EPS was precipitated and separated from the supernatant. Then it underwent further analysis using method given by [20]. A 50 mL conical centrifuge tube was filled with 10 mL of supernatant and two volumes (20 mL) of pre-chilled 95 percent ethanol to extract the EPS. The ethanol-supernatant mixture was then chilled at 4°C overnight to precipitate the EPS. The ethanol-supernatant combination was centrifuged the following day at 27°C for 10 minutes at 7000 rpm. After removing the supernatant, the EPS was dried at 80°C overnight before being weighed.

RESULTS

Two monosaccharides (glucose and xylose) and three disaccharides (maltose, sucrose, and lactose) were studied for their ability to support the production of CDW and pullulan. The results of CDW and pullulan production after five days of cultivation was presented in Figure 1. Control indicates cultivation medium without any C source. For CDW production, the highest yield was obtained from glucose (13.08 g L^{-1}), continued via maltose (11.73 g L^{-1}), sucrose (9.94 g L^{-1}), lactose (5.73 g L^{-1}), and xylose (5.51 g L^{-1}). For pullulan production, the highest yield was obtained from sucrose (13.38 g L-1), continued via maltose (12.13 g L^{-1}), glucose (5.70 g L^{-1}), xylose (2.35 g L^{-1}), and lactose (1.71 g L^{-1}). The highest cell productivity of 1.35 g g^{-1} was achieved by using sucrose as the sole C source, while the lowest was 0.30 g g^{-1} from lactose. The pH readings are ranging from 5.09 to 6.77.

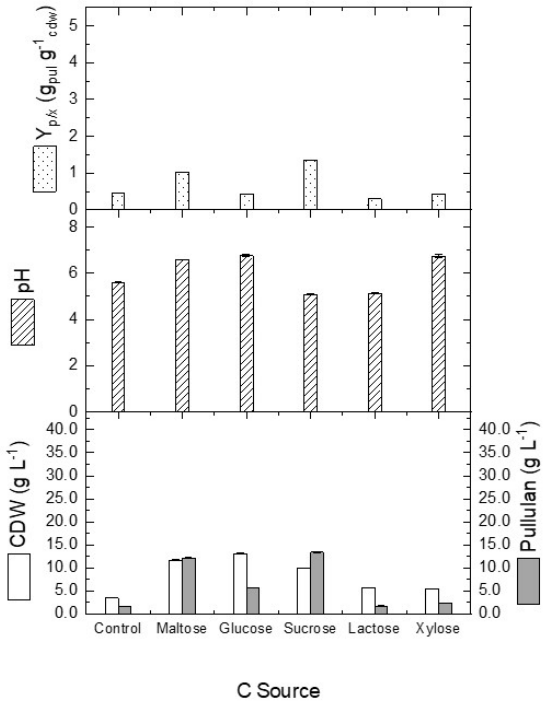

Figure 1 : The results of CDW and pullulan production after five days of cultivation.

Table I : Kinetic parameters for cell growth and pullulan production during batch cultivation of *Aureobasidium melanogenum* in shake flask culture (data were taken after 120 hours cultivation, and n=3)

Carbon Source	X_{max} [g L^{-1}]	P_{max} [g L^{-1}]	dx/dt [g^{-1} L^{-1} h^{-1}]	dp/dt [g^{-1} L^{-1} h^{-1}]	$Y_{P/X}$ [g g^{-1}]
Control	3.51±0.18	1.61±0.02	0.029	0.013	0.459
Maltose	11.73±0.35	12.13±0.1	0.098	0.101	1.034
Glucose	13.08±0.11	5.70±0.03	0.109	0.048	0.436
Sucrose	9.93±0.05	13.38±0.18	0.082	0.112	1.346
Lactose	5.72±0.04	1.71±0.02	0.048	0.014	0.299
Xylose	5.51±0.01	2.40±0.07	0.046	0.020	0.427

Notes: X_{max}: maximal cell dry weight; P_{max}: maximal pullulan production; dx/dt: growth rate; dp/dt: production rate; $Y_{P/X}$: Specific production.

To comprehend the impact of the carbon source on the kinetics of cell development and pullulan formation, Table I summarized the influence of carbon source on the overall primary and secondary kinetic parameters during cultivation at the shake flask level.

According to table I, the cell development rate and production rate were highly influenced by the type of sugar used during fermentation. The maximal pullulan production rate of 0.112 [g^{-1} L^{-1} h^{-1}] was obtained in sucrose culture whereas, the lowest value of 0.014 was in lactose culture. This suggests that sucrose provides favorable conditions for the microorganisms to produce pullulan at a faster rate compared to other sugars. In contrast, lactose resulted in the lowest pullulan production rate of 0.014. On the other hand, a maximal growth rate of 0.109 [g^{-1} L^{-1} h^{-1}] was attained in glucose culture followed by maltose culture. This implies that glucose and maltose are more efficiently utilized by the microorganisms for their growth during fermentation compared to other sugars.

DISCUSSION

Aureobasidium sp. was found to be able to utilize various C sources [20, 21]. Most of the studies found that the pullulan yield was higher when using sucrose as a C source [20, 22, 23]. Sucrose is the ideal C source for pullulan synthesis in A. pullulans CGMCC1234, according to Sheng et al [24]. In his research, he studied the relationships between different C sources and the β-fructofuranosidase enzyme's activity. In reduced concentrations of sucrose, this enzyme catalyzes sucrose to glucose and fructose. Furthermore, this enzyme can aid in kestose synthesis by combining fructose with sucrose at a higher sucrose concentration [25]. Sheng and associates found that the activity of -fructofuranosidase was highest when sucrose was employed as the carbon source, and the lowest value was noted when the strain was grown in media containing different carbon sources. The extracellular fluid's osmotic stress can be reduced by increasing the level of this enzyme, which promotes A. pullulans' growth rate and causes it to release more pullulan in the initial stages of fermentation. Besides that, sucrose is a disaccharide composed of glucose and fructose [26]. It has a relatively high carbon content, which makes it a suitable substrate for microbial growth and polysaccharide production [27]. Pullulan is a polysaccharide consisting of repeating maltotriose units (three glucose units), and sucrose provides the necessary carbon atoms for the biosynthesis of pullulan [28]. Sucrose is relatively inexpensive compared to other carbon sources, such as glucose or maltose [29]. Its low cost and ready availability make it an attractive choice for industrial-scale pullulan production, as it helps keep the production costs down.

However, higher pullulan production can be also be achieved from other C sources such as glucose [30, 31, 32]. Glucose, a monosaccharide, can also be utilized as a carbon source for pullulan production [33]. It can be obtained from various sources, such as corn syrup or glucose syrup. Some pullulan-producing microorganisms, such as Aureobasidium pullulans, have the ability to metabolize glucose efficiently for pullulan synthesis [34]. Elevated levels of glucosyltransferase, UDPG-pyrophosphorylase and α-phosphoglucose mutase activities remained observed when A. pullulans Y68 was cultured in the presence of glucose [14]. These enzymes were found to be involved in the pullulan synthesis process. Although glucose act as a potential C source to produce high CDW, the pullulan production from glucose is lower than sucrose. Hence, sucrose is used as a potential C source and undergoes further optimization.

The maximal specific production of pullulan of 1.34 [g g^{-1}] was obtained in sucrose culture followed by maltose culture. This indicates that sucrose and maltose promote higher pullulan production relative to the amount of biomass formed by the microorganisms. This finding showed similar result from previous in which high sucrose and maltose concentration was used to support high pullulan production [35].

These results suggest that different sugars provide varying conditions for pullulan production. Sucrose seems to be particularly effective in promoting both pullulan production rate and specific production, while glucose and maltose favor microbial growth rate. Lactose, on the other hand, appears to be less favorable for pullulan production based on the lower production rate observed. It is important to note that these results are specific to the study or experiment mentioned and may vary depending on the microbial strain, fermentation conditions, and other factors. Further research and experimentation are necessary to confirm and generalize these findings in different contexts

CONCLUSION

This study investigate the effect of various carbon sources on Aureobasidium melanogenum DSM 2404 growth and its production of pullulan in shake flasks. According to the findings, sucrose was the best carbon source for pullulan synthesis as demonstrated by Aureobasidium melanogenum DSM 2404's output of 13.38 g L^{-1} after 120 hours of growth. On the other hand, the maximal biomass of 13.08 g L^{-1} was obtained in glucose culture. Further studies are now

going in our laboratories for in-depth study of the biosynthesis regulation using mixed substrate at the bioreactor level for bioprocess industrialization of this important polysaccharide.

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