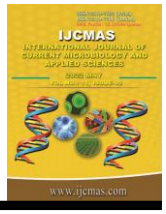
Int.J.Curr.Microbiol.App.Sci (2022) *11*(05): 319-324

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 11 Number 05 (2022)**
Journal homepage: http://www.ijcmas.com



Original Research Article https://doi.org/10.20546/ijcmas.2022.1105.036

Production of *Aureobasidium pullulan* by using Sugar Cane Molasses as Economical Media

Ranjan Singh[1]*, Shreyansh Singh[2] and Rajeev Gaur[1]

[1]*Department of Microbiology, DST-FIST Supported, Centre of Excellence, Govt. of U.P., India*
[2]*Department of Environmental Sciences, Dr. Ram Manohar Lohia Avadh University, Ayodhya, U.P., India*

**Corresponding author*

Keywords

Aureobasidium pullulans, linear α-D-glucan, tasteless, odourless, tenacious

Article Info

Received:
05 April 2022
Accepted:
30 April 2022
Available Online:
10 May 2022

A B S T R A C T

Aureobasidium pullulans, popularly known as black yeast, is one of the most widespread saprophytic fungi associated with wide range of terrestrial and aquatic habitats. The fungus has widely been employed in production of an economically important polysaccharide Pullulan. Pullulan is a linear glucan made mainly of maltotriose repeating units. This gives the polysaccharide structural flexibility and enhances solubility. This polysaccharide is of great economic importance with increase applications in food, pharmaceutical, agriculture, blood plasma substitute and chemical industry. Production of pullulan was observed in a novel media consisting of Sugar Cane Molasses (SCM) and distilled water. It was found that high amount of pullulan (2.5g /100ml) was obtained at 30°C, pH 5 at a concentration of 2.5% media in 96h. No synthetic minerals were used in the media for the growth of polysaccharide making it very economical.

Introduction

Aureobasidium pullulans is cosmopolitan yeast like fungus that occurs in diverse habitats, including the phyllo-sphere of many plants and also on various tropical fruits. *A. pullulans* is industrially important because of its capacity to produce the polysaccharide Pullulan. Pullulan is a transparent, colourless, tasteless, odourless, tenacious, resistant to oil and grease and unaffected by small thermal variations. It is soluble in cold and hot water and insoluble in organic solvents. It is a linear α-D-glucan, made mainly of maltotriose repeating units interconnected by -1, 6 linkages. The regular alternation of -1, 4 and - 1, 6 bonds results in two distinctive properties that is structural flexibility and enhanced solubility. This polysaccharide is of great economic importance with increased applications in food, pharmaceutical, agricultural, blood plasma substitute and chemical industries (Gaur *et al.*, 2010; Singh *et al.*, 2015). The cost of Pullulan primarily depends on the raw materials, especially of carbon

source, which play a major role in the economics of pullulan production. The sugars such as sucrose, glucose, fructose, maltose, starch support pullulan production by *A. pullulans* (Singh *et al.,* 2012; Singh *et al.,* 2016; Singh *et al.,* 2017). A number of complex carbon sources have been reported for pullulan production, including peat hydrolysate, cornmeal hydrolysates, corn syrup, fuel ethanol fermentation stillage, carob pod, grape skin pulp, olive oil, beet molasses, hydrolysed potato starch, spent grain liquor, jaggery, cashew fruit juice, coconut water and milk, Jerusalem artichoke andmixture of potato starch hydrolysate (Boa and LeDuy, 1984; Boa and LeDuy, 1987; Leather and Gupta, 1994; Roukas and Biliaderis, 1995; Isralidies *et al.,* 1998; Barnett *et al.,* 1999; Vijayendra *et al.,* 2001; Thirumavalan *et al.,* 2008; Thirumavalan *et al.,* 2009; Xia *et al.,* 2017; Chao *et al.,* 2017; Singh *et al.,* 2018). India is one of the biggest producers of Sugarcane around the world. Sugar Cane Molasses (SCM) contains different sugar and minerals and therefore it acts as a natural growth media for different microorganisms including *Aureobasidium pullulans*. It contains sugars like sucrose, glucose, and salts like potassium, calcium which is necessary for the growth. In India the molasses is available in all the sugar producing factories and is very cheap. Bedside India Sugar Cane is also grown in countries like Brazil, West Indies, Pakistan, U.S.A. and other tropical countries.

In the present study pullulan was grown on the media composed from sugar cane molasses (SCM) and distilled water. No other synthetic minerals were added in the media beside sugar cane juice. In this study SCM is exclusively used for the production of pullulan without adding any other synthetic mineral in the media therefore making it very cost effective.

Materials and Methods

Micro-organisms and Growth Conditions

Pullulans used in this work was isolated from Department of Environmental Sciences, Dr R. M. L. Avadh University, Ayodhya, Uttar Pradesh, India. Isolation was done by selective enrichment method (Singh *et al.,* 2012). The strain was grown on agar medium plates containing Glucose 2.0%, Ammonium Sulphate 0.06%, di-Potassium Hydrogen Orthophosphate 0.5%, Sodium Chloride 0.1%, Magnesium Sulphate 0.04% and Yeast Extract 0.04% with pH 5. Isolates were maintained on the same medium at 4°C and sub cultured every fortnight.

Inoculums Preparation

Cell suspension was prepared by inoculating 1 ml of 48h grown culture in 200 ml of the Sugar Cane Molasses broth (100ml cane molasses and 100ml distilled water) and incubated at 30°C for 24h to achieve active exponential phase of the culture.

Effect of Time on Pullulan Production

The effect of time on pullulan production by *A. pullulans* using cane molasses and distilled water as a medium was studied. The experiments were carried out at a time intervals of 24 h. (24h, 48h, 72h, 96h, 120h). Pullulan production was analysed.

Effect of pH

In order to investigate the influence of pH on pullulan production from *A. pullulans* utilizing cane molasses and distilled water, the initial pH of the medium was adjusted to 3.0, 4.0, 5.0, 7.0, and 9.0, individually, using either 1 N HCl or 1 N NaOH and left uncontrolled during the fermentation. Five ml of the inoculum was used to inoculate 100 ml sterile medium in 250 ml Erlenmeyer flasks and incubated for 96 h at 30°C. The broth was analysed for pullulan production.

Effect of Different Temperatures

The influence of different temperatures on pullulan production from *A. pullulans* utilizing cane molasses and distilled water was investigated. Five ml of the inoculum was used to inoculate 100 ml sterile medium in 250 ml Erlenmeyer flasks and incubated

for 96 h at different temperatures viz., 25ºC, 30ºC, 37ºC, 43ºC and 50ºC individually. The fermented broth was analysed for pullulan production.

Effect of different Concentration of Sugar Cane Molasses (SCM) and Distilled Water

The influence of different ratio of Sugar Cane Molasses (SCM) and Distilled Water on pullulan production from *A. pullulans* utilizing cane juice and distilled water was investigated using different concentrations of cane molasses in distilled water viz., 0.5%, 1%, 2%, 4%, 6%, 8% and 10% respectively. Five ml of the inoculum was used to inoculate 100 ml sterile medium in 250 ml Erlenmeyer flasks and incubated for 96 h at 30ºC. The broth was analysed for pullulan production.

Extraction and Estimation of Pullulan

After fermentation, the culture medium was heated at 100°C in water bath for 15 minutes cooled to room temperature and centrifuged at 12,000 rpm at 4° C for 10 minutes to remove cells and other precipitates. Three ml of the supernatant were transferred into a test tube and then 6ml of the cold ethanol was added to the test tube and mixed thoroughly and held at 4°C for 12h to precipitate the extracellular polysaccharide. After removal of the residual ethanol the precipitate was dissolved in 3 ml of deionized water at 80°C and the solution was dialyzed against deionized water for 48h to remove small molecules in the solution. The exopolysaccharide was precipitated again by using 6ml of the cold ethanol and the residual ethanol was removed the precipitate was dried at 80°C to a constant weight (Badr-Eldin *et al.*, 1994). Pullulan was measured using electronic balance and expressed in g/l.

Hydrolysis of the Purified Extracellular Polysaccharide and Assay of Reducing Sugar

To assay the component of the extracellular polysaccharide, the purified precipitate was vacuum desiccated to no alcohol by using a vacuum pump, then dissolved in 3ml deionized water at 80°C in water bath. The dissolved substrate was hydrolysed by incubating the mixture of 0.5 ml of the substrate, 0.4 ml of Na_2HPO_4 (0.2M), citric acid buffer 0.1M (pH 5.0) and 0.1 ml pullulanase (Sigma Aldrich Chemicals, U.S.A.) for 21 hours at 40°C. The released reducing sugar was determined by using the modified D.N.S. method (Singh *et al.,* 2012) for the conformation of pullulan.

Results and Discussion

Effect of Time Course on Pullulan Production and Biomass Yield

In order to find an optimum time for pullulan production using cane molasses as substrate, the experiments were carried out for different times. The effect of time on the kinetics of pullulan production by *A. pullulans* is shown in Table 1. The highest concentration of pullulan (2.5g/100ml) was obtained at a fermentation period of 96h.The pullulan concentration gradually increases when fermentation time increases and reaches a maximum for a fermentation period of 96 h. After which, the production becomes steady. This was mainly because the fungus did not produce pullulan degrading enzyme pullulanase. Similar trend in pullulan production was observed some other workers (Singh *et al.,* 2012; Singh *et al.,* 2016; Singh *et al.,* 2017; Singh *et al.,* 2018). Pullulan production is directly related to yeast phase of growth. Yeast-like cells are mainly responsible for pullulan production (Campbell *et al.,* 2004). Incubation period for pullulan production varies from strain to strain, therefore incubation period has been evaluated for pullulan production. Maximum pullulan production was achieved when the cells reached their stationary phase which was at 96h and beyond this no further growth in the cells were seen, thus production of pullulan became stable.

Effect of Initial pH on Biosynthesis of Pullulan

The effect of pH (3.0 to 9.0) on the production of pullulan from *A. pullulans* utilizing cane molasses

and distilled water is shown in Table 2. Pullulan concentration gradually increased with increasing initial pH up to 5 and then decreased. The highest pullulan concentration of 2.5 g/100ml was achieved at pH of 5.0. Beyond this the production decreased. It has been reported that pH has profound effect on both the rate of production and synthesis of pullulan. Different workers have reported pullulan production at different pH range (Thirumavalan *et al.,* 2008; Thirumavalan *et al.,* 2009; Singh *et al.,* 2012; Singh *et al.,* 2016; Singh *et al.,* 2017; Singh *et al.,* 2018). In this study maximum production of pullulan was obtained at pH 5. Optimal pH value for pullulan production depends on different yeast strain, composition of the fermentation medium and growth conditions. Therefore, the physiological function of *A. pullulans* varies from strain to strain in case of pH also. This is perhaps due to either special structure of the membrane and cell wall or transport system of the organism along with the change of cytosol pH due to medium constituent affecting the critical level at specific pKa value of the medium and ultimately affecting more or less hydrogen ion concentration which in turn affected cell growth or pullulan synthesis. At very low no growth was seen due to acid production in the medium by the yeast cells which negatively affects the growth of fungus and production of polysaccharide.

Effect of Temperature on Pullulan Production

In order to find an optimum temperature for pullulan production using cane molasses and distilled water as substrate, the experiments were carried out for different temperatures. The effect of temperature on pullulan production by A. pullulans is shown in Table-3. It is clearly indicated in Table -3 that strain was able to produce high amount of pullulan (2.5/100ml) at 30°C. Fermentation temperature is one of the most important factors for pullulan production. In *A. pullulans* yeast form is mainly responsible for pullulan production (Campbell *et al.,* 2004). Generally, fungus grows at temperature range of 28-32^{0}C. This particular strain also produced highest pullulan production at 30^{0}Cshowing the mesophilic nature of the strain. Beyond this temperature the production deserved. This is mainly due to inactivation of the enzymes and cells which were not able to cope up with the high temperature.

Effect of Different Concentration of Sugar Cane Molasses (SCM) and Distilled Water

Carbon sources play a vital role in the production of pullulan (Singh *et al.,* 2012; Singh *et al.,* 2016; Singh *et al.,* 2017; Singh *et al.,* 2018). Molasses along with distilled water was used for the production of pullulan. It was seen that maximum production was seen at 4g/100 ml of molasses concentration. Lesser concentration did not support the growth of fungus due to low concentration of sugar and minerals. Higher concentration also did not support the growth due to more concentration of sugar and minerals which caused a nutrient shock to the fungus.

This is the study in which molasses was used as a sole source of media for the production of pullulan. This can be used as one of the media for the production of this particular polysaccharide at industrial level without using any synthetic chemicals.

Table.1 Effect of Time on Pullulan Production

Time Duration	Biomass production per 100 ML
24 Hours	**0.9 g/100 ML**
48 Hours	**1.7 g/100 ML**
72 Hours	**2.1 g/100 ML**
96 Hours	**2.5 g/100 ML**
120 Hours	**2.3 g/100 ML**

Table.2 Effect of initial pH on Pullulan Production

Initial pH	Biomass Production per 100 ML
pH3	0.9 gram/100 ML
pH4	1.7 gram/100 ML
pH5	2.5 gram/100 ML
pH7	2.3 gram/100 ML
pH9	1.6 gram/100 ML

Table.3 Effect of Temperature on Pullulan Production

Temperatures (°C)	Biomass Production gram/100 ML
25°C	2.3 gram/100 ML
30°C	2.5 gram/100 ML
37°C	2.1 gram/100 ML
43°C	1.9 gram/100 ML
50°C	1.1 gram/100 ML

Table.4 Effect of different concentration of Molasses on Pullulan Production

Molasses concentration	Biomass Production
0.5 gram / 100 ML	0.6 gram/100 ML
1 gram / 100 ML	1 gram/100 ML
2 gram/100 ML	1.9 gram/100 ML
4 gram/100 ML	2.5 gram/100 ML
6 gram/100 ML	1.4 gram/100 ML
8 gram/100 ML	0.9 gram/100 ML
10 gram/100 ML	0.3 gram/100 ML

Acknowledgements

Shreyansh Singh is thankful to the Head, Department of Environmental Sciences, Dr. R.M.L. Avadh University, Ayodhya, U.P., India for the necessary laboratory facility provided to carry out the research. A thank is also extended to Professor Rajeeva Gaur for providing the necessary guidance and mentorship required to carry out the research work. Ranjan Singh would like to thank the department of Higher Education, Government of Uttar Pradesh, Lucknow, Uttar Pradesh, India for the financial support under the scheme of "Research and Development of State Universities of Uttar Pradesh". (47-2021/606/77-4-2021-4/56/2020; Lucknow Dated 30-03-2021). Rajeeva Gaur would like to thank the department of Higher Education, Government of Uttar Pradesh, Lucknow, Uttar Pradesh, India for the financial support under the scheme of "Research and Development of State Universities of Uttar Pradesh". (Letter No. 108/2021/2585/77-4-2021-4 (28)/2021; Lucknow Dated 28-12-2021).

References

Badr-Eldin, S. M., El-Tayeb, O. M., El-Masry, H. G., Mohamad, F. H. A., and Abd El-Rahman, O. A. (1994). Polysaccharide production by *Aureobasidium pullulans*: Factors affecting polysaccharide formation. World Journal of Microbiology and Biotechnology, 10,423-426.

Barnett, C., Smith, A., Scanlon, B., and Israilides, C. J. (1999). Pullulan production by *Aureobasidium pullulans* growing on hydrolysed potato starch waste. Carbohydrate Polymers, 38,203-209.

Boa, J. M., and LeDuy, A. (1984). Peat hydrolysate medium optimization for pullulan production. Applied Environmental Microbiology, 48,26-30.

Boa, J. M., and LeDuy, A. (1987). Pullulan from peat hydrolysate fermentation kinetics. Biotechnology Bioengineering. 30, 463-470.

Campbell, B. S., Siddique, A. B. M., McDougall, B. M., and Seviour, R. J. (2004). Which morphological forms of the fungus *Aureobasidium pullulans* are responsible for pullulan production? FEMS Microbiology Letters, 232,225-228.

Chao, A., Ma, S. J., Chang, F. and Xue, W. J. (2017). Efficient production of pullulan by *Aureobasidium pullulans* grown on mixture of potato starch hydrolysate and sucrose. Brazilian Journal of Microbiology. 48, 180-185.

Gaur, R., Singh, R., Gupta, M., and Gaur, M. K. (2010). *Aureobasidium pullulans*, an economically important polymorphic yeast with special reference to pullulan. *African Journal of Biotechnology, 9 (47)*, 7989-7997.

Israilides, C. J., Smith, A., Harthill, J. E., Barnett, C, Bambalov, G. and Scanlon, B. (1998). Pullulan content of the ethanol precipitate from fermented agro-industrial wastes. Applied Microbiology and Biotechnology, 49, 613-617.

Leather, T. D. and Gupta, S. C. (1994). Production of pullulan from fuel ethanol by-products by *Aureobasidium* sp. Strain NRRY-12,974. Biotechnology Letters, 16, 1163-1166.

Roukas, T. and Biliaridies, C. G. (1995). Evolution of carob pod as a substrate for pullulan production by *Aureobasidium pullulans*. Applied Biochemistry and Biotechnology, 55, 27-44.

Singh, R., Gaur, R., Tiwari, S., and Gaur, M. K. (2012). Production of pullulan by a thermotolerant *Aureobasidium pullulans* strain in non-stirred fed batch fermentation process. Brazilian Journal of Microbiology, 43,1042-1050.

Singh, R., Gaur, R., Bansal, S., Biswas, P., Pandey, P. K., Jamal, F., Tiwari S, and Gaur, M. K. (2015). *Aureobasidium pullulans*- an industrially important pullulan producing black yeast. International Journal of Current Microbiology and Applied Sciences, 4(10),605-622.

Singh, R., Gaur, R., Bansal, S., Biswas, P., Pandey, P. K., Jamal, F., Tiwari S, and Gaur, M. K. (2016). Production of pullulan from a high yielding strain of *Aureobasidium pullulans* isolated from Jabalpur region of Madhya Pradesh in Central India. Journal of Chemical and Pharmaceutical Research, 8(8),126-132.

Singh, R., Gaur, R., Bansal, S., Jamal, F., Pandey, P. K., Tiwari, S, Sarsaiya, S., Mishra, S., Chaturvedi, N., Singh, D. P., Gaur, M. K. and Arasu, G. V. (2017). Production of pullulan from a high yielding strain of *Aureobasidium pullulans* in non-stirred flask type fermentation system. Journal of Microbiology and Biotechnology Research, 7(1),26-32.

Singh, R., Gaur, R., Pandey, P. K., Jamal F., Pandey, L. K., Kewat, H. K., Biswas, P. and Gaur, M. K. (2018). A novel Media Optimized for Production of Pullulan in Flask Type Fermentation System. International Journal of Current Microbiology and Applied Sciences,7(04), 53-61.

Thirumavalan, K., Manikkandan, T. R. and Dhanesekar, R. (2008). Batch fermentation kinetics of pullulan from *Aureobasidium pullulans* using low-cost substrate. Biotechnology, 7, 317-322.

Thirumavalan, K., Manikkandan, T. R., and Dhanesekar, R. (2009). Pullulan production from coconut by-products by *Aureobasidium pullulans*. *African Journal of Biotechnology, 8(2),* 254-258.

Vijayendra, S. V. N., Bansal, D., Prasad, M.S. and Nand, K. (2001). Jaggery: A novel substrate for pullulan production by *Aureobasidium pullulans* CFR-77. Process Biochemistry. 37, 359-364.

Xia, J., Xu, J., Liu, X., Xu, J., Wang, X., and Li, X. (2017). Economic co-production of poly (malic acid) and pullulan from Jerusaleum artichoke tuber by *Aureobasidium pullulans* HA-4D. BMC Biotechnology,17 (20),1-10.

How to cite this article:

Ranjan Singh, Shreyansh Singh and Rajeev Gaur. 2022. Production of *Aureobasidium pullulan* by using Sugar Cane Molasses as Economical Media. *Int.J.Curr.Microbiol.App.Sci.* 11(05): 319-324.
doi: https://doi.org/10.20546/ijcmas.2022.1105.036