

Original Research Article

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A Novel Media Optimized for Production of Pullulan in Flask Type Fermentation System

Ranjan Singh^{1*}, Rajeeva Gaur², Prabhask K. Pandey³, Farrukh Jamal⁴,
Laxmi K. Pandey⁵, Sangram Singh⁴, Harish K. Kewat⁶, Soni Tiwari²,
Pritha Biswas¹ and Manogya K. Gaur⁷

¹Department of Botany and Microbiology, St. Aloysius College (Autonomous),
Jabalpur, M.P., India

²Department of Microbiology (DST-FIST Supported, Centre of Excellence, Govt. of U.P.), Dr.
R.M.L. Avadh University, Faizabad, U.P., India

³Genotoxicity Lab, Division of Toxicology, Central Drug Research Institute,
Lucknow, U.P., India

⁴Department of Biochemistry, Dr. R.M.L. Avadh University, Faizabad, U.P., India

⁵Department of Biotechnology, St. Aloysius College (Autonomous), Jabalpur, M.P., India

⁶Regional Office, I.G.N.O.U., Jabalpur, M.P., India

⁷D.G.M. Environment (ETP and Biocompost), Balrampur Distillery, Bhabhanan,
Basti, U.P., India

*Corresponding author

ABSTRACT

Aureobasidium pullulans, popularly known as black yeast, is one of the most widespread saprophytic fungus 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 Sugarcane juice (SCJ) and distilled water. It was found that high amount of pullulan ($6.4 \pm 0.04 \text{g}/100 \text{ml}$) was obtained at 37°C , pH 5 at a concentration of 50% media in 96h. This is a novel study in which pullulan is grown on media consisting of Sugar Cane Juice (SCJ) and distilled water. Further no synthetic minerals are used in the media for the growth of polysaccharide making it very economical.

Keywords

Aureobasidium pullulans, Pullulan,
Sugarcane juice,
Production

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Introduction

Aureobasidium pullulans (De Bary) Arnaud is cosmopolitan yeast like fungus that occurs in diverse habitats, including the phyllosphere 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, colorless, tasteless, odorless, 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, Sing, Gupta and Gaur, 2010; Singh, Gaur, Bansal, Biswas, Pandey *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, Gaur, Tiwari and Gaur, 2012; Singh, Gaur, Bansal, Biswas, Pandey *et al.*, 2016; Singh, Gaur, Bansal, Jamal, Pandey *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 and sucrose, beet molasses, hydrolyzed potato starch, spent grain liquor, jaggery, cashew fruit juice, coconut water and milk, Jerusalem artichoke and on mixture of potato starch hydrolysate and sucrose (Boa and LeDuy, 1984; Boa and LeDuy, 1987;

Leather and Gupta, 1994; Roukas and Biliaderis, 1995; Isralidies, Smith, Harthill, Barnett, Bambalov and Scanlon, 1998; Youseef, Biliaders and Roukas, 1998; Roukas, 1998; Roukas and Liakopoulou, 1999; Barnett, Smith, Scanlon, Israilides, 1999; Roukas, 1999; Vijayendra, Bansal, Prasad and Nand, 2001; Thirumavalan, Manikkandan and Dhanesekar, 2008; Thirumavalan, Manikkandan and Dhanesekar, 2009; Xia, Xu, Liu, Xu, Wang and Li, 2017; Chao, Ma, Chang and Xue, 2017).

India is one of the biggest producers of Sugarcane around the world. Sugar Cane Juice (SCJ) 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 juice is available readily and is very cheap. It is consumed as a sweet beverage and added in different food products. Beside 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 juice (SCJ) and distilled water. No other synthetic minerals were added in the media beside sugar cane juice. This is infect the first study in which SCJ is exclusively used for the production of pullulan without adding any other synthetic mineral in the media therefore making it very cost effective.

Hypotheses

The cost of any polysaccharide mainly depends on the cost of carbon and nitrogen sources used for its production. Due to this the cost of production generally goes high. Therefore it is required to use substances which are cheap and will reduce the cost of production at industrial level. This study was

mainly on the Hypotheses that by using cheap sources of nutrition we can reduce the cost of production of the polysaccharide in study.

Materials and Methods

Micro-organisms and growth conditions

A. pullulans used in this work was isolated from college campus of St. Aloysius' College, Jabalpur, Madhya Pradesh. 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 Juice broth (100ml Sugarcane juice and 100ml distilled water) and incubated at 37°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 juice and distilled water as a medium was studied. The experiments were carried out at a time intervals of 24 h. Pullulan and biomass production was analyzed.

Effect of pH

In order to investigate the influence of pH on pullulan production from *A. pullulans* utilizing cane juice and distilled water, the initial pH of the medium was adjusted to 2.0, 3.0, 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 a 250 ml Erlenmeyer flasks and incubated for 96 h at 37°C. The broth was analyzed for pullulan production.

Effect of different temperatures

The influence of different temperatures on pullulan production from *A. pullulans* utilizing cane juice and distilled water was investigated. Five ml of the inoculum was used to inoculate 100 ml sterile medium in a 250 ml Erlenmeyer flasks and incubated for 96 h at different temperatures *viz.*, 30°C, 37°C, 43°C, 50°C and 60°C individually. The fermented broth was analyzed for pullulan production.

Effect of different concentration of cane juice and distilled water

The influence of different ratio of cane juice and distilled water on pullulan production from *A. pullulans* utilizing cane juice and distilled water was investigated using different concentrations of cane juice in distilled water *viz.*, 20%, 30%, 50%, 70% and 90% respectively. Five ml of the inoculum was used to inoculate 100 ml sterile medium in a 250 ml Erlenmeyer flasks and incubated for 96 h at 37°C. The broth was analyzed 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 milliliters 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 exo polysaccharide 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 hydrolyzed by incubating the mixture of 0.5 ml of the substrate, 0.4 ml of Na₂HPO₄ (0.2M), citric acid buffer 0.1M (pH 5.0) and 0.1 ml pullulanase (Sigma 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.

Statistical analysis

Karl Pearson Method (Variability) was followed for statistical analysis. All the experiments were done in triplicate and mean were calculated using standard deviation.

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 juice 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 Figure 1. The highest concentration of pullulan (4.9±0.06 g/100ml) was obtained at a fermentation period of 96h. Similarly, highest biomass production (4.2± 0.05 g/100ml) was also obtained at 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.

Effect of initial pH on biosynthesis of pullulan

The effect of pH (2.0 to 9.0) on the production of pullulan from *A. pullulans* utilizing cane juice and distilled water is shown in Figure 2. Pullulan concentration gradually increased with increasing initial pH up to 5 and then decreased. The highest pullulan concentration of 4.2±0.04 g/100ml was achieved at pH of 5.0. Beyond this the production decreased.

Effect of temperature on pullulan production

In order to find an optimum temperature for pullulan production using cane juice 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 Figure 3. It is clearly indicated in Figure 3 that strain has able to produce high amount of pullulan (4.3±0.03g/100ml) at 37°C. Beyond this the production decreased significantly.

Effect of different concentration of cane juice 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) (Fig. 4).

Fig.1 Effect of fermentation time on biosynthesis of pullulan production

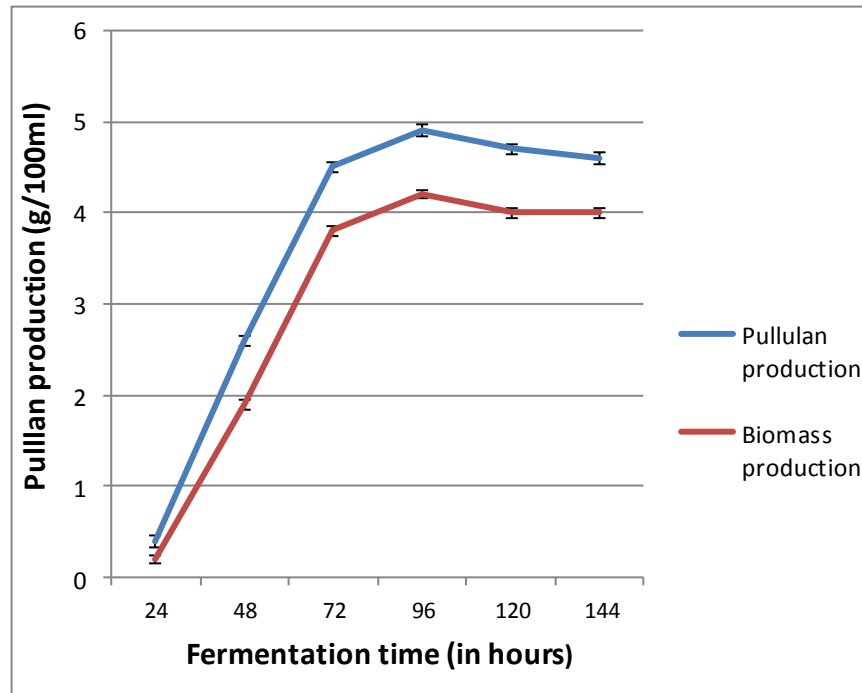


Fig.2 Effect of initial pH on biosynthesis of pullulan

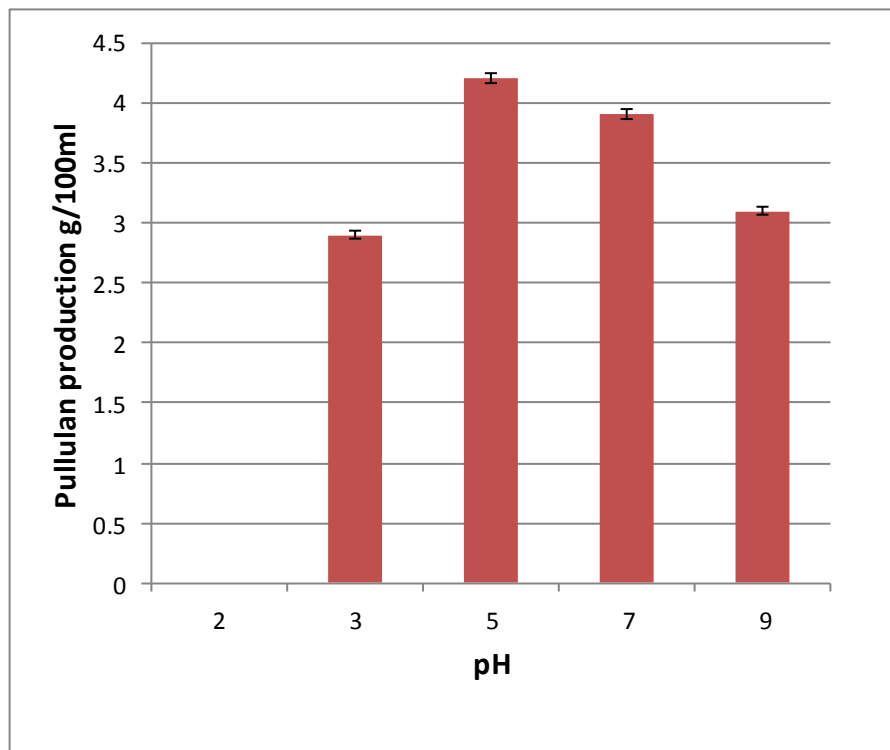


Fig.3 Effect of fermentation time on biosynthesis of pullulan production

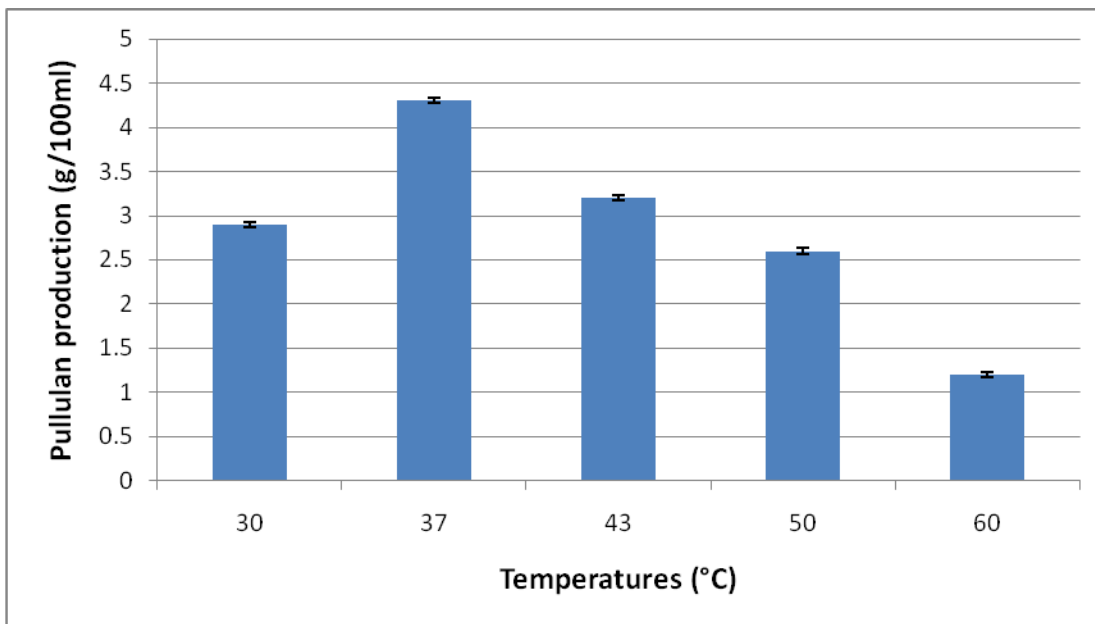
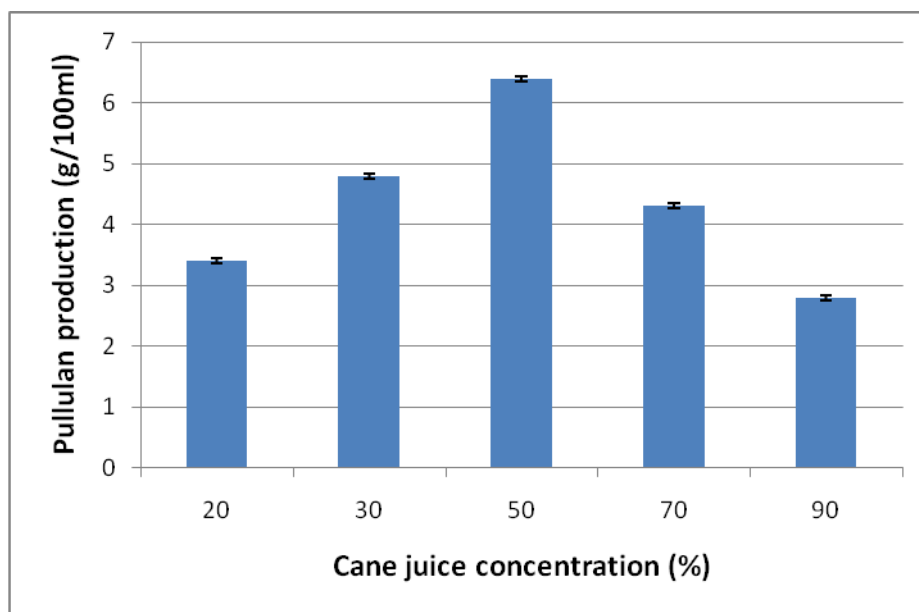


Fig.4 Effect of different concentration of cane juice and water



The influence of different ratio of cane juice and distilled water on pullulan production from *A. pullulans* was investigated. Among different ratio of cane juice and distilled water, 50% cane juice concentration showed best result (6.4±0.04 g/100ml). Beyond this ratio pullulan production dropped

significantly. Pullulan production is directly related to yeast phase of growth. Yeast-like cells are mainly responsible for pullulan production (Cambell *et al.*, 2004). Incubation period for pullulan production varies from strain to strain; therefore incubation period has been evaluated for pullulan and biomass

production. From the study it was revealed that highest production of pullulan was obtained at 96h of fermentation. Further after 96h of incubation the production of pullulan became stable. This was mainly because the fungus did not produce pullulan degrading enzyme pullulanase.

Similar trend in pullulan production was observed by in other works of same worker (Singh *et al.*, 2012; Singh *et al.*, 2016; Singh *et al.*, 2017). Similarly maximum biomass production was also observed at 96h, since formation of biomass directly depends on pullulan formation. 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.

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 (Singh *et al.*, 2012; Singh *et al.*, 2016; Singh *et al.*, 2017; Thirumavalan *et al.*, 2008; Thirumavalan *et al.*, 2009).

In this particular study maximum production of pullulan was obtained at a pH 5. Optimal pH values 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 constituents 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 pH 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.

Fermentation temperature is one of the most important factors for pullulan production affecting yeast phase of *A. pullulans* growth because change in morphology adversely affects pullulan production. In *A. pullulans* yeast form is mainly responsible for pullulan production (Campbell *et al.*, 2004). In this study highest pullulan production was seen at 37°C. This means that this particular strain is thermo-tolerant which can tolerate high temperature. Generally fungus grows at a range of 28-32°C but our strain can grow at much higher level than that. This is an important finding because in industry temperature goes up very high and only thermo-tolerant strains are able to tolerate it thus making it a suitable strain for industrial use.

Sugarcane juice along with distilled water was used for the production of pullulan. It was seen that maximum production was seen at 50% of sugarcane juice 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. Thus the best result was seen at 50% concentration. This is the first study done in which no synthetic mineral is used for the production of pullulan. Polysaccharide is grown on a media consisting of Sugar Cane Juice (SCJ) and distilled water thus making it very cheap and cost effective.

From the ongoing study it can be concluded the isolate of *Aureobasidium pullulans* was able to produce higher amount of pullulan (6.4±0.04 g/100ml) in a medium composed of Sugarcane juice and distilled water. This is

the first study done in which no synthetic mineral is used for the production of pullulan. Polysaccharide is grown on a media consisting of Sugar Cane Juice (SCJ) and distilled water thus making it very cheap and cost effective.

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Conflict of Interest

There is no conflict of interest among the authors related to the work.

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