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Full Length Research Paper

Pullulan production from coconut by-products by *Aureobasidium pullulans*

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The production of pullulan from coconut water and coconut milk by *Aureobasidium pullulans* was investigated. The highest production of pullulan in batch fermentation with coconut water yield of 38.3 (g/L) and coconut milk yield 58.0 (g/L). The effect of initial pH, fermentation time, and additional nitrogen source was investigated. The highest pullulan concentration of 58.0 g/L was obtained for a fermentation of period of 144 h with the initial substrate concentration equivalent to 50 g/L and pH 7. The chemical structure of the pullulan synthesized from coconut by-products exhibited was similar when it was compared using FT-IR spectroscopy with that of pullulan available commercially.

Key words: Pullulan, *Aureobasidium pullulans*, coconut water, coconut milk, characterization, FT-IR.

INTRODUCTION

Pullulan is an extra-cellular, linear, unbranched, water soluble, microbial polysaccharide. It is composed of linear α-(1→6) linked maltotriose units and a small number of α-(1→4) linked maltotetraose units (Catley, 1970; Catley and Whelan, 1971; Shingel, 2004; Carolan et al., 1983; Taguchi et al., 1973). It is produced by a yeast-like fungus *Aureobasidium pullulans*. Pullulan compounds are biodegradable in biologically active environments, have high heat resistance, and display a wide range of elasticities and solubilities. This versatility allows them to be utilized in many different ways. Pullulan has many uses as an industrial plastic. It can be formed into compression moldings that resemble polystyrene or polyvinyl chloride in transparency, gloss, hardness, strength, and toughness, but are far more elastic. It decomposes above 200°C, apparently without the formation of toxic gases. 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, except dimethylformamide and dimethylsulfoxide (Leathers, 2002; Sugimoto, 1978).

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, or malto oligosaccharides support pullulan production by *A. pullulans* (Badr-Eldin et al., 1994; Catley, 1971b; Imshenetskii et al., 1985). A number of complex carbon sources have been reported for pullulan production, including spent sulfite liquor, 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 and jaggery and cashew fruit juice (Zajic et al., 1979; Boa and LeDuy, 1984, 1987; West and Reed-Hamer, 1991; West and Reed-Hamer, 1993; Leathers and Gupta, 1994; Roukas and Biliaderis, 1995; Israilides et al., 1998; Youssef et al., 1998; Roukas, 1998; Roukas and Liakopoulou-Kriakides, 1999; Barnett et al., 1999; Roukas, 1999; Vijayendra et al., 2001; Thirumavalavan et al., 2008).

Conformation of carbohydrate chains were obtained from the analysis of these compounds by IR spectroscopy. In the IR spectrum of pullulan, the absorbance bands in the carbohydrate fingerprint region were identified (Catley, 1970).The co-existence of α-(1→4)- and α-(1→6)-glycosidic linkages in the pullulan structure can be established (Petrov et al., 2002; Yurlova and Hoog, 1997; Madi et al., 1997).

Coconut water is the naturally-occurring liquid found inside a coconut. Coconut milk is a sweet, milky white cooking base derived from the meat of a mature coconut. Coconut water is considered a waste product, especially

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of factories producing copra, dessicated coconut, and other coconut meat products. It is a very active pollutant because of its high biological oxygen demand. The pollution problem has increased the interest in coconut water and motivated its utilization in the production of such an industrially important biopolymer.

In this study, the production of pullulan was investigated using various coconut by-products and it has advantages such as reduction of production cost and recycling of natural sources. In addition, the effect of initial pH, fermentation time and nitrogen sources were investigated in batch fermentation. This is the first report using coconut by-products for the production of pullulan.

MATERIALS AND METHODS

Micro organisms and growth conditions

A. pullulans MTCC 2195 used in this work was obtained from MTCC, Chandigarh. The strain was maintained on agar slants at 4°C and subcultured every fortnight time interval. The composition of seed medium (g l^{-1}) is sucrose, 50.0; K_2HPO_4, 5.0; yeast extract, 3.0; $(NH_4)_2SO_4$, 2.0; NaCl, 1.0 and distilled water l litre. When required, agar, (20 g $litre^{-1}$) was added to the cultivation medium. The medium was autoclaved for 15 min at 121°C, cooled and the initial pH was adjusted to 7.0. A loop-full of culture was transferred to 250 ml conical flasks containing 50 ml culture medium. The flasks were incubated at 30°C for 36 h in a rotary shaker incubator at 200 rpm. These cultures at a level of 5% (v/v) were then used to inoculate the production medium for the fermentations.

Effect of pH

In order to investigate the influence of pH on pullulan fermentation from *A. pullulans* utilizing coconut water and coconut milk, the initial pH of the medium was adjusted to 4.0, 5.0, 6.0, 6.4, 6.8, 7.0, 8.0 and 9.0, individually, using either 1 N HCl or 1 N NaOH and left uncontrolled during the fermentation. Five percent (v/v) of the inoculum was used to inoculate 50 ml sterile medium in a 250 ml Erlenmeyer flasks and incubated on a rotary shaker (200 rev/min) for 156 h at 28°C. The fermented broth was analysed for biomass, pullulan content, pH and residual sugar content.

Effect of fermentation time

The effect of fermentation time on the kinetics of pullulan production by *A. pullulans* using both coconut water and coconut milk as a medium were studied separately. The experiments were carried out for different fermentation times on the kinetics of pullulan production by *A. pullulans* is conducted for the time intervals of 12 h. The cell mass, pullulan and glucose concentrations were analyzed.

Estimation of biomass and pullulan

At specific time intervals, the flasks were removed and the fermentation broth was analyzed for biomass and pullulan. Dry weight of total biomass (mycelia and yeast cells) was determined by centrifuging the fermentation broth (after appropriate dilution) at 10000 x g for 20 min. The collected cell mass was washed twice with saline and distilled water and dried at 90°C till the mass reaches consistent weight. The first supernatant was combined with the washing, and the pullulan was precipitated using two volumes of ethanol at 4°C for 12 h and filtered through a pre-weighed Whatman No.1 filter and dried at 90°C for constant weight.

Estimation of total sugar

Total sugar concentration was measured by phenol-sulphuric acid method based on reaction with hot acidic medium (Krishnaveni et al., 1984). Glucose was dehydrated to hydroxyl methyl furfural and forms a green coloured product with phenol. Absorbance of the resulting solution was measured at 490 nm using Spectronic – 20D Spectrophotometer.

Pullulan characterization

The characterization of EPS was carried out using IR spectroscopy. Fourier transform infrared (FTIR) spectra were recorded with a Perkin Elmer-Spectrum RX_1 spectrometer (32 scans; resolution, 4 cm^{-1}) over KBr pellet. Pullulan sample (2 mg) was manually well blended with 60 mg of KBr powder. These mixtures were then desiccated overnight at 50°C under reduced pressure prior to FTIR measurement.

RESULTS AND DISCUSSION

Studies coconut by-products as carbon on growth and pullulan production

The carbon source is mainly used in cellular constituent, synthesis of new cells, production of polysaccharide, and as an energy source (Seviour et al., 1992). The original reducing sugar present in the coconut by-products are 40 (g/L) of matured coconut water, 22 (g/L) of tender coconut water, and 48 (g/L) of coconut milk respectively. The coconut by-product such as matured coconut water, tender coconut water, coconut milk and sucrose (seed medium composition) were used with an initial sugar concentration of 50 (g/L) with the addition of sucrose. The highest concentration of pullulan (54.0 g/L) was obtained at a fermentation period of 144 h for the case of coconut milk, where as in sucrose, the highest pullulan concentration obtained was 28 g/L. Similar higher results were obtained for other substrates also. The results are in Figure 1. The coconut by-products are much suitable for pullulan production in large scale because of rich mineral sources and amino acids. The main important feature is it does not require any additional pre-treatment methods as they used in other crude substrates.

Similar result can be obtained with these findings, Jaggery supported good growth of *A. pullulans* and the pullulan produced was compared with derived from sucrose (Vijayendra et al., 2001). Sena et al (2006) described the *A. pullulans* fermentation process using in addition to sucrose as a carbon source, refined soybean oil (SBO) at different concentrations both to increase and to improve pullulan production.

Effect of initial pH on biosynthesis of pullulan in batch culture

An initial pH of the fermentation medium affects the mor-

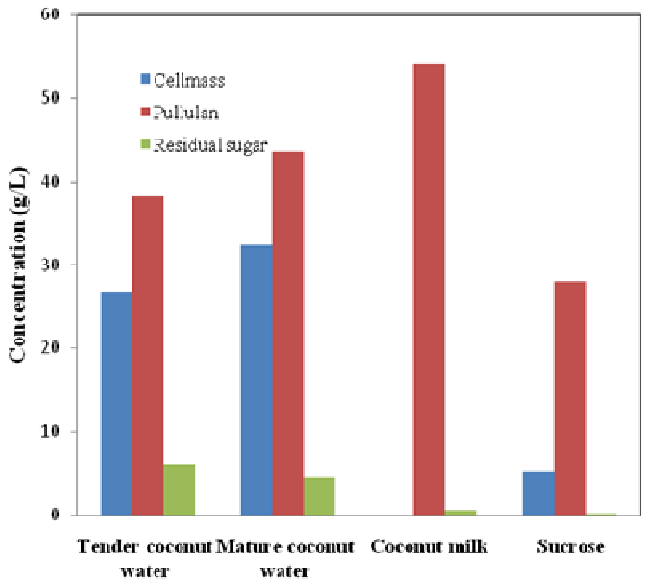

Figure 1. Studies on coconut by-products as carbon source on growth of *Aureobasidium pullulans* and pullulan production.

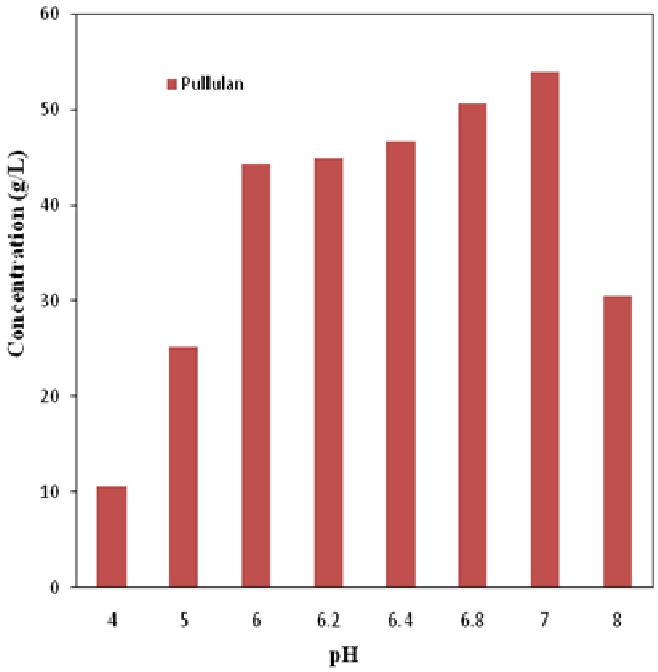

Figure 2. Effect of pH on pullulan production using coconut milk by *Aureobasidium pullulans*.

phology of an organism which influences pullulan synthesis (Thirumavalavan et al., 2008). The effect of initial pH (4.0 to 8.0) on the kinetics of pullulan from *A. pullulans* utilizing coconut water and coconut milk extract is shown in Figure 2. Pullulan concentration gradually increased with increasing initial pH up to 7 and then decreased. The relative concentrations of pullulan at pH 4 and 8 were 10.7 g/L and 30.5 g/L, respectively. The highest pullulan concentration of 58.0 g/L was achieved at pH of 7.0. A lower production of cell mass and pullulan was obtained at lower pH levels when compared to higher levels of pH. This is probably due to an influence of acidic pH on morphological character of the organism.

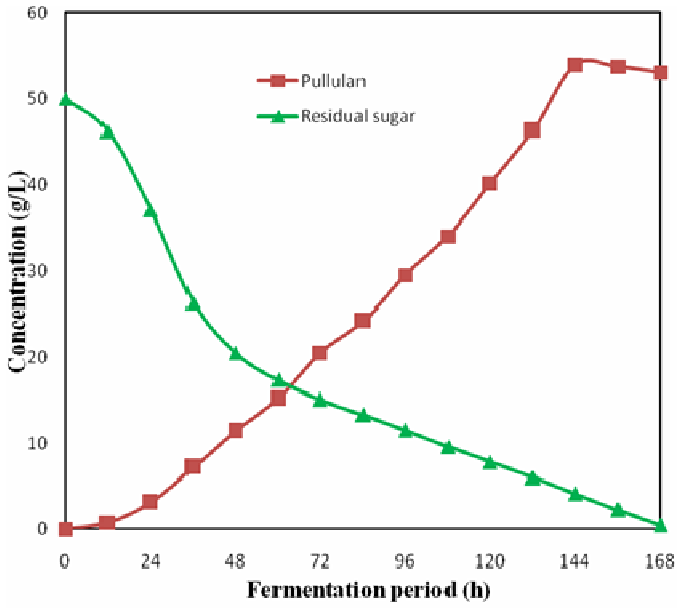

Figure 3. Effect of fermentation period during pullulan production from coconut milk in batch study using *Aureobasidium pullulans*.

Similar results were obtained in previous investigations. A maximum polysaccharide concentration at an initial pH of 7.0 was optimal for pullulan production in batch culture (Papon et al., 1989). A maximum polysac-charide concentration (6.5 g/L) was obtained in cultures of *A. pullulans* grown in carob pod extract at an initial pH of 6.5 (Israilides et al., 1998), whereas using synthetic medium, optimum polysaccharide production at a pH of 6.0 (Ono et al., 1977). An initial culture pH of 2.0 supported almost no pullulan formation, while maximum yields were obtained at pH 5.5 (Lacroix et al., 1985).

Effect of fermentation time on biosynthesis of pullulan in batch culture

In order to find an optimum fermentation time for pullulan fermentation using coconut water and coconut milk extract as substrate, the experiments were carried out for different fermentation times. The effect of fermentation time on the kinetics of pullulan production by *A. pullulans* is shown in Figure 3. The pullulan concentration gradually increases when fermentation time increases and reaches a maximum for a fermentation period of 144 h. After which, the production starts to decrease, this may be due to the hydrolysis of pullulan by endogenous glucoamylase-A, which may be released by the organism

Table 1. Comparative infra-red spectroscopy data of EPS from different source.

Assignment	Pullulan standard wavenumber (cm^{-1})	Pullulan from coconut water and cococnut milk wavenumber (cm^{-1})
O-H str.	3432.4	3386
C-H str.	2927.3	2929
O-C-O str.	1639.7	1654
C-O-H bend	1366.4	1342
C-O-C str.	1154.6	1125
C-O str.	1021.4	1032
α-configuration	851.0	860

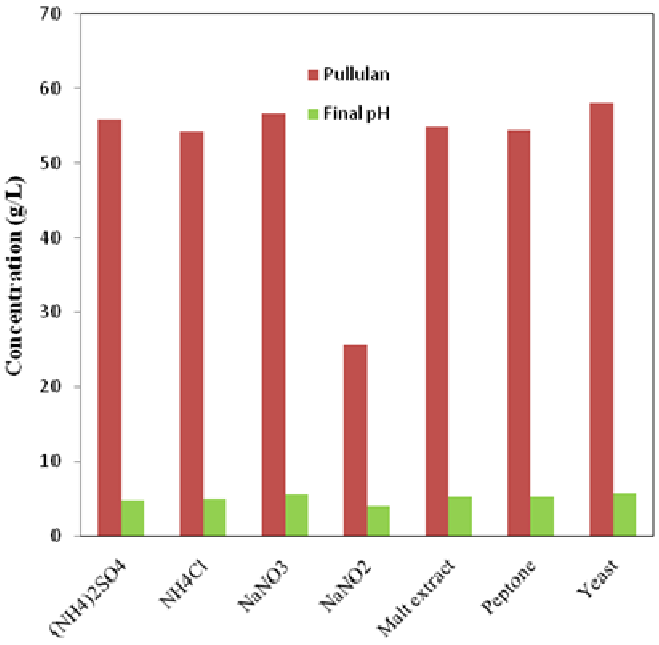

Figure 4. Effect of nitrogen (synthetic) source on pullulan production using *Aureobasidium pullulans*.

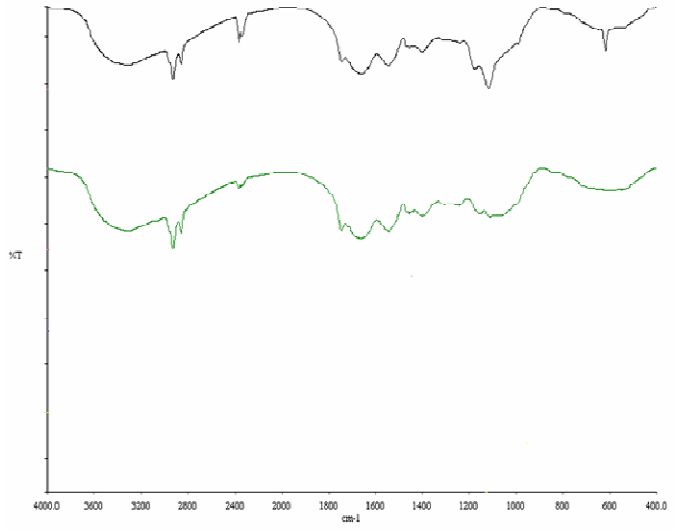

Figure 5. IR Spectra of pullulan produced by *Aureobasidium pullulans* from matured coconut water (top) and coconut milk (bottom).

at the latter stages of fermentation (Thomas and Strohfus, 1996). Their highest concentration of pullulan (54.0 g/L) was obtained at a fermentation period of 144 h. There may be several reasons for such variability in the production of pullulan including the type of organism used, chemical composition of the substrate, fermentation system and conditions employed during the fermentation.

Effect of nitrogen (synthetic) source on pullulan production

The nitrogen source affects the growth and metabolic activities of the organism. Several researchers have used yeast extract as nitrogen source, but it is expensive except malt extract and peptone. The nitrogen sources such as, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, $NaNO_2$, malt extract, peptone and yeast extract were tried. The experiments were conducted for all the nitrogen sources equivalent to 0.19g nitrogen/L. Yeast extract, $NaNO_3$ and $(NH_4)2SO_4$ results the highest pullulan concentration of 58.1g/L, 56.6 and 55.8g/L respectively. $NaNO_2$ suppresses the pullulan yield. No significant effect was noticed for the case of NH_4Cl, malt extract and peptone. The results are shown in Figure 4.

Structural characterisation of pullulans

FT-IR spectra for commercial pullulan (Sigma) used as a reference and those for crude pullulans obtained from the strains evaluated are compared in Table 1 and Figure 5. The strong absorption at 3386 cm^{-1} indicated that all the pullulans had some repeating units of –OH as in sugars. The other strong absorption at 2928 cm^{-1} indicated a sp^3 C–H bond of alkane compounds existed in all the samples. In the specific area (1500–650 cm^{-1}) which is

characteristic for the pullulan molecule as a whole, the spectra for commercial pullulan as well as those for evaluated samples exhibited similar features (Figure 5). Such results confirmed the identical chemical structure of the samples. Strong absorption in 860 cm^{-1} is characteristic of the α-D-glucopiranosid units. Absorption in 755 cm^{-1} indicates the presence of α- (1 → 4)-D-glucosidic bonds, and spectra in 918 cm^{-1} proved the presence of α-(1 → 6)-D-glucosidic bonds. Besides, in the areas for reference and evaluated samples the frequencies are the same (Seo et al., 2004; Singh and Saini, 2008; Gniewosz and Duszkiewicz-Reinhard, 2008).

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