PRODUCTION OF PULLULAN FROM DE-OILED *PONGAMIA* SEED CAKE BY USING *AUREOBASIDIUM PULLULANS*

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ABSTRACT

The efficiency of pullulan production using *Aureobasidium pullulans* from *Pongamia* seed cake, a byproduct of biodiesel industry was studied. Studies were conducted by using both acid pre-treated and untreated residual seed cake samples. The raw material used, although unconventional, supported the growth of *A. pullulans* yielding a maximum biomass of 2.48 g/L in acid pre-treated sample during the incubation period. Pullulan production was also found to be maximum in acid pre-treated sample reaching a concentration of 3.84 g/L. These findings reveal the possibility of pullulan production from biodiesel industry residues thus bringing down the cost of pullulan production and at the same time generating valuable product with commercial end uses from underutilized residue.

KEYWORDS

Pongamia* seed cake, acid hydrolysate, aqueous hydrolysate, biomass, pullulan, *Aureobasidium pullulans

1. INTRODUCTION

Microbial exopolysaccharides are the long chain polymers, that have been exploited for a lot of industrial applications especially in food industries such as usage as viscosity enhancing, thickening and gelling agents, stabilizing, emulsifying and water-binding agents **(Ramos *et al.,* 2016)**. Owing to their moisture retention capabilities, they find applications in food preservation **(Rekha *et al.,* 2007)**. They are also known to act as prebiotic agents by enhancing the development of beneficial microbiota **(Ramos *et al.,* 2016)**. In addition, they find other pharmaceutical applications including reduction of cholesterol levels, usage as antioxidants and anti-carcinogenic agents **(Ramos *et al.,* 2016; Rekha *et al.,* 2007; Scomparin *et al.,* 2011)**. One such exopoysaccharide that is being exploited for a variety of commercial applications is pullulan, produced by the yeast-like fungus *Aureobasidium pullulans*.

Pullulan is a natural, linear, water soluble, extracellular fungal homopolysaccharide consisting of repeating units of maltotriose. Three glucose units in maltotriose are connected by an α-1,4 glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-1,6 glycosidic bond **(Thirumavalavan *et al.,* 2008; Scomparin *et al.,* 2011)**. Owing to the beneficial properties of pullulan such as biodegradability, impermeability to oxygen, non-hygroscopicity, considerably good solubility in aqueous and a few non-aqueous solvents, low immunogenicity, film forming capacity, etc, it has been exploited for a variety of applications in food, pharmaceutical, cosmetics and chemical industries **(Thirumavalavan *et al.,* 2008; Scomparin *et al.,* 2011; Rajeeva *et al.,* 2010)**. Recently, it

is also being investigated for biomedical applications such as targeted drug and gene delivery, wound healing, tissue engineering, diagnostic imaging using quantum dots, surface modification, etc **(Rekha *et al.,* 2007)**.

Although pullulan offers attractive commercial applications, cost of pullulan production from synthetic media is found to be comparatively higher than other biopolymers **(Chaudhury *et al.,* 2012)**. In this regard, attempts have been made to utilize a variety of agro-industrial residues such as molasses, hydrolyzed potato starch waste, brewery waste, olive oil waste effluents **(Thirumavalavan *et al.,* 2008)** peat hydrolysate **(Boa *et al.,* 1984)**, soybean cake hydrolysate **(Chi *et al.,* 2003)**, coconut byproducts **(Thirumavalavan *et al.,* 2009)**, de-oiled *Jatropha* seed cake **(Chaudhury *et al.,* 2012)**, etc, as a means to alleviate production costs. Thus utilization of agricultural and industrial residues serves the dual purpose of effective waste treatment and simultaneous value addition to the wastes.

In connection to this, in the present work, focus was laid on exploiting and utilizing de-oiled *Pongamia* seed cake generated as a byproduct during biodiesel production, as a source for pullulan production. The de-oiled *Pongamia* seed cake hydrolysate was used for other studies in the Institution during which the cake sediment was generated as a waste. However, this sediment was further utilized to check if there could be any further value addition to it by using it for pullulan production. Preliminary investigations were performed to evaluate the pullulan production profiles and check the effectivity of this residual medium to support pullulan production from *A. pullulans*.

2. MATERIALS AND METHODS

2.1. Microorganism and culture conditions

Aureobasidium pullulans (NCIM 976) obtained from National Chemical Laboratories (NCL), Pune, was used for the study. The seed culture was prepared in YPD medium comprising of the following constituents (g/L): yeast extract, 10.0; Peptone, 20.0; Dextrose, 20. The cultures were maintained in agar slants at 4 ºC.

2.2. Collection and processing of the raw material

The de-oiled *Pongamia* seed cake to be used as the raw material was collected from the Bio-diesel Center of the Institution. The cake was dried and powdered. Following this, 5% (w/v) of the cake was hydrolyzed by heating it with distilled water on a low flame for 30 minutes with continuous stirring. The hydrolysate thus obtained was sedimented and the supernatant obtained was used for a different study. From the cake sediment that was left out, excess water was drained and dried in hot air oven. The dry cake was finely powdered and stored for further use as raw material for pullulan production.

2.3. Preparation of aqueous and acid hydrolysate of the residual cake

For the preparation of acid hydrolysate, 5% (w/v) of the raw material was treated with 2% sulphuric acid and autoclaved. Following this, the medium was sedimented, decanted and neutralized using NaOH flakes. On the other hand, aqueous hydrolysate was prepared by heating 5% (w/v) of the seed cake in water; sedimenting the mixture; decanting it and collecting the supernatant. Prior to usage as the study media, both acid and aqueous hydrolysate was sterilized by autoclaving the mixture.

2.4. Pullulan production studies

The growth curves and the pullulan production profiles were studied in shake flasks containing 100 mL each of the acid hydrolysate and the aqueous hydrolysate medium. 100 µL of the seed culture was transferred to the flasks containing sterilized media. The culture flasks were incubated at 30 ºC in a rotary shaker for 3 days.

2.5. Analytical procedures

Estimation of pullulan content in the medium was conducted according to the procedure described by **Badr-Eldin et al. (1994)**. Following incubation period, culture media was heated to 100°C in water bath for 15 minutes and then cooled to room temperature. It was then centrifuged at 12000 X g at 4°C for 15 minutes in order to precipitate the cells following which the biomass was estimated gravimetrically. 3 mL of supernatant was transferred into a test tube and was mixed with 6 mL of cold ethanol. This test tube was kept at 4°C for 12 hours to precipitate the extracellular polysaccharide. Residual ethanol was removed and filtered using a pre-weighed Whatman No.1 filter paper. Thus obtained precipitate was dried at 80°C to a constant weight. Pullulan dry weight was measured and expressed in g/L. In addition to this, the media used were analyzed for the contents of total sugars **(Dubois *et al.*, 1956)**, reducing sugars **(Miller *et al.*, 1959)**, proteins **(Lowry *et al.*, 1951)** and lipids **(Frings *et al.*, 1970)**.

3. RESULTS AND DISCUSSION

3.1. Nutrient estimation studies

The total sugars, reducing sugars, proteins and lipids utilization profiles obtained for the media used in the study were shown in figures 1 and 2. Total sugars in aqueous hydrolysate medium were 18.24g/L and in the acid hydrolysate was 21.02g/L. In both the medium, there was rapid utilization of total sugars compared to the other nutrients during the incubation period with the concentrations decreasing in the aqueous hydrolysate medium to 1.246 g/L and 5.5625 g/L in the acid hydrolysate medium respectively. On the other hand, there were significant difference in reducing sugars in the two media with the initial concentration being 2.93g/L in the aqueous hydrolysate medium and 20.43g/L in the acid hydrolysate medium. This significant difference in the initial concentration of the reducing sugars could be attributed to the hydrolyzing capability of the acid resulting in the effective release of reducing sugars from the residual cake into the medium. In accordance with this, in the acid hydrolysate medium, there was significant utilization of the reducing sugars observed with the final concentration getting reduced to 6.69 g/L. Although there was reduction in the reducing sugars content observed in the aqueous hydrolysate medium as well, it was not as significant as that observed in the initial medium with the final concentration reaching 1.26 g/L. There was no much utilization of proteins observed in both the media with the concentration reducing from 4.0916 g/L to 3.7183 g/L in aqueous hydrolysate medium and in acid hydrolysate medium from 7.6037 g/L to 6.1633 g/L. Lipid utilization was comparatively higher than that of proteins where the concentration reduced from 4.64 g/L at the beginning of the incubation period to 1.392 g/L towards the end of the cycle in aqueous hydrolysate medium while in acid hydrolysate medium, the concentration reduced from 3.48g/L to 1.392 g/L from the start to the end of the incubation period, respectively indicating significant utilization of lipids during the growth.

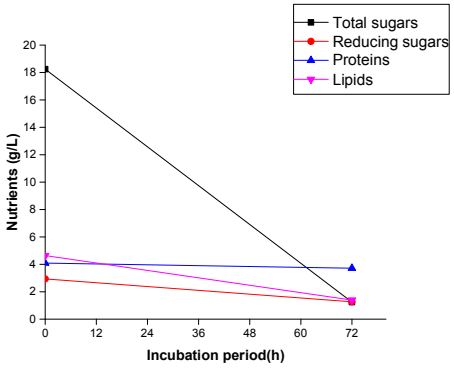

Fig.1. Nutrient utilization profiles for aqueous hydrolysate medium

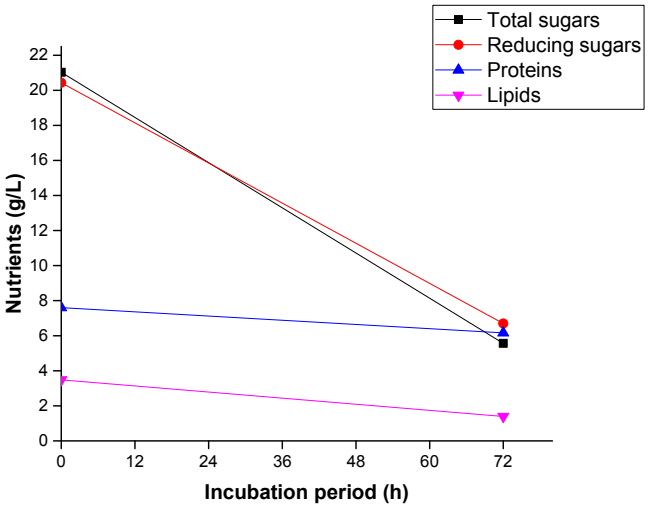

Fig.2. Nutrient utilization profiles for acid hydrolysate medium

3.2. Biomass and pullulan production profiles

Aureobasidium pullulans, when grown in the two different media, showed the biomass and pullulan productivity profiles during the end of the incubation period as represented in fig.3. Both biomass and pullulan productivities were higher in the acid hydrolysate medium when compared to aqueous hydrolysate medium. The difference in the biomass concentration between the two media was 0.68g/L with the biomass concentration in the aqueous hydrolysate medium being 1.8g/L while that in the acid hydrolysate medium being 2.48 g/L. However, on the other hand, there was a tremendous difference between the two media with respect to pullulan production levels with the concentration in the aqueous hydrolysate medium being 0.14 g/L while that in the acid hydrolysate medium being 3.84 g/L. This difference in the pullulan production levels could be attributed to the effective utilization of the reducing sugars in the acid hydrolysate medium as is evident from the fig.2. The result obtained is well supported by the literature that says that *A.pullulans* grows best in the medium containing monoscaacharides than that containing discaccharides or polysaccharides **(Israelides *et al.*, 1999)** considering that the acid hydrolysate medium contains highest proportion of monosaccharides when compared to aqueous hydrolysate medium. In the different studies conducted for the production of pullulan both using synthetic media and various agroindustrial residues, reports indicate that the pullulan concentration has varied from as little as 3 g/L to as high as 90 g/L **(Choudhury *et al.*, 2012; Thirumavalavan *et al.*, 2008; Thirumavalan *et al.*, 2009)**. Although the concentrations of the pullulan obtained in the present study were much lower compared to the values in literature, the work cannot be deemed insignificant owing to the fact that the raw material used in the present study is the residual seed cake remaining after the first extraction from the *Pongamia* seed cake. Thus the present results still can be considered as a valuable finding because they do not eliminate the possibility of pullulan production from the residual content of the seed cake but do reveal the production in the concentration range of 3.84 g/L for the acid pre-treated raw material thus indicating that the *Pongamia* seed cake has a potential to be exploited as an efficient medium for pullulan production using *A. pullulans* thus adding value to the unused raw material.

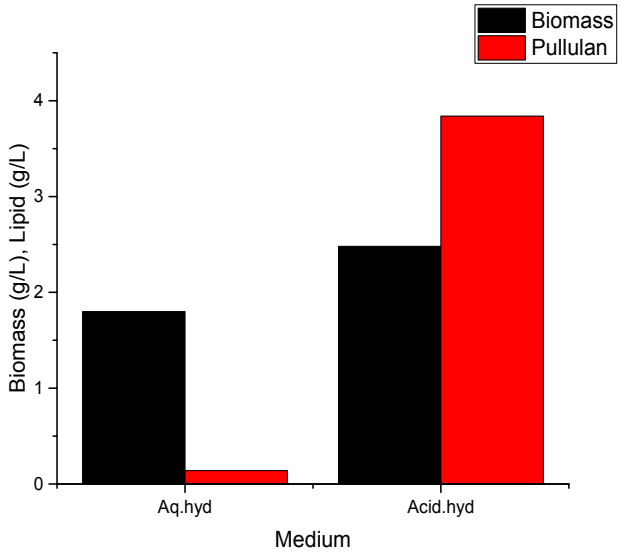

Fig.3. Biomass and pullulan productivities in aqueous hydrolysate and acid hydrolysate media

4. CONCLUSIONS

The present study reveals the potential of *Pongamia* seed cake, even in its residual concentration, to serve as a raw material for pullulan production. This unconventional medium, in addition to supporting the growth of *A. pullulans*, also yielded pullulan up to a concentration of 3.84 g/L. The present study also demonstrates that acid hydrolysis of the cake results in better pullulan yields when compared to the untreated sample. These findings from the study pave way for further exploitation of *Pongamia* seed cake for enhancing pullulan production by introducing modifications in the process parameters. The present study has made use of the residual seed cake; this suggests that the *Pongamia* seed cake, if used in its original form, could result in much higher yields with respect to pullulan production and can serve as a promising source for cost-effective pullulan production.

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