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Downstream processing of Pullulan recovery from Palm kernel hydrolysate

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Abstract: Pullulan, a microbial exopolysaccharide was produced by submerged fermentation of palm kernel hydrolysate. Besides pullulan, *Aureobasidium pullulans* synthesized some enzymes and they interfered with product purity. Pullulan was separated from the fermentation broth by sequential steps, involving heat treatment to denature enzymes and solvent precipitation to recover the product. Fermentation broth was centrifuged at 8000rpm for 15 min to harvest the cells. Supernatant was heated to 120 °C for 60 min and added with three volumes of isopropanol. Pure pullulan was obtained after precipitation time of 10hrs at 4 °C.
Keywords: Pullulan; downstream processing; heat treatment

Introduction

Pullulan, an extracellular polymeric substance produced by yeast like mesophilic (22-30 °C) fungal strain *Aureobasidium pullulans*[1] is composed of maltotriosyl units linked by α-1, 6 glycosidic bond. Internal glucose units are connected by α-1, 4 glycosidic bond[2]. Pullulan is available as a white, odorless, and tasteless powder and it is edible & non toxic[3]. Alpha 1, 6 linkages in pullulan can be hydrolyzed by pullulanase enzyme[4]. Pullulan is synthesized during late exponential phase and early stationary phase of cells[5].

A.pullulans is a polymorphic fungus belonging to ascomycota phylogeny[6]. They exhibit extreme morphological variability as swollen blastospores, yeast-like cells, mycelia, chlamydospores and young blastospores[7]. It is a ubiquitous fungus widely spread in soil, water, and air[8].

Borassus flabellifer (Palm kernel), one of the cheapest agricultural substrates, consists of polymeric forms of sugars mainly mannose, galactose. These sugars are converted to monomeric forms by hydrolysis of the substrate. Organism easily consumes these sugars and yields pullulan.

One of the major setback faced during recovery of pullulan is that, removal of enzymes mainly amylase, mannanase, and xylanase, synthesized by organism[9, 10]. These enzymes can be degraded by heating the fermentation broth. The following study was performed to find the effective downstream processing of pullulan.

Materials and Methods:

Strain specification and inoculum preparation:

Fungal strain *A.pullulans* (NCIM No. 1049*)* obtained from National Chemical laboratory, India, was preserved on potato dextrose agar slant at 4 °C and sub cultured once in a week in potato dextrose broth. Seed culture was prepared by inoculating a loop full of culture from agar slope into a sterilized medium having the

composition (g/l) as follows[11]: Sucrose-30; yeast extract -0.4; K_2HPO_4 -5; $(NH_4)_2SO_4$ -2; NaCl-1; $MgSO_4.7H_2O$-0.2 and pH 5.5. After inoculation, the culture was kept in shaking condition of 150 rpm at 30°C for 48 hrs. The above cell suspension was used to inoculate the fermentation medium.

Fermentation and extraction procedure of pullulan:

Palm fruits were collected and its outer layer and following hard shell were removed to get the inner kernel. They were cut into equal pieces and hydrolyzed by adding water at 121°C for 20 min. The resulting fluid was filtered and the filtrate was mixed with following basal medium (g/l): $(NH_4)_2SO_4$ (0.4), $MgSO_4.7H_2O$ (0.2), Yeast extract (0.4) and NaCl (1). The above mixture was autoclaved and inoculated with 5% (v/v) of 48 hr old seed culture[12]. After a period of fermentation, the broth was heated to inactivate the enzymes synthesized by organism and centrifuged to remove the cells. Equal volume of ice cold solvent was added to the supernatant and kept overnight at 4 °C [13]. Precipitated pullulan was centrifuged, dried and weighed. Yield was expressed as, ratio of dry weight of pullulan to dry weight of cells.

Downstream processing of pullulan:

Effect of heating temperature:

The fermented hydrolysate was centrifuged at 8000 rpm for 15 min to remove the cells. Supernatant containing pullulan and enzymes were heated at different temperatures from 50°C to 120°C for constant 60 min and cooled. Solvent was added to these supernatant and dry weight of the products were estimated. Protein concentration was determined using Lowry's assay[14]. The temperature at which maximum enzymes were degraded was studied.

Effect of heating time:

The supernatant from broth was heated for varying time from 10 to 60 min at optimum heating temperature and subsequently enzyme concentration and dry weight of product were analyzed.

Effect of different solvents:

After heating the supernatant for above resulted temperature and time, different solvents were added in equal amount and precipitated pullulan was dried and weighed. Solvents used were ethanol, acetone, isopropanol, diethyl ether, methanol and chloroform.

Effect of solvent ratio:

Supernatant was added with varying ratios of solvent from 1:1 to 1:8, to find the probable volume of solvent for obtaining maximum amount of product.

Effect of precipitation time:

To determine the time at which maximum pullulan was precipitated by solvent, samples were kept for precipitation up to 16 h at 4°C and analyzed at 2 h intervals.

Results and Discussion:

Aureobasidium pullulan synthesized some enzymes in addition to pullulan. At the later stage these enzymes were responsible for the degradation of pullulan. These enzymes were also be precipitated during solvent precipitation, hence they need to removed from fermentation broth. The decomposition temperature of pullulan was greater than enzymes, hence by heating the fermentation broth, enzymes were degraded and pure pullulan can be obtained.

Effect of heating temperature:

Fermentation broth was heated from 50 to 120 °C to inactivate the enzymes. Fig.1 shows that maximum enzymes were denatured at 120°C and hence it was employed to further treatment. Since enzymes were continuously denatured, they were not precipitated by the solvent. Hence the dry weight of the final product gradually decreases. Many researchers explained that in order to remove protein, broth must be subjected to 80 °C of heat treatment[15-17].

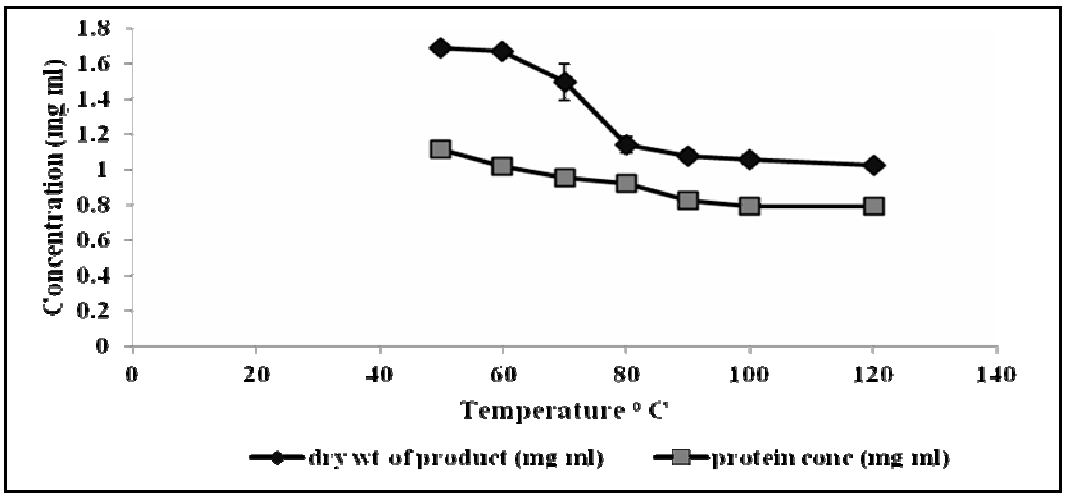

Fig.1 Effect of heating temperature on protein and product concentration

Effect of heating time:

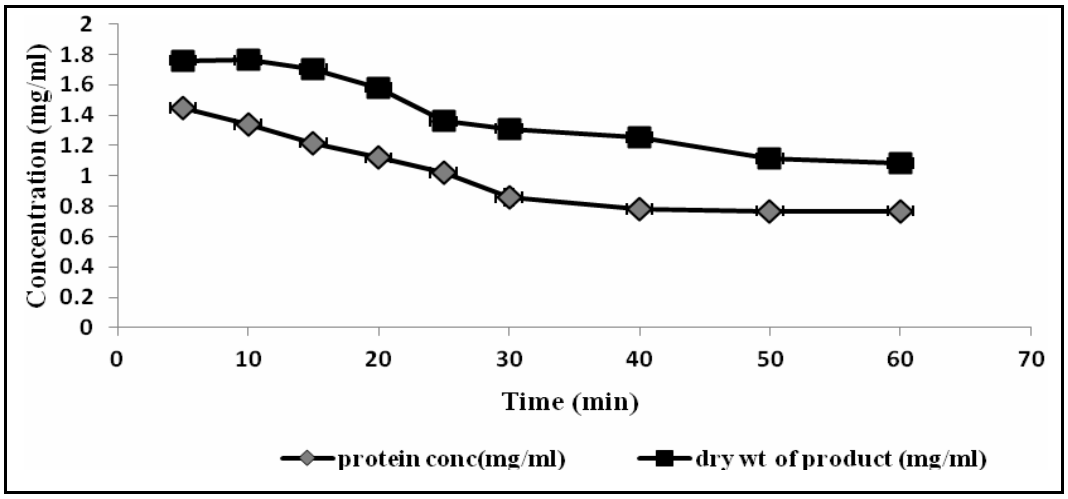

Fig.2 Effect of heating time on protein and pullulan concentration

Fig.2 elucidates that broth should be heated to 120 ºC for 60 min to denature the protein.

Effect of different solvents:

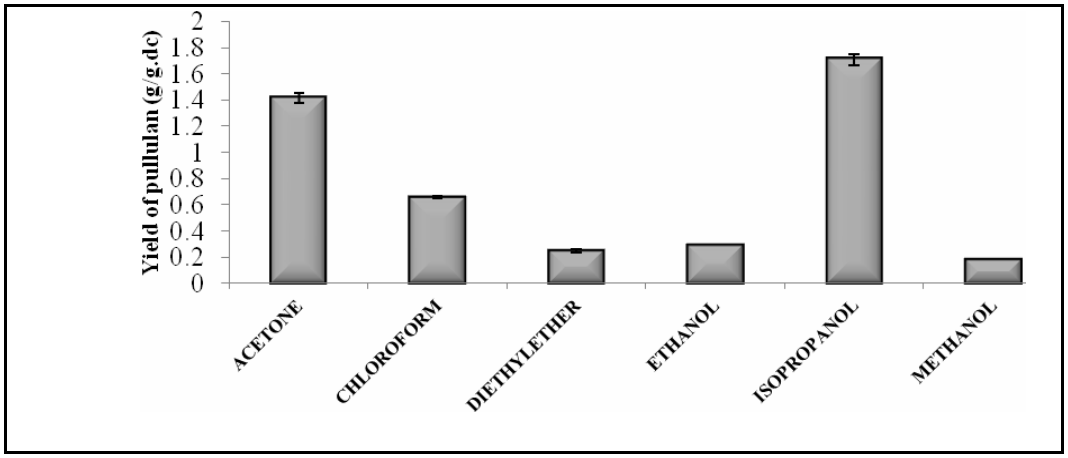

Fig.3 Effect of solvents on precipitation of pullulan

When solvent was added with fermented broth, solubility of polymer gets decreased in aqueous fermentation broth phase and precipitated in organic solvent phase. Downstream processing studies on pullulan

recovery demonstrated that, maximum amount of pure pullulan (1.709 g/gdc) was obtained when isopropanol was applied as solvent (Fig.3). Isopropanol have properties of low hydrophilicity and less volatility and also pullulan was less soluble in isopropanol compare to other solvents. All these enhanced maximum precipitation of pullulan in isopropanol. This result was in accordance with previous results of Kato & Nomura[18] and Pollock *et al*[19]. Various solvents such as ethyl methyl ketone, methanol, tetrahydrofuran, isopropyl alcohol, acetone and ethanol were compared for precipitation of pullulan and it was reported that ethanol was the best solvent[15]. Similarly ethanol was used to precipitate maximum amount of pullulan[20, 21].

Effect of solvent ratio:

When supernatant was added with isopropanol in different ratios from 1:1 to 1:8, all of the pullulan recovered in 1:3 ratio itself (Fig.4). Above this solvent ratio, there observed a constant dry weight of product. Bishwambar and suneetha [18], recovered the maximum pullulan using two volumes of isopropanol whereas Pollock *et al*[19] attained this using one volume of isopropanol. Three volumes of solvent were needed to precipitate maximum pullulan[22]. Maximum pullulan was precipitated by adding two volumes of isopropanol and the pellet was again washed with acetone[23]. Previous studies reported that two volumes of ethanol precipitated more amount of pullulan[15, 24.]

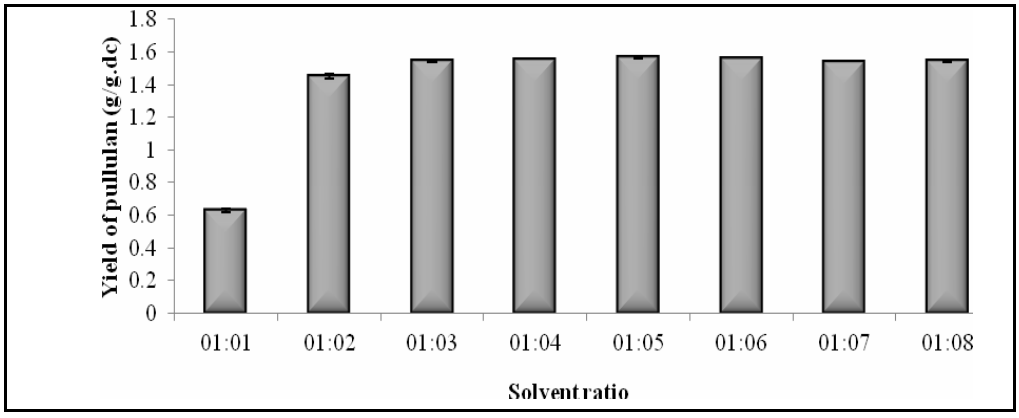

Fig.4 Effect of supernatant: solvent ratio on precipitation of pullulan

Effect of precipitation time:

Maximum amount of product recovered at 10 h of precipitation time and thereafter pullulan yield was constant (Fig.5). Thus it was concluded that 10 hr of precipitation time was effective for complete precipitation of product. Earlier studies explained that maximum polysaccharide precipitated after 12 hr [20]. Precipitation time of 6 hr was required to obtain maximum product [22].

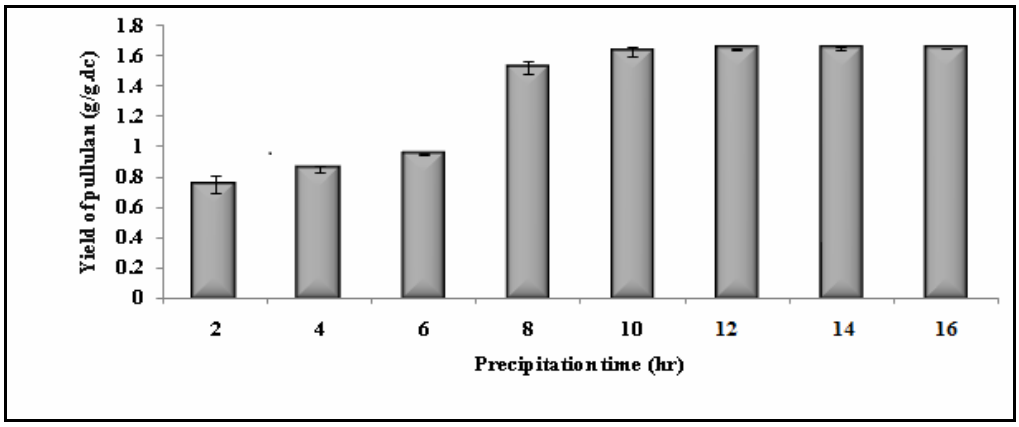

Fig.5 Effect of precipitation time on pullulan precipitation.

Conclusion

Palm kernel hydrolysate was employed as the substrate for pullulan production. The favorable downstream conditions to attain product recovery were obtained as: heating time to degrade proteins (1 hr), heating temperature (120 ºC), solvent (isopropanol), solvent ratio (1:3) and precipitation time (10 hrs).

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