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Bioprocessing of Areca husk in solid state fermentation for cellulase production using Trichoderma viride

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ABSTRACT

Solid-state fermentation (SSF) is an alternative to submerged fermentation for production of antibiotics, enzymes, biofuel, etc. Cellulases are a complex enzyme system, comprising endo-1,4-b-D-glucanase (EC-3.2.1.4), exo-1,4-b -glucanase (exocellobiohydrolase, EC-3.2.1.91) and b -D-glucosidase (EC-3.2.1.21). Cellulases find applications in areas like textile industry, retting process, for the production of feed, fuel etc. This actual waste, Areca catechu husk from agriculture was used as a nourishing base by Trichoderma viride in SSF for cellulase enzyme production. The optimum growth was seen in 5 day culture with inoculums size 5% on the areca husk particles of 180 microns and media of 5.75 pH. The enzyme was extracted and its activity was found out to be 500 mg/ml/min. The results demonstrated that areca husk can be used as an inexpensive source (carbon) for industrial production of cellulase enzymes in solid state fermentation by Trichoderma viride.

Keywords : Cellulase, Trichoderma viride, Solid-state fermentation, Areca catechu husk.

Introduction

Natural fibers like, cotton, coir, sisal jute and others have attracted the attention of scientists and technologists for applications in packaging, low-cost housing, and other structures. Among all the natural fibers, areca appears to be a promising material because it is inexpensive, availability is abundant and a very high potential perennial crop. It belongs to the species Areca catechu L. under the family Palmaceae. It constitutes 3045% of the total volume of the fruit. Areca husk fibers are predominantly composed of hemicelluloses and cellulose. Areca husks left in the plantation lead to bad odors and other decay-related environmental problems.

Cellulose, being an abundant and renewable resource, is a potential raw material for the microbial production of food, fuel and chemicals (Coughlan, 1985). Various bacteria, actinomycetes and filamentous fungi produce extra cellular cellulases when grown on cellulosic substrates though many actinomycetes have been reported to have less cellulase activity than moulds (Ishaque and Kluepfel, 1980; Kluepfel et al., 1986). Investigations on the extracellular cellulases of fungi have been concerted mainly on Trichoderma sp. (Jabbar and Ilahi, 1981; Ross et al., 1983; Ghosh et al., 1984).

Cellulases are important industrial enzymes and find applications in several industrial processes (Hanif et al., 2004; Jamil et al., 2005). Researchers have strong interests in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry, and textile industry (Gao et al., 2008; Zhou et al., 2008).

Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. Agro-industrial residues are generally considered the best substrates for the SSF processes. In solid state fermentation, aeration is facilitated through the spaces between the substrate (Lambraki et al 1994, Soccol et al 1994). Substrate agitation, when necessary, is discontinued (Senez et al 1980, Deschamps et al 1982). The absence of a liquid phase and a low water content permit a) reduction of fermentor volume of liquid effluents from the process, b) reagents saving during metabolites recovery, c) reduction of bacterial contamination and d) use of no sterile solid substrate in some cases. Culture media are simple mainly composed of agro-industrial residues (Lonsane et al. 1985 and Roussos et al. 1991).

Materials and Methods:

Substrate: Areca nut husks belonging to the variety 'Mangala' were procured from a farm in Sirsi, Karnataka, India. Dried areca nut husk was used as the solid substrate for cellulase production using Trichoderma viride. It was crushed to pieces, sun and oven dried, grounded and sieved to obtain 180μ particle size and was stored at room temperature.

Microorganism used for solid state fermentation: The pure culture of the fungi Trichoderma viride was obtained from IMTECH, Chandigarh. This was sub cultured and maintained on potato dextrose agar slants. The pure cultures were stored at 4ºC for further use.

Pre treatment of areca husk: 10g of powdered areca husk of was pre-treated with 2% Hydrochloric acid in an Erlenmeyer flask. This was left to stay at room temperature for 2 hrs, then autoclaved at 121ºC for 15 minutes. The slurry of substrate was filtered through muslin cloth and both filtrate and residue were saved. The filtrate was used for analysis of total sugars, reducing sugars, and protein content. (Ishtiaq Ahmed and et al; 2010).

Determination of percent moisture content: To determine the percent moisture content of areca husk, 1g of the areca husk was weighed in the fresh and moist state (W1), and dried for 2 weeks and again weighed (W2). The % moisture was determined by using the formula: Percent moisture = [(W1-W2) / (W1)] × 100. (Ishtiaq Ahmed et al; 2010).

Estimation of reducing sugar content: Reducing sugar was measured using 3, 5- dinitrosalicylic acid (DNS) (Miller, 1959). 1 ml of the sample was mixed with 3ml of DNS and kept in boiling water bath for 15 minutes. Optical density was checked at 540 nm to measure color intensity.

Estimation of protein content: Protein in the medium was estimated by the method of Lowry using bovine serum albumin as standard (O.H Lowry *et al*, 1951). Different concentrations of the standard were taken along with a known amount of sample in respective labeled test tubes. 5 ml of alkaline copper reagent was added to all the test tubes and incubated for 10 mins at room temperature. To this 0.5 ml of freshly prepared folin ciocalteau reagent was added and again incubated for about 15 minutes at room temperature. Optical density was checked at 660 nm to measure color intensity.

Fermentation protocol: The growth of the microorganism and synthesis of cellulase enzymes were performed in sterilized Petri dishes, with 1g areca husk of 180µ and with fermentation media containing different salts and nitrogen source. This was cultured and recorded for 5 days. The different salts used are as follows: $(NH4)_2SO_4$, [0.7 g/l]; KH_2PO_4[2.0 g/l; Urea [0.3 g/l]; $CaCl_2$ [0.3 g/l]; $MgSO_4.7H_2O$ [0.3 g/l]; Peptone [1.0 g/l]; $MnSO_4.H_2O$ [1.56 g/l]; $FeSO_4.7H_2O$ [5.00 g/l]; $ZnCl_2$ [1.67 g/l]; $CoCl_2$ [2.00 g/l]; Tween 20 [2.5 ml/l]. pH was set to 7 and autoclaved. A very small amount of this media was added just to wet the substrate (husk). Inoculation was done with loopful of spores and was incubated for 5 days at room temperature.

Enzyme extraction: The enzyme was extracted by simple contact method (C. Krishna, *et al* 1996). The clear solution thus obtained was used for further analysis. Phosphate buffer 0.05M was added to the conical flasks, along with the fully grown 5 day culture of *Trichoderma viride* on areca husk. The buffer was added in the ratio of 1:10(w/v) and kept in rotary shaker for 30 minutes at 120 rpm. Then it was filtered using whattman filter paper No. 1. The filtrate was centrifuged at 10,000 rpm (4ºC) for 15 minutes to obtain clear supernatant which was used for further analysis.

Enzyme activity: Cellulase enzyme activity was found out by the Ghose method. (T.K Ghose, 1987). One unit of enzyme activity which is the amount of enzyme required for liberating 1µg of glucose per milliliter per minute was expressed as µg/ml/min. 1% Carboxymethyl cellulose was dissolved in 0.05M of 0.5ml phosphate buffer, to this 0.5 ml of crude enzyme extract was added and kept for incubation at 50ºC for 1hr. An appropriate control was also run along with the test.

Incubation was done in boiling water bath for 5 min to arrest the reaction and DNS method by Miller was carried out to estimate the amount of glucose (Miller 1959). The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose.

Purification of the enzyme: Purification was performed in three stages. The first was using salting out technique, wherein ammonium sulphate salt was used in 45% and pellet was resuspended in phosphate buffer. In the second stage the extract was poured into the dialysis bag and dialyzed for overnight. The product obtained from this stage was further used in third stage of purification by column chromatography using sephadex g-25.

Effect of various carbon sources on cellulases production: T. viride was grown in fermentation medium on different substrates i.e. carbon sources like sugarcane bagasse, wheat bran, coconut coir, shredded paper to check their effect on the expression of cellulases.

Parameter analysis for cellulases production from Trichoderma viride:

Determination of optimal pH:

The optimum pH for cellulases production was estimated at various pH values of 7.9, 5.75, 6.4 with appropriate buffer at 28ºC. The mycelia and spores were seen in only 6.8 and 5.75 pH media when grown under the conditions mentioned above. Cellulases activities were determined from the culture filtrate.

Determination of temperature:

The temperature optimization was performed by incubating the cultures at 4 different places. The inoculated media was kept at room temperature, incubator, hot air oven, refrigerator which had an atmosphere of 29ºC, 37ºC, 60ºC, 4ºC respectively.

Determination of salting out percentage:

Salting out technique was optimized using ammonium sulphate salt where in different percentages of 43,45,47,49 were taken and dissolved in the crude filtrate and centrifuged to obtain pellet.

Determination of particle size of substrate:

The dried and powdered areca husk was sieved under different mesh sizes of 75µ, 90µ, 125µ, 150µ, 180µ. All these were later weighed, cultured and the substrate particle size was optimized.

Determination of inoculums size:

The optimum inoculums size was carried out using inoculums of different percentage like 1%, 2%, 3%, 4%, 5%. These were poured onto optimized husk size and cultured. Thus the inoculums size was optimized.

Determination of incubation time:

The incubation time was optimized using 6 cultures all of which was inoculated on the same day and incubated for 6 days. Every day a single culture was used for extraction of cellulase and its activity was estimated.

Results

Chemical Composition of Substrate

To investigate the suitability of areca husk for cellulose production under solid state fermentation condition, chemical composition of areca husk was determined. Result obtained showed protein, cellulose, fat, sugar content of areca husk were 5.2-5.76%, 65-72%, 5-5.2%, 39-43.2%. And the moisture content in wet green areca husk was 82-83%. The protein content in areca husk was estimated by Lowry's method. According to the graph plotted below the protein content was found to be 8mg/ml, similarly the sugar content was estimated through DNS method and the graph plotted below shows sugar content as 60mg/ml.\

Figure 1 : Sugar estimiation

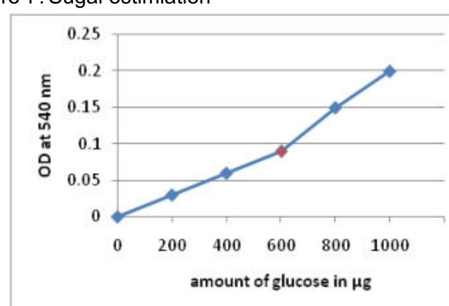
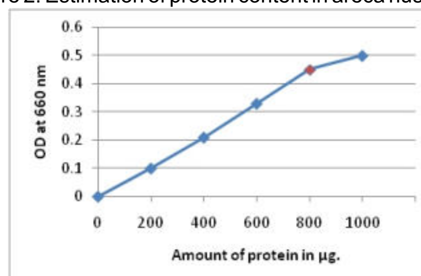


Figure 2: Estimation of protein content in areca husk

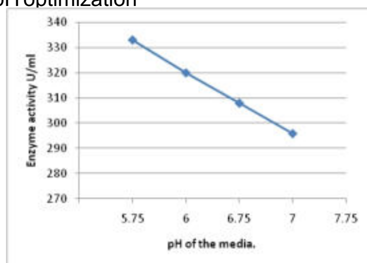


To produce cellulase through solid state fermentation, media with different salts, Peptone, Tween 20 and dry areca husk as carbon source was prepared. The Trichoderma viride subculture was inoculated over the media and on third day of incubation mycelium was observed and on 5th day spores were observed. The culture obtained was used for determining cellulose degradation activity to establish the presence of cellulase enzyme. Cellulase extraction was performed and crude enzyme solution was obtained. The crude enzyme was incubated with CMC to determine cellulose degradation activity by reading the amount of glucose formed by DNS method.

Parameter Optimization

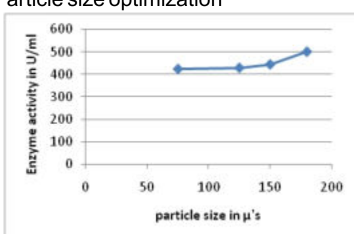
The optimization of number of days for incubation was done by incubating the culture for 3, 4, 5, 6, days and finding the crude enzyme activity. We saw, as the number of days increases, activity increases and reaches optimum, and further increases in incubation period activity declines. Thus optimal incubation time was found to be 5 days. And the activity was found to be 296 µg/ml/min at neutral pH. This is in accordance with article reported by Sibtain Ahmed et al. Similarly the pH was optimized, here we grown the culture at different pH 5.75, 6.0, 6.75, 7.0 pH and activity found to be 333,320,308 and 296U/ml.

Figure 3: pH optimization



To optimize the particle size, Sieve analysis technique was followed. After performing sieve analysis particles of different sizes 180,150, 125, 90, 75, 45microns were obtained. Fungi were grown on all the different size particles. Activity v/s particle size graph was plotted 180microns with highest activity of 494.22U/ml found to be optimal.

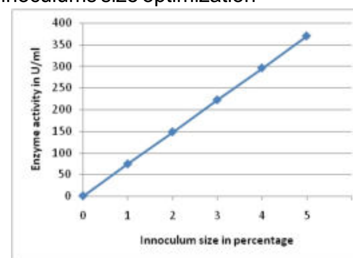
Figure 4: Particle size optimization



Innoculum size containing number of cells was optimized by inoculating the media with 1%, 2%, 3%, 4%, 5% inoculum. And the activity v/s inoculums size graph was plotted 5% innoculum with 370.67U/ml was found to be optimal.

To optimize the temperature, the culture was incubated at different locations incubator (35'C), hot air oven (55'C), at room temperature in laboratory (28'C-35'C). And the growth was seen only at room temperature thus room temperature was assumed as optimal. This is in accordance with article reported by Sibtain Ahmed et al.

Figure 5: inoculums size optimization



Comparison with other substrates

Trichoderma viride culture was inoculated to the media containing other substrates such as carbon sources like coconut coir, paper, sugarcane bagasse and wheat bran. And growth of fungi was compared with areca husk. Wheat bran and coconut coir showed totally no growth whereas sugarcane bagasse and paper showed little growth, though the growth was so less such that no complete utilization of substrates were observed. From this we understand that in solid state fermentation areca husk is an ideal substrate for the production of cellulase.

Figure 6: (a) five day cultured areca husk, Fig: (b) five day cultured coconut coir, paper, and sugarcane bagasse, in clock wise rotation.

Conclusions:

It was confirmed from the observation that the cellulase enzyme activity was increased after being grown for optimized number of days on the Areca husk. Thus results obtained were that at pH 5.75, particle size of 180 microns, inoculums size of 5 percent and at room temperature, the activity was maximum of 500 µg/ml/min also there was no growth or enzyme activity seen in different cellulosic substrates that were used.

We also came to know the areca husk composition and the optimum values at which there was maximum enzyme activity.

It was noticed that the Trichoderma viride grew very well on areca husk than other cellulose containing substrates like sugarcane bagasse or coconut coir or shredded paper. Thus this led to the confirmation in potential of using areca husk as the substrate that can be best utilized for production of cellulase enzyme with a considerably high enzyme activity in solid state fermentation using the most commonly found and easily growing fungi T.viride.

Solid state fermentation was the process which was followed here and it proved to be the best technique as it required very less amount of moisture or water content, there by chances of contamination of the cultures was almost negligible. It was inferred that Trichoderma viride can also be used as a bio pesticide, whereas the used husk could be beneficial in the production of alcohol, which is gaining importance as a bio fuel.

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