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Research Article

Production of Cellulase using *Trichoderma atroviride* ATCC® 28043™ by Solid State Fermentation from a Novel Mixture of Coir, Vegetable and Fruit Peels

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Abstract

Lignocellulosic bio-ethanol owes close to 40% of its production cost to the expensive cellulase production processes. *Trichoderma atroviride* ATCC® 28043™ is a filamentous fungi with previously reported cellulolytic activities. In this work, we have performed an optimized Solid State Fermentation (SSF) in an aerated tray reactor to produce cellulases (Koji type fermentation) using coir, potato peels and Sapodilla peels as the substrates. In an optimized 10 day SSF process the activities of FPases was 1.665 IU/mL, CMCase was 12.7 IU/ml, Xylanase was 676 IU/mL and Beta-glucosidase was 2825.9 IU/mL. Optimum conditions for saccharification were determined. The enzymes concentrated using a tangential flow filtration apparatus were used to saccharify ammonia pre-treated rice straw. The release 0.33 g glucose/g rice straw after 48 h at 50°C was obtained using cellulase loading of 35 FPU/g rice straw. The 43.67% saccharification of rice straw with 91.6% yield was obtained using produced cellulases.

Keywords: Cellulase; Coir; Saccharification; Optimization; Solid state fermentation.

Introduction

Cellulases are a group of three enzymes which act sequentially to hydrolyze the cellulose polysaccharide to individual beta-D-glucose units. Endoglucanases/CMCases (EC 3.2.1.4), exoglucanases/FPases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and β -glucosidase (BG) (EC 3.2.1.21) are the three enzymes. Using the mechanism of acid-base catalysis, these enzymes cleave the beta 1-4 beta glycosidic linkages in cellulose to glucose monomers. Endoglucanase, or CMCase, randomly cleave the β-1,4-bonds in the cellulose chains, creating new ends. Exoglucanases act on the reducing or nonreducing ends of cellulose polysaccharide chains, releasing cellobioses or glucose. β -Glucosidases (BG s) hydrolyze soluble cellodextrins and cellobiose to glucose [1, 2].

Solid State Fermentation is one of the major cultivation methods of micro-organisms for the production of value added products. The micro-organisms grow on a moist bed of substrates that has no water or no free-water [3-7]. Mushroom cultivation and soy bean koji fermentation for the production of soya sauces are good examples. The process may be carried out in aerobic, anaerobic or micro-aerophilic conditions. The substrates must provide the growing microorganisms with efficient carbon sources, nitrogen sources and nutrients [6, 8]. Yeasts and fungi in SSF can grow at a water activity well below 0.7. This low water requirement and presence of very less free water offers the advantage of very less contamination in SSF [3, 5]. Tray fermentor, rotating disc fermentor, rolling drum fermentor continuous screw fermenters and agitated tank fermenters are some major types of solid state fermenters. Microbial growth inside the SSF follows the usual three stage growth namely lag phase, log phase or exponential phase and Stationary phase [9-13]. The major advantage of SSF over Submerged Fermentation SMF is a slightly reduced catabolite repression caused by the availability of an easily metabolizable substrate.

The particle size of the fermentation substrates play a major role in the anchoring of the mycelia of the fungus [14]. The fungus may propagate in the aerial direction and on the surface by producing mycelia [15]. The bed

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height of the media used for fungal growth is maintained below 30 cm. Temperature is held constant from around 30°C for most applications [2, 4, 6, 15-17]. The moisture content is maintained by using minimal media components [14] around 30-37% [16, 17] and 60% to 90% in some special cases [6]. Efficient mass transfer between the particles needs to be maintained by using an internal aeration system. The aeration should be mild so as to not dry the medium which will cause the death of the mycelia. If the media dries up, sterile minimal media components are sprayed on top of the media to moisten it [4, 9]. Humidity needs to be maintained. Usually 85-90 % humidity is maintained by growing in a humidity chamber [15].

Heat generation in solid state fermentor will be high. The heat is maintained using a cooling jacket that exchanges heat or inside a temperature maintaining incubator. The surface area of the substrate used for fungal growth is kept high while compared to the depth [6]. Partial intermittent mixing maybe essential during some SSF's while some SSFs will require static growth [9]. A fed-batch kind of SSF is practiced in which a portion of the used substrate containing the microorganism is reused as the starter inoculum for the next batch of the solid state fermentation [18]. The initial liquid inoculum used for commencing the fermentation was varied between 10 to 20% [18]. The pH plays an important role in the production of the enzymes. However, the pH cannot be maintained throughout the process unlike a solid state fermentation. It needs to be checked and set during the start of the fermentation prior to autoclaving and maintained [2] at around 4-6 pH [4, 7, 9, 14, 15]. The batch SSF is complete when visible uniform fungal growth is observed throughout the surface of the substrate. After this, a step of extraction of the enzymes is followed by crushing the Koji components (SSF media with microorganism on top) and extracting the enzymes using 10-40 volumes buffer [6, 17, 18]. A stage of centrifugation is performed to remove the debris, if any. The enzyme activities are estimated then [6, 9-13, 19-21].

Materials and methods

The strains were purchased from the ATCC. The nutrient rich media Potato Dextrose Broth procured from M/s Hi-Media was used for the growth of fungi. It was prepared according to the manufacturer's instructions and autoclaved at 121°C for 20 min. Other reagents used below were procured from the brand Hi-Media.

The composition of Vogel's media (g/L) is as follows: Tryptone (1 g/L), Tri-sodium citrate (2.5 g/L), Di-Potassium hydrogen phosphate (5 g/L), Ammonium nitrate (2 g/L), Magnesium sulphate heptahydrate (1.4 g/L), Calcium chloride dehydrate 0.1 g/L, Tween 80 – 0.2 % (v/v). A Trace element solution containing Citric acid monohydrate 5 g/L, Zinc sulphate heptahydrate 5 g/L; ferrous ammonium sulphate 1 g/L, Copper sulphate 250 mg/L, Manganese sulphate 50 mg/L ; Boric acid 50 mg/L, Sodium molybdate 50 mg/L was prepared. 1 mL of trace elements per litre of the total media components was added [22]. The pH was set at 5.5 before autoclaving.

Preparation of the Coir, Potato peel and Sapodilla Peel Substrate

Coir was procured from coconut processing industry. Potato peels and sapodilla peels were procured from the market. The three substrates were individually sundried for 24 hours. After sun-drying they were washed three times with hot water to remove any residual content. They were then finely ground to a powder form using a mixer. Equal quantities of the three cellulosic substrates were mixed to yield a total weight of 200g cellulosic substrate.

Optimization of Solid State Fermentation using Vegetable, Fruit Peels and Coir

Potato and sapodilla peel powders were mixed in a constant ratio of (1:1). The ratio of potato-Sapodilla peels:coir was varied as (1:0.5), (1:1) and (1:1.25). The other parameter that was analyzed was the number of days of incubation which varied as 5 days, 10 days and 15 days. Potato Sapodilla powder were so fine that they stuck together when moisture was added and inhibited mass transfer and fungal growth. As they dried, the potato sapodilla peel mixture became solid with time due to drying. Hence coir which is a cellulosic substrate apart from serving as a substance that does not permit Sapodilla potato peels to stick together was used in an optimized ratio. The right proportion of coir would permit the mass transfer, equal spreading of moisture and growth of fungus evenly throughout all the layers without over drying of

the Koji components. A Response Surface Curve was plotted to observe the optimum condition.

Solid State Fermentation

Agar plates with the minimal media components of Vogel's media with 1% Microcrystalline cellulose were used for initial culture from PDA slants of *Trichoderma atroviride* ATCC® 28043™. A spore suspension of (2.0 x 10^9 /mL) was used to inoculate a primary culture Erlen meyer flask containing autoclaved 200 mL of Potato Dextrose Broth. The flask was incubated in an incubator shaker at 180 rpm and 28°C. After 3 days of incubation when the culture enters the log phase and multiplies rapidly, incubation was halted. In an autoclavable aerated tray reactor, 200 g (1:1 ratio of potato-Sapodilla:Coir) of the cellulose substrate was mixed homogenously with Vogel's media basal salts 50 (v/w %) at a pH of around 5.5. The peels and coir themselves serve as the carbon source. A moisture content of around 50-60 % was maintained in the process. The pH for the fermentation was maintained at 5.5 before autoclaving. The substrate bed were spread for a height of 5 cm above the aerated tray of the reactor of dimensions lbh (35 cm x 30 cm x 15 cm). The tray reactor along with the media mixture was autoclaved for 20 min at 121°C. After autoclaving, 60 mL of the starter culture in PDB was used to inoculate the 200 g SSF media. The inoculation and mixing were performed under sterile conditions. The tray reactor was placed inside a humidity chamber to keep the humidity around 80% and a temperature of 28° C. A constant aeration of around 0.5 vvm was maintained in the systems. Regular observations were done visually to check for the drying of the fermentation contents. If the components dried up due to aeration, a sterile pipette was used to moisten the fermentation components using autoclaved Vogel's minimal media components periodically under aseptic conditions. At the end of 10 days, a dark mat of the fungal hyphae entirely covered the surface and the total contents. An optimized time of 10 days was required for complete growth [19-21].

Extraction of Enzymes

The same method of extraction was followed for the 3 variants of the tray reactor media components. 2 volumes (400 mL) of Citrate buffer of 0.05M similar to the Koji volume was used to run through the buffer port in the top of the reactor. A harvest port in the bottom collected the buffer which flowed down. The citrate buffer was recycled into the system 4 times. In the second process, the mould Koji was pressed using a wooden presser under sterile conditions [18]. The remnant of the buffer and the enzymes flowed out. A metal wire gauze filter was used to filter out any residual components. A step of centrifugation was performed to remove the debris from the extract. The supernatant contains the enzyme [18]. The total volume of the buffer used was 400 mL. Around 390 mL of the buffer was recovered from the Koji. The enzyme activities for the produced cellulases were measured using the standard IUPAC DNSA [23] reducing sugar estimation method.

Concentration of the Enzymes

The supernatant obtained after centrifugation was subjected to a Tangential Flow Filtration using a TFF Cassette of Sartorius Viva flow 50R model. It was concentrated around 15 folds. A substantial reduction in the total volume of the enzyme supernatant broth was observed. For the extraction liquid of the Solid state fermentation, the 390 mL volume was reduced to 20-25 mL final volume after concentration. The activity was measured using the standard DNSA method of IUPAC [23].

Optimization of Saccharification using Cellulases

The produced cellulases were concentrated using a tangential flow filtration system. Once concentrated, they were optimized for loading into the reactor for saccharification. Temperature of saccharification (45°C, 50 °C and 55°C and three loading quantities (30 FPU/g biomass, 35 FPU/g biomass and 40 FPU/g biomass) were chosen and the optimum condition was evaluated after estimating the saccharifcation efficiency using HPLC analyses. 2 g/L Tween 80 and 0.01% (w/v) sodium azide were used in the saccharification process. The efficiency of the produced cellulases were calculated using the standard IUPAC formulae is given in equation (1) and (2).

$$\%\ Saccharification = \left\{\frac{(Total\ Sugars\ (g) \times 0.9)}{Weight\ of\ alkali-pretreated\ rice\ straw\ (g)}\right\} \times 100 \quad (1)$$

$$\%\ yeild\ of\ saccharification = \left(\frac{practical\ yeild}{theoritical\ yeild}\right) \times 100 \quad (2)$$

The factor is used to convert polysaccharides to monosaccharides accounting for water uptake during hydrolysis is 0.9.

Results and discussion

Optimization of Solid State Fermentation using Vegetable, Fruit Peels and Coir

The process of solid state fermentation was optimized for the best combination of the substrate ratios and days of incubation as shown in Fig. 1. The Sapodilla:potato peel ratio was maintained constant while the Sapodilla-potato peel:coir ratio was varied as (1:0.5), (1:1) and (1:1.25). The days of incubation were 5 days, 10 days and 15 days. 10 day incubation with 1:1 Sapodilla-potato peel:coir ratio was the optimum yielding significantly higher enzyme activities of 1.66 FPU/mL, 12.7 IU/mL CMCase, 676 IU/mL Xylanase, and 2825.9 IU/mL Beta-glucosidases.

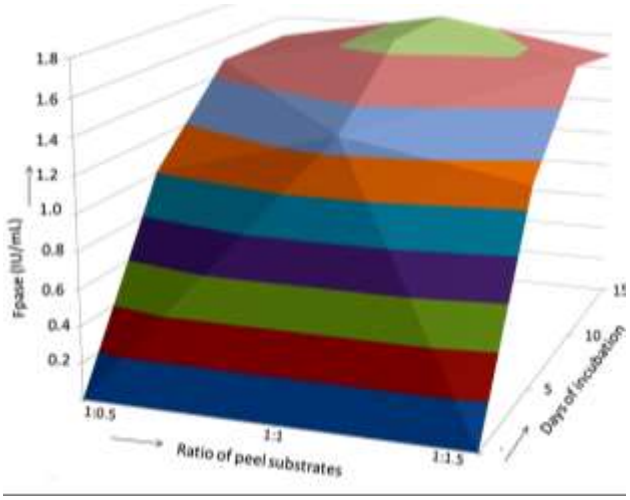

Fig. 1. Response Surface Curve for the optimization of Solid State Fermentation using Veg-fruit peel-coir mixture

When a (1:0.5) Sapodilla-potato peel:coir ratio was incubated for 5 days and 10 days, 1.01 and 1.11 FPU/mL activity was recorded. The lower activity may be due to lesser days of incubation and mass transfer hindrances to the growing fungi. Sapodilla-potato peel powders are so fine that they become pasty mass following moisture addition and autoclaving. As the moisture dries up due to uptake of minimal media by the fungus, the pasty mass dries to become solid. Aeration cannot efficiently happen within the pasty solid which is why the (1:0.5) Sapodilla-potato peel:coir ratio fails to give better enzyme activity. However in the (1:1.25) Sapodilla-potato peel:coir ratio, too much coir would cause improper adhering of the fungi to the major cellulose containing substrates Sapodilla and potato peels and quickening of drying of the minimal media moisture which results in a decreased activity. The above cited explanations may be the reason for a reduced enzyme activity for a 10 day and 15 day incubation period with (1:0.5) and (1:1.25) Sapodilla-potato peel:coir ratios. (1:1) ratio of Sapodilla-potato peel:coir ratio and 10 day and 15 day incubation periods gave the same enzyme activities of 1.66 FPU/mL. However, since there was no difference as the time of incubation was extended to 15 days, it was decided to consider 10 days and (1:1) Sapodilla-potato peel:coir ratio as the optimum condition.

Solid State Fermentation

The solid state fermentation gave the enzyme yield for FPases: 3.24 IU/g.ds, for CMCases: 24.8 IU/g.ds, for Xylanase: 1318 IU/g.ds and for Beta-glucosidase: 5508.7 IU/g.ds the enzyme activities are shown in the figures as follows: Fig. 2 shows the Enzyme activity of FPases, Fig. 3 shows the Enzyme activity of CMCases, Fig. 4 shows the Enzyme activity of Xylanases, Fig. 5 shows the Enzyme activity of Beta-glucosidases. The growth of the fungus commenced after 12 h like a spongy mass. Slowly the growth started intensifying in the bottom layers as well [15, 24, 25].

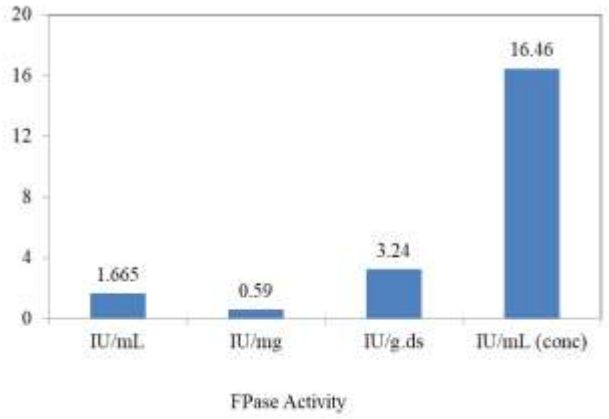

Fig. 2. FPases enzyme activity IU/mL, specific activity IU/mg, activity per gram dry solids IU/g.ds and Enzyme activity after concentration using Tangential Flow Filtration IU/mL

After 3 days, the fungus had developed nascent mycelia uniformly throughout the bed. Though the mycelia took time to initiate growth on the coir-peel substrate, it was observed that the growth intensified within 48 hours. A short lag that occurred may be attributed to the presence of the recalcitrant lignin coat. The hemicelluloses may pose a problem of catabolite repression decreasing the production of cellulases. After the consumption of hemicelluloses, the fungi may start secreting cellulases which may be why the coir peel

substrate produces lesser cellulases than a grain straw substrate. The growth was observed with the fungi absorbing the moisture from the solid state components [15].

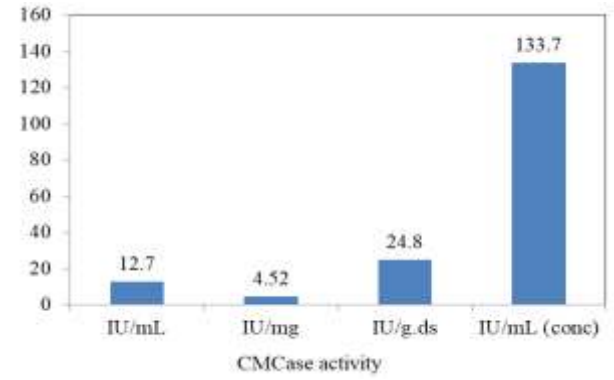

Fig. 3. CMCases enzyme activity IU/mL, specific activity IU/mg, activity per gram dry solids IU/g.ds and Enzyme activity after concentration using Tangential Flow Filtration IU/mL

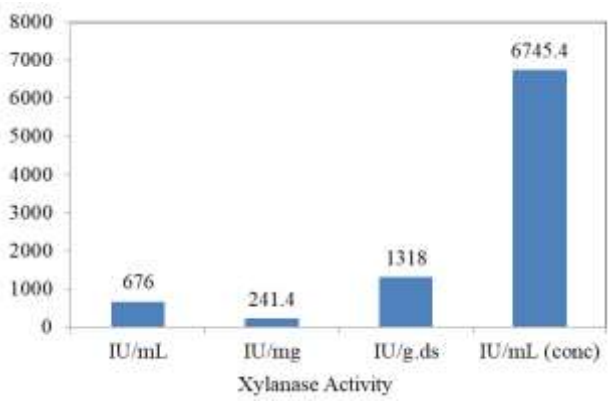

Fig. 4. Xylanases enzyme activity IU/mL, specific activity IU/mg, activity per gram dry solids IU/g.ds and Enzyme activity after concentration using Tangential Flow Filtration IU/mL

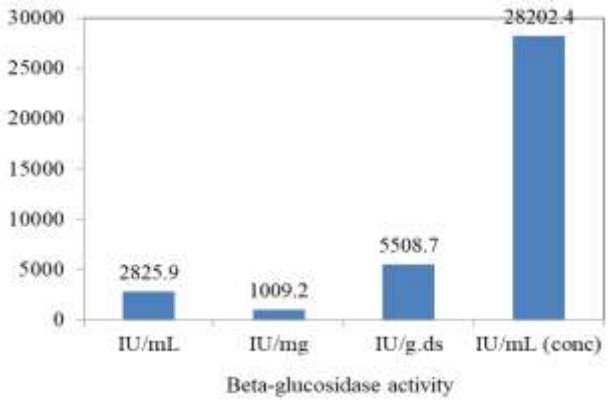

Fig. 5. Beta-glucosidases enzyme activity IU/mL, specific activity IU/mg, activity per gram dry solids IU/g.ds and Enzyme activity after concentration using Tangential Flow Filtration IU/mL

Mass transfer was maintained using an internal aeration line that passes through the solid state components. While working out the economics of the process, it has been observed that SSF gives higher cellulase activity in IU/mL, than SMF processes. SSF process incorporating such substrates is cheaper than the process and costly substrate used in submerged fermentation. SSF has performed better than SMF in yielding high IU/gds of cellulase activity. During our previous trials it has been observed that harvesting the enzyme from the mouldy Koji at the end of 10 days gave the enzyme with optimum activity. A further increase after 10 days did not show an increase in the enzyme activity. The increased enzyme activities obtained at the end of 10 days makes this an attractive process compared to the submerged fermentation [19-21].

Optimization of Saccharification using Cellulases

Saccharification was carried out using the produced and concentrated cellulases. The optimal condition was found to be 35 FPU/g rice straw loading and 50^{o}C saccharification temperature which released 0.33 g glucose /g pre-treated rice straw. The process lasted for 48 hours at a stretch. The response surface curve for the process of optimization of saccharification is given in Fig. 6. When 30 FPU/g rice straw loading and 45°C was used, around 0.28 g glucose / g rice straw was released which conveyed that higher FPU/g rice straw loading and a higher temperature maybe required. When 35 and 40 FPU/g rice straw were used at 45°C, around 0.30g glucose / g rice straw was released which showed that 35 and 40 FPU/g rice straw loading of the cellulase did not make a big difference in the saccharification efficiency.

At 50°C and 55°C and 30 FPU/g rice straw cellulase loading, 0.30 g glucose/g rice straw was released which indicated that temperature played a major role in the saccharification process. At 50 and 55°C and 35 and 40 FPU/g rice straw cellulase loadings, we obtained a release of 0.33g glucose/g rice straw which indicated that 35 FPU/g rice straw loading and 50°C were the optimum conditions as any further increase in the enzyme loading and temperature did not show any significant increase in the sugar release. The percentage Saccharification (% Saccharification) of the cellulases produced from the veg peel fruit peel

and coir substrates as determined by the standard IUPAC method was found to be 43.67% and the % yield of saccharification was found to be 91.66%. The process of cellulase production in submerged fermentation suffers a serious issue of catabolite repression due to the accumulation of glucose in the media which are hydrolyzed cellulose units from the substrate [27-29].

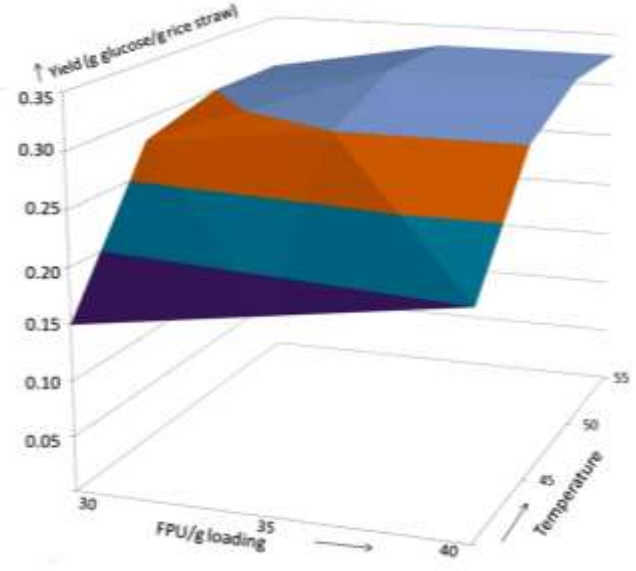

Fig. 6. Response Surface for the optimization for the process of saccharification using the produced cellulases

Conclusions

The SSF was carried out under optimized conditions using coir, Sapodilla peels and potato peels as the cellulosic substrates. The vegetable coir and fruit's cellulose content was made use for the production of cellullytic enzymes. Catabolite repression may happen in SSF but it doesn't happen to a much greater extent as it does in SMF operations. The process of scale-up may pose a problem when SSF methods are chosen industrially. The limited bed height and constant moistening of the solid state Koji media make the process of SSF cumbersome, when considered in large scale operations. In the perspective of cost efficiency, SSF definitely has an edge over the SMF systems. The substrate is inexpensive. The use of such cheap residual fruit, vegetable matter is economical and also an alternative approach to the use of just food grain straw as the preferred biomass for cellulolytic enzyme production. An optimal Condition for saccharification was determined and the efficiency of the produced *in-house* cellulases was assessed.

Conflicts of interest

The authors hereby declare that they have no conflict of interest with similar work being carried out elsewhere.

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