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Research Article

Production of Cellulase from Potato, Sapodilla, Kiwi peels and Coir using *Trichoderma atroviride* ATCC 28043 by Submerged Fermentation

T. R. Sambavi, R. Navnit Kumar, S. Jason Charles, S. Renganathan*

Centre for Biotechnology, A. C. Tech., Anna University, Chennai-600025. India.

*Corresponding author's e-mail: srenganathan@annauniv.edu

Abstract

Lignocellulosic bioethanol production cost owes close to 40% to the cellulase production processes. A novel combination of potato, Sapodilla, Kiwi peels and coir were used for cellulase production by an optimized submerged fermentation using *Trichoderma atroviride* ATCC 28043. Submerged Batch fermentation (SMF) were carried out individually using two substrates such as the novel veg-fruit peel, coir substrate and pulverized rice straw to produce cellulases. The activities of cellulase produced in SMF using the novel substrate mixture were FPase 1.66 IU/mL, CMCase 10.85 IU/mL, beta Glucosidases 2705 IU/mL and xylanases 609.9 IU/mL. Using rice straw as the substrate, the enzyme activities were FPase 0.28 IU/mL, CMCase 1.83 IU/mL, beta Glucosidases 456.3 IU/mL and xylanases 102.8 IU/mL. Percentage Saccharification of 43.6% and percentage yield of saccharification of 91.6% were observed for the cellulases produced from the novel substrate mixture.

Keywords: Lignocellulosic Biomass, Cellulase, Submerged fermentation, Saccharification, Tangential flow filtration.

Introduction

Cellulases are a group of three enzymes which act sequentially to hydrolyze the cellulose polysachharide 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].

Submerged fermentation and the conditions mentioned in this paper are in accordance with submerged cultivation of fungus. The submerged fermentation in a bioreactor is an extension of the microbial cultivation in an Erlen Meyer flask, the only difference being the controllable parameters such as temperature, aeration, pH, foaming and agitation [2]. Baffles are used to prevent the formation of vortex in the liquid broth. Prior to inoculation of the microbe in a bioreactor, a primary culture or the starter inoculum is essential [3]. The primary inoculum may be prepared in an undefined nutrient rich media such as the Nutrient Broth or the Potato Dextrose Broth. In some cases the spores from a pre-cultured agar plate are counted and they are transferred to a preliminary culture medium which may be a defined media [1, 3, 4]. The media components may be the same as used in the bioreactor. Once the starter culture is ready, the bioreactor is filled up to the required volume, up to or below the working volume. The pH probe is calibrated using two point standards such as pH 7 and pH 4 prior to autoclaving. The bioreactor after being filled up with the media, is autoclaved at 121°C for 20 min [3, 5]. After autoclaving is complete and the media reaches the temperature of operation, the DO (Dissolved Oxygen) probe is polarized for 6 h with continuous aeration of air. After the DO probe is polarized, the bioreactor is inoculated with the required volume percentage of the starter culture. After inoculation all the parameters such as the

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maintenance of temperature, antifoam, pH maintenance, aeration, agitation are controlled throughout the operation of submerged fermentation [1, 3, 5, 6].

Heat transfer and mass transfer rates are much higher in SMF than SSF [7-10] and Aeration and agitation are important parameters for growth. The impeller / agitator should be chosen carefully based on the nature of the culture broth. For cultures having moderate to highly viscous applications, anchor type impeller and propeller type impellers or impellers with low-shear axial flow are used The type of impeller suited for an operation may be designed using the Computational Fluid Dynamics studies CFD [11]. Newer fermenters use the intermixing impeller disc Fungal culture usually require better formation of mycelia and the impeller motion should be low enough to not cause shearing to the pelleted formation and hyphal tips [10]. Mature mycelia accumulate and produce larger volumes of the extracellular enzymes. However, filamentous fungi show a comparatively lesser production in SMF than SSF due to the lesser amount of mycelia formed in SMF operations [4, 9, 10].

The DO probe senses depletion in the oxygen inside the reactor which occurs with a natural increase in the biomass. Too much agitation and too much aeration cause the problem of foaming. Foams increase the mass transfer coefficient, but are not preferred owing to chances of contamination and overflow through the reactor ports. A stipulated quantity of antifoam such as silicone oil or polypropylene glycol is prepared and used to reduce the foam. The above mentioned parameters are controlled by the integrated system of the bioreactor based on the set pH, temperature, agitation and aeration. A foam sensor will send signals and the antifoam agent adds up into the bioreactor through special ports. To maintain pH, specific concentrations of acids and bases are used in separate ports. A harvest port in the bioreactor helps in the collection of samples at specified and required intervals from it. Scale up of the fermentation from a lab scale bio reactor to a pilot scale/ production scale bioreactor can be performed using the calculation of various data such as the yield coefficient, product per substrate consumption rate, Substrate utilization rate and Oxygen uptake rate of the growing culture along with the determination of the maintenance coefficient of the fungi in the culture broth [9].

Materials and methods

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.

Preparation of the novel substrate mixture

Coir was procured from a coconut processing industry. Potato peels, Kiwi peels and sapodilla peels were procured from the market. The four substrates were individually sundried for 24 h. 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 four cellulosic substrates were mixed. The vegetable, fruit peels and coir mentioned above have individual reports on the composition of cellulose, hemicelluloses and lignin in them [12-15]. In the present work, an investigation was performed on the cellulase production capabilities of the peels and coir.

Fermentation media

The present work aimed at investigating the growth and cellulase production of *Trichoderma atroviride* ATCC 28043 using two cellulosic substrates namely the novel peel-coir substrate mixture and rice straw. The rice straw was pulverized and ground to a fine powder prior to usage as the cellulosic substrate. The composition of Vogel's media 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), finely powdered peel-coir substrate 1% (w/v), 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 [16]. The pH was set at 5.5 before autoclaving. While using rice straw as the cellulose substrate, a compositional analysis was performed in advance to know the amount of

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cellulose per gram of the rice straw. Once known, around 32 g/L of rice straw was added to Vogel's media. For rice straw, 32 g/L constitutes 1% cellulose quantity as estimated by compositional analysis. Around 9.6 g cellulose was present in 32 g rice straw.

Submerged fermentation

The submerged batch fermentation of 1 L culture volume was carried out in a 3.2 L Bioengineering KLF Advanced Bioreactor with automated controllers. Two individual batch fermentations were carried for the two different substrates. 1% finely powdered peel-coir substrate in Vogel's media and 1% cellulose from rice straw in Vogel's media.

Primary inoculum

Two primary inocula for the two different submerged batch fermentations were prepared using microcrystalline cellulose. The primary inocula were prepared in autoclaved 200 mL Vogel's media in individual Erlen Meyer flasks containing 1% microcrystalline cellulose. A spore suspension containing ($3x10^9$ /mL) spores was used to prepare the starter cultures. The primary inocula were incubated in a rotary incubator at 28 degree Celsius for 5 days prior to inoculation into the bioreactors.

Optimization of submerged fermentation

The submerged fermentation process was optimized for the production of cellulases with significantly higher enzyme activities in flask level experiments before proceeding to the actual bioreactor cultivation. The cellulose volume % (v/v) and the inoculum % (v/v) were optimized at various levels and a response surface curve was plotted to observe the optimum levels. Apart from the two mentioned parameters considered for optimization, the rest of the conditions were constant as mentioned in cultivation in the bioreactor.

Cultivation in the bioreactor

A working volume of 1 litre (media + inoculum) was to be used in the bioreactor. 800 mL of Vogel's media containing 1% finely powdered peel-coir substrate / 1% rice straw cellulose was used as the media. 2N sodium hydroxide and 2N hydrochloric acid were used to maintain the pH. 5-10 ppm of polypropylene glycol was used as the antifoam. Prior to autoclave the pH probe was calibrated for two points of pH 7 and pH 4. The media components along with the acid base and antifoam components were autoclaved at 121°C for 20 min. An external supply of cold water was done by connecting to a chiller running at 20°C to maintain the temperature. Once the media was cool and the component lines were connected, a constant agitation of 50 rpm was maintained and the DO probe was polarized with a constant supply of 1 vvm of air for around 6 h. 20% (v/v%) inocula from the starter culture was used to inoculate the fermentor aseptically. The fermentor was set in the automatic mode with a set point of 5.5 for pH and a set temperature of 28 degree celsius. Antifoam would automatically dispense based on the response by the foam sensor. Aeration was varied manually from time to time between 1 vvm to 0.5 vvm based on the foaming. Too much antifoam addition would be detrimental to the production of cellulases by hindering the mass transfer within the fermentor. Agitation and the DO% were cascaded. The high point of agitation was 160 rpm while the low set point was 100 rpm. High set point of DO% was 80% and lowest was 60%. An anchor type impeller was used keeping in mind the broth's viscosity increase after 36 h. The same parameters and conditions were used for both the finely powdered peel-coir substrate containing batch and the 1% rice straw containing batch fermentations [17-19].

Enzyme harvest and enzyme activity estimation

Sampling was done at an interval of 24 h to estimate the enzyme activity. After the maximum enzyme activity was estimated at the end of 5 days, the fermentors were stopped and the broth was harvested through the bottom harvest port. The broth was centrifuged at 6000 rpm at 4°C for 20 min. The supernatant contains the secreted enzymes. The enzyme activity was measured using the standard IUPAC DNSA method [20].

Dry mycelial weight measurement

The dry mycelial weight was measured using a slightly modified method of [21, 22]. The residual substrate concentration was also measured in this method. The fermentation continued until the substrate concentration reached constancy and no further substrate was being taken up and no increase in enzyme activity was observed. The total protein content

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from time to time was measured using the Lowry's method.

Concentration of the Enzymes

The supernatant obtained after centrifugation was subjected to a Tangential Flow Filtration using a TFF Cassette of Sartorius Vivaflow 50R model. It was concentrated around 15 folds. A substantial reduction in the total volume of the enzyme supernatant broth was observed. 750 mL of the culture supernatant from submerged fermentation containing the enzyme was reduced to 50mL after concentration. The activity was measured using the standard DNSA method of IUPAC [20].

Saccharification using produced cellulases and HPLC analysis

15% ammonia pre-treated rice straw was saccharified using the produced and concentrated cellulases produced from the finely powdered peel-coir substrate. 35 FPU/g of concentrated enzyme produced by submerged fermentation was used in the process. 100 g of ammonia pre-treated rice straw along with 1:10 solid:liquid ratio of 0.05 M citrate buffer was used in the process that lasted for 48 h which was carried out with continuous stirring at 50 degree Celsius. 2.5g/L Tween 80 and 0.01% (w/v) sodium azide were added to the saccharification reactor. The amount of sugars released during the process of enzymatic saccharification was analyzed using a HPLC. The percentage of Saccharification was determined using eq. (1).

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

Where, 0.9 - The factor is used to convert polysaccharides to monosaccharides accounting for water uptake during hydrolysis. Percentage yield was calclulated using eq. (2).

$$\%\ \text{Yield of saccharification} = \left(\frac{\text{Practical yield}}{\text{theoretical yield}}\right) \times 100 \quad (2)$$

A HPLC analysis was performed using an Agilent 1290 Infinity HPLC with a Refractive Index detector. The mobile phase was sulfuric acid 0.005 M. 20 microlitre of appropriately diluted (using HPLC grade water) enzymatic saccharied hydrolysate was injected. Standards with known concentrations were run prior to analysis of unknown sample mixtures. The oven temperature was 60°C and the detector temperature was 55°C. The column was Agilent Hi-Plex H 7.7 mm x 300 mm x 8 mm.

Results and discussion

Optimization of submerged fermentation

The process of submerged fermentation was optimized for two conditions at three different levels as shown in Fig. 1. The inoculum 15 %, 20% and 25% (v/v) and the cellulose loading 0.5%, 1% and 1.5% (v/v) were optimized with rest of the conditions for cultivation being the same. 20% (v/v) inoculums and 1% (v/v) cellulose loading were found to the optimal condition yielding enzyme activities of 1.66 IU/mL FPases, 10.85 IU/mL CMCases, 609.9 IU/mL Xylanases and 2705.5 IU/mL Beta-glucosidases [1,17-19].

At 15%, 20% inoculum volume and 0.5% cellulose volume, a lesser activity of 1.11 FPU/mL was obtained which could be attributed to the reduced cellulose loading while a 1.5% cellulose loading with 15%, 20% inoculum volumes resulted in 1.20,1.29 FPU/mL respectively, which could be attributed to substrate inhibition by cellulose 15% volume of inoculum 1% Cellulose volume resulted in 1.38 FPU/mL which showed that of the other mentioned conditions, 1% cellulose produced cellulose with a slightly higher enzyme activity.

However, while using 15% inoculum volume, though the cellulose activity was moderate, a significant quantity of cellulose remained unconsumed which could be attributed to catabolite repression, where the glucose produced through saccharification by the produced cellulases will be chosen to be taken up by the fungus instead of secreting more cellulases [6]. To circumvent this problem, 20% inoculum volume was used with 1% cellulose, where a significantly higher FPU/mL of 1.66 was obtained of all the other combinations.

At 20% inoculum volume and 1.5% cellulose volume, substrate inhibition occurred to yield a FPU/mL of 1.29. At 25% inoculum volume and 0.5%, 1.5% cellulose loading, FPU/mL of 1.38 was observed which may be attributed to too less substrate:inoculums ratio and slightly higher substrate loading respectively along with mass transfer resistances playing a role [3]. At 25% inoculum volume and 1% cellulose loading a FPU/mL of 1.48 was observed. This could be due to problems associated with mass transfer to the fungus and its potential to secrete cellulases [3]. No residual cellulose was left behind in the flask while

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cellulases activity did not seem to increase in this condition.

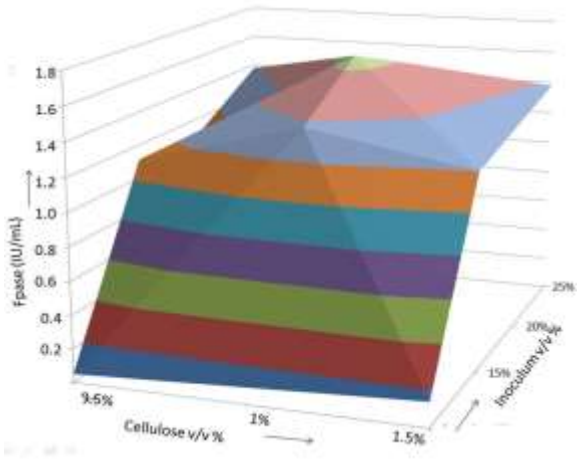

Fig. 1. Response surface curve for the optimization of submerged fermentation for cellulase production

Submerged fermentation

The FPase enzyme activities obtained using the batch SMF process at the end of 120 h was 1.66 IU/mL, 0.28 IU/mL for 1% finely powdered peel-coir substrate containing media and rice straw cellulose containing media respectively (Fig. 2). The starter culture in the Vogel's medium contained a significant amount of cellulases. This was used as the starter culture because the cellulases in it may act as an inducer to initiate biomass formation for the culture in the bioreactor's increased volume environment [1-8]. As expected, we observed quick adaptation and the mycelia started increasing in proportions [23]. Since the agitation was cascaded, it was observed that the agitation increased only to matchup the DO% set point. Agitation with a low shear rate did not cause considerable breakage of the growing hyphae [3].

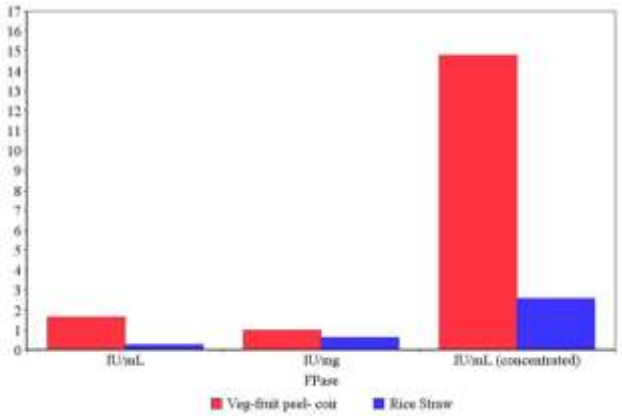

Fig. 2. FPase enzyme produced using 1% (w/v) vegetable-fruit peel-coir and 1%(w/v) rice straw as substrates in submerged fermentation

The aeration was varied manually between 0.5 vvm and 1 vvm to keep a check on foaming. Mild concentrations of Poly Propylene Glycol (5-10 ppm) antifoam were used. Addition of excessive antifoam decreases foam and hence influences negatively the K_La, the mass transfer coefficient. It is preferable to operate the reactor without antifoam. However, considering the unmonitored long time operation, a mild concentration of antifoam sufficient to kill the bubbles on the top of the foam was used. The media's viscosity increased after 48 h indicating the secretion of enzymes. The agitator did not cause maximum shear to the hyphae. The hyphal tips produce cellulases [24] and when these hyphae are broken down due to high shear rates, one cannot expect the production of more cellulases. Lengthy hyphae were produced and accumulated in the bottom and on the baffles. Partial solid state growth characteristics were observed when the hyphae accumulated on the baffles and consumed media by secreting more cellulases. The deposited hyphal mass increased in proportion with time. A serious issue of catabolite repression was observed in the fermentation [3].

During the DNSA reducing sugar estimation as the values of background sugar in the control sample having just the media along with cellulases was investigated, it was observed that after 72 h, cellobioses and glucose were produced considerably. Cellobioses themselves inhibit and cause catabolite repression during cellulase production [6]. It could be attributed to this reason that there happens to be constancy in the cellulase production after a point of time in the submerged fermentation. FPase activity of 1.66 IU/mL and 0.28 IU/mL for finely powdered peel-coir substrate and rice straw cellulose respectively were the maximum activity obtained at the end of 120 h. The catabolic repression also results in no further usage of rice straw cellulose in the media resulting in 6.1 g/L of cellulose being left as the residual cellulose in the fermentation media after 120 h.

A high cAMP level in the cell needs to be maintained in order to facilitate the expression of more cellulases from the promoter. The presence of a sugar such as glucose will inhibit the further synthesis of cellulases. Hence glucose and cellobioses are considered as negatively impacting substances during the production of cellulases. To utilize maximum amount of

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cellulose and produce cellulases with enhanced enzyme activities, the stress levels of the fungus needs to be kept high along with the removal of the produced cellobioses and glucoses to provide uninterrupted stress conditions to stimulate higher cellulase yields [25]. The enzyme activities of FPase, CMCase, Xylanase and Beta-glucosidase are shown in Fig. 2, 3, 4 and 5 respectively.

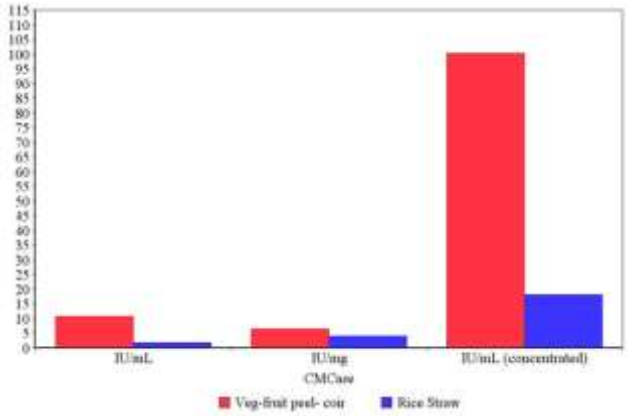

Fig. 3. CMCase enzyme produced using 1% (w/v) vegetable-fruit peel-coir and 1%(w/v) rice straw as substrates in submerged fermentation

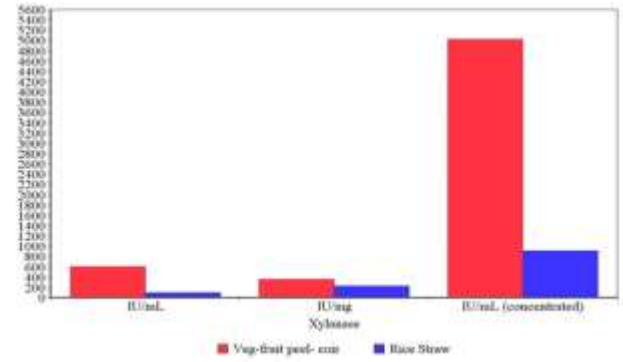

Fig. 4. Xylanase enzyme produced using 1% (w/v) vegetable-fruit peel-coir and 1%(w/v) rice straw as substrates in submerged fermentation

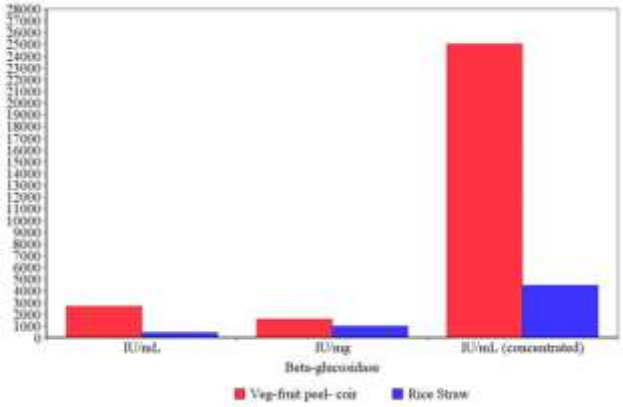

Fig. 5. Beta-glucosidase enzyme produced using 1% (w/v) vegetable-fruit peel-coir and 1%(w/v) rice straw as substrates in submerged fermentation

Growth characteristics of submerged fermentation

In the submerged fermentation using 1% (w/v) finely powdered peel-coir substrate a Biomass yield coefficient $Y_{x/s}$ of 0.4 g of biomass/ g of cellulose was obtained, a maintanence coefficient of 0.03 g/g.h was obtained, a maximum specific growth rate of μ_{max} 0.04g/h was measured. For the fermentation carried out using rice straw as a substrate, a Biomass yield coefficient $Y_{x/s}$ of 0.3 g of biomass/ g of substrate mixture was obtained, a maintanence coefficient of 0.01 g/g.h was obtained, a maximum specific growth rate of μ_{max} 0.02 g/h was measured [26]. The substrate was consumed fully in the 1% finely powdered peel-coir substrate containing process while the cellulose consumption did not reach completion in the rice straw containing process and it stalled after 120 h which may be due to the catabolite repression by the produced glucoses and cellobioses [6] and also due to accessibility of the hyphae to the cellulose within the recalcitrant lignin coat [19]. Around 6.1 g/L cellulose from the rice straw remained unconsumed in the fermentor .The maximum biomass yield was 3.9 g/L for the 1% finely powdered peel-coir substrate containing media and 1.24 g/L for the rice straw containing media. After 120 h, for the rice straw media though the cellulase production stalled, the biomass increase didn't halt [3] as the concentration of glucose and cellobioses helps the growth of the fungi. For both the processes the pH decreased initially to a very low point of pH 3.2 in the fermentation. This indicated that the carbon source was being used up first along with the usage of the nitrogen source. A higher C/N ratio in the media components decreases the pH [1]. This liberates free H+ in the broth leading to a decrease in the fermentation pH [24]. The DO% decreased to 20% during 36 to 72 h of growth. This was clearly in accordance with the observed increase in the biomass after 36 h [3]. The cascaded operation efficiently managed the foaming, and the DO% maintenance to yield cellulases with a considerably good activity. The cellulose consumption and the fungal biomass increase for the submerged fermentation using vegetable-fruit peel-coir mixture is depicted in Fig 6. The cellulose consumption and the fungal biomass increase for the submerged fermentation using rice straw is depicted in Fig. 7.

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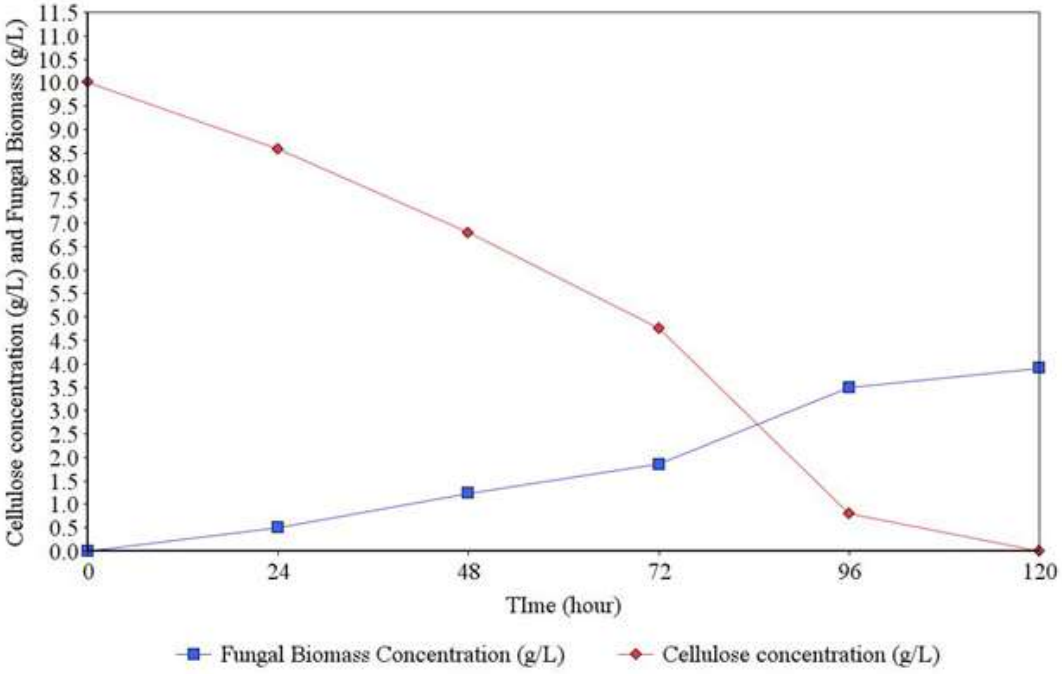

Fig. 6. Submerged Fermentation for Cellulase production using vegetable-fruit peel-coir mixture as substrate

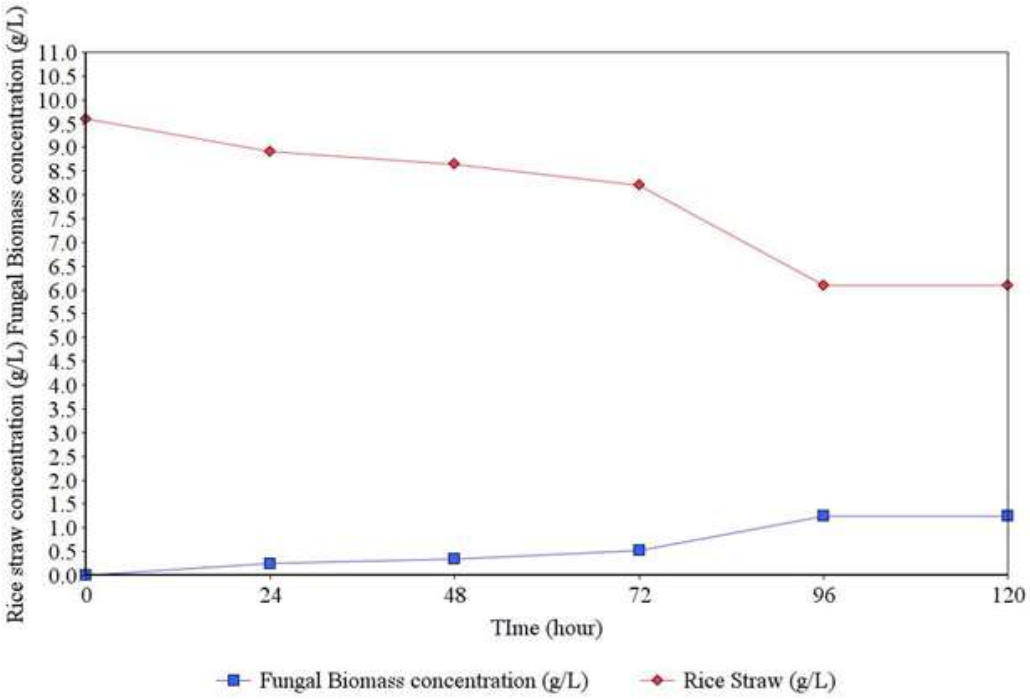

Fig. 7. Submerged Fermentation for Cellulase production using rice straw as substrate

Saccharification using produced cellulases

The process of saccharification inside a stirred reactor with 50 degree Celsius and 35 FPU/g rice straw loading of enzyme produced from the peel-coir mixture resulted in the release of 33 g/L glucose, 10g/L xylose and 0.08 g/L arabinose. Ammonia pre-treated rice straw with 70% of its lignin content removed was used for the process. The process of saccharification continued for 48 h. The percentage Saccharification (% Saccharification) as determined by the standard IUPAC method for the produced cellulases was found to be 43.67% and the % yield of saccharification was found to be 91.66%. The HPLC chromatogram of the saccharification process is as shown in Fig. 8.

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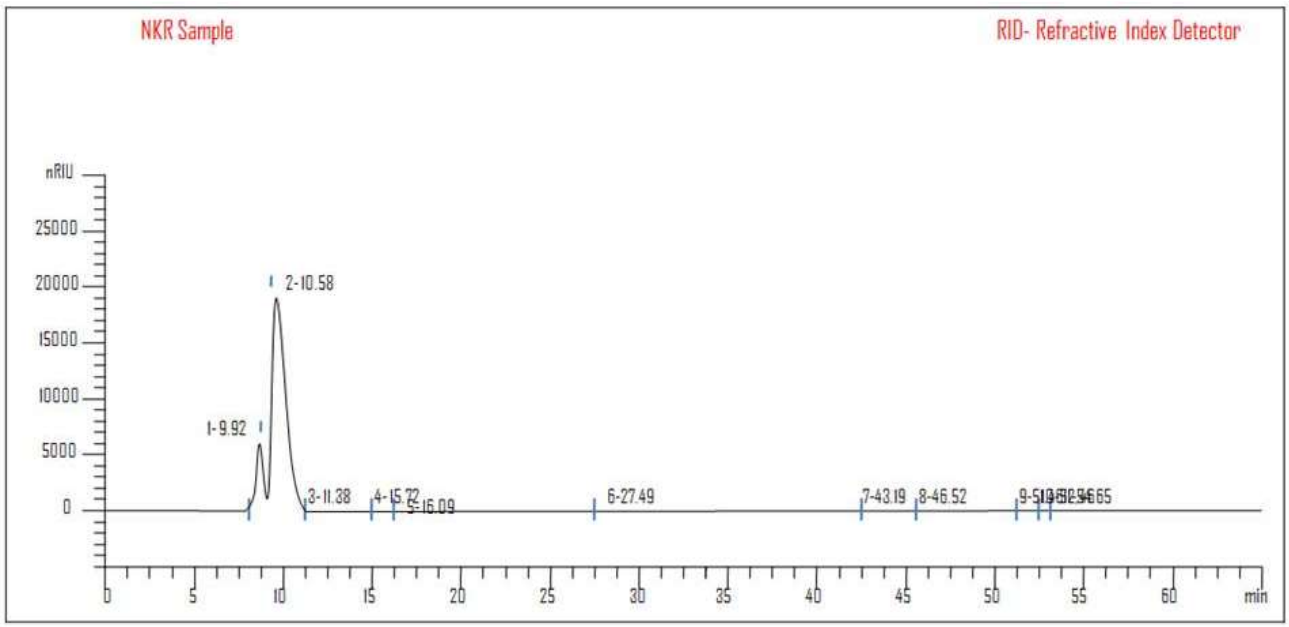

Fig. 8. A HPLC Chromatogram representing the presence of 33 g/L glucose at a retention time of 9.92 min and xylose 10 g/L at a retention time of 10.58 min and 0.08 g/L arabinose at a retention time of 11.38 min in the enzymatic saccharified hydrolysate

Conclusions

A novel substrate mixture was used for cellulase production. The activities are on par with the enzyme activities obtained using other substrates. The substrate has an advantage of being taken out of waste peels and coir. This waste substrate becomes a source for enzyme production that can be used in bio-ethanol production which is a major highlight of the work. In SMF operations, Catabolite repression happens to a much greater extent. The conventional SMF methods are the industrially preferred models and are easily scale able. The enzyme activity of the cellulase obtained using finely powdered peel-coir as a substrate was higher than that obtained using rice straw as a substrate. The Lignin coat and the presence of hemicelluloses in the rice straw reduce the production of cellulases while using them as substrates. The usage of pulverized pre-treated rice straw devoid of lignin and hemicelluloses may prove to be an option to produce cellulase in larger quantities. The efficiency of the produced cellulases was investigated using a batch saccharification process.

Conflicts of interest

Authors declare no conflict of interest.

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