

# Mixture of Potato, Sapodilla, Kiwi Peels and Coir as a Substrate for the Production of Cellulases using *Trichoderma Atroviride* ATCC® 28043™ by a Solid State Cyclic Fed-Batch Strategy and Evaluation of its Saccharification Efficiency

[<sup>1</sup>] Sambavi T R, [<sup>2</sup>] Navnit Kumar Ramamoorthy, [<sup>3</sup>] Renganathan Sahadevan  
 [<sup>3</sup>] Professor

[<sup>1</sup>][<sup>2</sup>][<sup>3</sup>] Biofuel Laboratory, Centre for Biotechnology, Anna University, Chennai

[<sup>1</sup>] Sambavi.ravi@gmail.com, [<sup>2</sup>] suryaramnavnit@gmail.com, [<sup>3</sup>] rensah@rediffmail.com

**Abstract--** The second generation Biofuel production process of Lignocellulosic ethanol conversion is gaining rapid interest owing to the accelerated depletion of fossil fuels. The work was performed with an aim to produce in-house cellulases and reduce the major cost incurred (around 40% of the total fuel production cost) towards commercial cellulase procurement. A novel mixture containing equal volumes of the peels of potato, Sapodilla, Kiwi and coir was subjected to compositional analysis by the standard NREL procedure. The mixture was pre-treated using 15% (v/v) ammonia which showed around 70% lignin removal. In-house Cellulases were produced by innovative fixed volume solid repeated fed-batch from the above stated mixture using *Trichoderma atroviride* ATCC 28043. The in-house cellulases were concentrated 15 folds using a Tangential Flow Filtration unit and showed a percentage saccharification of 45% and percentage yield saccharification of 94.6% in an optimized saccharification process, performed within a Continuous stirred-tank reactor. The percentage yield of saccharification was 4% lesser than commercial cellulases while the cost for production of in-house cellulases was 15 folds reduced than the market cost of commercially available cellulases.

**Keywords--** Fermentation, Pre-treatment, Saccharification, Tangential Flow Filtration

## 1. INTRODUCTION

### A. Stipulated Volume Repeated-Fed Batch Solid mode Fermentation

In a Solid mode repeated fed batch Fermentation, the sugar sources should suffice the emerging microbes' carbon and nitrogen supplementation [6], [7]. Low water requirement and presence of very less free water offers the advantage of very less contamination in SSF [8]. Tray type bioreactor, rotary disk type bioreactor, revolving bioreactor consistent helical bioreactor and distributed tank bioreactor are some major classifications of solid mode repeated fed batch fermenters. The salient feature of SSF over Submerged bioreactor operation is a mildly lessened catabolite repression owing to the presence of a digestible sugar source.

Potato peels, Kiwi peels and sapodilla peels were procured from the market. The four 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 four cellulosic substrates were used as the cellulosic substrate. The vegetable, fruit peels and coir mentioned above have individual reports on the composition of cellulose, hemicelluloses and lignin in them. [9]–[12].

### B. Media for Solid mode repeated fed batch fermentation

The nutrient rich media Potato Dextrose Broth procured from M/s Hi-Media was used for the growth of fungi as a primary culture for Solid mode repeated fed batch fermentation.

## 2. MATERIALS AND METHODS

### A. Preparation of the Novel Substrate Mixture

Coir was procured from a coconut processing industry.

**C. Solid mode repeated fed batch Fermentation**

Petri plates harbouring minimum constituents of Vogel’s culture media having 1% cellulose media were employed for starting microbial culture. A suspension of fungal spores of ( $2.0 \times 10^9$  /mL) was employed to make ready an initial culture conical flask having sterile 200 mL of Potato Dextrose Broth. The finely powdered peel-coir substrate mixture itself serves as the carbon source. A content of the moisture quantity of around 50-60 % was made available for the finely powdered peel-coir substrate mixture. The pH for the bioreactor was equated to 5.5 before autoclaving. The flat plate bioreactor in addition to media mixture was subjected to autoclaving for 20 min at standard temperature. After sterility performance, 60 mL of the primary microbial inoculum in PDB was employed to inoculate the 200 g SSF media. An optimized time of 10 x 24 hour was required for complete growth.

**D. Repeated Fed-Batch Solid mode Fermentation**

The batch process completed after 10 days To the volume  $V_{min}$  within the bioreactor a newly mixed quantity of finely powdered peel-coir substrate mixture mixed with minimum essential culture media constituents was introduced. The added media was mixed homogenously with the existing Fermentation mixture. The bioreactor apparatus was later incubated within a humidifier at 80 % humidity content 28 degree celsius till the completion of the initial operative cycle. The first operative cycle lengthened to 9 days to complete. 9 days to complete. [1], [3], [4].

*Extraction of Cellulases from SSF Reactor*

The activity for the enzymes synthesized were recorded applying the Ghose protocol[15] reducing sugar estimation method. The same process of extraction was performed for each cycle of the harvest obtained [1], [3], [4].

**E. Concentration of the Enzymes**

The soup obtained after centrifugation was co-currently concentrated using Tangential flow filtration. 15 fold concentration occurred. [15].

**F. Saccharification of Pre-Treated Peel-coir mixture**

15% ammonia pre-treated biomass was saccharified using the produced and concentrated cellulases. 35 FPU/g of concentrated enzyme produced by solid mode repeated fed batch bioreaction was used in the process. 100 g of ammonia pre-treated biomass along with 1:10 buffer was used in the process that lasted for 48 hours which was

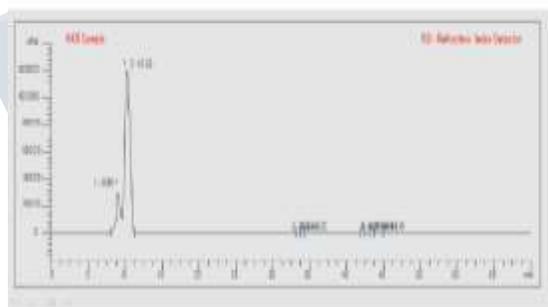
carried out with continuous stirring at 50°C. The percentage of Saccharification was determined by the standard IUPAC formula [15]:

$$\% \text{ Saccharification} = \left( \frac{\text{Total sugars (g)} \times 0.9}{\text{weight of alkali – pretreated biomass (g)}} \right) \times 100$$

$$\text{percentage efficacy saccharification} = (\text{Actual yield} / \text{calculated yield}) \times 100$$

**G. HPLC Quantification of the Cellulase Saccharified Stream**

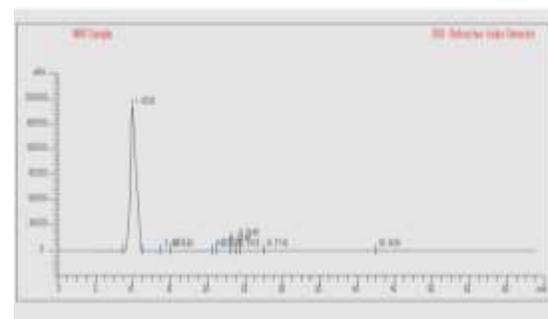
**3. RESULTS AND DISCUSSION**



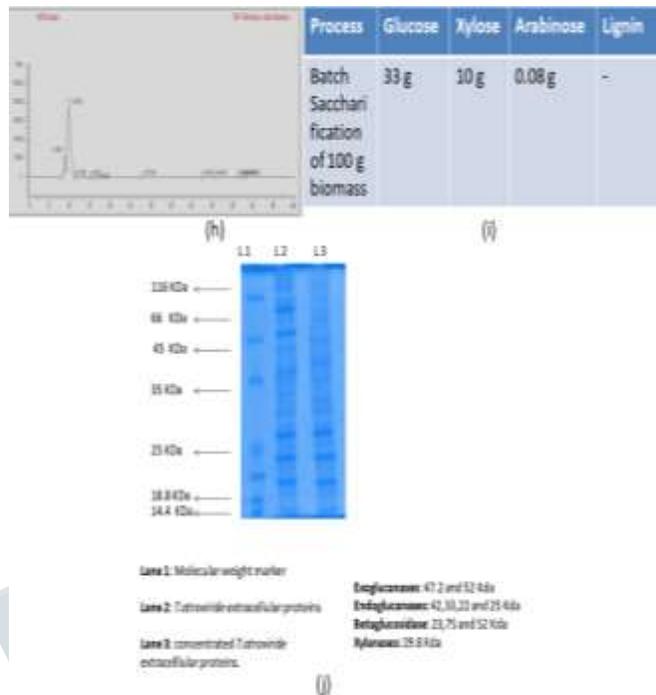
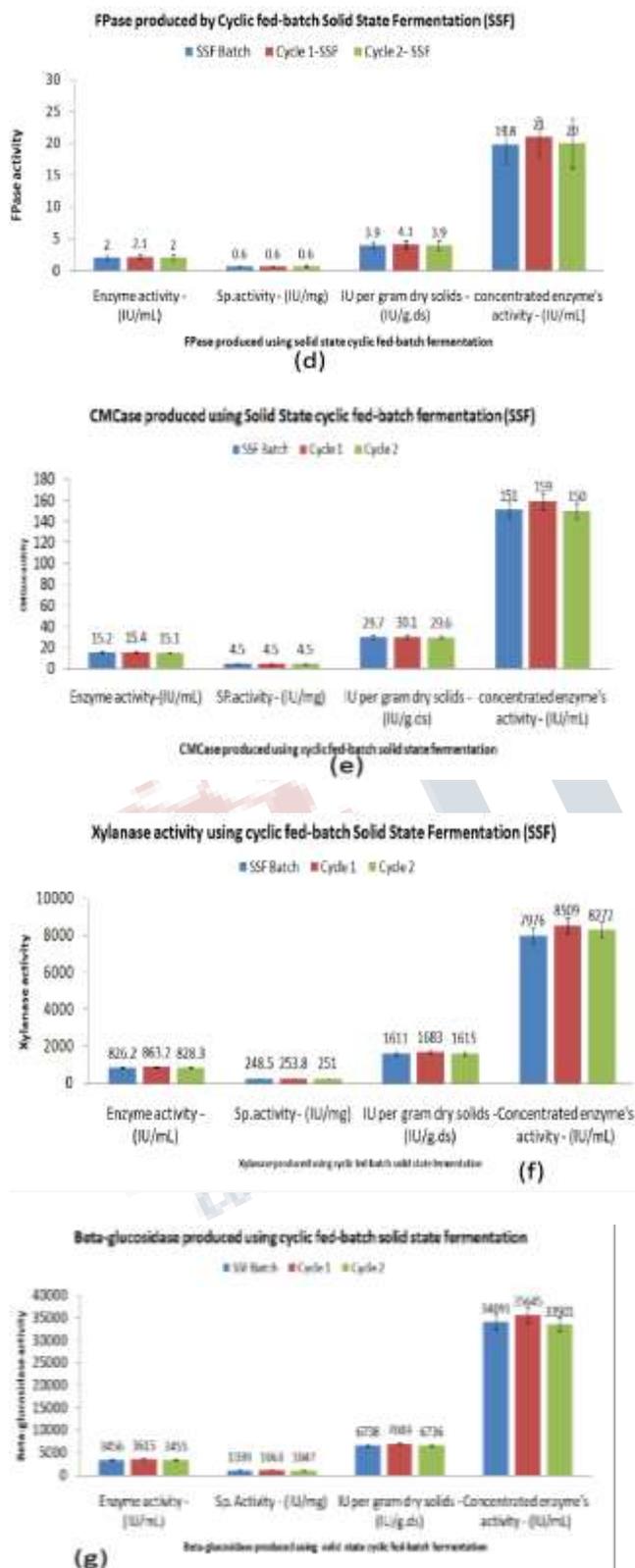
(a)

S.N	Glucose	Xylose	Arabinose	Lignin
0				
1.	0.35 g/g biomass	0.196 g/g biomass	Nil	0.167 g/g biomass

(b)



(c)



**Fig 1**

Fig 1(a) HPLC of the compositional analysis hydrolysate (b) components of the biomass mixture (c) HPLC of the 15% (v/v) ammonia pre-treated hydrolysate. Xylose in hydrolysate was 0.176 g/g biomass. No traces of Arabinose in the pretreatment hydrolysate. Lignin reduced to 0.05 g/g of biomass. Furfural was 0.15 mg/g biomass (library stds). HMF was 0.32 mg/g biomass (library stds).

Fig 1(d) FPase production by solid mode repeated fed batch cyclic fed-batch fermentation. (e) CMCase production by solid mode repeated fed batch cyclic fed-batch fermentation. (f) Xylanase production by solid mode repeated fed batch cyclic fed-batch fermentation. (g) Beta-glucosidase production by solid mode repeated fed batch cyclic fed-batch fermentation.

Fig 1(h) HPLC of the enzymatic saccharified hydrolysate (i) components in the saccharified hydrolysate (j) SODIUM DODECYL SULPHATE - POLY ACRYLAMIDE GEL ELECTROPHORESIS PAGE of the produced *in-house* cellulases.

The compositional analysis results and the hydrolysate of the HPLC compositional analysis procedure are given in Fig 1(a) and (b) respectively. 15% (v/v) of ammonia hydrolysates from the pre-treatment operation's HPLC is depicted in Fig 1(c). The suggested unique methodology of performing repeated fed batch in a solid mode is a novel

idea not reported elsewhere so far. A period of 10 x 24 hour required for the first cycle of the fed-batch to complete. Activities as represented in the figure 1(d),(e),(f) and (h) for FPases, CMCases, Xylanases and Beta-glucosidases respectively were not seen to increase much. [3].

#### **A. Saccharification of Pre-treated Peel-coir using Concentrated Cellulases**

Saccharification was carried out for 48 hours in a continuously stirred vessel at 50°C with 35 FPU/g of peel-coir loading. When analyzed using a HPLC, it was perceived that around 33g/L of glucose and 10g/L xylose were released after saccharification of the 100g ammonia pre-treated mixture. Saccharification was carried out to estimate the efficacy of the enzymes produced by solid mode repeated fed batch fermentation. 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 chromatogram of the HPLC saccharification is represented in the figure 1(h) and 1(i) shows the components of the batch saccharified hydrolysate.

#### **CONCLUSION**

A novel veg-fruit peel and coir substrate mixture used for cellulase production reduced the cost for cellulose source procurement by 60-70%. Ammonia Pre-treatment in an autoclave decreased the generation of inhibitors to a very great extent. Cyclic fed-batch decreased the time for cellulase production by 1-1.5 times. Saccharification efficiency was 45%. The idea proposed could be put to use by Biofuel researchers and manufacturers world wide.

#### **REFERENCES**

1. C. Henry Lim and Hwa Sung Shin "Fed-Batch Cultures: Principles and Applications of Semi-Batch Bioreactors" doi:10.1017/CBO9781139018777, Cambridge series in chemical engineering, Cambridge University Press.
2. Swapnil Bhargava, S. Kevin Wenger and R. Mark Marten. "Pulsed Feeding during Fed-Batch *Aspergillus oryzae* Fermentation Leads to Improved Oxygen Mass Transfer". *Biotechnology Progress*, vol. 19, no. 3, pp. 1091-1094. Sep. 1991.
3. PKR Kumar and BK Lonsane. "Potential of Fed-batch culture in Solid State Fermentation for Production of Gibberellic Acid". <https://doi.org/10.1007/BF01024560>, *Biotechnology Letters*. vol. 9, pp.179-182. March 1987.
4. Sun Tao, Liu Beihui & Li Zuohu. "Enhanced Cellulase Production in Fed-Batch Solid State Fermentation of *Trichoderma viride* SL-1", [http://dx.doi.org/10.1002/\(SICI\)1097-4660\(199708\)69:4<429::AID-JCTB732>3.0.CO;2-Q](http://dx.doi.org/10.1002/(SICI)1097-4660(199708)69:4<429::AID-JCTB732>3.0.CO;2-Q), *Journal of*

- Chemical Technology and Biotechnology*. vol. 69, pp 429-432. Aug 1997.
5. M. E. Bushell, M. Rowe, C. A. Avignone-Rossa, J. N. Wardell. "Cyclic Fed-Batch Culture for Production of Human Serum Albumin in *Pichia pastoris*", <https://doi.org/10.1002/bit.10616>, *Biotechnology and Bioengineering*. Vol. 82, pp. 678 – 683, June 2002.
6. Vidhya Rangaswamy. "Improved Production of Gibberellic Acid by *Fusarium moniliforme*". [10.5923/j.microbiology.20120203.02](https://doi.org/10.5923/j.microbiology.20120203.02). *Journal of Microbiology Research*. vol.2, pp. 51-55. 2012.
7. Behera, Sudhanshu & Ray, Ramesh. "Solid State Fermentation for Production of Microbial Cellulases: Recent Advances and Improvement Strategies", [10.1016/j.ijbiomac.2015.10.090](https://doi.org/10.1016/j.ijbiomac.2015.10.090). *International Journal of Biological Macromolecules*. Vol. 86. Nov 2015.
8. M A Shata, Hoda. "Extraction of milk-clotting enzyme produced by solid state fermentation of *Aspergillus oryzae*" *Polish journal of microbiology*, vol. 54 pp. 241-7. Feb 2005.
9. G. Arifuzzaman Khan, M Shamsul Alam. "Thermal Characterization of Chemically Treated Coconut Husk Fibre" *Indian Journal of Fibre and Textile Research*, Vol. 37, pp. 20-26. March 2012.
10. Feumba Dibanda Romelle , Ashwini Rani P and Ragu Sai Manohar. "Chemical Composition of Some Selected Fruit Peels", *European Journal of Food Science and Technology*, vol. 4, pp. 12-21. Sep 2016.
11. Igor Sepelev and Ruta Galoburd "Industrial Potato Peel waste Application in Food Production: A Review" *Research for Rural Development*, vol. 1, pp. 130-136. 2015.
12. Paramanandham, J & Ronald Ross "Lignin and Cellulose Content in Coir Waste on Subject to Sequential Washing" *Journal of Chemistry and Chemical Research*, vol. 1, no. 1, pp. 10-13. Dec 2015.
13. Nighat Aslam, Munir A Shiekh, Muhammad Ashraf and Amer Jamil. "Expression of *Trichoderma* Cellulases under Different Carbon Sources" vol. 42, pp. 2895-2902. 2010.
14. Liming Xia, Peilin Cen. "Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry" *Process Biochemistry*. vol. 34, pp. 909 – 912. Dec 1998.
15. TK. Ghose "Measurement of cellulase activities" <http://dx.doi.org/10.1351/pac198759020257>, *Pure and Applied Chemistry*, vol. 59, no.2, pp. 257-268. 1987.
16. Yi-Heng Percival Zhang and R. Lee Lynd. Regulation of Cellulase Synthesis in Batch and Continuous Culture of *Clostridium thermocellum*. *Journal of Bacteriology*. vol. 187, pp. 99-106. Jan 2005.
17. Chia-wen C. Hsieh, David Cannella, Henning Jørgensen, Claus Felby, and Lisbeth G. Thygesen, "Cellulase Inhibition by High Concentrations of Monosaccharides", *Journal of Agricultural and Food Chemistry*, vol. 62, pp. 3800 – 3805. 2014.