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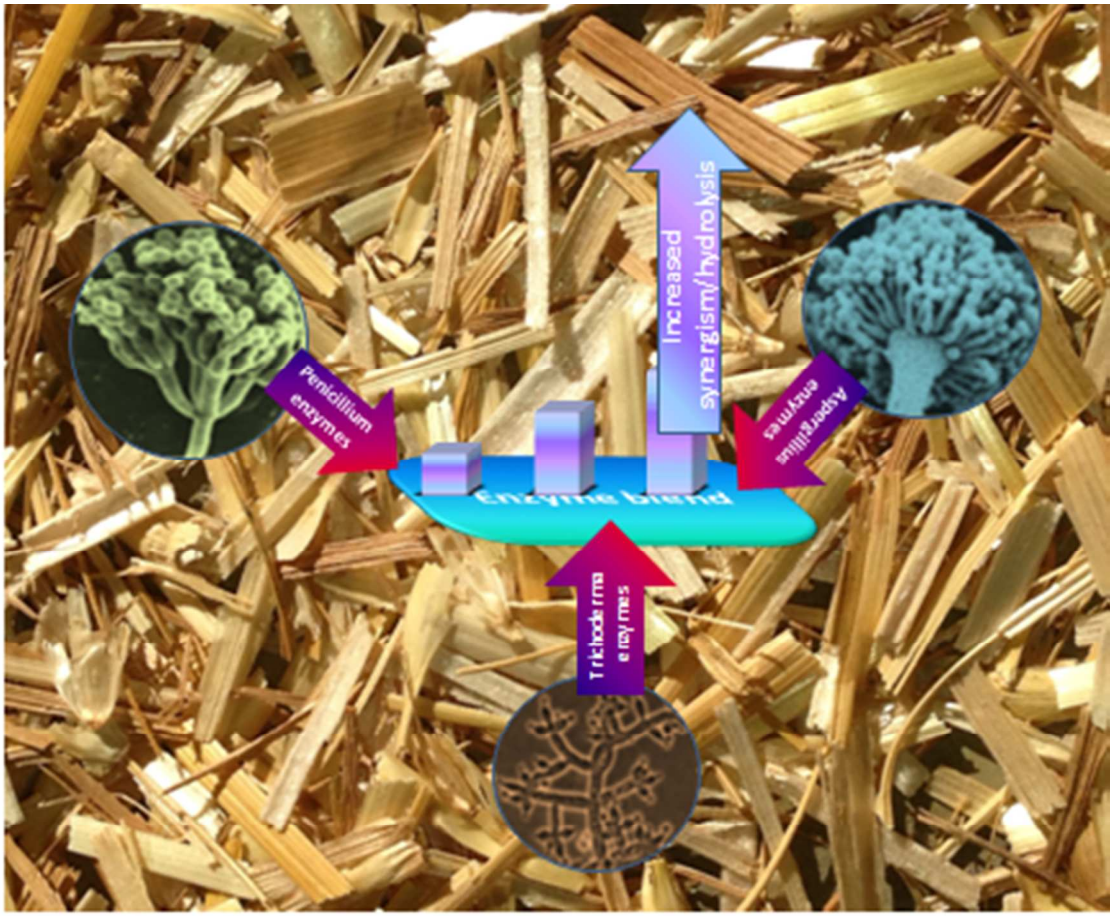

Prepared enzyme cocktail from different fungal enzyme preparations increases the hydrolysis of avicel/wheat straw by increasing synergism between the same or different types of cellulases.

Blending of cellulolytic enzyme preparations from different fungal sources for improved cellulose hydrolysis by increasing synergism

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Abstract

Cellulolytic enzymes were produced from the three fungal strains [*P. janthinellum* EMS-UV-8 *(E)*, *T. reesei* Rut C-30(R) and *A. tubingenesis (A)*] and used to prepare blends for the hydrolysis of avicel and acid treated wheat straw (A-WS). An enzyme blend prepared from three different crude preparations (E+R+A) on the basis of equivalent FPU or protein was found to be more synergistic and gave excellent hydrolysis of avicel or A-WS than the blend of two enzyme preparation (E+R, E+A and R+A) or individual enzyme preparation (E, R, and A). The triple blend gave two times higher hydrolysis of avicel or A-WS than the individual enzyme preparations at the same enzyme dosages. In all cases the individual or cumulative FPU or protein in blends was equal (10 FPU or 20 mg protein/gm of substrate). The increased enzyme activities (CMCase and FPU) were found in the blends than the sum of individual enzyme added for the blend preparation. This revels that the increased hydrolysis of cellulose by blends was a result of increased synergism between the same (endoglucanase) and / or different types of cellulases from different preparations. Enzyme blending is thus a facile, cost effective and sustainable approach for biomass saccharification for biofuels.

Keywords: biocatalysis; biorefinery/Biofuels; cellulase enzyme cocktail; Cellulose hydrolysis; synergism

1. Introduction

Lignocellulosic biomass (LCB) has a remarkable potential to act as an important renewable resource material for the production of commodity chemicals/fuels or other value added products. [1] LCB needs some pretreatment because of its recalcitrance nature. The holocellulose needs depolymerization to simple sugars for further easy biological conversion or fermentation. Green method to deconstruct cellulose in these materials is an enzymatic hydrolysis and the combination enzymes, called cellulases, play a major role in this process.[2] Many demonstration as well as pilot plants are underway to convert cellulosic biomass through enzymatic hydrolysis for biofuels production.[3] However, the major bottleneck to make this process economically feasible is the high cost of the enzyme production [4, 5]. As reported in literature the average off-site cellulase production cost ranges from $4.4 to $8.8/kg protein [4]. Enzymatic hydrolysis of cellulosic materials for biofuels showed better energy efficiency but its commercialization depends on lowering the cost of cellulases [6, 7]. There are number of ways to reduce the cost of enzyme for pretreated LCB hydrolysis and/or to improve the hydrolysis such as selection of hyper secreting mutant for higher production of cellulase,[8] enzyme recycling,[9, 10] selecting potent hydrolytic enzyme, supplementing the oxidative enzymes as well as non hydrolytic enzymes etc, [11] but all of them have some issues at commercial levels. Therefore a simple way to reduce the cost of the enzymatic hydrolysis process is a priority area of focus.

In this study, a very simple approach was used to increase the hydrolysis of avicel as well as dilute acid treated wheat straw (A-WS) through the use of blends of enzymes obtained from well known and commercially exploited fungal strains. This approach has led to the reduction of cost of biomass hydrolysis. Addition of one of the deficient enzymes in other preparations (e.g. addition of β-glucosidase in *Trichorderma sp*. enzyme preparations) is well known strategy but present study deals beyond it. In this study also deals with the use of different blends of enzymes obtained from cellulase producing fungal strains and their use in cellulose hydrolysis.

2. Experimental Methodology

2.1 Chemicals

Avicel PH101, p-Nitrophenyl β-D-glucopyranoside (pNPG) and 3, 5-dinitrosalysilic acid were obtained from Sigma-Aldrich Co. Carboxymethylcellulose (CMC) sodium salt-medium viscosity was obtained from S.D. fine-chem Ltd. India. Wheat straw was supplied by local farmers. All other chemicals used were AR grade.

2.3 Microbial strains

Fungal mutant strain *Penicillium janthinellum* EMS-UV-8 [8] was obtained from Dr. D.V. Gokhale, National Chemical Laboratory, Pune, India. *Aspergillius tubingenesis* MTCC 7956 was procured from Microbial type culture collection, IMTECH, Chandigarh, India. *Trichoderma reesei* Rut C-30 was a kind gift from Dr. Ashok Pandey, NIIST, Trivandrum, India.

Other cellulose degrading fungal strains used were isolated from soil and laboratory. Total seven fungal strains were isolated and designated as IODBF-1, IODBF-5, DBT-IOC-ASMA, PDI-6, PDI-8, A2-Old and MGA. A2-Old is a mutant strain generated from EMS-UV-8 strain through Ethylmethyl sulfonate and UV mutagenesis. These strains were further identified based on morphology. All these strains were maintained on PDA slant.

2.3 Enzyme production in shake flasks

The fungal strains were used for the production of cellulolytic enzymes in shake flasks. The Mendel and Weber [12] production medium used contained (g/L): KH_2PO_4 2; $CaCl_2.2H_2O$ 0.3; Urea 0.3; $MgSO_4.7H_2O$ 0.3; $(NH_4)_2SO_4$ 1.4; Peptone 0.25; Yeast extract 0.1; Tween-80 1ml; $FeSO_4$ $7H_2O$ 0.005; $MnSO_4$ H_2O 0.0016; $ZnSO_4$ 7H2O 0.0014; $CoCl_2$ $6H_2O$ 0.002; pH-5.0. For enzyme production 500ml flasks were used containing 150 ml of above medium with 1% avicel and 2.5% wheat bran. A loopful of spores from respective strains was inoculated in these flasks. These flasks were incubated at 30^0C with shaking (170 rpm). After 8 days of incubation the fermented broth was centrifuged and supernatant was used for the analysis of extracellular enzyme activities, proteins and the same was used as a source of extracellular enzymes for hydrolysis of avicel and wheat straw.

2.4 Enzyme production in 5 L fermentor by *P. janthinellum* EMS-UV-8 (EMS-UV-8-β)

Cellulase production by EMS-UV-8 was carried out in a 5 L BioFlo115 fermentor (New Brunswick Scientific, USA), with a working volume of 3 L using Mendel and Weber medium with 10 g/L Avicel PH-101 and 25 g/L wheat bran were used as substrates. Fermentor vessel along with medium components was sterilized at 121^0C for 30 min. Spores of *P. janthinellum* EMS-UV-8 were used as inoculum. Temperature during fermentation was set at 30^0C and pH was maintained between 3 to 5 for up to 72 hrs and then controlled at 6.0 by adding 1M HCl or 1M NaOH. During production agitation was set at 400 rpm and airflow of 2-3 vvm. Silicone based antifoam was added manually to control the foam. After 5 days of fermentation the whole broth was harvested and centrifuged at 5,000 *g* for 10 min and supernatant was used as crude enzyme preparation for enzymatic hydrolysis of avicel and wheat straw. This enzyme preparation was named as EMS-UV-8-β.

2.5 Enzyme assays

β-Glucosidase activity was analyzed by using pNPG as substrate. The total of assay mixture (1 ml) consisting 0.1 ml of suitably diluted enzyme and 0.9 ml of pNPG (1 mg/ml) which was prepared in 50mM citrate buffer(pH 4.8).This mixture was incubated at 50^0C for 30 min. After 30 min the reaction was stopped by adding 2 ml of sodium carbonate (2%). The color of liberated p-nitrophenol was measured at 410 nm. Enzyme activity was calculated by using molar extinction coefficient 18.3×10^3 of p-nitrophenol.[13]

Filter paper units (FPU) and CMCase activity were analyzed according to the method described by T. K. Ghose.[14] All activities were analyzed in international units/ml (IU/ml). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose or p-nitrophenol from the respective substrates per ml/min of crude supernatant under assay conditions.

2.6 Wheat straw pre-treatment in pilot plant

Wheat straw was pretreated in a continuous pilot-scale pretreatment reactor system having capacity of 250 kg/day. Wheat straw was first milled to 4-5 mm particle size. This wheat straw was soaked in the acid solution (2.5% w/w) for 30 min in a soaking chamber. The wet biomass

was hung for 2 h and further pressed for 15 min at a pressure of upto 100 bar in a hydraulic filter press to remove excess liquid. The biomass having 60% moisture was then transferred to a specially designed feed hopper to transfer biomass through a pressurized plug screw feeder. This biomass was subjected to pre-treatment in the reactor at temperature 140-180°C, pressure 6 bar and residence time of 5-30 min. Residence time was controlled by the screw speed of the reactor. The pre-treated biomass slurry was collected in the slurry tank, cooled and then transferred through a peristaltic pump to a high speed centrifuge for separating solids (cellulose and lignin) and liquid (hemicelluloses). The solid portion of this pre-treated wheat straw was washed with demineralised water and buffer. Pretreated sample was air dried and analyzed for cellulose content [15] which was found to be 70%. This pretreated wheat straw (A-WS) from single batch was used for all hydrolysis studies and had approximately 70% cellulose and 30% lignin.

2.7 Enzymatic hydrolysis

The hydrolysis experiments were performed in 100 ml conical flasks containing 1 g of avicel or dilute acid pretreated wheat straw and 25 ml mixture of citrate buffer (50mM, pH 4.8) and enzyme. In case where the enzyme preparations having low enzyme activity, the high morality of citrate buffer was used to get final 50mM concentration in 25 ml buffer. The total 10 FPU or 20 mg protein was used to hydrolyze 1 g of substrate. The total FPU activity used per gram of substrate is 10 FPU whether enzyme preparation is used singly (10 FPU) or in combination(5+5 combination from two different preparations or 3.3+3.3+3.3 combinations from three different preparations). Similarly, the total 20 mg protein used per gram of substrate whether preparation used singly (20 mg) or in combination (in combination of 10+10 from two different preparations or in 7+7+7 combinations from three different preparations). After mixing the substrate and enzyme in buffer the flasks were kept at 50^0C with shaking. The reducing sugar/Glucose or cellobiose was analyzed after suitable time interval to check the hydrolysis performance by enzyme mixture.

2.8 Analytical methods

The total reducing sugar as glucose equivalent was estimated by dinitrosalicylic acid (DNS) method.[16] Protein was estimated according to the method of Lowry et al.[17] using bovine serum albumin as a standard.

3. Results and discussion

First experiment performed was the hydrolysis of avicel (4% w/v) and A-WS cellulose (4%) using three different enzyme preparations from three different fungal strains (Fig. 1). The three fungal strains used were *Penicillium janthinellum* EMS-UV-8 (E), *Trichoderma reesei* Rut C-30 (R) and *Aspergillius tubingenesis* (A). The cellulolytic enzymes were produced from all these three strains in a similar medium using only 1% avicel and 2.5% wheat bran under similar conditions. Produced enzyme was used for the hydrolysis of two substrate i.e. highly crystalline avicel and A-WS prepared in pilot plant. The importance of using acid treated wheat straw is that the economic analysis showed that the combined process of dilute acid pretreatment and enzymatic hydrolysis was found to have best economic potential.[7] Avicel and cellulose of A-WS were hydrolyzed using 10 FPU from each strain and it was found that, after 72 hrs of hydrolysis the avicel was hydrolyzed up to 36%, 32% and 24% by crude enzyme preparations of E, R and A respectively. However, cellulose of A-WS was hydrolyzed up to 24%, 27% and 24% by E, R and A respectively. Thus enzyme E was good for avicel hydrolysis and enzyme R was good for A-WS hydrolysis but the overall hydrolysis of both the cellulose i.e. avicel as well as A-WS cellulose was still poor. This low level of hydrolysis may be because of the individual enzyme preparations may not have complete or all necessary cellulolytic enzymes for complete hydrolysis of cellulose. So the mixture of enzyme preparations was prepared using individual enzymes to get the final FPU of 10. The mixture/blend prepared are of two compositions, first one was having 5 FPU of one strain and 5 FPU of another and other mixture contained 3.3 FPU each from three enzyme preparations i.e. 3.3+3.3+3.3. The hydrolysis was carried out using these blends and significant boost in the hydrolysis of avicel as well as A-WS cellulose was observed. The 5+5 FPU combination of E+R, E+A and R+A gives higher hydrolysis than individual enzyme of 10 FPU and the hydrolysis was 47%, 49% and 58% of avicel respectively. However, the hydrolysis of A-WS cellulose was 28%, 31% and 37% by E+R, E+A and R+A blend respectively. Another enzyme combinations from three strains (E+R+A) having 3.3+3.3+3.3 FPU worked much better than individual enzyme and even better than two enzyme combination described earlier. The final hydrolysis of avicel and A-WS cellulose observed by this combination was 70% and 50% respectively which was approximately more than two times higher than the individual enzyme preparation.

There may be some discrimination by using same amount of FPU because the same FPU from different enzyme preparation may not have same amount of protein. Additionally, there may be some differences of using equal FPU for hydrolysis, as FPU indicates overall enzymes activity or cumulative effect of all cellulolytic enzymes (endo/ exo-cellulases, β-glucosidase, etc) and may not be suitable because some enzyme preparation may not have all necessary enzymes. So, the hydrolysis was also carried out at equal protein level (Fig. 2) using same enzyme combinations. Here, 20 mg protein was used from each preparations to hydrolyze avicel and A-WS cellulose. The avicel was hydrolyzed up to 27%, 21% and 26 % by E, R and A respectively after 72 hrs. However, A-WS cellulose was hydrolyzed up to 28%, 20% and 21% by E, R and A respectively. The two types of enzyme blends were prepared in the same way as described earlier i.e. protein content of 10mg+10mg and 7mg+7mg+7mg. Both blends gave higher hydrolysis of avicel as well as A-WS cellulose. 10+10 mg combination of E+R, E+A and R+A hydrolyses avicel up to 41%, 45% and 41 % respectively which was higher than the individual 20 mg protein. Similarly A-WS cellulose hydrolyzed up to 29% 30% and 33% by E+R, E+A and R+A respectively. Again, the hydrolysis of three enzyme combination (7+7+7 mg) was found to be better than individual enzymes (20 mg) as well as two enzyme (10+10mg) combinations. The maximum hydrolysis of avicel by this combination was 55% and A-WS cellulose was 38%, an approximately two times higher than individual enzyme protein at 20 mg. From above results it can be concluded that blending of enzyme preparation to get 10 FPU or 20 mg protein works more efficiently than the individual enzyme at 10 FPU or 20 mg protein.

To check what happens to enzyme activity after mixing or to find out the reason of increased hydrolysis, the enzyme activity analysis of blend was performed and compared with the sum of individual enzymes activities. Three enzyme activities were compared i.e. FPU, carboxymethyl cellulase (CMCase) and β-glucosidase (Table 1). During hydrolysis in all cases 10 FPU was added for 1 g of dry substrate, 10 FPU may be from single enzyme preparation or cumulative of two or three enzyme preparations. The analysis showed that FPU in most of the blends were more than the sum of FPU of individual enzymes added. There was large difference observed between the CMCase of actual added and analyzed in blend. Approximately twice increase in the CMCase activity in all cases were observed except R+A. Both activities i.e. FPU and CMCase were 1.3 times and two times higher respectively than the actual added in the E+R+A combinations. Similar increased hydrolysis was observed in avicel and A-WS cellulose by this

triple combination (Fig. 1). It was interesting to note that E+R combinations (Table 1) do not have much β-glucosidase (total 1.7 units) even then it gives hydrolysis more than the individual E and R (Fig. 1). It means that there was a synergism between the endo-glucanases of the two (E and R) strains and that's the reason why the blend E+R gives higher CMCase activity than the individual E and R at same concentration (table 1).

One more enzyme preparation of EMS-UV-8 was used in this study which was designated as E-β or EMS-UV-8-β, because this enzyme preparation has more β–glucosidase which is equivalent to *A. tubingenesis* enzyme preparation. This enzyme was prepared in 5L fermentor with controlled pH. Because of the controlled pH during enzyme production this fungal strain produces more β-glucosidase as well as more endoglucanase titer. This enzyme preparation has all necessary (FPU, CMcase and β-glucosidase) enzymes in good proportions. It produces pink-brown color pigment along with enzyme at pH above 6, which is the indication of having more β-glucosidase in the preparation. Again similar enzyme activity analysis experiments were performed using E-β enzyme preparation to check what happen when one enzyme preparation is of having all necessary cellulolytic activities. The results obtained (table 1, EMS-UV-8-β) are in a similar way but the analyzed FPU was increased than the previous enzyme preparation (E). The FPU in 5+5 and 3.3+3.3+3.3 combinations was 1.5 and 1.7 times higher respectively than actual added. The CMCase was again observed higher in blend of triple combination. In both above cases β-glucosidase activity do not change much by blending, which was found approximately same values as sum of individual enzyme.

Results obtained from the enzyme activities analysis of enzyme blends gives us a clear indication that FPU or CMCase assay could be used for the high-throughput screening of other potentials enzyme candidates for the preparation of blends or cocktails for higher hydrolysis of cellulose using same amount of individual enzymes. As the analyzed enzyme activities was found higher than the added it means that there were increased synergisms within the enzyme present in the blend, which consequently increase the hydrolysis of avicel as well as A-WS cellulose.

Further to investigate the hydrolysis performance of E-β (having more β–glucosidase), similar experiments were performed for hydrolysis of avicel and A-WS cellulose with enzyme preparations (A and R) and compared with E-β. It was found that E-β was more efficient and even better than E. The increased hydrolysis by E-β than E may not only because of higher β–glucosidase but could be because of some other enzymes/protein which have not been analyzed

but are present in the preparation. The difference in the enzyme activities was observed in fungal strains when conditions, substrate, media and process changes were made during enzyme production. Figure 3 summarizes the comparision of hydrolysis performance of E-β with A and R. The E-β hydrolyzes avicel up to 57% and cellulose of A-WS up to 45% which is much better than the E, A and R.

In 5+5 blend, the avicel was hydrolyzed up to 64% and 67% and 73% by R+A, E-β+R and E-β+A respectively. The pretreated A-WS cellulose was hydrolyzed up to 49%, 50% and 54% by R+A, E-β+R and E-β+A respectively. The blend of 3 enzymes having cumulative 10 FPU of E-β+R +A hydrolyse avicel and pretreated A-WS cellulose up to 83% and 63% respectively which was better than the previous 3 enzyme blend (E+R+A). Overall it proves that, enzyme blends always help to improve the hydrolysis performance even though the individual enzyme preparation may have all the necessary enzymes (e.g. in EMS-Uv-8-β).

The above stated results are from three fungal strains only, and to investigate it again whether that is true for other fungal strains from different sources, same more fungal stains were studied. Total seven fungal strains from soil, laboratory isolate or through mutagenesis were isolated. A2-old is a mutant strain generated from EMS-UV-8. IODBF-1, IODBF-5, PDI-6 and PDI-8 were isolated from soil samples. DBT-IOC-ASMA and MGA are laboratory isolates. The cellulolytic enzymes were produced from seven strains using earlier described method and substrate (1 % avicel and 2.5% wheat bran). The enzyme produced from all these strains were analyzed and found that the FPU was in the range of 0.6-1.6 IU/ml. DBT-IOC-ASMA shows highest FPU as well as β-glucosidase among these strains i.e. 1.6 IU/ml and 3.7 IU/ml respectively (Table S1). The mutant strain A2-old produced highest CMCase i.e. 27.4 IU/ml which is even higher than EMS-UV-8.

All seven enzyme preparations were used for the hydrolysis avicel and A-WS cellulose using 10 FPU/ g of substrate. Figure S1 (supplementary information) shows the hydrolysis pattern of both the substrates. Among all, three strains i.e. IODBF-1, DBT-IOC-ASMA and MGA showed higher avicel hydrolysis compared to others and the values are 44%, 42% and 43% respectively. But the pretreated A-WS cellulose hydrolysis was more by the enzyme preparation of DBT-IOC-ASMA, A2-Old and MGA than other strains and that was 32%, 33% and 34% respectively. Figure S2 shows the hydrolysis by blend using 5+5 FPU combination. In all blends, 5 FPU from EMS-UV-8(E) was common and 5 FPU from other seven strains was

used. The hydrolysis pattern indicates that again blending improves the hydrolysis at cumulative 10 FPU. The highest hydrolysis of avicel was observed in two blends of E+ IODBF-1 and E+MGA and that was 63% & 62% respectively. The maximum A-WS cellulose was hydrolyzed by E+ IODBF-1, E+IODBF-5 and E+DBT-IOC-ASMA blends which was around 50%. From these results it is again clear that blending of different enzyme produced from different fungal sources increases the hydrolysis of cellulose or cellulosic materials. Enzyme preparations used were crude as produced in broth and without any purification which could be a better way than adding purified cellulase. Again, it was observed that A-WS cellulose hydrolysis was slower than avicel and that may be attributed to the presence of inhibitors or pseudo-lignin [18] or irreversible cellulase binding to lignin.[19-22]

We studied three fungal strains, used widely in the industry as well as in scientific community; these strains are *Penicillium janthinellum*, *Trichoderma reesei* and *Aspergillius tubengenesis*. Their commercial preparations are easily available for making blends for industrial purpose through same basic optimization or straightforward mixing. This is the best way of reducing the cost of enzyme through just blending of different enzymes for more sugar production and this sugar could be utilized for the production of values added products such as ethanol, butanol, lactic acid etc.[1, 23] It was also proved that the combination of bacterial and fungal cellulases improves the cellulose hydrolysis [24, 25] but the enzyme titer in bacteria is very low and hence seldom used

The ideal method to prepare enzyme cocktail is the addition of enzyme components which are limiting in some preparations were generally preferred e.g. addition of β-glucosidase enzyme from *Aspergillius sp*. in to the enzyme preparation of *Trichoderma sp*. which is deficient in β-glucosidase.[26, 27, 28] But in this study equal amount of enzymes were mixed to get same FPU in all blends. The increased hydrolysis as well as increased enzyme activities in blends suggests that there is an increased synergism within all enzymes and proteins from the different sources which may not be possible in individual enzyme preparations. However, the synergism is a very complex phenomenon in case of cellulose hydrolysis and may be dependent on many factors. [29] There may be a possibility of other non hydrolytic proteins which may helps in increased synergism of blend too. For example there are recent reports about non hydrolytic enzymes such as AA9 or other recombinant proteins which increases the hydrolysis performance if added with canonical cellulases. [24, 25, 30] However, addition of recombinant may add to cost the enzyme

blend. Additionally, AA9 has few limitations such as production of gluconic acids (inhibit ethanol fermenting yeast), requirement of oxygen (may not be suitable for anaerobic simultaneous saccharification and fermentation), need of some electron donor (which could be celllobiose dehydrogenase or some chemicals) without this enzyme may not work. AA9 containing cocktail are available and work better with minimum amount of protein but their cost is not available in public domain. Addition of recombinant protein or enzymes one by one to make cocktail/blend may not become sustainable as well as economic for industrial purpose. So the blending of commercially cheaper or in-house enzyme preparations will be better choice to get higher hydrolysis yield with low enzyme loading.

A lot of scope exists to develop a suitable enzyme blend depending on the type of enzyme preparation, type of fungal strains, different growth conditions and the type of biomass as well as method of pretreatment but the present study establishes that blending always improves the hydrolysis performance. [26] From this study it is indicated that combination of enzyme preparation from different genus was always better than the combination of enzyme preparation within the genus. (E.g. EMS-UV-8 and A2-Old combination does not improve much). Blending seems to be a better way because it is difficult to have all cellulase components in a single organism enzyme preparation. Insertion of each enzyme gene into the genome in single organism is difficult. So blending may be a better way to increase hydrolysis performance at lower enzyme dose. There are number of possible explanations for increase in the hydrolysis of cellulose by enzyme blending and these are the role of non hydrolytic proteins, presence some accessory enzymes, resistance to feed back inhibition, stability of enzyme etc. One more possible reason in case of R+E is that cellobiohydrolase from *Trichoderma sp.* may face the traffic jam on cellulose fibre but addition of more endoglucanases from EMS-UV-8 may reduce the traffic jam by increasing more ends for cellobiohydrolase and increases the hydrolysis.[31, 32]

4.0 Conclusion

In summary, blending of enzymes from different fungal sources is a facile and sustainable approach to increase the cellulose hydrolysis performance which in turns reduces the cost of enzymatic lignocellulosic biomass deconstruction. This study proves that the hydrolysis performance increases through increased synergism within the different type of cellulases as well as same type of cellulases from different sources. Blending of three enzyme preparations was

found to be better than the blending of two enzyme preparations which in turn was better than that of individual enzyme preparations at equivalent filter paper units (FPU) or protein level. This study may have an immediate industrial significance.

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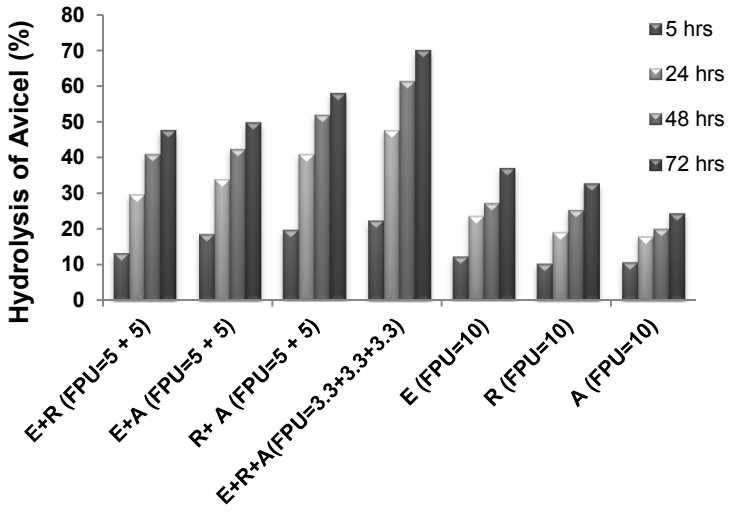

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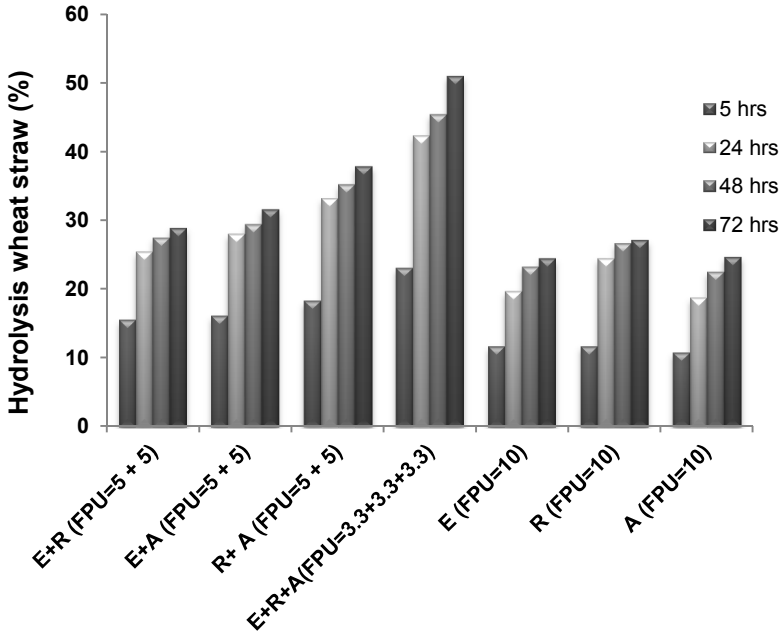

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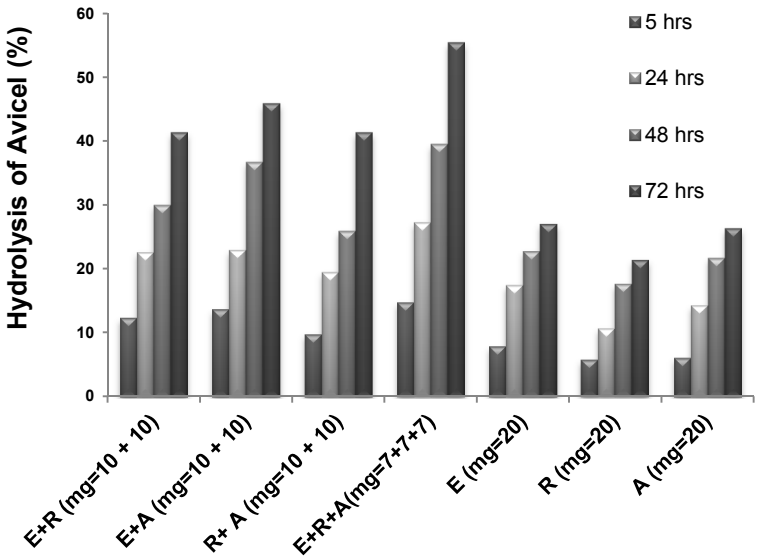
5 **Fig. 1** Hydrolysis of avicel and A-WS cellulose (4% w/v) using 10 FPU (filter paper units) as a
6 final enzyme concentration. E+R (FPU=5+5) means 5 filter paper units from E and 5 filter paper
7 units from R. Enzyme activities of enzyme preparations are: *Penicillium janthinellum* EMS-UV-
8 8 (E) enzyme preparation contains 2 IU/ml FPU, 10.5 IU/ml CMCase and 0.11 IU/ml β-
9 glucosidase. *Trichoderma reesei* Rut C-30 (R) enzyme preparation contains 0.45 IU/ml FPU, 3.3
10 IU/ml CMCase and 0.12 IU/ml β-glucosidase. *Aspergillus tubingenesis* (A) enzyme preparation
11 contains 0.4 IU/ml FPU, 2.1 IU/ml CMCase and 3.3 IU/ml β-glucosidase. The values reported
12 in graph are the average values of three independent experiments.



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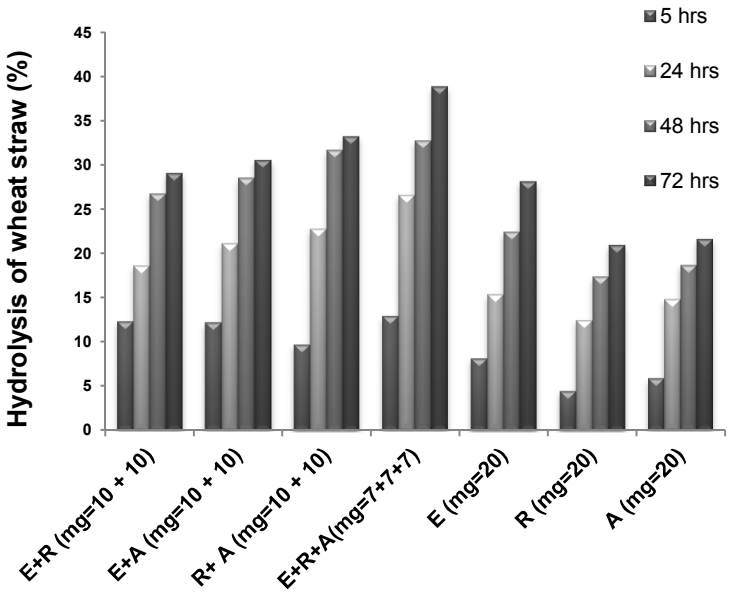

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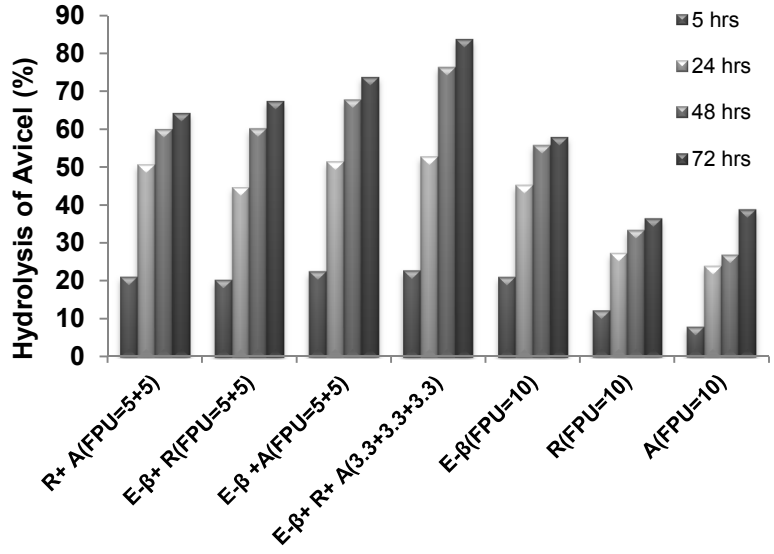
8 **Fig. 2** Hydrolysis of avicel and A-WS cellulose (4% w/v) using 20 mg protein as a final
9 concentration. Protein content of enzyme preparations are: *Penicillium janthinellum* EMS-UV-8
10 (E) contains 5.4 mg/ml protein. *Trichoderma reesei* Rut C-30 (R) contains 3 mg/ml protein.
11 *Aspergillus tubingenesis* (A) contains 2.1 mg/ml protein. The values reported in graph are the
12 average values of two independent experiments

Table 1 Enzyme activity analysis in enzyme blend.

Enzyme preparation	Total FPU		Total CMCase		Total β-Glucosidase	
	FPU added	FPU analyzed in blend	CMCase added	CMCase analyzed in blend	β-glucosidase added	β-glucosidase analyzed in blend
EMS-UV-8 + Rut C-30	5+5	10.4	26+38[b]	120	0.27+1.38	1.70
EMS-UV-8 + *A. tubingenesis*	5+5	12.1	26+26	112	0.27+41	35.5
Rut C-30 +*A. tubingenesis*	5+5	11.1	38+26	70	1.38+41	37
EMS-UV-8 + Rut C-30+*A. Tubingenesis*	3.3+3.3+3.3	13	17.8+24+17.4	122	0.18+0.87+27	25.5
EMS-UV-8-β[a] + Rut C-30	5 + 5	14.9	42.2+38	133	6.2+1.38	9.2
EMS-UV-8-β+ *A. tubingenesis*	5 + 5	15.1	42.2+26	108	6.2+41	43.5
EMS-UV-8-β+ Rut C-30 + *A. tubingenesis*	3.3+3.3+3.3	16.9	29.5+24+17.4	162	4.34+0.87+27	33

[a]Enzyme activities of *P. Janthinellum* EMS-UV-8-β (produced in 5L fermentor at controlled pH) enzyme preparation are 2.5 IU/ml FPU, 19 IU/ml CMCase, 3.1 IU/ml β-glucosidase and 6 mg/ml protein.
Other enzyme activities are same as stated in Figure 1.The values reported (analyzed enzyme activities in blend) here are the average values of two independent experiments with 8-10% variation.
[b]CMCase 26+38 means 26 CMCase units from EMS-UV-8 and 38 CMCase units from Rut C-30 was present while mixing 5+5 FPU respectively and similarly for other combinations.



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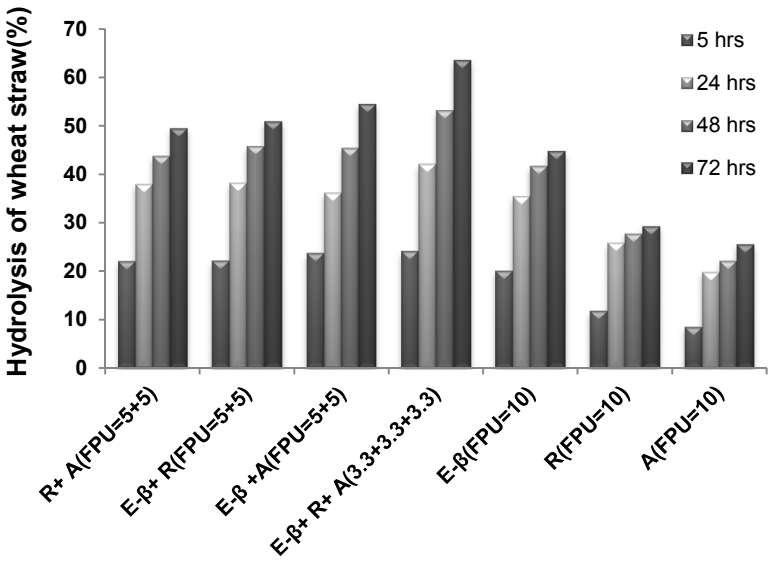

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3 **Fig. 3** Hydrolysis of avicel and A-WS cellulose (4% w/v) using enzyme preparation from *P.*
4 *janthinellum* EMS-UV-8 produced in 5L fermentor at controlled pH (EMS-UV-8-β or E-β).
5 Other enzyme preparations used to prepare blend are from different batch which were produced
6 in shake flasks. The enzyme activities used in this experiment are: EMS-UV-8-β or E-β enzyme
7 preparation contains 2.5 IU/ml FPU, 19 IU/ml CMCase, 3.1 IU/ml β-glucosidase and 6 mg/ml
8 protein. Rut C-30 enzyme preparation contains 0.55 IU/ml FPU, 5 IU/ml CMCase, 0.15 IU/ml β-
9 glucosidase and 2.5 mg/ml protein. *A. tubingenesis* enzyme preparation contains 0.59 IU/ml
10 FPU, 5.6 IU/ml CMCase, 3.6 IU/ml β-glucosidase and 2.3 mg/ml protein. The values reported
11 here are the average values of two independent experiments.