Augmentation of Cellulase Cocktail with β-glucosidase for Enhanced Sugar Yields

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Abstract—*Biofuels provide the answer to the energy crisis faced by majority of the countries today. Economic production of biofuel from cheap and easily available substrates can only lead to its commercialisation on large scale. The cost of production can be further cut down by increasing the efficiency of the hydrolytic enzymes or by using enzymatic cocktails in the saccharification step. Supplementation of β-glucosidases to cellulases can be done to enhance sugar recovery. Study was conducted to evaluate the effect of addition of β-glucosidases to cellulases on hydrolysis of α-cellulose and filter paper (Whatman no.1). Three commercial enzymes viz. Novozyme 188, Celluclast®1.5L and Accelerase®1500 were used for the hydrolysis of substrate. Maximum saccharification efficiency of 88.08% was observed when Novozyme was supplemented to Celluclaston hydrolysis of α-cellulose. Optimumsubstrate loading of α- cellulose for saccahrification by hydrolytic enzyme was determined. In the case of filter paper, maximum saccharification efficiency of 88.86% was obtained when Novozyme188 was added to Celluclast. This study shows, that β-glucosidase is one of the key enzyme involved in the hydrolysis step and that it can increase saccharification efficiency and sugar yield and help in making the economics of the process more favorable.*

1. INTRODUCTION

Production of biofuels and value added chemicals from lignocellulosic biomass depends on efficiency of conversion of biomass carbohydrates to fermentable sugars. Glucose which is the universal fermentation feedstock substrate for production of bioethanol and other value added chemicals is primarily derived from cellulose and hemicelluloses. However, saccharification of cellulose requires high amount of costly enzymes and this restricts its industrialization. To reduce the cost, it is imperative to develop highly efficient enzyme preparations and optimise the hydrolysis conditions in respect of pH, temperature, enzyme and substrate loading [1].

For commercialization of this technology, it is also important to get concentrated glucose slurries which demands higher cellulose loading and its efficient break down to glucose. In recent years, the enzymatic preparations for industrial use are being developed from *Trichodermareesei* which have various component enzymes mostly with glycosyl hydrolase activity and certain other proteins like swollenins and expansins which show synergism with hydrolases but have little cellulolytic activity [2]. The classical model for degradation of cellulose to glucose involves the cooperative action of endocellulases (EC 3.2.1.4), exocellulases (cellobiohydrolases, CBH, EC 3.2.1.91; glucanohydrolases, EC 3.2.1.74), and betaglucosidases (EC 3.2.1.21). Endoglucanases cleave cellulose chains at random and work synergistically with exoglucanases. β-glucosidase completes the process of saccharification of cellulose by hydrolysingcellobioses to glucose. The cellulases from *Trichoderma*have high endo and exoglucanase components with lower β-glucosidase levels, and hence limited efficiency in cellulose hydrolysis [3]. In order to overcome product inhibition of cellobiohydrolases (CBHs), cellulase mixtures must be optimized for β-glucosidase activity. Hence, reconstitution of cellulase cocktails with β-glucosidase and other accessory enzymes is required to considerably enhance the effectiveness of lignocellulose bioconversion.

The present study was, thus, carried out to evaluate the enhancement in performance of commercial cellulases in terms of sugar yield and saccharification efficiency upon augmentation with β-glucosidase enzyme.

2. MATERIALS AND METHODS

2.1 Substrate and enzymes used for hydrolysis

Two substrates α-cellulose (Sigma) and filter paper (whatman no. 1) were selected for saccharification.

Three commercial enzymes were used for saccharification. They were **Accelerase®1500** (Endoglucanase- 2200-2800 IU/gm; β-glucosidase- 450-775 IU/gm) a commercial cellulase enzyme complex with high β-glucosidase activity supplied by Genencor, **Celluclast® 1.5L** (Endoglucanase- 700 IU/gm) a commercial cellulase sourced from *Trichodermareesei* ATCC 26921 (Sigma Aldrich) and **Novozyme 188** (β-glucosidase- >250 IU/gm) a commercial β-glucosidase sourced from *Aspergillusniger*(Sigma Aldrich). They were used in two separate sets experiments for hydrolysis of the substrates, α-cellulose and filter paper.

2.2 Saccharification of α-cellulose and filter paper using commercial enzymes

Different combinations of commercial enzymes were added to these substrates at different loading rate and the role of the enzymes on their hydrolysis was studied. Saccharification of α-cellulose and filter paper (whatman no. 1) was carried out by procedure described [4]. One gram of substrate (10% substrate loading; w/v) was taken in a 50 ml plastic bottle to which different enzyme mixtures were added and the volume was made to 10 ml using citrate buffer (pH- 4.8). The bottles were incubated in shaking water bath at 50˚C for 72 hours. An aliquot 0.5 ml was withdrawn periodically after 24 hours for 3 days and heated in boiling water bath for 2 minutes to stop the reaction. Total reducing sugars were estimated by DNSA method as described earlier. The enzyme and substrate combinations used have been mentioned in figure. All the treatments have been carried out in triplicates.

2.3 Effect of substrate loading (α-cellulose) on sugar yield and saccharification efficiency

Optimum substrate loading for saccharification of α-cellulose was determined by using the substrate at different rates i.e.10%, 15%, 20% w/v with different combinations of commercial enzymes in a similar manner as described earlier.The enzyme and substrate combinations used have been mentioned in figure.All the treatments have been carried out in triplicates.

After suitable incubation periods 0.5ml aliquots were withdrawn from reaction mixtures and reducing sugars were estimated.

2.4. Estimation of reducing sugar

The concentration of reducing sugars released by hydrolytic enzymes was estimated by the DNSA (dinitrosalicylic acid) method [5]. Aliquots were taken out periodically from the reaction mixture and the amount of reducing sugars released was quantified and used to calculate the efficiency of saccharification by the following formula.

$$\%\ \text{Saccharification Efficiency} = \frac{\text{Reducing sugars released} \times 0.9 \times 100}{\text{Glucan content}}$$

3. RESULTS AND DISCUSSION

Economical biofuel production requires efficient conversion of lignocellulosic biomass to ethanol which greatly depends on improved conversion of these raw carbohydrate substrates to simple sugars. Saccharification efficiency has been found to be improved by supplementation of β-glucosidase to commercial cellulases like Celluclast®. The effectiveness of the β-glucosidase loading on the hydrolysis of complex substrate is found to vary with the type of substrate, pretreatment method, substrate loading and time of hydrolysis.

In this study, hydrolysis of α-cellulose and filter paper substrates with different combinations of commercial enzymes were done and the role of β-glucosidase in catalysing the rate limiting step leading to an increase in sugar yields was clearly demonstrated.

3.1. Evaluation of commercial cellulaseas well as β-glucosidasehydrolytic activity for releasing sugars from reference substrate α-cellulose

The release of reducing sugars from α-cellulose by the action of hydrolytic enzymes differed widely with different combinations of enzymes used. The sugar yield increased during 72 hrs of incubation in all the treatments. However, the reducing sugars released at 72 hrs of incubation with Accelerase®1500 (889.0 mg) was less as compared to those released by action of Novozyme 188 supplemented to Celluclast®1.5L (978.6 mg). Reducing sugar yield increased by 19.97%on supplementation of the commercial β-glucosidase, Novozyme 188 to Celluclast®(Fig. 1). Beneficial effect of supplementation of β-glucosidases on the relative amount of glucose produced and residual cellobiose on hydrolysis of amorphous cellulose have also been demonstrated by researchers [6].

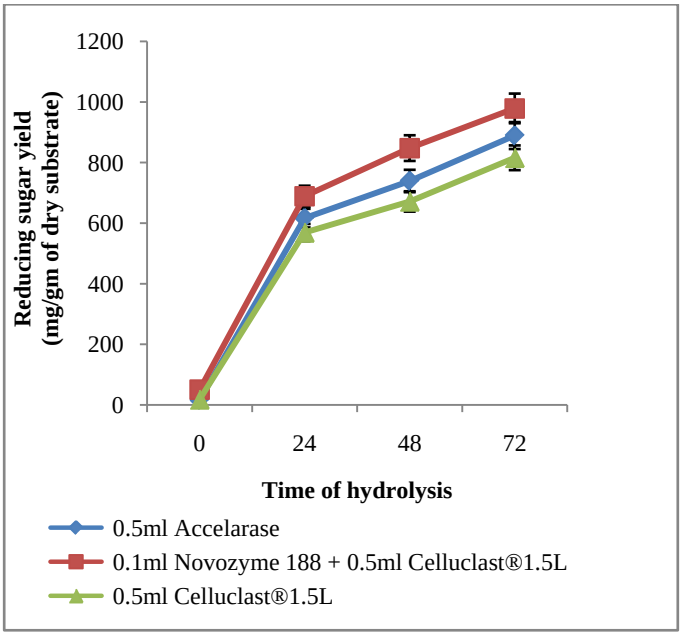

Fig. 1: Enhanced release of reducing sugars on supplementation of β-glucosidase to cellulases using α-cellulose (10% substrate loading)

Maximum saccharification efficiency of 88.07% was observed when Novozyme 188 was supplemented to Celluclast® (Fig. 2). The results, therefore, indicate that β-glucosidase has

important application in enhancing cellulase efficiency and sugar yield.

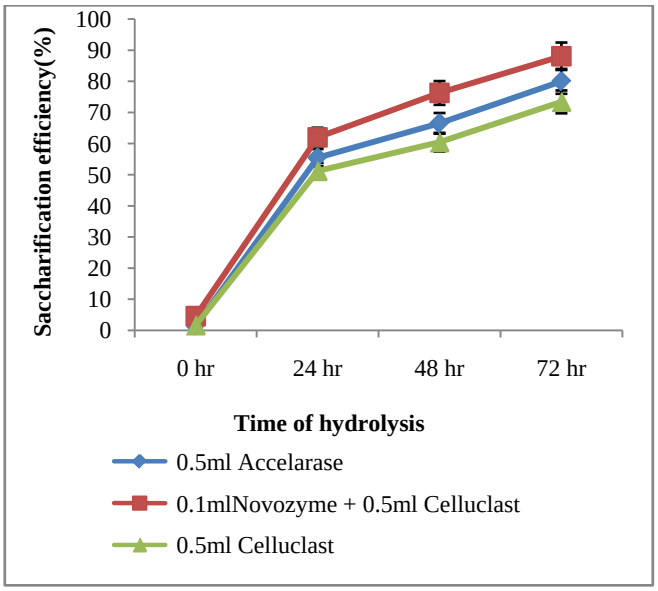

Fig. 2: Enhanced saccharification efficiency on supplementation of β-glucosidase to cellulases using α-cellulose (10% substrate loading)

3.2. Determination of optimum substrate loading (α-cellulose) for hydrolysis using commercial enzymes

Substrate loading and enzyme loading are the major determinants of the amount of fermentable monomeric sugars produced by hydrolytic enzymes. Maximum sugar yield of 1316.92 mg was obtained at 20% substrate loading but the saccharification efficiency was found to be highest at 10% substrate loading (Table 1). The increase in substrate loading from 10% to 20% increased the release of reducing sugars but a decrease in saccharification efficiency was observed which is explained due to lack of substantial increase in sugar yield as compared to substrate added. Increase in yield of reducing sugars but decrease in saccharification efficiencyon increasing substrate loading of xylose-extracted corn cob residues from 15% to 35% has also been reported [7]. Improper mixing due to high substrate loading may hinder the hydrolysis process, resulting in lower saccharification efficiency. It can be concluded, that mixing of hydrolytic enzymes and substrates in appropriate ratio is of high importance while preparing glucose rich slurries.

Table 1: Determination of optimum substrate loading for maximising sugar yields and saccharification efficiency

Treatment	Sugars released (mg/10 ml of reaction mixture)			
	0 hour	24 hours	48 hours	72 hours
α-cellulose 10% + 0.5ml Celluclast®1.5L + 0.1ml Novozyme 188	50.80±2.37 (4.57)*	689.00±8.73 (62.01)*	847.62±10.20 (76.29)*	978.58±9.24 (88.07)*
α-cellulose 15 % +0.5ml Celluclast®1.5L + 0.1 ml Novozyme 188	52.28±2.45 (3.14)*	839.83±6.90 (50.39)*	1045.95±11.02 (62.76)*	1144.833±10.23 (68.69)*
α-cellulose 20 % + 0.5ml Celluclast®1.5L + 0.1ml Novozyme 188	33.03±1.24 (1.49)*	970.25±9.20 (43.66)*	1192.42±12.10 (53.66)*	1316.92±12.67 (59.26)*
α-cellulose 15 % + 0.5ml Accelerase®1500	24.11±1.09 (1.45)*	775.67±8.45 (46.54)*	1000.75±9.43 (60.04)*	1206.08±9.72 (72.36)*
α-cellulose 20 % + 0.5ml Accelerase®1500	32.14±1.75 (1.47)*	785.67±7.92 (35.35)*	1038.25±10.87 (46.72)*	1274.42± (57.34)*

*Data in paranthesis indicate saccharification efficiency (%)

3.3. Evaluation of commercial cellulase as well as β-glucosidase hydrolytic activity for releasing sugars from reference substrate filter paper

Hydrolysis of filter paper (whatman no. 1) was carried out with different commercial enzymes. Maximum sugar yield of 987.33 mg was obtained when Novozyme was added to Celluclast®, with a saccharification efficiency of 88.86% (Fig. 3, 4). The sugar released on addition of Novozyme 188 to Celluclast® (987.33 mg/g of filter paper) was more as compared to that with Accelerase® (909.0 mg/g of filter paper)(Fig. 3). The result is in accordance with the hydrolysis of α-cellulose signifying that the β-glucosidase from Novozyme added to Celluclast® is superior combination to Accelerase® in terms of filter paper hydrolysis.Release of reducing sugars on hydrolysis of various substrates is greatly affected by the kind of substrate and enzyme used for their saccharification or the proportions of enzymes in cocktails. Therefore, having a diverse library of cellulases and other complementary enzymes will enable tailoring enzymatic cocktails for wide range of biomass feedstocks at reduced costs.

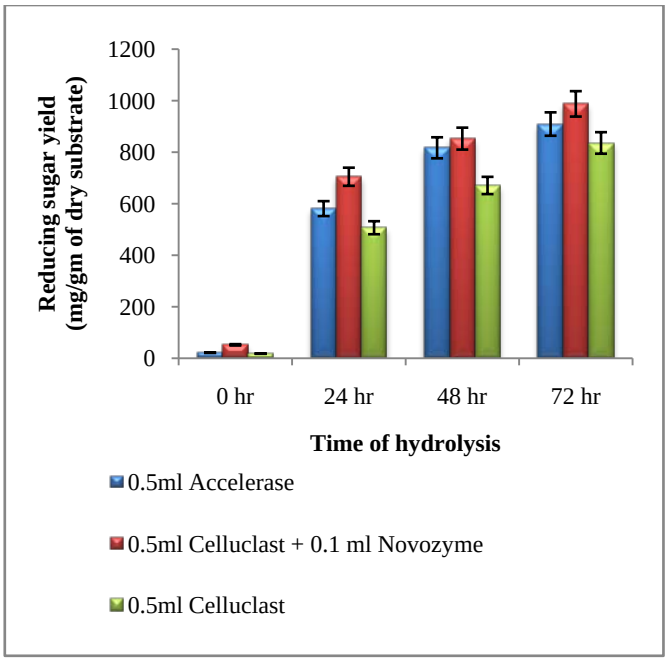

Fig. 3: Enhanced release of reducing sugars on supplementation of β-glucosidase to cellulases using filter paper (10% substrate loading)

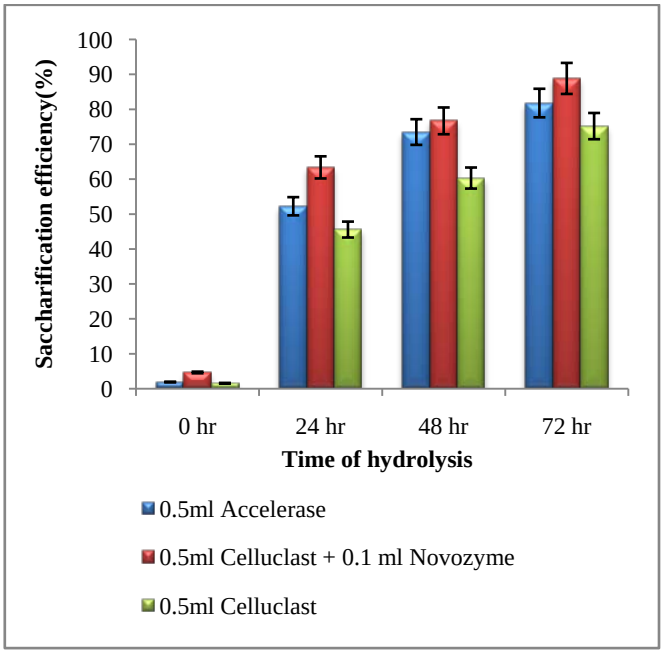

Fig. 4: Enhanced saccharification efficiency on supplementation of β-glucosidase to cellulases using filter paper (10% substrate loading)

4. Conclusion

The study investigates the role of β-glucosidase in augmenting the performance of cellulases in hydrolysis of cellulose in biomass for improved sugar yields. Our study, confirms that β-glucosidase is one of the key enzymes involved in the hydrolysis step and that it can augment cellulase performance resulting in increase of sugar yields and saccharification efficiency. This helps increase the cost effectiveness of enzymatic saccharification step in the overall process of commercial ethanol production. The need for optimisation of reaction conditions in terms of substrate loading and enzyme loading has also been emphasised.

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