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Feasibility studies with lignin blocking additives in enhancing saccharification and cellulase recovery: Mutant UV-8 of *T. verruculosus* IIPC 324 a case study

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ABSTRACT

The process economics of fermentable sugar production is dependent on the performance of cellulase cocktail on realistic lignocellulosic biomass and their capability to be recovered and recycled. Feasibility studies were conducted to enhance the digestibility of acid pretreated sugarcane bagasse using novel cellulase cocktail obtained from stable mutant UV-8 of *Talaromyces verruculosus* IIPC 324 in presence of lignin blocking additives. PEG 6000 was shortlisted as the best additive as it could simultaneously enhance saccharification and overall cellulase recoveries namely cellobiohydrolase, endoglucanase and cellobiase. Addition of 0.3 g PEG 6000/g acid-insoluble lignin content, resulted in 55% and 49.2% saccharification yields in terms of reducing sugars and glucose respectively using this cellulase cocktail (25 mg protein/g cellulose content) after 72 h from acid pretreated sugarcane bagasse loaded at 7.5%. The study also suggested that the endoglucanase of this mutant was unique with high desorption capability as 85% activity was observed in the saccharified broth devoid of any lignin blocking additive. At its optimum concentration, PEG 6000 was able to retain 94 ± 0.79% cellobiohydrolase I and 97.97 ± 1.16% cellobiase enzyme in the saccharified broth which were otherwise lost in residual biomass by ~80%, in the absence of this polymeric additive. These results suggest that PEG 6000 was the most promising facilitator for recycling of cellulases obtained from mutant UV-8 of *Talaromyces verruculosus* IIPC 324 in particular. It paved a way towards the production of cheaper fermentable sugars which serve as a starting raw material for the production of green chemicals and fuels.

1. Introduction

Bio-refining is an integrated process, which may be chemical, biological, thermo-chemical or their combination to convert biomass to valuable products such as liquid fuels, commodity chemicals and industrial materials. The successful commercial deployment of lignocellulosic feedstock for production of biofuels and bio-based chemicals is largely dependent on a number of factors.

Biomass recalcitrance, which is the natural resistance of plant cell walls to biological deconstruction, remains one of the major roadblocks for economic production of fermentable sugars via the biotechnological route. Among the various structural factors in biomass, lignin significantly contributes to biomass recalcitrance resulting in high operating costs for during processing [1–3]. Lignin, a complex aromatic polymer, is the most significant non-carbohydrate component (15–40% dry weight) in most terrestrial plants whose primary function is to provide structural integrity, facilitate water and nutrient transport, and confer microbial resistance to plant cell wall [3,4]. Recently, Liu et al have comprehensively reviewed the role of lignin as a technological hurdle in biomass enzymatic digestibility [5]. Three major covalent forces namely hydrophobic interactions, ionic and hydrogen bonds have been attributed to playing a vital role in triggering interactions of lignin moieties with cellulases during saccharification process. As a result lignin in all the forms (liquid, solid or monomeric) not only hinders the accessibility of enzyme to cellulose but also adversely affects the enzyme recyclability [5].

Various surfactants and additives such as Tween 20, Tween 80, Triton X-100, polyethylene glycol (PEG) 4000, PEG 6000, and bovine serum albumin (BSA) have been explored to overcome the problem of unproductive binding of cellulases on lignin surface and mechanism by which these additives work [6–10]. However most of the researchers have used these additives either as enzyme stabilizers or lignin blocking

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agents reporting improved saccharification rates, but most of the studies in state of art have screened these additives based on the dry matter basis [6] or cellulose content [7,11] present in the lignocellulosic biomass.

The present study was investigated with intent to screen the best lignin blocking additive (LBA) for concentrated cellulase enzyme (CCE) obtained from a stable mutant UV-8. This mutant was selected after UV mutagenesis of hypercellulolytic soil isolate *Talaromyces verruculosus* IIPC 324 using a rational approach as described previously [12]. During screening studies, all the additives were dosed on the basis of acid-insoluble lignin (AISL) content (0.15 g/g AISL) present in the acid pretreated sugarcane bagasse during enzymatic saccharification. The best additive was adjudged not only on the basis of enhanced saccharification yields but overall cellulase (endoglucanase or EG, cellobiohydrolase or CBH I and cellobiase or CBU) recoveries in saccharified broth for their efficient reuse. Cellulase recoveries were specially considered in the present study based on the report of Gao et al, where lignin after dilute acid pretreatment was found to be more detrimental and resulted in most significant losses of cellulases in the corn stover biomass when compared to other pretreatment strategies [13]. To establish that each enzyme cocktail from different microbial origin has a unique composition with differential affinity for lignin moieties, Cellic CTec2 was chosen as the reference enzyme during screening studies.

After short-listing the best LBA, the effect of increasing its dosage (0.075–0.375 g/g AISL) was studied with respect to saccharification and cellulase recovery of cellulase cocktail from mutant UV-8. Further, the effectivity of shortlisted LBA was also assessed at higher substrate loadings (5–15%).

2. Materials and methods

2.1. Lignocellulosic substrates for enzymatic saccharification and cellulase production

For enzymatic saccharification studies, raw sugarcane bagasse (SCB) was procured from Doiwala sugar mill, Dehradun, India. Pretreatment of SCB was conducted by the method described previously [14]. The compositional analysis and ash content of the acid pretreated SCB were carried out as per the method of National Renewable Energy Laboratory (NREL) described by Sluiter et al. [15,16]. The SCB consisted of 58.9 ± 1.0% glucan, 5.15 ± 0.19% xylan, 30.9 ± 0.3% acid insoluble lignin (AISL), 3.3 ± 0.09% ash content. Wheat bran was used as substrate for solid state fermentation (SSF) and was purchased from local market.

2.2. Lignin blocking additives (LBA)

The various lignin blocking additives that were screened for the present experimental set up include Tween 20, Tween 80, Triton X-100, Polyethylene glycol (PEG) 6000, Bovine serum albumin (BSA). All these chemicals were either procured from Sigma Aldrich, USA or Hi-Media Laboratories (Mumbai, India) and were of analytical or laboratory grade.

2.3. Microorganism, cultivations conditions and enzyme concentration

A stable mutant of *Talaromyces verruculosus* IIPC 324 namely mutant UV-8 was used for the present study as described previously [12]. This mutant was routinely maintained on PDA slants at 4 °C, and simultaneously the glycerol stocks (25% w/v) were also made and stored at −80 °C. The cellulase production of the said mutant was carried out under SSF conditions as described earlier [12].

For the preparation of the concentrated cellulase enzyme (CCE), 120 g of dry moldy bran was extracted with 1500 ml of deionized water and filtered. The spores present in the filtrate were removed by centrifugation at 10,000 rpm for 30 min at 4 °C. 1300 ml of the filtrate was subjected to ammonium sulfate precipitation (40–75% saturation), and the precipitated product was reconstituted in 50 mM citrate buffer followed by desalting using 10 kDa membrane (Macrosep Advance Centrifugal devices with Omega Membrane, Pall Make).

2.4. Reference commercial cellulase enzyme

Cellic® CTec2 was kindly gifted by Novozymes A/S (Bagsværd, Denmark). However, it was used a positive reference only for the screening experiments. The performance of Cellic CTec2 was observed with and without addition of lignin blocking additives in terms of enzymatic saccharification and cellulase recoveries.

2.5. Enzyme assays and protein determination

For CCE of mutant UV-8, the endoglucanase assay (EG) was carried out incubating them with 1% sodium salt of carboxymethyl cellulose (Sodium salt of CMC; Fluka- 21,902) in 50 mM citrate buffer at 55 °C for 10 min. Reducing sugars were determined by the 3,5, dinitrosalicylic acid (DNS) method [17]. One unit of endoglucanase activity was defined as the amount of enzyme which released 1 μmole of glucose/ min under the conditions indicated. Cellobiase assay (CBU) was carried out as per IUPAC protocol described earlier [18]. One unit of cellobiase activity was defined as the amount of enzyme which released 2 μmoles of glucose/min under the optimized conditions. Cellobiohydrolase I (CBH I) assay was carried out at 55 °C with 10 mM p-nitrophenyl β-D-cellobioside (pNPC) as substrate in 50 mM citrate buffer. After 20 min of incubation, the reaction was stopped by adding 1 ml of 2 M sodium carbonate solution. The 4-nitrophenol liberated was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmole of p-nitrophenol/min from pNPC under optimized assay conditions carbonate [19]. The protein concentration was measured by the Bradford Assay using bovine serum albumin (BSA) Fraction V as the protein standard [20].

The cellulase activities of Cellic CTec2 were also determined by the same procedure at its optimum temp and pH (50 °C; 5.2). Protein content was also measured by Bradford assay only.

2.6. Enzymatic saccharification studies

Hydrolysis of acid pretreated sugarcane bagasse was carried out at shake flask level and all experiments were performed in duplicates on one gram lignin basis. The initial substrate loading was 5% whereas the enzymes were added at 25 mg protein/g cellulose content during the entire study.

All the saccharification studies were performed at respective temperature (Cellic CTec2-50 °C; CCE UV-8 -55 °C) and pH optimum (Cellic CTec2-5.2; CCE UV-8 -4.5) of the enzymes for 72 h.

The amount of reducing sugars released was determined by 3, 5, Dinitrosalicylic acid (DNS) method using glucose as standard [17]. Glucose release in the saccharified broth was quantified by GOD-POD kit (Accurex Biomedical Pvt Ltd, India). To assess the complete release of sugars and glucose, after removal of saccharified broth the residual biomass was washed with water and both the sugar assays were conducted in the wash as well. Percentage saccharification was calculated using the following formula:

$$\%\ Saccharification = \frac{(Total\ reducing\ sugar/glucose\ released\ in\ g) \times 100}{Cellulose\ content\ in\ the\ substrate\ (g) \times 1.\ 11}$$

2.7. Cellulase recoveries after enzymatic saccharification

Overall cellulases recoveries were estimated in the saccharified broth, to assess the affinity of the various enzyme components present in the CCE of mutant UV-8 towards lignin (soluble and insoluble) and