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Improvement of wheat straw hydrolysis by cellulolytic blends of two *Penicillium spp.*

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ABSTRACT

Co-culture of fungal strains *Penicillium janthinellum* EMS-UV-8 (E), *Penicillium funiculosum* strain P (P) and *Aspergillus* sp. strain G (G) and blending of their crude cellulase were evaluated for improvements in cellulase activities as well as for enhanced hydrolysis of dilute acid pretreated wheat straw (PWS). The blending of crude enzymes of P and E enhanced the hydrolysis of PWS more effectively due to synergism in cellulolytic enzyme activities. Here, three types of blends were made on the basis of equal FPUs, equal protein content or fixed volume containing different proportions of individual enzymes, the former blend hydrolyzed 42.6% of PWS due to the 98%,62%, 64% and 34% synergistic enhancement in endo-glucanase, cellulase (FPU), β-glucosidase and xylanase activities, respectively. Hydrolysis at 10% solid loading of PWS in roller bottle reactor with this blend further enhanced hydrolysis yield to 74% within 24 h, which was much better than the corresponding hydrolysis yields of individual (38.1% by E and 61.5% by P) or the commercial enzyme (62.3%). This study proved that synergistic blends of cellulases from two *Penicillium* spp. are cost-effective tools for efficient wheat straw hydrolysis for on-site biofuel production.

1. Introduction

The breakdown of cellulosic biomass to its glucose monomers requires the action of cellulolytic enzymes in a sequential and synergistic manner. Endo-glucanase, exo-glucanase and β-glucosidase are the three major enzymes of this multicomponent cellulolytic system that play central role in lignocellulose hydrolysis [1]. Enzymatic hydrolysis step is still considered as bottleneck of the biochemical route for lignocellulose to ethanol conversion owing to higher cost and/or of low efficiencies of cellulolytic enzymes [2]. Till date many potential indigenous cellulolytic microorganisms have been discovered, yet none of them is known to possess complete set of ideal enzyme machinery for total biomass hydrolysis. Therefore, many strategies have been adopted to decrease the costs of enzyme production and/or hydrolysis including strain improvement through mutagenesis [3] or genome shuffling [4], use of microbial co-cultures to produce more diverse set of enzymes [5,6], application of crude enzyme extracts produced on-site in place of commercial enzymes [7], use of recombinant DNA technology for enhanced cellulase production, obtaining an ideal enzyme cocktail by mixing or blending of enzyme extracts with complementing activities so as to achieve maximum cellulose hydrolysis [8,9] and enzyme recycling [10] etc. Of these available techniques, use of microbial co-cultures and blending of on-site/in-house produced crude enzyme extracts are practically the simplest and comparatively cheaper ways to reduce the cost of enzymatic hydrolysis of lignocelluloses. Co-culture fermentations involve two or more organisms and may lead to better substrate utilization, increased productivity, and increased resistance to contamination by unwanted microbes as compared to monocultures. Moreover, synergetic interactions between compatible fungi may overcome nutritional limitations allowing higher cellulolytic enzyme production. This synergistic effect is a result of sequential, cooperative action between the three enzyme components, where the product of one enzyme reaction becomes the substrate for another. In order to achieve maximum cellulose-to-glucose conversion efficiencies and thereby reduce biofuel production costs, highly efficient cellulolytic enzyme cocktails are required that should be based upon maximum enzyme synergy i.e. increased combined activity than the sum of the individual enzyme activities [11–13]. Possible

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approach for supplementing the lacking enzymatic activities is the mixing of either indigenous or recombinant enzymes. Blending of crude enzyme extracts can improve the efficiency of lignocellulose hydrolysis due to enzyme synergy in the main cellulase activities as well as addition of accessory enzymes. When cellulase enzymes act synergistically, their collective activity is higher than that of their individual enzymes. Synergism is related to enhancement of either substrate hydrolysis or cellulase activities, or both [14–16]. There are four forms of synergism in cellulase mixtures, including endo–exo, exo–exo, endo-b-glucosidase, and intramolecular activities [17]. At least three key factors affect the extent of synergism among cellulases: the ratio and concentrations of the cellulases in the reaction mixture, access to binding sites for the cellulases, and heterogeneity in the substrate [16]. Many different fungal strains such as those belonging to *Aspergillus* and *Trichoderma* and their combinations have been used in past to prepare synergistic cellulase blends either by co-culture or by mixing of broths. Though *Penicillium* spp. are now well known for their cellulolytic potential but reports on their synergistic cellulase preparations are fewer in comparison to other fungal species.

The objective of this work was to study the increased lignocellulose hydrolysis by synergistic enhancement of cellulolytic activities of fungal enzyme preparations through co-cultures or blending of on-site/in-house crude enzyme extracts. Different blends of crude cellulases were prepared and evaluated for their hydrolytic efficiencies on dilute acid pre-treated wheat straw by performing saccharification assay. The approach used in this study has the potential for on-site or in-house application in biomass hydrolysis for biofuel purpose.

2. Materials and methods

2.1. Materials

Microcrystalline cellulose Avicel PH-101, p-Nitrophenyl β-D-glucopyranoside (pNPG) and 3, 5-dinitrosalysilic acid were procured from Sigma–Aldrich and carboxymethylcellulose (CMC) sodium salt-medium viscosity was from S.D. Fine Chemicals Ltd. India. SacchariSeb C6, a commercial cellulase powder, was purchased from Advanced Enzymes Inc, India. All other chemicals used were of analytical grade. Wheat straw and wheat bran were obtained locally.

2.2. Microorganisms and inoculum preparation

Fungal strain *Penicillium janthinellum* EMS-UV-8 (E), a cellulase hyper-producing mutant derived from *P. janthinellum* NCIM-1171 was obtained from Dr. D.V. Gokhale, National Chemical Laboratory, Pune, India. *Penicillium funiculosum* NCIM 1228 strain P and *Aspergillus* sp. strain G were kindly provided by Dr. Syed Shams Yazdani, ICGEB, New Delhi, India. All fungal strains were maintained on potato dextrose agar (PDA) slants and sub-cultured once in a month.

Medium used for inoculum development contained (g/L): glucose 5; 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; $FeSO_4$ $7H_2O$ 0.005; $MnSO_4$ H_2O 0.0016; $ZnSO_4$ 7H2O 0.0014; $CoCl_2$ $6H_2O$ 0.002; Tween-80 1 mL and pH was 5.0. The flasks containing 100 mL of above medium in 500 mL Erlenmeyer flasks were inoculated with approximately 1×10^7 spores from 7 to 10 d old slants of the fungal cultures. These flasks were incubated for 2 d at 30 °C with shaking at 150 rpm and the developed mycelial culture was used as inoculum for enzyme production.

2.3. Enzyme production in shake flasks

Shake flask studies on production of cellulolytic enzymes were carried out in 500 mL Erlenmeyer flasks containing 140 mL of above medium (section 2.2) except that the glucose was replaced with 1% Avicel and 2.5% wheat bran for strain E and G. co-culture study was also performed in this medium. Enzyme production medium used for strain P contained (g/L): wheat bran 21.4; Avicel 24; KH_2PO_4 5.9; $CaCl_2.2H_2O$ 0.05; $(NH_4)_2SO_4$ 3.12; Soya peptone 24; Yeast extract 0.05; and pH 5.0. The flasks were inoculated with 2 d old mycelial seed culture (10%, v/v) and incubated at 30 °C and 150 rpm for 8 d. For enzyme production in co-cultures, equal proportion of seed cultures of fungal strains was used. After incubation period, supernatants containing crude enzymes were collected by centrifugation at 5000 × g, analyzed for extracellular enzyme activities and protein contents and stored at 4 °C for subsequent experiments.

2.4. Enzyme production in fermentor

Submerged fermentation for cellulase production by fungal strains *P. janthinellum* EMS-UV-8 and *Penicillium fellutenum* strain P was carried out in 3 L of their respective medium as described by Singhania et al. [18], in a 7.5 L BioFlo115 fermenter (New Brunswick Scientific, USA). Sterilization of the fermentor vessel along with enzyme production medium was done at 121 °C for 30 min. Seed culture of the fungal strains were used as inoculum, as described in Section 2.2. Temperature during fermentation was set at 28 °C and pH was maintained at 5.0 by addition of 1 M HCl or 1 M NaOH. Airflow of 1 vvm was maintained which was increased to 1.5 vvm after 72 h. Pitch blade impellers were used to maintain agitation speed of 150 rpm. Frothing was controlled by addition of sterilized antifoam (2%, v/v) solution manually. Sampling was done at regular intervals and the centrifuged samples (5000 × g for 10 min) were used for analysis of cellulase activities and protein content.

2.5. Enzyme assays

Filter paper cellulase (FPase), endoglucanase (CMCase), xylanase and β-glucosidase activities were determined as reported earlier [9]. Filter paper units (FPU) were analized by incubating the suitably diluted enzyme (0.1 mL) with 1.4 mL citrate buffer (50 mM, pH 4.5) containing Whatman no. 1 filter paper strip (50 mg, 1 × 6 cm). The reaction mixture was incubated at 50 °C for 60 min. Endoglucanase (CMCase, Endo-1, 4-b-D-glucanase; EC 3.2.1.4) activity was carried out in the total reaction mixture of 1 mL containing 0.5 mL of suitably diluted enzyme and 0.5 mL of 2% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5). This mixture was incubated at 50 °C for 30 min. Xylanase (1,4-b-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined under similar conditions using 1% xylan solution as substrate. After incubation period, reaction (for FPase/FPU, CMCase and Xylanase) was stopped by addition of 3 mL of DNS solution and boiling for 5 min. After boiling, 20 ml de-ionized water was added and tubes were mixed by inverting and released reducing sugars were measured at 540 nm β-glucosidase activity was analyzed by using pNPG as substrate. The assay mixture comprised 0.5 mL of suitably diluted enzyme in 0.5 mL of pNPG (3 mg/mL, prepared in 50 mM citrate buffer of pH 4.8). This mixture was incubated at 50 °C for 30 min after which the reaction was stopped by adding 2 mL of 1% (w/v) sodium carbonate solution. 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.

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of glucose, xylose or p-nitrophenol produced from the appropriate substrates per min at