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Enhanced cellulase production by *Penicillium oxalicum* for bio-ethanol application

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HIGHLIGHTS

- A potent cellulolytic fungal strain was isolated and identified as *P. oxalicum*.
- Media engineering enhanced cellulase production by 1.7-fold.
- In bioreactor incubation time was reduced for producing same cellulase titer.
- In-house cellulase showed equal hydrolysis potential as that of commercial ones.

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ABSTRACT

Present study was focused on cellulase production from an indigenously isolated filamentous fungal strain, identified as *Penicillium oxalicum*. Initially, cellulase production under submerged fermentation in shake flasks resulted in cellulase activity of 0.7 FPU/mL. Optimization of process parameters enhanced cellulase production by 1.7-fold and resulted in maximum cellulase activity of 1.2 FPU/mL in 8 days. Cellulase production was successfully scaled-up to 7 L fermenter under controlled conditions and incubation time was reduced from 8 days to 4 days for achieving similar cellulase titer. Optimum pH and temperature for activity of the crude enzyme were pH 5 and 50 °C, respectively. At 50 °C the produced cellulase retained approximately 50% and 26% of its activity at 48 h and 72 h, respectively. Hydrolytic efficiency of *P. oxalicum* was comparable to commercial cellulase preparations which indicate its great potential for application in the lignocellulose hydrolysis.

1. Introduction

Bioethanol produced from biomass is foreseen as one of the major alternatives to petroleum based fuels due to abundant biomass availability and ubiquity in nature (Sukumaran et al., 2005). Its production via enzymatic route is the most popular way being sustainable and eco-friendly. Bioethanol production from biomass requires four major steps: biomass pretreatment, enzymatic hydrolysis, fermentation and distillation. Cellulase is a multienzyme complex that is mainly responsible for hydrolysis of lignocellulosic biomass into its monomeric sugars that can be fermented further to ethanol. Three major components of cellulases namely, endo-glucanase, exo-glucanase and β-glucosidase (BGL) act synergistically during enzymatic hydrolysis. Cellulases have remained a major thrust area of research from several decades and their application for bioethanol production has given a major push to the subject. Enzymatic hydrolysis step has been previously cited many times as a major bottleneck during biochemical conversion of biomass to bioethanol (Singhania et al., 2010; Visser et al., 2013; Saini et al., 2014). Multi directional research is being done worldwide comprising isolation of efficient and high titer cellulase secreting microorganisms, strain improvement of available isolates for hyperproduction of cellulase (Adsul et al., 2007), process development for cheaper production technology (Singhania et al., 2007, 2010), engineering of cellulase protein for further improvement in its efficiency (Kellermann and Rentmeister, 2014), etc. Though efficient cellulases are available for cellulosic biomass hydrolysis with major global enzyme players such as Novozymes and Genencore but bioethanol production technologies need indigenous enzyme production technology to ensure continuous supply of enzyme. This can be easily achieved by on-site/in-house production of cellulases which have activities and/or hydrolysis efficiencies comparable to commercial cellulase preparations.

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At present, almost all commercially available cellulases are derived from fungal sources due to their comparative higher cellulase titer as compared to bacteria and extracellular nature which makes extraction easier. Most common fungal strains employed for cellulase production are mutants derived from *Trichoderma reesei*. However, prior research indicates that *T. reesei* produces cellulases that have very low levels of BGL activity. Lower BGL/FPA ratio results in higher accumulation of cellobiose during hydrolysis which further inhibits cellulase enzyme activity by feedback inhibition mechanism. Therefore, several other filamentous fungi like *Penicillium*, *Aspergillus*, *Chrysosporium*, *Acremonium*, etc. have been tested as alternatives to *T. reesei* for production of cellulases (Gusakov, 2013). Several strains of *Penicillium* spp. have been reported to produce comparable or even better cellulase titer along with higher BGL as well as other necessary enzyme activities (Adsul et al., 2007; Gusakov, 2013). On-site/in-house cellulase production by potential cellulase producers could help in bringing down the cost of biomass to bioethanol process. Cellulases produced in this manner can help in decreasing the dependence on commercial sources and also ensure a continuous supply at or near the site of their application. Enzyme production can be enhanced by optimizing the process parameters through response surface methodology (RSM). RSM is a statistical tool involving several designs such as central composite design, Box–Behnken design, etc. and has been successfully applied in the past for enhancing the enzymatic hydrolysis, production of enzymes or several other bioprocesses (Saini et al., 2013).

The objective of the present study was to evaluate the application of a potential fungal strain *Penicillium oxalicum* IODBF5 in cellulase production under submerged fermentation. For enhancing the cellulase production by this strain, important process parameters were optimized using response surface methodology and cellulase production was successfully scaled-up to 7 L fermenter. To prove the potential of crude cellulase of this strain in the lignocellulose hydrolysis its cellulose hydrolysis efficiency was compared with that of commercial preparations.

2. Methods

2.1. Microorganism and culture conditions

The fungal strain *IODBF5* was isolated from soil containing decayed wood from Chamba, Himachal Pradesh, India. It was isolated as a pure culture among several other fungal strains and was maintained on potato dextrose agar slants. The culture sporulated fully after 5 days. Spores were used as inoculum and were obtained by dislodging them from the surface of a fully grown PDA slant into sterile 0.05% tween 80 solution. Spores were counted on haemocytometer and 10^8 spores/100 mL were inoculated into the shake flasks for enzyme production.

All the chemicals used in the medium were reagent grade from HiMedia (India) and Sigma (USA).

2.2. Morphology and molecular identification

The color and pattern of the fungal growth on PDA plates was examined after 8 days incubation. The hyphal morphology of the fungal isolate was studied by bright field microscopy (Nikon eclipse Ni, Tokyo, Japan) after staining with lacto-phenol cotton blue. For molecular phylogenetic studies, the genomic DNA of fungus was extracted and purified using HiPurA™ Fungal DNA Purification Kit (HiMedia, India). For fungal DNA analysis, universal primers ITS1 and ITS4 targeting fungal ITS region were used (White et al., 1990). The 5.8S-internal transcribed spacer region of the rDNA gene was amplified by polymerase chain reaction (PCR). PCR was performed in a total reaction volume of 25 μL using 12.5 μL of Dream Taq PCR 2× master-mix (Thermo Fisher Scientific, India) along with 0.4 μL each of forward and reverse primers (10 μM) and 5–10 ng DNA template. The amplification was performed by initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 30 s, and elongation at 72 °C for 1 min and then final elongation step at 72 °C for 10 min. The sequencing was carried out by Shrimpex biotech services Pvt. Ltd., Chennai, India. The ITS-5.8s rRNA gene sequence of strain IODBF-5 and that of its closest phylogenetic neighbors retrieved by NCBI BLAST (n) analysis were aligned using the multiple alignment program CLUSTALW. Phylogenetic tree was then constructed by neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. The evolutionary analysis was done using MEGA4 (Tamura et al., 2007).

2.3. Enzyme production in shake flask

Medium used for enzyme production had following composition in g/L: KH_2PO_4-2.0, $CaCl_2{\cdot}2H_2O$-0.3, urea-0.3, $MgSO_4{\cdot}7H_2O$-0.3, $(NH_4)_2SO_4$-1.4, peptone-0.25, yeast extract-0.1, tween 80–0.1 mL/L, $FeSO_4{\cdot}7H_2O$-0.005, $MnSO_4{\cdot}H_2O$-0.0016, $ZnSO_4{\cdot}7H_2O$-0.0014, $CoCl_2{\cdot}6H_2O$-0.002, Avicel-10, and wheat bran-25 unless changes are mentioned (Adsul et al., 2007). Enzyme production was carried out in 250 mL Erlenmeyer flasks. For enzyme production, flasks were incubated at 28 °C and 180 rpm for 8 days.

2.4. Enzyme production in 7 L stirred tank bio-reactor

The medium used for enzyme production in 7 L stirred tank reactor Bioflow-115 (New Brunswick Scientific, USA), had similar composition as described in Section 2.2 above. Sterilization of fermenter vessel along with enzyme production medium was done in autoclave at 121 °C for 30 min. Temperature was maintained at 28 °C and the pH was controlled at 5.0 by addition of 1 M HCl or 1 M NaOH. Spores were used as inoculum at the rate of 10^9 spores/L and 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 was maintained. Antifoam (2%, v/v) was autoclaved separately and approximately 0.5 mL was added manually during frothing. Sampling was done at regular intervals for analysis of cellulase activities. Extracellular enzyme was extracted by centrifugation of the sample at 8000 rpm for 10 min.

2.5. Enzyme and protein assays

Filter paper cellulase (FPase), endoglucanase, xylanase and BGL activities were determined as reported earlier (Adsul et al., 2007). Filter paper activity was assayed by incubating the suitably diluted enzyme (0.1 mL) with 1.9 mL citrate buffer (50 mM, pH 4.5) containing Whatman No. 1 filter paper (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 1% (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, except that 1% xylan solution was used as substrate in place of CMC. BGL activity was