New isolate of *Trichoderma viride* strain for enhanced cellulolytic enzyme complex production

Xinde Jiang,[1,2] Anli Geng,[2,*] Ning He,[1] and Qingbiao Li[1,3]

Department of Chemical and Biochemical Engineering, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, People's Republic of China,[1] School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, Singapore,[2] and The Key Lab for Chemical Biology of Fujian Province, Xiamen 361005, People's Republic of China[3]

A new *Trichoderma viride* stain was isolated from Singapore soil samples. Its mutants were developed by using ethyl methyl sulfonate (EMS) treatment and UV-irradiation followed by a semi-quantitative plate clearing assay on phosphoric-acid-swollen cellulose plates. Mutant EU2-77 proved to be the most promising extracellular cellulase producer among 20 mutants in a screening program performed in shake flask fermentation after plate screening. Soluble protein content, filter paper cellulase (FPase) activity, β-glucosidase activity and endoglucanase (CMCase) activity of the fermentation broths of the mutant strain were increased to 1.67, 2.49, 2.16, and 2.61 folds, respectively, compared with the wild strain. This enzyme complex produced by mutant EU2-77 contained FPase (2.19 IU/ml), CMCase (16.46 IU/ml), β-glucosidase (4.04 IU/ml), xylanase (42.37 IU/ml), and β-xylosidase (0.12 IU/ml). The soluble protein concentration in the enzyme complex was 1.69 mg/ml. The hydrolytic capacities of fermentation supernatants of *T. reesei* Rut-C30, the wild strain *T. viride* NP13a and mutant *T. viride* EU2-77 were compared with the commercial enzymes on the hydrolysis of waste newspaper. The crude enzymes prepared by *T. viride* EU2-77 showed much higher hydrolysis performance than that from the commercial strain Rut-C30 and demonstrated much comparable hydrolytic performances with the commercial enzyme mixtures. *T. viride* mutant EU2-77 produced high levels of extracellular cellulases as well as β-glucosidase, rendering the supplementation of β-glucosidase unnecessary in waste newspaper hydrolysis.

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Recently, conversion of biomass to bio-fuels has been the subject of intense research efforts and gained significant political and scientific momentum, owing to concerns about the shortage of fossil fuels and the emission of green house gases (1–3). Global energy usage is projected to be almost doubled in the next two decades (http://www.eia.doe.gov/oiaf/ieo/pdf/highlights.pdf), and biological fuel production might serve as a sustainable, carbon-neutral energy source compatible with current engine technology. Lignocellulosic biomass is by far the most abundant renewable source of sugars that can be fermented to bio-fuels like ethanol. These include hardwood, softwood, grasses and agricultural residues. The additional raw materials of potential interest are newsprint, office paper, municipal solid wastes, etc. It has been estimated that lignocellulose accounts for about 50% of the biomass in the world according to Classen et al. (4). While fermentation of cornstarch or sugar cane juice by *S. cerevisiae* is a well-established technology, fermentation of lignocellulose is rather challenging. It involves a pretreatment process to release cellulose, hemicellulose and lignin from the lignocellulose matrix, hydrolysis to

produce reducing sugars and fermentation to convert sugar mixtures to ethanol.

Cellulose, one of the major components of lignocellulosic materials, is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1–4)-glycosidic bonds. Cellobiose is the smallest repetitive unit of cellulose and can be converted into glucose residues. The cellulose-hydrolyzing enzymes (i.e. cellulases) are divided into three major groups: endoglucanases (CMCases), cellobiohydrolases (exoglucanases), and β-glucosidases. The CMCases catalyze random cleavage of internal bonds of the cellulose chain, while cellobiohydrolases attack the chain ends, releasing cellobiose. β-glucosidases are only active on cello-oligosaccharides and cellobiose, and release glucose monomers units from the cellobiose.

As the production of cellulolytic enzymes is a major factor in lignocelluloses hydrolysis, it is important to make the process economically viable, because large-scale application in the degradation lignocelluloses demands microorganisms with improved activity and productivity and cellulases with better resistance to product inhibition. A variety of microorganisms including bacteria and fungi have the ability to secrete cellulase to degrade the lignocelluloses to glucose monomers. Filamentous fungi, typically *Trichoderma reesei* (5), have an excellent capacity for extracellular protein production. It is well known that *T. reesei* produces the cellobiohydrolase (CBH) and

* Corresponding author. School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, 535 Clementi Road, Singapore 599489. Tel.: +65 64608617; fax: +65 64679109.

E-mail address: gan2@np.edu.sg (A. Geng).

endoglucanase (EG) components of cellulolytic enzyme complex in large quantities. However, the amount of β-glucosidase secreted by *T. reesei* is insufficient causing the accumulation of cellobiose, which produces repression and end product inhibition to the enzymes (6,7). Other important cellulase producing fungi such as *Penicillium* (6,8) and *Aspergillus* (9) species, produce cellulolytic enzyme complex showing a higher β-glucosidase activity than *T. reesei*, but the total cellulase (FPase) activity is relatively low. As a result, production of glucose from waste cellulose is still not commercially feasible.

Wild strain *Trichoderma viride* NP13a was isolated from the soil samples in the campus of Ngee Ann Polytechnic Singapore in 2007. It is a cellulose-degrading fungus and it secretes large amount of cellulases and the ratio of β-glucosidase/FPase in its enzyme complex is higher than that in the enzyme mixture of *T. reesei* strains (10). In this study, successive rounds of random mutagenesis using ethyl methyl sulfonate (EMS) treatment and UV-irradiation were conducted to improve strain *T. viride* NP13a in order to obtain a high-yield cellulase producer and in turn a better enzyme complex for lignocellulosic biomass hydrolysis. Phosphoric-acid-swollen cellulose plates were used to screen the potential mutants. Liquid cultivation was conducted to evaluate the activities of the enzyme complex produced from the selected mutants. The potential of the crude enzyme complex produced by the potential mutant, the commercial enzymes and their mixtures, was demonstrated by the hydrolysis of waste newspaper.

MATERIALS AND METHODS

Microorganisms and culture media   The cellulase hyper-producing fungus *T. reesei* Rut-C30 (ATCC 56765) was obtained from the American Type Culture Collection (ATCC). Wild strain *Trichoderma viride* NP13a was isolated from the soil samples in the campus of Ngee Ann Polytechnic Singapore according to methods described by Kader et al. (11) and it was kept in the biocatalysis laboratory of Ngee Ann Polytechnic. The wild type strain *T. viride* NP13a, its mutants and *T. reesei* Rut-C30 were maintained on Potato-Dextrose-Agar (PDA) plates and sub-cultured every two weeks. The plates were prepared by dissolving 39 g of PDA powder (Merck, Germany) in 1 l of distilled water and autoclaved at 121°C for 15 min.

Selection medium contained Basal Medium (BM) described by Mandels and Weber (12) and (w/v) 0.1% Triton X-100, 2.0% agar, 0.4% sorbose (Sigma) and 1.0% phosphoric-acid-swollen cellulose (SM1), supplemented with 0.2% 2-deoxy-D-glucose (SM2) or 1.5% 2-deoxy-D-glucose (SM3). 2-deoxy-D-glucose and micro-granular cellulose were purchased from Sigma. Inoculum medium contained Basal Medium and 1.0% lactose (Merck) as the carbon source. The medium was autoclaved at 115°C for 20 min. Fermentation medium (enzyme production medium) contained Basal Medium and 1% cellulose (Sigma). The medium was autoclaved at 121°C for 20 min and pH was adjusted to 6.0.

Enzyme production in shake flasks   Shake flask experiments were carried out in 250-ml Erlenmeyer flasks. Seed cultures were prepared by inoculating 2 ml spore suspension (10^8 spores/ml) to 100 ml inoculum medium and they were incubated at 30°C and 200 rpm for 48 h. Eleven milliliters aliquots of the inoculums were then used to initiate the growth in 250-ml Erlenmeyer flasks containing 100 ml enzyme production medium and the cultures were incubated at 30°C and 200 rpm for 7 days. Samples were withdrawn daily and were centrifuged at $14,972 \times g$, at 4°C for 5 min. Supernatants were then assayed for enzyme activities and the soluble protein content.

Mutagenesis and screening of mutants   Wild strain *T. viride* NP13a was grown on PDA plates for 8 days at 30°C for spore development. Spores were then washed using 0.05% Tween 80 solution and they were counted under the microscope. Spore suspension was adjusted to 10^8 spores/ml. Random mutagenesis was conducted using ethyl methyl sulfonate (EMS) treatment followed by UV-irradiation (at 254 nm and 10 cm distance) according to the method described by Asdul et al. (6) with modifications. The scheme of obtaining the mutants is depicted in Fig. 1. Fifty microliters of ethyl methyl sulfonate (EMS) was added to 10 ml of the spore suspension and it was kept at room temperature for 24 h followed by UV-irradiation at 254 nm for 20 min corresponding to a lethal rate of 84%. Two hundred microliters of the treated spore suspension was subsequently spread on the screening plates. The cultures were then incubated at 30°C for 5 days and potential mutants were selected on the basis of clear zones appeared on SM1. The selected mutants were then further screened on SM2 plates and the fast growing colonies were chosen for further screening on SM3 plates. The final selected mutants from SM3 plates were cultured in shake flasks and the supernatants of the fermentation broth were assayed for enzyme activities. The most promising enzyme producing mutants were selected for further studies.

Enzyme assay   Filter paper activity was assayed as described by Ghose (13) by incubating 0.5 ml of the suitably diluted enzyme samples with 1.0 ml 0.05 M citrate buffer (pH 4.8) containing one Whatman No.1 filter paper strip (50 mg, 1×6 cm). The reaction mixture was incubated at 50°C for 60 min.
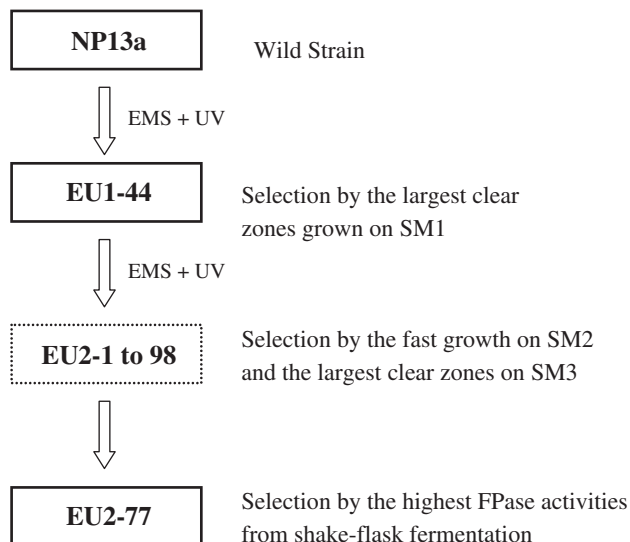

FIG. 1. Schematic presentation of strain improvement and screening for the obtaining of the most potential cellulolytic mutant.

CMCase activity was carried out in the total reaction mixture of 1.5 ml containing 0.5 ml of suitably diluted enzyme samples and 1.0 ml 2% (w/v) carboxymethylcellulose (CMC 7L2, degree of substitution = 0.7, Hercules Inc., USA) solution in 0.05 M citrate buffer (pH 4.8) and this mixture was incubated at 50°C for 30 min (14). Xylanase activity was determined by catalyzing of 1.0 ml of 1% xylan in 0.05 M phosphate buffer (pH 6.5) by 0.5 ml of suitably diluted enzymes at 40°C for 10 min (14). Xylan from Birchwood was obtained from Sigma (USA).

β-Glucosidase activity was estimated using *p*-nitro phenyl β-D-glucopyranoside (pNPG) as the substrate according to Berghem and Pettersson with modifications (15). The total of assay mixture (1 ml) consisted of 0.9 ml of 1 mM pNPG and 0.1 ml of suitably diluted enzyme samples and it was incubated at 50°C for 10 min. The reaction was terminated by the addition of 0.5 ml 1 M sodium carbonate. The p-nitrophenol liberated was measured at 400 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). β-Xylosidase activity was estimated in an analogous manner except that p-nitro phenyl β-D-xylopyranoside (pNPX) was used as the substrate.

The reducing sugars were determined by dinitrosalicylic acid (DNS) method (16). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of the product from their respective substrate per min under the assay conditions. The soluble protein, an indicator of the enzyme released, was measured by the Lowry Protein Assay using bovine serum albumin as the standard (17).

Enzymatic hydrolysis of waste newspaper   The crude enzyme mixtures of *T. reesei* Rut-C30, wild strain *T. viride* NP13a, and EU2-77 were recovered from the fermentation broth after 7 days of growth. Waste newspaper was collected from the office and its composition was characterized before (18). It was cut into approx 0.5 × 0.5 cm pieces after drying at 90°C for 2 days. Enzymatic hydrolysis of waste newspaper was carried out at a biomass loading of 2.5% (w/v) in 20 ml of 0.05 M citrate buffer (pH 4.8) in 50-ml Falcon tubes. The experiments were performed in triplicate at 50 ± 1°C in a shaking water bath (Memmert GmbH + Co. KG, Schwabach, Germany) with maximum strokes for 96 h. The commercial *T. reesei* cellulase (Celluclast 1.5 L), β-glucosidase (Novozyme 188) and cellulolytic enzyme cocktail (Celic CTec) were used as the controls. Celluclast 1.5 L, Novozym 188, and Cellic CTec were kindly donated by Novozymes Malaysia sdn bhd. The enzyme activity for FPase was 10 IU/g newspaper and that for β-glucosidase was 30 IU/g newspaper in the mixture of Celluclast 1.5 L and Novozym 188. Enzyme loading for the crude enzyme mixture from the fermentation broth and for Cellic CTec was the same, i.e.10 IU/g substrate of FPase. Samples were withdrawn every 24 h. They were centrifuged at $2105 \times g$, 4°C for 5 min. The amount of reducing sugars released from enzymatic hydrolysis was measured using the 3,5-dinitrosalicylic acid (DNS) method (16). Concentration of each individual sugar was measured using High Performance Liquid Chromatography (HPLC) on an Aminex HPX-87H column (Bio-Rad, Richmond, CA, USA) at 75°C with 0.6 ml/min eluent of 5 mM sulfuric acid. Such analysis was conducted on a 1200 Series HPLC system (Agilent Technologies Inc.) equipped with a Refractive Index Detector.

RESULTS

Mutagenesis and screening of mutants   Wild strain *T. viride* NP13a was subjected to successive mutagenic treatment with EMS followed by UV-irradiation. After each round of mutagenesis, enzyme