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Strategy for screening metagenomic resources for exocellulase activity using a robotic, high-throughput screening system

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ABSTRACT

Exocellulases play a key role in cleaving the accessible ends of cellulose molecules to release soluble glucose and cellobiose. To date, there have been no screens for exocellulase owing to assay protocol limitations, the high cost of substrates, and low activity of exocellulases compared with endocellulases. This study is the first to demonstrate direct screening for exocellulase activity using a robotic, high-throughput screening (HTS) system. Cell growth in 96-well plates was measured by monitoring optical density over 11–14 h at 37 °C with agitation. Fluorescence of methylumbelliferyl groups released from 4-methylumbelliferyl-β-D-cellobioside was determined using a VICTOR3 microplate reader. This new HTS system enabled activity verification of more than 10^4 clones per day. As a result, we obtained four exocellulases clones (CelEx-SF301, CelEx-SF309, CelEx-BR12 and CelEx-BR15) from 29,006 metagenomic fosmid clones that had previously been prepared from sweet potato field soil microbes and rumen fluid. This powerful approach could be effectively applied to screen various metagenomic resources for new enzymes.

1. Introduction

Enzymes are used in a wide range of biotechnological applications, and an increasing number of bio-based materials have been produced using one or more enzymes (Beilen and Li, 2002). Typically, industrial enzymes have been developed by screening pure cultured microorganisms using activity-based screening methods for application-specific performance such as activity, specificity, and stability under operational conditions based on previously characterized enzymes (Lorenz and Eck, 2005). However, it is very difficult to screen for novel enzymes using traditional methods owing to limitations in resources and screening methods.

Cellulose can be degraded to glucose through the synergistic action of three classes of glycoside hydrolases (GHs): (1) endo-β-1,4-glucanase (EG; EC 3.2.1.4), which randomly attacks the cellulose polymer by endo action; (2) exo-β-1,4-cellobiohydrolases, CBH I and CBH II (EC 3.2.1.91), which remove cellobiose from the non-reducing and reducing ends, respectively, of the cellulose chain; and (3) β-glucosidase (BGL; EC 3.2.1.21), which hydrolyzes cello-oligosaccharides and cellobiose to glucose (Beguin and Aubert, 1994; Clarke, 1997; Henrissat et al., 1985).

Hydrolases, such as esterases, lipases, proteases and GHs, are enzymes that catalyze the hydrolysis of a chemical bond. In general, activity-based screens can visually detect most hydrolases by the appearance of a halo around microorganism-producers. Endocellulase activity, for example, is easily detected in this manner on plates using dye-labeled substrates. For example, adding a reducing sugar of carboxymethyl cellulose (CMC) or cellulose stained with Congo red and Gram's iodine to agar plates or polyacrylamide gels has been used to screen for cellulolytic microorganisms (Béguin, 1983; Kasana et al., 2008; Zhang et al., 2006). However, such approaches are not quantitative or sufficiently sensitive owing to a poor correlation between enzyme activity and halo size (Sharrock, 1988).

A major limitation of most such traditional, activity-based screening methods is the difficulty in applying them to screen for enzymes such as exocellulase. Exocellulases cleave the accessible ends of cellulose molecules, liberating glucose and cellobiose. *Trichoderma reesei* choloylglycine hydrolases. (CBH) I and II act on the reducing and non-reducing cellulose chain ends, respectively (Teeri, 1997; Teeri et al., 1998; Zhang and Lynd, 2004). The modes of actions of endocellulase and exocellulase enzymes differ in that the intramolecular cleavages produced by endocellulases significantly decrease the specific viscosity of CMC, even with a small amount of hydrolysis, whereas exocellulases hydrolyze long chains from the ends in a processive manner (Irwin et al., 1993, Teeri, 1997; Zhang and Lynd, 2004). A selective assay for exoglucanases in the presence of endoglucanases and β-glucosidases has been reported. This assay is based on an exoglucanase that specifically hydrolyzes the aglyconic bond of p-nitrophenyl-β-D-cellobioside to yield cellobiose and p-nitrophenol (Deshpande et al., 1984). Efficient screening using such

Abbreviations: HTS, High-throughput screening; GHs, Glycoside hydrolases; EG, Endo-β-1,4-glucanase; CBH, Exo-β-1,4-cellobiohydrolases; BGL, β-Glucosidase; $MeUmbG_2$, 4-Methylumbelliferyl-β-d-cellobioside; MeUmb, Methylumbelliferyl; TLC, Thin-layer chromatography; ORF, Open reading frame; PFGE, Pulsed-field gel electrophoresis; LB, Luria-Bertani; G1, Glucose; G2, Cellobiose; G3, Cellotriose; G4, Cellotetraose; G5, Cellopentaose.

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assays requires measuring the activity of colonies one by one. Thus, these methods are typically slow and difficult to implement for high-throughput screening (HTS) of large libraries. A major limitation of most traditional assays for screening exocellulase is that they are difficult to use in a plate format, and therefore are labor- and time-intensive.

Direct screening for novel enzymes has been limited to rational design and/or strategies that are dependent on activity-screening approaches. Screening metagenomic resources for new enzymes is difficult compared with hydrolase-based on-plate due to assay protocol limitations, the high cost of substrate, and low sensitivity.

We previously described the development of a HTS system for screening various environmental sources, including metagenomes, for enzymes (Ko et al., 2012b). This HTS system consists of a colony picker, liquid handler, multilabel microplate reader, automated incubator, microplate washer, and F3 robotic arm. In the present paper, we describe a rapid, simple, and efficient method for screening exocellulases using a robotically controlled HTS system. Ultimately, an optimized screening protocol for this HTS system enabled us to perform activity verifications on more than 10^4 clones per day and identify four distinct clones with exocellulase activity, demonstrating the effectiveness of this strategy. This HTS system is a model system for sensitive, multiplex screening of metagenomic resources for enzymes that holds promise in providing new enzymes for bioindustrial applications.

2. Materials and methods

2.1. Chemicals

Analytical-grade 4-methylumbelliferyl-β-D-cellobioside ($MeUmbG_2$) and cello-oligosaccharides were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Restriction enzymes were the products of New England Biolabs Inc. (Beverly, MA, USA). Unless specified otherwise, all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Construction of a metagenomic library

Metagenomic DNA was isolated from microorganisms of a sweet-potato field and rumen fluid of a rumen-fistulated Korean cow (Hanwoo) using a modification of a sodium dodecyl sulfate (SDS)-based DNA extraction protocol (Zhou et al., 1996). A sample (10 g) was suspended in 27 ml of extraction buffer (2% [*w*/*v*] CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris–HCl [pH 8.0]) containing 100 μl of proteinase K (10 mg/ml), and shaken for 30 min at 200 rpm. Six milliliters of 10% (*w*/*v*) SDS was added, and each sample was incubated at 65 °C for 2 h with gentle inversion. Samples were centrifuged at 16,000 ×*g* for 20 min at 4 °C, and each supernatant was mixed with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1, *v*/*v*/*v*). The upper phase was recovered by centrifugation, mixed with 1 volume of isopropanol, and held at −20 °C for 1 h. A pellet was obtained by centrifugation at maximum speed (20,000 ×*g*) for 20 min at 4 °C, washed with 70% (*v*/*v*) ethanol, and resuspended in 200 μl of Tris–EDTA buffer.

Metagenomic DNA was purified using a modified single-step purification method employing Q-Sepharose to remove phenolic and humic acid contaminants (Sharma et al., 2007). The sheared DNA was end-repaired to yield 5′-phosphorylated blunt ends. Pulsed-field gel electrophoresis (PFGE) using 1% (*w*/*v*) low melting-point agarose was employed to size-select end-repaired DNA. PFGE was performed at 6 V/cm for 16 h using a CHEF-DR II clamped homogeneous electrical field system (Bio-Rad Laboratories, Hercules, CA, USA), and DNA fragments approximately 30–40 kb in size were isolated using the GELase system (Epicentre Technologies, Madison, WI, USA). A fosmid library of isolated DNA was constructed using the Copy Control Fosmid Library Production Kit (Epicentre Technologies). After PFGE, size-resolved DNA (30–40 kb) was ligated into the pCC1FOS fosmid vector using Fast-Link DNA ligase. Lambda packaging extracts were added to ligation mixtures and used to infect phage EPI300-T1^R. Packaged fosmid clones were stored at 4 °C.

2.3. Optimal conditions for screening a metagenomic library for exocellulase using HTS

Escherichia coli XL1-Blue strain pHSGC16 containing the exocellulase gene *celEdx16* cloned into the pHSG298 vector using a shotgun method (Ko et al., 2011) was used as positive control for optimizing conditions for screening exocellulase from a metagenomic library using the HTS system. Strains pHSG298 (*E. coli* XL1-Blue containing empty pHSG298 vector) and pCC1FOS (*E. coli* EPI300 containing empty pCC1FOS fosmid) were used as negative controls. The optimal concentration of $MeUmbG_2$ substrate for the HTS system was determined by testing concentrations ranging from 0 to 100 μM. Experimental conditions were also varied to establish the simplest possible HTS system.

2.4. Screening a metagenomic library for exocellulase using a robotic HTS system

The robotic HTS system (Fig. 1A) comprises the following elements: (1) a robot manipulator system, consisting mainly of a six-axis Thermo CRS F3 robot manipulator (Thermo Electron co., Franklin, MA, USA), a robot controller and a computer, all at the remote robot site; (2) plate and lid handling, performed by the CRS F3 robot; (3) liquid handling, performed by a Janus Liquid Handler (Perkin Elmer, Boston, MA, USA), which holds and distributes large amounts of liquids used during processing (e.g., culture medium, substrate); (4) a carousel (Thermo CRS, Canada) for processing and storage of large numbers of plates undergoing analysis; (5) a K3 colony picker (KBiosystems, Basildon, Essex, UK), which transfers bacterial colonies to growth medium in multiwell plates. The K3 colony picker (KBiosystems) images culture plates by illuminating from beneath the plate. A computer then processes the image to locate colonies and instructs a robot arm tipped with a pin to stab the selected colonies and transfer bacteria to growth medium in a particular chamber in a 96-well plate. The pin is then sterilized in ethanol or peroxide and with heat, and the cycle begins is repeated; (6) a Liconic STX40 Automated Incubator (Woburn, MA, USA), which supports a wide temperature and humidity range; and (7) a 1420 VICTOR multilabel counter (PerkinElmer Life Sciences, Wallac Finland Oy, Turku, Finland) for measuring fluorescence and detecting positive samples ("hits").

The strategy and operation protocol for screening various metagenomic resources for novel exocellulases using our novel robotic HTS system is shown in Fig. 1B. To screen for cellulolytic hydrolases, we spread the metagenomic fosmid library onto Luria-Bertani (LB) agar plates containing 12.5 μg/μl chloramphenicol. All liquid-handling steps were performed using a Janus Automated Workstation (Perkin Elmer, Boston, MA). LB media containing 10 μM $MeUmbG_2$, arabinose, and 12.5 μg/μl chloramphenicol were dispensed into 96-well plates using the Janus Liquid Handler (Perkin Elmer, Boston, MA). Cell growth was monitored for 12–14 h at 37 °C with agitation in a Liconic STX40 Automated Incubator. Exocellulase activity of the metagenomic library was assayed by measuring the release of the methylumbelliferyl (MeUmb) group upon incubation of an aliquot of enzyme with $MeUmbG_2$ in the HTS system. The reaction in the 100 μl culture broth was terminated by addition of 100 μl of 500 mM glycine buffer (pH 10.4). Fluorescence of MeUmb groups released from $MeUmbG_2$ was determined using a 1420 VICTOR multilabel counter ($\lambda_{excitation}$ =365 nm, $\lambda_{emission} \geq$ 460 nm) with integrated stacker and barcode reader and analyzed using Workout software (Perkin Elmer, Waltham, MA).

2.5. Shotgun cloning of hit clones for exocellulase activity and sequencing analyses

Fosmids from clones yielding positive values with 10 μM $MeUmbG_2$ were purified using an alkaline lysis method (Birnboim and Doly, 1979). Clones isolated from the metagenomic library exhibiting exocellulase