Journal of Industrial Microbiology & Biotechnology (2019) 46:1603–1610
https://doi.org/10.1007/s10295-019-02221-2

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BIOTECHNOLOGY METHODS - ORIGINAL PAPER

Ultra-high-throughput picoliter-droplet microfluidics screening of the industrial cellulase-producing filamentous fungus *Trichoderma reesei*

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Received: 10 May 2019 / Accepted: 16 July 2019 / Published online: 2 August 2019

Abstract

The selection of improved producers among the huge number of variants in mutant libraries is a key issue in filamentous fungi of industrial biotechnology. Here, we developed a droplet-based microfluidic high-throughput screening platform for selection of high-cellulase producers from filamentous fungus *Trichoderma reesei*. The screening system used a fluorogenic assay to measure amount of cellulase and its activity. The key effectors such as cellulase-inducing medium, spore germination, droplet cultivation time, droplet fluorescence signal detection, and droplet cell sorting were studied. An artificial pre-mixed library of high- and low-cellulase-producing *T. reesei* strains was screened successfully to verify the feasibility of our method. Finally, two cellulase hyperproducers exhibiting improvements in cellulase activity of 27% and 46% were isolated from an atmospheric and room-temperature plasma (ARTP)-mutated library. This high-throughput screening system could be applied to the engineering of *T. reesei* strains and other industrially valuable protein-producing filamentous fungi.

Keywords Filamentous fungi · Cellulase · High-throughput screening · Droplet microfluidics

Introduction

Lignocellulosic biomass is the most abundant renewable source for the production of biofuels. However, the development of an inexpensive method to convert lignocellulose into soluble sugars remains the primary bottleneck associated with second-generation bioenergy production [31]. In this process, the high cost of enzymatic lignocellulose saccharification is the major limiting factor in the bioconversion of lignocellulosic biomass. Here, cellulase undoubtedly plays a vital role in the conversion of cellulose to fermentable monosaccharide [7].

Currently, most commercial cellulolytic enzymes are produced by ascomycete fungi, such as *Trichoderma*, *Penicillium*, and *Aspergillus* species [10]. Of all ascomycete fungal species that produce cellulolytic enzymes, *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is the most commonly used in the production and preparation of commercial cellulolytic enzymes [24]. In the past 4 decades, enormous efforts have been made towards improving cellulase production in *T. reesei*, with an overall goal of cost reduction. Strain modification has been implemented as one of the main strategies for improving cellulase production from *T. reesei* [27]. Notably, strain-specific characteristics are essential to

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10295-019-02221-2) contains supplementary material, which is available to authorized users.

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any by-product derived from a microorganism. Therefore, the success of industrial biotechnology depends on improvements to the production capacities of microbial strains.

To date, both genetic engineering and random mutagenesis have been used to modify *T. reesei* and thus improve the cellulase productivity. The application of classic mutagenesis methods has yielded remarkable improvements in *T. reesei* and led to the considerable modification of industrial strains [13, 21, 22]. Still, these classical random mutagenesis methods are limited by various factors, including a time-consuming and laborious nature. The publication of the *T. reesei* genome sequence and the availability of state-of-the-art gene manipulation methods have enabled the introduction of molecular genetic engineering methods into microbial improvement programs [18]. For *T. reesei*, genetic engineering strategies mainly involve manipulating the expression of key regulatory genes [28, 36] and multiple host genes (e.g., enhanced expression of positive transcriptional activators and decreased expression of transcriptional repressors) [32] and promoting the over-expression of genes encoding cellulases [37] at transcriptional repressor loci [20]. Regardless of the methods used, isolation of the desired mutants from a mutant library requires an efficient high-throughput screening strategy. However, the construction of such a system for *T. reesei* is challenging, given the filamentous nature of this fungus and the fact that cellulase is secreted extracellularly.

Fluorescence-activated cell sorting (FACS) is a high-throughput method for cell sorting and analysis. However, FACS requires fluorescent marker(s) which must remain either within or on the surface of the cells to be sorted. Recently, a new high-throughput screening method that couples FACS with red fluorescence protein DsRed expression was established for the isolation of cellulase-hyperproducing *T. reesei* strains. Host strains expressing *DsRed* under a control of the major cellulase *cbh1* promoter both intracellularly and on the surfaces of growing hyphae should first be constructed and subjected to further studies [8]. Therefore, a complex genetic manipulation was required to couple cellulase production with the fluorescence protein DsRed. In contrast, droplet-based microfluidic systems can encapsulate non-genetic fluorescence labeled single cells and fluorogenic assay reactions in droplets with picoliter- to nanoliter-scale volumes. The amount of cellulase and its activity of cell were represented by fluorescence signal from the fluorogenic assay as previous reported [15]. These cells with improved fluorescence signal can be isolated using fluorescence-activated dielectrophoresis or by fluorescence-activated electrocoalescence with an aqueous stream [23] The droplet microfluidic technologies developed in recent years have enabled screening capabilities in the range of millions per day and have been widely used in strain modification and screening applications [4, 25, 29]. Compared with flow cytometry, the greatest advantage of droplet microfluidics is the potential ability to screen secreted products [1]. This advantage could enable the screening of cellulase-hyperproducing strains of *T. reesei*.

In this study, a novel high-throughput screening approach for cellulase-producing *T. reesei* strains was proposed and established using a droplet-based microfluidic platform. We explored the single-spore droplet preparation conditions, adjusted the spore germination rate and enzyme activity in the droplets, controlled the droplet size, and optimized the detection and sorting parameters to establish a screening method for cellulase-producing filamentous fungi with a throughput rate of 10,000 strains per hour. We further demonstrated the ability of our newly designed platform to successfully screen both pre-mixed and random mutagenesis libraries of *T. reesei*. We expect that this high-throughput screening system could be used to improve the efficiency of strain engineering protocols for *T. reesei* and other filamentous fungi that produce industrially valuable proteins.

Materials and methods

Strains, media, and cultivation conditions

T. reesei DES-15 (China General Microbiological Culture Collection Center; CGMCC 9644) [12] and *T. reesei ΔTrhfb3-cp* [11] were used as high- and low-cellulase production reference strains in the study. For temporary conservation, all strains were maintained on potato dextrose agar (PDA) plates at 4 °C. For strain recovery, mycelia agar disks were inoculated on fresh PDA and cultivated at 30 °C for 7 days until conidia formed.

The cellulase-inducing culture medium in water–oil droplets contained 0.2% lactose, 0.1% polypeptone, 0.06% $MgSO_4$, 0.5% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, and 0.1% trace elements at a pH of 4.8 (adjusted using 2 M H_2SO_4). For cellulase production via shake-flask fermentation, 250-mL flasks containing 30 mL of fermentation medium were inoculated with 1 mL of spore suspension (10^6 spores/mL). The fermentation medium included 3.3% avicel, 1.7% corn-steeping liquor, 0.5% $(NH_4)_2SO_4$, 0.6% KH_2PO_4, 0.1% $MgSO_4{\cdot}7H_2O$, 0.25% $CaCO_3$, 0.25% glycerol, and 0.1% Tween-80. The initial pH of the fermentation medium was adjusted to 5.5 using 2 M H_2SO_4. The flasks were incubated in a rotary shaker (200 rpm) at 26 °C for 120 h.

Cellulase activity assay

The filter paper activity (FPA) is represented by the filter paper unit and was measured as described by Ghose [9]. Cellobiohydrolase levels were measured at $Ab_{420\ nm}$ using *p*-nitrophenyl-β-D-cellobioside as the substrate for a rapid detection [33]. One unit of cellobiohydrolase activity was

defined as the amount of enzyme required to produce 1 μmol of product per min under the assay conditions. The fluorogenic assay of cellulase activity was performed in droplet using a fluorogenic substrate fluorescein-di-β-cellobioside (Gering, China) and detected at ex488 nm/em520 nm [15].

Spore germination

The spore germination rates were examined to accurately determine the appropriate incubation time prior to microfluidic analysis and sorting. First, the spores were collected and adjusted to a density of 10^5 spores/mL in liquid cellulase-inducing medium. Next, 10 μL of spore suspension was placed on glass slides and incubated in a humid chamber at 30 °C. The germination rates of at least 300 spores were examined every 2 h under a microscope (DM5000B, Leica, Germany). To induce spore germination, the generated single-spore droplets were cultured at 30 °C for desired period. Spore germination in the droplets was observed microscopically by pipetting 10 μL of droplets into a Countess TM cell counting chamber (ThermoFisher Scientific, USA) at different time points.

Droplet generation and microfluidic screening

Droplets were produced using a surfactant-containing fluorinated oil [14] as the oil phase and a suspension of 10^6 spores/mL in media supplemented with 40 μM cellulase fluorogenic substrate fluorescein-di-β-cellobioside as the aqueous phase. The flow rates were 650 and 1000 μL/h for the aqueous and oil phases, respectively. The uniform droplets were generated using the microfluidic drop-making device (kindly provided by Harvard University) at a speed of hundred–thousand per hour with a diameter of 100 μm (Supplementary Movie 1). Droplets were then collected into 1-mL syringes (BD Biosciences, USA) and moved to 30 °C culture incubator for desired period.

After incubation, the droplets were reinjected into the microfluidic sorting device (kindly provided by Harvard University) at optimized flow rates of 20 μL/h and 1000 μL/h for droplets and spacing oil (HFE7500, 3M, Minnesota, USA), respectively. In the sorting device, the droplets were excited with a laser, and the resulting florescence signals were detected by the droplet microfluidic system constructed in the previous study [29]. Droplets exhibiting high levels of fluorescence, which suggested high-cellulase activity, were sorted for subsequent experimental verification.

Verification of mutants by PCR

The sorted colonies were verified through PCR assays. The sorted colonies were inoculated into liquid PDA media and cultivated at 30 °C for 48 h. Then, the cultivated mycelium was collected for genomic DNA isolation. Genomic DNA was extracted from mycelia using fungi DNA isolation kit (Tiangen, China) following the manufacturer's manual. PCR assays were performed in an Eppendorf Master Cycler PCR System (Eppendorf, Germany) according to the manufacturer's instructions. Each reaction mixture contained 1 μL genomic DNA (50 ng/μL), 10 μL mix (Vazyme, China), 0.2 μL each PCR forward and reverse primers (10 μM), and nuclease-free water to a final volume of 20 μL. The PCR protocol was as follows: 30 s of initial denaturation at 95 °C, 30 s of annealing at 55 °C, and 30 s of extension at 68 °C for 25 cycles. Actin primers [11] or hygromycin primers (hph-F: 5′-CGTTGCAAGACCTGCCTGAA-3′; hph-R: 5′-CTCCATACAAGCCAACCACGG-3′) were used to verify the sorted colonies.

ARTP mutation

Atmospheric and room-temperature plasma (ARTP) mutations were induced according to the following procedure. Spores from 7-day-old fungal culture plates were collected using sterilized distilled–deionized H_2O and filtered through three-layer lens paper. The resulting spore suspension was diluted to a density of 10^7 spores/mL. Next, 10 μL of each diluted spore suspension was spread on a sterilized steel dish and exposed to a helium gas flow for 90 s at a distance of 2 mm from a plasma reactor and then transferred into 1 mL of distilled water. The ARTP mutation procedure was repeated ten times until a total spore suspension volume of 100 μL was treated, and all treatments were eluted altogether into 1 mL of distilled water. Following ARTP treatment, the spores were pre-cultured at 30 °C for 6 h prior to droplet encapsulation.

Results and discussion

Detection of enzymatic activities during initial cultivation stages

The stability and fluorescence signal of the droplets are the key to establish droplet-based microfluidic screening. In *T. reesei* cultures, cellulase activity is generally detectable after 24 h cultivation in insoluble liquid medium [33], at which time hyphae from the germinated spores might exit the encapsulated droplets. The long induction time required for enzyme secretion conflicts with the rapid growth of filamentous fungal hyphae in picoliter- or nanoliter-range droplets. Therefore, we developed a culture medium that induced enough cellulase production during the very early stage of cell cultivation at which time the hyphae is shorter. Avicel is commonly used to induce protein production in *T. reesei* [35]. However, it is insoluble and may block the channel of

microfluidic devices. Therefore, it could not be uniformly encapsulated in microfluidic droplets. Soluble inducers for cellulase, namely cellulose-generated disaccharides such as sophorose, cellobiose, L-sorbose, and gentiobiose, are suitable to microfluidic system, but they are expensive in industrial application. In contrast, lactose is the best cost-effective carbon source among the soluble inducers to induce cellulase expression in *T. reesei* [2]. However, compared with cellulose, lactose is associated with lower cellulase yields and initial cellulase-producing rates. Therefore, the cellulase activities in lactose-based medium during the very early cultivation period need to be evaluated. Additives such as Tween-80 were also reported to improve cellulase productivity in *Trichoderma* [34]. Thus, we added Tween-80 to the cellulase induction medium to accelerate cellulase production during the very early cultivation stage. As shown in Fig. 1, cellobiohydrolase activities were detectable at the 12-h cultivation time point when the cellulase-inducing medium only had 0.1% or 0.2% lactose as the carbon source. The cellobiohydrolase activities were further increased when additional Tween-80 was added to the medium. Notably, the cellobiohydrolase activities induced by 0.2% lactose and 0.1% Tween-80 were at least 43% higher than by other media at 16 h cultivation time point. These results indicate that a modified cellulase induction medium might be used to induce enough cellulase production during very early cultivation time for picoliter-droplet-based microfluidic detection and screening.

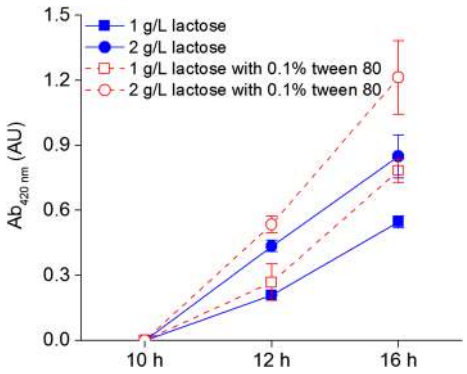

Fig. 1 Detection of cellobiohydrolase activities at the very early cultivation stage. Cellobiohydrolase activities were measured using *p*-nitrophenyl-β-D-cellobioside as the substrate and assayed by measuring the absorbance at 420 nm at the indicated time points. Error bars indicate SDs from three independently grown cultures

Spore germination and fluorescence signal detection in picoliter-range droplets

Filamentous fungi primarily secrete proteins at the hyphal tips [26]. During droplet cultivation, the hyphal tips grow rapidly after spore germination and puncture the droplets, leading to uncontrolled coalescence [4]. The strict incubation time presents a major limitation on the secretion of a sufficient amount of enzyme for screening. Therefore, both spore germination and hyphal growth must be monitored before sorting to prevent hyphae blocking channels of the microfluidic device. To ensure accurate control of the spore cultivation time in the droplets, we first examined the spore germination rates in a humid chamber. Here, 10 µL of each diluted spore suspension was placed on a slide, cultivated at 30 °C, and observed microscopically at different incubation times. Many DES-15 spores began to germinate at 6 h post-incubation (hpi), and the germination rate increased to nearly 100% at 10 hpi (Fig. 2a).

Next, we observed spore germination in the droplets. Due to the instability of droplets, we found that it is very difficult to encapsulate spores into the droplets when using a modified induction medium containing the surfactant Tween-80.

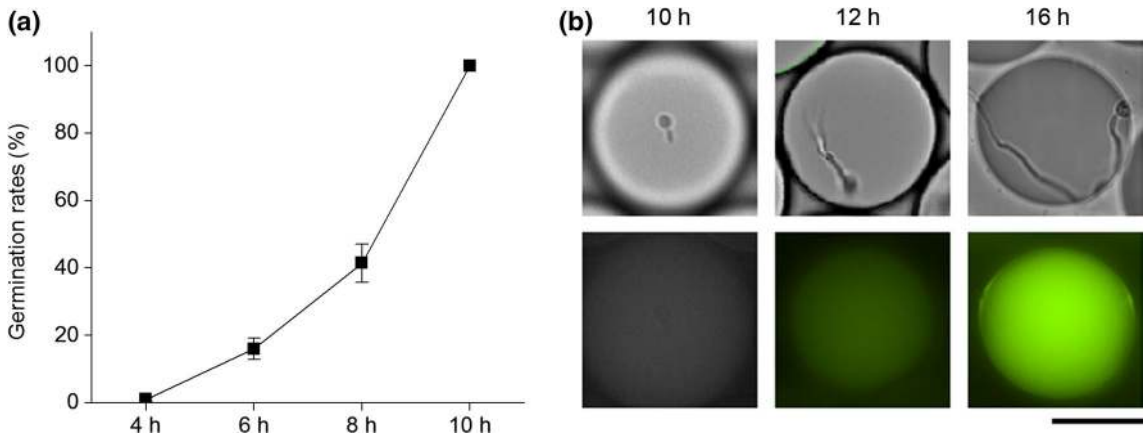

Fig. 2 Spore germination and fluorescence signal detection in picoliter-range droplets. **a** Spore germination rates in a humid chamber. **b** Fungal hyphal growth and fluorescence signal detection in droplets at different cultivation times. Scale bar, 50 µm

Surfactants diffuse in water and adsorb at interfaces between air or oil and water [30]. Therefore, the cellulase induction medium including 0.2% lactose was used for further experiments. Subsequently, spores were encapsulated in water-in-oil droplets containing modified induction medium and cultivated at 30 °C. The germination patterns of spores in the droplets were similar to those of spores germinated in a moist chamber. After 10 hpi, all spores had germinated and the hyphae continued to extend until the tips reached the edges of the droplets at 12 h. As shown in Fig. 2b, green fluorescence signals became detectable at 12 h of cultivation. An observation of a 16-h culture revealed more obvious fluorescence signals and no extension of hyphae beyond the droplets (Fig. 2b).

Studies of *T. reesei* revealed that the process from spore germination to the detection of enzymatic activity requires approximately 24 h [17]. This long incubation time conflicts with rapid hyphal growth and thus presents a challenge. In this study, the cellulase-inducing medium was optimized and cellobiohydrolase activity could be detected at very early cultivation stages. Furthermore, obvious fluorescence signals and a lack of hyphal exit from the droplets were observed at 16 h of cultivation. Taken together, these results indicate that cellulase-producing strains could feasibly be screened using a droplet-based microfluidic method.

Proof-of-concept screening of an artificial mixed library

Next, a mixed library containing the high- and low-cellulase production strains obtained in previous studies was pre-screened to verify the feasibility of this droplet-based microfluidic platform. The high-cellulase production strain DES-15 was obtained from *T. reesei* RUT C30 using diethyl sulfite mutagenesis [12] and the low-cellulase production strain *ΔTrhfb3-cp* was the complementary transformant derived from *T. reesei* RUT C30 carrying the hygromycin B phosphotransferase (*hph*) gene [11]. The *ΔTrhfb3-cp* strain had about 60% lower cellulase activity than that of DES-15 (data not shown). Besides, the *ΔTrhfb3-cp* strain has a selectable hygromycin resistance marker which is easy to validate. The proportion of DES-15 in this mixed library was less than 10%. After 12 h cultivation at 30 °C, fluorescence signals were detectable in the droplets. Subsequently, the droplets were reinjected into the sorting device and excited with a laser at the sorting junction to enable florescence signal detection. Droplets with high levels of fluorescence were steered into the sorting channel using an electrical field, while unwanted droplets were moved to the waste channel and discarded.

The sorted droplets were spread on PDA plates and recovered at 30 °C for 24 h. Next, grown colonies were picked and placed onto PDA with or without hygromycin to determine if cells were from strain *ΔTrhfb3-cp* or from strain DES-15. As shown in Fig. 3a, b, colonies that grew on PDA without hygromycin did not grow on PDA with hygromycin, indicating that these colonies were derived from DES-15 rather than *ΔTrhfb3-cp*. The colonies were further confirmed by PCR using *hph* gene primers, which were unable to amplify the *hph* gene from the sorted colonies (Fig. 3c, d). The results indicated that this high-throughput screening methods based on a droplet-based microfluidic platform could feasibly screen cellulase-producing filamentous fungi.

Library mutagenesis and screening and shake-flask fermentation assays

In the field of modern biotechnology, classical mutagenesis has been used to improve many producing strains [3]. Random mutagenesis has been proved efficient for the improvement of *T. reesei* strains [27]. The selection of improved producers among the huge number of variants in mutant libraries is a key issue. Based on the proof-of-concept screening results, we further tested the feasibility of the droplet-based microfluidic system for screening a library of random *T. reesei* mutants. A whole-genome-mutated *T. reesei* library was constructed using an ARTP mutation protocol. In our previous study, the high-cellulase producer DES-15 was obtained by DES mutagenesis using the lethality of 85% [12]. Therefore, the ARTP lethality rate was also optimized to reach approximately 85% of spores at a processing time of 90 s. After mutation, the spores were initially cultured in 1.5-mL Eppendorf tubes for 6 h, followed by droplet encapsulation and cultivation at 30 °C for 12 h. Subsequently, the droplets were examined using fluorescence microscopy and sorted through the microfluidic system at a throughput rate of approximately 10,000 variants per hour (Supplementary Movie 2). The sorted droplets containing spores (including high-yield cellulase producers) were demulsified and spread onto PDA for verification.

Forty-one colonies were picked randomly and subjected to further cellulase determination. All 41 colonies were inoculated into fermentation medium, and the enzyme activities were determined. Twenty-two of the forty-one colonies exhibited higher enzyme activity levels than the parent strain DES-15 (Fig. 4a); the remainder did not (data not shown). Of the 22 improved strains, two mutants with the highest cellulase activities, A1 and A2, were subjected to further detailed analyses. The strain A1 and A2 exhibited improvements of cellulase activity and extracellular protein when compared with the parent strain DES-15. After a 6-day cultivation, they had about 27% and 46% of cellulase activity increased than the DES-15, respectively, (Fig. 4b, c).

The results of this study suggest that a strong green fluorescent signal in the droplets at the very early stage of germination does not necessarily indicate a

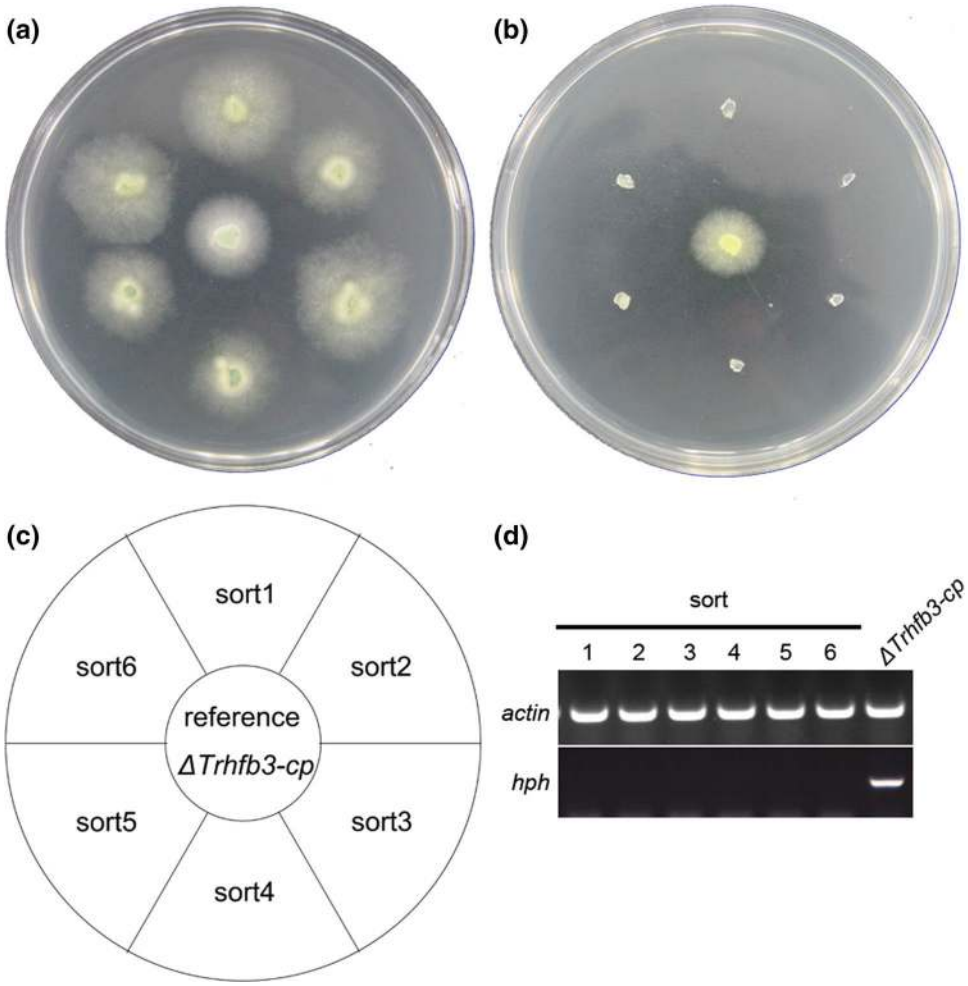

Fig. 3 Pre-screening of an artificial mixed library. **a** Growth of sorted colonies (sort1-6) and reference strain *ΔTrhfb3-cp* on potato dextrose agar (PDA) plates. **b** Growth of sorted colonies (sort1–6) and reference strain *ΔTrhfb3-cp* on PDA plates containing 100 mg/mL hygromycin. **c** Diagram of colony distribution on the plate. Sort1–6 represent isolated DES-15 colonies from the artificial mixed library. **d** PCR confirmation of the sorted colonies. Lane 1–6 represent the sorted colonies. Top, PCR using actin primers; bottom, PCR using hygromycin primers

high-cellulase-producing ability. The coupling of fungal growth, enzyme secretion, and fluorogenic substrate-derived cellobiohydrolase activity makes it difficult to reliably distinguish mutants exhibiting different levels of enzyme activity at the assay endpoint. A similar phenomenon was reported in the studies of screening strategy-coupled fluorescence signal in other filamentous fungi [4, 8]. In addition, if the differences in enzyme activities between the parent and mutant strains are small, deviations in single-droplet detection may also increase the possibility that mutant strain may exhibit a similar or lower enzyme activity than that of the parent strain after sorting. To overcome this limitation and obtain the best-performing strains, the separated variants can be re-encapsulated in droplets for several rounds of sorting. In some cases, however, the differences in enzyme activities between the parent and mutant strains are sufficiently obvious that the percentage of producing strains after sorting may reach 100%, as observed when the high-cellulase-producing strain DES-15 was sorted from an artificial library containing DES-15 and the low-cellulase-producing strain *ΔTrhfb3-cp*. Therefore, in a modern industrial biotechnology setting, this high-throughput screening method is more suitable for isolating filamentous fungal strains with high-yield improvements.

In the past 40 years, tremendous efforts have been made towards improving the traditional mutagenesis methods and isolating enhanced filamentous fungal strains capable of producing high yields of cellulase. Normally, hyperproducing strains of major cellulase-producing filamentous fungi, such as *Trichoderma* and *Penicillium*, were identified using a combination of visible halo examination and one-by-one enzymatic activity determination methods [5, 6, 16, 21, 22]. However, this random screening method is labor-intensive and time-consuming and is used for the blind selection of hundreds of mutants. The lack of high-throughput and efficient screening methods has hindered the improvement of cellulase-producing filamentous fungal strains. Our newly constructed, droplet-based, microfluidic high-throughput screening method could potentially accelerate the improvement of filamentous fungi. In the present study, we have provided a proof-of-concept of this droplet-based microfluidic method by demonstrating the ability to sort strains with a higher cellulase-producing capacity from both artificial pre-mixed and ARTP-mutated libraries at a throughput rate

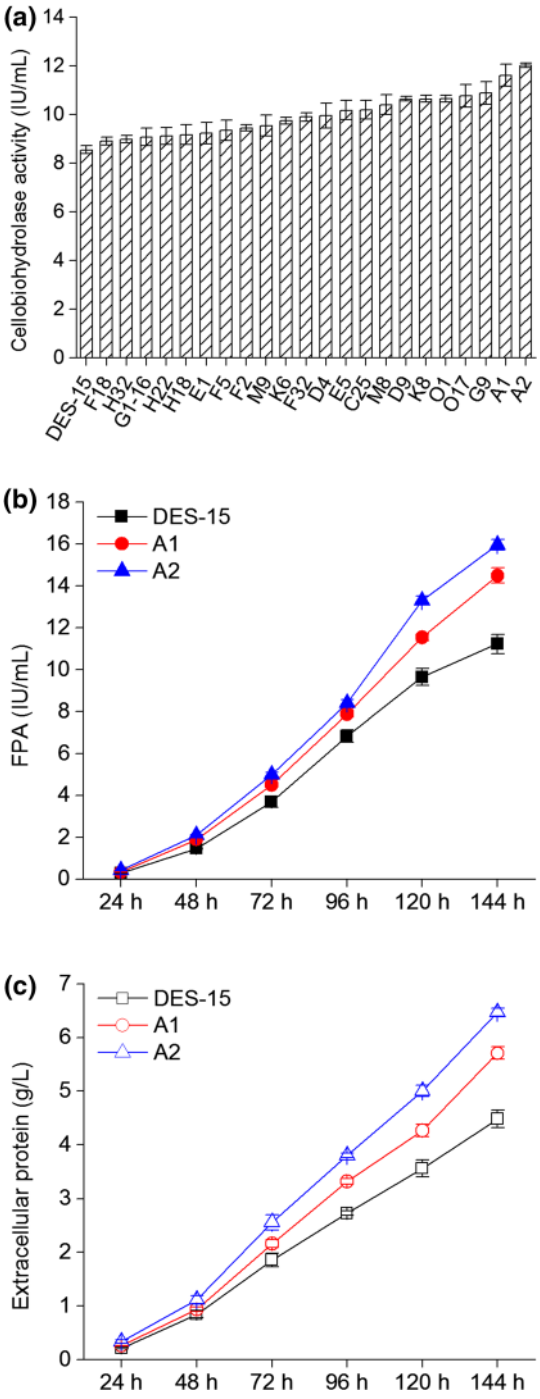

Fig. 4 Cellulase activities of the sorted mutants. **a** Cellobiohydrolase activities of mutants. **b** Filter paper activities of the parent strain DES-15 and two sorted cellulase hyperproducers, A1 and A2. **c** Extracellular protein from parent strain DES-15 and the two sorted cellulase hyperproducers, A1 and A2

of 10,000 strains per hour. The validity of this method was further confirmed through shaking-fermentation assays. The entire screening process could be reduced from weeks to hours while decreasing the costs of related reagents and consumables by a million-fold, compared to the traditional methods [19]. Compared with a recently published FACS-based method for screening *T. reesei* [8], our newly developed droplet-based microfluidic method avoids the requirement for extra gene manipulation to express exogenous fluorescent proteins either within or on the surfaces of cells prior to sorting. This change could improve the production of strains that are difficult to manipulate genetically or of industrial strains that should not be modified through genetic engineering.

Conclusions

In this study, a novel cellulase high-throughput screening approach involving a droplet-based microfluidic platform was proposed and established in *T. reesei*. Compared with the traditional screening methods, this method greatly increases the screening speed while significantly reducing labor, time, and other experimental costs. More importantly, this high-throughput screening strategy could be applied to the industrial modification of other filamentous fungal strains.

Acknowledgements We are grateful to Prof. David A. Weitz and Dr. Lloyd W. Ung (School of Engineering and Applied Science (SEAS), Harvard University) for valuable discussion on droplet microfluidic technology and the kind of providing microfluidic devices. This work was supported by the National Key R&D Program of China (2016YFD0501405) and the Science and Technology Service Network Initiative of the Chinese Academy of Sciences (KFJ-SW-STS-165). Ran Tu is supported by China Scholarship Council (201404910306) during visiting studies at Harvard University. This work was performed in part at the Center for Nanoscale Systems (CNS), a member of the National Nanotechnology Infrastructure Network (NNIN). CNS is part of Harvard University.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Abatemarco J, Sarhan MF, Wagner JM, Lin JL, Liu L, Hassouneh W, Yuan SF, Alper HS, Abate AR (2017) RNA-aptamers-in-droplets (RAPID) high-throughput screening for secretory phenotypes. Nat Commun 8:332
2. Aro N, Pakula T, Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev 29:719–739
3. Baltz RH (1986) Mutagenesis in *Streptomyces*. In: Demain AL, Soloman NA (eds) Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, DC, pp 184–190
4. Beneyton T, Wijaya IP, Postros P, Najah M, Leblond P, Couvent A, Mayot E, Griffiths AD, Drevelle A (2016) High-throughput

screening of filamentous fungi using nanoliter-range droplet-based microfluidics. Sci Rep 6:27223
5. Cheng Y, Song X, Qin Y, Qu Y (2009) Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. J Appl Microbiol 107:1837–1846
6. Dillon AJ, Bettio M, Pozzan FG, Andrighetti T, Camassola M (2011) A new *Penicillium echinulatum* strain with faster cellulase secretion obtained using hydrogen peroxide mutagenesis and screening with 2-deoxyglucose. J Appl Microbiol 111:48–53
7. Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM (2006) Ethanol can contribute to energy and environmental goals. Science 311:506–508
8. Gao F, Hao Z, Sun X, Qin L, Zhao T, Liu W, Luo H, Yao B, Su X (2018) A versatile system for fast screening and isolation of *Trichoderma reesei* cellulase hyperproducers based on DsRed and fluorescence-assisted cell sorting. Biotechnol Biofuels 11:261
9. Ghose TK (1987) Measurement of cellulase activities. Pure Appl Chem 59:257–268
10. Gusakov AV (2011) Alternatives to *Trichoderma reesei* in biofuel production. Trends Biotechnol 29:419–425
11. He R, Li C, Feng J, Zhang D (2017) A class II hydrophobin gene, *Trhfb3*, participates in fungal asexual development of *Trichoderma reesei*. FEMS Microbiol Lett 1:364
12. He R, Li C, Ma L, Zhang D, Chen S (2016) Effect of highly branched hyphal morphology on the over-production of cellulase in *Trichoderma reesei* DES-15. 3 Biotech 6:214
13. Herpoël-Gimbert I, Margeot A, Dolla A, Jan G, Mollé D, Lignon S, Mathis H, Sigoillot JC, Monot F, Asther M (2008) Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol Biofuels 23:18
14. Klein AM, Mazutis L, Akartuna I, Tallapragada N, Veres A, Li V, Peshkin L, Weitz DA, Kirschner MW (2015) Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. Cell 161:1187–1201
15. Korfer G, Pitzler C, Vojcic L, Martinez R, Schwaneberg U (2016) In vitro flow cytometry-based screening platform for cellulase engineering. Sci Rep 6:26128
16. Liu YT, Luo ZY, Long CN, Wang HD, Long MN, Hu Z (2011) Cellulase production in a new mutant strain of *Penicillium decumbens* ML-017 by solid state fermentation with rice bran. N Biotechnol 28:733–737
17. Ma LJ, Li C, Yang ZH, Jia WD, Zhang DY, Chen SL (2013) Kinetic studies on batch cultivation of *Trichoderma reesei* and application to enhance cellulase production by fed-batch fermentation. J Biotechnol 166:192–197
18. Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D, Danchin EG, Grigoriev IV, Harris P, Jackson M, Kubicek CP, Han CS, Ho I, Larrondo LF, de Leon AL, Magnuson JK, Merino S, Misra M, Nelson B, Putnam N, Robbertse B, Salamov AA, Schmoll M, Terry A, Thayer N, Westerholm-Parvinen A, Schoch CL, Yao J, Barabote R, Nelson MA, Detter C, Bruce D, Kuske CR, Xie G, Richardson P, Rokhsar DS, Lucas SM, Rubin EM, Dunn-Coleman N, Ward M, Brettin TS (2008) Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nat Biotechnol 26:553–560
19. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA (2013) Single-cell analysis and sorting using droplet-based microfluidics. Nat Protoc 8:870–891
20. Meng Q, Liu C, Zhao X, Bai F (2018) Engineering *Trichoderma reesei* Rut-C30 with the overexpression of *egl1* at the *ace1* locus to relieve repression on cellulase production and to adjust the ratio of cellulolytic enzymes for more efficient hydrolysis of lignocellulosic biomass. J Biotechnol 285:56–63
21. Montenecourt BS, Eveleigh DE (1977) Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Appl Environ Microbiol 34:777–782
22. Morikawa Y, Ohashi T, Mantani O, Okada H (1995) Cellulase induction by lactose in *Trichoderma reesei* PC-3-7. Appl Microbiol Biotechnol 44:106–111
23. Najah M, Calbrix R, Mahendra-Wijaya IP, Beneyton T, Griffiths AD, Drevelle A (2014) Droplet-based microfluidics platform for ultra-high-throughput bioprospecting of cellulolytic microorganisms. Chem Biol 21:1722–1732
24. Nevalainen H, Suominen P, Taimisto K (1994) On the safety of *Trichoderma reesei*. J Biotechnol 37:193–200
25. Obexer R, Godina A, Garrabou X, Mittl PR, Baker D, Griffiths AD, Hilvert D (2017) Emergence of a catalytic tetrad during evolution of a highly active artificial aldolase. Nat Chem 9:50–56
26. Peberdy JF (1994) Protein secretion in filamentous fungi—trying to understand a highly productive black box. Trends Biotechnol 12:50–57
27. Peterson R, Nevalainen H (2012) *Trichoderma reesei* RUT-C30—thirty years of strain improvement. Microbiology 158:58–68
28. Pucher ME, Steiger MG, Mach RL, Mach-Aigner AR (2011) A modified expression of the major hydrolase activator in *Hypocrea jecorina* (*Trichoderma reesei*) changes enzymatic catalysis of biopolymer degradation. Catal Today 10:122–128
29. Qiao Y, Zhao X, Zhu J, Tu R, Dong L, Wang L, Dong Z, Wang Q, Du W (2018) Fluorescence-activated droplet sorting of lipolytic microorganisms using a compact optical system. Lab Chip 18:190–196
30. Shea JJ (1998) Surfactants and polymers in aqueous solutions. IEEE Electric Insul Mag 14:42–43
31. Sheldon RA (2014) Green and sustainable manufacture of chemicals from biomass: state of the art. Green Chem 16:950–963
32. Wang S, Liu G, Wang J, Yu J, Huang B, Xing M (2013) Enhancing cellulase production in *Trichoderma reesei* RUT C30 through combined manipulation of activating and repressing genes. J Ind Microbiol Biotechnol 40:633–641
33. Wang M, Zhao Q, Yang J, Jiang B, Wang F, Liu K, Fang X (2013) A mitogen-activated protein kinase Tmk3 participates in high osmolarity resistance, cell wall integrity maintenance and cellulase production regulation in *Trichoderma reesei*. PLoS One 8:e72189
34. Wang Y, Yan J (2002) Effect of surfactant on cellulase production by *Trichoderma*. Biotechnology 12:37–38 **(in Chinese)**
35. Wilson DB (2009) Cellulases and biofuels. Curr Opin Biotechnol 20:295–299
36. Zhang F, Zhao X, Bai F (2018) Improvement of cellulase production in *Trichoderma reesei* Rut-C30 by overexpression of a novel regulatory gene *Trvib-1*. Bioresour Technol 247:676–683
37. Zou G, Shi S, Jiang Y, van den Brink J, de Vries RP, Chen L, Zhang J, Ma L, Wang C, Zhou Z (2012) Construction of a cellulase hyper-expression system in *Trichoderma reesei* by promoter and enzyme engineering. Microb Cell Fact 8:21