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Gel microdroplet–based high-throughput screening for directed evolution of xylanase-producing *Pichia pastoris*

Chunxuan Ma,[1,2,‡] Zheng Lin Tan,[1,2,3,4,‡] Ying Lin,[5] Shuangyan Han,[5] Xinhui Xing,[1,2,6] and Chong Zhang[1,2,6,*]

Institute of Biochemical Engineering, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China,[1] Key Laboratory for Industrial Biocatalysis of the Ministry of Education, Beijing 100084, China,[2] School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8503, Japan,[3] Laboratory of Future Interdisciplinary Research and Science Technology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8503, Japan,[4] Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Biology and Biological Engineering, South China University of Technology, Guangzhou 510006, China,[5] and Center for Synthetic and Systems Biology, Tsinghua University, Beijing 100084, China[6]

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Xylanases have useful applications in a wide range of industries. In this regard, *Pichia pastoris* has become one of the most attractive host platforms for large-scale production of xylanases. However, genomic engineering is still required for overexpression and efficient secretion. In this paper, we applied droplet-based method to screen directed evolved extracellular xylanase producing *P. pastoris* strain. Xylanase-producing *P. pastoris* cells were encapsulated in gel microdroplets with a fluorogenic reporter substrate. Improved production of xylanase increases fluorescence intensity of gel microdroplets, enabled accurate selection of evolved clones by droplet sorting. The screening strategy was validated by identifying yeast with improved xylanase production from a mixed sample with a positive selection accuracy of up to 98%. After three rounds of mutagenesis and selection, approximately 10^8 variants were screened, and a *P. pastoris* clone with more than 1.3-fold increase in xylanase activity was identified, representing cellular functions improvement of the production host. The throughput of this approach was at least 10^3-fold higher than that of the robot-assisted microtiter plate reader, and reagent consumption was reduced by $\sim 10^6$-fold. Furthermore, the greatly shortened incubation time prior screening significantly accelerated the process of directed evolution.

[**Key words:** Droplet microfluidics; Gel microdroplet; Xylanase; *Pichia pastoris*; Directed evolution]

In recent years, rising awareness about environmental pollution and concerns on the quality of downstream production have increased industrial interest in green biotechnology. The discovery of the use of xylanase in pulp bleaching by Viikari et al. (1) brought attention to the industrial application of various microbial xylanases. These enzymes degrade xylan, the second-most-abundant carbon source found in nature, and are expected to bring great economic value to the food (2), paper and pulp (3,4), and biofuel (5) industries. Recombinant DNA techniques have been employed to clone and express isolated xylanase genes in suitable hosts (e.g., *Escherichia coli*, *Saccharomyces cerevisiae*, and filamentous fungi) for xylanase production at industrial levels (6–8). Yeasts are currently the most promising expression systems because of their ability to grow to very high densities, perform eukaryotic post-translational modifications, and secrete large extracellular proteins (9–12). Among yeasts, methylotrophic *Pichia pastoris* has become one of the most commonly used hosts, owing to its ability to efficiently express and secrete recombinant proteins, and its repertoire of strong and tightly controlled promoters (13–15). *P. pastoris* is not only a stable platform for biosynthesis, but also possess the potential to significantly increase xylanase expression levels through genetic modification, further reducing costs for industrial applications (13,16).

The rapid development of systems biology has improved our ability to quantitatively assess the functions of the diverse arrays of eukaryotic cellular machinery (17,18). Strategies involving genetic and process engineering of *P. pastoris* that have improved innate xylanase expression levels include optimization of codon usage and gene dosage as well as engineering of promoters, protein secretion pathways, and methanol metabolic pathways (12,15,19–22). Nonetheless, the metabolic system of *P. pastoris* is complicated. Our limited understanding has confined our ability to genetically modify *P. pastoris* for advanced metabolic engineering (23).

On the other hand, directed evolution has provided a set of effective tools (24,25), by which various methods such as random mutagenesis and semi-rational mutagenesis, can be used to introduce genetic diversity to the entire *P. pastoris* genome as an alternative to rational design approach (26–28). This approach generates mutant libraries with diverse genotypic variants with rich phenotypic expression, yet it requires a reliable, robust,

* Corresponding author at: MOE Key Laboratory for Industrial Biocatalysis, Institute of Biochemical Engineering, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China. Tel.: +86 010 62772249; fax: +86 010 62787472.
E-mail address: chongzhang@tsinghua.edu.cn (C. Zhang).
‡ The first two authors contributed equally to this work.

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sensitive and reproducible high-throughput screening (HTS) method to efficiently explore and screen the desired mutants (29–31). Conventional xylanase activity screening methods, such as halo and microtiter plate assays, require specialized apparatus with sizeable, costly, and intricate colony pickers to compensate for their limited throughput (31–33). Fluorescence-activated droplet sorting (FADS) has been reported as a useful HTS method for enzymes evolution (34,35) and has been successfully applied for screening heterologous xylanases secreted by the yeast *Yarrowia lipolytica* (36). These studies focused primarily on improving protein structure rather than evolutionary engineering of the protein production host. Moreover, the complex aspects of system construction and lack of commercial availability have limited the widespread use of on-chip FADS (37). Therefore, a convenient and feasible HTS system is needed to identify yeast mutants with improved xylanase production capacity.

Gel microdroplet (GMD)–based HTS system, which is compatible with fluorescence-activated cell sorting (FACS), provides a robust, controllable, and flexible means of selecting cells that overproduce targeted enzymes (38,39). The immiscible boundaries of the gelified microdroplets ensure single cell along with its secreted product to be contained in individual compartments (35). GMD-based HTS system has been used for evolution of *Yersinia mollaretii* phytase (40), discovery of natural-product antibiotics (37), and function-based screening of a metagenomic library (41), but GMD has not been applied to directed evolution of xylanase-producing hosts.

Here, we present a GMD–based HTS method to screen *P. pastoris* host with improved xylanase production capability (Fig. 1). Atmospheric and room temperature plasma (ARTP) mutagenesis (42) was adopted to induce random mutations throughout the xylanase producing *P. pastoris* genome. Mutants generated were encapsulated in GMDs together with a fluorogenic substrate to couple the desired phenotype (improved production of secreted xylanase) with increased fluorescence, which enables efficient selection of the desired *P. pastoris* strains by droplet sorting. We have successfully identified several yeast clones with higher xylanase production than the parent strain, demonstrating the feasibility of the system.

MATERIALS AND METHODS

Strains and materials *P. pastoris* GS115/XynA-4 was constructed by one of us (12,43). *S. cerevisiae* strain BY4741/POT2-pGPD-sfGFP-tADH1 was produced via chemical transfection of *S. cerevisiae* BY4741 cells with plasmid POT2-pGPD-sfGFP-tADH1 developed in our laboratory. The transformed strain was grown and induced in synthetic complete medium without uracil (SC –Ura medium) to express superfolder green fluorescent protein (sfGFP).

Epoxy resin SU-8 2015 and SU-8 developer were obtained from Microchem (Westborough, MA, USA). Polydimethylsiloxane (PDMS) was obtained from Dow Corning (Midland, MI, USA). Dimethyldichlorosilane was obtained from Macklin (Shanghai, China). Xylanase from *Penicillium*, xylose, dinitrosalicylic acid solution, and Congo Red were obtained from Yuanye Biological Technology (Shanghai, China). Xylan from beechwood was obtained from HarveyBio (Beijing, China). The EnzChek Ultra Xylanase Assay kit was obtained from Invitrogen (Eugene, OR, USA). Agarose was obtained from Sigma–Aldrich (St. Louis, MO, USA). 2-(Perfluorohexyl) Ethyl Alcohol was obtained from Ark Pharm (Arlington Heights, IL, USA). Fluorinated oil (Droplet Generation Oil for EvaGreen) was obtained from Bio-Rad (Hercules, CA, USA). Other chemicals were obtained from Sinopharm Chemical (Shanghai, China) unless otherwise noted.

Fabrication of droplet microfluidic device A microfluidic device was fabricated using the soft lithography method (44). A thin film of photosensitive epoxy resin SU-8 2015 with a thickness of 14 μm was coated on a clean silicon substrate and exposed with i-line (365 nm ultraviolet) exposure (URE2000/35; Institute of Optics and Electronics, Chinese Academy of Sciences) through a chrome photomask (Changsha Heheng Optoelectronics Technology Company Ltd., Changsha, China; Fig. S1). The mold was developed and silanized with dimethyldichlorosilane. Curing agent was added to PDMS monomer (base) to a final concentration of 9.09% (w/w), degassed until all tiny bubbles were removed, and then cast on the mold. Curing was carried out by heating at 80°C. PDMS device was then peeled off the mold and the inlets and outlets were punched with a 0.5-mm puncher. The contact surfaces of the PDMS device and glass substrate were treated with air plasma using a plasma cleaner (PDC002; Harrick Plasma, Ithaca, NY, USA) before bonding.

Xylanase activity measurements in droplets with purified enzyme Xylanase from *Penicillium* was diluted in reaction buffer contained in EnzChek Ultra Xylanase Assay kit. Fluorogenic xylanase substrate (100 μg/mL) was mixed with 4% (w/v) agarose solution at 1:1 ratio. Emulsification was carried out on microfluidic device with fluorinated oil, agarose-substrate mixture, and xylanase solution each at a flow rate of 1 μL/min. All syringes were controlled by syringe pumps (Harvard Apparatus, Holliston, MA, USA) to introduce solutions into the chip inlets through polytetrafluoroethylene tubing. A heat blower set at 50°C was used to keep the agarose in liquid form throughout GMD generation process (Fig. S2). The emulsion was collected in a PCR tube for 20 min, then kept in a 42°C water bath for 10 min for the xylanase reaction. The emulsion was hardened into microbeads at 4°C. The microbeads were demulsified and suspended in phosphate-buffered saline, filtered through a 50-μm-mesh filter, and analyzed on BD FACSAria SORP.

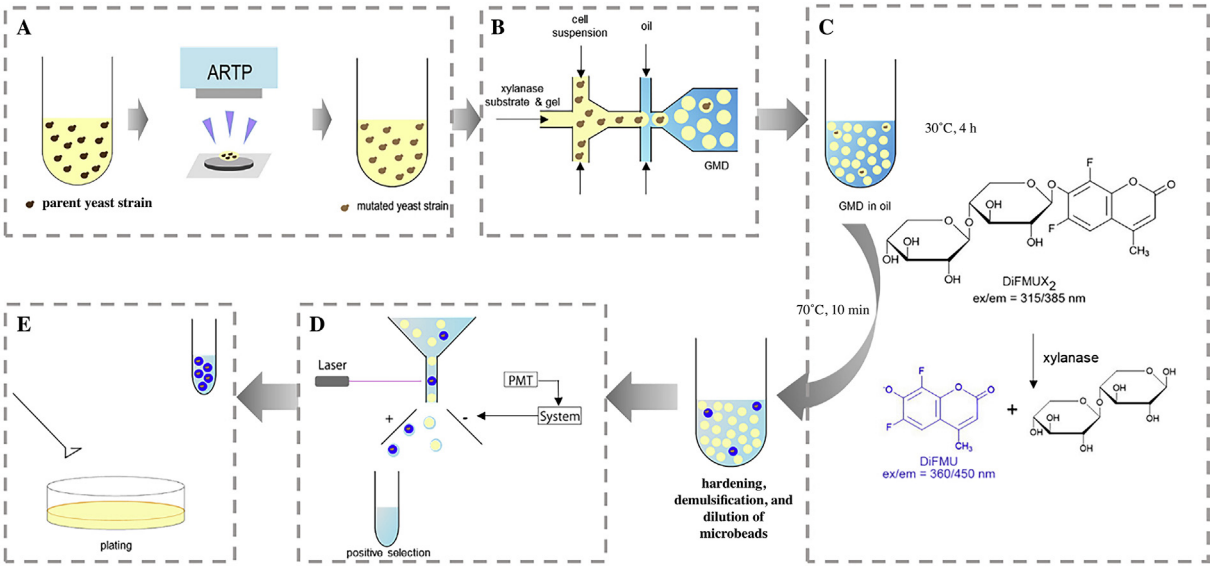

FIG. 1. Schematic workflow for gel droplet microfluidics-based screening of a *P. pastoris* library for increased production of secreted xylanase. (A) Library generation by ARTP mutagenesis. (B) Single-cell encapsulation with a fluorogenic reporter substrate and agarose solution. (C) Xylanase hydrolyzes the substrate sealed in the droplets, breaking the fluorogenic molecule free from the xylobiose. (D) Droplet sorting using FACS. (E) Validation of xylanase production capacity of sorted strains.

Preparation of mutant yeast library All mutant strains used in this study were generated from *P. pastoris* GS115/XynA-4 by ARTP mutagenesis using the ARTP mutation breeding system (ARTP-IIs; Tmaxtree Biotechnology Co. Ltd., China). A colony of *P. pastoris* GS115/XynA-4 parent strain was inoculated in BMGY culture medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, and 1% glycerol) in a conical flask and incubated at 30°C and 220 rpm for more than 24 h. Then, 10 μL of cell suspension was coated evenly on a small round sterilized metal sheet and exposed to ARTP for 180 s, with an output power of 120 W and helium flow rate of 1.667×10^{-4} m^3/s. Random mutations throughout the genome were induced by ARTP. Treated cells were transferred to Eppendorf tubes containing 1 mL of BMGY medium and grown overnight at 30°C and 220 rpm before encapsulation in GMDs (Fig. 1A).

Screening for increased xylanase production Suspensions of mutated yeast were washed five times by centrifuging (Kubota 3700; AF-5004CA rotors, Kubota Corporation, Tokyo, Japan) and exchanging with fresh BMGY medium. They were sonicated for 3×20 s with 5-s rest intervals, then resuspended in fresh BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, and 4×10^{-5}% biotin) with 1% (v/v) methanol. The yeast suspensions were diluted to 10^6 cells/mL to maintain the cell-to-droplet ratio at 0.1 (45) and transferred to a syringe. Fluorinated oil and the 1:1 mixture of agarose and fluorogenic substrate (refer to the Xylanase activity measurements in droplets with purified enzyme section) were extracted into syringes and injected intomicrofluidic device. The flow rate of cell suspension, fluorinated oil, and agarose solution was set to 1 μL/min (Fig. 1B). The emulsion generated was collected in a PCR tube for 30 min and incubated at 30°C for 4 h before being transferred to a 70°C water bath to react for 10 min (Fig. 1C). Subsequently, the emulsions were hardened into microbeads at 4°C. The microbeads were demulsified and suspended in phosphate-buffered saline, passed through a 50-μm-mesh filter, and sorted using BD FACS Aria SORP with AccuDrop technology and a Sweet Spot system (Fig. 1D). Calibration was performed relative to the parent strain. Sorted yeast cells were recovered by spreading on xylan-BMMY agar plates containing 1% (w/v) xylan and 1% (w/v) methanol and incubating at 30°C until colonies formed (Fig. 1E).

Xylanase activity assays in selected yeast clones Approximately 10 individual clones were picked from each xylan plate, and xylanase activity was analyzed by flask fermentation using the parent strain as a control. Seed cultures were prepared in 50-mL conical flasks containing 5 mL of BMGY medium and incubated at 30°C and 220 rpm for more than 24 h. The suspensions were centrifuged at 7809 rpm for 5 min, the supernatants were removed, and the pellets were transferred to 25 mL of BMMY medium in 250-mL conical flasks for flask fermentation at 30°C and 220 rpm agitation. Every 24 h, 0.5-mL samples were collected, the OD_{600} was measured, and culture supernatants were collected by centrifugation at 9017 rpm for 10 min at 4°C. The xylanase activity of the supernatants was measured using the dinitrosalicylic acid reduction method. Methanol was added at a final concentration of 1% (v/v) every 24 h for 4 days. Appropriately diluted crude enzyme (supernatant) was added to 1% (w/v) beechwood xylan dissolved in glycine/NaOH buffer (pH 9.0) and incubated at 70°C for 30 min, after which the reaction was stopped by adding dinitrosalicylic acid reagent (12,43). All analytical measurements were performed at least in triplicate. Clones exhibiting the highest secreted xylanase activity were selected for the next round of ARTP mutagenesis, single-cell encapsulation, and screening.

RESULTS

Xylanase activity assay Our screen was designed to assay xylanase activity using fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl β-D-xylobiose ($DiFMUX_2$) contained in the EnzChek Ultra Xylanase Assay kit (46). When xylanase hydrolyzes $DiFMUX_2$, the fluorophore DiFMU is released from the xylobiose moiety (Fig. 1C) and the fluorescence intensity increases. Although $DiFMUX_2$ offers a broad working pH range (pH 4–10) and excellent temperature tolerance, a more specific understanding of its properties was required. Thus, the dynamic range, sensitivity, and resolution of the enzyme assay were tested in agarose microdroplets containing fluorogenic substrate and a concentration range of purified xylanase from *Penicillium* (Figs. 2B and S3B). It was found that the fluorescence of droplets correlated with xylanase activity. When the results obtained in microdroplets were compared with those in 96-well microplates (Figs. 2A and S3A), the high consistency further validated the positive correlation between fluorescence and enzyme concentration. Furthermore, the xylanase-producing *P. pastoris* strain GS115/XynA-4 was induced for various times in 96-well microplates (Fig. S4) and droplets (Fig. 2C) to find the optimal induction time for sorting. It was found that the fluorescence of droplets correlated with induction time and that a 2-fold increase

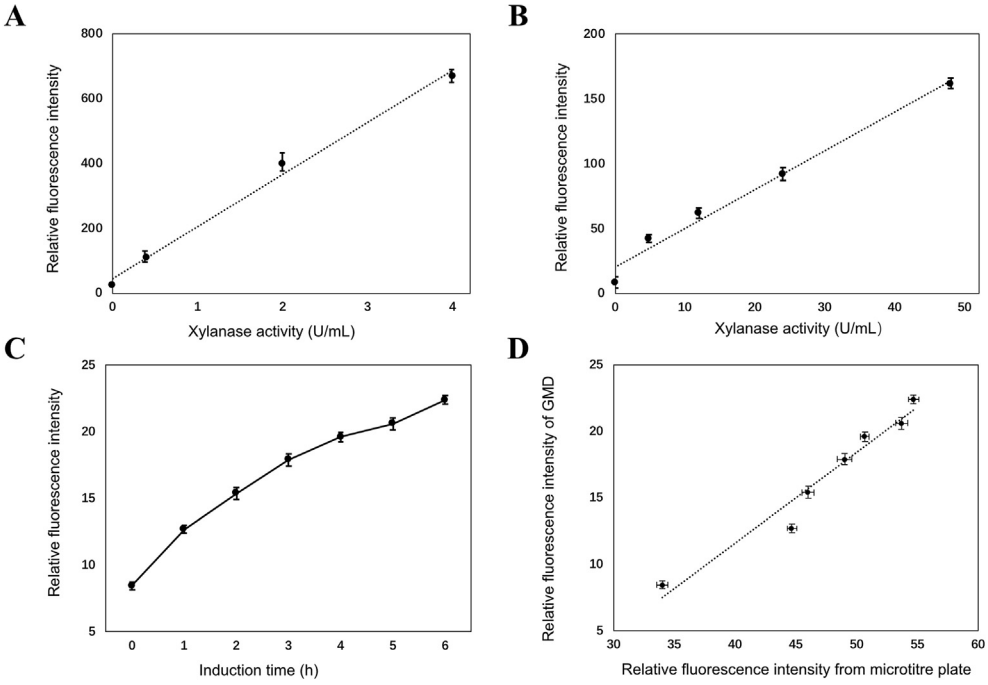

FIG. 2. (A) Various concentrations of xylanase were combined with fluorogenic substrate in 96-well microplates, and fluorescence was followed over time using a microplate reader. Using data at 60 min of incubation, fluorescence was correlated with enzyme concentration. (B) Droplets were generated containing fluorogenic substrate and different concentrations of xylanase, with subsequent FACS. The mean droplet fluorescence was correlated with xylanase concentration. (C) Droplets were generated encapsulating single cells of GS115/XynA-4 with xylanase substrate, with subsequent incubation for 0–6 h. By 4 h, there was a more than 2-fold increase in mean droplet fluorescence. (D) Fluorescence generated at various incubation times was also evaluated in 96-well microplates, which showed a high correlation with the microdroplet results. All experiments were conducted in triplicate. Error bars indicate standard deviation.

in fluorescence intensity could be resolved after a 4-h induction. This confirmed that 4-h induction could be adopted to ensure the activity of secreted xylanase in droplets was within the dynamic range of enzyme assay and the corresponding change in fluorescence intensity was sufficient for subsequent sorting.

Model selection for xylanase expression To validate the directed evolution workflow, microdroplets encapsulating xylanase-producing *P. pastoris* GS115/XynA-4 were mixed at different ratios with microdroplets encapsulating the non-xylanase-producing *S. cerevisiae* strains BY4741 and BY4741/POT2-pGPD-sfGFP-tADH1 (Fig. 3A). A cell-to-droplet ratio of 0.1 was used to achieve predominately single-cell encapsulation based on a Poisson distribution (45). Cells from each strain were processed and ultrasonicated to prevent clustering and then encapsulated in droplets with a fluorogenic substrate and agarose solution. The emulsions were incubated off-chip for 4 h at 30°C to allow xylanase induction, then at 70°C for 10 min to activate enzyme to hydrolyze the substrate. The temperature elevation did not have a significant effect on the shape of agarose GMDs and the distribution of fluorescence intensity (Fig. S5). After hardening emulsion at 4°C, the microbeads from the three strains were mixed at ratio of approximately 1:1:1 prior to FACS. As shown in Fig. 3, the green fluorescence of BY4741/POT2-pGPD-sfGFP-tADH1 was used as a control for population gating, and the gated region was sorted as positive samples (Fig. 3C). The collected beads were spread on a SC−Ura agar plate and incubated at 30°C for 48 h. The survival rate of *P. pastoris* GS115/XynA-4 was estimated to be $>$ 5% (Fig. S6). On the other hand, the negative strain BY4741 cannot survive on this medium due to its autotrophy, and strain BY4741/POT2-pGPD-sfGFP-tADH1 was distinguished by green fluorescence. As shown in Fig. S7, only 1 of 43 colonies expressed sfGFP, indicating a positive selection accuracy of 98%.

The selection model was further improved by reducing the amount of target strain to 0.01% of the total cells. Sorted droplets were spread on two BMMY screening plates containing 1% (w/v) xylan and incubated at 30°C for 48 h. The Xylan-Congo red clearance plate assay was adopted to qualitatively show that the clones were xylanase-producing strains. The clear zones around all the colonies indicated that xylanase was secreted, thereby confirming the feasibility of our screening strategy (Fig. S8).

Yeast library screening for improved xylanase production Mutations were randomly introduced throughout the *P. pastoris* genome by ARTP mutagenesis, and cells with elevated xylanase production were sorted based on relative fluorescence intensity in GMDs. Approximately 10^7 droplets were sorted in the first round of screening, and the fluorescence intensity of droplets encapsulating parent strain were used as control for population gating and recovery (Figs. S9A and B). We verified that colonies from sorted fraction plates had higher xylanase production in units of xylanase activity per milliliter compared with the original library, demonstrating the effectiveness of screening based on GMD-based HTS method (Figs. S9C and D). For every round of mutagenesis, the clone that exhibited the highest xylanase activity relative to the previous round of screening was chosen for the next round of mutagenesis (Fig. 4).

Because the yield of the desired mutants in any mutagenesis strategy is limited by the probability of mutation, an enrichment strategy was employed in the next two rounds of mutagenesis and screening to ensure that improved strains could be obtained from the library. For every round, the sorted library was incubated and encapsulated for second sorting. The ratio of improved cells in the subsequent round of sorting was reliably higher compared with the control sample of current round (Fig. S10), verifying the availability of enrichment strategy. After three rounds of mutagenesis and screening, approximately 10^8 variants were screened and 60 clones were picked and analyzed for xylanase production following flask fermentation. Several *P. pastoris* strains with improved production were successfully identified (Fig. 4), and the top-performing clone was found to have more than 1.3 times the xylanase production of the parent strain (Fig. 5). The genetic stability of the top-performing mutant strain was tested. As shown in Fig. S11, after six generations, the strain still maintained its production of secreted xylanase which was $>$ 1.3 times that of the parent strain.

DISCUSSION

We propose a GMD-based HTS method to screen *P. pastoris* strains with improved xylanase production at a throughput of up to 10^8 variants per day. The method presented could have substantial industrial value, as the throughput was $>$ 10^3-fold higher and

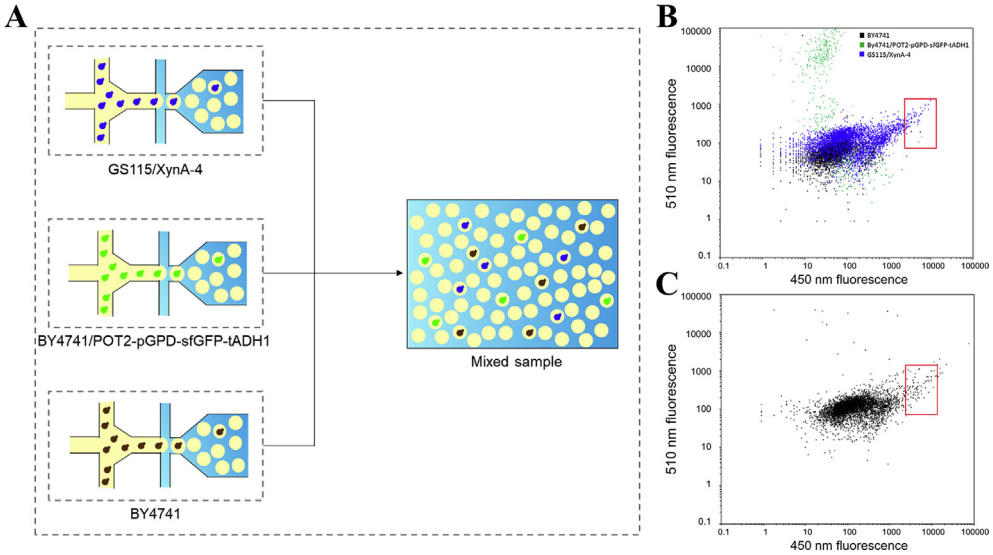

FIG. 3. (A) Screening model for validation of directed evolution workflow. (B) Flow cytometry data from analysis of three strains, differentiating blue fluorescent droplets (450 nm fluorescence; xylanase-producing strain GS115/XynA-4) from green fluorescent droplets (510 nm fluorescence; strain BY4741/POT2-pGPD-sfGFP-tADH1) and non-fluorescing droplets (strain BY4741). The gated area was identified based on the fluorescence of the two non-xylanase-producing strains. (C) Flow cytometry data from the sample mixed at a ratio of 1:1:1. The gated area was sorted as positive samples.

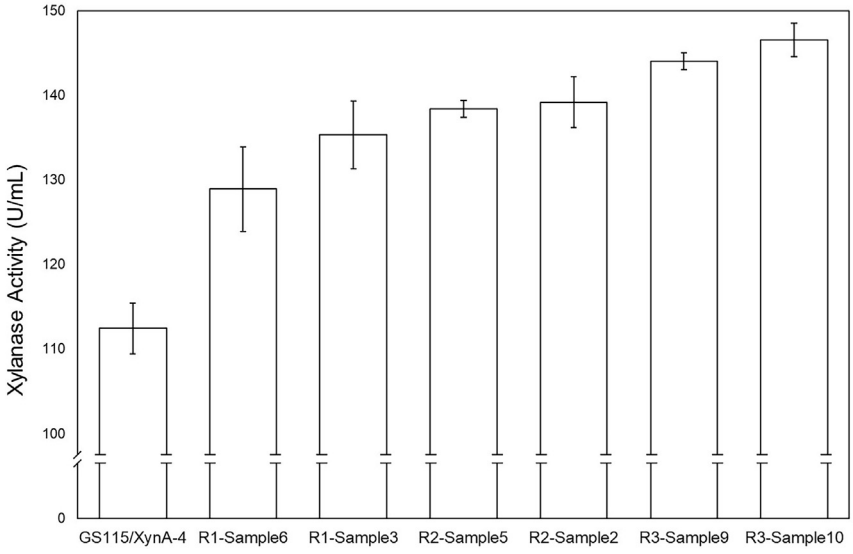

FIG. 4. Analysis of xylanase activity in the two top-performing strains from each round (R1 to R3) of mutagenesis and screening. All analytical measurements were performed in triplicate. Error bars indicate standard deviation.

reagent consumption was 10^6-fold lower compared with methods using robot-assisted microtiter plate reader (35). This method was readily applied to directed evolution, yielding a *P. pastoris* strain with 1.3-times higher xylanase production compared with the parent strain after three rounds of whole-genome mutagenesis and screening. In addition, it took 8 h to complete a round of generation, induction and sorting of approximately 10^7 mutants. The greatly shortened incubation time prior to screening significantly accelerated the process of directed evolution.

GMD-based HTS system is an attractive technology with incredible potential for single-molecule or single-cell analysis, offering flexible manipulation in a robust system (38–41,47). It is also more compatible with FACS compared with double-emulsion microdroplet-based methods. Although water-in-oil-in-water double emulsions make it possible to screen droplets using FACS (48–53), the method is limited by its throughput and complex emulsion modification. FADS has emerged as a powerful technology for HTS (32,36,54–56), but the inherent complexity of the system construction and lack of commercial availability have limited its application (37).

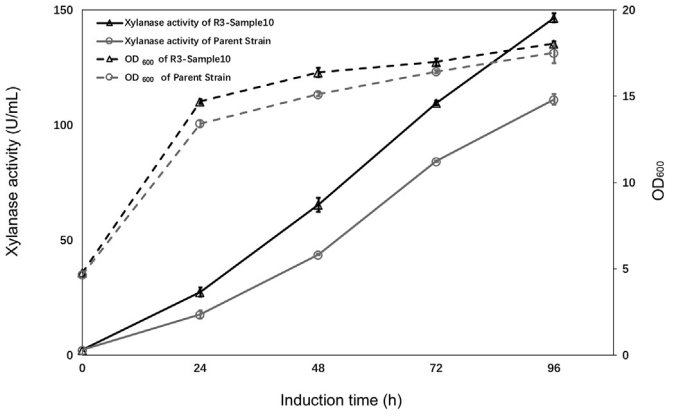

FIG. 5. After 96 h of fermentation in flasks, the top-performing clone (R3-sample 10) had > 1.3 times the xylanase production of the parent strain. For comparison, the cell density was not significantly higher. Analytical measurements were performed in triplicate. Error bars indicate standard deviation.

P. pastoris strains with improved production of secreted xylanase, as sorted from whole-genome mutant libraries, could also be used as hosts for expression of other engineered xylanase genes. In addition, whole-genome sequencing can be used to identify mutations that bring about the improved phenotype (55), and these mutations can potentially be transferred to other production hosts to improve their expression by reverse metabolic engineering to construct efficient microbial cell factories for xylanase secretion (32,57). Furthermore, the GMD-based HTS system is applicable to hosts of other secreted enzymes or even secreted metabolites as long as they can be coupled to an applicable fluorescence assay. The combination with whole-genome sequencing to map the mutations associated with the improved phenotype can also be easily adapted for other products and cell types to identify novel engineering targets, broadly facilitate design of novel microbial cell factories.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.008.

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