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Droplet-based microfluidic platform for heterogeneous enzymatic assays†

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Heterogeneous enzymatic reactions are used in many industrial processes including pulp and paper, food, and biofuel production. Industrially-relevant optimization of the enzymes used in these processes requires assaying them with insoluble substrates. However, platforms for high throughput heterogeneous assays do not exist thereby severely increasing the cost and time of enzyme optimization, or leading to the use of assays with soluble substrates for convenient, but non-ideal, optimization. We present an innovative approach to perform heterogeneous reactions in a high throughput fashion using droplet microfluidics. Droplets provide a facile platform for heterogeneous reactions as internal recirculation allows rapid mixing of insoluble substrates with soluble enzymes. Moreover, it is easy to generate hundreds or thousands of picoliter droplets in a small footprint chip allowing many parallel reactions. We validate our approach by screening combinations of cellulases with real-world insoluble substrates, and demonstrate that the chip-based screening is in excellent agreement with the conventional screening methods, while offering advantages of throughput, speed and lower reagent consumption. We believe that our approach, while demonstrated for a biofuel application, provides a generic platform for high throughput monitoring of heterogeneous reactions.

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Introduction

Many industrial processes, such as manufacturing of paper, textiles, detergents, food, pharmaceuticals and biofuels, rely on heterogeneous enzymatic catalysis involving soluble enzymes acting on insoluble substrates. A prominent example is the production of cellulosic biofuel where solid lignocellulosic biomass is hydrolyzed by chemical and enzymatic treatment to form simple sugars before they are fermented into liquid biofuel. The current capacity of biofuel production in the U.S. is 15 billion gallons per year and is projected to reach 36 billion gallons per year by 2022.¹ The key steps in cellulosic biofuel production involve the chemical deconstruction of biomass, enzymatic saccharification of the pretreated biomass, and fermentation of the resulting sugars into ethanol or other biofuels. Chemical pretreatment is achieved using dilute acids,² ammonia fiber explosion³ or, more recently, using ionic liquids (ILs).^{4–6} Ionic liquid pretreatment, the method used here, is attractive because of the IL's ability to efficiently dissolve biomass and produce a higher yield of cellulose compared to other methods.⁷ The cellulose in

pretreated biomass is further broken down into simple sugars by the synergistic hydrolytic activities of three families of cellulases (β -glucosidases, cellobiohydrolases and endoglucanases) which form the core components of an industrial cellulase cocktail. Enzymatic hydrolysis remains the most expensive step in the biofuel production and recent estimates have identified the enzyme cost (as much as \$0.68–1.47/gallon) as the single largest contributor to the high price of cellulosic biofuel.⁸ Consequently, significant efforts are made on discovering new cellulases⁹ and genetically modifying existing ones for improving the yield, reducing the time required for hydrolysis, and withstanding harsh pretreatment conditions (such as extreme pH, high temperature). Advances in genetic engineering have made it routine to express thousands of cellulase variants, but the screening of these enzymes and their combinations using insoluble biomass remains a major bottleneck. Cellulases (and other enzymes with insoluble substrates) offer a unique challenge for activity measurements, because their substrates are both insoluble and structurally variable, making it hard to develop standardized, high throughput platforms. Consequently, the screening of cellulase cocktails has been done mostly using soluble substrates, or by simple, qualitative insoluble substrate assays such as filter-paper assays.^{10,11} Assays with soluble substrates typically are not relevant industrially and filter-paper assays are too slow, low throughput and not quantitative. A few recent reports have attempted to improve the throughput of filter-

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paper assays by adapting them to microtiter plates and robotics;^{12–14} nevertheless, they remain slow and suffer from poor quantification and reproducibility. Recently, assay formats have been proposed using insoluble cellulose,¹⁵ but they are not easily adaptable to high throughput screening due to the practical issues associated with the precise and reproducible metering of small amounts of solid substrates. We developed a microtiter plate-based heterogeneous assay platform¹⁶ using *in situ* regeneration of a solid substrate to obviate the metering of solid biomass; however, the assay is manual and low throughput.

In this study, we report the development of a droplet-based microfluidic chip for carrying out hundreds of heterogeneous enzymatic reactions in parallel, requiring small amounts of reagents and capable of hands-free operation. A major challenge in performing heterogeneous reactions is to keep the reactants, especially the solid substrate, suspended and mixed. Encapsulation of the reactants in a droplet flowing through a circuitous channel, easily overcomes this challenge as internal recirculation continuously mixes the contents of the droplet. The other key advantages of the droplet-based platform include a) the ability to perform hundreds of discrete reactions in parallel and b) the significant cost savings as an exceedingly small amount of enzyme is required per reaction (the droplet volume is typically a few pL).¹⁷ Because of these features, droplet microfluidics have been used extensively for carrying out highly parallel reactions in a multitude of applications by many groups including Weitz,^{18–20} Ismagilov,^{17,21–25} Griffiths,^{18,26–28} and others.²⁹ Some of the applications include the cytotoxicity screening assay of mammalian cells,²⁹ screening reagents and conditions for the protein crystallization efficacy,²³ and the elucidation of quorum sensing mechanisms in bacteria.²⁵

Materials and methods

Microfluidic chip fabrication

The microfluidic chips were fabricated using soft lithography techniques. The master of the microfluidic channels was fabricated in SU-8 2025 photoresist (MicroChem, Inc) on a 4-inch silicon wafer. The structure of the master had a height of 25 μm , which generated a 25 μm -deep channel in the following PDMS replication step. The master was silanized with trichloro(1H,1H,2H,2H-perfluorooctyl) silane (97%, Aldrich) for 1 h. PDMS (Sylgard 184) mixed at a ratio of 10 to 1 (base to curing agent) was poured onto the master, cured at 80 °C for 1 h, and peeled away. The inlet and outlet holes were punched into the layer of channels using a Harris Uni-Core with a tip diameter of 0.75 mm. We exposed the PDMS and a glass substrate to oxygen plasma for 30 s at a RF power of 25 Watts and chamber pressure of 0.2 torr, and brought the PDMS and substrate in contact to form a permanent seal. The fusion electrodes were formed by injecting PELCO® Colloidal Silver (Ted Pella, Inc.) into the microfluidic channels. The colloidal silver liquid filled the channel and dried in ~ 1 min.

Microfluidic chip controls

The pressure-driven flow was controlled by electronic pressure regulators (Parker Life Sciences) through a Tygon® microbore tubing connection (0.01 inch inner-diameter and 0.03 inch outer diameter) with 1.5 mL pressure reservoirs, which allowed less reagent usage and more steady and responsive flow control. The pressure controllers were made in-house and were controlled by a computer *via* a LabVIEW interface. The heater for the incubation was also made in-house with a thermocouple probe for temperature monitoring.

The pressure used for controlling the IL-solubilized biomass was in the range of 2–3 psig, for the carrier oil 3–5 psig, for the aqueous phase 1–2 psig, and for the injected oil-surfactant mixture 3–5 psig. For electro-coalescence the optimal electrical field across the fusion electrodes was generated at 100–120 V and 10 kHz.

Fluorescence detection

For the microplate-based method, fluorescence was recorded using a spectrofluorophotometer (SpectraMax M2, Molecular Devices). The excitation wavelength was at 535 nm and the emission at 590 nm. For the microfluidic approach, we used an inverted Olympus IX71 microscope for fluorescence imaging of the droplets. A scientific grade CCD camera (iXon EMCCD, Andor Technology, South Windsor, CT, USA) was used to capture the images at various magnifications (1.25 $\times$, 4 $\times$, and 10 $\times$). For each droplet passing through the detector, the droplet size was first examined to ensure that proper coalescence of the droplets occurred, and then the fluorescence signal was analyzed to determine the average fluorescence of each droplet.

Ionic liquid pretreatment

We used switchgrass and microcrystalline cellulose (Avicel) as the substrates for developing the heterogeneous assay. Switchgrass was milled with a Thomas-Wiley Mini Mill fitted with a 40-mesh screen (Model 3383-L10 Arthur H. Thomas Co., Philadelphia, PA, USA). We used 1-ethyl-3-methylimidazolium acetate (EMIMAc) (Sigma-Aldrich, St. Louis, MO, USA) as the IL solvent. The switchgrass pretreatment was performed by incubating a slurry of milled switchgrass and EMIMAc in the oven (Thelco Laboratory Oven, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 160 °C for 6 h. The samples were incubated in 50 mL autoclave vials. For Avicel, the pretreatment was performed at 120 °C for 3 h. Under these conditions, both biomass substrates were completely solubilized in the IL. Three samples were prepared for both substrates at biomass loadings of 0.5% to 3% (w/v).

Enzymatic saccharification

For the microplate-based method, the enzymatic saccharification was carried out following the steps described in our previous work.¹⁶ Glucose produced was then measured using the BioVision Glucose Assay Kit. For the microfluidic method, the enzymatic saccharification and the detection of glucose were performed in one step at 37 °C for 30–60 s. An assay buffer, provided by the glucose assay kit at pH of 7.02, was used for the whole experiment. The purified enzymes for the

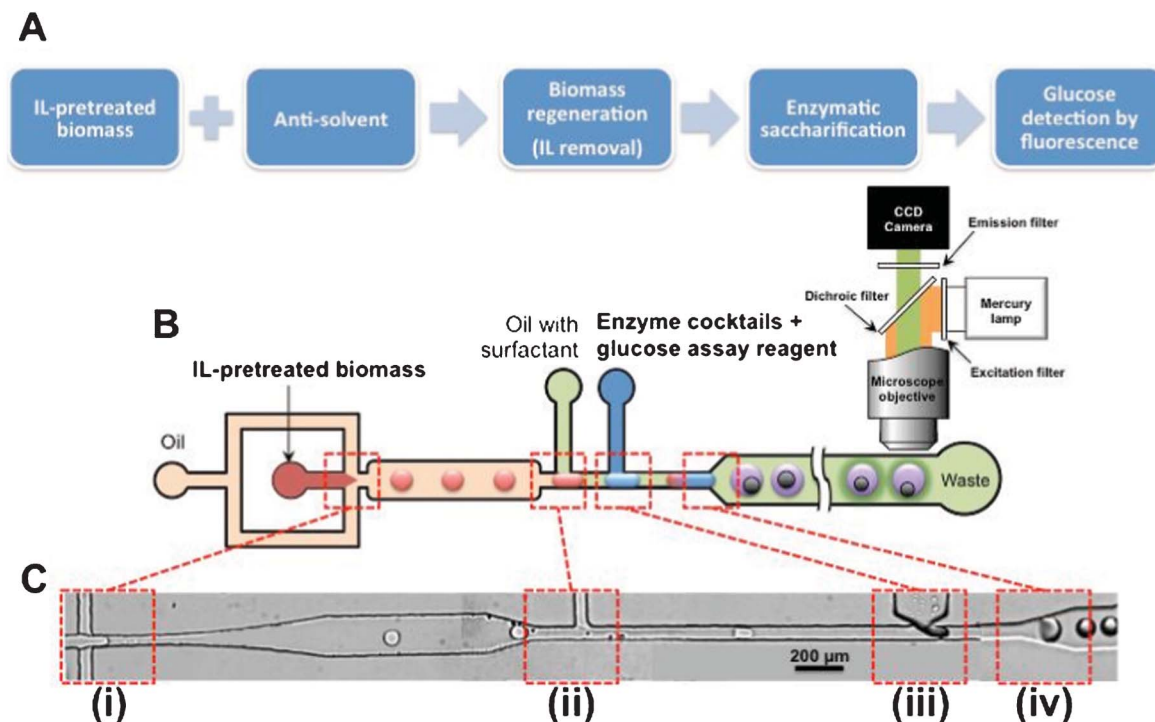


Fig. 1 (A) Workflow of the droplet-based microfluidic regeneration–saccharification approach. (B) Schematic of the droplet-based microfluidic system for high throughput heterogeneous assay. (C) Optical images showing the (i) flow-focusing IL droplet generation, (ii) surfactant injection, (iii) T-junction aqueous droplet generation, and (iv) droplet coalescing using expanded channel. Alternatively, a pair of electrocoalescence electrodes can be placed under the T-junction for enzyme mixtures droplet generation to assist droplet coalescing.

cocktail development experiments were purchased from Megazyme International Ireland Ltd. (Co. Wicklow, Ireland). The catalogue numbers for the β -glucosidase, cellobiohydrolase-I, and endoglucanase were E-BGLUC, E-CBHI, and E-CELAN, respectively.

Residual IL measurement

The residual IL amount was estimated by a conductivity measurement reported in our previous work.¹⁶ Droplets from the biomass regeneration chip were collected and then centrifuged to obtain the aqueous phase. We used a micro-scale conductivity probe (B-137 Compact Conductivity Meter, HORIBA Instruments Inc., Irvine, CA, USA) to measure the conductivity of the aqueous phase, and then obtained the IL concentration from the standard curve of electrical conductivity as a function of volume fraction of IL in water.

Results and discussion

Fig. 1A depicts the microfluidic workflow integrating the steps of biomass introduction, regeneration, enzymatic saccharification and fluorescence assay of glucose released in one microfluidic chip. It is worth noting that the microfluidic integration greatly simplifies the workflow compared to the tedious conventional method, shown in Fig. S1,[†] requiring multiple steps of washing, centrifugation, drying and metering of solid biomass. The steps in performing a heterogeneous

enzymatic assay in a droplet microfluidic chip are as follows (Fig. 1A): generation of pL-sized droplets encapsulating the IL-pretreated biomass, merging of these with the droplets encapsulating the enzymes and glucose assay reagents, incubation of the droplets for biomass regeneration and enzymatic hydrolysis, and fluorescence detection of glucose to assess the yield. A key challenge in droplet microfluidics has been the addition of reagents to existing droplets and a number of recent reports^{19,28} describe electrocoalescence methods to accomplish this. We used a slight modification of a method described by Mazutis *et al.* to merge droplets.²⁸ When a droplet containing IL-solubilized biomass merges with a droplet containing aqueous reagents, water in the aqueous droplet serves as the anti-solvent leading to the precipitation (regeneration) of cellulose in the biomass.³⁰ Controlling the volume of the IL-pretreated biomass droplets allows the precise metering of biomass, thereby improving the consistency and reproducibility. The schematic in Fig. 1B shows the detailed chip design that incorporates various microfluidic functionalities, including droplet generation (Fig. 1C-i, iii), surfactant injection (Fig. 1C-ii), droplet merging and mixing (Fig. 1C-iv), droplet incubation, and fluorescence detection. First, droplets containing the IL-pretreated biomass solution are generated in the continuous carrier flow (mineral oil) using flow-focusing geometry (Fig. 1C-i). IL-pretreated biomass is highly viscous and hence, a flow-focusing design (pinching from two sides) is adopted for better control over droplet

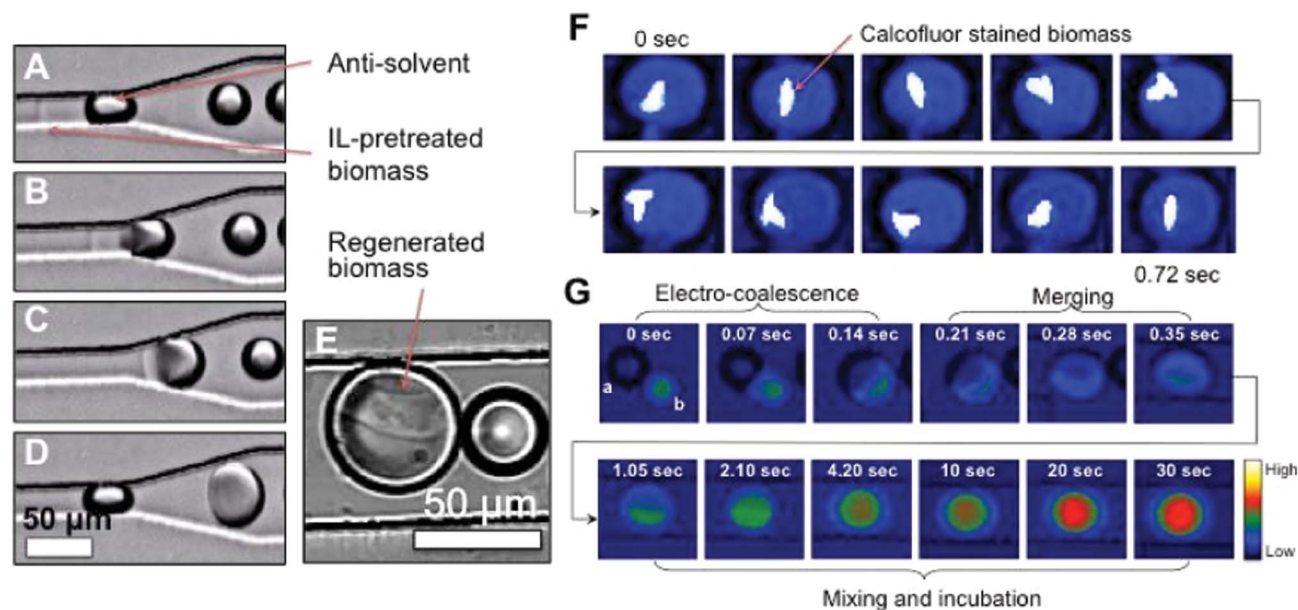


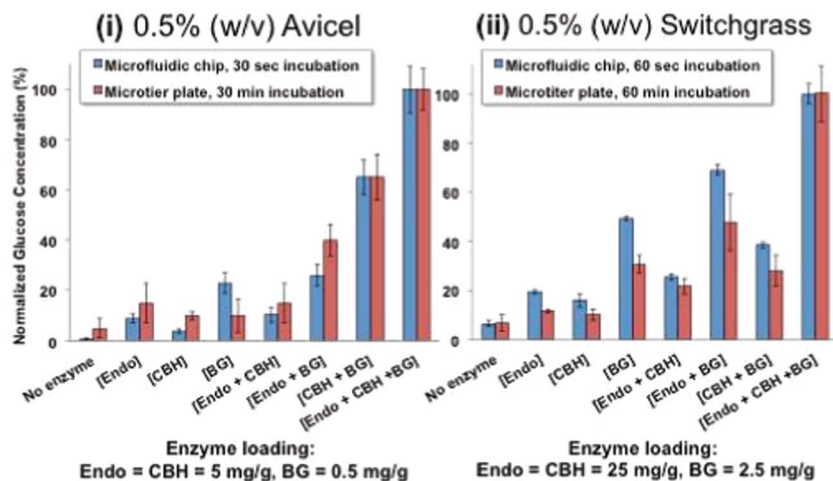
Fig. 2 (A–D) Time-lapse optical images showing the coalescence of the droplets containing the IL-pretreated biomass and anti-solvent. (E) Optical image showing the biomass regeneration inside a droplet. (F) Time-lapse fluorescence microscopy images showing the rapid chaotic advection within a droplet. The biomass is stained by calcofluor for easier visualization. The time interval between each image is 0.08 s and the droplet has a diameter of around 55 μm. (G) Time-lapse fluorescence microscopy images showing the production of glucose resulting from the enzymatic hydrolysis of carboxymethyl cellulose during the droplet coalescence, merging, mixing, and incubation (total time elapsed = 30 s). Droplet (a) contains the CMC substrate and droplet (b) contains the enzyme cocktails and glucose assay reagent mixture. The chip design for the soluble substrate is shown in Fig. 1B and C. The merged droplet has a diameter of approximately 70 μm.

generation and biomass metering. Although a surfactant is usually needed in droplet-based microfluidic systems to stabilize the droplets from coalescing, no surfactant is added in the oil phase at this stage. This is because the oil with surfactant will dissolve the IL-pretreated biomass droplets, causing them to wet and adhere to the channel wall. While we mitigated the problem of IL partitioning into the oil phase by removal of surfactant in the first step, it is also possible that this issue can be eliminated or minimized by changing the oil-surfactant combination. In the next stage (Fig. 1c-iii), aqueous droplets containing the enzyme components and glucose-assay reagents are generated using a T-junction geometry. An oil phase that consists of a stream of oil-surfactant mixture (4.5% Span 80, 0.4% Tween 80, and 0.05% Triton X-100) is injected right before the aqueous droplet generation to stabilize the droplets downstream (Fig. 1C-ii). The next step is the coalescence of the IL-pretreated biomass droplet with the aqueous enzyme droplet (Fig. 1C-iv). The amount of injected oil-surfactant is optimized to allow the facile merging of the IL-pretreated biomass droplet with the enzyme cocktail droplet, while allowing for the merged droplets to stay discrete during transportation and incubation. An expansion in the channel width is incorporated to assist in the droplet coalescence; when the droplet enters the expansion region, it slows down and merges with the next droplet (Fig. 2A–D). Biomass regeneration takes place immediately in the merged droplet with the water in the enzyme cocktail droplet acting as the anti-solvent and precipitating the cellulose. It is important to note that we add non-ionic surfactant Triton X-100 at 0.01%

into the enzyme cocktail mixture to decrease the surface tension of water to around 40 mN m^{−1}. This ensures that the biomass is regenerated inside the droplet (Fig. 2E), and not at the surface causing precipitation of biomass in the channels of the chip. We have shown that the presence of a small amount of surfactant does not significantly alter the cellulase activity.¹⁶ The IL content of the merged droplet is ~6% as measured by monitoring the conductivity of droplets (see ESI†), and at this concentration it does not interfere substantially with the cellulase activity.³¹

Following the biomass regeneration, the droplet is transported into a serpentine channel for temperature- and time-controlled incubation (37 °C for 30–60 s). This step involves three unit operations: (i) mixing of the regenerated, solid cellulose by chaotic advection, (ii) hydrolysis by the enzyme cocktail, and (iii) generation of a fluorescent product indicating production of glucose. Chaotic advection within the droplets moving through the serpentine/windy channels has been described before²³ and we utilize it for keeping the solid biomass suspended and well mixed. As shown in Fig. 2F, the regenerated biomass (stained with calcofluor for visualization) moves and rotates rapidly around the inside of a droplet. The rapid mixing is further confirmed by the fact that a detectable signal is generated in the droplets in 30 s (Fig. 2G), while it takes the macroscale approach (in 96-well format) 30 min for enzymatic hydrolysis and another 30 min for detectable glucose concentration. Other reasons for faster detection in the chip can also be the small size of the regenerated biomass (~ few μm) allowing easier access to the enzyme compared to

A. Heterogeneous substrates



B. Soluble substrate

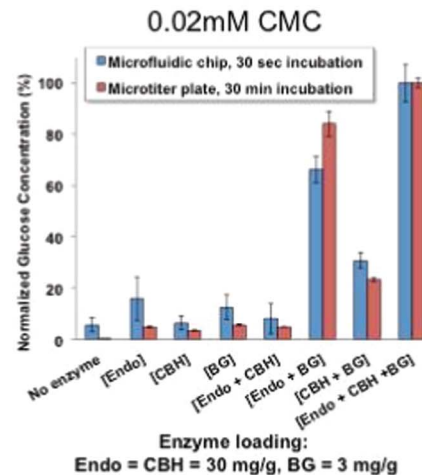


Fig. 3 Synergistic effect of the combination of cellulases on (A) heterogeneous substrates: (i) Avicel and (ii) switchgrass, and (B) soluble substrate: carboxymethylcellulose. The glucose yield obtained in a microchip at various enzyme combinations is in excellent qualitative agreement with those obtained in a microtiter plate.

the ~mm-sized biomass in the microtiter wells and the higher detection sensitivity of a fluorescence microscope compared to a plate-reader.

We demonstrated the utility of our droplet-based microfluidics approach to the screen activities of enzymatic cocktails using an industry-accepted model heterogeneous substrate (Avicel) and a real biomass sample (Switchgrass) (Fig. 3A). The breakdown of cellulosic biomass involves the synergistic action of three families of cellulases: endoglucanase (Endo), cellobiohydrolase (CBH), and β -glucosidase (BG). Endo hydrolyzes the intramolecular β -1,4-glucosidic bonds of the cellulose chains randomly to produce smaller cellulose oligomers; CBH processively cleaves the cellulose chains from either end to release soluble cellobiose; and BG hydrolyzes cellobiose to glucose. For Avicel, the three-part enzyme cocktails show the highest glucose production due to the synergistic activities among the three enzymes (Fig. 3A-i). In the case of the two-part enzyme cocktails, the CBH+BG shows higher activity than Endo+BG. Our results agree with previous reports that demonstrate that Avicel is a better substrate for CBH than for Endo because it has a low degree of polymerization.³² For the pretreated switchgrass substrate, as expected, the three-part enzyme cocktail shows the highest glucose yield (Fig. 3A-ii). However, the activities of the enzyme cocktails are lower for switchgrass compared to Avicel. This can be explained by the scanning electron microscopy images (Fig. S2†) of the substrates that show that the internal structure of Avicel is highly porous, whereas switchgrass has a dense layered structure leading to lower enzyme accessibility and hence, slower hydrolysis.

To ensure that the microfluidic results are consistent with the conventional approaches, we compared the results with those obtained using a 96-well microplate approach¹⁶ and, as

shown in Fig. 3A, the chip results are in excellent qualitative agreement.

Both the microfluidic and microplate methods show a similar pattern of glucose yield for different enzyme combinations validating the microfluidic platform (note: the comparison is qualitative as the reaction kinetics are different in the two formats). Application of our droplet platform is not necessarily limited to insoluble substrates, but it is also useful for homogeneous reactions. We tested the chip (Fig. 1B-C) to screen cellulase cocktails with a widely-used soluble substrate, carboxymethyl cellulose (CMC) and found that the three-part cocktails produced the highest amount of glucose, followed by the two-part cocktails ([Endo+BG], [CBH+BG]) (Fig. 3B). The microfluidic results are once again in excellent qualitative agreement with the microplate-based assays.

Conclusions

Our droplet approach offers a number of unique advantages for heterogeneous reaction screening: 1) droplets provide an ideal platform for the mixing of insoluble substrates with soluble enzymes owing to the internal recirculation. 2) Hundreds or thousands of pL-sized droplets can be generated in a chip allowing HTP screening of hundreds of conditions, such as substrate concentration, enzyme loading, and combination of enzymes. 3) pL-sized droplets require very small amounts of expensive enzymes thereby reducing cost. 4) The approach enables a hands-free, automated workflow without requiring expensive robotic stations. Although the chip was used to demonstrate the screening of enzymes for biomass hydrolysis, it could be useful as a screening platform for applications in other industrial processes, such as manufacturing of paper, textiles, detergents, food and pharmaceuticals

that rely on heterogeneous reactions involving enzymes such as chitinases,³³ xylanases,³⁴ amylases,³⁵ lignin-degrading enzymes,³⁶ pectinases,³⁷ proteases³⁸ and lipases.³⁹ An important point to note is that testing of our chip with IL-pretreated biomass does not preclude its general applicability to other solid substrates as long as the substrate can be introduced as a dilute suspension.

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