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Fast Screening of Bacterial Suspension Culture Conditions on Chip

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Culture conditions including pH, nutrient concentration and temperature strongly influence the properties of a microbial strain by affecting many factors such as microbial membrane and metabolism. We present a microfluidic chip for screening of pH and nutrient content with a concentration gradient generator connected to eight parallel suspension culture loops and another chip for screening of temperature with four different temperature zones under suspension culture loops. Bacteria grow much faster on chip than in test tubes, and yet interestingly, on-chip screening of culture conditions for *E. coli* yields results similar to those from culture in test tubes, demonstrating the validity of the on-chip screening approach. The microfluidic chips were applied to study the growth conditions of two wild *Bacillus subtilis* strains isolated from polluted water. The on-chip screening experiments show advantages of nano-liter scale screening units, high-throughput and require only one-fourth of the time.

Introduction

Industrial biotechnology widely uses microorganisms for processes in large-scale chemical, pharmaceutical, food, feed, textile, and energy production. Microorganism strain improvement, which includes two aspects: strain selection and culture condition optimization, is crucial in industrial biotechnology.[1] Culture conditions strongly influence enzymatic activity and membrane structural stability, and thus alter microbial metabolism and overall properties of the strain.[2] Proper conditions need to be selected via numerous culture condition screening tests before large-scale cultivation can take place. Therefore, screening of culture conditions is also an emphasis of industrial microbiology research. For example, Somporn Tanskul and co-workers optimized culture conditions such as pH, peptone concentration etc. of culture media as a necessary step for commercial industrial applications.[3] Traditional strain selection and culture condition screening processes using shaking flasks or test tubes are labor-intensive and time-and-reagent-consuming.[4] It is important to reduce the culture volume in order to improve the throughput and efficiency of screening, as well as to enable screening on scarce materials such as biomolecules only available on the order of micrograms or nanograms. 96-well microtiter plates have been used in place of shaking flasks and test tubes to reduce the culture volume in screening tests.[5, 6] However, the volume of each well in microtiter plates is still about hundreds of microliters, which on one hand limits their application towards screening of scarce materials and on the other hand also starts to show sub-optimal mixing effects at this volume. Further reduction in volume of microtiter wells will result in poor mixing of liquid via shaking, and is thus unsuitable for suspension culture for microbes. In addition, many operations of screening with microtiter plates such as the preparation of solutions with various concentrations and inoculation of microbes are still performed manually.

Microfluidic technology potentially offers an alternative approach towards miniaturized and high-throughput microbial screening.[7,8,9,10] While microfluidic chips for mammalian cell culture and screening have been reported,[11, 12] those for microbial screening are yet to be demonstrated. The major challenge lies in the need for mechanical mixing techniques in typically nano-liter scaled volume that are highly parallel and can be scaled up as the degree of integration of microfluidic chips improves. Recently, we published two microfluidic chips composed of culture chamber loops with active mixing micropumps for the demonstration of highly parallel suspension culture of microbiological organisms such as *E.coli* and yeast[13, 14]. However, the function of these chips is limited to microbial culture only. It is crucial to demonstrate the capability of carrying out realistic tasks. In this paper, a complete microfluidic system for microbial screening is constructed by combining parallel suspension culture units with concentration gradient generator or temperature reservoirs. Screening of culture conditions for model microbe *E. coli* and wild type *Bacillus subtilis* strains isolated from environment is carried out with high efficiency.

Three most important variables in bacterial culture are pH, nutrient content and culture temperature. Two separated chips are designed for the screening of the concentration of soluble substance (including pH and nutrients) in growth medium and culture temperature, respectively. For the screening of pH and nutrient content, a Christmas tree type gradient generator[15,16] is constructed on chip to produce different concentrations of tested nutrient or pH. And three parallel microbial culture loops are connected to each of the outputs of the gradient generator. The effect of pH or nutrient content on bacterial growth can thus be monitored as the culture proceeds in each loop. Screening of culture temperature is achieved by creating four temperature zones on the chip. An extra layer of PDMS with four temperature reservoirs are attached to the bottom of the culture chip; and water with different temperatures is circulated through the reservoirs to create different temperature zones.

We first screened the culture conditions for *E. coli* growth on chip. The optimal conditions including pH, nutrient content and temperature obtained from on-chip screening agree well with those from traditional screening methods, which demonstrates the validity of the screening-on-chip approach. The chips were then applied for the screening of culture conditions for two wild *Bacillus subtilis* strains isolated from polluted water. The work on both the model microbe *E. coli* and wild *Bacillus subtilis* strains demonstrated the advantage of on-chip screening technology as being high throughput, high growth rate, and reduced cost due to miniaturization and automation of the process.

Experimental

Chip fabrication

Polydimethylsiloxane (PDMS) microchannels with height of about 10 μm were fabricated by following the general soft lithography procedures as published before[17,18]. In preparation for the mold of the chip, photoresist was spun-coat on substrate at 2000 rpm; and then the resist on substrate was soft baked at 95 ℃ for 6 minutes. After exposure and development, the mold was hard baked at 120 ℃ for 5 minutes to crosslink the resist. A 10:1 mixture of PDMS oligomer and the crosslinking agent (Sylgard 184, Dow Corning) was poured onto the mold after being degassed under vacuum. The PDMS was then removed from the mold after 30 minutes of curing at 90 ℃ to yield the patterned channels. The different PDMS layers of one chip were bonded to each other after air plasma treatment of the bonding surfaces. Channels surface was hydrophilic modified by soaking of 0.2% solution of Pluronic F-127 (Sigma-Aldrich) in channel for 30 minutes.

Microfluidic setup

The microfluidic chips were connected to external flow with Teflon tubes inserted at the inlet and outlet reservoirs. Syringe pumps (PHD 2000, Harvard Apparatus) were used to control volume flow rates.[19] The pneumatic control channels were filled with water as pressure transferring medium and the inlets of the control channels were connected to compressed air cylinders through Teflon tubes. The actuation of the two-stage peristaltic pump during culture process was carried out using the same procedure as in previously reported work.[20]

For the screening of pH and nutrient concentrations on chip, the generated concentrations in culture loops were determined by optical absorption of dyes. 0.05 mol L^{-1} methylene blue solution and pure water were separately injected into the two inlets of gradient generator at flow rates of 0.2 μL s^{-1}. After balancing for 20 min, micrographs of all culture loops were taken under microscope (AZ100, Nikon) equipped with CCD camera and compared against blank images. The optical density in each loop was measured on the micrograph, and the concentration was calculated according to Beer-Lambert law.

For the temperature-controlled culture on chip, constant-temperature water was circulated through heat bath attached to the bottom of the culture chip by using a peristaltic pump. Temperature of culture zones was measured with an infrared thermometer after equilibrium for half an hour. The distribution of temperature on chip was determined by thermal infrared imager (SC325, FLIR Systems).

Microbial culture

E. coli TOP10 used in this study was laboratory preserved. Two strains of *Bacillus subtilis* were isolated from animal breeding waste water in suburb of Chengdu, China. Unless otherwise specified, the medium for *E. coli* culture was a standard laboratory growth medium consisted of 1% peptone, 0.5% yeast extract, 1% NaCl in tap water with pH 7.0; the medium for *Bacillus subtilis* strain culture consisted of 1% peptone, 0.3% beef extract, 0.5% NaCl in tap water with pH 7.4-7.6.[21] Medium with different pH were configured by adding a certain percentage of 0.02 mol L^{-1} Na_2HPO_4 solution and 0.01 mol L^{-1} citric acid solution. Microfluidic chips and culture medium were sterilized by autoclaving at 121 ℃ for 20 min.

All microbes separately transferred to fresh medium in test tubes and well mixed. A small portion of liquid was then separately injected into channels on chips and kept the left cultured in test tubes as the control group. Unless otherwise specified, then the chips were placed into water bath at 37 ℃ when microbe was cultured. The cell concentration was measured after culture for 6 hours by counting the cell numbers from the pictures taken by CCD camera of a microscope. Error bars were calculated based on data obtained from different parallel culture loops.

Results and discussion

Chip design for fast screening of bacterial suspension culture conditions

The 7.5 cm × 5.0 cm chip fabricated by three-layer PDMS for screening of pH and nutrient content is composed of a gradient generator and suspension culture loops array (Fig. 1a, 1b, 1c). The solution with different concentration obtained at each output of the Christmas-tree type gradient generator[22] flows into three actively pumped culture loops in parallel. A near-linear concentration gradient is beneficial for acquisition of growth state under different culture conditions and effectively screening for the suitable parameters. By increasing the length of each S-shape channel to 8.4 mm and reducing the flow rate to 0.2 μL s^{-1} for sufficient mixing, linear concentration gradient is achieved in our gradient generator with a correlation coefficient r of 0.996 (Fig. S1).

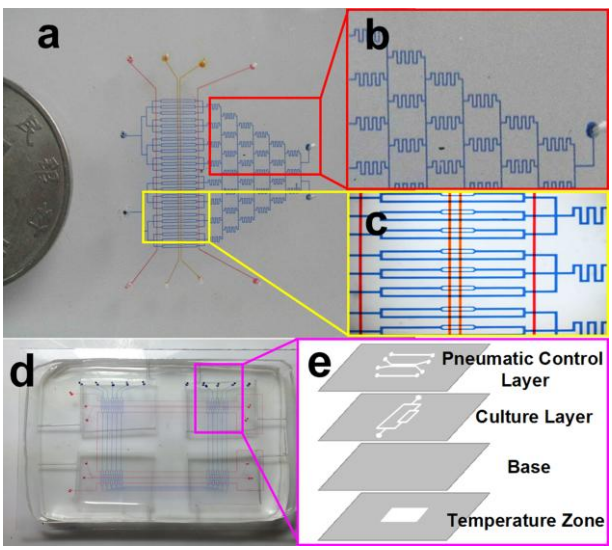

Fig. 1 (a) Photograph of a chip for screening of pH and nutrient content; (b) Zoom-in photograph of the gradient generator; (c) Zoom-in photograph of the culture loops (blue channels are for gradient generating and microbial culture, red channels are pneumatic control channels); (d) Photograph of a chip for screening of culture temperature with 4 temperature zones; and each zone contains 10 culture loops; (e) A schematic diagram showing four-layer structure of chip for screening of culture temperature.

The microfluidic chip for screening of culture temperature is made from a special four-layered structure (Fig. 1d, 1e): the top layer is a PDMS layer for pneumatic control, the middle layer above the glass base is the PDMS layer of culture loops and the bottom layer under the glass base is a PDMS layer with four large reservoirs. The culture chamber loops in the middle layer are aligned to the position of reservoirs at the bottom layer. Growth status of different bacteria at different temperature can thus be observed on the chip simultaneously. The temperature of each temperature zones is measured with infrared thermometer and separately controlled by adjusting the temperature of circulating water to each of the water reservoirs. Thermal micrograph of the temperature-controlling chip in Fig. S2 shows even distribution of temperature in each zone and the variation in each culture area is less than ±1 ℃.

Screening of suspension culture conditions for *E. coli* on chip

A series of experiments were carried out on the model bacterium *E. coli* to test the validity of microfluidic on-chip screening of culture conditions. pH, nutrients content and temperature are three main factors of concern in *E. coli* culture.

pH influences microbial growth and metabolism by affecting charge states of cell membrane as well as the activity of enzymes; so suitable pH is essential for microbial culture.[23, 24] The growth of *E. coli* showed big difference when pH of culture media varied from 3.0 to 7.2 (Fig. 2a). The best pH range for *E. coli* culture was between 7.2 and 6.6. *E. coli* growth started to slow down slightly as the pH decreases to the 6.0 to 4.8 range; at even more acidic pH of the 4.2 to 3.0 range, *E. coli* growth was severely impaired. The morphology of *E. coli* showed the phenomena of growing partly in cluster when pH came close to 3.0 (Fig. S3). It can be concluded from the on-chip screening experiments that near neutral pH is best suited for *E. coli* culture. Test tube culture experiments in the pH range from 3.0 to 7.2 showed similar results (Fig. S4a). These results are fully consistent with the result obtained from traditional screening techniques.[25]

Nutrients in culture media such as peptone provide microorganism carbon source, nitrogen source and growth factors. Screening for adequate but not excessive or wasteful concentration of nutrients is thus also very important. On-chip experiments of *E. coli* culture in media with different peptone content showed that the growth rate of *E. coli* increased by 13 times as peptone content increased from 0.22% to 0.89% (Fig. 2b). But further increase of peptone content from 0.89% to 1.78% did not affect *E. coli* growth. This indicates that below 0.9% peptone is the limiting nutrient for *E. coli* growth in this media, which is consistent with previous report.[26]

Temperature profoundly influences microbial physiology by affecting the thermodynamics and kinetics of biochemical reactions.[27] It is highly relevant to investigate the effect of temperature on bacterial growth. In the on-chip experiment, temperatures of the four independent zones were set to 22, 25, 28 and 37 ℃, respectively. The results in Fig. 2c show that *E. coli* growth is highly sensitive to the temperature. Less than 22 ℃, *E. coli* almost stopped growing for 6 hours. At 25 ℃ and 28 ℃, it showed much slower growth rate than at 37 ℃. The cell concentration of *E. coli* at 28 ℃ was about one sixth of that at optimal growth temperature of 37 ℃ (Fig. S5). The results demonstrate that the four temperature zones on the chip can be independently controlled for the screening of bacterial culture conditions. Due to miniaturization of culture chambers, on-chip screening of temperature can be accomplished in a single chip, which guarantees better parallel culture conditions (other than temperature) than traditional method utilizing simultaneous suspension culture in multiple shaking incubators set at different temperatures. Furthermore, microbial metabolism and fermentation are exothermic processes. Thus, the heat accumulated from miniaturized culture chips can be better dissipated and the temperature better controlled than in traditional systems with large culture or fermentation volume.

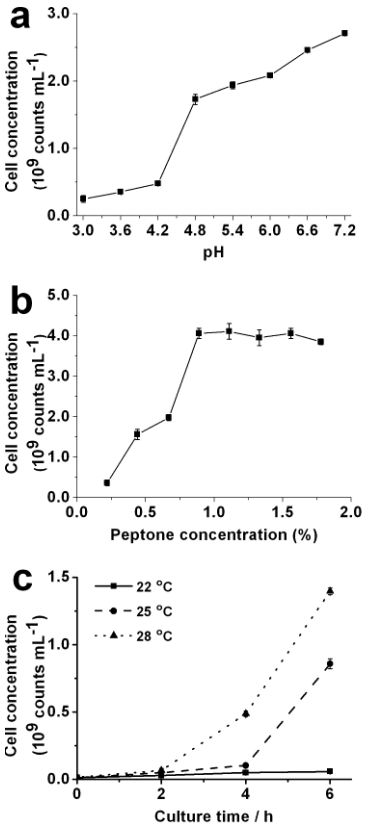

Fig. 2 Screening of culture conditions for *E. coli*. The effects of (a) pH, (b) peptone content, and (c) temperature on the growth of *E. coli* on chip.

Screening of culture conditions for wild *Bacillus subtilis* strains isolated from environment

Environmental microbe *Bacillus subtilis* can degrade environmental pollutants, such as organic macromolecules, into small molecules such as amino acids, glucose and so on.[28] The two wild *Bacillus subtilis* strains in these experiments were isolated from natural environment and proven to be cellulose-decomposing microorganisms. For realistic applications, it is crucial for microbes to have high growth rate in order to dominate over other competing microorganisms and to accelerate the degradation of pollutants. Although the optimal growth conditions for bacteria are always close, the growth rate of different strains within the same species in adverse conditions may vary significantly. The resistance toward adverse conditions often determines whether the strain can be practically applied in realistic environments. Here we aim at testing the adaptive capability of the two wild *Bacillus subtilis* strains in adverse conditions such as pH, nutrient composition and temperature using the microfluidic chips described above.

It is important to study the growth of *Bacillus subtilis* under different pH in order to learn whether this bacterium can be applied in an acidic environment.[29] The results in Fig. 3a show that two *Bacillus subtilis* strains grow fast at the pH 7.2 to 6.6 range, and slightly slower at the pH 6.0 to 4.2 range. The *Bacillus subtilis* strains nearly stop growth at the pH 3.6 to 3.0 range. Compared to

Bacillus subtilis #1, *Bacillus subtilis #2* is more sensitive to environmental pH, which exhibits sharp decrease in growth rate when the pH is below 6.0. *Bacillus subtilis #1* is more suitable for application in weakly acidic environments at the pH 6.0 to 4.2 range with cell concentration over 10^9 counts mL^{-1}. Control experiments of *Bacillus subtilis #1* suspension culture in test tubes reflect the same trend (Fig. S4b) but exhibit much slower absolute growth rate, confirming that the on-chip screening of pH conditions is credible.

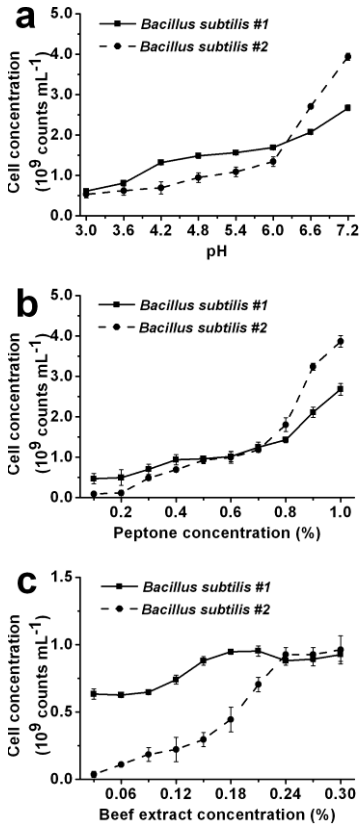

Fig. 3 Screening of culture conditions for *Bacillus subtilis* strains. The effects of (a) pH, (b) peptone content, and (c) beef extract content on the growth of two *Bacillus subtilis* strains cultured on chip.

Similar to the behavior of *E. coli*, both *Bacillus subtilis* strains exhibit significant changes in growth rate as the peptone content in media increases from 0.1% to 1% (Fig. 3b). The growths of the two *Bacillus subtilis* strains are severely restricted when the peptone content is below 0.8%.

Although conventional culture media containing 0.3% beef extract can satisfy the growth of *Bacillus subtilis*,[21] it is still unknown that below what concentration will beef extract restrict microbial growth. Fig. 3c shows the biomass of the two *Bacillus subtilis* strains cultured on chip when beef extract content is varied from 0.03% to 0.30%. *Bacillus subtilis #1* is less dependent beef extract, showing reasonable growth at beef extract concentration as low as 0.03%. The saturation concentration of beef extract for *Bacillus subtilis #1* is around 0.18%, beyond which the growth rate does not accelerate further. On the other hand, *Bacillus subtilis #2* is more sensitive to and dependent on beef extract. *Bacillus subtilis #2* growth is severely suppressed when beef extract concentration is below 0.18% and the saturation concentration is around 0.24%.

Microbial grow that different temperature is important for practical applications since natural environmental temperature changes. Although most reported optimum growth temperature for *Bacillus subtilis* is 30 ℃ to 37 ℃, natural water environment can rarely reach such a high temperature. We study the growth of the *Bacillus subtilis* strains in the temperature range of 20 ℃ to 30 ℃ which is commonly encountered in environment. Results in Fig. 4 showed that the two strains of *Bacillus subtilis* both grew fast at 28 ℃, slower at 25 ℃, but almost stopped growth at 22 ℃. Compared to *Bacillus subtilis #2*, *Bacillus subtilis #1* exhibited stronger growth ability at 25 ℃. For both strains, their application in environmental temperature below 25 ℃ is limited because of slow growth.

Based on these results, we conclude that *Bacillus subtilis #1* has better adaptability than *Bacillus subtilis #2* in adverse conditions such as low pH, low temperature and insufficient beef extract contents, and thus may be more widespread in realistic applications.

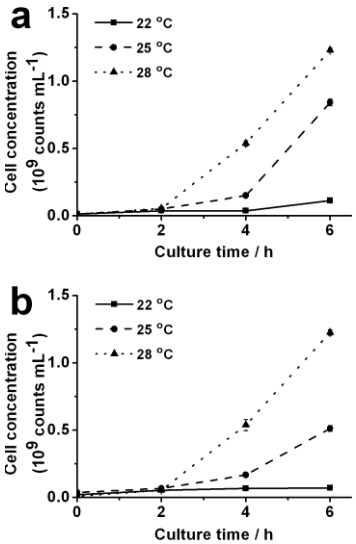

Fig. 4 Growth curves of (a) *Bacillus subtilis #1*, and (b) *Bacillus subtilis #2* on chip under different temperatures.

Fast screening of bacterial culture conditions on chip

Microbe counts need to reach 10^8 or even 10^9 counts mL^{-1} in many cases of practical applications.[2,30] It is necessary for microbial screening process to reach such high microbial concentrations, which means that the growth time for obtaining high microbial concentration determines the efficiency of entire screening process, and high growth rate of microbes can shorten the total time of microbial screening process. Limited by the microbial growth rate, culture experiments in test tubes usually needs 24 hours or longer for providing enough cell concentration to screen for suitable culture conditions. Interestingly, bacteria are consistently found to grow faster on microfluidic chips than in test tubes. Consequently, it takes only 6 hours or less for on-chip suspension culture to reach required cell concentration, which significantly improves efficiency of screening.

Bacteria grew much faster on chip than in test tube when other parameters were kept the same (Table 1). The growth rate of *E. coli* and *Bacillus subtilis* may even increase to ten times in some cases when the culture environment changed from test tubes to microfluidic channels. This is most likely due to the high specific surface area of the microfluidic channels and the good gas permeability of PDMS, which are both beneficial to the diffusion and dissolution of oxygen into the media.[31] This feature is particularly important for applications requiring high microbial cell

density.[32] Fast on-chip screening for high microbial density culture conditions will have broad applications in the future.

Table. 1 Average microbial growth rate in suspension culture on chip and in test tube

Strain	Conditions		Growth Rate (10^8 counts/mL/h)	
			On chip	In test tube
E. coli	pH	7	3.2	0.2
		4	0.7	0.1
Bacillus subtilis #1	Peptone	0.1%	1.6	0.8
		1%	9.1	1.6
	Beef extract	0.03%	0.9	0.3
		0.3%	2.0	0.4

Conclusions

A chip for screening of pH and nutrient content with a concentration gradient generator connected to eight parallel suspension culture loops and another chip for screening of temperature with four different temperature zones were presented for fast screening of bacterial suspension culture conditions. Suitable pH and nutrient content for *E. coli* were screened and growth status of *E. coli* at different temperature was observed on chip. The growth of two wild *Bacillus subtilis* strains isolated from polluted water was also studied on chip with different culture conditions, which yielded valuable information for future environmental applications.

Compared to conventional experiments in test tubes, screening of microbial culture conditions on microfluidic chips largely simplifies the operation by accomplishing the whole process on a single chip including gradient generation, microbial suspension culture and observation of microbial morphology. The small volume, high specific surface area, and good gas permeability of microfluidic culture also result in better control of culture temperature and higher growth rate. Although only one independent parameter is screened at one time in the current design, it is also feasible to combine multiple screening parameters in the future to further improve screening efficiency. Overall, screening of bacterial culture conditions on microfluidic chips exhibits advantages of high-throughput, high efficiency, and low cost. We believe this approach will have great impact and broad applications in microbial biotechnology.

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Notes and references

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1. M. J. Waites, N. L. Morgan, J. S. Rockey and G. Higton, *Industrial microbiology: an introduction*, Wiley. com, 2009.
2. G. Sreekumar and S. Krishnan, *Afr. J. Biotechnol.*, 2010, **9**, 8078-8084.
3. S. Tanskul, K. Amornthatree and N. Jaturonlak, *Carbohydr. Polym.*, 2013, **92**, 421-428.
4. S. Parekh, V. A. Vinci and R. J. Strobel, *Appl. Microbiol. Biotechnol.*, 2000, **54**, 287-301.
5. F. Kensy, E. Zang, C. Faulhammer, R. K. Tan and J. Buchs, *Microb. Cell. Fact.*, 2009, **8**, 17.
6. J. Tan, J. Chu, Y. Y. Hao, Y. X. Guo, Y. P. Zhuang and S. L. Zhang, *Appl. Biochem. Biotechnol.*, 2013, **169**, 1683-1695.
7. D. B. Weibel, W. R. DiLuzio and G. M. Whitesides, *Nat. Rev. Microbiol.*, 2007, **5**, 209-218.
8. R. Gomez-Sjoberg, A. A. Leyrat, D. M. Pirone, C. S. Chen and S. R. Quake, *Anal. Chem.*, 2007, **79**, 8557-8563.
9. F. K. Balagadde, L. C. You, C. L. Hansen, F. H. Arnold and S. R. Quake, *Science*, 2005, **309**, 137-140.
10. P. N. Nge, C. I. Rogers and A. T. Woolley, *Chem. Rev.*, 2013, **113**, 2550-2583.
11. Y. H. Jang, M. J. Hancock, S. B. Kim, S. Selimovic, W. Y. Sim, H. Bae and A. Khademhosseini, *Lab Chip*, 2011, **11**, 3277-3286.
12. B. Y. Xu, S. W. Hu, G. S. Qian, J. J. Xu and H. Y. Chen, *Lab Chip*, 2013, **13**, 3714-3720.
13. M. Gan, J. Su, J. Wang, H. Wu and L. Chen, *Lab Chip*, 2011, **11**, 4087-4092.
14. M. Gan, Y. Tang, Y. Shu, H. Wu and L. Chen, *Small*, 2012, **8**, 863-867.
15. J. A. Burdick, A. Khademhosseini and R. Langer, *Langmuir*, 2004, **20**, 5153-5156.
16. N. N. Ye, J. H. Qin, W. W. Shi, X. Liu and B. C. Lin, *Lab Chip*, 2007, **7**, 1696-1704.
17. J. C. McDonald and G. M. Whitesides, *Acc. Chem. Res.*, 2002, **35**, 491-499.
18. D. C. Duffy, J. C. McDonald, O. J. A. Schueller and G. M. Whitesides, *Anal. Chem.*, 1998, **70**, 4974-4984.
19. Y. X. Gao and L. W. Chen, *Lab Chip*, 2008, **8**, 1695-1699.
20. J. M. Berg, R. Anderson, M. Anaya, B. Lahlouh, M. Holtz and T. Dallas, *Sens. Actuators, A*, 2003, **104**, 6-10.
21. T. Zhang, Z. Q. Shi, L. B. Hu, L. G. Cheng and F. Wang, *World J. Microbiol. Biotechnol.*, 2008, **24**, 783-788.

22. N. L. Jeon, S. K. W. Dertinger, D. T. Chiu, I. S. Choi, A. D. Stroock and G. M. Whitesides, *Langmuir*, 2000, **16**, 8311-8316.
23. T. Warnecke and R. T. Gill, *Microb. Cell. Fact.*, 2005, **4**, 8.
24. P. D. Cotter and C. Hill, *Microbiol. Mol. Biol. Rev.*, 2003, **67**, 429-+.
25. L. K. Cheng, J. Wang, Q. Y. Xu, C. G. Zhao, Z. Q. Shen, X. X. Xie and N. Chen, *World J. Microbiol. Biotechnol.*, 2013, **29**, 883-890.
26. S. Jebri, F. Hmaied, J. Jofre, MariemYahya, J. Mendez, I. Barkallah and M. Hamdi, *Water Res.*, 2013, **47**, 3673-3678.
27. E. K. Hall, G. A. Singer, M. J. Kainz and J. T. Lennon, *Funct. Ecol.*, 2010, **24**, 898-908.
28. M. Pacwa-Plociniczak, G. A. Plaza, Z. Piotrowska-Seget and S. S. Cameotra, *Int. J. Mol. Sci.*, 2011, **12**, 633-654.
29. C. Zhang, H. W. Diao, F. X. Lu, X. M. Bie, Y. F. Wang and Z. X. Lu, *Bioresour. Technol.*, 2012, **126**, 80-86.
30. L. Korsten and N. Cook, *South African Avocado Growers' Association Yearbook*, 1996, **19**, 54-58.
31. F. Garcia-Ochoa and E. Gomez, *Biotechnol. Adv.*, 2009, **27**, 153-176.
32. D. Riesenberg and R. Guthke, *Appl. Microbiol. Biotechnol.*, 1999, **51**, 422-430.

