Lab on a Chip

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PAPER

A scalable microfluidic chip for bacterial suspension culture†

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Microfluidic systems could, in principle, enable high-throughput breeding and screening of microbial strains for industrial applications, but parallel and scalable culture and detection chips are needed before complete microbial selection systems can be integrated and tested. Here we demonstrate a scalable multi-channel chip that is capable of bacterial suspension culture. The key invention is a multi-layered chip design, which enables a single set of control channels to function as serial peristaltic pumps to drive parallel culture chamber loops. Such design leads to scalability of the culture chip. We demonstrate that *E. coli* growth in the chip is equivalent or superior to conventional suspension culture on shaking beds. The chip could also be used for suspension culture of other microbes such as *Bacillus subtilis*, *Pseudomonas stutzeri*, and *Zymomonas mobilis*, indicating its general applicability for bacterial suspension culture.

Introduction

Microbes and their metabolic products are widely used in the chemical, food, pharmaceutical, and health care industries as well as new industries such as environmental remediation, green chemistry, sustainable manufacturing, biomass energy and resources, and low-carbon biodiesel. Improvement in the microbial production strains offers great opportunity for elevated profits and sustainability of relevant industries without significant capital outlay.[1] While many modern bioengineering approaches such as random or directed genetic mutation, genetic recombination, and rationalized breeding have been employed for this purpose, the success of obtaining improved strains still relies on efficient culture and screening of microbes.[2,3] Shake flasks and tubes account for 90% or more of all microbe culture experiments.[4] However, current flask culture techniques require large numbers of flasks, bulky shaking beds, large fluid volume, intensive human labor and expensive equipment, and thus cause poor screening efficiency.

Microfluidic chips bring new capabilities to microbiological research and have been applied in many key areas in microbiology.[5] They offer a potential alternative to overcome the limitations and accelerate microbial screening processes. Due to miniaturization of culture chambers in microfluidic devices, high throughput mammalian cell culture has been progressively realized by integrating hundreds of culture chambers on microfluidic chips.[6,7] However, most mammalian cell culture chips are not suitable for microbial culture due to the lack of an active mixer. Mixing is one of the most important operations in suspension cultivation of microbes as well as in industrial fermentation.[8,9] The quality of mixing directly influences the distribution and suspension of substrates and microorganisms in fermentation reactors, and thus affects the growth characteristics (*e.g.*, the availability of oxygen and nutrients and removal of waste products).[10] In a conventional fermentor, mixing is realized *via* mechanical stirring or air bubble lifting; in a lab scale flask and tube culture, it is realized through shaking. These methods are simple and effective in macroscopic worlds but become extremely difficult to implement because of unique fluid dynamics in microscale fluidic channels.

Few microfluidic culture chips integrated with an active mixer aimed at suspension bacterial cultivation have been reported. One example used micro magnetic stirrers in microbioreactors to mimic a conventional fermentor.[11] But the stirrer required high precision fabrication, and the 10 mm diameter chamber limits the integration density of the chip.[10] Balagadde *et al.* reported a microfluidic chip with six independent but identical bioreactors for bacterial suspension culture.[12] Each bioreactor consists of a culture loop and three independent control channels as the peristaltic micropump to drive the circulation of culture media and the suspension of bacteria in the media. It laid the groundwork for future bacterial suspension culture in chip.

For scalable high throughput bacterial suspension culture, it is imperative to limit the number of control channels as the number of parallel culture loops in a chip increases. This is a challenging task in a 2-dimensional design of single-layered channel layouts due to geometrical constraints. In this work, we present a novel

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3-dimensional multi-layered design that solves the problem. In this device, culture media in several parallel and identical culture chamber loops were driven by a series of micropumps formed with a single set of control channels. The culture media in different culture loops were demonstrated to cycle at the same speed, which ensures identical growth environments in all culture loops. The suspension culture of *E. coli* in the chip was compared to that in a shake flask and in static conditions. Using a chip with 8 × 4 culture units, growth of *E. coli* under different micropumping rates was investigated. Suspended cultivations of other strains such as *Bacillus subtilis*, *Pseudomonas stutzeri* and *Zymomonas mobilis* were also carried out to demonstrate general applicability of the chip for microbial culture.

Materials and methods

Design and fabrication of microbial culture chip

The microfluidic chip consists of an array of culture units organized in groups. Fig. 1 illustrates the structure of eight parallel culture units in a column. The device is designed in four layers. Culture chamber loops are located at the two stacked layers at the bottom, with an L shaped half loop in the lower bottom layer, and an inverted L shaped half loop in the upper bottom layer. The two layers are aligned to join the two half loops in a head-to-tail configuration to form a complete culture chamber loop. The third layer from the bottom is a thin elastic membrane. Together with the control channels in the top layer, these two top layers form the peristaltic micropumps that drive the culture media in the two bottom layers. The key innovation in the design is the arrangement of culture loops in two layers instead of in a single layer. This allows the pneumatic peristaltic micropumps to drive only one side of the culture loop, thus resulting in uni-directional flow in the loop. The media inlets and outlets of the eight culture units are connected to the common inlet and outlet channel at the bottom layer, and two extra control channels are used as stop valves to isolate the culture chamber loop from the outside after inoculation. All the channels and loops are 100 μm wide and 12 μm deep.

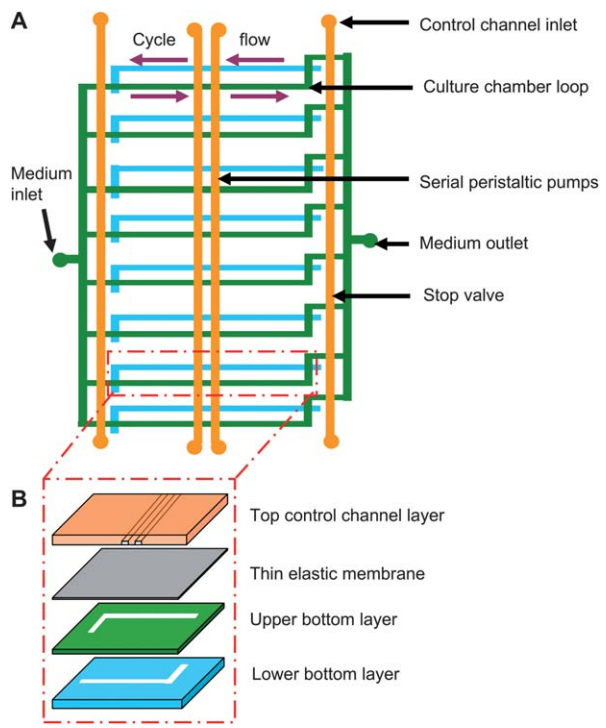

Fig. 1 Schematic diagram of the scalable microfluidic culture chip. (A) Top view with overall channel and connection layout. (B) Zoom-in illustration of a culture loop unit showing the multi-layered design.

The culture chips were made of PDMS (Sylgard 184, Dow Corning Corp) using soft lithography techniques with minor modification.[13–15] PDMS prepolymer, mixed at a 10 : 1 (w/w) ratio with a curing agent was poured onto the glass mould of the top layer and lower bottom layer and subsequently cured at 90 °C for 20 min. To fabricate the upper bottom layer and the thin elastic membrane, PDMS prepolymer was spin-coated at 3500 rpm for 60 s on the glass mould and then baked for 10 min at 80 °C, resulting in a ~40 μm thickness. The top layer was then aligned on the thin elastic membrane and baked for 20 min at 90 °C to bond together. A 23 gauge flat-head needle was used to punch through the three layers to reach the inlet and outlet holes. Finally, the PDMS pieces containing the top three layers and the bottom layer were both treated using air plasma for 30 s and then aligned and bonded. To strengthen the bonding between layers, the chip was post-baked at 90 °C overnight.

Biological experiments

Escherichia coli (*E. coli*) strain TOP10 was kindly provided by Prof. Qiangbin Wang, Suzhou Institute of Nano Tech and Nano Bionics, Chinese Academy of Sciences. *Bacillus subtilis* CICC 23591, *Pseudomonas stutzeri* CICC 31616 and *Zymomonas mobilis* CICC 10232 were obtained from the China Center of Industrial Culture Collection. The medium for bacterial culture consisted of 0.5% peptone, 0.5% yeast extracts, 1% beef extracts, 5% glucose and 0.5% NaCl with pH 7.4. The microfluidic chip was sterilized by autoclaving at 121 °C for 20 min. Bacterial culture was suspended in fresh medium and then the suspension was injected into each culture chamber loop in the chip. Then the medium outlets and inlets were sealed by epoxy glue. The chip was placed in a water bath at 37 °C for the bacteria culture. Each control channel was filled with water and connected to external air pressure using solenoid valves (Zhejiang Yongjing Technical Co., Ltd.). The frequency and sequence of solenoid valves were controlled by computer.[16] To reduce the complexity of the chip, a two-staged peristaltic micropump instead of the typical three-stage pump was adopted from a previously published report.[17] The actuation pressure was 75 kPa. As control tests, conventional bacterial shaken cultures were placed in a 250 mL shake flask with a rotation speed of 100 rpm at 37 °C, while the conventional static cultures were placed in a static incubator at 37 °C. For the flow rate measurement, *E. coli* suspended in water was used as a tracer particle and monitored under an optical microscope (AZ 100, Nikon). The flow rate was determined by measuring the time that *E. coli* spent traveling a known distance in the culture chamber loops. The cell concentration of the bacteria was determined by counting the number of bacteria in a known volume at regular time intervals using an optical microscope equipped with a digital camera (DS-Ri1, Nikon).

Results and discussion

Flow rate in each chamber

Peristaltic pumps have been demonstrated for driving fluids in microfluidic chip. By applying pressure through two or three control channels in sequence, the PDMS membrane between control and fluid channels deflect to extrude the fluid in one direction.[14,17] Generally, each bioreactor may consist of a culture loop and three independent control channels as the peristaltic micropump to drive the circulation of culture media. However, an increase in integration density may lead to excessive control channels and overly complicated systems. Two features in our chip design lead to simplification of chip layout and scalability for a large number of parallel culture chambers. Firstly, two-staged peristaltic micropumps were used instead of the typical three-staged pumps. By adapting a pumping sequence from the published literature,[17] a directional flow with intermittent back flow was achieved (see ESI†). Since the back flow does not compromise or may even benefit the purpose of solid-liquid mixing in culture channels, this feature allowed us to reduce the number of control channels from three to two. Secondly, the two-layered design of the culture channel loop (Fig. 1) allows a single set of control channels to form serial micropumps and drive parallel culture channel loops. For each culture unit in the chip, the control channels cross both L-shaped half loops of the culture chamber, creating two peristaltic micropumps. The two micropumps drive the circulation of culture media in opposite directions (one clockwise and the other counter-clockwise), but the pumping efficiency of the two micropumps is different. In one of the micropumps, the wall thickness between the control channel and the culture channel is just the thickness of the thin elastic membrane (~28 μm), while in the other micropump, it is the thickness of the thin elastic membrane plus that of the upper culture loop layer (~40 μm). Thicker walls in micropumps lead to lower occulation and lower pumping efficiency. Therefore, the overall action of the two micropumps results in uni-directional circulation in the culture chamber loop.

To compare the growth or fermentation of different cultures, the parameters of each culture chamber loop including the length, width and height of the culture chamber loops as well as the flow rate for the cell suspension should be identical. While the size of the culture chamber loop is fixed after device fabrication, the rate of the cycling flow in the culture chamber loop is a critical parameter for microbial culture because it relates to gas diffusion, the degree of mixing, and the shear force on microbes. We measured the flow rate of each chamber under an optical microscope. As shown in Fig. 2, the flow rates in the eight chambers are very similar, with an average of 47.8 ± 2.0 μm s^{-1}. The similarity in flow rates is attributed to the synchronous operation of the serial micropumps across the whole column of culture loops. A systematic trend is observed that the flow rate is slightly higher at the ends of the column and slightly lower in the middle of the column. With longer distances between micropumps and control channel inlets, the pump becomes less efficient. While the variation in flow rate is acceptable for the current design of 8-channeled columns, modifications could be adopted if a greater number of channels needs to be integrated. For example, the growth loops could be placed closer to each other to increase the density of culture loops without increasing the length of control channels; inlets of control channel could be added at some intervals as relays if long control channels are inevitable; or the diameter of the control channel could be gradually enlarged from inlets to the middle area to compensate for weaker pumping efficiency.

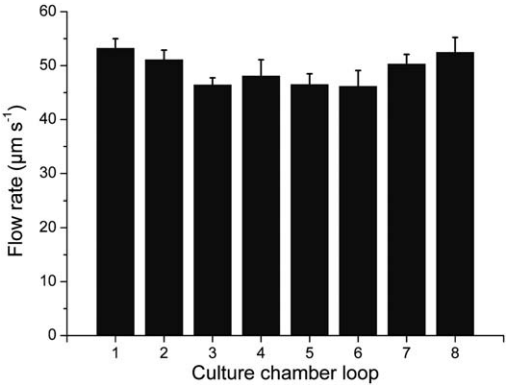

Fig. 2 Flow rate of each culture chamber loop in one column at pumping frequency of 0.62 Hz. Each loop was numbered in sequence from one side to the other.

E. coli suspension culture in chip

To demonstrate the applicability of the chip for microbial culture, we grew *E. coli* as a model bacterium in an 8 × 2 chip. One column of eight chambers was pumped during culture while the other was kept static. A movie illustrating the suspension culture of *E. coli* is provided in the ESI.† The growth of *E. coli* in the two types of culture methods was monitored during the six hour long cultivation experiment (Fig. 3A). The final cell concentration at the end of 6 h cultivation was 2.9×10^9 counts mL^{-1}, which was close to the cell concentration at stationary phase in macroscopic culture. In contrast, the growth in static microfluidic channels without pumping was very slow. The cell concentration was 0.6×10^9 counts mL^{-1} at the end of 6 h, merely about one fifth of that in the pumped in-chip culture. Static culture in an ordinary flask yielded similar results as static in-chip culture, which suggested little difference between millilitre- and nanolitre-volume culture. Furthermore, the cells in the pumped suspension culture showed uniform distribution in the whole chamber loop but the static culture showed clustered cells at many regions along the culture loops (see Fig. 3B–C).

Considering the identical culture loops and inoculation method in the experiment above, it is clear that the cycling medium flow in the culture loops plays a crucial role for rapid cell growth. In microfluidic channels, mixing is greatly limited because the fluid flow is usually laminar. Liquid–liquid mixing in microfluidics has attracted much attention.[18] However, cell suspension is a typical liquid–solid mixing process. Although some bacteria such as *E. coli* have good motility, uniform distribution of bacteria in microfluidic chambers is still difficult to achieve. Micropumps offer an excellent tool for liquid–solid mixing without adverse effects on bacterial growth.[12] Here we showed that cycling flow resulted in uniform distribution of cells in the chamber. It had the same function as a stirrer or gas

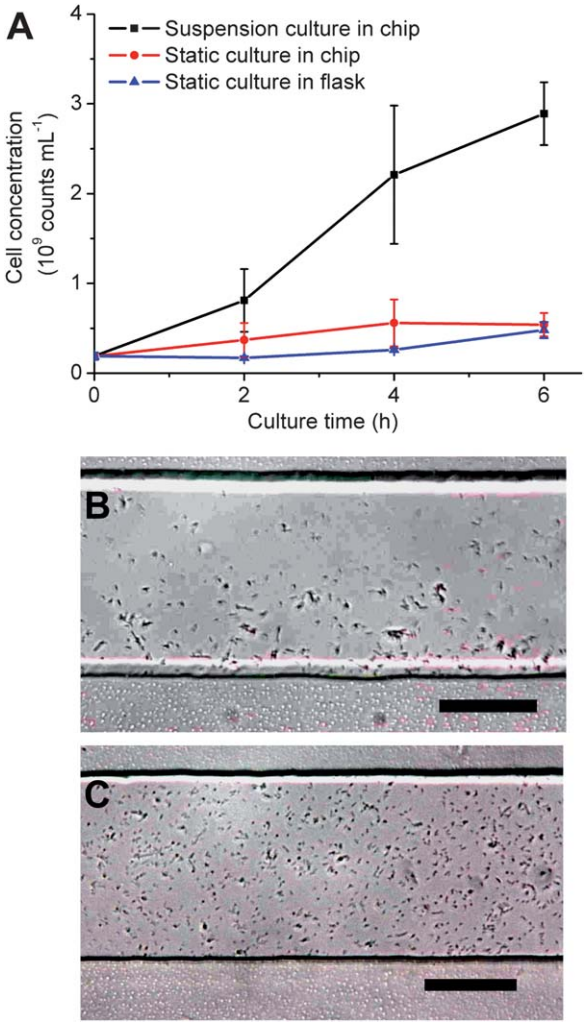

Fig. 3 (A) Growth curve of *E. coli* in chip under static culture and suspension culture with 2.50 Hz pumping frequency. (B) Optical micrograph of the static culture of *E. coli* in chip at 6 h. (C) Optical micrograph of the suspension culture of *E. coli* in chip at 6 h. Scale bar: 50 μm.

bubbles in ordinary suspension culture but was relatively simple and effective in microchannels. To further scale up in degrees of integration, more culture chamber loops can be replicated and integrated into the column of culture chamber loops without increasing the number of control channels.

Effect of micropump frequency on the growth of *E. coli*

The degree of mixing affects important factors in bacterial growth such as the mass transfer, oxygen transfer and shear force, and thus plays a vital role in microbial culture.[19,20] We investigated the effect of flow rates in culture loops under various micropump frequencies. As shown in Fig. 4A, the flow rate rose from 48 μm s^{-1} to 103 μm s^{-1} as the pumping frequency increased from 0.62 Hz to 2.50 Hz. Further increase in pumping frequency did not result in higher flow rate. The flow rate remained nearly constant at 100–110 μm s^{-1} for the frequency range between 2.5 and 9 Hz. The flow rate at frequency over 9 Hz decreased rapidly. This dependence qualitatively agrees with previous reports.[14] To evaluate the impact of micropump frequency, an 8 × 4 chip was

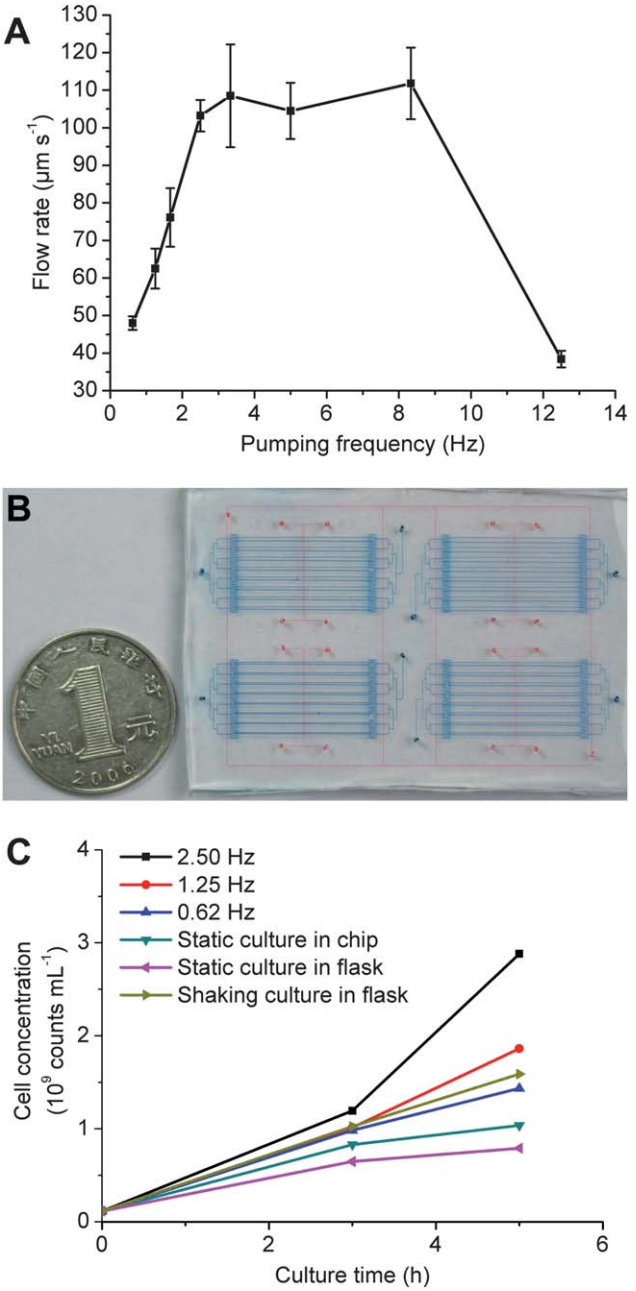

Fig. 4 (A) Flow rate at different pumping frequencies. Actuation pressure: 75 kPa. (B) Photograph of suspension culture chip with 8 × 4 identical culture loops. A red dye solution was loaded in control channels and a blue dye solution was loaded in culture chamber loops. The diameter of the coin is 25 mm. (C) Growth of *E. coli* in chip under pumping frequencies of 2.50 Hz, 1.25 Hz and 0.62 Hz.

used for *E. coli* culture, in which the four columns of culture chamber loops were pumped at 0 Hz (static), 0.62 Hz, 1.25 Hz, and 2.50 Hz, respectively. The multi-channeled chip ensured identical environmental factors except the flow rate (Fig. 4B). The results of *E. coli* growth under these conditions are shown in Fig. 4C. The higher the micropump frequency applied, the faster the growth attained. The microfluidic culture at 2.50 Hz pumping frequency showed faster *E. coli* growth than in conventional shaking culture in flasks, and *E. coli* growth at 1.25 Hz

micropump frequency was approximately the same as that in shaking culture. In conventional flask and fermentor cultures, high agitation usually results in higher biomass growth rate due to many factors such as mass transfer of nutrients and diffusion of oxygen and carbon dioxide; shear force may also affect cell growth.[21–23] Varied flow rate due to pumping frequency in our culture chips may have similar effects. The pumping frequency could then be used as an experimental handle to adjust the *E. coli* growth rate just like shaking or stirring speed in conventional culture methods.

Suspension culture of other strains

To testify its general applicability, we employed the microfluidic chips to suspension culture of other microbial strains. *Bacillus subtilis* is an important bacterium in molecular biology and industrial applications.[24] We cultivated *Bacillus subtilis* in parallel in the chip under suspension and static modes for 5 h (Fig. 5A–B and videos in the ESI†). Unlike *E. coli*, *Bacillus subtilis* was prone to chaining in static culture. The length of the cell chains could reach several tens of micrometres. It stuck to certain regions of the channel wall and formed clusters. In suspension culture, micropumps helped to disperse strains uniformly in the whole loops, mainly in single bacterium or pairs. Thus, micropump mixing also benefited the growth of *Bacillus subtilis*. The cell concentration in suspension culture at 5 h was 1.67×10^9 counts mL^{-1} while it was only 0.13×10^9 counts mL^{-1} in shaken flask culture. Surface modification of PDMS channels was necessary in these experiments to reduce bacterial adhesion to the channel walls and maintain continuous circulation of the culture media.[25,26]

Pseudomonas stutzeri is commonly used in pollutants biodegradation.[27,28] *Zymomonas mobilis* has been of considerable interest in recent years for bioethanol production.[29] We also cultured the two bacteria on the designed chip separately (see Fig. 5C–D and videos in the ESI†). Parallel micropumps combined with surface modification of the microfluidic chip were shown to be effective for suspension culture of these two strains. The cell concentration of *Pseudomonas stutzeri* was 2.81×10^9 counts mL^{-1} at 5 h, while only 0.17×10^9 counts mL^{-1} was achieved in conventional shaken flask culture at that time. The cell concentrations of *Zymomonas mobilis* at 5 h were 1.22×10^9 counts mL^{-1} in chip culture and 0.92×10^9 counts mL^{-1} in shaken flask culture. These results indicated that the designed microfluidic chip is widely suitable for bacterial suspension culture. Comparing the suspension growth results in chip to that in shaken flasks, aerobic bacteria *Bacillus subtilis* and *Pseudomonas stutzeri* reproduced much faster in chip while facultative anaerobes *E. coli* and *Zymomonas mobilis* had relatively higher proliferation rates. High concentration of dissolved oxygen in well-mixed phases has been found to be beneficial to cell growth.[19] It was also reported that effective oxygenation in a static microchannel results in a superior *E. coli* growth rate to that in flask culture.[30] We speculate that the gas permeability of PDMS may lead to higher dissolved oxygen in channels and faster discharge of CO_2. Both factors are beneficial for aerobic bacterial growth but less influential for facultative anaerobes.

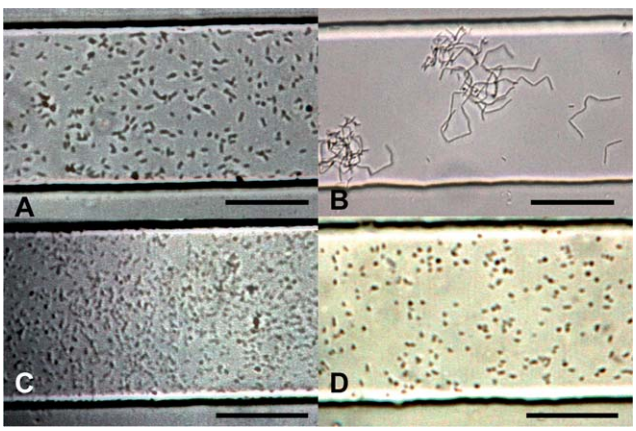

Fig. 5 Micrographs of chip cultures of strains at 5 h. (A) *Bacillus subtilis* in suspension culture. (B) *Bacillus subtilis* in static culture. (C) *Pseudomonas stutzeri* in suspension culture. (D) *Zymomonas mobilis* in suspension culture. Scale bar: 50 μm.

Conclusions

A scalable multi-channel microfluidic chip for bacterial suspension culture was described. Nearly equal cycling flow rates were achieved in identical parallel culture chamber loops. The culture loops could be replicated in large numbers for high throughput suspension culture of bacteria in the chip. The results showed that the growth of *E. coli* in the chip was equivalent or superior to conventional suspension culture on shaking beds. Suspended cultivation of other strains such as *Bacillus subtilis*, *Pseudomonas stutzeri*, *Zymomonas mobilis* in the chip was also demonstrated. The chip design is easy and low-cost for fabrication, which allows for high throughput cell suspension culture and other liquid–solid mixing tasks in microfluidic devices.

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