Electronic Supplementary Information (ESI)
Microfluidic Microbioreactor for Lower Cost and Faster Optimisation of Protein Production

Mayur Parekh[a], AbdulAziz Ali[a], Simon Bateson[a], Fathi Abugchem[a], Leon Pybus[b], Christopher Lennon [b], Zulfiqur Ali[a†]

[a]Healthcare Innovation Centre, Teesside University, Middlesbrough, Tees Valley, TS1 3BX, UK

[b]FUJIFILM Diosynth Biotechnologies, Belasis Avenue, Billingham, TS23 1LH, UK

Corresponding author: Z. Ali[†]

Materials and Methods

Media

0.02% biotin- 20 mg biotin (Merck, cat. no. **B4501)** is dissolved in 100 mL deionised (DI) water. The solution was sterilised by filtration and stored at 4 ^{o}C.

10XGY- 100 mL glycerol (Merck, UK, cat. no. G5516) is dissolved to 900 mL DI water. The solution was then sterilised by autoclaving and stored at room temperature.

10X Yeast Nitrogen Base (YNB) –16.8 g yeast nitrogen base (Millipore, UK, cat. no. 51483) is dissolved in 200 mL DI water. The solution was sterilised by filtration and stored at 4 ^{o}C.

Buffered minimal glycerol media prepared by adding 100 mL 1 M potassium phosphate buffer, 100 mL 10X YNB, 100 mL 10X glycerol, 2 mL 0.02% biotin, 1 mL trace metal solution A and 1 mL trace metal solution B was added and make up to 1000 mL by DI water. The solution was sterilised by filtration and stored at room temperature.

Table 1 Composition of trace elements solution A and B

Trace metal solution A	Amount	Cat. no
Calcium sulphate ($CaSO_4.2H_2O$)	0.9 gL^{-1}	C3771 (Sigma-Aldrich, UK)
Magnesium sulphate ($MgSO_4.7H_2O$)	11.17 gL^{-1}	M2773 (Sigma-Aldrich, UK)
Zinc sulphate ($ZnSO_4.7H_2O$)	20.0 gL^{-1}	Z0251 (Sigma-Aldrich, UK)
Manganese sulphate ($MnSO_4.H_2O$)	3.0 gL^{-1}	M7899 (Sigma-Aldrich, UK)
Sulphuric acid (H_2SO_4)	5 mL	339741 (Sigma-Aldrich, UK)
DI water	Up to 1000 mL	
Trace metal solution B		
Ferrous sulphate ($FeSO_4.7H_2O$)	6.5 gL^{-1}	F8633 (Sigma-Aldrich, UK)
copper sulphate ($CuSO_4.5H_2O$)	6.0 gL^{-1}	C8027 (Sigma-Aldrich, UK)
DI water	Up to 1000 mL	

Buffered minimal methanol media prepared the same way by replacing 10X glycerol with 20.87 gL^{-1} methanol. Buffered minimal glycerol-methanol or sorbitol-methanol or glucose-methanol media were prepared in the same way by replacing the 10X glycerol with respectively:10 gL^{-1} methanol and 10 gL^{-1} glycerol; 10.44 gL^{-1} methanol and 9.89 gL^{-1} sorbitol; 10.44 gL^{-1} methanol and 9.78 gL^{-1} glucose.

Microbioreactor system

The pressurised fluid driving system comprised an electronic pressure regulator (ITV 0010-2 UBL, SMC Pneumatics, UK) controlled by voltage supply for specific flow requirements and a flow sensor (SLI 0430, Sensirion, Switzerland). The media reservoirs were connected with PTFE tubing (0.5 mm, internal diameter Sigma, UK) to the microbioreactor element and flow sensor via a i3DP printed fluidic manifold with a series of normally closed solenoid valves (LVM09R3Y1-5A-3-Q, SMC pneumatics, UK) and a four-way rotary valve (Omnifit, UK) controlled by the GUI for fluidic selection and continuous feeding of media for cultivation. To prevent the clogging of tubing, media was pre-sterilized with 0.2 µm syringe filter to the media reservoir in aseptic condition.

The pH and dissolved oxygen (DO) were optically measured with Presens sensor spots and electro-optical modules (EOM-O2-mini and EOM-pH-mini). The data for the OD, pH and DO are recorded through a GUI written for the Raspberry Pi microcomputer. The DO was controlled by varying the oxygen concentration in the headspace chamber over the growth reactor using gas mass flow controller (MC-500SCCM-D, Alicat Scientific, Netherlands). The DO level was recorded every 2 sec by the optical DO sensor and received by Qt software. The required amount of pure oxygen was supplied in the top headspace chamber of the microbioreactor and this diffuses through the silicone membrane and into the culture medium to maintain the required set point.

The microbioreactor contents are stirred with a magnetic stainless-steel bar (VP 736-1) having a central silicone 'O' ring for support. This was spun at reproducible speeds up to 1500rpm with an external magnet attached to a miniature stepper motor controlled by a PIC16F1829 microcontroller and LB1909 (ON semiconductor) motor driver. The support keeps the rotating bar (0.5 mm) above the chamber bottom, preventing shear damage to cells. The temperature was controlled by maintaining an ITO glass heater plate (Cell Microcontrols, Norfolk, US, cat. no. HI 711Dp) digital mTCII 2 channel micro-temperature controller (Cell microcontrols, Norfolk, US). Reactor effluent samples were collected in 1.5 mL Eppendorf tube that was held in a Peltier cooler. The pneumatic fluid driving system comprised an electronic pressure regulator (ITV 0010-2 UBL, SMC Pneumatics, UK) controlled by voltage supply for specific flow requirements and a flow sensor (SLI 0430, Sensirion, Switzerland). The interface between microbioreactor and reservoir was accomplished through 0.5 mm (internal diameter) polytetrafluoroethylene (PTFE) tubing (Sigma, UK) and 5-way 3D printed manifold. The media selection was realised by using normally closed solenoid valves (LVM09R3Y1-5A-3-Q, SMC pneumatics, UK) controlled by software.

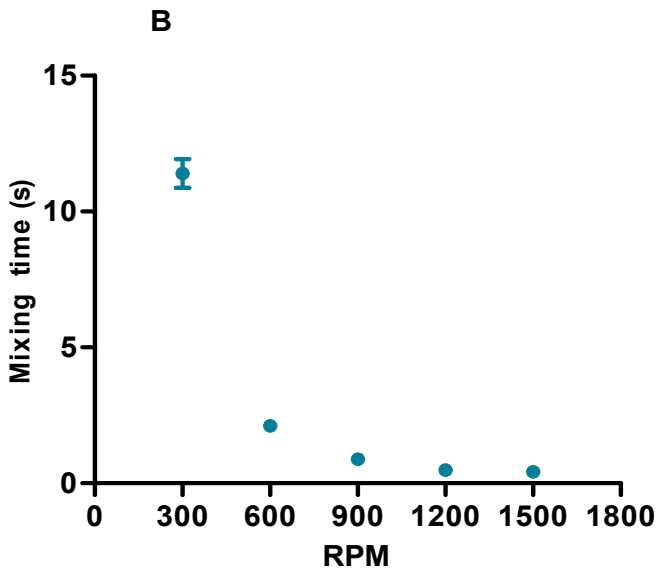

Fig. 1 Mixing times at different speed in 1 mL microbioreactor, data are for three repeat experiments

Sterilisation of the microbioreactor

The sterilisation and cleaning of the microbioreactor is an important step for running cultivation without contamination and increasing the reproducibility of data. All large-scale bioreactors are sterilised by autoclaving at 121 ^{o}C as they are mostly stainless steel and glass. However, this is not applicable with polymer, such as PMMA, VeroWhite plus and silicone elastomer which are used for the microbioreactor construction. Each material or polymer has its own specific resistance toward different chemicals, which makes sterilisation of microbioreactor difficult. After examining different characteristics of the used materials in the microbioreactor system, different methods for sterilisation were used for the different elements.

The microbioreactor cleaning is performed as follow:

1. The microbioreactor is disassembled to parts.
2. Each reservoir bottles, sample collecting bottle and tubing for inlet and outlet for manifold were sterilised by autoclaving at 121 ^{o}C for 15 minutes.
3. The microfluidic manifold was submerged in 70% ethanol for 30 minutes in Biosafety cabinet class II. The manifold was then rinsed with sterile water to remove 70% ethanol and then left to dry inside the Biosafety cabinet class II bench.
4. The fluorescence sensor spot for measurement of DO and pH is glued to the transparent bottom layer of the microbioreactor and all three layers were bonded by using double sided adhesive tape in Biosafety cabinet class II. The DO and pH sensor are pre-calibrated.
5. The Microbioreactor is assembled in the Biosafety cabinet class II bench.
6. The reservoir bottles were connected to microfluidic manifold and microbioreactor including sample collector. The flow sensor is also connected between microbioreactor and sample collector to provide feedback control to flow rate for continuous cultivation. Each reservoir bottle was filled with 100 mL sterile media.
7. The sterile and airtight microbioreactor is properly positioned on the platform outside of in Biosafety cabinet class II.
8. The pressurised air supply is connected to reservoirs and temperature sensor is connected to transparent heater.

Optical density sensor

Optical density was used to monitor biomass by transmission measurement at 600 nm. Light from 600 nm LED (HLMP-C423, Broadcom, San Jose) and which was placed in Nylon holder to avoid stray light was passed through the microbioreactor through a 600 µm optical fibre. After passing through the sample, the transmitted light is collected by an optical fibre (M29L01, Thorlabs, UK) and sent to a photodiode (VTB8441BH, Excelitas Technologies, Massachusetts) through 600 µm optical fibre. To maintain a consistent path length, optical density was measured through a rigid section of the growth chamber through glass window (Thorlabs, UK). The OD was determined using Equation 1.

$$OD = \log I_{reference}/I_o \quad \text{..... [1]}$$

where $I_{reference}$ is the intensity of the medium (blank) for a given experiment and I_0 *is* the intensity of the signal. These measurements are only valid in the linear region. The curvature of the silicone membrane and path length are important parameter which affect the optical density measurements.

Correlation of Optical Density with cell density biomass

E. coli

This was done by first cultivating *E. coli* BL21 using specified medium in shake flask overnight. The cultures were then harvested and concentrated by centrifugation (3900 rpm, 10min). The OD of different cell culture sample were measured both in the microbioreactor and in a laboratory spectrophotometer at 600 nm (CamSpec 108,

Leeds, UK). For the spectrophotometer, highly concentrated samples were diluted 10 or 50 times and the final OD_{600} was calculated by multiplying with dilution factor.

To correlate higher cell density biomass with scattered light intensities, the *E. coli* BL21 strain was cultivated in LB media in two 250 mL shake flask overnight (50 mL, 37 °C, 150 rpm shaking). The cultures were harvested and concentrated ten times by centrifugation for 10 min at 3900 rpm. For calibration, different cell biomass concentrations (0-15 OD) were placed inside the microbioreactor and absorbance measured. Correlation between OD and dry cell weight (gL^{-1}) was achieved by taking five 2 mL samples from a 100 mL shake flask and the OD measured. The samples were then centrifuged and washed with deionised water. Samples were dried for more than 24 hours at 105 ^{0}C oven and weighed. The conversion factor for OD to g_{dcw} is found to be 0.30 ± 0.08 g-dcw/OD.

Pichia pastoris

To correlate higher cell density biomass with scattered light intensities, the *P. pastoris* CLD804 aprotinin strain was cultivated using Buffered minimal glycerol media in shake flask overnight (50 mL, 30 °C, 225 rpm). The cultures were harvested and concentrated by centrifugation (3900 rpm, 10min). The OD of different cell culture sample were measured in the microbioreactor and in the spectrophotometer at 600. For spectrophotometer, highly concentrated samples were diluted 10 or 50 times to linear range, and the final OD600 was calculated by multiplying with dilution factor. This can help to establish correlation of OD of cell culture in microbioreactor and standard conventional offline techniques.

The measured OD value is converted to cell density (DCW gL^{-1}) with previously calibrated value of cell biomass. These DCW were then used to calculate the μ:

The measured OD value is converted to cell density (DCW gL^{-1}) with previously calibrated value of cell biomass.

These DCW were then used to calculate the μ:

$$\mu = \frac{lnC_2 - lnC_1}{t_2 - t_1} \quad \text{..... [2]}$$

Where: C = dry cell weight (gL^{-1})

t = sampling time (h)

Postprocessing of 3D printed parts

All 3D printed parts were produced in matt finish in which outer surface of parts were covered with support material which was required to be removed by hand or washing with water. Removal of support material from the threaded area and microchannel required cleaning with a tip, use of pressurized water and sonication in 2% NaOH solution for a few hours.

Preparation of extraction medium

3D printed microstructure (figure S1) with dimension of 10×45×2 mm (with pore size of 0.8 mm) were printed from Objet24 (VeroWhiteplus). This simple design was cleaned with water and followed by caustic soda solution to remove support material and dry under normal air. The microstructure was sterilized by 70% ethanol followed by 30 min UV irradiation. After sterilisation, this design was dipped in DMEM media and CD OptiCHO (1.25 cm^2/mL). This media was incubated at 37 ^{o}C from 24 to 72 hrs at 140 rpm shaking for maximum extraction. This extracted media is used for MTT cell viability assay (J774) and growth curve study (CHO) to assess any toxic effect of photopolymers on cells.

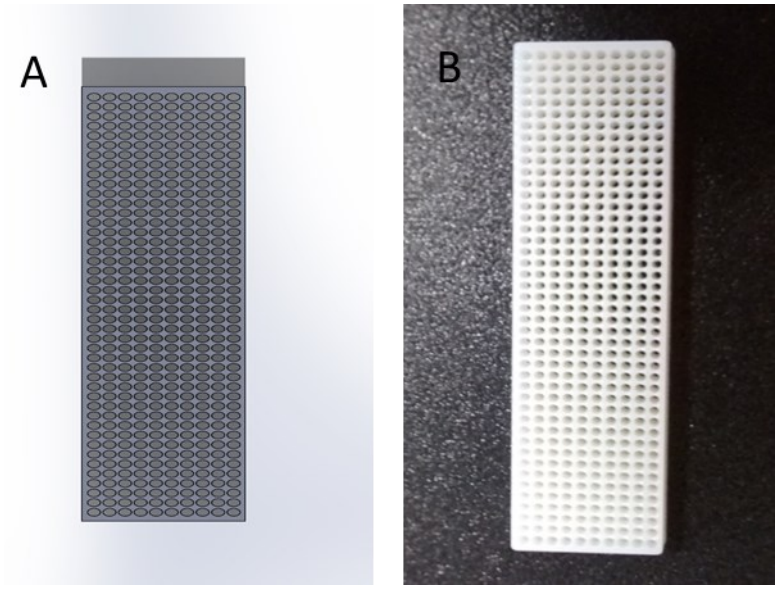

Fig. 2 (A) CAD image of Mesh like structure (B) photo of the printed structure from VeroWhiteplus

MTT assay

To detect any possible toxic effect of photopolymers, MTT assay were carried out in standard 96 well plate (Sarstedt, Germany). J774 cells were seeded at 2.5×10^4 cells/well with 200 µl final volume in each well and maintained in a CO_2 incubator at 37 °C, 5%CO_2 for 24 hrs. The normal media was removed, and extraction media was added to each well for a 24 hrs cultivation period. MTT reagent (10 µl) was added after incubation and plate was incubated overnight. The next day, medium was removed and 100 µl solubilisation buffer was added to dissolve insoluble formazan crystals and plate was incubated overnight. The absorbance was measured at 562 nm with a plate reader and the relative cell viability (%) was calculated as follows.

% cell viability = $A_{test}/A_{control} \times 100$ …… [3]

The same procedure was followed for control (normal media).

Growth curve study

Biocompatibility was carried out by growth curve study. For this, cells were cultured in 125 mL shake flask with initial density of 0.2×10^6 cells/mL using extracted medium from VeroClear, VeroWhiteplus plus and double-sided adhesive tape and incubated into CO_2 incubator at 37°C, 5% CO_2 and 140 rpm. Cells were counted everyday by withdrawing 1 mL sample from flask of each condition. The experiments were carried out up to 6 days and graph was plotted (i) days vs viable cell density (ii) days vs % viability. Moreover, specific growth rate for each condition were calculated by assuming first order growth during initial stage of exponential growth (equation 4).[1]

$$\frac{dX}{dt} = \mu[X] \quad \text{…….} \quad [4]$$

Equation 5 is an integrated version of Equation 4.

$$\ln[X] = \mu t + \ln[X]_0 \quad \text{……} \quad [5]$$

Where [X] = cell concentration at a given t time

$[X]_0$= initial cell concentration at time t=0

µ= specific growth rate (h^{-1})

µ was calculated from the slop of the plot of ln[X] versus time t.

Equation 6 is used to calculate doubling time for CHO cell to each condition.

$$t_d = \frac{0.693}{\mu} \quad \text{……} \quad [6]$$

Where t_d= doubling time

Table 2 Biocompatibility study of CHO cells in extracted media from materials in shake flask after 4 days; TCD= total cell density ($\times 10^6$ cells/mL), μ= growth rate (h^{-1}), t_d= doubling time

Material	TCD	% viability	μ	Avg μ	t_d	Avg t_d
Control	5.7	92.46	0.025	0.027	27.2	25.19
	6.22	98.23	0.029		23.51	
	6.82	98.9	0.028		24.87	
VeroWhiteplus	5.53	97.74	0.026	0.025	27.83	29.16
	5.25	96.19	0.024		30.2	
	5.55	95.86	0.024		29.46	

Analytics

The release of aprotinin protein from *P. pastoris* CLD804 fermentation were qualitatively assessed by NuPAGE® Novex 4-12% Bis-Tris 12 well 1 mm pre-cast gel kits from Invitrogen (Paisley, UK, cat. no. NP0322BOX). For SDS-PAGE electrophoresis, 40 µL of fermentation supernatant, 10 µL reducing agent, 25 µL sample buffer (NuPAGE® LDS, Invitrogen, Paisley, UK, cat. no. NP0007) and 25 µL ultra-pure H_2O were mixed in PCR sample tube and heated for 15 minutes at 90°C. A 5 µL protein ladder (Mark 12™ unstained, Invitrogen, Paisley, UK) was first loaded onto the gel followed by the 10 µL samples and gaps were left between samples, ladder and between different cultivations. The gel was run for 35 min at 200 V in MES (2-(*N*-morpholino) ethane sulphonic acid) buffer. Gels were then removed from their casing, washed in deionised water, then fixed in a 15 mL Instant Blue stain per gel and incubate until the bands are resolved. Following the gels were washed with deionised water and scanned with GS-900 calibrated densitometer (Bio rad, UK).

The protein (total soluble protein) quantification was carried out by first using 1 g/L bovine serum albumin (BSA) stock solution to generate a standard calibration curve. The standards and samples were diluted with filtered Bradford reagent (1:50) and incubated at room temperature for 10 minutes. 2 µL of standards were pipetted on pedestal for calibration curve. For the samples, 2 µL were loaded into pedestal. For accurate measurement, sample volume kept constant throughout experiment. Absorbance were recorded at 595 nm and values were compared to the calibration curve in order to calculate protein concentration.

For DNA quantification, DNA standards were first generated by serial dilution with ultra-pure water from stock DNA solution. 10 µL of each DNA standards were loaded into 500 µL Eppendorf tube in triplicate. PicoGreen® dsDNA reagent was diluted 200-fold in TE buffer to create the working reagent (WR) for the assay in dark because of photosensitivity of the reagent. 10 µL of samples were then also added into a clear, polypropylene 500 µL Eppendorf tube in triplicate and 190 µL of WR was then added to all Eppendorf tubes and incubated at room temperature for 5 minutes in dark to exclude light. The fluorescence was measured by Qubit 4 fluorometer (Invitrogen, UK) with fluorescence settings for the excitation and emission wavelengths at respectively 480 nm and 520 nm. The DNA concentration in the samples were then calculated by using a standard calibration curve.

Protease quantification was carried out by first preparing a FTC-casein stock solution (5 mg/mL), by dissolving 2.5 mg of FTC-casein (fluorescein isothiocyanate labelled casein) in 500 µL ultra-pure water, and preparing 12 aliquots of 40 µL. The working reagent (WR) generated by 500-fold diluting 20 µL BupHTM TBS buffer. Lyophilised TPCK Trypsin (50 mg) was dissolved in TBS buffer (1 mL) and aliquoted into 40 µL. TBS buffer is used

to create a range of trypsin standards by seral dilution. 10 µL of samples were added to clear polypropylene Eppendorf tube in triplicate. 10 µL of TBS diluted trypsin was added into the clear polypropylene Eppendorf tube in triplicate to generate standard curve. Finally, 190 µL of WR was added to each Eppendorf tube and was incubated for 5 minutes at room temperature and analysed by using a Qubit 4 fluorometer with fluorescence settings at an excitation/ emission wavelength of 480 nm / 520 nm.

Sorbitol in the supernatant was determined by dissolving the sorbitol enzyme mix in 220 µL DI H_2O. The sorbitol developer was dissolved in 1 mL DI H_2O and solution was kept on ice while using. Sorbitol standards were created by diluting 10 µL of 100 mM stock with 990 µL of DI H_2O. 0, 2, 4, 6, 8 and 10 µL of sorbitol standards were added into 96 well plate in triplicate and volume adjusted with assay buffer (50 µL/well) to produce 0, 2, 4, 6, 8 and 10 nmol/well of the sorbitol standard. The sample are diluted with DI H2O 1:20 and centrifuged to remove any insoluble particles and 10 µL of sample was added to 96 well plate in triplicate and volume adjusted to 50 µL with assay buffer. The 50 µL sorbitol reaction mix was created for each well by mixing 36 µL assay buffer, 2 µL probe, 2 µL enzyme mix and 10 µL developer. The 50 µL reaction mix was added to each well containing the sorbitol standard and test samples. The 96 well plate was incubated for 60 min at 37 ^{o}C in the dark to protect the assay mix from light.

i3DP Manifold

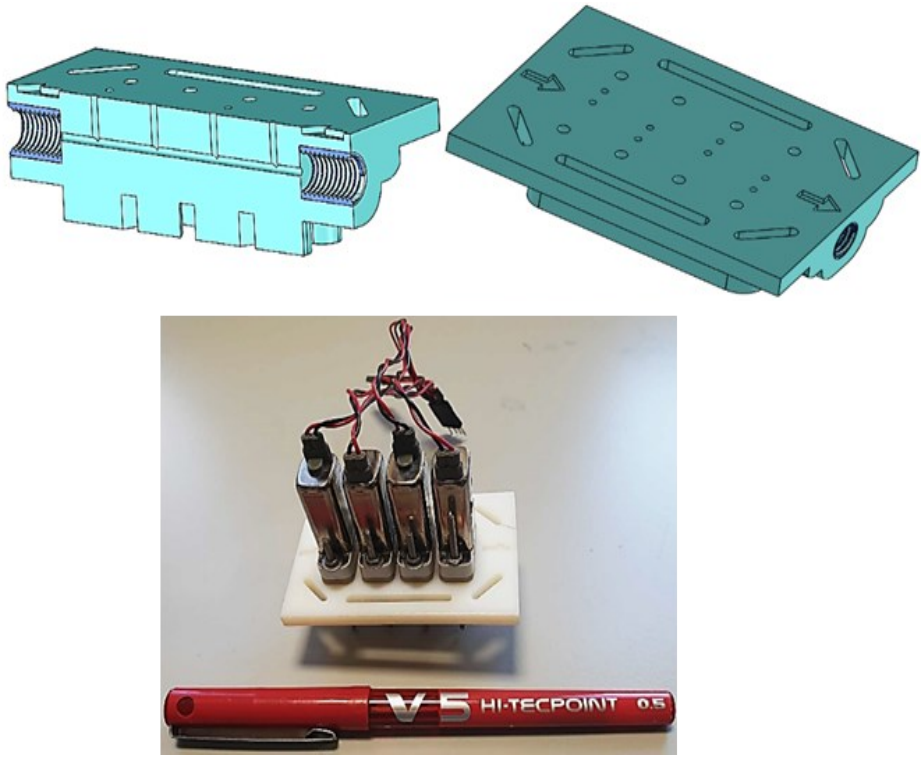

Fig. 3 (Top Left) CAD sectional view of the microfluidic manifold showing the common microchannel (horizontal) and the four inlets (uprights); (Top Right) isometric view of the manifold. Bottom, a photograph of the 3D printed manifold with the SMC NC solenoid valves on.

i3DP supporting platform for microbioreactor

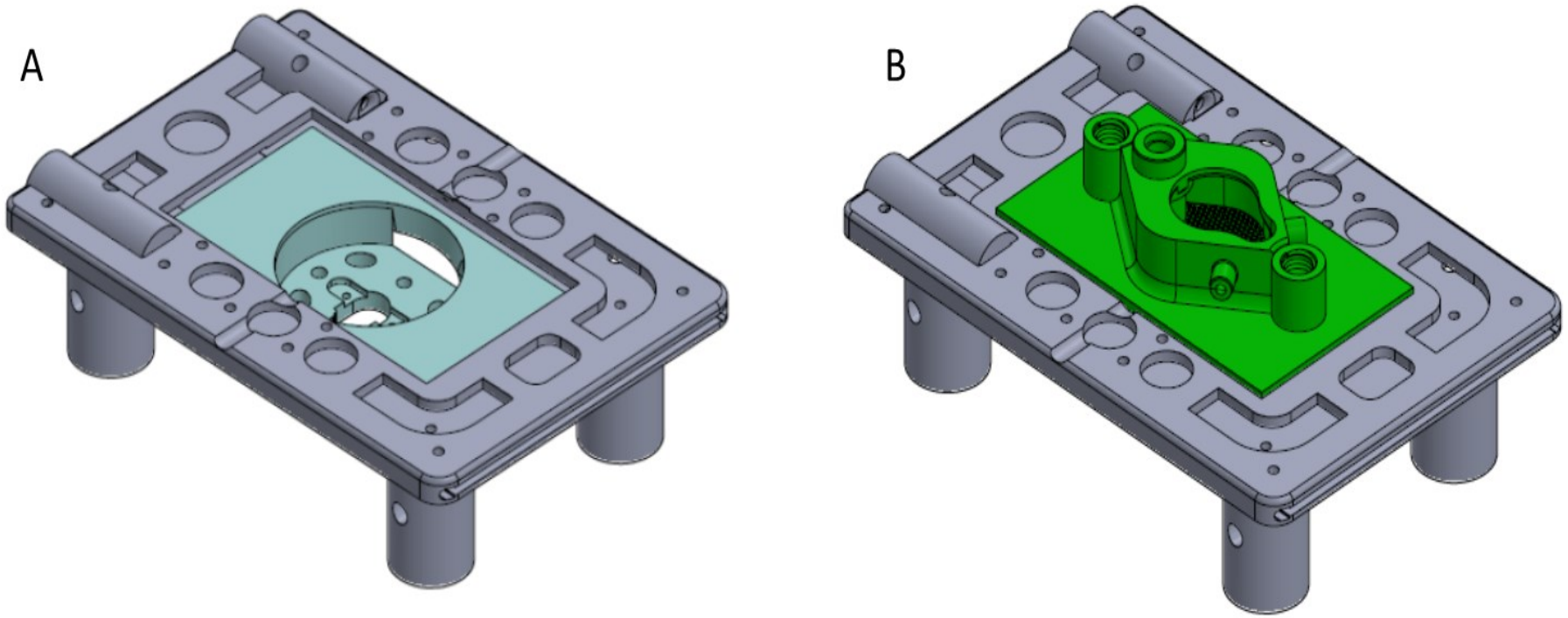

Fig. 4 (A) and (B) i3DP microbioreactor supporting platform

References

1 J. López-Meza, D. Araíz-Hernández, L. M. Carrillo-Cocom, F. López-Pacheco, M. del R. Rocha-Pizaña and M. M. Alvarez, *Cytotechnology*, 2016, **68**, 1287–1300.