Microfluidic Microbioreactor to Reduce the Cost and Speed Up Optimisation of Protein Production

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ABSTRACT

Optimisation of bioprocesses relies heavily on use of microtitre plates, shake flasks, bench scale bioreactors or the use of expensive automated robotic systems. These are used with a variety of analytical tools to ensure that the bioprocess is robust with consistent product quality. There is a need for a low-cost and flexible system that can be used for fluidic processing and integrated with existing bioprocess analytics to aid the development and optimisation of bioprocesses. Here we demonstrate a low-cost polymer microfluidic microbioreactor with a working volume of 1 mL, integrated with optical sensors for pH, oxygen and cell density, and maintained at constant set temperature for the optimisation of recombinant protein production from *Pichia pastoris*. The microbioreactor comprises functional layers which are joined using a pressure sensitive adhesive tape, and incorporates a miniaturised stirrer as a mimic for larger scale bioreactor. Oxygen is transferred from a headspace enclosure, formed using inkjet 3D printing, through a gas-permeable membrane within the microbioreactor with a K_La of 90 at 1500 rpm. A pressurised fluid driving system is used with flow rates controllable to 0.7 µL/min with fluid switching from four reservoirs performed off the microfluidic microbioreactor element so that this can be produced at low cost using high replication techniques. Three repeat batch cultivations of *P. pastoris* showed good correlation for cell density, pH and DO with a maximum dry cell weight of 10±1 g/L. For the conditions of continuous cultivations used it was found, from the normalised aprotinin SDS-PAGE band intensity, that the recombinant protein production was higher in pure methanol (314±23) than methanol-sorbitol (202±17) feed, whilst it reduces over time with the methanol-glucose mixed feed. We show here that the microfluidic microbioreactor offers the potential to reduce cost and increase the speed of bioprocess development. This could also offer a flexible approach for a range of applications including within synthetic biology, cell and gene therapy and organ-on-chips.

Key words: Microfluidics; Microbioreactor; *Pichia Pastoris*; Continuous cultivation; Protein production; Cell culture

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1.0 Introduction

Cell culture derived biotherapeutics are important for the development of new medicines. A frequent requirement in the development of a bioprocess is initial screening of production strains, growth medium and production strategy (e.g. feeding strategy for recombinant protein production). Variations in the bioprocess parameters can change the molecular and cellular response of cells to the media in terms of cell growth that can affect the product yield, quality and production costs. It is, therefore, important to understand the main factors that produce high concentrations of recombinant proteins, and to be able to control the culture conditions during the bioprocess (Zepeda et al., 2018). This can involve large numbers of initial batch experiments in a combinatorial experimental design followed by iterative strain and process development. The current approaches, which typically use shake flask or micro-titre plates are often limited to single point measurements as well as being labour intensive, time consuming and expensive. There is a limited ability to learn optimised process parameters for process development and production scale up. The choices made in these experiments either remain fixed in the final process and/or entail expensive changes in process parameters at a larger scale. There is therefore a need for the development of new types of tools that can dramatically speed up the process of biotherapeutic protein production.

The methylotrophic yeast *Komagataella phaffii* also known as *P. pastoris (P. pastoris*) can grow to very high concentrations and is widely used for heterologous protein production within the biopharmaceutical sector. *P. pastoris* expresses recombinant proteins under regulation of alcohol oxidase promoters and the respective enzymes, alcohol oxidase 1 (AOX1) and alcohol oxidase 2 (AOX2)(Stratton et al.,). When methanol enters the cell, the transcription activity leads to expression of the enzyme which in turn leads to production of the desired protein. Given that the metabolism of methanol in methylotrophic yeast can be regulated by derepression, repression and induction mechanisms then the growth and expression from *P. pastoris* can be affected by a variety of factors including methanol concentration, temperature, pH, dissolved oxygen concentration, carbon source and feeding method (Zepeda et al., 2018). The use of pure methanol as the sole carbon source for continuous cultivation requires the necessary enzymes to be synthesised as soon as the methanol is fed to the culture. This can have a number of disadvantages at large scale including: high flammability, high enthalpy of combustion leading to heat generation and increase in the oxygen requirement; and methanol accumulation which can cause oxidative stress and loss of cell viability(Capone et al., 2015; Niu et al., 2013; Zahrl et al., 2017).

There is increasing effort on the development of miniaturised bioreactors, typically at the milliliter (mL) scale, as part of a broader drive to increase the chemical and biological information about bioprocesses (Marques and Szita, 2017; Pasirayi et al., 2011). Previously, we have used an ultra-low cost approach with a simple PTFE tube and a syringe pump for inoculum and nutrient feed (Rahman et al., 2009, Rahman et al., 2010). The large surface area to volume ratio can offer rapid heat and mass transfer but the approach is limited in the complexity of bioprocessing and with no integrated sensing. Parallel cultivations at the 10mL scale has been carried out using a single-use stirred tank bioreactor with gas-inducing impellers and headspace with sterile air saturated with water (Schmideder et al., 2015). The approach offers high oxygen transfer but there is a requirement for a separate liquid handling system, with limitations of size and cost for automation of fluidic operations. Buchenauer et

al., (2009) have developed an array of microbioreactors based on microtitre plates, with the microbioreactor comprising four reaction wells and two reservoirs which provide the feed and pH control. Pressure connection on the reservoirs and pneumatically actuated valves underneath the reservoirs and reaction wells are used to move fluid from the reservoirs to the reaction wells. A drawback to this approach is that mixing requires the microbioreactor to be placed on a shaker and more sophisticated fluid handling would require an external liquid handling system.

Microbial continuous culture has been demonstrated in a polymer-based microfluidic 150µl microbioreactor which is aerated through a PDMS membrane and with integrated optical density (OD), pH and dissolved oxygen (DO) measurements for prolonged periods of cultivation of *E. coli* (Zhang et al., 2006). An alternative approach of pneumatic bubble aeration to ensure sufficient oxygen transfer has been demonstrated within a multiphase microbioreactor for batch cultivation of *Saccharomyces cerevisiae*(Lladó Maldonado et al., 2019). A microfluidic device has been demonstrated for perfusion culture of yeast cells for continuous production of invertase (Brás et al., 2017). The device was operated over a 32 hr period, with potential limitations on the operation time from microbiological contamination and the viability of cells within a stationary phase culture. Moreover, it should be noted that the cell microenvironment is different to the macroscale environment; this may lead to differences in the cell behaviour. A number of other interesting approaches have been taken including a digital microfluidic system which has been used for culturing of mammalian cells over four days and used impedance measurements to determine proliferation (Shih et al., 2013).

Halldorsson et al., (2015) have described some of the advantages and challenges of using PDMS for the development of microfluidic devices for cell culture. We have previously used PDMS for the integration of normally closed pneumatically actuated valves within a microfluidic device to allow sophisticated processing for culturing of cells for cytotoxicity assay (Pasirayi et al., 2014). Lee et al., (2011) have used microfabrication processes with PDMS valves on rigid plastics to develop an elegant microbioreactor, with a 1mL working volume, that integrates multiple fluid inputs and outputs, on-chip peristaltic pumping and an oxygenating peristaltic mixer. This offers a means for control of fluids, oxygen and mixing for flexible applications within synthetic biology and programmable production of biologics (Perez-Pinera et al., 2016). The approach does, however, inherently require a complicated microfluidic chip design as well as considerable additional external peripherals. More generally, PDMS is widely used for prototyping of microfluidic devices but is less amenable for higher volume manufacturing of devices. We have shown the potential for the development of low-cost polymer microfluidic devices using high volume roll-to-roll manufacture, or other sheet processes, along with other high replication approaches such as hot embossing (Lakey et al., 2019).

Here we report a 1mL microfluidic microbioreactor with elements fabricated using inkjet 3d printing (i3DP) but amenable to manufacturing processes such as moulding, to reduce the overall cost of the device. The complexity of fluidic operations is carried out off-chip with non-disposable hardware elements using a flexible and controllable pressure driven system. The developed microfluidic microbioreactor system has been demonstrated for cultivation of *P. pastoris*, which has requirement for high oxygen transfer and grows to high cell density. We

show that the microfluidic microbioreactor system can be used with existing bioprocess analytics to investigate feeding strategies and gain valuable bioprocess information faster and with reduced cost.

2.0 Materials and Methods

2.1 Materials

P. pastoris CLD804 Mut$^+$ Aprotinin has been used and was gifted by Fujifilm Diosynth Biotechnologies (Billingham, UK). Strain maintenance was carried out in buffered minimal glycerol complex media with Zeocin (100 µg/mL) as selection marker for sterility during cultivation. The media used included 0.02% biotin (Merck, cat. no. B4501), 10XGY glycerol (Merck, UK, cat. no. G5516) and 10X Yeast Nitrogen Base (YNB) (Millipore, UK, cat. no. 51483) and buffered minimal glycerol media (SI, 1).

2.2 Microbioreactor element

The microbioreactor comprises a number of functional layers, joined with a pressure sensitive adhesive (Adhesive Research Inc, UK), and a working volume of 1 mL with a single inlet and outlet. Sensors are integrated within the microbioreactor for pH, oxygen and optical density (Fig. 1A, 1B)). The bottom optical sensing layer incorporates sensor spots for DO and pH (Presens) that are joined to a poly(methyl methacrylate) (PMMA) substrate using adhesive, There is an access window for optical density measurements. The middle growth chamber layer and top layers are fabricated using i3DP (Stratasys) with VeroWhitePlus material. The middle layer incorporates a stainless-steel stirrer bar with a centre O-ring. The top layer provides the chip to world interface for the fluidics and optical sensors. It also incorporates a gas headspace (2mL) that is separated from the growth chamber by a gas-permeable membrane and linked with a low pressure oxygen inlet and outlet gas stream to provide oxygen to the growth chamber. The top layer has a mesh structure integrated to support the gas-permeable membrane.

2.3 Microbioreactor control platform

The microbioreactor control platform (Fig. 2A, 2B) provides the fluidic control using a combination of pressurised fluidics, pneumatics, electroactuation, flow measurement, PID control and fluidic architecture for the delivery cell culture media and reagents. The platform with the graphical user interface, on a single board computer, allows control and monitoring of the key parameters including fluid flow rate, pH, oxygen, optical density, cell viability and temperature.

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) PTFE tubing (Sigma, UK) and 5-way manifold valve (Kinesis, UK). The media selection was realised by using normally closed solenoid valves (LVM09R3Y1-5A-3-Q, SMC pneumatics, UK) controlled by software. We used PMMA optical fibres (Thorlabs, UK, cat. no. M29L01) with 600 µm excitation and collecting fibres to measure optical density by monitoring the transmission though the flat microbioreactor window parts; this allowed linear correlation between OD and cell dry weight up to 15 g/L. Dissolved oxygen (EOM-tO2-mini, Presens, Precision sensing, Germany) and pH (EOM-pH, Presens, Precision sensing, Germany) were measured by fluorescence

spectrophotometry with optical fibres provided by the manufacturer. The temperature was controlled by maintaining an ITO glass heater plate (Cell Microcontrols, Norfolk, US, cat. no. HI 711Dp) at 30^{o}C with digital mTCII 2 channel micro-temperature controller (Cell microcontrols, Norfolk, US). Reactor effluent samples were collected in 1 mL Eppendorf tube that was held in a Peltier cooler.

2.4 Batch and continuous cultivation

All relevant parts of microbioreactor platform, including the microbioreactor element, were sterilised (SI, 1.2). The medium was pumped through the microbioreactor to remove air and fill with media for optical density reading before starting experiment. A frozen vial of *P. pastoris* CLD804 Aprotinin was thawed in 5 mL BMGY or defined medium containing 100 mg/L of Zeocin (Thermo Fisher Scientific, UK, cat. no R25001) in a test tube and incubated overnight at 30^{o}C. The Optical Density (OD) at 600nm was monitored until the culture reached 1-2. It was then transferred to 50 mL of fresh medium (250-mL shake flask) with a starting OD of 0.2. The inoculated shake flasks were incubated on shakers (225 rpm) at 30^{o}C. OD was monitored until the OD within the flasks reached 6-10. At this point, the culture was used to inoculate the microbioreactor. Batch cultivation is started with initial conditions of pH: 6.9-7.1, aeration: 0-25 mL Oxygen, stirring: 0-1800 rpm, 0.652 C-mol-L^{-1} glycerol and methanol (50% C-molC-mol^{-1} glycerol-methanol) as a carbon source, temperature: 30°C. Stirring rate and aeration were automatically controlled according to the amount of dissolved oxygen available during the cultivation. Completion of the batch phase was followed by continuous cultivation of *P. pastoris* at a 0.07 h^{-1} dilution rate using three different media with different carbon source, specifically: 100% methanol C-mol (0.652 C-mol-L^{-1}); 50% methanol sorbitol C-molC-mol^{-1} (0.652 C-molL^{-1}) and 50% methanol glucose C-molC-mol^{-1} (0.652 C-mol-L^{-1}).

2.5 Analytics

SDS-PAGE electrophoresis was used for determination of release of aprotinin protein from *P. pastoris* CLD804 cultivation. The Bradford assay (Bio-Rad protein assay) was used to quantify total secreted protein in supernatant using a Nanodrop One (Thermo Fisher Scientific, UK) by measuring absorbance at 595 nm. The total DNA release during the cultivation of *P. pastoris* CLD804 aprotinin was measured using a Quant-iTTM PicoGreen® dsDNA reagent assay kit from Invitrogen (Paisley, UK, cat. no. P7589). Fluorescence was measured using a Qubit 4 fluorometer (Invitrogen, UK) with excitation and emission at respectively 480 nm and 520 nm and the DNA concentration in the samples determined from a standard calibration curve. The protease released into the cell culture supernatant was quantified using a Pierce® fluorescent protease assay kit (Thermo Fisher Scientific, UK, cat. no.23266) and determination of fluorescence in a similar manner as for the DNA quantification. Analysis of the substrate and metabolites was carried out by first collecting the culture sample in an Eppendorf kept at 0-4^{o}C in sample holder and stored at -20^{o}C. Methanol was determined by YSI (Yellow Spring Instruments, UK). Cedex Bio HT analyser (Roche Diagnostics, Germany) was used to determine the glucose, acetate and ammonia concentrations. Sorbitol in supernatant was determined by colorimetric assay kit (Abcam, UK, cat. no. ab118968) and the absorbance was measured in microwell plate at 560 nm.

3.0 Results and Discussion

3.1 Microfluidic microbioreactor system

The microbioreactor system has been designed to minimise complexity and increase the reliability of operation. The device is developed using a layered approach, with each layer having a particular function and with the layers being joined using a biocompatible pressure sensitive adhesive. Fabrication of the middle growth chamber and top layers is carried out using i3DP for ease of prototyping but the microbioreactor design is amenable for volume manufacturing with e.g. micro-injection moulding. The growth chamber is diamond shaped with rectangular inlet (500x600μm) and outlet (500x750μm) channels to allow a smooth fluid flow. The microbioreactor connectors are compatible with standard upchurch fittings for connection with tubing, as well as a push-fit connection port to fix an optical fibre cable for OD measurements. This allows quick world to chip connection for long term cultivation and reduces the risk of contamination.

Homogeneous mixing within the growth chamber was carried out using a magnetic stirrer bar, at 1200-1500 rpm, to provide a closer mimic to larger scale bioreactors and the microbioreactor temperature was kept constant at 30±0.2°C. The pH, OD and DO were recorded in real time with the DO concentration of the cultivation medium being dynamically controlled by changing the pure oxygen feed rate with a PID controller to match the DO setpoint. The media reservoirs were connected with PTFE tubing to the microbioreactor and flow sensor via a fluidic manifold and a four-way rotary 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.

Fluid driving is carried out using a pressurised system comprising a compressed air source (0.1MPa) connected to an electronic pressure regulator, driven by a voltage supply. The pressure regulator reduces the pressure down to ≈0.01 Mpa, necessary for the low rates, and controls the pressure in each of the media reservoir elements. Given that the fluid level in each of the media reservoirs may vary then there is need for dynamic control. An ADAM system generated the electrical signal (0V to 5V) for the electronic pressure regulator and simultaneously monitored the flow rate from the flow sensor. The fluid driving system was able to provide pulseless and continuous flow to the microbioreactor for continuous operation with a flow operating range of 0 to 80 μL/min and control to 0.7 μL/min. The entire microfluidic microbioreactor system is controlled by Raspberry Pi with a graphical user interface.

Microfluidic microbioreactor systems could be useful tools in a range of applications within synthetic biology including for cell free synthesis (Tinafar et al., 2019), engineered CAR-T cell therapy (Guedan et al., 2019) as well as personalised organs-on-chips for precision medicine (Van Den Berg et al., 2019). The developed microbioreactor system is flexible and can be customised to various applications that require accurate control and rapid dynamic response of flow rate. Here we have used the microbioreactor system for the continuous cultivation of *P. pastoris* - which is a commonly used system for protein expression and has a requirement for high oxygen transfer efficiency - with three different feeding strategies (pure methanol; 50% sorbitol-methanol; and 50% methanol glucose). Prior to continuous cultivation we first carry out a batch culture within the microbioreactor system using a mixed feed of methanol (10 gL^{-1}) and glycerol (10 gL^{-1}).

3.2 Batch cultivation

The batch cultivation was carried out with a mixed carbon source methanol (10 gL^{-1}) and glycerol (10 gL^{-1}) and with a DO setpoint of 30% air saturation to achieve the necessary biomass for continuous culture. Figure 3 shows triplicate cultivations within the microbioreactor at 1200 rpm stirring speed with buffered minimal media with 0.652 C-mol/L containing 50:50% glycerol and methanol as a mixed feed. The observed values of pH, DO and biomass (g/L dcw) were recorded every 10 seconds within the microbioreactor. The starting pH of the batch cultivation was 6.0±0.1 and the measured end point pH was around 5.5±0.1, with no pH control being implemented. The temperature and stirring speed were kept constant, whilst the DO was maintained through a feedback control loop to keep the concentration above 30%. The batch cultivations for the *P. pastoris* growth profiles showed that an increase in the cell biomass (g_{DCW}/L) is associated with a fall in the DO concentration, arising from oxygen consumption by cells.

All cultivations had an initial biomass concentration with OD 0.3 ±0.1 and a lag phase that ranged from one to three hours. There were some differences in the DO profile for the different cultivations and this could have arisen from differences in the inoculum. Efforts were made to standardise the procedure for inoculum preparation. It should, however, be noted that any differences in frozen vials or irregular mixing in the shake flask culture could affect the cells in the pre-culture and subsequently the inoculum.

Fig. 3 shows that the growth phase for *P. pastoris* on glycerol (0.20±0.02 h^{-1}) is higher than that on methanol (0.02±0.04 h^{-1}) since the cells first need to adapt to the methanol media and synthesize the enzymes responsible for methanol metabolism. This diauxic growth was exhibited on two carbon sources, shift occurred after 10-11 hrs of cultivation, and a lag period lasted for 1-2 hours, where the cells are adapting to the use of methanol and producing the alcohol oxidase (AOX) enzyme. Cultivation using both carbon sources, methanol and glycerol, resulted in an increase in the biomass and associated consumption of oxygen. After the consumption of glycerol, the DO concentration goes above 100 %, seen as a spike in the DO from lower metabolic activity, and remains high until cells adapt to methanol. When the cells become adapted to the methanol carbon source they then start to consume oxygen again and the DO is maintained at 30% setpoint value. The mixing speed was kept constant throughout the batch cultivation to monitor oxygen consumption, and check whether mixing and aeration in the microbioreactor is able to provide sufficient oxygen to meet the requirements for a high cell density culture at small scale. Towards the end of the cultivation, as the carbon source is consumed and is no longer available for the cells to use, the oxygen concentration is seen to increase and remains at a constant high level. Fig. 3 shows good growth agreement between the three batch cultivations for glycerol and methanol and a dry cell weight of 10±1 g/L for the three batch cultivations. The reproducibility of these cultivations within the microbioreactor demonstrates the system's utility for real world applications.

3.3 Continuous cultivation

Batch cultivation of cells with the mixed carbon source was used to achieve a good level of biomass and when the methanol is completely consumed, indicated by a spike in the DO curve, then continuous cultivation is started with a specific feed medium. The dilution rate for all of the continuous cultivation experiments were set to 0.07 h^{-1} (1.1 µL/min) which was the minimum flow rate that could be controlled within the system. The profile of growth with dry cell weight concentrations, DO and pH for continuous cultivation is shown in Figure 4 for constant feeds of (A) methanol (B) 50% methanol-sorbitol and (C) 50% methanol-glucose.

Fig. 4 shows that the DO profile is maintained at 30% air saturation, but with spikes at 2hr intervals arising from taking samples from the microbioreactor. In all three of the feeding cases, after three residence times the cells reach steady state with a relatively stable biomass density of around 10 gL^{-1} of dry cell weight. In the case of a mixed feed of methanol-sorbitol, the constant feeding rate (0.07 h^{-1}) is between the maximal specific growth rates on methanol and sorbitol, 0.140 h^{-1} ± 0.007 h^{-1} and 0.032 ± 0.002 h^{-1} respectively (Jungo et al., 2007). Initially cells are washed out, as seen by the decrease in biomass in Fig. 4B, since the dilution rate is higher than the maximum specific growth rate on sorbitol. This is in contrast to the pure methanol feed, shown in Fig. 4A, where no washout of cells is observed since the dilution rate is lower than the maximum specific growth rate on methanol.

For the methanol-sorbitol mixed feed, the cells are primarily consuming methanol since the dilution rate is higher than the maximum specific growth on sorbitol. The cells consume methanol and sorbitol simultaneously but once the methanol is completely consumed by the cells then the increase in biomass is constrained by the limited ability of cells to consume sorbitol. For the methanol-glucose feed, the dilution rate (0.07 h^{-1}) is lower than the maximal specific growth rates on glucose (0.28 ± 0.01 h^{-1}, Paulová et al., 2012) and methanol (0.140 ± 0.007 h^{-1}, Jungo et al., 2007) so that cells can consume both of the substrates. There is some wash out of cells observed (Fig. 4C), since there is a lag period required for adaptation of cells to the methanol-glucose mixed feed medium.

At steady state, the specific production and consumption rates *qi* were calculated from:

$$q_i = \frac{D(Ci\ in - Ci\ out)}{X} \quad \text{..... (1)}$$

where x is biomass, Ci *in* (g/L) is the concentration of substrate in inflow medium while Ci *out* (g/L) is the concentration of substrate inside the bioreactor, D (h^{-1}) is the dilution rate.
The yield (R) of substance j on substance i was calculated as a reconciled yield coefficient (g biomass per g of substrate) by

$$Y_{\frac{j}{i}} = \frac{Rj}{Ri} \ (g/g) \text{ ..... (2)}$$

The concentration of the substrate, metabolites and product in the microbioreactor were determined by collecting samples, at steady state, periodically from the microbioreactor. The specific consumption rate for each substrate and reconciled yield coefficients were calculated using respectively eq. (1) and (2). For the different media feeds, the reconciled yield

coefficients for biomass and ammonia produced; specific consumption rates of substrate as well as the residual substrate are shown in Fig. 5. Values for these, along with the concentrations for the substrate and ammonia at harvest are given in Table 1.

Methanol starts to accumulate inside the microbioreactor after 83 hrs (Fig. 5A) but with no effect on the biomass (Fig. 4A). For the methanol-sorbitol feed, the consumption of methanol does not change significantly during the cultivation and the residual methanol concentration is very low in the culture medium with a modest increase over time (Fig. 5B). This contrasts with a decrease in the consumption of sorbitol towards the later stage of the cultivation and an accumulation of the residual sorbitol within the culture medium. The accumulation of sorbitol in the spent medium arises from a lower maximum specific growth rate on sorbitol than the dilution rate for cultivation (Egli et al., 1986). For the methanol-glucose mixed feed, there is a decrease in the methanol consumption over the course of 124 hrs of cultivation (Fig. 5C) whilst this is maintained for both the methanol and methanol-sorbitol mixed feeding (Fig. 5A and 5B). It can also be seen from Fig. 5C that the residual glucose concentration is very low in the culture medium but that there is an accumulation of the residual methanol within the culture medium. In this case, after the cells have consumed all of the glucose then a further increase in biomass is limited since the cells have limited ability to consume methanol.

SDS page densitometry was used for quantification of the aprotinin product in the supernatant (Fig. 6A), where the error bars represent the standard deviation of the product band density over the period of the cultivation. The normalised aprotinin SDS-PAGE band intensity was higher for pure methanol induction (314±23) than for co-feeding with methanol-sorbitol (202±17). Moreover, the aprotinin production was fairly constant during cultivation with pure methanol and sorbitol-methanol co-feeding, but was found to reduce over time with methanol-glucose co-feeding as indicated by the larger error bar. A few studies have shown some minor improvement in product yield by directly replacing methanol with sorbitol in terms of C-mol (Jungo et al., 2007; Niu et al., 2013). Our work suggests that product formation is more sensitive to methanol induction rather than cell biomass, as indicated by the sharp drop in production of aprotinin during methanol-glucose mixed feeding. The relationship between recombinant protein production and biomass is complex and will depend on the cultivation conditions (pH, temperature, oxygen), physiology of cells, presence of repressor/inducer molecules as well as the target construct and product.

Methanol induction increases oxidative cellular stress due to the production of formaldehyde. Mixed feeding can improve cell viability by reducing the cellular burden of methanol metabolism on cells. An increase in the cell viability can potentially be indicated by an increase in the total protein production (Figure 5B). In the case of methanol induction, the total protein produced increases over a range of 12 mg g^{-1} DCW to 18 mg g^{-1} DCW. This compares with a gradual increase of total protein released reaching a maximum to 14.6 mg g^{-1} DCW for sorbitol co-feeding, but a gradual decrease of total protein released with glucose co-feeding from accumulation of methanol inside the microbioreactor. The total protein produced at harvest was found to be highest for sorbitol-methanol co-feeding, followed by pure methanol and lowest for methanol-glucose (Table 1). The gradual decrease in the total protein produced for methanol-glucose co-feeding (Fig. 5B) is ascribed to glucose providing an excess carbon source and being preferentially consumed over methanol by the cells which leads to

methanol accumulation in the media. In contrast to this, under carbon limited continuous culture - using 60:40 % carbon from glucose and methanol (11.7 gcarbonL^{-1}) – the repressive effect of glucose can be eliminated with improvement on biomass and productivity (Paulová et al., 2012). Some studies have found that co-feeding can improve protein production but these have used different conditions, e.g. using a mixed feed technique where the methanol concentration is kept fixed and sorbitol is fed as a large pulse during the cultivation (Gao et al., 2012; RAMON et al., 2007; Wang et al., 2010; Xie et al., 2005). In this study the sorbitol is added in a continuous manner since sorbitol accumulation has no effect on AOX1 (Çalık et al., 2013).

The total protein produced is not necessarily the most appropriate measure for cell viability since *P. pastoris* will naturally secrete some proteins and the amount of protein released is small (Lopes et al., 2012). Better measures of cell viability are a release of either DNA or protease into the supernatant since these are only released on cell death (Zhang et al., 2007). Fig. 6C shows that the residual DNA concentration increases with co-feeding of methanol-glucose and, in contrast to this a flat or decreased DNA release with respectively co-feeding of methanol-sorbitol and with methanol alone. We also find that the proteases released for the pure methanol feeding and the methanol-sorbitol co-feeding are broadly similar and flat against increased levels of protease released for co-feeding of methanol-glucose mixed. Moreover, the protease concentration in the harvest was approximately similar for the pure methanol and methanol-sorbitol mixed feeds, but was higher for the methanol-glucose feed. This indicates that the methanol-sorbitol mixed feed has similar cultivation to that of pure methanol, but that the methanol-glucose mixed feed, under the conditions used, has a relatively negative effect on productivity and quality.

4.0 Conclusion

A low-cost microfluidic microbioreactor with integrated optical sensors and pressurised fluid driving as well as off-chip fluidic switching can be used for batch and continuous cultivations with different feeds, facilitating the optimisation of recombinant protein production. Batch cultivation of *P. pastoris* within the microfluidic microbioreactor showed good reproducibility of cell density, pH and DO for three successive batch cultivations and a dry cell weight maximum of 10±1 g/L. Continuous cultivation showed that, under the conditions used, the aprotinin product was higher in pure methanol and methanol-sorbitol mixed feeds than with a methanol-glucose mixed feed. The use of a methanol-glucose mixed feed also showed over the period of the cultivation a decrease in the total protein produced with increases in DNA and proteases released, indicating a decrease in cellular viability. In the case where glucose is part of the feed, the cells preferentially consume glucose over the methanol and are unable to induce protein production.

Future work will focus on integration of information from the microfluidic microbiorector, as a miniaturised physical model, with computational models for the understanding of cellular processes. We will also seek comparison of performance of the microfluidic microbioreactor with larger scale microbioreactors and application for novel bioprocesses.

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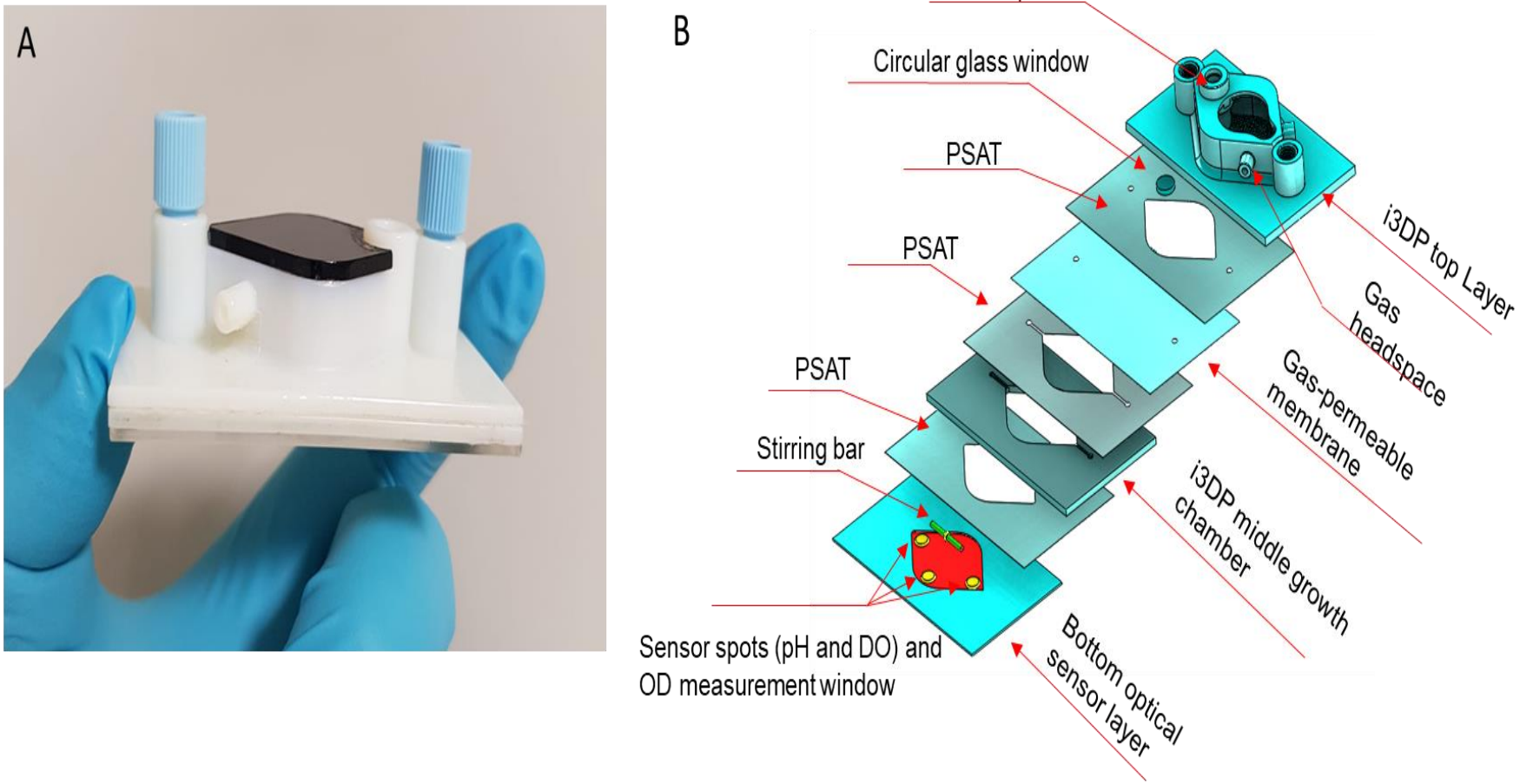

Figure 1 (A) photograph of the assembled microfluidic microbioreactor (B) different functional layers of microfluidic microbioreactor using inkjet 3D printing (i3DP) and joined with pressure sensitive adhesive tape (PSAT)

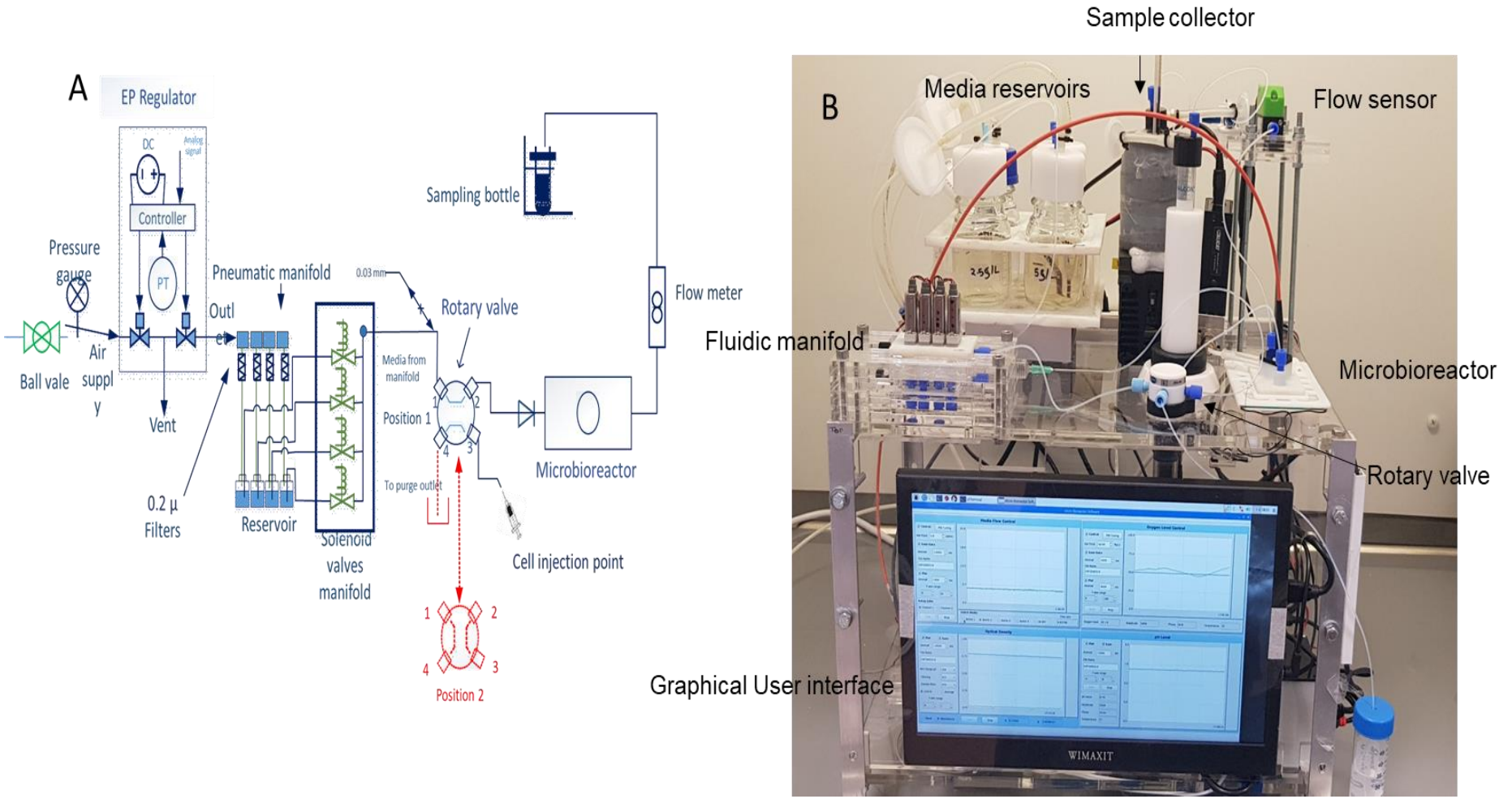

Figure 2 (A) the schematic of microbioreactor platform showing pressurised fluid driving system with media reservoir bottle, solenoid valve for media selection, cell injection port, microbioreactor chip, flow sensor and sample collector, (B) a photograph of the microbioreactor platform

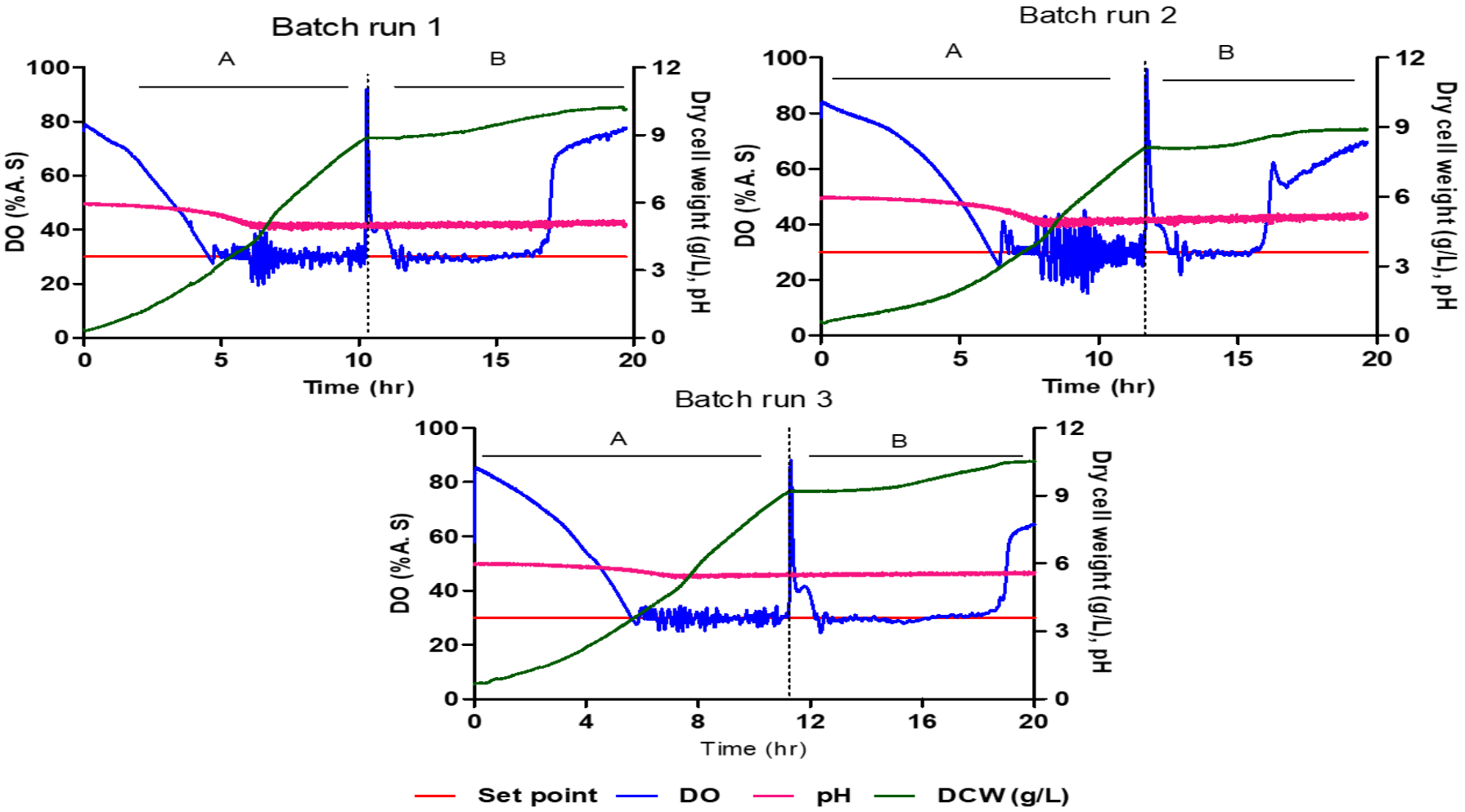

Figure 3 Diauxic growth of *P. pastoris* CLD804 Mut^{+} with glycerol (A) and methanol (B) with triplicate batch cultivation in microbioreactor with the growth curves showing profiles for DO, pH and cell density DCW (g/L)

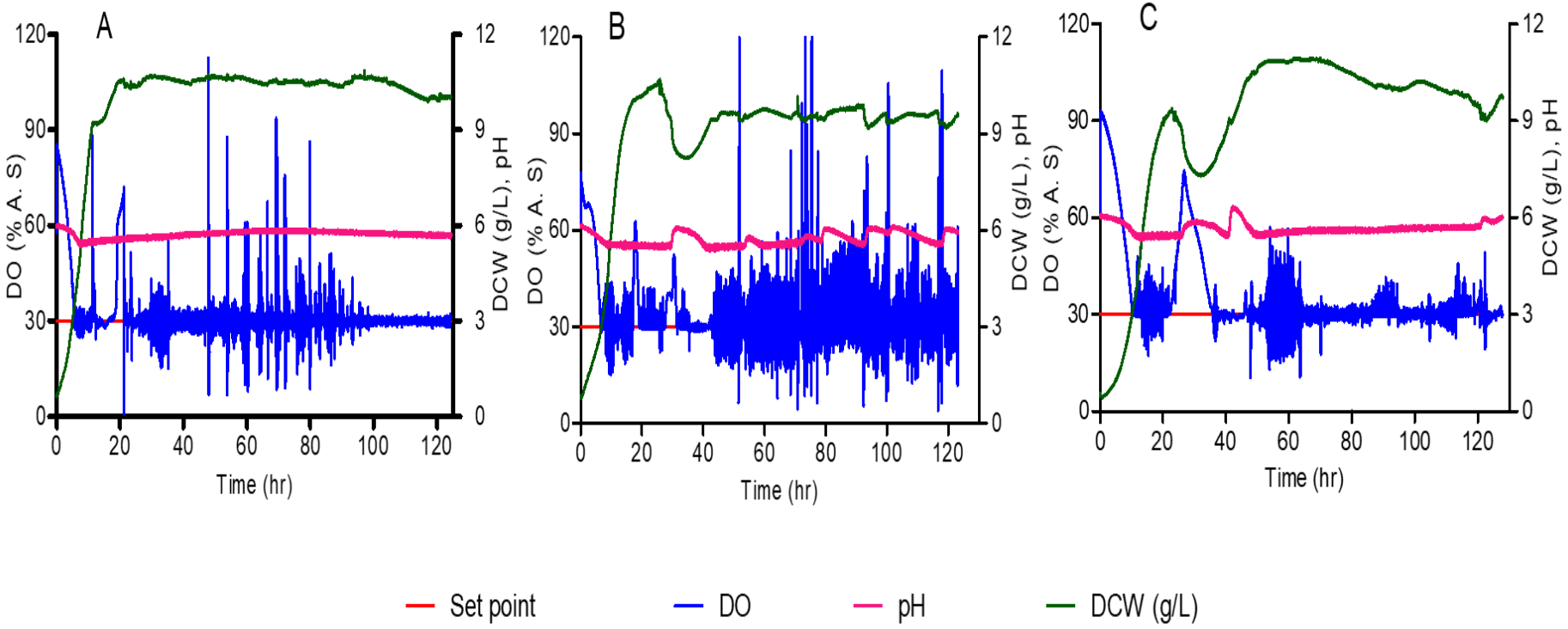

Figure 4 Dry cell weight in relation to dissolved oxygen and pH for continuous fermentation of P. pastoris CLD804 in microbioreactor at D= 0.07 h^{-1} for different feeding medium (A) 100 % pure methanol (0.652 C-mol$L^{-1,}$ 20.87 g/L) (B) 50% sorbitol- methanol (0.652 C-molL^{-1} ,10.44 gL^{-1} methanol and 9.89 gL^{-1} sorbitol) (C) 50% methanol-glucose (0.652 C-molL^{-1}, 10.44 gL^{-1} methanol and 9.78 gL^{-1} glucose)

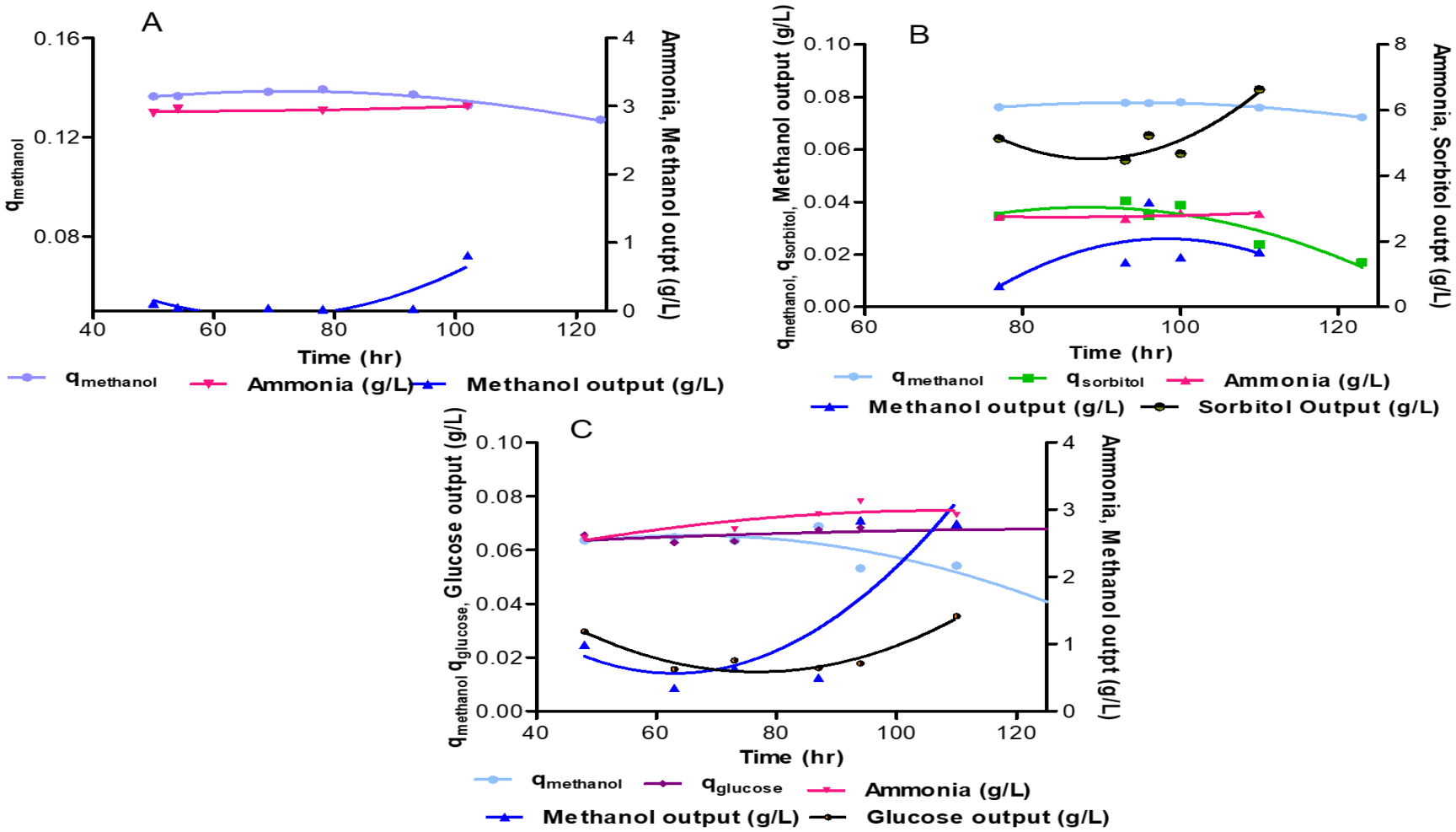

Figure 5 Specific consumption rate of substrate ($g_{substrate}g_{DCW}^{-1}h^{-1}$), NH_3 production and residual substrate concentration during continuous cultivation in microbioreactor with different feeding medium at D= 0.07 h^{-1} of (A) 100 % pure methanol (0.652 C-molL^{-1}, 20.87 g/L) (B) 50% sorbitol- methanol C-molC-mol^{-1} (0.652 C-molL^{-1} ,10.44 gL^{-1} methanol and 9.89 gL^{-1} sorbitol) (C) 50% methanol-glucose C-mol/C-mol (0.652 C-molL^{-1}, 10.44 gL^{-1} methanol and 9.78 gL^{-1} glucose). Lines of best fit were constructed for the datasets.

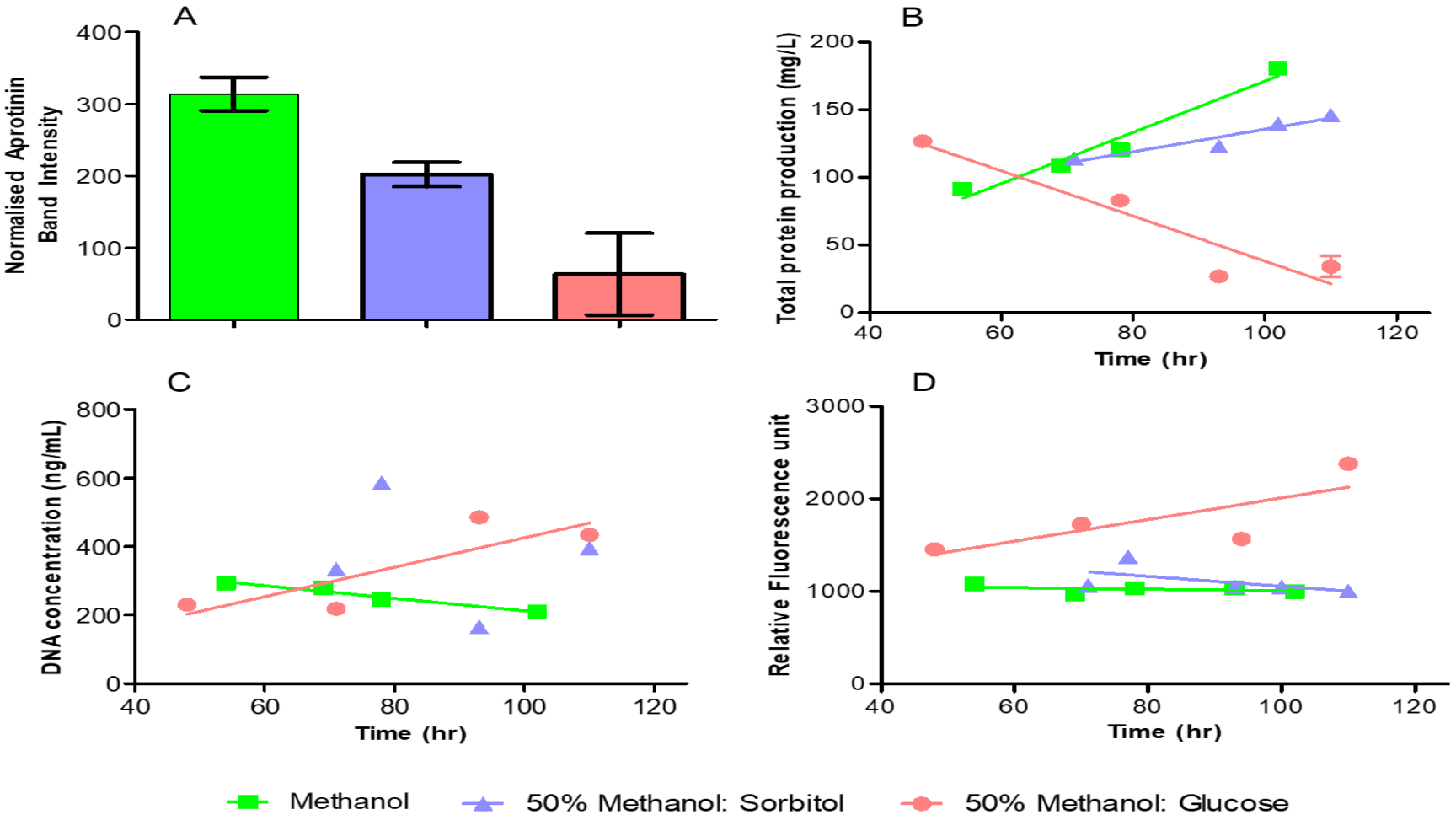

Figure 6 Supernatant analysis over the cultivation time with induction feed of pure methanol (green), 50% sorbitol-methanol (blue) and 50% glucose-methanol (red) by P. pastoris CLD804 in microbioreactor (A) densitometric analysis of the aprotinin bands, the intensity of bands were normalised by loaded sample concentration and the error bars are a result of the standard deviation between product band density over the period of the cultivation (B) total protein release (C) DNA release and (D) protease release.