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Development of a simple and low cost microbioreactor for high-throughput bioprocessing

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Abstract A simple microbioreactor for high-throughput bioprocessing made from low cost polymer polytetrafluoroethylene (PTFE) tubes with a working volume of 1.5 ml is described. We have developed a microfluidic system that handles a small population of cells of a model microorgnism, *Pseudomonas aeruginosa* DS10-129. Under the conditions of the microbioreactor, the organism produced extracellular secondary metabolites by using nutrient broth modified with glycerol. Pyocyanins were particularly isolated from the fermented medium as the metabolite of interest and was characterised by Infrared spectroscopy and quantified by microbiological assay. Antibiotic properties of pyocyanin were effective against a number of microorganisms such as *Staphylococcus aureus, S. epidermis, Bacillus subtilis, Micrococcus luteus* and *Saccharomyces cerevisiae*. Batch fermentation of the model organism in the microbioreactor was compared to shake-flask and conventional bench fermenter methods. Results obtained from the microbioreactor compared favourably with the conventional processes.

Introduction

With the advent of miniaturisation technology a decade ago, the development of microfluidic devices has witnessed a remarkable growth. Today microfluidic technology incorporating micro total analysis systems (µTAS) has become widely used to study a wide range of biological and chemical processes. The development of µTAS has seen researchers focus on scaling down grandiose expensive equipment to miniature scale with the overall aim of improving throughput, optimising operations and cutting down on resources to manageable levels. Currently, microfluidic systems are being employed to study diverse processes such as bioprocessing, chemical synthesis, clinical diagnostics and genetic engineering. Research has shown that microfluidic systems are a promising technology that has the potential to improve analytical performance, reduced laboratory safety requirements and costs, shorter analyses time and above all reduced reagent use (Chin et al. 2007).

Bioprocessing operations such as fermentation has been widely used to realize a number of products of economic importance such as antibiotics, vaccines, therapeutic proteins and many others. However the current mode of operation for these processes is the use of conventional stirred tank bioreactors with typical volumes between 0.5-10 l and shake-flasks (Zhang et al. 2007). These conventional tools have the following limitations: (i) process procedures are laborious and time-consuming; (ii) higher equipment maintenance cost (cleaning and assembling equipments), (iii) systems are not amenable to performance of experiments in parallel, (iv) samples are at risk of contamination during sampling procedures, (v) use of large amounts of reagents and generation of large volumes of waste, and (vi) low throughput.

On the other hand, in clinical diagnosis, patients are subjected to biochemical analyses of their biological fluids. Usually the analyses are carried out in clinical laboratories and the results become available after several days. As a consequence, a reliable diagnosis cannot be performed within the consultation time (Minas et al. 1995). In view of this, there is therefore a need to develop a microbioreactor that would address the following: process control, waste reduction, and high-throughput, speed-up testing, parallel investigation and optimisation. Previous work (Kostov et al. 2001; Zanzotto et al. 2004) has shown that

microbioreactors provide unique environments, which are ideal for rapid and efficient biosynthesis due to the scale dependence of thermal and mass transfers.

A silicon microfermentor chip that used electrodes to measure cell density, pH and dissolved O_2 was developed, while a low cost microbioreactor fabricated from poly-dimethylsiloxane (PDMS), polymethyl methacrylate (PMMA) and glass integrated with sensors for measurement of the same parameters was developed (Zanzotto et al. 2004). Optical density was monitored by transmittance, while fluorescence lifetime–based sensors measured pH and dissolved O_2. They demonstrated that *E. coli* was able to grow in the microbioreactor channels. A portable anaerobic microbioreactor to study the optimum growth conditions for the methanogen, *Methanosaeta concilii,* a methane-producing obligate anaerobic archaebacterium was developed (Steinhaus et al. 2007). A 6 ml bioreactor made from Plexiglas was reported (Lamping et al. 2003) and measurements of optical density, dissolved O_2 and pH were made optically.

In this work, our approach is to develop a simple device made from low cost polymer materials. The configuration of the device will be capable of sustaining the cells of the model microorganism DS10-129. The working volume of the bioreactor would be scaled down to 1.5 ml and the fermentation carried out in the microbioreactor will be compared to the shake-flask and bench fermenter.

Materials and methods

Microbioreactor design

The microbioreactor was made from a synthetic polymer polytetrafluoroethylene (PTFE). A PTFE tube (length 3 m, 0.8 mm diameter) was made into coils of diam. 4.2 cm to give a microbioreactor of working volume 1.5 ml. PTFE was chosen because its performance was equally comparable to PDMS and it allows rapid gaseous exchange (CO_2 and O_2), tubes were of lower costs and easily sterilisable at higher temperatures.

Bacterial strain and culture conditions

Pseudomonas aeruginosa DS10-129 (AM419153), isolated from diesel-contaminated sites (Rahman et al. 2002) was used for all the experiments. To promote pyocyanin production DS10-129 was cultured in glycerol supplemented nutrient broth (GSNB) medium. The composition of the GSNB medium was (g/l): Lab-lemco powder 1, Yeast extract 2, peptone 5, NaCl 5, 30 ml glycerol and 100 ml deionised water. Single colonies of DS10-129 were used to inoculate the sterile GSNB medium and were incubated at 30°C with shaking at 150 rpm. When the OD_{600} of the culture medium was between 0.8 -1, it was used to inoculate the microbioreactor, shake-flask and the bench fermenter.

Fermentation in the Microbioreactor

By using aseptic techniques, the sterile GSNB medium was inoculated with DS10-129 suspension to give a starting OD_{600} of 0.123, equivalent to approximately 5.6×10^8 cells/ml. The inoculated media was withdrawn with a 10 ml syringe and mounted on a syringe pump (Razel Scientific, USA) and was used to inoculate the microbioreactor as shown in Figure 1. After the inoculation procedure, the inlet and outlet ports of the microbioreactor were each connected to a 0.2 μm Whatman sterile syringe filter. This was to prevent contamination and allow diffusion of gases in and out of the microbioreactor. A total of three microbioreactors were prepared and inoculated in the same manner and allowed to run in parallel and incubated at 30°C with shaking at 150 rpm for 24 h. At intervals, all the three microbioreactors had their contents sacrificed and monitored for cell growth by measuring the OD_{600} of the metabolised GSNB medium. The metabolites produced by the organism were collected by centrifuging the culture broth at 8000 g at 4°C for 20 min to remove bacteria. The supernatant was filtered through a 0.2 μm syringe filter, protected from light and kept refrigerated for subsequent analyses.

Fermentation in a fermenter and shake- flask

Parallel batch fermentations of DS10-129 were carried out in a bench fermenter and shake-flask. A 7.5 l bioreactor (BioFlo 110, New Brunswick Scientific, USA) was filled with 3 l sterile GSNB medium and

inoculated with a suspension of DS10-129 with optical density 0.8-1 to give a starting OD_{600} of 0.123. The temperature, pH and agitation speed were maintained at 30°C, 7 and 200 rpm, respectively, but the levels of O_2 were not monitored. Samples of the metabolised culture medium were periodically withdrawn for OD_{600} measurements and subsequent down-stream processing as described for the microbioreactor. Similarly, a 250 ml conical flask containing 100 ml sterile GSNB medium was inoculated with DS10-129 and incubated at 30°C for 24 h with shaking at 150 rpm. Samples were withdrawn periodically and treated as described for the other devices.

Antimicrobial effect of pyocyanin

The microbiological assays for pyocyanin were carried out by using the agar diffusion method using: *S. epidermis, B. subtilis, Staph. aureus, M. luteus, Pseudomonas teessidea* PR6.5 (AM419154), *P. clemancea* PR22.1 (AM419155), *P. aeruginosa* DS10-129 (AM419153)*, Aspergillus niger and Sacc. cerevisiae*. 60 µl suspensions of each microorganism with an optical density of 1± 0.2 were used to inoculate 20 ml sterile molten Antibiotic Medium Number No.1 Agar (ANTBA1) and the agar plates were allowed to solidify at room temperature. The composition of ANTBA1was (g/l): peptone 6; tryptone 4; yeast extract 3; Lab-lemco powder 1.5; glucose 1; and agar 11.5. Holes of 7 mm diam. were punched on the solidified inoculated media using a cork borer. Then 60 µl of the concentrated cell free supernatant, harvested at different times were added to the agar wells and incubated at 37°C for 18-24 h. For *A. niger* and *Sacc. cerevisiae* Sabouraud's Dextrose Agar (SDA) was used and incubated at 30°C for 18-48 h respectively. The suspension of *B. subtilis* was prepared from a culture that had previously been incubated for 5-7 days to allow for the formation of spores. The antimicrobial activities of pyocyanin against each organism were determined by measuring the diam. of the zones of inhibition using a pair of vernier callipers. Pyocyanin was also extracted from culture supernatant and characterised by FTIR spectroscopy using the method described by Weibel and Whitesides (2006).

Results and discussion

Growth and pyocyanin production

The progress of the batch fermentation process of DS10-129 in the microbioreactor was compared with parallel fermentations carried out by the organism in the shake-flask and the fermenter (Fig.2). Growth of DS10-129 in the microbioreactor was slightly higher than the other two systems which could be attributed to the miniaturised devices having very small cross sectional dimensions thereby giving rapid heat and mass transfer because of the very large surface area to volume ratio (Zhang and Haswell, 2007). In this work we did not monitor O_2 concentrations due to the fermentation process being limited to a short period and the dissolved O_2 levels in the media and the porous nature of PTFE tubes were adequate to sustain the growth of bacteria for the duration of the fermentation. Previous studies (Costerton and Anwar 1994; Lederberg et al 2000), however, have shown that *P. aeruginosa* can synthesise arginine and can proliferate in anaerobic conditions which make it a very ubiquitous microorganism found in nearly every environment such as soil, water, humans, animals, plants, sewage, and hospitals. Agitation was unique in all the bioreactors to allow mixing and distribution of O_2 in all the processes. Previous reports (Schultz 1964) have shown that the speed of shaking affects mass transfer of O_2. McDaniel and Bailey (1969) investigated the effect of speed of shaking on a biosynthetic process involving the production of candidin by *Streptomyces virodoflavin* and demonstrated that increased shaking speeds resulted in increased oxygen absorption rates.

Fourier Transform Infra-red (FTIR) spectroscopy

Pyocyanin were characterised by Fourier Transform Infrared spectroscopy (supplementary Fig 1.). Our results are comparable with those previously reported by Von Saltza et al. 1969.

Antimicrobial effect of pyocyanin

The results of the antimicrobial effect of pyocyanin on various bacterial and fungal species are shown in Table 1. The diam. of the zones of inhibition was directly proportional to the concentration of pyocyanin. Gram-positive microorganisms *(B. subtilis, Staph. aureus, Staph. epidermis and M. luteus)* were more susceptible to pyocyanin than Gram-negative bacteria (*P. teessidea, P. clemancea and* DS10-129) and the eukaryotes (*A. niger* and *Sacc. cerevisiae)*. DS10-129 and *A. niger* were resistant to the highest concentration of the pyocyanin. Our results are comparable to the previous reports (Waksman and Woodruff, 1942; Baron and Rowe, 1981) that have shown that Gram-positive organisms are more susceptible to pyocyanin than Gram negative organisms. The resistance to pyocyanin by the Gram-negative bacteria is due to the presence of an outer membrane composed of mainly lipopolysaccharides (Ferguson et al. 2007). Previous findings have demonstrated that the outer membrane confers resistance to antibiotics, detergents and disinfectants by the Gram-negative species. Reports by Fridovich et al. (1995) suggested that organisms, which have high levels of superoxide dismutase enzyme are resistant to the action of pyocyanin. The inhibition of pathogenic bacteria and fungi suggests that pyocyanin could be used as effective antibiotics in the medical fraternity as well as biological control agents in agriculture. The report by Vukomanovic et al. (1997) has shown that pyocyanin have a variety of pharmacological effects on both eukaryotic and prokaryotic cells.

The production of pyocyanin by *P. aeruginosa* is thought to endow the organism with competitive advantage against other organisms (Parsons et al. 2007). In their work they described pyocyanin as biologically active phenazines whose toxic nature is largely due to its ability to engage in oxidation – reduction reactions that deplete cells of NADH, glutathione, and other oxidants. The results also suggest that actively growing cells of DS10-129 produce extracellular metabolites, which enhance them to their survival potential in harsh environments. This development is consistent with earlier studies (Reszka et al. 2004).

Conclusion

In this work we have demonstrated that the simple low cost microbioreactor can speed-up bioprocessing operations, use less reagents and samples, reduce waste streams, can be easily disposed of or reused and above all it is cheaper and easy to develop. The results for the fermentations carried out in the microbioreactor were analogous with those for the shake flask and the fermenter. The microbioreactor has the potential of replacing the conventional scale processes as well as offering high-throughput and being an efficient analytical tool in addressing some of the challenges encountered in bioprocessing. The construction of this simple low cost device for culturing bacteria has proved that very simple laboratory based work involving microfluidic technology can be taught to young scientists in colleges and high schools. This development will enable upcoming young scientists to learn and appreciate the merits of microfluidic and miniaturisation technologies reigning the 21st century.

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List of Figures and Table

Table 1 Antimicrobial effects of pyocyanin on microorganisms

Fig. 1 Schematic set up of the microbioreactor. The GSNB and the inoculum were batch pumped and fed into the microbioreactor by using a 10 ml syringe driven by a syringe pump. Sterile 0.2 µm Whatman syringe filters were fitted at the outlet and inlet of the microbioreactor after the inoculation

Fig. 2 Growth of *P. aeruginosa* DS10-129 in Glycerol Supplemented Nutrient Broth (GSNB) medium in three bioreactors. The OD_{600} values of the fermented culture from each bioreactor were determined at various times at 30°C**.** All data represents a mean of three fermentation measurements in each bioreactor (■ - Microbioreactor; ♦ - Shake-flask; ▲ – Fermenter)

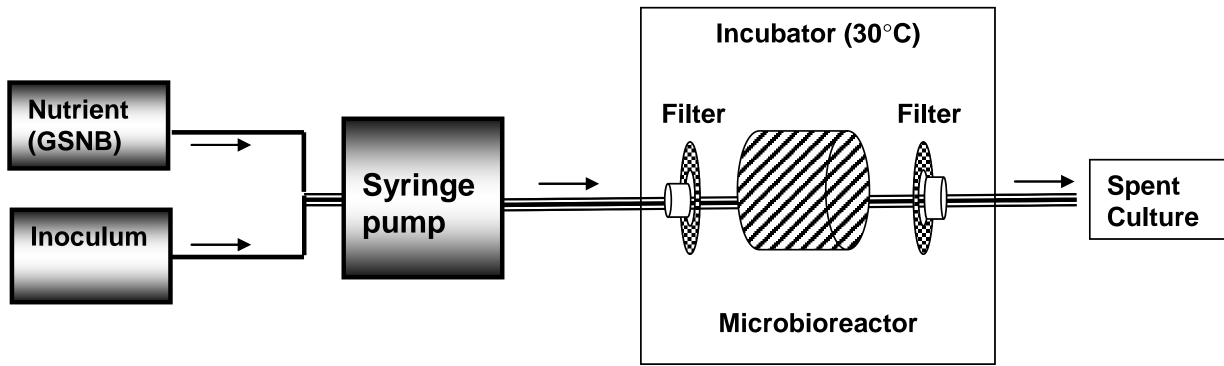
Supplementary Fig. 1 Infrared spectrum of pyocyanin from *P. aeruginosa* DS10-129 culture grown in microbioreactor. Aliphatic chain stretching and bending (2990-2800, 1470-1350 cm^{-1}), Aromatic compound C=C stretching vibrations (1600-1500 cm^{-1}), C-O stretching (1320- cm^{-1}), Medium band stretching of C-N (1250-1000 cm^{-1})

Organism	Antimicrobial activity in terms of diam. of zone of inhibition (mm)								
	8 h*			16 h*			24 h*		
	μBR	SF	F	μBR	SF	F	μBR	SF	F
Staph. epidermis	24	23	24	27	26	28	31	30	32
Bacillus subtilis	18	16	16	21	19	19	23	21	20
Micrococcus luteus	16	15	16	18	17	19	21	20	22
Staph. aureus	13	12	11	16	15	14	18	17	16
Saccharomyces cerevisiae	10	9	11	12	11	14	16	15	17
Pseudomonas teessidea	9	9	8	11	10	9	14	13	12
Pseudomonas clemancea	8	9	9	10	11	11	15	13	13

μBR= Microbioreactor; SF= Shake-flask; F = Fermenter; * = Incubation time

Footnotes: *Aspergillus niger*: initially small zones were observed, but disappeared after 48 h,

Pseudomonas aeruginosa: No zones of inhibitions were observed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Microbioreactor
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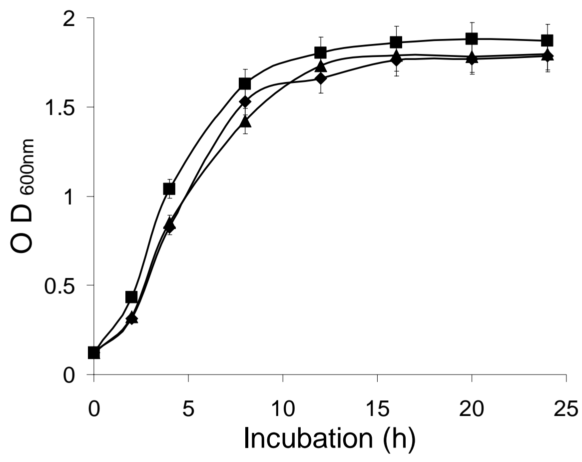
Culture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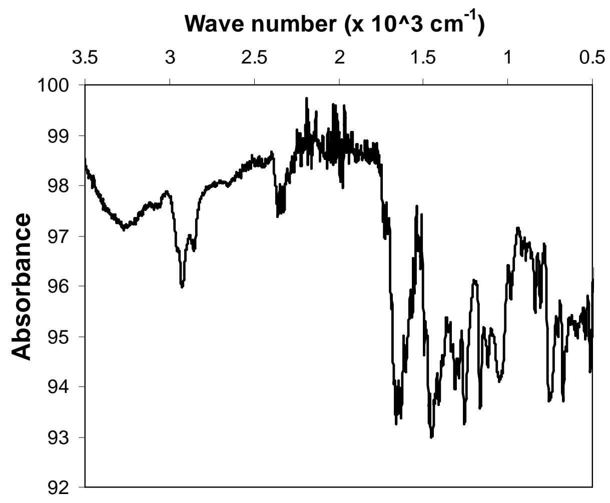

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1
0.5
100
99
98
97
96
95
94
93
92
Absorbance