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T.M. Ndlovu, A.C. Ward, J. Glassey, J. Eskildsen, G. Akay

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**Bioprocess intensification of antibiotic production by *Streptomyces coelicolor* A3(2) in
micro-porous culture**

T. M. Ndlovu¹, A.C.Ward³ J. Glassey², J. Eskildsen¹ and G. Akay²

¹ NUTRISS Limited, INEX, Herschel Annex, Kings Road

Newcastle upon Tyne, NE1 7RU (UK)

² School of Chemical Engineering and Advanced Materials

³ School of Biology and Psychology

Newcastle University, Newcastle upon Tyne NE1 7RU (UK)

Corresponding author: Dr Teresa M Ndlovu, (tm.ndlovu@nutriss.com)

1. Abstract

A novel functionalized micro-porous matrix was developed with well-controlled physicochemical properties such as pore size and surface chemistry. The matrix was used as a solid support in the growth of *Streptomyces coelicolor*"A3(2) to enhance the production of antibiotics. The results shown support a higher production of prodigiosin and actinorhodin with overall production increase of 2-5 and 6-17, respectively, compared to conventional submerged liquid culture, offering a potential improvement in volumetric productivity.

Scanning Electron Microscopy was used to evaluate pore size as well as bacterial adhesion, penetration, proliferation and migration within the micro-porous matrix.

[**Keywords:** Polymerization, Functionalization, Micro-porous matrix growth, Submerged growth, *Streptomyces coelicolor*" A3(2) and Antibiotic].

2. Introduction

Process intensification is one of the most significant trends in today's chemical engineering and biotechnology process development. It is the development of innovative equipment and techniques in order to achieve significant improvements in manufacture and processing, radically decreasing equipment volume and energy consumption and finally leading to cheaper, safer and sustainable technologies. The approach was first introduced during the 1970s by Colin Ramshaw and his co-workers; representing a novel design strategy with the aim of achieving a reduction in the processing volume compared with existing technology, without a reduction in process output (.Ramshaw, 1983), (Ramshaw, 1995) and (Stankiewicz & Moulijn, 2000).

2.1 Antibiotic overview

It has long been known that certain microbes are beneficial when it comes to secondary metabolite activity such as the production of antibiotics. In 2001 the WHO reported on the worldwide emergence of antibiotic resistance and the serious threat it poses to public health. Therefore, the development of new drugs is of paramount importance. For this to happen “both innovative cultural procedures and culture-independent methods have a role to play in unravelling the full extent of prokaryotic diversity in natural habitats”(Bull *et al.*, 2000). New antibiotics of higher potency are needed to combat infectious disease in human, animals and plants.

In 1947 the mycologist Selman Waksman defined *antibiotic* as “a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms”. The action of an antibiotic against micro-organisms is selective in nature (Waksman, 1947).

2.2 Synthesis of antibiotic by *Streptomyces coelicolor* A3(2)

Antibiotics produced by *Streptomyces* are typically synthesized in small amounts at the transition phase in colonial development when the growth of the vegetative mycelium is slowing as a result of nutrient exhaustion and the aerial mycelium is about to develop at the expense of nutrients released by breakdown of the vegetative hyphae (Migulez *et al.*, 2000).

“*S. coelicolor*” A3 (2) is the most extensively studied organism in terms of genetics and the regulation of secondary metabolite production in streptomycetes (Hopwood, 1999; Thompson *et al.*, 2002; Hopwood, 2003). In 2002 Bentley and co-workers published the complete genome sequence establishing it as a reference strain for post genomic studies and provides a comprehensive overview of its secondary metabolism (Bentley *et al.*, 2002), (Huang *et al.*, 2001). This well-known microorganism produces four known antibiotics (Kieser *et al.*, 2000) as a result of secondary metabolism; actinorhodin, prodigiosin, calcium dependent antibiotic (CDA) and methylomycin. The production of each of these antibiotics can be selectively enhanced by specific media and growth strategies.

In this study we investigated the production of blue-pigmented actinorhodin and red-pigmented antibiotic prodiginine. The latter is a member of a family of tripyrrole red pigments and it is attracting increased interest because of its immunosuppressive and anticancer activities. In addition it has been shown to have antifungal, antibacterial, antiprotozoal and antimalarial activities (Williams & Quadri, 1980; Demain, 1995).

Rudd and Hopwood first discovered this red antibiotic during genetic studies of actinorhodin biosynthesis (Rudd & Hopwood, 1979) as the synthesis of red antibiotic occurred first before actinorhodin. Due to their visible colour and the genetically amenable nature of some producer strains of *Serratia* and *Streptomyces* species, red-pigmented biosynthesis has been examined as model for bacterial secondary metabolites and the sequence of the entire red

cluster for prodiginine biosynthesis is currently available as part of the *S. coelicolor* genome-sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor).

2.3 Productivity

Our first understanding of productivity was founded on the Gaden report published in 1959, and early efforts by Wells, Moyer and Gastrock (Moyer *et al.*, 1937; Wells *et al.*, 1937; Gastrock *et al.*, 1938; Moyer *et al.*, 1940) in the late 1930s to study the production of gluconic acid. Recently much progress has been made in improving antibiotic producers (Chater, 1990.; Lai *et al.*, 1996; Lee & Rho, 1999; Haifeng & Kozo, 2001). However the expressions for average rate of production described by Gaden are still useful nowadays

2.4 Micro-porous matrix as an inert substrate growth

Solid state Fermentation (SSF) on impregnated inert supports offers the potential for improved process control and monitoring, owing to a more-defined environment (Ooijkaas *et al.*, 2000). Antibiotics, such as penicillin (Barrios-Gonzalez *et al.*, 1993), cephalosporin (Jermini & Demain, 1989), cyclosporin A (Balakrishnan & Pandey, 1996; Ramana *et al.*, 1999), tetracycline (Yang & Ling, 1989.), oxytetracyclines (Yang & Wang, 1996.), rifamycin (Krishna *et al.*, 2003) and iturin (Ohno *et al.*, 1993) , have been produced with higher productivity, defined as product concentration per volume, in SSF when compared to liquid culture.

During the course of the research on the development of an enhanced environment to mimic the natural habit of filament bacteria reported here, the use of a micro-porous matrix known as PolyHIPE, was explored as an attractive alternative for antibiotic production. It has been previously reported that a solid support is a better alternative during cultivation of

Streptomyces and fungi because the production of secondary metabolite is coupled with the formation of spores by the arial mycelia (Hölker *et al.*, 2004.).

The micro-porous matrix, polyHIPE was first created by pioneering investigators at Unilever (Barby & Haq, 1982) and has been studied in detail by different groups around the world. The material is described as open-cellular with an extremely low dry bulk density, approximately 0.1 g cm^{-3} . This is a consequence of a higher level of pore structure and a complete interconnection between all neighbouring cells (Akay, 1995; Cameron & Sherrington, 1997; Mercier *et al.*, 2000). The techniques and monolithic architecture of this material was described by (Akay, 1995; Akay *et al.* 2004; Akay *et al.*, 2005) and recently (Akay *et al.*,. 2006; Ndlovu, 2008 and Greco, 2014).

3. Material and Method

3.1 Preparation of micro-porous (PHP)

3.1.1 Reagents

All the chemicals (reagent grade) were used without further purification: styrene; 2-vinyl pyridine; divinylbenzene (DVB); sorbitan monooleate (Span 80); potassium persulphate ($K_2S_2O_8$); and isopropanol and they were purchased from Sigma Aldrich.

3.1.2 Preparation

The micro-porous matrix was formed by polymerisation of High Internal Phase Emulsion (HIPE) as described in (Akay *et al.*, 2005). The monomers, crosslinking agent and surfactant were dissolved in the continuous oil phase and this reactive mixture placed in the reactor (Figure1).

Figure 1: Schematic diagram of the preparation micro-porous matrix known as polyHIPE (Ndlovu 2008)

The aqueous dispersed phase containing polymerisation initiator and modifying chemicals was dosed into the continuous phase whilst stirring at 300 rpm using 2 flat panels (9 cm diameter) placed at right angles to each other and placed as close to the base of the vessel as possible. Mixing time after the complete addition of the dispersed phase was varied to control pore size. The emulsion was transferred to fill a 50 ml mould (internal diameter 2.6 cm) and polymerised in a preheated oven. After polymerisation, solidified blocks were cut into 0.5 cm thick disks.

3.1.2.1 Preparation of 2-vinyl-pyridine micro-porous matrix

For the production of 2-vinylpyridine micro-porous matrix the oil phase consisted of 70.2% w/w styrene and 7.8% w/w 2-vinylpyridine (monomers), 8% w/w divinylbenzene (cross-linking agent) and 14% w/w Span80 (surfactant). The aqueous phase consisted of 1% w/v potassium persulphate. 25 ml (for 90% phase volume) of the reactive oil phase was placed in the reaction vessel 225 ml of aqueous phase dosed in, with continuous stirring at 300 rpm over 5 minutes. Emulsion was continue stirred for (5, 10, 15 minutes) at 300 rpm to control pore sizes. The emulsion was transferred to a mould and polymerised at 40°C in a preheated oven for 6 hours and then heated to 60°C for a further 6 hours to complete polymerisation.

Disks were then washed with double distilled dried and stored for use (Ndlovu, 2008).

3.1.2.2 Preparation Sulphonated micro-porous matrix

For production of sulphonated micro-porous (PHP) the oil phase consisted of 78% w/w styrene (monomers), 8% w/w divinylbenzene (cross-linking agent) and 14% w/w Span80 (surfactant). The aqueous phase consisted of 1% w/v potassium persulphate and 5 w/w % of sulphuric acid (H_2SO_4).

25 ml (for 90% phase volume) of the reactive oil phase was placed in the reactor (Figure 1). 225 ml of aqueous phase was dosed in, with continuous stirring at 300 rpm, over dosing time equal to 5 minutes and the mixing time was varied per batch production stirred for 5, 10 or 15 minutes, at 300 rpm to control pore size. The emulsion was transferred to a mould and polymerised at 60°C for 24 hours to complete the polymerisation.

The disks were sulphonated by soaking in 97% sulphuric acid for a period of 1-2 hour and irradiated in a 1.4 kW microwave oven at maximum power, typically for eight periods of 30 seconds (4 minutes in total). The sulphonated disks were then

neutralised with 25% ammonium hydroxide solution (Figure 2). The neutralised polyHIPE was then washed again to remove excess base. Disks were then dried and stored for use.

Figure 1:

3.2 Spore Inoculum Preparation

3.2.1 Strain

“*Streptomyces coelicolor*” A3(2) JI1147 and “*Streptomyces coelicolor*” A3(2) MT1110 were obtained from NRRL (NRRL B-16638 =JI 1147=Hopwood strain A3(2)) and N.E.E. Allenby (University of Surrey), respectively.

3.2.1.1 Strain maintenance

The strains were maintained on oatmeal agar, grown at 28°C for 7-14 days, at 4°C and as glycerol suspensions (20% v/v) at –20°C. The glycerol suspensions were prepared in cryotubes by scraping growth from sporulating strains that had been grown on oatmeal agar plates at 28°C for 14 days. The frozen glycerol suspensions served for long-term preservation.

3.2.2 Media

Bennett’s and Oatmeal flour agar were used for growth and spore production. Growth in flask experiments used modified R5 medium and was prepared as described previously (Kieser *et al.*, 2000; Huang *et al.*, 2001).

3.2.3 Spore preparation

A spore suspension was inoculated onto Oatmeal agar plates and incubated for 14 days at 28°C to sporulate. Sterile Ringer’s solution (5 ml) was added to each plate, and the surface was gently scraped to release the spores. Sterile glass wool in a syringe was used to filter out hyphal biomass and the spores were collected by centrifugation. The spores were washed with sterile water and then washed in ¼

strength Ringer's solution (Kieser, *et al.*, 2000), before being used for inoculation the spore suspension was re-suspended in phosphate buffered saline (PBS). The spore density was determined by plate count and the concentration of spores adjusted to 2×10^7 spores per ml. Spores were stored frozen at -20°C in glycerol (20% v/v).

3.2.4 Colony count

A standard procedure for colony count (Jett *et al.*, 1997) was used in this research. Nine dilution tubes were prepared and 0.9 ml of sterile PBS was added aseptically to each of the dilution tubes.

Serial dilutions of the suspensions were prepared aseptically till 10^{-9} dilutions. 0.05 ml of each of the dilutions prepared were pipetted and plated on new plates of modified Bennett's agar (Jones, 1949) and incubate at 28°C until visible colonies were detected and the colonies were counted.

Dilution factor = Initial dilution x Subsequent dilutions x Amount plated
 $1/\text{Dilution factor} \times \text{Colonies counted} = \text{colony forming unit/gram (cfu/g)}$.

The spore density was calculated as $\text{cfu} \times 20 \times 1/\text{dilution spores ml}^{-1}$.

3.2.5 Pre-germination

Pre-germination was carried out following the procedure described by (Kieser *et al.*, 2000). The spores were re-suspended in 1 ml of PBS ($\sim 10^9 \text{ ml}^{-1}$) and pelleted by centrifugation at 6000 rpm. Glycerol from the spore suspension was completely removed by washing so that it did not impair spore viability during heat shock. The spore suspension was heat shocked at 50°C for 10 minutes, cooled under cold tap water, followed by centrifugation at 6000 rpm for 5 minutes, and then the pellets were re-suspended in an equal volume of Double Strength Germination medium and incubated in a rotary shaker at 300 rpm and 37°C for 2-3 hours.

3.3 Immobilising *S. coelicolor* A3(2) on the micro-porous matrix

3.3.1 Sterilisation of micro-porous matrix

Micro-porous discs were weighed and wrapped in aluminium foil, sealed with autoclave tape and autoclaved at 121°C for 30 minutes.

3.3.2 Inoculation of micro-porous matrix

The micro-porous matrix was prepared as described above and 0.5 ml of pre-germinated spore suspension ($\sim 0.5 \times 10^9$ spores) was inoculated into 50 ml of modified R5 medium and then aseptically, 2 ml inoculated onto the sterile substrate matrix, and then incubated at 28 °C.

3.4 *S. coelicolor* A3(2) liquid submerged culture

Pre-germinated spores were used to inoculate triplicate flasks. A total of 0.5 ml of pre-germinated spore suspension ($\sim 0.5 \times 10^9$ spores) was inoculated into sterile 50 ml of medium in 250 ml flasks with a stainless steel spring to create dispersed growth (Kieser *et al.*, 2000) and incubated on rotary shaker (160 rpm) at 28 °C.

3.5 Analysis of cultivation

3.5.1 Protein determination

3.5.1.1 Bradford Calibration Curve

Protein concentration was determined using the Bradford reagent (Bradford, 1976). Samples were diluted with buffer to an estimated concentration of 20-200 µg/ml. Samples of BSA at concentrations of 20-200 µg/ml were prepared for the calibration curve. 0.2ml of each of the diluted samples was added to 1ml of Bradford reagent and incubated for 45 minutes. The Absorbance at 600

nm was read. Protein concentration was calculated using the conversion factor determined from the slope of the calibration graph.

3.5.1.2 *Protein extracted from liquid culture*

Biomass from 1.0 ml of culture was collected by centrifugation and protein was extracted by dissolving in 1.0 ml of 1M NaOH for 1h at room temperature, cleared by centrifugation and neutralised with 1.0 ml of 1M HCl. An aliquot of extracted protein was assayed using the Bradford reagent (Bradford, 1976).

3.5.1.3 *Protein extracted from micro-porous cultures.*

Aseptically a polyHIPE-A(2) disc was sliced into small pieces and 1 ml of 1M of NaOH was added to 1/4 of the total weight (representing the equivalent of 1ml liquid culture as the total volume hold up of a disc was 4 ml) and then incubated for 1 hour at room temperature to dissolve the protein. PHP and undissolved biomass were removed by centrifugation. The protein supernatant was neutralised with 1.0 ml of 1M HCL. An aliquot of the protein was assayed using the Bradford reagent (Bradford, 1976).

3.5.2 Antibiotic Assay

3.5.2.1 *Prodigiosin production by S.coelicolor A3(2) grown liquid culture*

Prodigiosin was assayed by modifying the protocol described by (Kieser *et al.* 2000; Williams *et al.*, 1956) . 1 ml sample of biomass plus supernatant from liquid culture was placed at – 80 °C for about 3 hours, and then freeze dried overnight. 1 ml of methanol was added to the dried sample and incubated at room temperature for 24 hours with constant mixing; the supernatant was acidified with 0.1 ml of 5 M HCl and the concentration of prodigiosin determined by reading the absorbance at 530nm.

3.5.2.2 *Prodigiosin by S.coelicolor A3(2) grown in micro-porous matrix*

1/4 of total weight of a PHP-A3(2) disc was placed at -80°C for about 3 hours, freeze dried overnight, then 2 ml of methanol was added and incubated at room temperature for 24 hours with constant mixing. 1ml of supernatant was acidified with 0.1 ml of 5M HCl. The concentration of prodigiosin was then determined from the absorbance read at 530 nm.

3.5.2.3 *Actinorhodin from liquid culture*

A 1 ml sample from 50ml culture was taken and the pH adjusted to pH 8.0. After centrifugation at $1,100 \times g$ for 5 min, the amount of the blue-coloured antibiotic actinorhodin was determined by measuring the absorbance of the supernatant at 600 nm.

3.5.2.4 *Actinorhodin from micro-porous culture*

1/4 of the total weight of PHP disc was placed at -80 °C for about 3 hours and then freeze dried overnight. 1ml of NaOH was added and adjusted to pH 8.0. After centrifugation was carried out at $1,100 \times g$ for 5 min the actinorhodin was determined by measuring the absorbance of the supernatant at 600 nm.

3.5.3 **Scanning electron microscopy**

Micro-porous matrix samples, containing bacteria, were placed in a Petri dish and pre-washed with phosphate buffered saline (PBS), then post-fixed overnight in approximately 3 ml solution containing 2 % glutaraldehyde (Shim & Yang 1999) in PBS.

Before the SEM analysis, polymer samples containing the fixed bacteria were washed in phosphate buffered saline and then dehydrated in an ethanol series with 10, 25, 50, 75 and 100% ethanol (each step for 30 min.). At the end of the dehydration process

the samples were treated twice for 1 h with absolute ethanol. After 100% ethanol the sample was left in 100% ethanol and then finally dehydrated with carbon dioxide in a Samdri 780. Specimens were mounted on aluminium stubs with Acheson's Silver allectroDag (then dried overnight) or onto sticky carbon discs. Specimens were coated with gold (Shim & Yang 1999; Erhan *et al.*, 2004.) (Standard 15 nm) using a Polaron SEM Coating Unit or Bio-RAD SC500 Sputter Coater and the coated specimens were examined using a Stereo scan S40 Scanning Electron Microscope (EM Unit at Medical school) or the SEM (FEI XL30 ESEM FEG) at the Chemical Material Unit.

3.5.4 Concentration and Productivity Analysis

Equation 2 represents the average protein concentration obtained using the calculated conversion factor from the slope of the calibration graph, Equation 1.

$$Protein (C)_{\mu g/ml} = 39.458 \times (A_{600nm}) + 0.147 \quad \text{Equation (1)}$$

The amount of Prodigiosin and actinorhodin concentration was determined by measured the A_{530nm} and A_{600nm} converted at μmol using the molar extinction coefficient ($\epsilon M_{530nm} = 100150 M^{-1}cm^{-1}$) and ($\epsilon M_{600nm} = 12944.13 M^{-1}cm^{-1}$) respectively (Hobbs *et al.*, 1990; Ndlovu, 2008).

Then

$$[Red] = \frac{A_{530}}{0.100150} \mu mol \quad \text{Equation (2)}$$

$$[Blue] = \frac{A_{600nm}}{0.012944} \mu mol \quad \text{Equation (3)}$$

Note that equation 4 was rearranged from equation 2 and 3.

$$\frac{\mu mol}{ml} = [Antibiotic] \times vt \quad \text{Equation (4)}$$

vt in equation 4 is the total volume used in the extraction of 1ml including the amount of 1 M acid (0.1 ml) used to acidify 1 ml of 'extracted prodigiosin, in the liquid culture $vt = 1.1ml$ in plyHIPE $vt = 2.2ml$ per $\frac{1}{4}$ of total weight of the disk

4. Results and Discussion

Two growth conditions were compared in this research: conventional liquid culture in shake flasks and growth on micro-porous matrix (known as a polyHIPE) with two different surface chemistries, inoculated in the same manner. The incubation time was kept constant for all conditions.

4.1 Growth in liquid culture and micro-porous matrix

A pre-germinated spore suspension of “*Streptomyces coelicolor*” A3(2) was inoculated in modified R5 media and grown in 250 ml conical flask in order to determine protein and antibiotic concentration (Figures 5 and 6) and productivity (Tables 2 and 3). This standard growth strategy was then used as a reference, to compare with results obtained using solid state fermentation using micro-porous matrix.

“*Streptomyces coelicolor*” A3(2) was grown in 2.6 cm diameter and 0.5 cm thick micro-porous discs with different pore sizes, micro-architecture and surface chemistries. High Internal Phase Emulsion (HIPE) was polymerised in 50 ml plastic tubes and after polymerisation 5 mm disks were cut from the solid block (Figure 2a) as shown in Figure 2b. In order to obtain a hydrophilic micro-porous support, the vinyl pyridine co-PolyHIPE Polymer (PHP-VP) and the sulphonated polyHIPE was prepared (Figure 3b) with 5% sulphuric acid added in aqueous phase prior to the irradiation of (Figure 3a).

Figure 2: (a) Cast cylinder of PHP and (b) PHP-VP disks cut from the cylinder.

Figure 3: (a) PHP disc before sulphonation, (b) PHP disc after sulphonation by microwave irradiation and (c) PHP disc after sulphonation and neutralisation

The neutralisation of micro-porous matrix was important to adjust pH to optimum pH for “*Streptomyces coelicolor*” A3(2) growth, i.e. pH 7. The addition of base also helped to bleach out the excess of carbon and as result changed from the colour black carbonised micro-porous (Figure 3b) into a lighter brown (Figure 3c). This colour transformation of the material made the visual observation of red and blue pigmented biosynthesis possible.

In this study the colour changing of the support during the functionalization (sulphonation/neutralisation), was only evaluated through a visual observation. The influence of the excess of carbon upon the antibiotic synthesis will be studied in the future.

4.2 Importance of water absorbing capacity of micro-porous matrix

The change in physiochemical properties of the polymer created more interconnected channels and makes highly hydrophilic and permeable material which facilitated migration of bacteria in the vicinity and an increase in the adhesion cells to the material. The decreased kinetic rate of absorption of the fluid led to an increase in the absorbed amount (2008). This micro-porous matrix can absorb fluid at 21-35 times its dry weight and as a result it can facilitate the penetration, proliferation, migration and interaction between the 3D micro porous matrix and cells and allows this cells to grow homogeneously throughout the matrix and not only on the top surface of the support (Jimat, 2011). These features will enable the 3D micro porous matrix to find important application within tissue engineering, biotechnology and agro-technology (Bokhari, 2003; Erhan, *et al.*, 2004; Akay, *et al.*, 2006; Ndlovu, 2008; Jimat, 2011, Burke, 2007 and Fleming, 2012). Moreover, the high porosity of these matrices prevents clogging, which often occurs with other matrices (Jimat 2011).

Figure 8 shows the extent of the colonisation of the filamentous bacterium (*Streptomyces coelicolor* A3(2)) in the micro porous matrix which mimics the natural environment (Ndlovu, 2008).

4.3 Effect of the micro porous matrix in the biosynthesis of secondary metabolite

A micro-porous with a suitable morphology and pores structure was developed to support bacteria growth and enhance the secondary metabolite production. This has been reported in previous studies (Akay *et al.*, 2004, Akay, 2005, Bokhari *et al.*, 2007) that the matrix structure affects the growth pattern of the immobilized cells and influences their metabolic activity (Jimat; 2011). But until now there have been no reports in the use of this material to enhance the antibiotic production. The results

of this study is a novel discovery that describes the influence of the surface functionalization of the micro-porous matrix with vinyl pyridine (PHP-VP) and sulphuric acid (PHP-S/N) in the growth of *Streptomyces coelicolor* A3(2) as model organism. The growth in the inner porous matrix was measured using protein concentration determined by Bradford assay. The antibiotics extracted in this work were prodigiosin and actinorhodin.

4.3.1 Protein concentration

The overall growth of *S. coelicolor* on PHP-VP was 50% higher at 48 hours but very similar at 72 hours whereas growth on PHP-S/N was slightly low at 48 hours but double at 72 hours (Figure 4).

Figure 4: Growth and protein production (by Bradford) in the Liquid culture (Flask growth) and in two different surface chemistry of PHP, in Vinyl Pyridine PHP

(PHP90%-7.8VP5'5' (22 μm)), Sulphonated and Neutralised (PHP90%-5S/N5'15' (19 μm)).

4.3.2 Antibiotic Extraction

As shown in the Figures 5 and 6; the production of antibiotic in micro-porous matrix was demonstrated to exceed that in liquid growth.

a) Prodigiosin production

The overall prodigiosin production was 2-3 times higher at 48 hours in PHP-S/N and PHP-VP and 4-5 times better at 72 hours in PHP-VP and PHP-S/N compared to liquid growth (Table 1).

Table 1: Rate of productivity of Prodigiosin.

Micro-porous matrix	Rate of Prodigiosin production compared to flask growth ($\mu\text{mol.ml}^{-1}.\text{h}^{-1}$)		
	24 h	48 h	72 h
Vinyl Pyridine (22 μm)	1.2	2.7	3.7
Sulphonated and Neutralised (19 μm)	0.6	2	5.5

Figure 5: Growth and prodigiosin production in the Liquid culture (Flask growth) and in two different surface chemistry of PHP, in Vinyl Pyridine PHP (PHP90%-7.8VP5'5' (22 μm)), Sulphonated and Neutralised (PHP90%-5S/N5'15' (19 μm)).

b) Actinorhodin production

Actinorhodin production (Figure 6) was 13-15 times higher at 48h and 6-17 times higher at 72 hours in PHP-VP and PHP-S/N than those achieved in the liquid growth (Table 2).

Table 2: Rate of productivity of Actinorhodin.

Micro-porous matrix	Rate of Actinorhodin production compared to flask growth ($\mu\text{mol.ml}^{-1}.\text{h}^{-1}$)	
	48 h	72 h
Vinyl Pyridine (22 μm)	13.4	6.3
Sulphonated and Neutralised (19 μm)	15.1	17

Figure 6: Actinorhodin in the Liquid culture (Flask growth) and in two different surface chemistry of PHP, in Vinyl Pyridine PHP (PHP90%-7.8VP5'5' (22 μm)), Sulphonated and Neutralised (PHP90%-5S/N5'15' (19 μm))

4.4 Scanning electron microscopy (SEM) of micro-porous matrix

In this study SEM was an important analytical tool in order to determine pore morphology, pore size and interconnect structure of the material. Bacterial adhesion, penetration, proliferation and migration in the pores was also observed by examining the cross section.

a) SEM of micro-porous matrix

Discs were washed and dried. ¼ of blank disk (before inoculation) was taken to the SEM (Figure 7) to examine desirable pores size and topography (Figure 7 stages 3) of the functionalised micro-porous matrix processed.

Figure 7: SEM of functionalized micro-porous matrix, (A & B) stage 1 before sulphonation; (C & D) stage 2 after sulphonation; and (E & F) stage 3 after neutralisation designated as PHP-S/N.

b) SEM of *Streptomyces coelicolor* A3(2) immobilised in micro-porous matrix.

The images revealed significant microbial growth in the micro-porous matrix with the morphology of *S. coelicolor* A3(2). Filamentous arrangement of cells in a mycelial network could be clearly observed (Figure 8).

Figure 8: SEM Images of *S. coelicolor* A3 (2) growing with in micro-porous matrix (A & B) Surface, (C &D) Cross-Section. The white arrows indicate the surface of PHP Skin and the blank arrow indicates the fractured site of micro-porous matrix

S. coelicolor A3 (2) growing in the micro-porous matrix did not differentiate to form aerial hyphae or spores (Figure 8). It also did not exhibit the strict surface – associated growth shown for *Pseudomonas syringae*, when monolayer growth films were

formed (Erhan, *et al.*, 2004) or the tight integration shown with filamentous growth of *Salinispora arenicola* on natural surfaces (Jensen *et al.*, 2005).

Antibiotic production is in general it is subject to the suppressive effects caused by the limitation of nutrients such as carbon, nitrogen, and phosphate sources (Hobbs, *et al.*, 1990). In particular, ammonium and phosphate both appear to be major regulators of antibiotic production in *S. coelicolor* A3(2) and their control systems may be interrelated in some way (Hobbs, *et al.*, 1990). Considering the above statement, our results revealed that the antibiotic production by the *Streptomyces* strain studied was more or less environmental and physiological stress dependent.

The feasibility of fermentation using the same spore inoculant of *Streptomyces coelicolor* A3(2), loaded on to the micro-porous support was also examined in this research. The support created a suitable environment for the microorganism to grow and supported the production of prodigiosin and actinohodin at equivalent or better rate than in liquid culture. The micro-porous culture increased the yield, decreased production volume and labour in which has made the production of antibiotics more attractive.

5. Conclusions

The main focus of this research was the production of two well-known antibiotics, polyketide actinorhodin (Blue) and the tripyrrole prodigiosin (Red). The results of this experimental study demonstrate that solid state fermentation on inert micro-porous matrix significantly enhances the red and blue antibiotic production from *Streptomyces coelicolor* A3(2). On both types of micro-porous supports tested in this research the antibiotic production was higher than in conventional liquid culture. Process intensification of blue antibiotic production was achieved resulting in higher concentration using functionalised (sulphonated) micro-porous matrix at pore size of

19 μm . Rapid production of red antibiotic was achieved on functionalised (vinyl pyridine) micro-porous matrix at pore size of 22 μm . In order to understand how morphological differentiation and secondary metabolite formation are controlled in this micro-environment, proteomic studies were carried out and discussed elsewhere. Growth on the micro-porous matrix has shown the potential to improve the secondary metabolite production for process intensification. For further studies the development of multi micro-porous disks system and further optimisation will be essential (Ndlovu, 2008).

The support system allowed for antibiotic production with higher specific production rate and easy purification than when a liquid culture system was used. The micro-porous matrix supported growth and secondary metabolite production by “*Streptomyces coelicolor*” A3(2) which exceeded that in liquid submerged culture and was shown to be an alternative method for the production of secondary metabolites giving a more concentrated product.

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ACCEPTED MANUSCRIPT

Diagram of the experimental growth of *Streptomyces coelicolor* A3(2) in Micro-porous matrix (known as polyHIPE)

Graphical abstract

Highlights

- Preparation of novel micro-porous matrix with different physiochemical proprieties
- *S. coelicolor* A3(2) was cultured in those micro-porous and antibiotics was enhanced.
- Matrix pore sizes and surface chemistry .influenced bacterial signalling.
- Bacterial signalling has a profound effect in the overproduction of Prodigiosin and actinorhodin
- Prodigiosin and actinorhodin production within micro-porous was 5-17 times higher compared with liquid growth.