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Antibiotic Production Through Solid-State Fermentation Under a Novel Fixed-Bed Micro-Reactor

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Abstract: In the present study, solid state fermentation (SSF) was evaluated using a novel PolyHIPE Polymer (PHP) matrix. This matrix was developed with an approach to improve the production of antibiotics. For the production of the matrix, a batch reactor was operated with a mixing speed of 300 rpm at differents mixing times (5, 10, 15 min) as a consequence, differents pores sizes was Obtained (55, 39, 19 µm) with a sulfonated/neutralized surface chemistry. After functionalization and purification, the matrix was placed in a fixed-bed micro-reactor, designed specifically for this project; which had 16 fixed beds for the production of antibiotics in the solid state using a model filamentous bacteria *Streptomyces coelicolor* A3(2). The growth conditions such as the size of the pores of the matrix, were investigated in relation to the growth time (From 0 to 168 hours), and two extracts were produced, Prodigiosin a member of the family of red pigment tripyrrol and Actinorhodin a benzoisochromoquinone dimeric antibiotic that belongs to a class of aromatic polykets. The concentration of the extracted antibiotics and their activities were examined by the disc diffusion method. The two compounds produced were tested against microbial strains (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens, and Penicillium notatum*) and the inhibition effects were measured. The results concluded that the highest specific production rate of prodigiosin (3.02 µmol $ml^{-1}h^{-1}$) and actinorhodin (26.08 µmol $ml^{-1}h^{-1}$), was achieved within the PHP matrix, with pore sizes of 39 µm and 19 µm in diameter respectively. On the other hand, the assay revealed a larger inhibition halo (diameter in mm) that was observed in the plate inoculated with DSM 10 strains (*Bacillus subtilis*), inhibited by Prodigiosin extract.

Keywords: PolyHIPE Polymer (PHP), Solid State Fermentation (SSF), *Streptomyces coelicolor* A3(2) Prodigiosin, Actinorhodin, Antimicrobial Screening

1. Introduction

Solid-state fermentation (SSF) has been defined as the fermentation process that occurs in a solid matrix, (inert support or support/substrate), in the absence or near absence of excess water, [20, 21, 28]. In recent years this process has been well used in the production of secondary metabolites in the presence of moisture and nutrients, to support the growth and metabolic activity of microorganisms [31].

Two main types solid support are considered SSF system. Firstly, natural solid substrates, such as starch or lignocellulose residues, or agro-industrial sources, such as grains and grain by-products, cassava, potatoes, rice and beans. In these cases, substrates are used as a support and source of carbon and nutrients for microbial growth, [22, 30]. Secondly, inert substrates, which can be natural or synthetic solid supports, such as sugarcane bagasse, perlite, amber, polyurethane foam, etc. These last supports are used only as a support matrix for the growth of the culture after the impregnation of defined media. From an engineering point of

view, inert supports are better because they do not change their geometric and physical properties with microbial growth and allow better control of heat and mass transfer [4, 25].

SSF produces a high concentration of the product (in a short period of time), and has a relatively low energy requirement [19, 33]. It also needs a reactor of smaller volume, so it can be considered as an intensified process. Due to the lack of excess water, mycelial growth on solid surfaces does not encounter problems associated with media such as viscosity. Highly viscous liquid media are necessary for successful metabolite production, but this can interfere with oxygen transfer; consequently the production of secondary metabolites by SSF becomes attractive, as this technology has the potential for better oxygen circulation [7] and SSF in inert supports impregnated with adequate media facilitates reproducibility [21], offering the potential to improve process control and monitoring due to the well-defined fermentation environment [25].

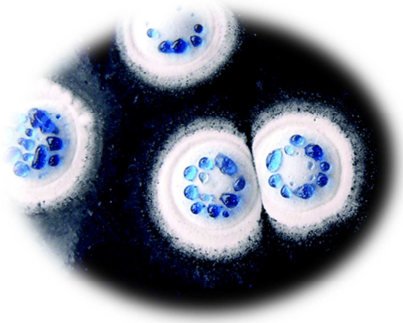
The use of a porous support with well-defined characteristics, (such as pore size, size of the interconnected or interconnected hole, porosity of the pore walls, as well as the chemical activity of the walls), can only be achieved if synthetic supports are developed [4]. Polymeric matrices such as polyurethane were also used to increase the production of antibiotics by "*Streptomyces coelicolor*" A3(2) by [26].

The objective of SSF is to put the cultured microorganism in close contact with the insoluble substrate that mimics the natural environment and achieves the highest concentration of nutrients and production of metabolites [4, 20, 21, 38], which can be achieved if the substrate is present as an open porous structure, with good mass transfer to the external world. The importance of the SSF system for the production of enzymes and secondary metabolites has gained recognition [12], but the main challenge is to develop a solid matrix suitable for microbial growth and formation of products such as antibiotics [4]. This is the main driver of this study.

Antibiotics are low molecular weight compounds, usually obtained from microorganisms that can inhibit growth or destroy certain other microorganisms. In this work *Streptomyces Coelicolor* A3 (2), was used as a model organism because it is the best studied organism for the genetics of Streptomyces and the regulation of the production of secondary metabolites in streptomycetes [3, 13, 14, 32]. The entire sequencing of the genome of the model organism, making it a reference strain for post-genomic studies and providing a complete view of its secondary metabolism [15].

Streptomyces coelicolor A3(2), produces four well-known antibiotics, such as actinorhodin, prodigiosin, calcium-dependent antibiotic (CDA) and methylomycin [2, 3, 13]. The typical colonies of *Streptomyces coelicolor*" A3(2) are represented in the following photograph (Figure 1); (a) colonies producing droplets of the blue-pigmented antibiotic actinorhodin. The droplets stay on the surface of the colonies due to the hydrophobic nature of the aerial hyphae and spores (b) a drop of antibiotic secreted from a colony of "*S. coelicolor*".

Streptomyces coelicolor as a multicellular organisms, the colonies development, is also maintained by a constricted equilibrium between cell proliferation and cell death processes [18].



a)

b)

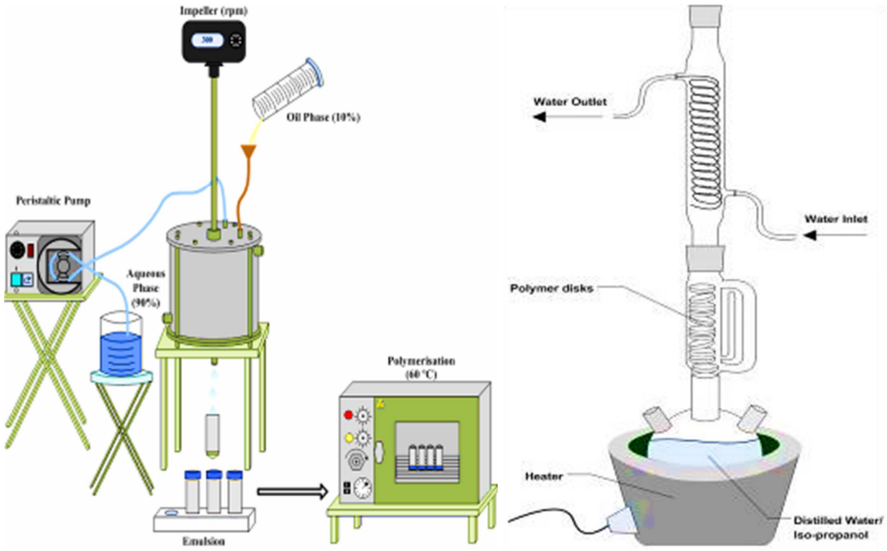
Figure 1. *Photo courtesy of Dr Paul A. Hoskisson, John Innes Centre, Norwich, UK [39].*

The growth and antibiotic production of "*Streptomyces coelicolor*" can be varied depending on the growth medium, growth conditions, and inocula [6, 8, 11, 24]. As the biosynthesis of antibiotics is subject to complex regulatory processes, influenced by the dynamics of growth and differentiation and by the environment, any variability is amplified both in the synthesis of antibiotics and in their visual detection [4, 20, 29].

This study is based on determining, to what extent solid state fermentation can be employed to achieve process intensification in the production of prodigiosin and actinorhodin, in the initial and final stationary phase respectively. PolyHIPE production has been modified from that previously used for cell growth, [1, 17]. This modification of the functionalization process altered its porosity, hydrophilicity and surface roughness, making the material a good candidate for microbial growth and production of secondary metabolites [20]. Inoculation of pre-germinated spores into the material has been shown to have a permanent effect on the growth and production of secondary metabolites [21].

This study examined antibiotics extracted from different pore materials and antimicrobial sensitivity tests were

performed. There are three approaches commonly used as antimicrobial sensitivity testing methods that are shown to consistently provide reproducible and repeatable results [34], such as the disc diffusion method, broth dilution method, and agar dilution method. The disc diffusion assay of antibiotics employing a disc plate method has the great advantage of convenience, simplicity, sensitivity, efficiency and reliability [10, 27, 34]. Given the disadvantages of more accurate and consistent methods on broth and agar, it would be advantageous to improve the disc diffusion method, possibly by combining with the intensification of SSF technology.



(a) (b)

Figure 2. *Schematic diagram, a) apparatus used in PHP production, b) soxhlet used for PHP washing.*

2. Materials and Methods

2.1. Preparation of Polyhipe Polymers

PolyHIPE was produced using a standard protocol, previously developed [1]. A high internal phase emulsion (HIPE) was made from an oily-aqueous mixture in a batch mixing reactor (Figure 2a). The aqueous phase used was 5% sulfuric acid by weight with a phase volume (VP) of 90%. The composition of the oil phase with a phase volume of 10%. The aqueous phase was slowly dosed in the oil phase, for a dosing time of 5 minutes under continuous agitation and an additional homogenization time of (0), (5) and (15 minutes). The mixing speed was 300 rpm. The emulsion was then transferred to falcon tubes and polymerized at 60^{o}C for 8h. After polymerization, the PHP disks PHP disks were washed (Figure 2b) and functionalized.

2.2. Fixed-Bed Micro-Reactor Design

A 16-bed micro-reactor to be filled with PHP (Figure 3), was designed in this work and to be used as a solid-state fermenter. This was built from Teflon with a stainless steel mesh and spacer ring, and an O-ring seal. The humidifier was made of glass with 16 outlets to supply the individual beds.

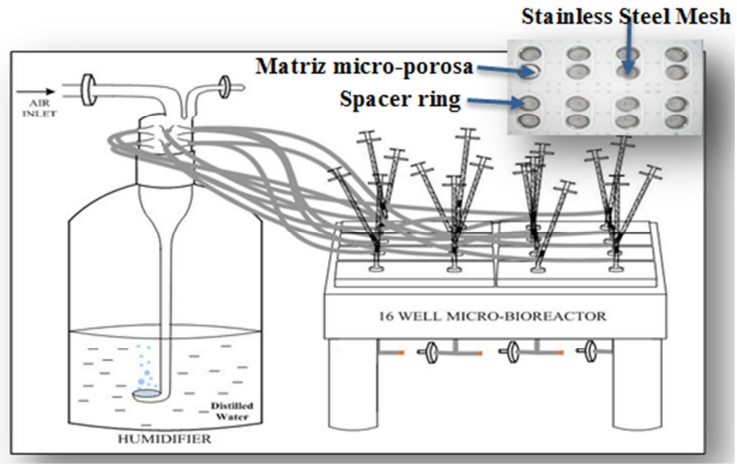


Figure 3. *Schematic design of 16 fixed-bed micro-reactor.*

2.3. Preparation of the Inoculum

"*Streptomyces coelicolor*" A3(2) was obtained from Nick E.E. Allenby (Newcastle University, UK). The maintenance of the strain was maintained in oat agar, cultivated at 28°C for 7–14 days, at 4°C and in glycerol suspension (20% v/v) at -20°C. Glycerol suspensions were prepared in cryotubes by scraping the growth of sporulating strains grown in oat agar plates at 28°C for up to 14 days. The modified R5 medium was prepared as previously described [15, 16] and then used, to impregnate the inert microporous matrix coined as PolyHIPE polymer [1, 21].

2.3.1. Spore Preparation and Pre-Germination

The spore suspension was inoculated in oat agar plates and incubated for 14 days at 28°C to sporulate. A Ringer's solution (5 ml) was added to each plate, and the surface was gently scraped to release the spores which were collected by centrifugation and washed in Ringer's 1/4 solution at concentration [16]. Spore density was determined by plate count and spore concentration was adjusted to 2×10^7 spores per ml. The spores were stored and frozen at -20°C in glycerol (20% v/v) [21]. Pre-germination was performed following the procedure described by [16].

2.3.2. Sterilization and Inoculation of Inert Microporous Matrix

The microporous matrices were weighed and wrapped in aluminum foil, sealed with autoclave tape and autoclaved at 121°C and aseptically embedded in the microreactor (Figure 3). An inoculum concentration of 0.32 ml of pre-germinated spore suspension (~0.3×10^9 spores) was inoculated in 32 ml of modified R5 medium, 2 ml of 32 ml inoculum were inoculated in each of the sterilized inert matrices. The inoculated matrix embedded in the microreactor, incubated at 28°C, for 168 hours [20, 21].

2.4. Scanning Electron Microscopy

In this study, SEM was an important analytical tool to determine matrix morphologies, pore size, interconnection structure. The PolyHIPE matrix containing "*Streptomyces coelicolor*" A3(2) was placed in a petri dish and pre-washed with saline solution (PBS), and post-fixed overnight in approximately 3 ml of solution containing 2% gluteraldehyde on PBS. Before the SEM analysis, the fixed samples were washed in a saline solution and then dehydrated in a series of ethanol with 10, 25, 50, 75 and 100% (each step for 30 min.). At the end of the dehydration process, the samples were treated twice for 1 h with absolute ethanol. After dehydration, the samples were left with carbon dioxide in a critical point dryer; Samdri 780. The specimens were mounted in aluminum tubes with Acheson's silver alectroDag. The specimens were coated with gold [21, 35] using a Polaron SEM coating unit or a Bio-RAD SC500 sputter coater. The coated specimens were examined using a Stereoscan S40 scanning electron microscope or the ESEM (FEI XL30 ESEM FEG).

2.5. Protein Determination

2.5.1. Bradford Calibration Curve

An aliquot portion of the protein extract was tested using Bradford's reagent [5]. Bradford's reagent was used to measure protein concentration and to evaluate growth based on optical absorption of the solutions. The spectrophotometer was heated 15 minutes before use. The samples were diluted with buffer to an estimated concentration of 20-200mg/ml. Samples of bovine serum albumin (BSA) at concentrations of 20-200 mg/ml were prepared for the calibration curve. 0.2ml of each of the diluted samples were added to 1ml of Bradford reagent and incubated for 45 minutes. The Absorbance was read at 600 nm (A600nm) and a calibration curve was determined. Deriving equation 1 [20]. The protein concentration was obtained from the calibration curve using Equation 1.

$$\mathrm{Pr}otein(C)_{\mu g/ml} = 39.458 \times (A_{600nm}) + 0.147 \quad (1)$$

An aliquot portion of the protein extract was tested using Bradford's reagent [5].

2.5.2. Protein in Polyhipe Matrix

A polyHIPE disc after growth was cut aseptically into small pieces and 1 ml of 1M NaOH was added to 1/4 of the total weight and then incubated for 1 hour at room temperature to dissolve the protein and then the PHP and undissolved biomass removed by centrifugation. The protein supernatant was neutralized with 1.0ml of 1M HCL. An aliquot of the protein was tested using Bradford's reagent [5] as before using the same protein calibration curve [21].

2.6. Procedure for Antibiotic Extraction

The extraction of prodigiosin was modified by modifying the protocol described in [16, 36]. 25% of the 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 were added and incubated at room temperature for 24 hours with constant mixing. 1ml of the supernatant was acidified with 0.1ml of 5M HCl. The concentration of prodigiosin was then determined from the absorbance at 530 nm. [21].

For actinorhodin extraction**,** 1/4 of the total weight of the PHP disk 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 at 1,100 × g for 5 min, actinorhodin was determined by measuring the absorbance of the supernatant at 600 nm [21].

2.6.1. Production Rate Analysis

The concentration obtained in equation 1 was used to determine the average volume of the product and, consequently, the average rate of production:

$$AVp = \frac{t_{P1} + t_{P2}}{2} \quad (2)$$

The amount of Prodigiosin and Actinorhodin was determined by measuring the A_{530nm} and A_{600nm}, converted to $\varepsilon M_{530nm} = 100150 M^{-1} Cm^{-1}$ μM using the Molar extinction coefficients and ($\varepsilon M_{(600nm)} = 1294413 M^{-1} cm^{-1}$), respectively (37)

So:

$$Antibiotic[Mol] = \frac{A_{530nm}}{0.100150} \mu mol \quad (3)$$

and

$$Antibiotic[Mol] = \frac{A_{600nm}}{0.012944} \mu mol \quad (4)$$

$$\mu mol / ml = Antibiotic[C] \times vt \quad (5)$$

vt In equation 5 indicates the total volume used in the extraction of 1ml, including the amount of 1 M of acid (0.1 ml) used to acidify the 1 ml of extracted prodigiosin. In liquid culture $vt = 1.1ml$ whereas in plyHIPE $vt = 2.2ml$ por 1/4 of the total weight of the disc.

2.6.2. Bioassay

The biological assays (tests) in the different strains, mentioned in table 1, were performed using the standard protocol of Professor Hans-Peter Fiedler's group in Tübingen, Germany. The microbial test strains used in this study were obtained from the DSMZ by Professor Emeritus Dr Michael Goodfellow at Newcastle University's School of Biology.

Plate Preparation and Screening Procedures

Bacillus Subtilis subsp. subtilis e Pseudomonas fluorescens

Bacterial biomass was inoculated with nutrient agar (AN) and the plates were incubated at 30°C overnight. Bacterial growth was determined by optical density measured at 600nm (OD_{600}). One milliliter of cell suspension (OD_{600} = 1.5) was added to 50 ml of NA maintained at 47°C and 4 ml of the mixtures superimposed on NA plates. The plates were left to dry. Sterile discs of filter paper soaked with 10 µl of extract were placed on the agar surface and the plates incubated at 30°C overnight. The diameter of any inhibition zones was observed and recorded the next day, after 24 hours.

Escherichia coli and Staphylococcus aureus

Assay plates for these strains were prepared as described for *B. subtilis* and *P. fluorescens*, but NB, NA and NA were replaced by LB broth, unused LB agar and LB agar, respectively, for *E. coli* and the incubation temperature was 37°C.

Penicillium notatum

The test plates for this strain were prepared by means of a giant colony plate. The fungal spores were inoculated with PF agar and incubated at 28°C for 2 days. Subsequently, agar caps were prepared using a 6 mm cork drill and placed on their heads tolow on the surface of the PF agar. The buffered plates were incubated at 28°C for 3 days and the giant colony was formed in all cases. Filter paper discs soaked with 10 µl of extract were placed on the edge of each giant colony and the plates incubated for another 15 days.

The presence of an inhibition halo was observed by light microscopy and recorded as positive (+) or negative (-).

The OD_{600} of 1.5 for bacteria and 2.8 for yeast wasdetermined empirically.

3. Results and Discussion

"*Streptomyces coelicolor*"A3(2) cultured in PolyHIPE matrix produced potential antibiotics and their activity was examined. The extracts were screened against the five bacterial strains listed in Table 1. The results of each of the tests are summarized in table 1, where the inhibited strain is indicated by a positive (+) sign and an uninhibited strain is indicated with a negative (–) sign. Figure 10 (c & d) illustrates the large diameter of the inhibition halo (in mm) observed in the plate inoculated with DSM 10 strains (*Bacillus subtilis*), inhibited by prodigiosin extract.

3.1. Synthesis of the Antibiotic Produced in a Solid State Culture

The main focus of this work was to demonstrate the synthesis capacity of two colored antibiotics (Prodigiosin and Actinorhodin) grown in solid state using functionalized PHP matrix, which have been the object of continuous efforts for the development of new drugs [1, 20, 21].

IUPAC of Prodigiosine shows 5-[(E)-[(5Z)-3-methoxy-5-pyrrole-2-ylidene-pyrrole-2-ylidene] methyl]-2-methyl-3-pentyl-1H-pyrrole and molecular weight of 323,432 daltons with molecular formula: $C_{20}H_{25}N_{3O}$.

The molecular formula of the compound Actinorhodin is $C_{33}H_{30}O_{14}$ Given a large molecular weight of 650.59 daltons higher when compared to prodigiosin, this explains the kinetic order of the two metabolic synthesis routes of the compound we extracted. Throughout our experimental growth we observed prodigiosin (red) first and actinorrodin (blue) this kinetic order is in accordance with the available literature. In the case of antibiotic production by filamentous microorganisms, there is subjective evidence that solid matrix growth can increase antibiotic production, as illustrated in (Photo a & b). In this sense, an innovative approach has been successfully developed in order to improve the synthesis of antibiotics by "*Streptomyces coelicolor*"A3(2). The growth of "*Streptomyces coelicolor*" A3(2) in the matrix in three different pore sizes; (55, 39 and 19µm) were cultivated in 16 beds fed with continuous air flow, using an air humidifier and incubated at 28°C.

3.2. Morphology and Immobilization of PHP

The porosity of the material wall, as well as the adhesion, penetration, proliferation and migration of bacteria within the pores was observed by scanning electron microscopy of the cross-section of the exposed material (Figure 4a). PHP inoculated with pre-germinated spores (as shown in Figures 4 f-h) showed growth on the surface and inside the pores as extensive filamentous growth with no signs of sporulation

observed [20]. There was no significant difference in the morphology of bacteria for growth with different pore sizes (19, 39 and 55µm).

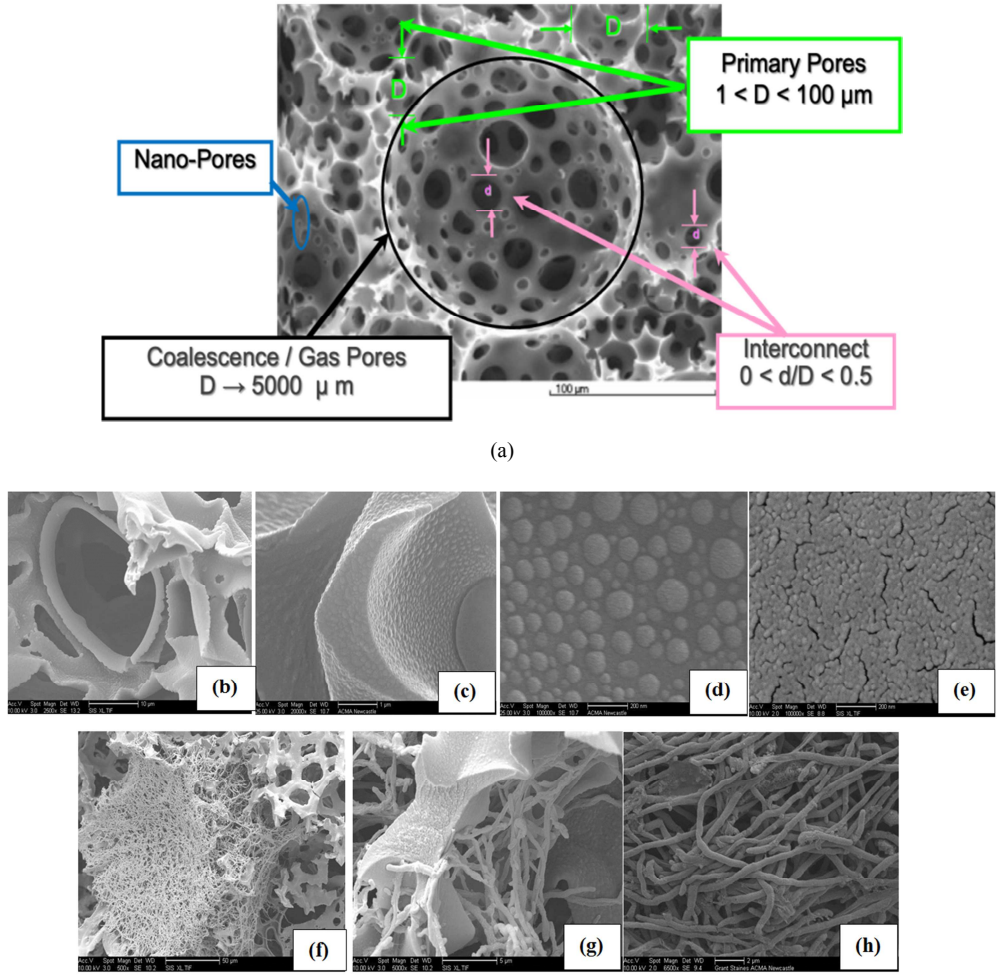


Figure 4. *SEM images a) PHP structure before functionalization, b-e) PHP after functionalization f-g) immobilization of Streptomyces coelicolor A3 (2) in Microconnective matrix surface and Cross section.*

3.3. Biomass Concentration

To quantify the biomass, samples were collected at 24, 36, 48, 72 and 168 h. Growth was determined by measuring absorbance in a spectrophotometer (OD_{600nm}) and using equation 1, readings were converted in to protein concentration as shows in (Figure 5).

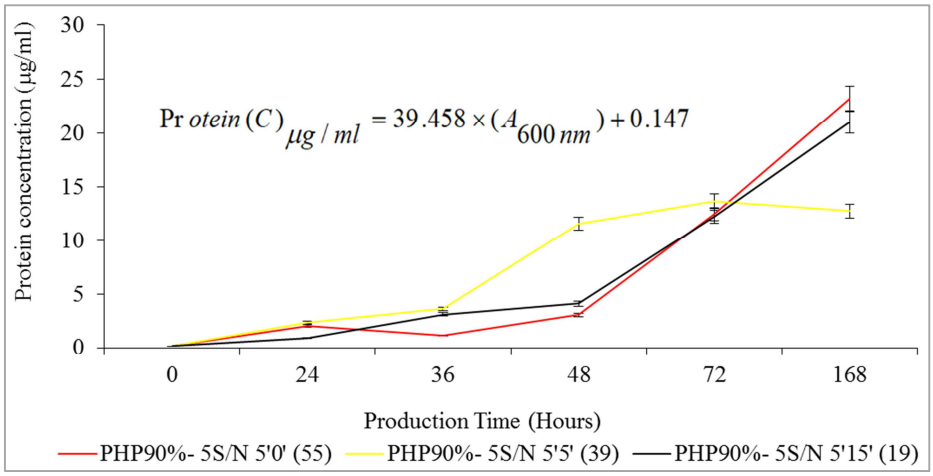


Figure 5. *Growth and protein production (by Bradford) in sulphonated/neutralised PolyHIPE Polymer, pore size 55 µm, 39 µm and 19 µm.*

3.4. Antibiotic Production

Actinomycetales bacteria are widely recognized as a very prolific source of biologically active natural compounds such as antibiotics, enzymes, immunosuppressants, and herbicides. "*Streptomyces coelicolor*"A3(2) is a model organism for the study of polyketide antibiotic production in Actinomycetales.

The spore of "*S. coelicolor*"A3(2) was inoculated in PHP with three different pore sizes PHP- S/N at levels 55, 39 and 19 µm diameter. The growth time was up to 168 hours, when the actinorodin was fully expressed in the PHP matrix.

3.4.1. Antibiotics Extraction

For the extraction of antibiotics, the cultured PHP was washed with PBS solution and submitted to the freeze-drying process in which the water is evaporated and thus the dry PHP was obtained. After drying, the concentrations antibiotics were measured by extracting, with 2 ml of acidified methanol pH 2, (Figure 6) and actinorhodin was extracted with NaOH (Figure 7).

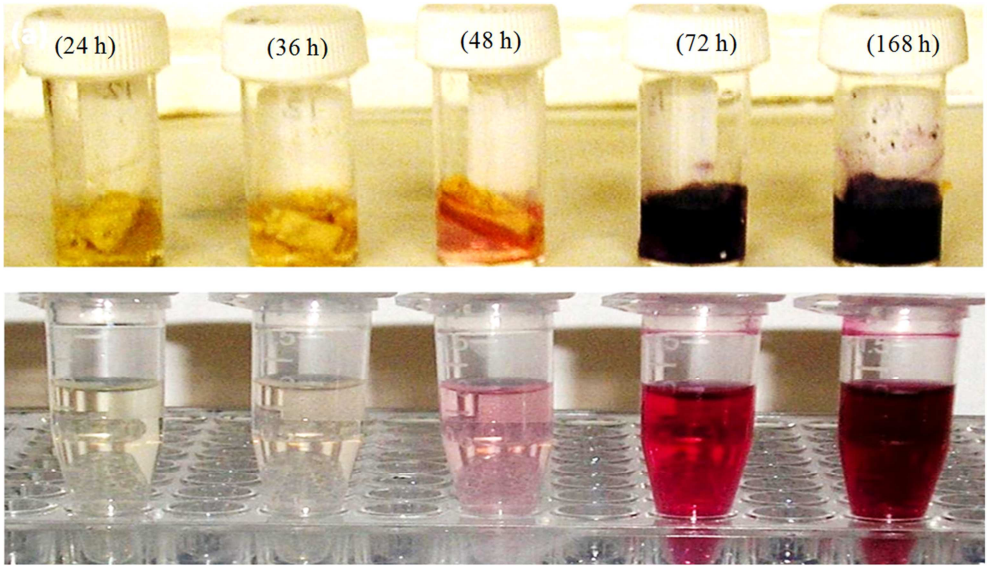


Figure 6. *Photograph of prodigiosin extract in sulphonated/neutralised PolyHIPE Polymer, 39 µm pore size a) crushed PHP disc in 2 ml methanol and b) purified sample from (a) and acidified at pH 2.*

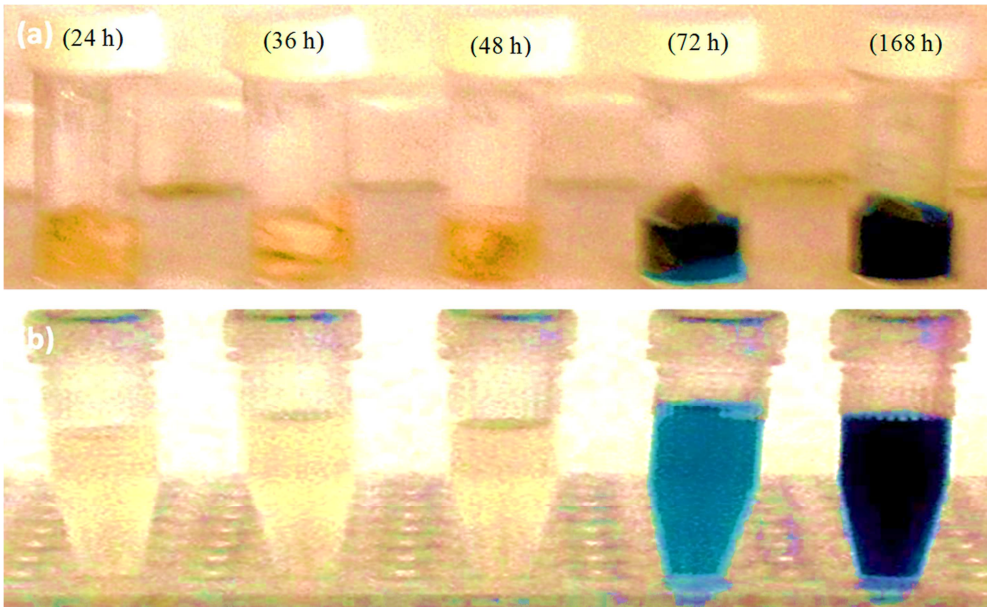
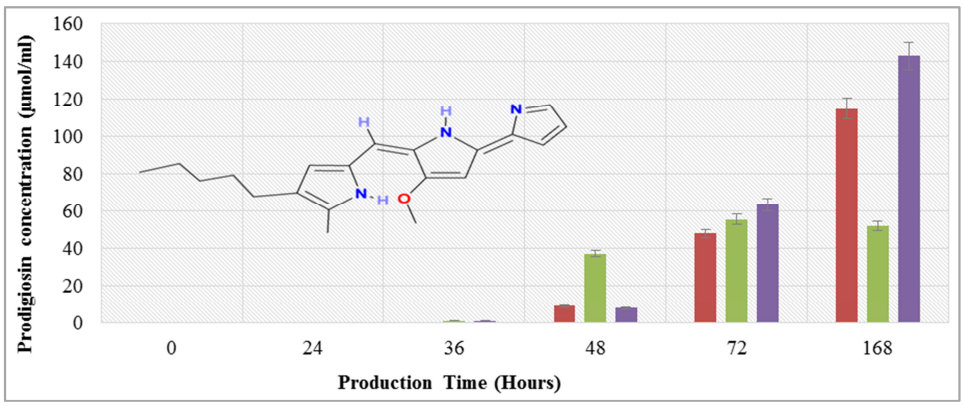
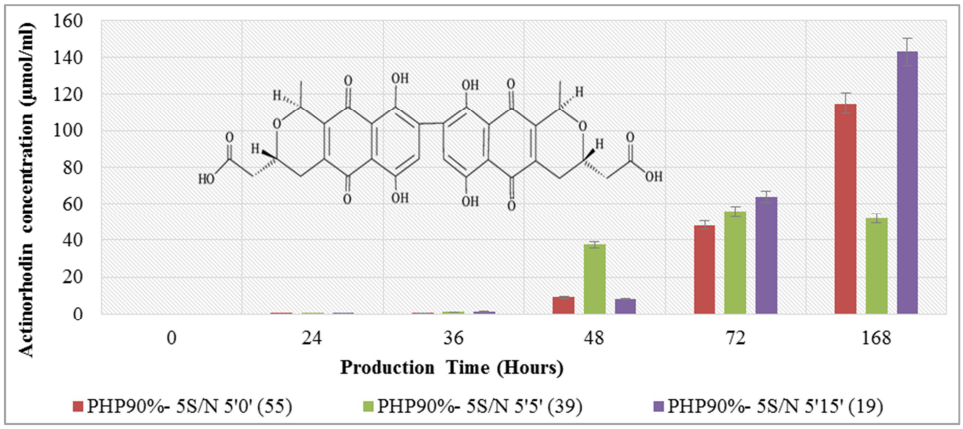


Figure 7. *Photograph of Actinorhodin extract in sulphonated/neutralised PolyHIPE Polymer, 19 µm pore size a) crushed PHP disc in 2 ml methanol and b) purified sample from (a) and acidified at pH 2.*

The purified antibiotics, was read with the extinction coefficient at 530nm for prodigiosin (Figure 8a) and actinorhodin read at 600nm converted into molar coefficient (Figure 8b).



(a)

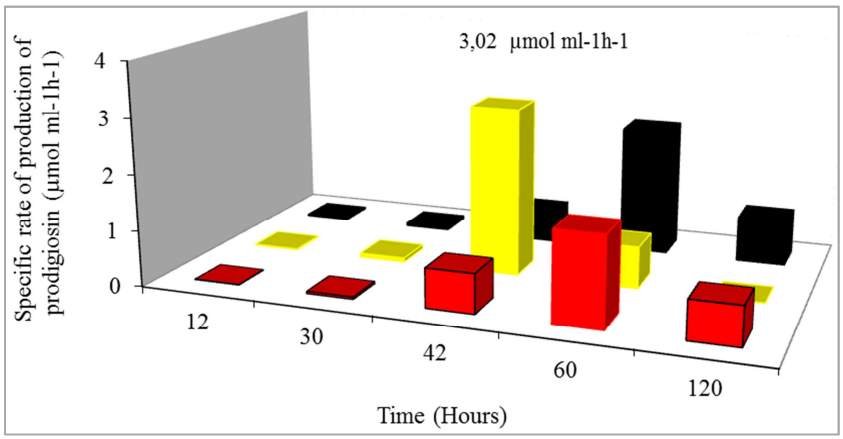


(b)

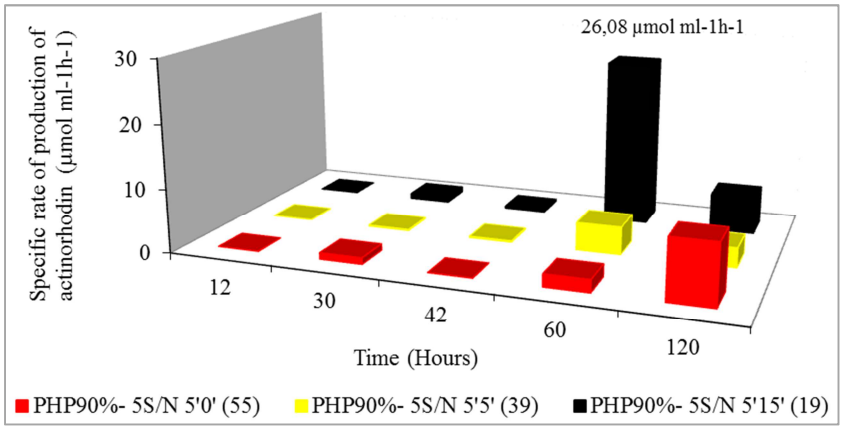
Figure 8. *Growth, antibiotic production in sulfonated/neutralized polyHIPE polymer (PHP90%-5S/N), pore sizes 55 µm, 39 µm and 19 µm, a) prodigiosin concentration, b) actinorhodin concentration.*

3.4.2. Production Rate Antibiotics

Consider the evolution of the rate of antibiotic production in the two antibiotics produced under three different porous-sized conditions. The natural process, begins with the production of prodigiosin (red), but after 72 hours the production changes from prodigiosin to actinorhdin (blue); once the production of prodigiosin began to show extinction, the synthesis of actinorhodin increases (as shown in Figure 8b). The overall production of prodigiosin was 4 times higher in 48 h when cultivated in pores of 39 µm and 2 times better in 168 hours when in 19 µm, the results demonstrate that for prodigiosin the highest production can be achieved before 72 hours using PHP matrix of 39 µm. (Figures 8a and 9a). More over the total production of actinorhodin was 6 times higher after 72 h grown in 19 µm pores. It can be observed that for actinorhodin, the highest production rate is achieved after 72 hours using a PHP matrix with a pore size of 19 µm. (Figures 8b and 9b).



(a)



(b)

Figure 9. *Specific rates of antibiotics production in sulfonated/neutralized polyHIPE polymer (PHP90%-5S/N), pore sizes 55 µm, 39 µm and 19 µm, a) prodigiosin, b) actinorhodin.*

3.4.3. Extracts Tested on Five Strains

The extracted antibiotics were tested and both extracts showed the ability to inhibit the strains DSM 10 (*Bacillus subtilis*) and DSM 20231 (*Staphylococcus aureus*). On the other hand, prodigiosin did not inhibit DSM 50090 strains (*Pseudomonas fluorescens*), but these three tested strains were inhibited by actinorhodin.

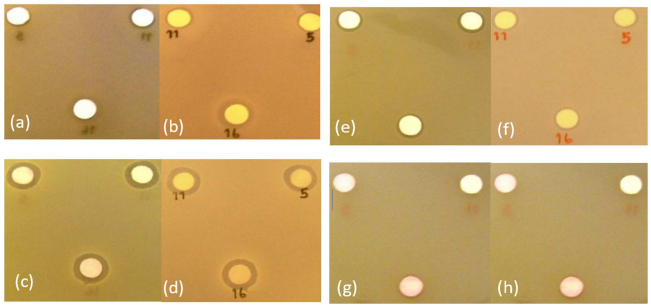


Figure 10. *Illustrates the large zone of inhibition diameter (in mm), and was observed in the plate inoculated with strains of DSM 10 (Bacillus subtilis), inhibited by prodigiosin extract.*

In Figure 10, *Bacillus subtilis* was tested with actinorhodin (a, b) and prodigiosin, (c, d) extracted from PHP-S/N in different pore sizes. *Staphylococcus aureus* was tested with actinorhodin (e, f) and prodigiosin (g and f).

Extracts tested on Penicillium notatum Tu136 = *Penicillium notatum,* was not inhibited by any extract as shown in Figure 11 no halo of inhibition was observed.

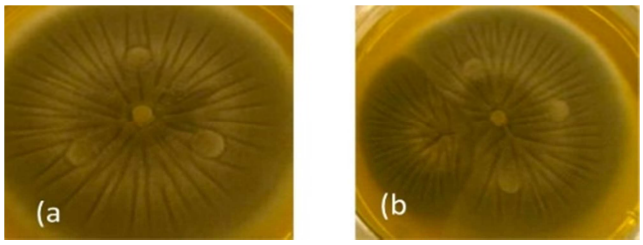


Figure 11. *The zone of inhibition for Penicillium notatum. (a) Prodigiosin and (b) Actinorhodin extract.*

The diameter of the inhibition zone (in mm) is summarized in Table 1 for all samples.

Table 1. *Summary result of the antimicrobial susceptibility test (disk diffusion method).*

Disc number	PHP description	Prodigiosin tested against					Actinorhodin tested against				
		10^T	20231	5698	50090	Tü 136	10^T	20231	5698	50090	Tü 136
5	PHP90%-5S/N 5' 0'	+	+	-	-	-	+	+	+	+	-
11	PHP90%-5S/N5' 5'	+	+	-	-	-	+	+	+	+	-
16	PHP90%-5S/N 5' 15'	+	+	-	-	-	+	+	+	+	-

DSM 10^T =*Bacillus subtilis subsp. subtilis*
DSM 20231=*Staphylococcus aureus*
DSM 5698 = *Escherichia coli*
DSM 50090 = *Pseudomonas fluorescens*
Tü 136 = *Penicillium notatum*

We investigated the production of antimicrobial metabolites by *Streptomyces coelicolor*"A3(2) in an inert solid support composed of polymeric material in three different pore sizes. The physicochemical approach improved the yields of Prodigiosin and actinorhodin. The SSF using the microporous matrix, with desirable miniaturized monolithic format, hydrophilic with greater absorption of nutrients and mimicking the natural environment of the model microorganism was achieved. It can be considered as an alternative method of this nature that, multiple steps can be achieved, such as the process of fermentation to extraction [4].

The two antibiotics investigated, prodigiosin and actinorhodin, produced in three different pore sizes (19 µm,

39 μm and 55 μm), and were evaluated for production rate. The highest specific production rate of prodigiosin (3.02 μmol $ml^{-1}h^{-1}$), was achieved, within the PHP matrix, with porous sizes of 39 μm in diameter and for actinorhodin, the highest specific production rate (26.08 μmol $ml^{-1}h^{-1}$) was achieved, within the PHP matrix, with a pore size of 19 μm.

4. Conclusions

It is common knowledge that antibiotics are secondary metabolites, and parameters such as substrate concentration, dissolved oxygen concentration in the fermentation medium, biomass concentration, culture volume, carbon dioxide concentration in the medium, pH, fermentation temperature, aeration rate, agitator power used for homogenization of the reaction medium, are important factors, and has to be closely monitored and optimised to ensure that higher antibiotic concentration and maximum production yield is obtained [23].

Although, on an industrial scale, antibiotics continue to be produced by a conventional fermentation process, where the microorganism is grown in large reactors of 50 to 1000 m^3 [23, 38], containing a liquid growth medium.

It was possible to obtain Prodigiosin and actinorhodin from the solid state fermentation process using functionalized PHP matrix. The process resulted in high antibiotic yield of Prodigiosin and atinorhodin. Futhermore, did not generate effluents or any environmental impacts compared to the submerged fermentation method that generates large amounts of liquid effluents with a high microbial load and containing antibiotics.

Although there are some limitations in the SSF process such as difficulties in controlling parameters (mixing and homogenization of the medium), thus not allowing the operation of large volumes in a single batch and therefore requiring the use of smaller units in parallel. But the physicochemical properties of PHP have overridden these limitations. The PHP fixed bed, It's microporous material, elastomers with high structured porous hirarchy, which mimics the natural environment of filamentous bacteria that inhabit the soil, such as *Streptomyces coelicolor* A3(2), allowing the proliferation, migration and differentiation of microorganisms within the pores, and consequently, increases bacterial signaling and improves the production of secondary metabolites [21].

This work revealed that the optimal pore size for the production of podigiosin was 39 μm and actinorhodin of 19 μm. Antibiotic production is increased when bacteria are confined to small pores, so the process can be intensified by confinement in the PolyHIPE material [21].

PHP, allowed an easy purification of the extract with a higher concentration, compared to conventional liquid fermentation, the mitigation of wastewater production with a lower denoted risk of bacterial contamination was another notable advantage [4, 9, 21].

In the present work the extracted antibiotics (Prodigiosin and actinorhodin) were tested and both extracts showed the ability to inhibit the strains DSM 10 (*Bacillus subtilis*) and DSM 20231 (*Staphylococcus aureus*). On the other hand, the largest inhibition halo (by diameter) was observed in the plate inoculated with the DSM 10 strain (*Bacillus subtilis*), inhibited by the prodigiosin extract (Figure 10, Table 1). None of the extracts (prodigiosin or actinorhodin) tested was able to inhibit the growth of the Tü 136 test strain (*Penicillium notatum*) (Figure 11). The methodology developed in this work is very innovative, so that in the literature there are almost no similar works that serve as a comparison regarding kinetic parameters, biological, etc.

Aknowlegments

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