**Universidade do Minho**
Escola de Engenharia

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# Optimization of fungal pigments production through different types of fermentation

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# Optimization of fungal pigments production through different types of fermentation

Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação do
**Professor Doutor José Texeira**
e da
**Doutora Sara Silvério**

outubro de 2019

## DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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## STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

## ABSTRACT

### OPTIMIZATION OF FUNGAL PIGMENTS PRODUCTION THROUGH DIFFERENT TYPES OF FERMENTATION

Pigments are organic or inorganic compounds with the ability to provide color, and therefore, are widely applied in several industrial products. Once public awareness concerning environment preservation and safety, sustainability and health issues have been increasing, industries began to call for different pigments from natural sources, obtained by cleaner processes and with less ecological impact. The production of natural pigments through microbial fermentation is considered a very promising alternative. Fungi are known to naturally synthesize and secrete several classes of pigments. Recently, some *Penicillium* species have been reported as effective pigment producers.

The use of fungi cultures to obtain products of interest is generally performed under two types of fermentation: submerged fermentation (SmF) and/or solid-state fermentation (SSF), with SmF being the most industrially used. For fermentation processes to become economically viable, the extractive fermentation (FE) has been proposed as an emerging option which allows the integration of fermentation and selective separation of the product of interest.

In this work, the pigments production using alternative low-cost media containing agroindustrial residues was evaluated under SmF (with and without biomass immobilization), SSF and EF conditions. For that purpose, 6 different culture media composed of cheese whey (CW) and/or corn steep liquor (CSL) in different ratios (culture media B-G) were prepared and compared with a reference synthetic medium (A). Regarding the production under SmF conditions, the results revealed no significant differences between the alternative media D, F and G and the reference synthetic medium A. The immobilization of the fungus on a natural support (corn cob, SmFn) under SmF conditions led to higher amount of pigments obtained than with an inert support (nylon sponge, SmFi). Furthermore, most of the culture media tested under SmFn proved to be a suitable alternative to the reference medium A. On the other hand, better pigments yields were achieved using the inert support under SSF conditions. Overall, the best conditions found for pigments production were media D and G both under SmFn conditions. For all types of fermentation processes considered in this work, pigments mixtures were obtained with yellow, orange and red pigments present. The pigments production was also attempted under EF conditions using a PEG8000-NaCit aqueous two-phase system (ATPS) and their recovery and concentration in top phase was observed.

In conclusion, the use of alternative low-cost culture media composed of agroindustrial residues to produce pigments by *P. brevicompactum* was successfully demonstrated.



**Keywords:** agroindustrial residues; fungal pigments; immobilization supports; *Penicillium brevicompactum*

## RESUMO

### OTIMIZAÇÃO DA PRODUÇÃO DE PIGMENTOS FÚNGICOS ATRAVÉS DE DIFERENTES TIPOS DE FERMENTAÇÃO

Os pigmentos são compostos orgânicos ou inorgânicos com a capacidade de conferir cor sendo amplamente usados em vários produtos industriais. Com o aumento da consciencialização do público sobre a preservação e segurança do meio ambiente, a sustentabilidade e a saúde, as indústrias começaram a exigir pigmentos de fontes naturais, mais limpos e com menos impacto ecológico. A produção de pigmentos naturais por fermentação microbiana é considerada uma alternativa promissora. Sabe-se que os fungos sintetizam e secretam naturalmente várias classes de pigmentos. Recentemente, algumas espécies de *Penicillium* foram reportadas como produtoras de pigmentos.

A utilização de culturas de fungos para obtenção de produtos de interesse é geralmente realizada recorrendo a dois tipos de fermentação: fermentação submersa (SmF) e/ou fermentação sólida (SSF), sendo a SmF a mais utilizada industrialmente. Para tornar os processos de fermentação economicamente viáveis, a fermentação extrativa (EF) tem sido proposta como uma opção emergente que permite a integração da fermentação e a separação seletiva do produto de interesse.

Neste trabalho, a produção de pigmentos utilizando meios alternativos de baixo custo contendo resíduos agroindustriais foi avaliada em condições de SmF (com e sem imobilização de biomassa), SSF e EF. Para isso, foram preparados 6 meios de cultura diferentes, compostos por soro de queijo (CW) e/ou milhocina (CSL) em diferentes proporções (meios de cultura B-G) e comparados com um meio sintético de referência (A). Usando condições de SmF, os resultados não revelaram diferenças significativas entre os meios alternativos D, F e G e o meio de referência A. A imobilização do fungo em suporte natural (espiga de milho, SmFn) em SmF permitiu obter uma maior quantidade de pigmentos do que com o suporte inerte (esponja de nylon, SmFi). Além disso, a maioria dos meios de cultura testados em SmFn provou ser uma alternativa adequada ao meio A. Por outro lado, foram obtidos melhores rendimentos de produção de pigmentos usando o suporte inerte em SSF. No geral, as melhores condições encontradas para a produção de pigmentos foram os meios D e G, ambos em SmFn. Todos os tipos de fermentação estudados neste trabalho originaram misturas de pigmentos, constituídos por pigmentos amarelos, laranjas e vermelhos. A produção de pigmentos foi também testada em EF usando um sistema de duas fases aquosas (ATPS) PEG8000-NaCit e verificou-se a recuperação e concentração dos pigmentos na fase superior.

Em conclusão, foi demonstrado com sucesso o uso de meios de cultura alternativos de baixo custo, compostos por resíduos agroindustriais, para produção de pigmentos por *P. brevicompactum*.



**Palavras-chave:** resíduos agroindustriais; pigmentos fúngicos; imobilização em suportes; *Penicillium brevicompactum*

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## ABBREVIATION LIST

**ATPS-** Aqueous Two-Phase Systems

**CSL-** Corn Steep Liquor

**CW-** Cheese whey/Cheese whey powder

**EE-** Ethanolic extract

**EF-** Extractive fermentation

**M-** Medium

**MC-** Medium change

**MEA-** Malt extract agar

**MPA-** Mycophenolic Acid

**P-YE-** Peptone and yeast extract mixture

**SmF-** Submerged fermentation

**SmFi-** Submerged fermentation with immobilization on inert support

**SmFn-** Submerged fermentation with immobilization on natural support

**SSF-** Solid-state fermentation

**SSFi-** Solid-state fermentation using inert support

**SSFn-** Solid-state fermentation using natural support

# 1. State of art

## 1.1. PIGMENTS AND THEIR IMPORTANCE IN DIFFERENT INDUSTRIES

Color has always attracted humans and plays an important role in the choice of various products of commercial value, since consumers tend to evaluate the quality of a product or choose it, by its visual appearance. Thus, the use of pigments as color-conferring molecules has been widely explored by various industries[1].

Pigments can be classified as organic or inorganic, both classes being subdivided into natural or synthetic pigments. Natural pigments can be obtained from living organisms such as plants, animals, fungi and other microorganisms and by ores, while the synthetic ones are obtained by chemical synthesis in the laboratory (**Figure 1**)[2,3].

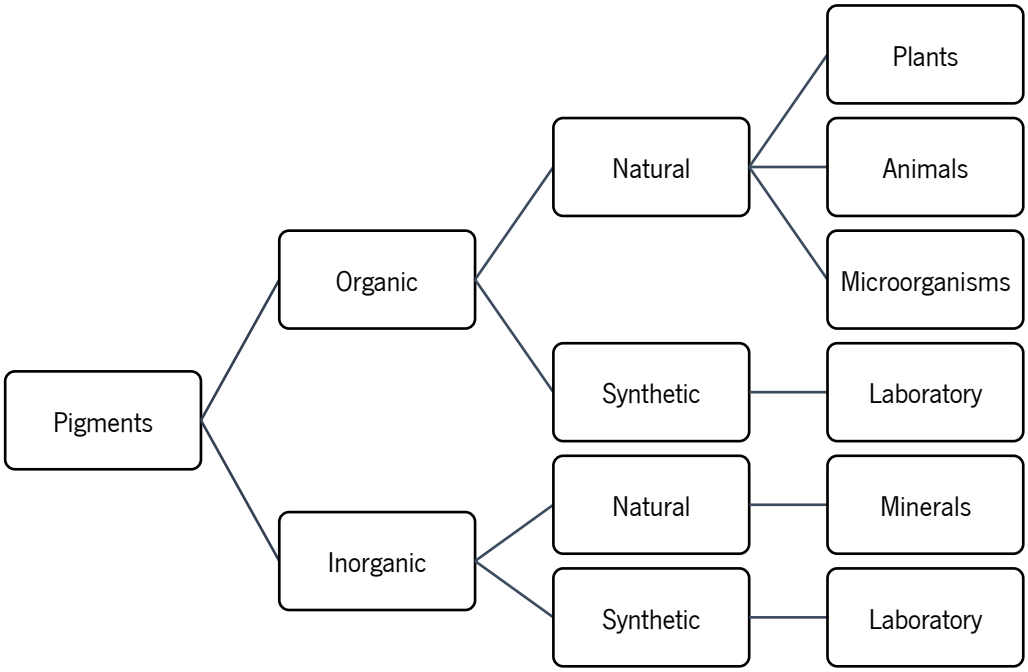


**Figure 1. Representative scheme of the classification of pigments and their sources of production.**

Pigments are compounds with important characteristics to several industries, such as food, cosmetics, textile, pharmaceutical and tanneries. Among these characteristics stands out the wide variety of existing colors, which led many pigments to begin to be produced, isolated and characterized[4].

Currently, approximately 43 pigments are allowed as food additives in the European Union, while in the United States approximately 30 are approved. Of these 43, a large number belongs to the class of organic natural pigments and are essentially obtained from plants[5].

In the food industry, pigments are used as color additives[6]. Color additives are mainly applied as: antioxidants, compensating the loss of color due to the exposure to light, air, temperature and storage

conditions; color enhancers, to turn food more attractive; suppliers of color to colorless foods[7]. Carotenoids, which are responsible for the attractive color of most of the fruits and vegetables and present diverse functions and biological properties, are a typical example of pigments used as color aditives[8].

Some studies have shown the potential of application of some pigmented secondary metabolites in the pharmaceutical industry, especially in the treatment of several diseases, since some pigments also present antibiotic, anticancer and immunosuppressive properties[9–11]. An example of such compounds are the anthocyanins, which present a wide range of biological properties such as potential to reduce cancer and inflammatory risks, and modulate the inflammatory response[12].

The textile industry is another industrial sector with great interest in pigments. Data found in the literature suggest that in 2013 approximately 1.3 million tons of dyes, pigments and color precursors are used in this sector per year, valued at around 19 billion euros, being almost all produced synthetically[13]. Currently, synthetic pigments derived from non-renewable resources, such as fossil fuels, are mainly used in this industry due to their simple application, reproducibility and possibility to obtain quality at a reasonable price to consumers. However, the biosynthesis of natural pigments for use in this industry has increased considerably in the recent years, mostly due to the issues associated with the synthetic dyes[14]. Among these issues, the production dependency on non-renewable petroleum resources, their environmental toxicity, sustainability matters and, particularly, the increasing concerns regarding their danger to human health can be highlighted. Due to all these factors, in the recent years there has been an increase in the preference for organic natural pigments obtained from sustainable processes[15].

## 1.2. ORGANIC NATURAL PIGMENTS AND THEIR PRODUCTION

Public consciousness of ecological preservation and safety, sustainability and health issues has been increasing. Thus, the development of biological, non-toxic and biocompatible pigments has become necessary. All these aspects have contributed to put pressure on the industries to take the steps needed to invest in the research and development of new types of pigments and to explore new sources for their production. In this way, various pigments derived from natural sources, cleaner and more ecological began to be reported and applied[16].

In 2010, natural pigments represented about 31% of the world market of pigments, and its application is growing and may even exceed the synthetic pigments in the near future[17].

Organic natural pigments are obtained and/or extracted from natural sources, mainly from plants. In most cases, they are mixtures of varied compositions, which depend on the climatic conditions

and form of cultivation. Therefore, they are not easy to obtain or characterize. Also, a great variation in the stability and functionality of different classes of existing organic natural pigments can be observed, being the pigments often affected by light, pH and temperature. Once certain products require the use of stable pigments, this lack of stability can limit their range of use[18].

Despite the disadvantages mentioned above, there are also undoubted advantages that justify the preference for these pigments. In terms of human metabolism, substances of natural origin are more easily metabolized by the body, since there are many metabolic pathways in common with other organisms. In addition, there are several organic natural pigments that have antioxidant activity, a biological property of extreme importance and that accompanies the trend of development of food with health benefits[19].

Thus, replacing artificial pigments by natural pigments, two desired effects are obtained, on the one hand, color matching (an important feature for the food industry) and on the other its biological properties, such as protection against free radicals, having a two in one effect[20].

Traditionally, organic natural pigments are extracted from plants with flowers, such as the blue color pigment indigo obtained from *Indigofera tinctoria,* or from insect tissues, such as red cochineal obtained from *Dactylopius coccus*[4,21]. However, most of the pigments obtained from these sources are produced in insufficient amounts for industrial use, being dependent on the supply of raw materials (due to seasonality) and being also affected by ethical reasons, particularly when considering an animal source[22].

Organic natural pigments obtained through microbial fermentation are considered a very promising alternative, namely to overcome the associated availability and ethical issues of the raw materials. Many bacteria, fungi and yeasts are known to produce a wide variety of natural pigments[23–26].

Pigments obtained from microorganisms are of industrial interest because these organisms are generally more accessible, can grow rapidly and still have the potential to be genetically modified, which consequently can provide better yields and productivities. Efforts have been made to reduce the costs associated with the production of pigments by microbial fermentation, to efficiently compete with the production of synthetics and derivatives from other natural sources, such as plants and animals[22,27].

Among the different microorganisms, fungi have been reported as the major pigment producers due to their versatility in producing pigments of different colors and easy large-scale cultivation. So far,

there are several fungi species well studied to produce pigments, and some examples will be presented and briefly discussed below[27–29].

## 1.3. PIGMENTS PRODUCED BY FILAMENTOUS FUNGI

Fungal secondary metabolites, such as pigments, can be divided into four different classes depending on their structural properties: terpenes, polyketides, non-ribosomal peptides, and amino acid-derived compounds[30]. These metabolites, also known as exometabolites, are characterized as bioactive small molecules that are not essential for the growth of the host organism but instead aid survival in harsh environments, resisting desiccation and UV stress and improving competition with other microbes[31,32].

Exometabolites are produced during morphological and chemical differentiation and then are secreted, deposited or accumulated in the cell wall[32]. Most of the exometabolites produced by ascomycetous filamentous fungi are polyketides. Polyketides are typically synthesized by multifunctional polyketide synthases (PKA) from acetylcoenzyme A (CoA) and malonyl-CoA[33,34].

Polyketides represent an array of structurally complex natural products such as anthraquinones, hydroxyanthraquinone, naphthoquinone and flavonoid pigments. Some species of ascomycetous filamentous fungi belonging to the families *Monascaceae*, *Trichocomaceae*, *Pleosporaceae* and *Nectriaceae* are responsible for the production of polyketide-based pigments with different colors (red, orange, yellow and/or brown)[27,35].

Certain pigments produced by filamentous fungi also belong to the terpene class. Terpene synthases and terpene cyclases generate terpenes from activated isoprene units. Carotene and antheraxanthin are examples of secondary metabolites belonging to this class[36].

This two class of secondary metabolites are derived from central metabolic pathways and primary metabolite pools, with acyl-CoAs being the critical initial building block (**Figure 2**).

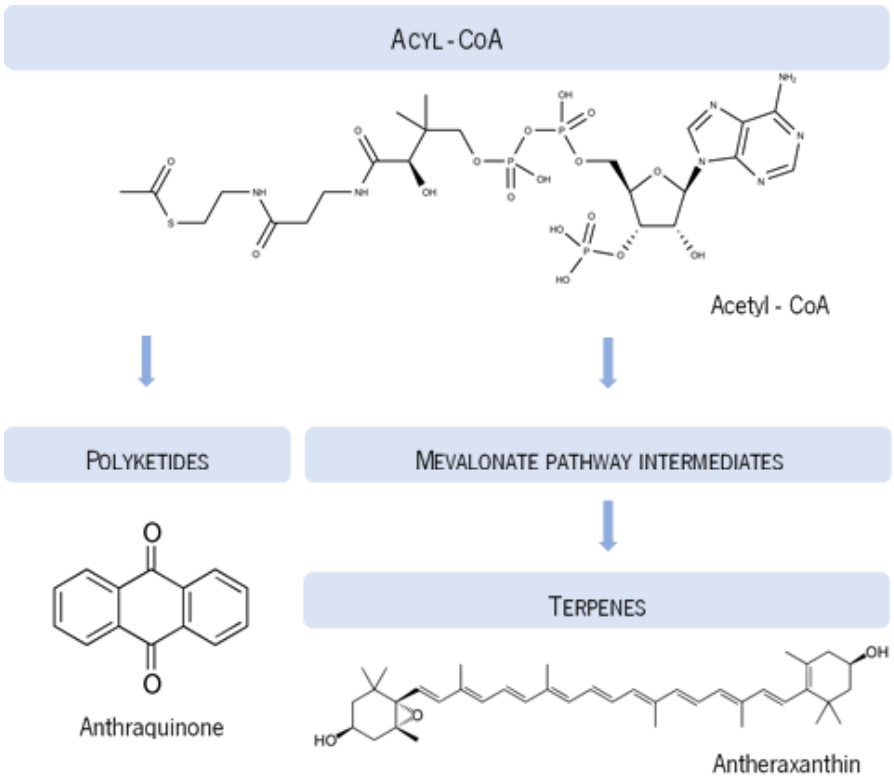


**Figure 2. Acyl-CoAs as critical initial building block for polyketides and terpenes.** Adapted from Keller[36].

Fungi, particularly filamentous fungi, are known for their ability to produce a wide range of pigments that are generally more soluble than plant-derived pigments[27,37]. Some pigments produced by filamentous fungi, and their respective color and class, are shown in **Table 1**.

**Table 1. Pigments produced by filamentous fungi and their respective color and class.** Adapted from Caro *et al.*[30], Mapari *et al.*[38] and Rao *et al.*[39].

| Filamentous Fungi | Pigment | Colour | Class |
|---|---|---|---|
| ***Monascus* spp.** | Ankaflavin | Yellow | Polyketides |
| | Rubropunctatin | Orange | |
| | Monascorubramin | Red | |
| ***Aspergillus versicolor*** | Asperversin | Yellow | Terpene |
| ***Fusarium* sp. *JN158*** | Benzoquinon | Yellow | Polyketide |
| ***Fusarium oxysporum*** | Anthraquinone | Yellow | Polyketide |
| ***Stemphylium lycopersici*** | Anthraquinone | Yellow | Polyketide |
| ***Ashbya gossip*** | Riboflavin | Yellow | Polyketide |
| ***Cordyceps unilateralis*** | Naphthoquinone | Deep blood red | Polyketide |
| ***Penicillium oxalicum*** | Anthraquinone | Red | Polyketide |
| ***Blakeslea trispora*** | Lycopene | Red | Terpene |
| ***Blakeslea trispora*** | ß-carotene | Yellow–orange | Terpene |
| ***Mucor circinelloides*** | ß-carotene | Yellow–orange | Terpene |
| ***Penicillium freii*** | Viomellein | Reddish-brown | Polyketide |
| | Xanthomegnin | Orange | |
| | Mitorubrin | Yellow | |
| ***Penicillium purpurogenum*** | Mitorubrinol | Orange-red | Polyketide |
| | Purpurogenone | Yellow-orange | |
| ***Penicillium rugulosum*** | Rugulosin | Yellow | Polyketide |

The first fungal pigment production known is ang-kak (red rice) using *Monascus purpureus*, which consists in fermenting rice with this fungus getting the rice a bright purplish red color.

Currently, more than 50 pigments produced by *Monascus* species have been identified and studied. Also, more than 50 patents have been issued worldwide for the use of *Monascus* pigments in

foods[40,41]. In addition to the variety of available colors, pigments produced by this genus of fungus have been shown to have antimicrobial[42,43], anticancer[44,45], antimutagenic[44] and antiobesity properties[46,47].

Although there are several fungal pigments reported in the literature, their practical use is conditioned by regulatory approval criteria concerning their toxicity and stability. In addition to these criteria, capital investment should also be considered, which represents the main bottleneck for transposing Petri dish products to the market[48].

The best example of these approval issues is the pigments obtained from *Monascus* which have been used in Asia for centuries as food pigments, but banned in Europe and in the United States due to the presence of mycotoxins in the final formulations[40]. Several methods have been developed to avoid obtaining mycotoxin-contaminated pigments such as: (a) selection of non-pathogenic strains (several fungal strains have been tested for toxicity and it was found that toxin production is only present in some lineages); (b) control of metabolites biosynthesis (the study of metabolic pathways has shown that toxin production can be controlled through the biosynthesis process); (c) selection of the culture media (it has been observed that the addition or removal of metal ions, carbon sources and nitrogen sources may affect toxin production)[40,49].

In addition to the absence of toxins during production and commercialization, microbial pigments must be stable under extreme pH and temperature conditions so that they can be used at industrial level. It is currently known that there are several strains of non-pathogenic filamentous fungi that can be used as potential producers of non-toxic and non-pathogenic pigments for humans, such as *Talaromyces*, *Cordyceps unilateralis*, *Herpotrichia rhodosticta*, *Curvularia lunata*, several species of *Drechslera* and some species of *Penicillium*[5,50,51].

### 1.3.1. *PENICILLIUM* SPP. AS POTENTIAL PIGMENT PRODUCERS

As mentioned previously, pigments obtained from fungi are produced as secondary metabolites of known or unknown function. Although *Monascus* pigments are the most commercially available, especially in the East as food pigments, it is known that several strains of *Monascus* produce, among other potentially toxic metabolites, the mycotoxin citrinin[18]. This mycotoxin is known to be nephrotoxic, hepatotoxic and possibly carcinogenic in humans and animals[52]. Therefore, the search for alternative microorganisms to produce safe pigments has become an important and demanding field[53].

Recently, *Penicillium* species have been identified as potential pigment producers. The pigments produced by these microorganisms are homologous to the those produced by *Monascus*, with similar

chromophores[4]. Also, these fungal pigments can be more easily accepted in the food industry since they come from microorganisms that are known to not produce the mycotoxin citrinin. However, the presence of other mycotoxins should be carefully evaluated prior their practical application at industrial level. Several studies have demonstrated the production of pigments by *Penicillium* species by fermentation, thus evidencing the potential of using this genus as an industrial pigment producer[4,54,55].

#### 1.3.1.1. *PENICILLIUM BREVICOMPACTUM*

*P. brevicompactum* is a mold usually found inside the production chambers of the food industry[56]. This specie of *Penicillium* is able to grow in environments with low water activity[57]. This fungus has the following lineage: *Eukaryota*, *Fungi*, *Dikarya*, *Ascomycota*, *Pezizomycotina*, *Eurotiomycetes*, *Eurotiomycetidae*, *Eurotiales*, *Aspergillaceae*, *Penicillium*.

According to the literature, this specie is able to produce mycophenolic acid (MPA), an immunosuppressive compound that is increasingly associated with cases of allergic and pulmonary fibrosis[58]. This compound is reported as a mycotoxin, which is synthesized during the secondary metabolism of the fungus while contaminating foods and feeds[56].

However, when found in contaminated foods, the levels of MPA are lower in comparison to the therapeutic doses commonly administered to humans, so its presence should not be a health concern. Some authors reported that MPA should not be classified as a mycotoxin since the risk and the severity of MPA effects after consuming potential contaminated foods are both reduced[59].

Until today, there are no reports in the literature about the ability of *P. brevicompactum* to produce pigments, but this fungus showed a high potential to produce this type of compounds from a yellow to a red color range, depending on the growth conditions. Recently, this strain was isolated from the air of a cheese curing chamber in Ponte de Lima, Portugal. Previous studies of our research group showed that *P. brevicompactum* has great potential for pigment production[60,61].

## 1.4. PRODUCTION OF FUNGAL PIGMENTS BY FERMENTATION

Fermentation is an important biological technology that allows to produce several chemical or pharmaceutical compounds by the cultivation of microorganisms such as bacteria and fungi in the presence of complex substrates. In the course of the metabolic decomposition of the substrates, various secondary compounds (e.g. pigments) are released in addition to the usual fermentation products, such as carbon dioxide or alcohol.

Besides pigments, many other secondary metabolites from microbial origin such as enzymes, antibiotics, peptides and growth factors have been obtained by fermentation and they are widely used by different industries. In order to efficiently use microorganisms to produce these metabolites at industrial level, it is necessary to scale-up the techniques used in the laboratory. However, performing the scale-up of fermentation processes presents some challenges. It is necessary to ensure that the growth of the microorganisms and consequently the obtention of the desired products correspond to that achieved at the laboratory scale, avoiding adverse conditions that may result in the production of undesirable compounds[62,63].

Since fungal pigments are fermentative products, their production can be significantly affected by variations of certain parameters like temperature, pH, carbon and nitrogen sources, aeration, agitation and the type of fermentation (solid or submerged)[62].

### 1.4.1. SUBMERGED FERMENTATION

Submerged fermentation (SmF), also called liquid fermentation, is a process in which microorganisms grow in liquid medium, which is essentially water containing dissolved nutrients (**Figure 3**).

In SmF, bioactive compounds can be excreted into the fermentation broth containing the dissolved nutrients. Depending on the microorganism, these substrates can be quickly consumed, being sometimes necessary to consider a replacement/supplementation of nutrients throughout the fermentation process[62,64].

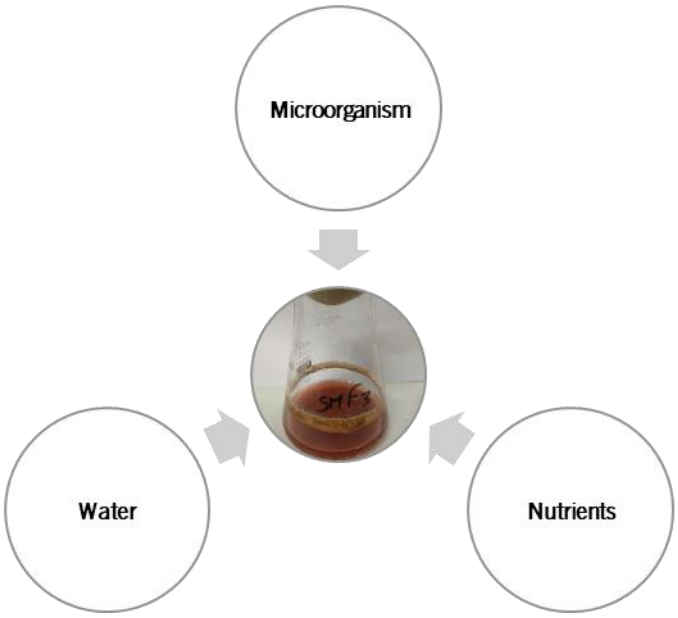


**Figure 3. Schematic representation of the main constituents of a submerged fermentation.**

This process is suitable for microorganisms, such as bacteria and fungi, which require a high moisture content. Also, SmF is more frequently used to produce secondary metabolites that are commonly utilized in liquid formulations[62].

The biological processes carried out by SmF have notable advantages in terms of instrumentation, parameters control (pH monitoring, dissolved oxygen, temperature, concentration of water-soluble molecules), biomass separation after fermentation (purification), agitation (in terms of mixing), aeration and scale-up[64].

Although the fungal production of products of interest, such as pigments, may be performed either through SmF or solid-state fermentation (SSF), the type of fermentation generally applied at industrial level is the SmF[65].

In SmF it is important to take into consideration different parameters, namely the carbon source, nitrogen source, pH, temperature, oxygen concentration, salt concentration, agitation and aeration.

Carbon and nitrogen sources are two important factors that can affect cell growth and development[65]. A liquid fungal culture is generally known to require carbohydrates, nitrogen, zinc and magnesium sulfate[40].

As fungi are heterotrophic, they require an exogenous carbon source. Some fungi use complex carbon-containing compounds, but others are more selective in their demands. Thus, many compounds can be used as a carbon source for fungal growth.

Santos-Ebinuma *et al.*[55] using *P. purpurogenum* DPUA 1275 demonstrated that is possible to produce red pigments through SmF conditions, which can be applied in different industries after toxicological examinations. In this work, different carbon sources, such as monosaccharides (glucose and fructose), disaccharides (sucrose and maltose) and starch were tested in the pigments production by fungus and it was concluded that the use of starch and sucrose enhanced the pigments production.

Gunasekaran and Poorniammal[66] determined that by optimizing the culture conditions of *Penicillium* spp., there was a seven-fold improvement in the production of a red-colored pigment using SmF and starch as carbon source.

Méndez *et al.*[4] demonstrated that the use of Czapek-Dox modified broth also allows the pigments production by *P. purpurogenum* GH2 under SmF conditions.

### 1.4.2. SOLID-STATE FERMENTATION

In recent years, SSF also called semi-solid fermentation, has presented great potential in the development of different bioprocesses. SSF is a process in which the growth of microorganisms occurs on solid materials (solid support) in the absence or near absence of free water. In this fermentation process, a natural substrate or an inert substrate can be used as solid supports (**Figure 4**)[67,68].

In SSF, the substrate must contain enough moisture to ensure a suitable water activity for the microorganism growth and metabolism. However, the amount of water must not exceed the maximum binding capacity of the solid matrix, i.e. no free water should be present in the fermentation medium[69].

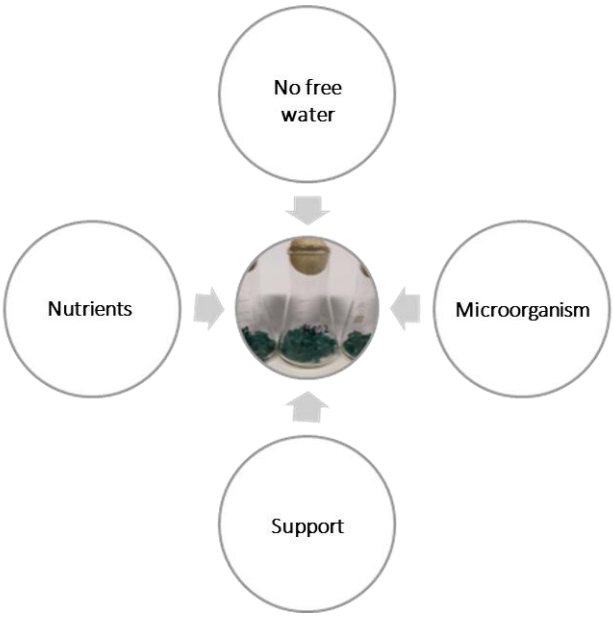


**Figure 4. Scheme representative of the main requirements of a solid-state fermentation.**

SSF presents several advantages when compared to SmF, such as generally higher production yields, lower energy requirements, easier aeration, less generation of liquid waste, reduced bacterial contamination and easier product recovery[68]. This type of fermentation mimics the natural habitat of many microorganisms, especially fungi. In addition to the advantages already mentioned, SSF has several environmental advantages, as it allows the use of agroindustrial solid wastes as substrates and/or energy sources in their natural form[70].

Among the factors that typically affect the efficiency of SSF, the microorganism strain, substrate selection, and process parameters (physical, chemical, and biochemical) selection are considered the most relevant[68]. Anyway, the moisture content is a key variable that influences the efficiency of SSF. This parameter is specific to each microorganism and support and it is essential to maximize metabolites production and promote favorable growth conditions[64,71].

Padmapriya and Murugesan[72] optimized the production of red pigments by *P. purpurogenum* using cassava waste as substrate/support under SSF conditions and a considerable amount of pigments was obtained.

Also using an industrial waste, namely orange processing waste, Kantifedaki *et al.*[14] demonstrated the pigments production by *M. purpureus* and *P. purpurogenum* under SFF conditions and concluded that *P. purpurogenum* successfully produced *Monascus*-like pigments from waste orange peels, in the absence of mycotoxins.

### 1.4.3. AGROINDUSTRIAL RESIDUES AS ALTERNATIVE CULTURE MEDIA IN FERMENTATION PROCESSES

In the last decades one of the biggest concerns of the modern society is the valorization of the wastes. Recently, there has been a significant increase in the worldwide generation of organic wastes. However, international entities have promoted a shift in waste classification from pollutants to secondary renewable resources, as an attempt to control the environmental damages.

In this context, the use of agroindustrial residues as low-cost substrates in biotechnological processes can result in recognized economic and environmental benefits. The incorporation of these residues in bioprocesses allows the processing of great amounts of wastes and by-products and the use (or reuse) of them as energy sources or valuable materials[73,74]. As these residues contain large amounts of organic compounds, they can replace conventional sources of carbon, nutrients and/or micronutrients[75]. Some examples of agroindustrial residues with potential to be used in fermentation processes will be presented and briefly discussed below.

Cheese whey (CW) is a liquid mixture obtained after the precipitation of milk casein in the cheese making process and represents the main by-product of dairy industry[76]. CW contains considerable amounts of lactose and can be used as low-cost substrate in culture media for microorganisms able to metabolize lactose and produce added-value compounds such as food colorants[76] and enzymes[75].

Lopes *et al.*[77] showed that *M. purpureus* produces red pigments on soy protein and cheese whey and that filamentous fungi that have capacity to growth on available agro-industrial residues may represent an interesting alternative to obtain pigments or pigmented extracts.

Corn steep liquor (CSL) is the major by-product generated during corn wet-milling milling industry. CSL has been identified as a potential nitrogen source for the biochemical industries and it is another example of an inexpensive substrate. This residue is a source of proteins, amino acids, minerals, vitamins, and trace elements. It can be used as a rich effective nutritional substitute for expensive complex media like yeast extract or beef extract, and peptone in fermentations[78].

Silbir and Goksungur[79] investigated different nitrogen sources, including CSL, for pigments production by *M. purpureus* and these authors found that CSL was a promising alternative source for the production of such natural pigments. However, the optimization of the process for pigments production when CSL is used as nitrogen source is required.

The corn cob is a worldwide abundant agricultural residue that represents 30 % of agricultural wastes derived from maize, whose valorization still needs to be investigated[80]. Although this residue has a low nutritional value, it contains considerable amounts of polysaccharides (such as cellulose and hemicellulose), which can promote the growth of several fungi[81]. Thus, depending on the fungal source used as pigment producer, corn cobs could be a highly economical support/substrate for SSF.

Velmurugan *et al.*[81] used corn cob as substrate for pigments production by *M. purpureus* under SSF conditions and determined that with this support high yields of pigments can be produced.

## 1.5. EXTRACTIVE FERMENTATION USING AQUEOUS TWO-PHASE SYSTEMS

Once the more suitable fermentation process to obtain a desired bioproduct is selected, it is important to consider the use of the most efficient method for the recovery and purification of the target product.

Furthermore, the integration of bioreactor fermentation and highly efficient separation processes in a single step is often a suitable approach to make fermentation processes economically viable[82].

Extractive fermentation (EF) or *in situ* product recovery is a technique that allows selective recovery of the product from the fermentation broth during the fermentation process. Therefore, the investment in this type of fermentation can be economically advantageous. EF greatly contributes to product enrichment (thus reducing downstream processing costs), and to increase production yields, by removing the target product from the medium, thus making it unavailable for secondary reactions[83].

One of the most commonly extraction techniques used for product recovery that can be coupled with a fermentation process is the Aqueous Two-Phase Systems (ATPSs) methodology. This technology provides an efficient alternative to the classical extraction by organic solvents, since product purification is performed by partitioning between two liquid phases mostly composed of water[84]. ATPSs result from the aqueous mixture of two hydrophilic polymers or a polymer and a low molecular weight compound, such as a salt, above certain critical conditions[85,86].

Since ATPSs generally contain about 80 to 90 % of water, they provide a biocompatible environment for biologically active microorganisms, cell organelles and biologically active substances[87].

In EF using ATPSs, cells (and other contaminants) are retained in one of the equilibrium phases and the desired product is expected to have affinity for the opposite phase. For the successful use of ATPSs in FE, certain conditions should be verified: (a) the phase constituents (polymers and/or salt)

should not be used in concentrations that are toxic or inhibit cell growth; (b) the physicochemical nature of the two-phase system, such as polymer integrity and stability, should not be affected by the fermentation process; (c) the cells and the substrates should be fully or predominantly split into one of the phases (preferably the opposite phase of the product accumulation); (d) feed constituents such as mineral salts or substrates should not drastically affect phase behavior; (e) the system should be cost effective in terms of the recycling and reuse of its constituents (polymer and/or salt)[87].

In **Table 2,** some selected examples of extractive fermentation using ATPSs for the recovery of different products of interest, including pigments, are presented.

**Table 2**. **A list of some selected examples of extractive fermentation using ATPSs.**

| ATPS | Product | Reference |
|---|---|---|
| **PEG[(1)]8000/NaCit[(2)]** | Protease | Silva *et al.*[88] |
| **Thermo-separating EOPO[(3)]** | Polyhydroxyalkanoates | Leong *et al.*[89] |
| **PEG3350/Dextran 66900** | β-carotene | Chavez-Santoscoy *et al.*[90] |
| **PEG8000/Dextran 66900** | Lutein | Chavez-Santoscoy *et al.*[90] |
| **PEG8000/Phosphate salts** | Clavulanic acid | Viana Marques *et al.*[91] |

[(1)]PEG - polyethylene glycol; [(2)]NaCit - sodium citrate; [(3)]EOPO - copolymer of ethylene oxide-propylene oxide.

### 1.5.1. POLYMER-POLYMER AQUEOUS TWO-PHASE SYSTEMS

When mixing two hydrophilic polymers in water a partially immiscible aqueous two-phase system (ATPS polymer-polymer) is formed above certain critical conditions[87]. The composition of the two phases depends, among others, on the amount of polymer added, their molecular weights and the equilibrium temperature. The concentration of each polymer in the equilibrium phases of a given system can be determined by a phase diagram, which is specific for the temperature used[92].

Originally, these systems were based on aqueous mixtures of polymers such as polyethylene glycol (PEG), dextran and/or maltodextrin. The combination of dextran and PEG represents one of the most studied systems, where dextran, the most dense and hydrophilic polymer, predominantly accumulates in the bottom phase and PEG, more hydrophobic and less dense, forms the top phase[93]. However, the major limitations of this biphasic system are the high cost and viscosity of dextran, which economically and technically impairs its application at industrial scale. Therefore, new and cheaper polymers have emerged as an alternative to dextran such as crude dextran, waxy starch, hydroxypropyl starch or polyvinyl alcohol[68,94]. However, polymer-polymer ATPS performance is not as promising for the separation of compounds of interest because they usually have low selectivity[95].

Additionally, thermoseparating copolymers such as Ucon (an ethylene oxide and propylene oxide copolymer, EOPO) were proposed as phase forming components for cheaper and reusable systems. An aqueous solution containing 10-40 (% w/w) of this copolymer, forms a biphasic system with the Ucon predominantly concentrated in the bottom phase, when heated above 47 °C. The advantage of these copolymers is that at the end of the process, about 95 % of the initial copolymer can be recovered by increasing the temperature, thus allowing its recycling and potential reuse[96–98].

The PEG-polyacrylic acid ATPSs were also reported as an interesting alternative to the PEG-dextran systems, since they present a lower cost, constituting a suitable biphasic system for the industrial separation of biomolecules[99].

### 1.5.2. Polymer-Salt Aqueous Two-Phase Systems

ATPSs formed by polymers and electrolytes (salt) have a vast range of applications due to the significantly different chemical environments achieved in top and bottom phases, which consequently results in different physicochemical properties. Therefore, separation processes are generally more efficient in such ATPSs with the additional advantage that they are relatively cheaper and thus more suitable for large scale operations[100].

Different salts, such as phosphates, citrates, tartrates, succinates and oxalates may form an ATPS when combined with polymers, namely PEG[101]. Polymer/salts systems generally present higher selectivity in the partitioning process, with high yields in first extraction step[87].

PEG-potassium phosphate ATPSs are ones of the most common biphasic systems due to several advantages such as extensive physicochemical characterization, low cost and wide range of applications. However, the use of phosphates represents an environmental threat, particularly their waste disposal, because they are responsible for the eutrophication of several watercourses[87,102].

Thus, alternative ATPSs such as PEG-citrate and PEG-formate may be used, representing a lower environmental risk due to their higher biodegradability[103,104].

## 2. Objectives of the Thesis

Previously results obtained by our research group demonstrated the potential of *P. brevicompactum* to produce pigments under submerged fermentation using a synthetic culture medium. Thus, the present work aimed to improve pigment production using alternative low-cost media containing agroindustrial residues. Culture media composed of residues offer recognized economic advantages and therefore can

result in a more attractive approach for pigment production at industrial level. Additionally, other alternative strategies for pigment production, such as solid and extractive fermentations, were explored and compared in terms of production effectiveness.

In order to successfully achieve the proposed main goals, the following secondary objectives were established:

- pigment production under submerged fermentation conditions (with and without immobilization support) using fermentation media composed of CW and CSL;
- pigment production under solid fermentation conditions using fermentation media composed of CW and CSL;
- scale-up of pigment production under submerged fermentation conditions using synthetic medium in a 3.7 L bioreactor;
- pigment production under extractive fermentation conditions using a PEG-salt ATPS.

# 3. Materials and Methods

## 3.1. MICROORGANISM

*P. brevicompactum* (MUM 02.07) was obtained from the Mycology collection of University of Minho (MUM). Stock cultures were maintained at room temperature (around 25 °C) in spore suspensions of semi-solid agar medium (2 g/L). For inoculum preparation, stock cultures were then sub-cultured on Petri dishes containing the so-called malt extract agar (MEA) medium (g/L): malt extract (20), glucose (20), peptone (1) and agar (20), and grown at 25 °C for 7 days.

## 3.2. AGRO-INDUSTRY RESIDUES

Cheese whey powder (CW) was kindly provided by Lactogal Produtos Alimentares S.A. and corn steep liquor (CSL) by COPAM (Companhia Portuguesa de Amidos, S.A.), Portugal. The composition (% w/w) of CW was 58.5 % lactose, 12.6 % protein, less than 0.2 % fat and 1.2 % moisture. The composition of CSL was 75 g/L sugars and 5 g/L proteins[105].

## 3.3. SUPPORTS

### 3.3.1. INERT SUPPORT

Cubes (0.125 $cm^3$) of commercial nylon sponge (Vileda Ultra Fresh®, Freudenberg) were used as inert supports. Prior to use, the nylon sponge cubes were pre-treated by boiling for 15 min and washing with distilled water. After that, the cubes were dried at 60 °C. Prior to use, the support was autoclaved at 121 °C for 15 min. **Figure 5** shows the inert support before and after cutting into cubs.

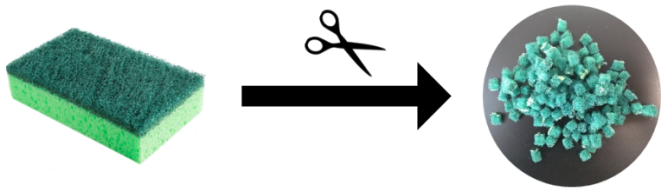

**Figure 5. Inert support before (left side) and after cutting into cubs (right side).**

### 3.3.2. NATURAL SUPPORT

Corn cobs were obtained from local harvest (Lousada, Portugal). Corn cobs were cut into small pieces with approximately 0.1 $cm^3$. The prepared material was soaked in deionized water at 80 °C for 6 h to wash and increase porosity[81]. Then, the material was dried at 60 °C. Prior to use, the support was autoclaved at 121 °C for 15 min. **Figure 6** shows the natural support before and after cutting into pieces.

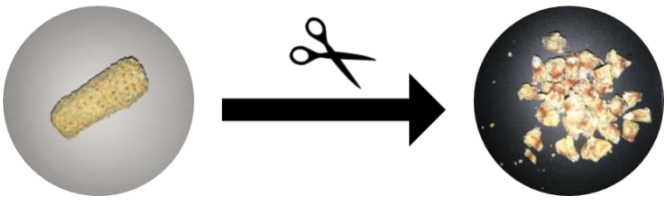

**Figure 6. Natural support before (left side) and after cutting into pieces (right side).**

## 3.4. INOCULUM PREPARATION

Inoculum for fermentations was prepared by adding 1 - 2 mL of a sterile saline solution (8.5 g/L NaCl) containing 0.1 g/L Tween 80 to fully sporulated agar plate culture of *P. brevicompactum*[75]. The spores were then scraped from the agar plates under aseptic conditions and the conidia suspension was used as the inoculum. Conidia density was adjusted to $10^6$ conidia/mL using a Neubauer chamber.

## 3.5. PIGMENTS PRODUCTION

The experiments were performed in triplicate using 250 mL cotton-plugged Erlenmeyer flasks containing an initial density of $10^6$ conidia/mL. Several fermentation media were prepared as indicated in **Table 3**. Prior to use, all the media and material were autoclaved at 121 °C during 15 min.

**Table 3. Different culture media studied and their composition.**

| Culture media | Composition (g/L) |
|---|---|
| **Synthetic (A)** | Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20) |
| **Cheese whey (CW) (B)** | CW (34.6) |
| **CW synthetic supplementation 0.5 (C)** | CW (34.6), yeast extract (0.5) and peptone (0.5) |
| **CW synthetic supplementation 4 (D)** | CW (34.6), yeast extract (4) and peptone (4) |
| **Corn steep liquor (CSL) (E)** | CSL (12.55) |
| **CW CSL supplementation 1 (F)** | CW (34.6) and CSL (1) |
| **CW CSL supplementation 8 (G)** | CW (34.6) and CSL (8) |

The different culture media (A, B, C, D, E, F and G) were studied under different types of fermentation.

Initial tests were performed in submerged fermentation (SmF) using a volume of 50 mL for each type of medium presented in **Table 3**.

Submerged fermentation tests were also carried out with fungus immobilization on an inert support (nylon sponge; SmFi) and a natural support (corn cob; SmFn). SmFi and SmFn were performed with 50 mL of each medium and approximately 20 pieces of support (corresponding to 0.8 g of nylon sponge and 2 g of corn cob, respectively).

Solid-state fermentation using inert (SSFi) and natural support (SSFn) was also tested. Initial moisture content was previously evaluated using 3 g of nylon sponge cubes (75 cubes) and synthetic medium (A). The moisture % (w/w) contents tested were 75, 80, 85 and 90 % since the maximum volume that this support can absorb without resulting in visible free medium, corresponds to 90 % moisture. The

amount of support used was selected according to the volume occupied by the cubes in the flask. The moisture content (wet basis) was calculated according Ahmad and Munaim[106] and is shown in **Eq. (1)**.

$$\text{Moisture content} = \frac{\text{Mass of liquid component}}{\text{Total mass (liquid + support)}} \times 100\ \% \quad \textbf{(1)}$$

All the media present in **Table 3** were studied under SSFi conditions using 3 g of nylon sponge cubes and 85 % moisture content.

For SSFn, the amount of support used was the same used in SSFi, 75 pieces of corn cobs, corresponding to 7 g. For this support the moisture content used was 65 %, being this the maximum that it can absorb.

All the fermentations were performed in an orbital shaker at 23 °C and 150 rpm for 12 days. These experimental conditions were determined in a previous work performed by our research group to optimize pigment production by *P. brevicompactum* in synthetic fermentation medium[60]. Samples were taken on days 0, 3, 5, 7, 9 and 12 days in aseptic conditions for SmF, SmFi and SmFn.

### 3.6. PIGMENTS RECOVERY

At the end of the SmF, the culture media containing the extracellular pigments were recovered through vacuum filtration using a 0.45 µm Whatman filter. The biomass was dried at 60 °C until constant weight (dry weight).

For SmFi and SmFn, the culture media with the extracellular pigments were treated as described above for SmF. Additionally, the support containing biomass was taken for intracellular pigment extraction using a solution with 95 % (v/v) ethanol and 5 % (v/v) water (20 mL total volume). The mixture was kept on a rotary shaker at 150 rpm for 24 h at room temperature. The support was squeezed with the aid of a syringe and the liquid was filtered through a 0.45 µm Whatman filter. The ethanol was then evaporated at 60 °C.

For SSF, pigments were also extracted from supports using a solution of 95 % (v/v) ethanol and 5 % (v/v) water. For that purpose, all the content removed from the Erlenmeyer was submerged in ethanol. The volume of ethanol solution used corresponded to three times the culture medium volume initially added[107]. Apart from the volume of ethanol solution, the extraction method was performed similarly to that described above for intracellular pigment extraction in SmFi and SmFn.

All the mixtures containing the extracellular pigments collected by vacuum filtration were freeze-dried. The dry extracts obtained by ethanolic extraction were diluted in 2.5 mL of distilled water and their absorbance was measured (section 3.10) before freeze-drying.

## 3.7. BIOMASS REUSE UNDER SUBMERGED FERMENTATION CONDITIONS

To evaluate if biomass can be reused to increase the pigments production, two types of fermentation were carried out: SmF and SmFi.

For each type of fermentation, the experiments were performed in triplicate using 250 mL cotton-plugged Erlenmeyer flasks containing 50 mL of culture medium G (**Table 3**) and an initial density of $10^6$ conidia/mL.

This test was divided in 3 cycles of fermentation with 12 days. After 12 and 24 days of fermentation (first and second cycle, respectively), the medium present in the Erlenmeyer was removed with aid of a sterile net and replaced by 50 mL of fresh culture medium G.

Simultaneously, for each condition (SmF and SmFi) parallel fermentation studies were performed for 36 days without replacing the fermentation medium. All the material and media were previously autoclaved at 121 °C during 15 min. The fermentations were performed in an orbital shaker at 23 °C and 150 rpm for a total of 36 days. In the three cycles, samples were aseptically taken at 0, 3, 5, 7, 9 and 12 days. In the fermentation without replacing the culture medium, samples were aseptically taken at 0, 3, 5, 7, 10, 12, 14, 24, 26 and 36 days.

For each fermentation condition, pigment recovery was performed as described in **section 3.6.**.

## 3.8. EXTRACTIVE FERMENTATION

The EF was carried out using culture medium A and a polymer-salt ATPS, composed of polyethylene glycol average molecular weight 8,000 (PEG 8000) purchase from Sigma Aldrich (Lot# BCBS0176V) and sodium citrate (NaCit). To find the more suitable composition, different mixtures of PEG 8000 and NaCit were previously prepared by weighting appropriate amounts of each solid component into 2mL tubes. Then adequate amounts of culture medium A were added to obtain the required final systems compositions (PEG 8000-NaCit: 10-10, 10-8, 10-6, 9-9, 8-8, 7-7 % (w/w)) with 1 g total weight.

The EF was performed in 250 ml cotton-plugged Erlenmeyer flasks containing a 9 % wt. PEG 8000 and 9 % wt. NaCit ATPS with 50 g total weight. Two strategies to inoculate this fermentation medium

with an initial spore density of $10^6$ conidia/mL were investigated: the direct addition of the spore suspension to the medium or the addition of biomass from a 2-day pre-inoculum. Furthermore, other two initial spore densities ($10^2$ and $10^4$ conidia/mL) were tested. Prior to use, all the material and media were autoclaved at 121 °C during 15 min. **Figure 7** represent a scheme of the extractive fermentation process.

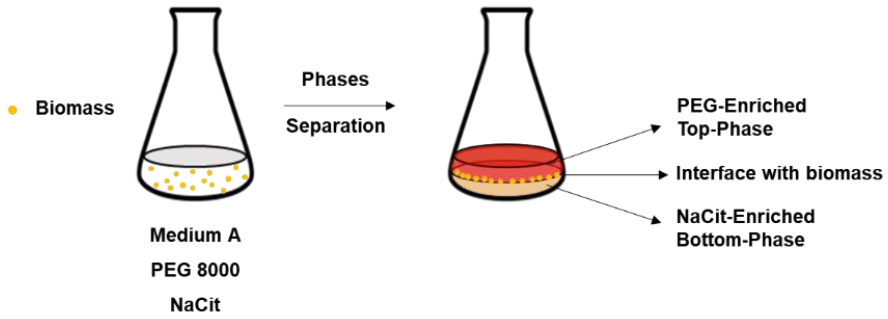


**Figure 7. Scheme of a typical extractive fermentation process.**

The fermentations were performed in an orbital shaker at 23 °C and 150 rpm for 12 days. Samples from the top phase were taken at 0, 2, 5, 7, 9 and 12 days in aseptic conditions and centrifuged at 4500 rpm during 5 min before reading the absorbance (section 3.10).

### 3.9. SUBMERGED FERMENTATION IN BIOREACTOR

Pigment production by SmF was performed in a bioreactor Bioengineering RALF 3500 mL containing 2500 mL of culture medium A. A pre-inoculum was prepared with the same medium composition, initial density of $10^6$ conidia/mL and growth for 48 h. The culture medium and the bioreactor were autoclaved at 121 °C during 30 min. The medium used for pre-inoculum was autoclaved at 121 °C during 15 min. The pre-inoculum was added to the bioreactor under aseptic conditions.

The fermentation was performed at controlled temperature of 23 °C, agitation of 150 rpm and aeration rate of approximately 2 vvm (volume flow per unit of liquid volume per minute). Additionally, another fermentation trial was performed under the same experimental conditions but without mechanical agitation.

### 3.10. PIGMENT ANALYSIS

The samples taken during fermentations were used to spectrophotometrically monitor the production of pigments. For all the conditions tested, the samples were centrifuged prior the absorbance

read. Absorbance was measured at wavelengths of 400, 470, and 500 nm (corresponding to the yellow, orange and red regions of the visible spectrum, respectively)[14,108].

To compare and evaluate the best fermentation conditions for pigments production, freeze-dried media (collected at the end of submerged fermentations) and dried ethanolic extracts (obtained by extraction from immobilization supports with biomass) were dissolved in 2.5 mL of distilled water and the absorbance was measured at the same wavelengths.

To determine the best conditions for pigment production, the sum of the three absorbances (at 400, 470 and 500 nm) was considered.

### 3.11. LACTOSE QUANTIFICATION

Lactose concentration in the fermentation media was obtained through HPLC analysis using a Jasco chromatograph equipped with refractive index detector (K-2300, Knauer) and a Prevail Carbohydrate ES column (5 μm, 250 × 4.6 mm, Alltech) fitted with a pre-column using the same stationary phase.

A mixture of acetonitrile-water (70:30 %(v/v)) pumped at 0.9 mL/min was used as mobile phase. The injection volume was defined as 20 μL[75]. A calibration curve was previously prepared with standards of lactose in the range 1-15 g/L.

The samples collected during submerged fermentations were analyzed to monitoring the consumption of lactose during fermentation time. Samples were centrifuged and filter through a 0.45 μm sterile syringe filter prior to HPLC analysis.

### 3.12. QUALITATIVE ANALYSIS OF PIGMENTS

The fermentation media and the ethanolic extracts obtained from fermentations were qualitatively analyzed by Thin Layer Chromatography (TLC). For each fermentation condition, 3 μL of a pigment solution with a concentration of 100 g/L was loaded on a silica plate (Macherey-Nagel, DC-Fertigfolien ALUGRAM SIL G/UV254, Macherey-Nagel GmbH & Co., Germany). Pigments were separated using water:ethanol (50:50) as eluent[60]. The plates were exposed to UV light (254 nm and 366 nm).

### 3.13. STATISTICAL ANALYSIS

All the experiments were performed in triplicate and the values obtained are presented with the means and respective standard deviations. One-way ANOVA and two-way ANOVA tests were performed

using GraphPad Prism 6.0 software (GraphPad Software, California, USA) to estimate significant differences ($p<0.05$) among samples in graphics of columns with a confidence interval of 95%.

# 4. RESULTS AND DISCUSSION

## 4.1. PIGMENTS PRODUCTION BY SUBMERGED FERMENTATION

In a previous work performed by our research group, promising results were obtained concerning pigments production by *P. brevicompactum* through submerged fermentation. In the referred study, different carbon sources were tested and a considerable amount of pigments was obtained using a synthetic medium containing lactose (here called medium A)[60,61].

Since promising results were obtained using a SmF approach, and because it is a widely used technique, both laboratory and industrially, it was decided to firstly test the production of pigments in alternative media under SmF conditions.

Initially, CW was selected as an alternative fermentation medium (medium B) due to its high lactose content (58.5 % w/w). In the preparation of medium B, the lactose concentration used in medium A (20 g/L) was maintained.

As reported by Babitha *et al.*[28] the nitrogen source represents an important factor for cellular growth and pigment production.

Santos-Ebinuma *et al.*[109] demonstrated that the addition of peptone and yeast extract to the culture medium has a positive effect on pigment production by *P. purpurogenum*. Also Gunasekaran and Poorniammal[66] reported that the use of an yeast extract-peptone mixture as nitrogen source improved the pigments production by *Penicillium* sp.

Based on these facts, it was decided to use peptone and yeast extract (P-YE) to supplement medium B. Therefore, different concentrations of the P-YE supplements were tested to analyze the effect of the nitrogen source concentration on pigments production by the fungus. The selected P-YE concentrations were 0.5 g/L and 4 g/L, corresponding to medium C and medium D, respectively.

As the main objective of this work was the use agroindustrial residues to reduce the costs associated to pigment production, CSL was also studied as alternative culture medium (medium D). In this case, CSL was diluted to a final concentration of 12.55 g/L in order to decrease its contribution to the color of the medium and thus minimize interferences in the pigment quantification.

CSL was also used as a supplement in the culture medium containing only CW as it has been widely reported as a well-known source of nitrogen[78]. Therefore, two different CSL concentrations were tested for supplementation (1 g/L and 8 g/L, corresponding to media E and G, respectively), which were

selected to keep the same concentration of nitrogen sources as in the previous media (C and D) supplemented with the P-YE mixtures.

It has been shown that carbon and nitrogen are two elements essential for cellular metabolism, being also related to the formation of biomass and to the type of pigment (or other metabolites) produced as well. So, the optimization of the culture media used to obtain the fungal pigments is necessary since these nutrients may regulate the expression of genes of interest and activate important metabolic pathways[110].

The results obtained for each media tested were expressed in relative absorbance (%) and compared with the medium A, being this medium the so-called "reference medium" and thus corresponding to 100 % of absorbance (**Figure 8**).

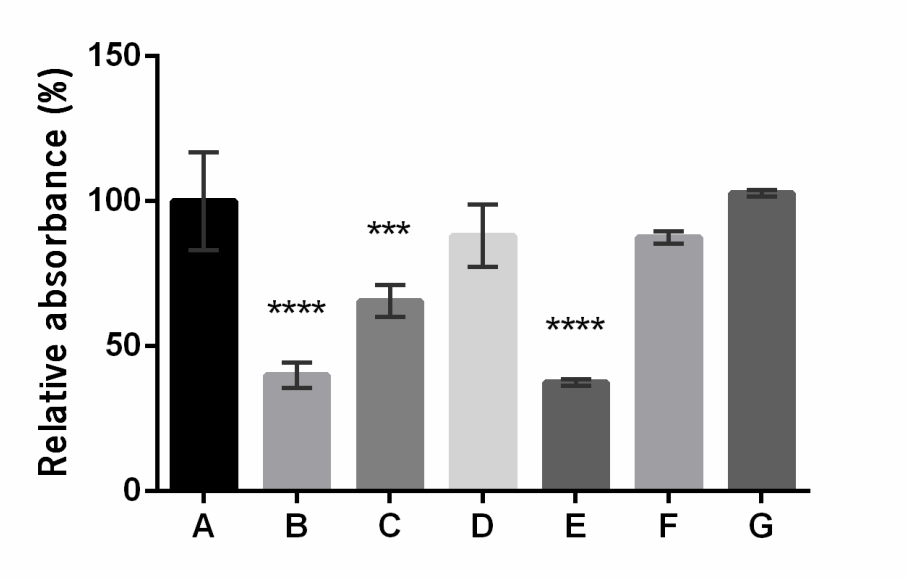


**Figure 8. Relative absorbance (%) of pigment production for all the media tested under submerged fermentation conditions.** The composition of media (g/L) was: A) Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20); B) CW (34.6); C) CW (34.6), yeast extract (0.5) and peptone (0.5); D) CW (34.6), yeast extract (4) and peptone (4); E) CSL (12.55); F) CW (34.6) and CSL (1) and G) CW (34.6) and CSL (8). Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. ***p<0.001, ****p<0.0001

The growth of the biomass was observed in all the culture media tested, indicating that the alternative media prepared are suitable substitutes of the synthetic medium A regarding the growth of *P. brevicompactum*. The different media originated different colors in both the fermentation broth and biomass suggesting that the supplementation with different nitrogen sources may also be responsible for activating the metabolic pathway responsible for pigments production.

Considering the results presented in **Figure 8**, it is possible to verify that there are no statistically significant differences between the medium A and the media D, F and G, showing that the use of agro-industry residues is a viable alternative for pigments production by *P. brevicompactum*. It is also found that, in general, supplementation with nitrogen sources results in a higher pigment yield.

Analyzing the results for media B, C and D it is possible to observe that there is an increase of 25.5 % in pigment production from medium B to medium C and about 48 % from medium B to D. Therefore, it can be assumed that the concentration of supplements (peptone/yeast extract) used significantly influences the pigment production under SmF conditions.

When CSL was used as culture medium (medium E), it was found that this residue *per se* yields the lowest pigment production. However, using it as a supplement in a culture medium containing CW (medium F and G), the same pigment production is obtained as in the reference medium A, even at the lowest CSL concentration (1g/L). Thus, CSL had a positive effect in pigment production, but the amount of CSL used seemed to not influence the yield of pigments obtained.

Silbir and Goksungur[79] determined that to obtain the same amount of nitrogen in a peptone/yeast extract mixture and CSL, approximately, twice of the concentration of CSL has to be used. The results obtained for media F and G, both presenting less amount of nitrogen in comparison with media C and D, showed that even reducing the amount of nitrogen it is possible to use CSL as an effective supplement. However, it should be emphasized that CSL is a complex residue and so some of its additional constituents may be favoring fungal growth and consequently pigment production.

Moreover, it was observed that in media F and G color appeared in the fermentation broth on third day while in the remaining media it appeared only on the fifth day. These results may suggest that pigment production can be associated with nitrogen depletion. However, further studies are necessary to allow us to take any final consideration.

When the profiles of pigment production *versus* fermentation time are analyzed, it is verified a decrease on absorbance for all the media containing agroindustrial residues on the early days of production (**Appendix A**). This may be due to the reduction of the initial turbidity of the medium as a result of the consumption of some medium constituents. Interestingly, all the media led to the production of more yellow pigments than orange or red (**Appendix A**).

In order to assess whether lactose consumption influenced pigment production, samples corresponding to the initial time (0 days), to the time at which color appeared in the culture broth (3/5

days) and samples corresponding to the final fermentation time (12 days) were analyzed by HPLC (**Appendix B**). For media A and F, lactose was completely consumed during the fermentation process. The media C, D and G presented a lactose concentration lower than 1 g/L at the final time. For medium B, the concentration of lactose was higher than 8 g/L. Through this analysis it was also possible to verify that the medium E had no lactose in its composition. However, it is known that CSL has other sugars in its composition which may contribute to the fungal growth[105]. This is in line with a previous work performed by our research group which concluded that lactose is the best carbon source for *P. brevicompactum* pigment production under SmF conditions[60].

Thus, the results suggest that lactose supplementation with an adequate nitrogen source is important to pigment production, since its absence (medium E) yielded in a lower pigment production. On the other hand, its total or almost total consumption led to a higher pigment production. Surprisingly it was observed a possible association with the timing of lactose initial consumption and color appearance in the fermentation broth, which led us to assume that the use of this carbon source by the fungus may be favoring or activating secondary metabolic pathways and consequently promoting pigment production.

The work performed by Gmoser *et al.*[111] aiming the production of carotenoids by *Neurospora* sp. suggested that the change from glucose to mannose as carbon source could direct the metabolic pathway for to acetyl-CoA pool (the most important precursor for pigments production) rather than cell growth pathways.

However, additional studies are needed to unequivocally conclude about this specific effect of the carbon source in pigments production by *P. brevicompactum*.

In order to study if the biomass originated could influence the pigment production, the amount of biomass was gravimetrically determined (dry weight) (**Table 4**).

**Table 4. Dry weight of biomass obtained for all the culture media studied under submerged fermentation conditions.** Values are the mean ± SD (n=3).

| | Biomass (g) |
|---|---|
| **A** | 0.445 ± 0,043 |
| **B** | 0.442 ± 0,017 |
| **C** | 0.547 ± 0,012 |
| **D** | 0.655 ± 0,099 |
| **E** | 0.080 ± 0,001 |
| **F** | 0.529 ± 0,007 |
| **G** | 0.641 ± 0,010 |

The results in **Table 4** show that for media D and G the maximal cellular growth was achieved, which also correspond to the media where higher production of pigments was obtained. However, with medium A (also with the highest pigment production), the cellular growth was not so high. These results seem to be concordant with Méndez *et al.*[4] who showed that does not exist a direct relationship between maximal growth and pigment production by *P. purpurogenum*.

It is important to mention that more biomass was obtained in media with the maximal supplementation with nitrogen sources (media D and G), probably because the microorganism can easily assimilate the nutrients present in the supplements and use them to grow, since they are more available (higher concentrations).

According to Lebeau *et al.*[112] an increase in the biomass content is attributed to the richness of nutrients in the medium and the pigment production is related to stress conditions, as a way of protection.

Pigments mixtures obtained from the several fermentations media studied were qualitatively analyzed by Thin Layer Chromatography (TLC) using a silica plate. Pigments were separated using a (50:50) mixture of water:ethanol as eluent, which was shown in previous studies to be the best eluent for their separation[60]. The separation obtained by TLC under visible light and exposition to UV light at 254 and 366 nm is presented in **Figure 9**.

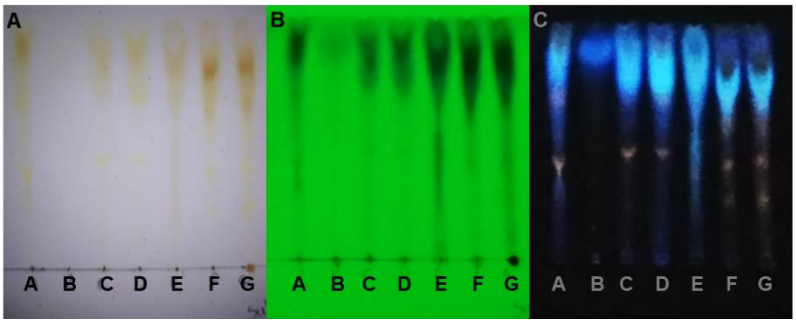


**Figure 9. Silica gel TLC showing the pigments separation obtained for the extracellular culture medium from submerged fermentation: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The results obtained by TLC suggested that different pigments mixtures were produced according with the fermentation medium used.

The migration pattern for CW culture media supplemented with P-YE (C and D) was very similar. For CW culture media supplemented with a different nitrogen source, CSL (F and G), also a similar migration pattern was observed (but distinct from the C and D). This result suggests that different nitrogen supplementation can probably originate different pigments mixtures.

### 4.1.1. EFFECT OF THE FERMENTATION TIME IN THE ALTERNATIVE MEDIUM

In previous studies it was determined that 12 days of fermentation was the suitable time for pigments production using the culture medium A[60,61].

In order to evaluate if the same fermentation time would be appropriated for media composed only by agroindustrial residues, a 36 days fermentation was performed using medium G. Medium G was chosen preferentially to medium F because it presented higher pigments production (**Figure 8**) and larger amounts of residues are used in its preparation, which may represent an environmental advantage. Two tests were performed: (i) a continuous 36 days fermentation and (ii) a 3x12 days fermentation with culture medium replacement by a fresh one (with the same initial composition) at the end of each 12 days cycle.

The sum of the absorbances obtained for these two assays, with medium change (MC) and without medium change (M) is presented in **Figure 10**. The bar corresponding to MC includes the absorbance obtained after each 12 days cycle of fermentation.

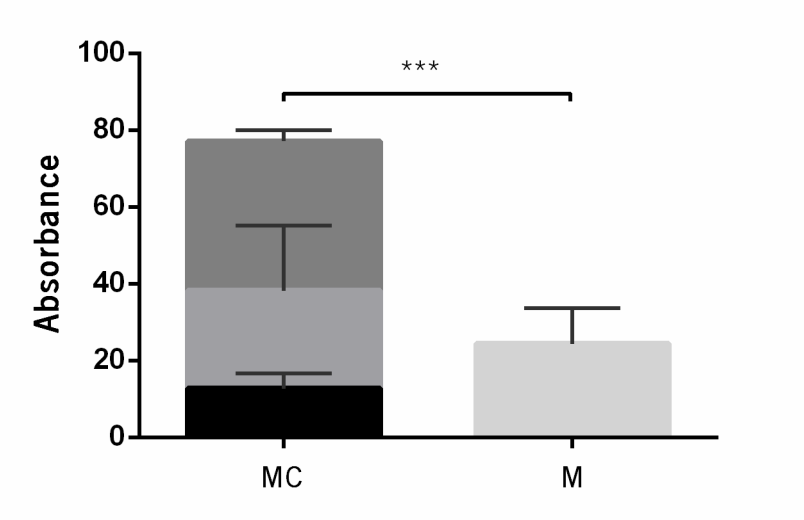


**Figure 10**. **Absorbances obtained for the 36 days submerged fermentations: medium change condition (MC) and condition without medium change (M).** In medium change condition it was represented the absorbance measured after culture medium recovery in the 12 days of each cycle: first cycle (black); second cycle (gray); and third cycle (dark gray). Values are the mean ± SD (n=3). Statistical analysis was performed between each media recovery and with the total absorbance for MC by one-way ANOVA. ***p<0.001

Regarding test (ii) (MC bar in **Figure 10**) no significant differences were found between each of the 3 cycles of biomass reuse. Furthermore, the absorbance values obtained indicate that a fresh medium replacing led to a higher pigment production in comparison with the approach performed in test (i).

It is known that secondary metabolites may be produced under stress conditions[31,32]. However, some authors referred that the secondary metabolites production in fungi depends on some factors, such as nutrient, concentration sources and their adequate ratio in the culture media[40,113]. The obtained results suggest that the presence of adequate carbon and nitrogen sources in the culture media lead to higher pigments production than in their absence.

Thus, these results allowed to establish 12 days of fermentation as the minimum suitable fermentation time for pigment production using media composed by CW and CSL. This conclusion was supported by the lack of statistically differences between the production after the first 12 cycle (MC) and 36 days fermentations (M).

However, the substitution of the medium allowed a higher total pigments recovery at the end of 36 days of fermentation. This can be explained by the fact that in MC condition biomass always has nutrients available, producing pigments whenever the fermentation medium is renewed. In contrast, in M condition the biomass probably consumes all the nutrients supplied in the first 12 days. These results

also support the assumption that the carbon and the nitrogen sources in the medium are essential to stimulate the metabolic pathways involved in pigments production.

Since the best pigments production was observed following the fresh medium replacing and biomass reuse approach, it was decided to immobilize the biomass on an inert support, nylon sponge[114], in order to facilitate its handling and transfer. Therefore, the two previous assays were repeated under submerged fermentation conditions with fungus immobilization in nylon sponge. However, it is important to mention that this type of fermentation (SmF with immobilization) will be discussed in detail in **section 4.2.1**, being presented here only the effect of the fermentation time on pigment production for direct comparison with the SmF with free *P. brevicompactum*.

The sum of absorbances for the two conditions evaluated, medium change (MC) and without medium change (M), is present in **Figure 11**. The bar corresponding to MC includes the absorbance obtained for each fermentation cycle.

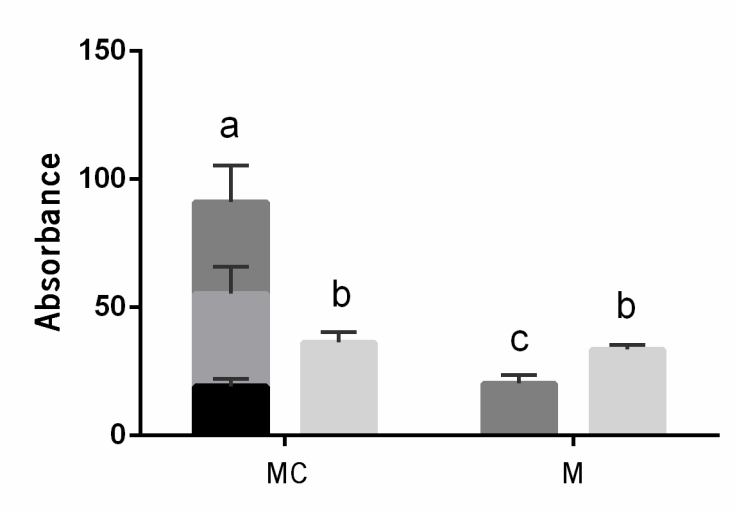


**Figure 11. Absorbance for medium change condition (MC) and for condition without medium change (M) with immobilization of biomass on inert support.** In medium change condition was represented the absorbance from medium recovery (left) in first (black), second (gray) and third 12 days cycle (dark gray) and ethanolic extract obtained in the end of 36 days. In medium change condition was represented the absorbance from medium (left) and from ethanolic extract obtained from immobilized biomass. Values are the mean ± SD (n=3). Statistical analysis was performed between each media recovery and with the total absorbance for MC (letters) by one-way ANOVA. Bar graphs which have the same letter are significantly equal therefore p is not bellow 0.05. On contrary bar graph with different letters are significantly different from each other ($p<0.05$).

Similarly the previous test, the absorbance values obtained indicate that a fresh medium replacement led to a higher pigment production in comparison with the approach performed without medium change. Also, for this type of fermentation, there are no statistical differences between the

production after the first 12 cycle (MC) and the 36 days fermentations (M) for medium. Thus, 12 days of fermentation seems to be the minimum suitable fermentation time.

Interestingly, the absorbance of the ethanolic extracts was equal for MC and M and it was higher than the absorbance of the medium after 36 days of fermentation (when statistical test was performed with the sum of absorbances of each cycle in MC).

Comparing the two tests performed, the SmF with immobilization of biomass (**Figure 11**) allows to obtain more extracellular pigments than submerged fermentation without immobilization (**Figure 10**) under MC conditions. Under M conditions the absorbance values for the mixtures of extracellular pigments were similar for both types of fermentation.

## 4.2. PIGMENTS PRODUCTION BY SUBMERGED FERMENTATION WITH IMMOBILIZATION

### 4.2.1. THE USE OF AN INERT SUPPORT

The immobilization of filamentous fungi is a relatively simple procedure since this kind of microorganism adheres easily to the surfaces of several materials. Fungal immobilization can be advantageous since it facilitates the recovery and reuse of the cells and, consequently, the bioproducts[115]. In a study conducted by Domínguez-Espinosa and Webb[116] the immobilization of *Monascus* on an inert support (foam) was tested and resulted in an enhanced production of red pigments compared with submerged fermentation using the free fungus.

To investigate whether biomass immobilization influenced pigment production, all the media (A-G) were tested under submerged fermentation conditions with immobilization of the fungus on an inert support (SmFi), namely nylon sponge. The amount of support used, 20 pieces, was chosen based on the amount of support that could be used and still be completely submerged in the total volume of culture medium.

Since the extraction of pigments from biomass was necessary, ethanol was used due to its well-recognized classification as GRAS solvent[105]. Moreover, ethanol can be easily recovered and recycled using distillations. This solvent was used in all the extraction of pigments from biomass procedures performed in the present work. The ethanolic extraction of pigments was carried out in these fermentation conditions because the biomass obtained at the end of the fermentation presented a strong coloration, thus indicating a considerable amount of (intracellular) pigments.

The results of pigment production were expressed in relative absorbance (%) for the pigments mixtures obtained from extracellular medium (M) and from ethanolic extraction (EE) from biomass. All the alternative media were compared with the medium A, used as reference and corresponding to 100 % absorbance (**Figure 12**).

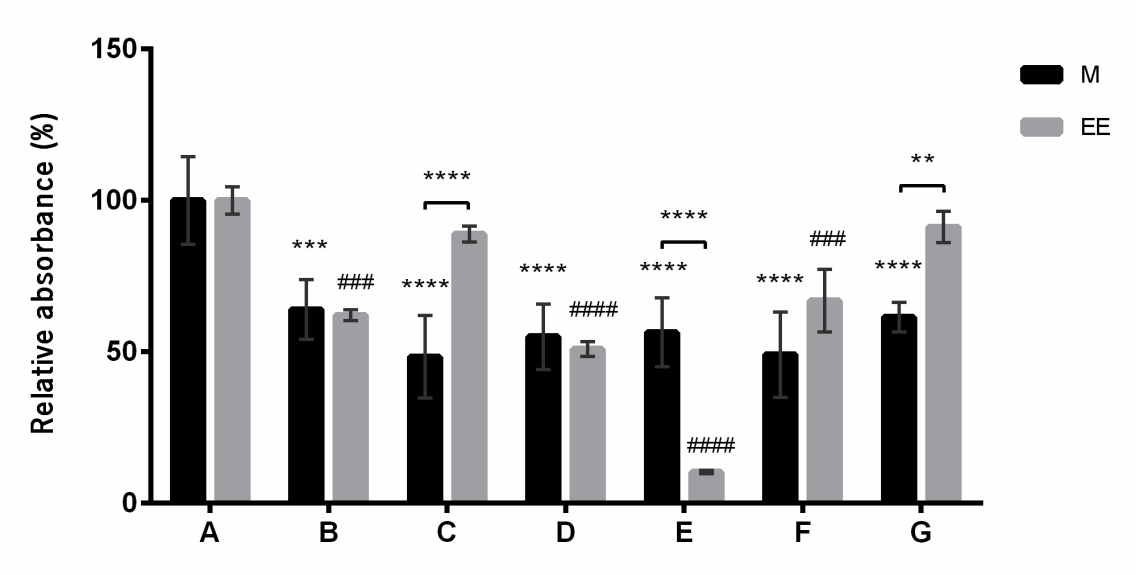


**Figure 12. Relative absorbance (%) obtained for each culture media (A-G) under submerged fermentation conditions with immobilization on an inert support, extracellular medium (M) and ethanolic extracts (EE) from biomass.** The composition of media (g/L) was: A) Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20); B) CW (34.6); C) CW (34.6), yeast extract (0.5) and peptone (0.5); D) CW (34.6), yeast extract (4) and peptone (4); E) CSL (12.55); F) CW (34.6) and CSL (1) and G) CW (34.6) and CSL (8). Values are the mean ± SD (n=3). Statistical analysis was performed for medium samples, ethanolic samples and with each other separately by two-way ANOVA. **p<0.01, ***p<0.001, ****p<0.0001, ###p<0.001 and ####p<0.0001

Analyzing the results corresponding to M samples it can be observed that none of the alternative media tested allowed to reach the same relative absorbance obtained with the reference medium A. On the other hand, examining the results of the EE it is verified that the media C and G do not present statistically significant differences comparing with medium A, which means that with these alternative media the same amount of pigments can be obtained by ethanolic extraction from the immobilized biomass.

Comparing the relative absorbances measured for the same culture medium (M and EE) it is possible to establish that there is only a significant difference in media C, E and G. In the media C and G, there is a higher content of pigments in the ethanolic extract (EE) than in the fermentation broth (M). In contrast, in medium E higher amount of pigments was obtained in the fermentation broth (M).

The differences observed between M and EE for the same culture medium might indicate that the amount of pigment excreted may vary with the fungal immobilization. It was visually verified that the immobilization did not occur evenly in all media. Plus, in some cases, the biomass immobilization suffered variations among the replicates (all biomass immobilized and/or some suspended biomass).

In addition, it was found that in media A, D and E pigment production started on the third day, while in the others it happened on the fifth day of fermentation. Based on the absorbance measurements, it was also observed that in all the media a higher amount of yellow pigments was produced than orange or red (**Appendix C**).

Similarly, to SmF, samples from culture medium corresponding to the initial time (0 days), the time at which color appeared in the culture broth (3/5 days) and the final fermentation time (12 days) were analyzed by HPLC to assess the consumption of lactose over the fermentation (**Appendix D**). The results showed that the medium which allowed the best pigment production (medium A) presented less lactose concentration in the final fermentation time (less than 5 g/L). All the other media presented a lactose concentration in the final time over 5 g/L.

As the nylon sponge is a considerable porous material, the growth of biomass occurs both inside of the support and on its surface, forming a dense coating. This fact can probably explain why lactose was not completely consumed. The biomass inside the support did not have the same access to the medium nutrients as the free biomass or the biomass on the support surface.

Pigments mixtures corresponding to the fermentation media (M) and to the ethanolic extracts (EE) obtained from the several fermentations were qualitatively analyzed by TLC using a silica plate. The produced pigments mixtures were separated using water:ethanol (50:50) as eluent, as previously described. The separation obtained by TLC under visible light and exposition to UV light at 254 and 366 nm is presented in **Figure 13**.

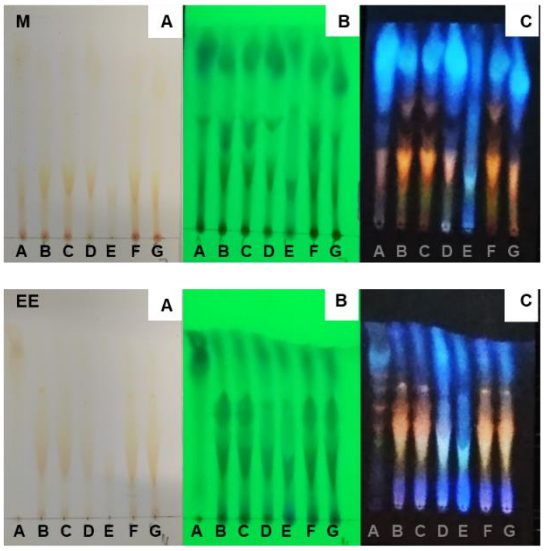


**Figure 13. Silica gel TLC showing the pigments separation obtained for the extracellular culture medium (M) and ethanolic extracts (EE) from submerged fermentation with immobilization on inert support: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The results obtained by TLC suggested that different pigments mixtures were produced according with the fermentation medium used.

Also, it was possible to verify that the migration pattern of M samples was different from that of EE. The migration pattern in M samples suggests that the migration order of pigments was a blue spot (visible at 366 nm) followed by yellow-orange-red. In EE, initially a blue spot (visible at 366 nm) can be detected, which is followed by yellow-orange-red spots and finally a new blue/purple spot. It is important to mention that the yellow-orange-red migration order was observed for all the conditions.

### 4.2.2. IMMOBILIZATION IN A NATURAL SUPPORT

Some studies showed the potential of corn cob hydrolysate to be used both as a substrate in SmF as well as a support/substrate in SSF[81,117].

In this way, it was decided to test all the culture media defined before under submerged fermentation using the corn cob as a natural immobilization support (SmFn). Similarly, to SmFi, the amount of support used in these experiments was 20 pieces of corn cob.

The results were expressed in relative absorbance (%) for culture medium samples (M) and ethanolic extract samples (EE), always comparing the alternative media with the reference medium A, corresponding to 100 % of absorbance (**Figure 14**).

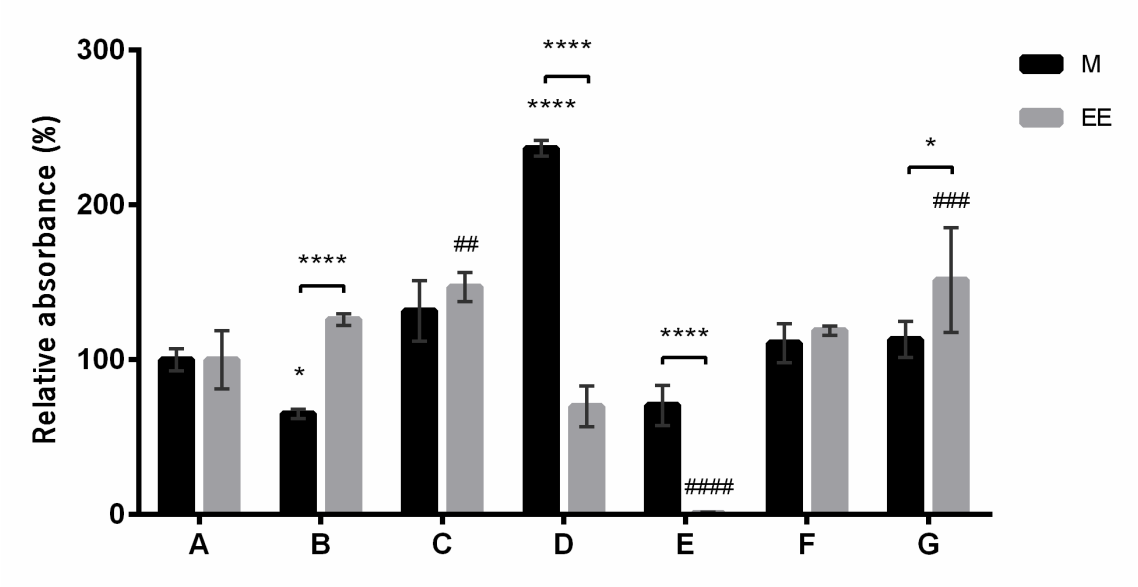


**Figure 14. Relative absorbance (%) obtained for each culture media (A-G) under submerged fermentation conditions with immobilization on natural support, extracellular medium (M) and ethanolic extracts (EE) from biomass.** The composition of media (g/L) was: A) Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20); B) CW (34.6); C) CW (34.6), yeast extract (0.5) and peptone (0.5); D) CW (34.6), yeast extract (4) and peptone (4); E) CSL (12.55); F) CW (34.6) and CSL (1) and G) CW (34.6) and CSL (8). Values are the mean ± SD (n=3). Statistical analysis was performed for medium samples and ethanolic samples separately by two-way ANOVA. *p<0.05, ****p<0.0001, ##p<0.01, ###p<0.001, ####p<0.0001

Analyzing the results corresponding to M it can be seen that all the alternative media promoted the production of the same amount of pigments (media C, E, F and G) or even more (medium D) than the medium A, exception for medium B. Regarding EE, the media B, D and F yielded the same amount of pigments than the medium A; and media C and G allowed to produce more pigments than medium A. Medium E is the only one that did not reach the same pigments production obtained with the reference medium A.

Comparing M and EE from the same culture medium, significant statistical differences were found between media B, D, E and G. For media B and G more pigments were obtained in EE conditions. In contrast, for media D and E more pigments were obtained in M conditions.

A remarkable result was observed in M samples from medium D where the relative absorbance exceeds 136 % the absorbance obtained with the reference medium (A), indicating that this medium is an effective alternative for the synthetic one.

The use of corn cob as immobilization support showed more potential than the use of nylon sponge (**Figure 13**). As the corn cob is less porous than the nylon sponge, the fungus probably adhered mostly on the surface of this support. In this way, all or almost all the biomass was in contact with the

nutrients present in the fermentation broth, what may justify the differences in pigment production compared to the results obtained in SmFi.

Similarly to SmF and SmFi, M samples (from all media) corresponding to the initial time (0 days), the time at which color appeared in the medium (3/5 days) and the final fermentation time (12 days) were analyzed by HPLC to evaluate the consumption of lactose (**Appendix F**). It was found that only in media A and D lactose was completely metabolized. In media B and F, a considerable amount of lactose (approximately 6 g/L and 4 g/L, respectively) remained in the fermentation broth after 12 days. In media C and G, a small amount (approximately 1 g/L) was quantified in the final fermentation time.

The fermentation media (M) and the ethanolic extracts (EE) obtained from fermentations were qualitatively analyzed by TLC using a silica plate. The separation obtained by TLC under visible light and exposition to UV light at 254 and 366 nm is presented in **Figure 15**.

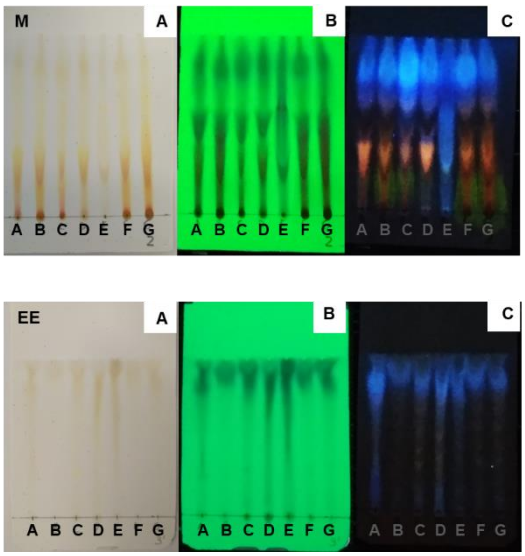


**Figure 15. Silica gel TLC showing the pigments separation obtained for the extracellular culture medium (M) and ethanolic extracts (EE) from submerged fermentation with immobilization on natural support: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The results obtained from the TLC demonstrate that there are differences between the pigment migration pattern for both types of samples analyzed (M and EE). This seems to indicate that some of the pigments obtained from the biomass (EE) may not be excreted into the fermentation medium, but may be retained in the fungal cell wall, and an extraction step is required to get access to them.

Similar to the other TLCs, the results obtained suggest that a mixture of pigments was produced. The migration order of pigments of the M samples was a blue spot followed by yellow-orange-red spots.

For EE, it was possible to verify some different spots, but their colors intensities are considerable low. However, the presence of different spots also indicates that a mixture of pigments was obtained.

## 4.3. PIGMENTS PRODUCTION BY SOLID-STATE FERMENTATION

### 4.3.1. SSF WITH AN INERT SUPPORT

In SmFi experiments, higher amounts of pigments were extracted from the immobilized biomass, suggesting that SSF could be a promising fermentation process for pigments obtention.

SSF presents some advantages over submerged fermentation. In this approach only the basic nutrients for fungal growth are provided but most important, it mimics the natural growth environment, which can led to higher pigment productions with high yields and relatively low process costs[118].

Among others, the moisture content is a key variable that influences the efficiency of SSF to obtain a desired product. Using the most adequate moisture content for each substrate and microorganism is crucial to promote favorable growth conditions and to maximize metabolites production[64,71]. If the moisture content used is too high, gas diffusion will be restricted due to the occupancy of the cavities of the supports with water. On the other hand, if the moisture content is too low, fungal growth will be severely affected[119].

Thus, preliminary tests were performed with medium A to optimize the moisture content for pigment production under SSFi. The amount of support used (75 pieces corresponding to 3 g of nylon sponge) was selected according to the volume occupied by it in the fermentation flask. The relative absorbance (%) determined for each media is represented in **Figure 16**.

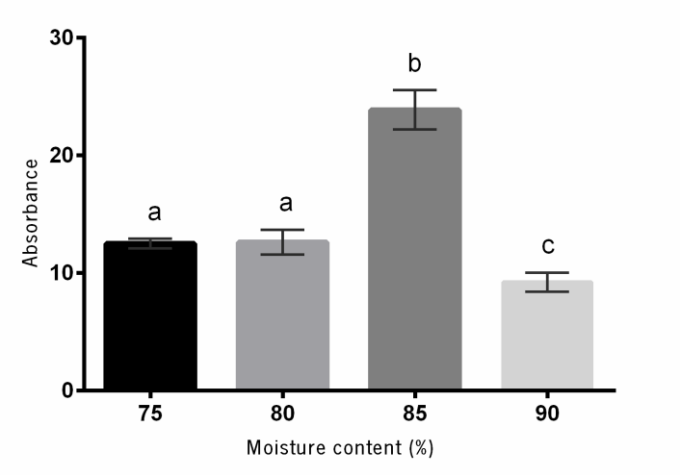


**Figure 16. Evaluation of moisture content (%) for solid-state fermentation using an inert support and culture medium A.** Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. Bar graphs which have the same letter are significantly equal therefore p is not bellow 0.05. On contrary bar graph with different letters are significantly different from each other ($p<0.05$).

**Figure 16** shows that by using a moisture content of 85 % it was possible to obtain 2 to 2.5 times more pigments than using the other moisture contents examined.

Therefore, to evaluate the performance of the different media by SSFi, 85 % of moisture content was use with the aim to favor pigments production. The results obtained for the different culture media are presented in **Figure 17**.

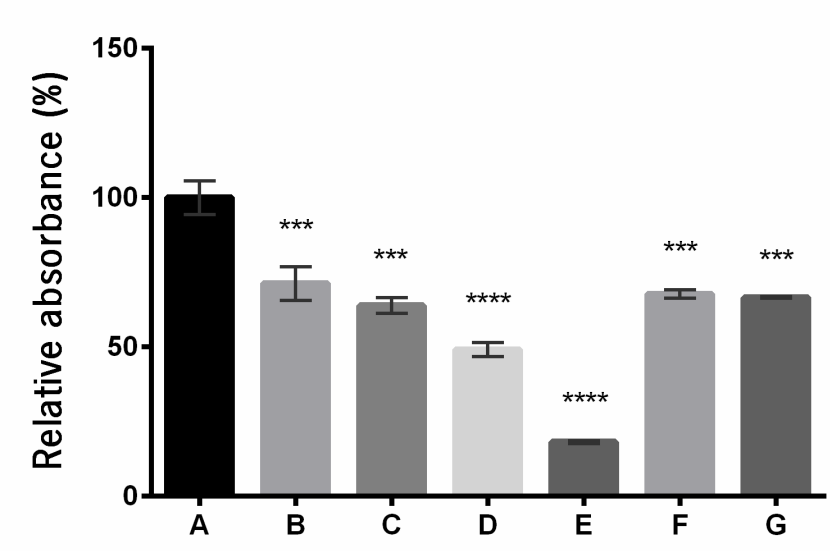


**Figure 17. Relative absorbance obtained for pigment production in all the culture media tested under solid-state fermentation using an inert support**. The composition of media (g/L) was A) Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20); B) CW (34.6); C) CW (34.6), yeast extract (0.5) and peptone (0.5); D) CW (34.6), yeast extract (4) and peptone (4); E) CSL (12.55); F) CW (34.6) and CSL (1) and G) CW (34.6) and CSL (8). Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. ***$p<0.001$, ****$p<0.0001$

The analysis of **Figure 17** shows that none of the alternative media tested allowed to achieve the pigment yield obtained with medium A.

Contrary to the results obtained for SmF, the increase of the supplementation of CW with nitrogen sources (media B, C and D) resulted in less pigments produced. In fact, pigments production suffered a reduction of 22 % with supplementation of 4 g/L P-YE compared with medium B, without nitrogen supplementation.

The supplementation of CW with CSL did not affect the pigments production on this type of fermentation as there are no statistically significant differences between medium B and the media F and G.

The pigments mixtures obtained under SSFi conditions were qualitatively analyzed by TLC using a silica plate and the results obtained are presented in **Figure 18**.

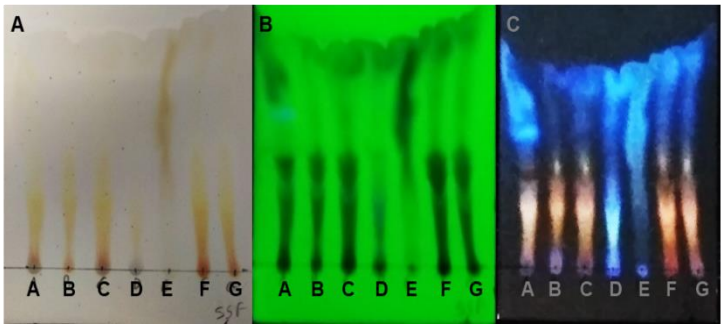


**Figure 18. Silica gel TLC showing the pigments separation obtained for the ethanolic extract from solid-state fermentation using an inert support: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The results obtained by the TLC indicated that with SSFi were also produced mixtures of pigments. Moreover, different pigments mixtures were produced depending on the fermentation medium used. However, in media containing CW it is possible to observe similar migration patterns, with exception of medium D (CW supplemented with 4 g/L P-YE).

The migration order seems to be a blue spot (visible at 366 nm) followed by the yellow-orange-red spots and finally a new blue/purple spot.

### 4.3.2. SSF WITH A NATURAL SUPPORT

As corn cob showed a great potential in SmFn it was decided to use this natural support, which is also considered an abundant agro-residue, to produce pigments under SSF.

In these experiments a moisture content of 65 % was used, because it was shown to be the maximum amount of culture medium that this support was able to absorb. A control using only corn cob and water was also performed to evaluate if corn cob could provide color to the extract and interfere with pigment monitoring. The absorbance obtained for this control was then discounted in the absorbance of all the other media studied. For this type of fermentation, it was also evaluated if corn cob *per se* could be a suitable substrate for *P. brevicompactum* growth and pigment production.

The relative absorbance (%) obtained for each of the media tested was represented in **Figure 19**.

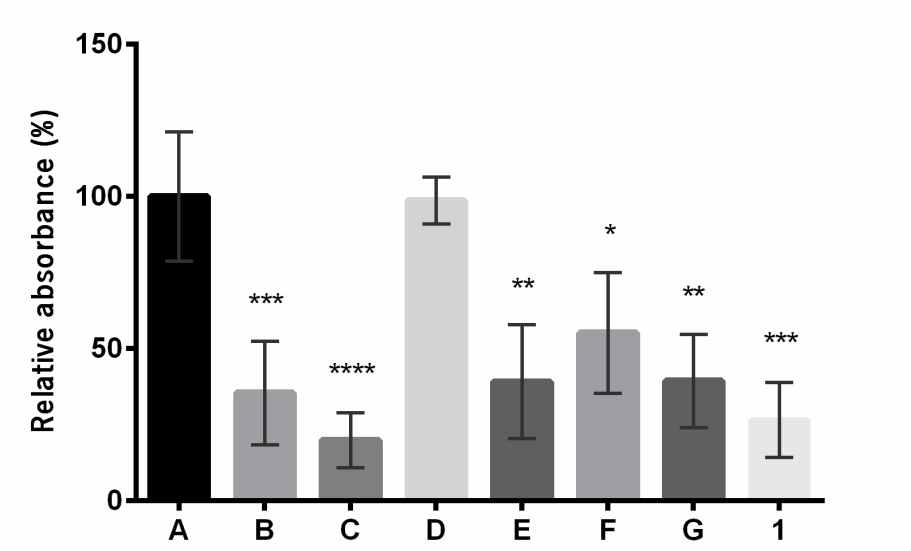


**Figure 19. Relative absorbance obtained for pigments production in all the culture media tested under solid-state fermentation using a natural support.** 1 represents the additional test only with the natural support, water and the fungus. The composition of media (g/L) was: A) Peptone (8), yeast extract (8), $KH_2PO_4$ (2), $Na_2HPO_4.12H_2O$ (8), $MgSO_4.6H_2O$ (0.25) and lactose (20); B) CW (34.6); C) CW (34.6), yeast extract (0.5) and peptone (0.5); D) CW (34.6), yeast extract (4) and peptone (4); E) CSL (12.55); F) CW (34.6) and CSL (1) and G) CW (34.6) and CSL (8). Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. *p<0.05, **p<0.01 ***p<0.001, ****p<0.0001

It is possible to verify that only medium D presented the same relative absorbance (%) as medium A. No relationship between nitrogen source supplementation and pigment production was verified, however the medium showing the best results is the one supplemented with the higher concentration of peptone and yeast extract (medium D).

Moreover, the use of corn cob as substrate (without additional nutrients) promoted pigments production, however with a low productivity yield. This result indicates that the corn cob may be used as an alternative substrate/support for pigment production, however an additional optimization of the growth conditions is required.

The pigments mixtures obtained under SSFn conditions were qualitatively analyzed by TLC using a silica plate and the results are presented in **Figure 20**.

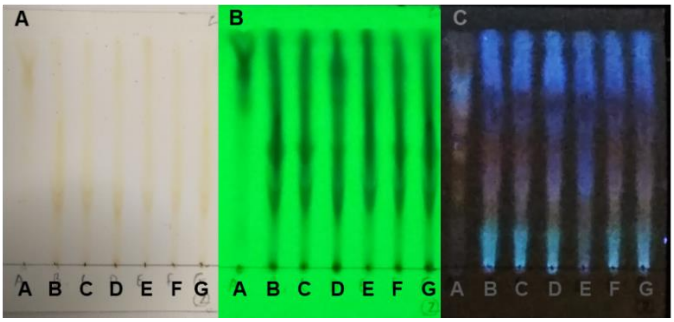


**Figure 20. Silica gel TLC showing the pigments separation obtained for the ethanolic extracts (EE) from solid-state fermentation using natural support: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The results obtained by the TLC indicated that, with exception for medium A, all the media show similar pigment migration patterns. Thus, it was found that, under these conditions, all the media containing agroindustrial residues also led to the production of similar pigments mixtures.

In media with residues it is possible to verify a blue spot in the beginning and at the end of the TLC migration, analogous to the ones observed after EE samples analysis from submerged fermentation with fungus immobilization.

## 4.4. COMPARISON OF THE FERMENTATIONS PERFORMANCE

Attempting to disclosure which type of fermentation (and medium) is the most suitable for pigments production a direct comparation, between the best results belonging to all the conditions tested, was performed. The best conditions selected were the media A, D, F from SmF, medium A (M) and media A, C and G (EE) from SmFi, medium D (M) and media C and G (EE) from SmFn, medium A from SSFi and media A and D from SSFn. The absolute absorbances obtained for each one of the best conditions achieved in the different types of fermentation are summarized in **Figure 21.**

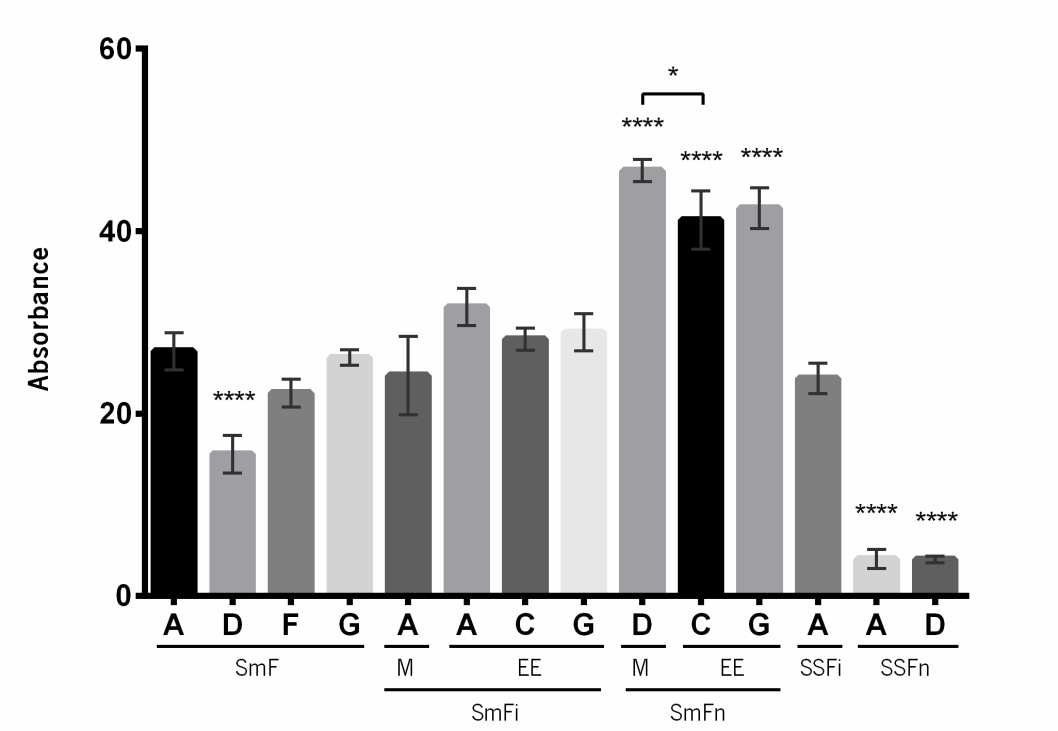


**Figure 21. Sum of the absorbances (400, 470 and 500 nm) obtained for the best conditions found for pigments production using the respective culture media tested under: submerged fermentation (SmF); submerged fermentation with immobilization on an inert support (SmFi) and on a natural support (SmFn), which include the extracellular pigments present in the fermentation broth (M) and the intracellular pigments extracted from biomass using ethanol (EE); solid state fermentation using an inert support (SSFi); and solid state fermentation using a natural support (SSFn).** Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. *p<0.05, ****p<0.0001

The data presented in **Figure 21** shows that from all the selected conditions the best ones belong to the SmFn, namely with the use of the medium D when pigments amount in the fermentation broth is considered and the media C and G when ethanolic extracts are evaluated.

In order to verify whether there were significant differences between these three media, a one-way ANOVA ($p < 0.05$) was performed. The results obtained through this statistical analysis showed that there are differences between media D and C, but not between media D and G. Thus, based on all the studies performed, media D and G can be indicated as the best conditions for pigments production using *P. brevicompactum* under SmFn. These are considered a promising result because the use of agro-residues as alternative media allows the reuse of wastes generated from the agroindustry sector, for the production of valuable products. In this way, the pigments production is more environmentally friendly and more interesting economically.

In a previous work developed by our research group, a fraction containing a red pigment was chromatographically separated from an ethanolic extract of the mycelium grown in agar plate (P). After

some structural characterization studies, it was found that this fraction could be an antheraxanthin-type pigment. Thus, the best condition obtained in this work and a P sample were qualitatively analyzed by TLC using a silica plate and the results are presented in **Figures 22**. Medium D (SmFn) and ethanolic extracts C and G (SmFn) correspond to 1, 2 and 3, respectively.

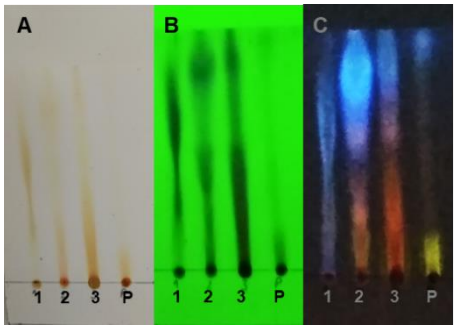


**Figure 22. Silica gel TLC showing the pigments separation obtained for the best fermentation conditions (1, 2 and 3) and for P sample: visible light (A) and exposed to UV light at 254 (B) and 366 nm (C), respectively.**

The samples obtained in the present work show a more complex composition, since they correspond to a mixture of pigments, as already mentioned.

Although this TLC analysis showed not to be conclusive, the pigments samples obtained in this work seem to present some similarities, mainly at visible light, with the P sample. Further studies are needed to obtain more isolated fractions in order to determine their chemical structures and then conclude about the presence of antheraxanthin-type pigments in the mixtures produced in this work.

## 4.5. PIGMENTS PRODUCTION BY EXTRACTIVE FERMENTATION

In this study a polymer-salt ATPS was used to perform extractive fermentation. The biodegradable and non-toxic NaCit was the selected salt mainly due to its high biocompatibility and considering its lower environmental impact and toxicity issues[120]. Regarding the polymer choice, PEG was selected once it is the most well-studied and used polymer engaged in polymer-salt ATPS formation. Besides, this polymer is known to be non-toxic, being frequently used in several bioapplications.

Before performing the extractive fermentation, the most suitable composition for PEG 8000/NaCit ATPS was determined. It was verified that a system composed by 9% wt. PEG 8000 and 9% wt. NaCit was the most appropriate due to the stability of both phases and the lower amounts of PEG and NaCit required as well.

The best way to inoculate the medium was also tested and the direct method was chosen (instead of using 2 days pre-inoculum) as it allowed a more controlled biomass growth that grant the collection of samples. However, even by adjusting the amount of inoculum (which needs to be optimized in the future) sampling from the bottom phase was impossible since biomass steered to this phase of the biphasic system being completely spread over it.

The sum of the absorbances in the top phase at the end of the fermentation for each initial spore density is presented in **Table 5**.

**Table 5. Sum of absorbances (400, 470 and 500 nm) for top phase sample in the end of the fermentation for each initial spore density (conidia/mL) tested.** The initial absorbance of medium was subtracted to final absorbance. Values are the mean ± SD (n=3). Statistical analysis was performed by one-way ANOVA. Values which have the same letter are significantly equal therefore P is not bellow 0.05. On contrary bar graph with different letters are significantly different from each other ($p<0.05$).

| Initial spore density (conidia/mL) | Absorbance (Top phase) |
|---|---|
| $10^6$ | $0.719 \pm 0.043^a$ |
| $10^4$ | $1.284 \pm 0.105^b$ |
| $10^2$ | $1.273 \pm 0.130^b$ |

The results indicate that PEG 8000-NaCit ATPS can be a suitable biphasic system for extractive fermentation that allows the growth of *P. brevicompactum* and the production of pigments. The reduction of the initial spore density from $10^6$ to $10^4$ conidia/mL resulted in a higher total absorbance (meaning that more pigments were produced). The reduction of the initial spore density from $10^4$ to $10^2$ does not present statistical differences regarding pigments production. Although the sampling of bottom phase was not possible, it was possible to verify that the top phase had color, being the pigments concentrated in that equilibrium phase. The use of EF for pigments production requires an additional optimization but the present results show that this technique is a promising alternative to reduce downstream processes for pigments extraction.

However, this result is not conclusive, since the clogging of the bottom phase with biomass was verified. Therefore, this approach should be optimized to avoid clogging of bottom phase. Increasing the volume of the culture medium (i.e. the total volume of the biphasic system) and using a thinner and taller flask may help to overcome the issues faced. In this way, biomass can have room to accumulate at the interface of the ATPS (as frequently reported in the literature) instead of occupying all the bottom phase.

## 4.6. PIGMENTS PRODUCTION BY SUBMERGED FERMENTATION IN BIOREACTOR

It is well-known that scaling up a bioprocess aiming the production of a desired product is easier when this one is performed under submerged fermentation[70].

In order to increase the amount of fungal pigments, a scale up of their production by *P. brevicompactum* under submerged fermentation was carried out in a bioreactor using the synthetic culture medium A. Two experiments were carried out, one using a mechanical agitation of 150 rpm and another one without mechanical agitation (only aeration).

It was verified that when the fermentation was performed with mechanical agitation the shear stress caused by the bioreactor blades compromise the biomass integrity, increasing the viscosity of the culture broth. The fermentation with mechanical agitation was carried out for 12 days, the same time used for the production tests in Erlenmeyer flasks, and no pigment production was observed.

This result suggested that the morphology of the fungus, the viscosity of the broth after some cell disruption and/or the design/configuration of the bioreactor negatively influenced the pigment production. However, even with cell disruption the growth of fungus was verified. These observations seem to be corroborated by the work carried out by Lu *et al.*[121] who demonstrated that the viscosity triggered by shear stress affects negatively the production of desirable products.

In order to avoid shear stress and the disruption of the biomass a fermentation without mechanical agitation was carried out instead. After 5 days it was observed pigment production. However, the increase of broth viscosity was also verified.

At the end of the fermentation, the absorbance was measured and the sum of absorbances was considered. The total absorbance obtained was 0.787 ± 0.052.

In this test it was possible to verify that the type of agitation influenced the fungus morphology and that the type of morphology can be related with pigments production.

Although some pigment production was observed in the bioreactor fermentation, the absorbance value is around 2.5 times lower than that obtained under submerged fermentation in Erlenmeyer flasks (**Appendix A**). Thus, to perform an effective scale up for pigments production by *P. brevicompactum* more studies involving the optimization of some operational parameters such as the amount of the inoculum, temperature, fermentation time, agitation and aeration is needed.

# 5. CONCLUSIONS AND FUTURE PERSPECTIVES

Fungi, particularly filamentous fungi, are known for their ability to produce a wide range of pigments. It was demonstrated in this work that *P. brevicompactum* is a promising pigments producer using alternative media composed of agroindustrial residues.

For SmF, no direct relationship between maximal growth and pigment production was established. It was demonstrated that 12 days of fermentation is the minimal suitable fermentation time for pigments production. An increase in pigments production was observed after the replacement of the culture medium after 12 days of fermentation. Under these conditions the immobilization of the biomass (3 cycles of 12 days of fermentation) allowed to obtain more pigments than with free biomass.

It was also shown that the immobilization of the fungus on a natural support (corn cob) allowed the production of a larger amount of pigments than obtained with an inert support (nylon sponge). In the future it would be interesting to optimize the SSFn conditions using only corn cob and water, since pigments production in this condition was observed.

The most suitable moisture contents were shown to be 85 and 65 % for SSFi and SSFn, respectively.

The highest pigments productions were achieved with medium D (CW supplemented with 4 g/L P-YE, extracellular pigments mixture) and medium G (CW supplemented with 4 g/L CSL, ethanolic extract), both under SmFn conditions.

It was also concluded that for all the types of fermentation studied, a pigment mixture was produced possibly containing antheraxanthin-type pigments.

Under EF conditions, promising results were obtained since the pigments production and their concentration in top phase was noticed. Therefore, process integration using a PEG 8000/NaCit ATPS can be a suitable alterative to produce and separate pigments from the biomass and the remaining contaminants of the fermentation broth. However, the optimization of some parameters such as fermentation volume, fermentation apparatus, fermentation time and type of inoculum are necessary. Optimization of this process may allow to produce interesting amounts of pigments, reducing downstream processes and making pigment production by *P. brevicompactum* more environmentally friendly and more industrially attractive.

Under SmF conditions in bioreactor, the amount of pigments produced was 2.5 lower than using the 250 mL Erlenmeyer. To scale up the pigments production by *P. brevicompactum*, additional studies involving the optimization of several operational parameters such as the amount of the inoculum, temperature, fermentation time, agitation and aeration are needed. Additionally, it would be interesting to investigate the pigments production using an air-lift bioreactor, where no mechanical agitation is present and thus the biomass morphology (which is probably related with pigments production) would not be negatively affected by the harsh action of the impellers.

Overall, this work demonstrated alternative low-cost culture media composed of agroindustrial residues as an efficient substitute of the synthetic ones to produce pigments by *P. brevicompactum*. These results also address the potential of profuse agroindustrial residues to be used as suitable resources of nutrients for fungal growth and metabolites biosynthesis. In the future, diverse residues should be evaluated aiming to develop an efficient environmentally sustainable process, thus meeting the industrial claims for greener solutions.

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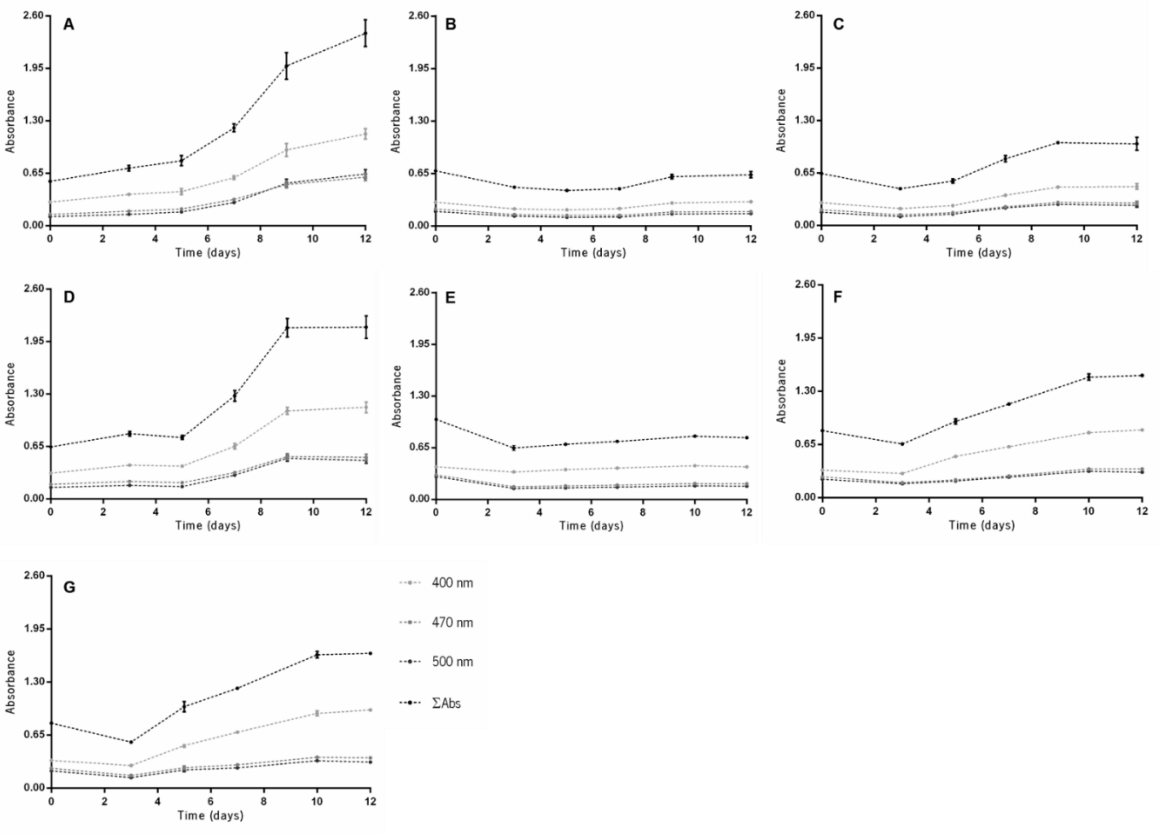
## APPENDIX

This section includes all the additional results not shown in the section of Results and Discussion. The appendix results are organized in three subsections, namely the pigment production by submerged fermentation, submerged fermentation with immobilization on inert support and submerged fermentation with immobilization on natural support. Lactose concentration over the fermentation time is also present in specific tables for a better understanding of the results explained in the section mentioned before.

### 1. SUBMERGED FERMENTATION

**Appendix A** contain all the results for absorbances and the sum of the absorbances obtained through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) under SmF conditions.




**Appendix A. Absorbance and the sum of absorbances *versus* fermentation time for each culture medium tested under submerged fermentation conditions.** Values are the mean ± SD (n=3).

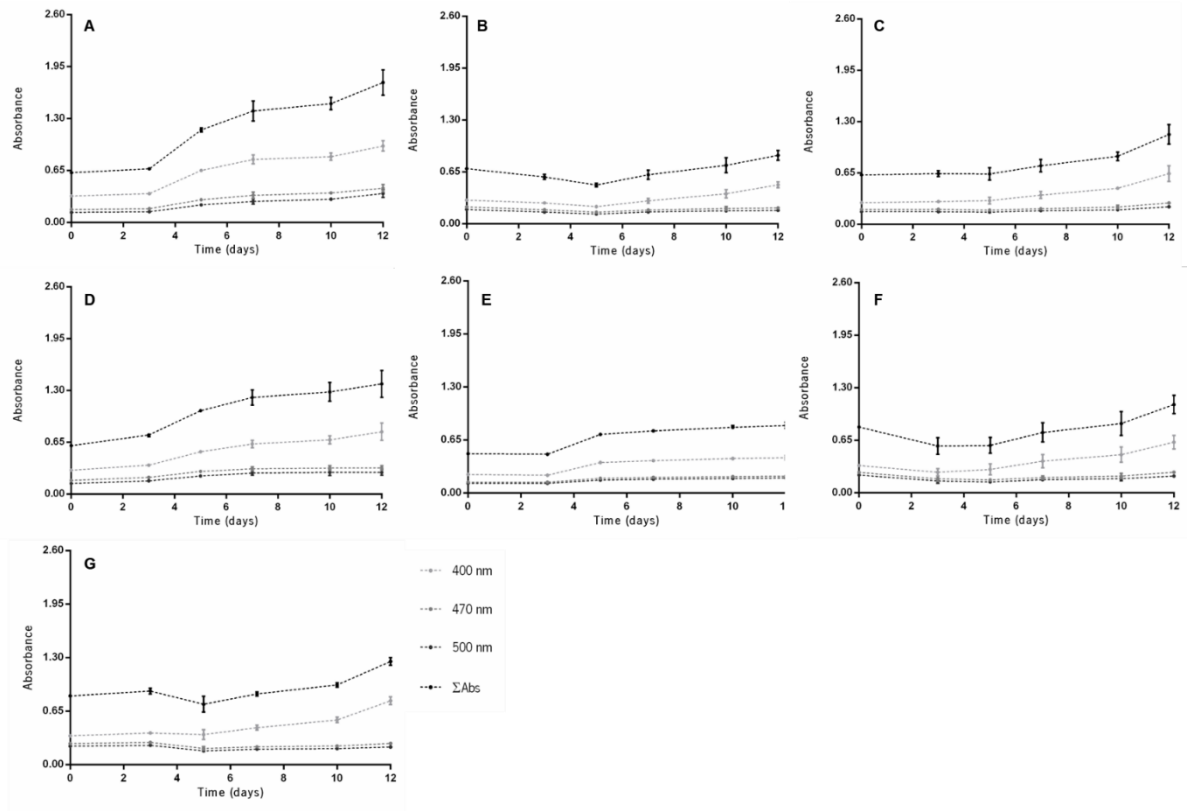
**Appendix B** shows the lactose concentration determined by HPLC for all the culture media at initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under submerged fermentation conditions.

**Appendix B. Lactose concentration determined by HPLC for all the culture media at initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under submerged fermentation conditions.** Values are the mean ± SD (n=3).

| | [Lactose] (g/L) | | |
|---|---|---|---|
| | It | Pt | Ft |
| **A** | 19.203 ± 1.382 | 17.046 ± 3.116 | 0.000 ± 0.000 |
| **B** | 23.068 ± 1.923 | 17.294 ± 3,737 | 8.961 ± 1,049 |
| **C** | 22.815 ± 0.263 | 17.084 ± 4,943 | 0.508 ± 0,443 |
| **D** | 22.648 ± 0.730 | 16.733 ± 0.496 | 0.966 ± 0.043 |
| **E** | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| **F** | 24,091 ± 0,638 | 19.672 ± 0,344 | 0.000 ± 0.000 |
| **G** | 25,528 ± 0,564 | 11.398 ± 0,434 | 0,184 ± 0,730 |

## 2. SUBMERGED FERMENTATION WITH IMMOBILIZATION ON INERT SUPPORT

**Appendix C** contain all the results for absorbances and the sum of the absorbances obtained through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) under SmFi conditions.




**Appendix C. Absorbance and the sum of absorbances *versus* fermentation time for each culture media tested under submerged fermentation conditions with fungus immobilization on inert support.** Values are the mean ± SD (n=3).

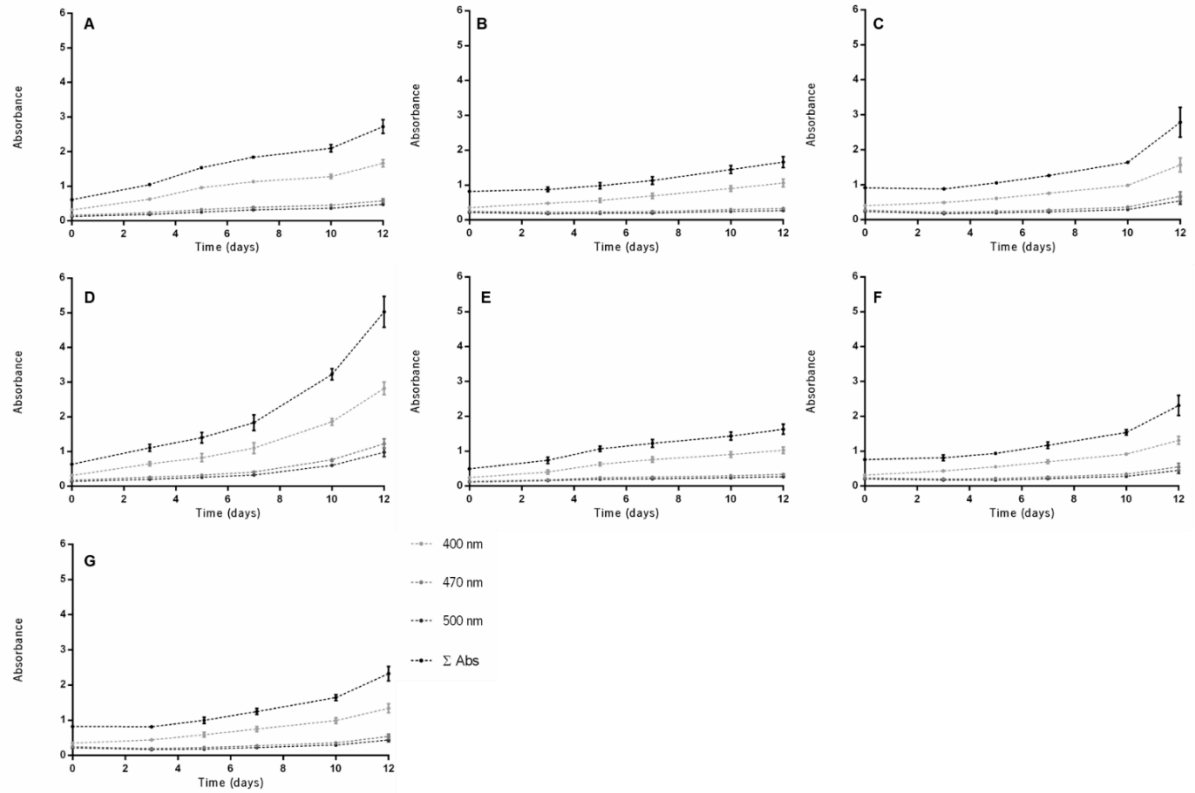
**Appendix D** shows the lactose concentration determined by HPLC for all the culture media at initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under SmFi conditions.

**Appendix D. Lactose concentration determined by HPLC for all the culture media in initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under submerged fermentation conditions with fungus immobilization on inert support.** Values are the mean ± SD (n=3).

| | [Lactose] (g/L) | | |
|---|---|---|---|
| | It | Pt | Ft |
| **A** | 19.203 ± 1.382 | 17.789 ± 0.395 | 4.259 ± 2.901 |
| **B** | 23.068 ± 1.923 | 18.595 ± 1.033 | 9.834 ± 1.359 |
| **C** | 22.815 ± 0.263 | 18.359 ± 0.252 | 9.399 ± 1.358 |
| **D** | 22.648 ± 0.730 | 20.157 ± 1.391 | 7.997 ± 3.257 |
| **E** | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| **F** | 24.091 ± 0.638 | 22.371 ± 0.329 | 10.685 ± 1.628 |
| **G** | 25.528 ± 0.564 | 23.978 ± 0.914 | 12.727 1.394 |

## 3. SUBMERGED FERMENTATION WITH IMMOBILIZATION ON NATURAL SUPPORT

**Appendix E** contain all the results for absorbances and the sum of the absorbances obtained through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) under SmFn conditions.




**Appendix E. Absorbance and the sum of absorbances *versus* fermentation time for each culture media tested under submerged fermentation conditions with fungus immobilization on natural support.** Values are the mean ± SD (n=3).

**Appendix F** shows the lactose concentration determined by HPLC for all the culture media at initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under SmFn conditions.

**Appendix F. Lactose concentration determined by HPLC for all the culture media in initial time (It), the time where pigment was visible in the medium (Pt) and final time (Ft) under submerged fermentation conditions with fungus immobilization on natural support.** Values are the mean ± SD (n=3).

| | [Lactose] (g/L) | | |
|---|---|---|---|
| | It | Pt | Ft |
| **A** | 19.203 ± 1.382 | 17.565 ± 0.250 | 0.000 ± 0.000 |
| **B** | 23.068 ± 1.923 | 19.804 ± 0.885 | 5.644 ± 0.795 |
| **C** | 22.815 ± 0.263 | 18.359 ± 0.252 | 0.760 ± 0.219 |
| **D** | 22.648 ± 0.730 | 14.319 ± 1.820 | 0.000 ± 0.000 |
| **E** | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| **F** | 24.091 ± 0.638 | 17.662 ± 1.166 | 3.739 ± 0.772 |
| **G** | 25.528 ± 0.564 | 17.698 ± 1.233 | 1.244 ± 0.241 |