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Production, extraction and characterization of a natural fungal pigment

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and
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DECLARAÇÃO

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Abstract

Natural pigments can be used for different purposes, namely in the textile industry for dyeing cloths, or in the food industry, as coloring agents. Nowadays, some food grade pigments produced by filamentous fungi, namely *Monascus* pigments, Arpink red™ from *Penicillium oxalicum*, riboflavin from *Ashbya gossypii*, as well as lycopene and β-carotene from *Blakeslea trispora* can be found in the market. In the European Union there are approximately 43 colorants authorized as food additives, whereas approximately 30 are approved in the United States, several of them from natural sources. Pigments can also be used in cosmetics, leather or in the pharmaceutical industry. More recently, other applications were found for pigments like in histological staining, in solar cells or as pH indicators. Additionally, important biological properties such as antibacterial, antifungal and herbicidal activities have been reported for natural pigments.

Fungi are reported as potent pigment producers due to the rapid growth capacity of microorganisms and the fact that there is no seasonal effect as in plants. In this work, we optimized the production of extracellular pigments by *Penicillium* sp. under submerged fermentation. A mixture of three pigments (yellow, orange and red with λ_{max}=400, 470 and 500 nm, respectively) was obtained under the optimal conditions (23 °C, 150 rpm, 20g/L lactose, 8 g/L yeast extract, 8 g/L peptone, and initial pH=7.0). The pigments were recovered from the fermentation broth using an alternative extraction approach based on Aqueous Two-Phase Systems (ATPSs) composed of two polymers or a polymer and a salt. PEG-salt ATPSs proved to be suitable to separate the pigments (top phase) from other components (proteins) present in the fermentation broth (bottom phase). Biomass (12 days fermentation) and Mycelial (7 days growth) pigments were also extracted with an organic solvent (ethanol).

Finally, the pigments were characterized regarding their antioxidant and biological activity (antibacterial and antifungal) and potential use as pH indicators (yellow in the acid range and dark red at alkaline pH). All the extracts showed antioxidant potential and the mycelial ethanolic extract (MEE) was the only extract that presented antimicrobial activity against bacteria, fungi and yeast.

Keywords: *Penicillium brevicompactum*; fungal pigments; aqueous two-phase systems; antioxidant; antimicrobial.

Sumário

Os pigmentos naturais podem ser utilizados para diferentes fins, nomeadamente na indústria têxtil para tingir panos ou na indústria alimentar, como corantes. Atualmente, alguns pigmentos de grau alimentício produzidos por fungos filamentosos, nomeadamente pigmentos produzidos por *Monascus*, Arpink red™ produzido por *Penicillium oxalicum*, a riboflavina através de *Ashbya gossypii*, bem como licopeno e β-caroteno de *Blakeslea trispora* podem ser encontrados no mercado. Na União Europeia existem aproximadamente 43 corantes autorizados como aditivos alimentares, enquanto aproximadamente 30 são aprovados nos Estados Unidos, vários deles de fontes naturais. Os pigmentos também podem ser usados em cosméticos, couro ou na indústria farmacêutica. Mais recentemente, outras aplicações foram encontradas para pigmentos como na coloração histológica, em células solares ou como indicadores de pH. Além disso, importantes propriedades biológicas, tais como atividades antibacterianas, antifúngicas e herbicidas foram relatadas para pigmentos naturais.

Os fungos são relatados como potentes produtores de pigmentos devido à rápida capacidade de crescimento dos microrganismos e ao facto de que não existe o efeito sazonal como nas plantas. Neste trabalho, otimizámos a produção de pigmentos extracelulares por *Penicillium sp.* sob fermentação submersa. Uma mistura de três pigmentos (amarelo, laranja e vermelho com λmax = 400, 470 e 500 nm, respetivamente) foi obtida sob condições ótimas (23 ° C, 150 rpm, 20 g/L de lactose, 8 g/L de suplementos e inicial pH = 7,0). Os pigmentos foram recuperados do meio de fermentação usando uma abordagem alternativa de extração baseada em Sistemas de Duas Fases Aquosas (ATPSs) compostos por dois polímeros ou um polímero e um sal. Os ATPSs PEG – sal provaram ser adequados para separar os pigmentos (fase superior) de outros componentes presentes no meio de fermentação (fase inferior). Os pigmentos obtidos a partir da biomassa (12 dias de fermentação) e do micélio (7 dias de crescimento), foram extraídos com um solvente orgânico (etanol).

Finalmente, os pigmentos foram caracterizados quanto à sua atividade antioxidante e biológica (antibacteriana e antifúngica) e uso potencial como indicadores de pH (amarelo na faixa ácida e vermelho escuro em pH alcalino). Todos os extratos apresentaram potencial antioxidante e o MEE foi o único extrato a mostrar atividade antimicrobiana contra bactérias, fungos e leveduras.

Palavras-chave: *Penicillium brevicompactum*; pigmentos fúngicos; sistemas de duas fases aquosas; antioxidante; antimicrobiano.

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Abbreviation List

ABTS- 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt

AF- Aflatoxin

ATPS- Aqueous Two-Phase Systems

DON- Deoxynivalenol

DPPH- di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium

F- Fumonisin

FBEE- Fermentative Biomass Ethanolic Extract

FRAP- Ferric Reducing Antioxidant Power

Kpig- Pigment partition coefficient

Kprot- Protein partition coefficient

LFE- Lyophilized Fermentation Extract

MBC- Minimal Bactericidal Concentration

MEA- Malt-Extract broth

MEE- Mycelial Ethanolic Extract

MFC- Minimal Fungicidal Concentration

MHB- Mueller-Hinton Broth

MIC- Minimal Inhibitory Concentration

MPA- Mycophenolic Acid

MRS- De Man, Rogosa and Sharpe broth

MYPGP- Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, pyruvate

OPA- *O*-phthaldialdehyde

OTA- Ochratoxin A

PDA- Potato Dextrose Agar

R- Volume Ratio

SMZ- Specific Medium *Zimomonas*

SMW- Specific Medium *Wickerhamomyces*

YPD- Yeast Extract-Peptone-Dextrose

ZEA- Zearalenone

%η_{TOP}- Recovery in the Top Phase

%MB- Mass balance

1. Introduction

1.1. Molecules that produce color: Pigments

Color is an important property when choosing a product of commercial value as well as in several aspects of human life. Nature, for example plants and animals, is surrounded by color and there is a variety of compounds responsible for that. These compounds are called pigments and generally comprise the red, purple, orange, brown, yellow, green, blue and their various shades (Benjamin K. Simpson, Soottawat Benjakul 2012).

The term pigment becomes from the Latin word *pigmentum* and it was initially used for the designation of color (in the sense of colored matter), being later extended to indicate colored objects. In the Middle Ages, the term was also used to relate diverse plant and vegetable extracts, especially those used for the food coloring. Nowadays, depending on the source, structure and application of the pigments, these can be called as dyes (textile industry) or colorants (food and pharmaceutical industry) (Singh Nee Nigam and Pandey 2009).

Pigments are chemical compounds (Figure 1.1) that through the absorption and refraction of light, in the wavelength range of the visible spectrum, give our world a variety of colors.
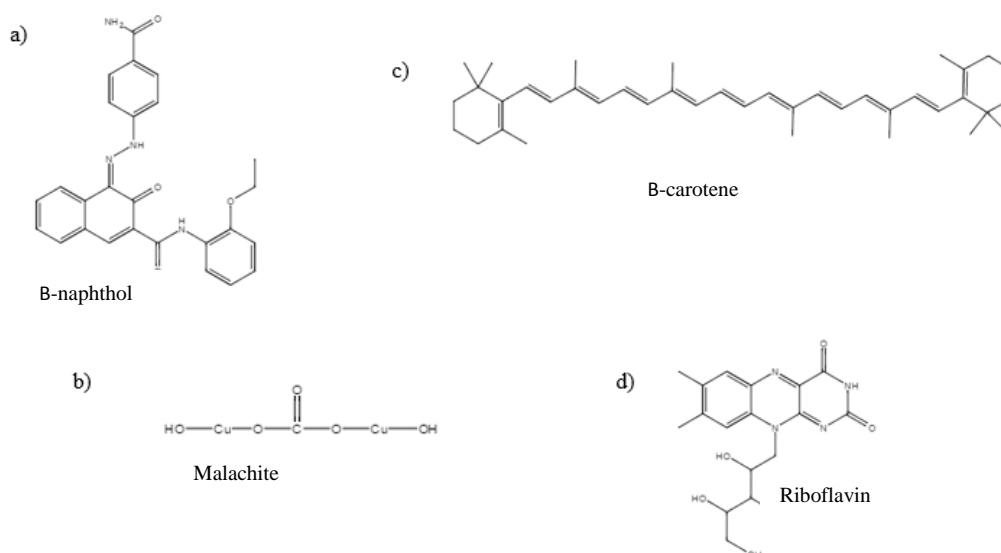
Figure 1.1- *Chemical structure of some pigments. a) Organic synthetic pigment; b) Inorganic natural pigment; c) and d) Organic natural pigments.*

The color that we see from pigments is due to the presence of a chromophore which is a specific part of the molecule responsible for its color. The energy captured by this structure causes the

1

excitation of an electron from an external orbital to a higher one. The non-absorbed energy is reflected and/or refracted and consequently can be captured by the eye, showing us the color (Figure 1.2) (Cordero and Casadevall 2017; Delgado-Vargas, Jiménez, and Paredes-López 2000; Méndez *et al.* 2011).
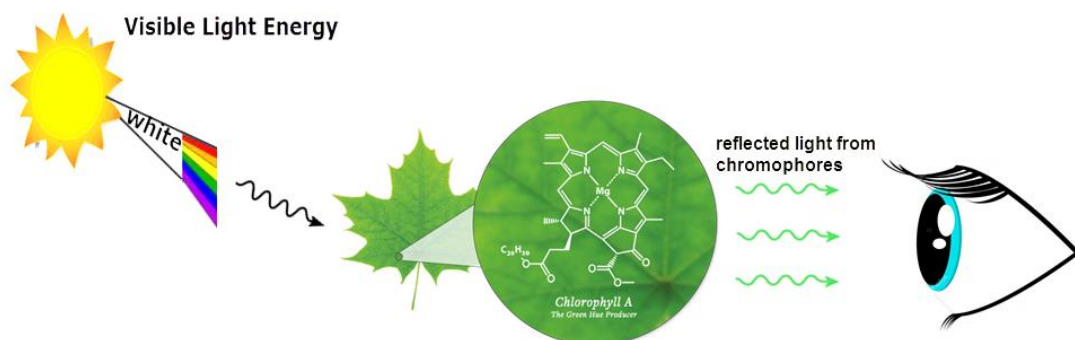
Figure 1.2*- Schematic representation of color captured by the eye.*

1.2. History and role of Pigments

Humankind knows pigments and their value since the prehistoric times. Natural pigments were used to mark trees and rocks to signal important sources of food and water or even delimitate the territory. Important prehistoric paintings found in caves also demonstrate the knowledge and practical application of different kinds of pigments. Additionally, pigments were used in body painting to indicate *status* and they were found being associated to superstitious and religious practices. The Egyptians were important color manufacturers improving the workability and the stability of pigments by mixing them with gums and animal glue. These compounds were also useful to several ancient civilizations to color different daily objects made of glass, clay, wood or wool. The application of pigments as cosmetics probably started by Greco-Roman ladies, who often used white pigments as face powder. Later, in the Middle Ages, pigments gained special attention from painters and an assorted palette became available.

The first chemically synthesized pigment (Prussian blue) was obtained in Germany in 1704. The rapid development of chemistry as a natural science during the 19th century, led to the synthesis of several new pigments in large scale (Barnett, Miller, and Pearce 2006). More recently, pigments gained additional importance due to the promising biological activities described for some of them. Therefore,

their range of application has been gradually extended beyond the coloring purposes (Gandía-herrero, Escribano, and García-carmona 2014).

Pigments also play an important role in the human body. For example, melanin is responsible for skin pigmentation and it has a recognized photoprotective effect against sun-induced skin cancers (Brenner and Hearing 2008). Opsins are cone visual pigments which enable us to discriminate the color of materials (Imamoto and Shichida 2014). Other example is hemoglobin, the most widespread respiratory pigment, which has a crucial contribution in the oxygen transport (Marengo-rowe 2006). The bile pigments, such as bilirubin and biliverdin, can also act as endogenous tissue protectors (Nakagami and Toyomura 1993).

Besides humans, also animals benefit from their own natural pigments. Several classes of pigments impart noticeable colors on animals and are useful to attract potential mates or avoid a possible predator (McGraw 2005). Similarly, plant pigments such as anthocyanin, betalains and carotenoids, act as visible signals to attract insects, birds and animals for pollination and seed dispersal. Chlorophyll is other important plant pigment which plays a decisive role in photosynthesis. Pigments can also protect plants from damage caused by UV and visible light (Yoshikazu Tanaka, Nobuhiro Sasaki 2008). In the same way, the production of pigments by microorganisms is generally associated with protective functions. For example, pigments produced from fungi, gives protection to them against bacterial and insects attacks and against harmful effects of sunlight and ultraviolet radiation (UV) (Pagano and Dhar 2015).

1.3. Types of pigments

Pigments can be classified as organic and inorganic, both including natural and synthetic molecules/compounds. Natural organic pigments are produced by plants, animals and some microorganisms (Santos-Ebinuma, Francisca, and Teixeira 2015) while the synthetic organic ones are produced in laboratories through chemical reactions. Inorganic pigments can be found both in nature or produced by synthesis (Delgado-Vargas *et al.* 2000). Figure 1.3 summarizes pigment's classification and some examples of each group.
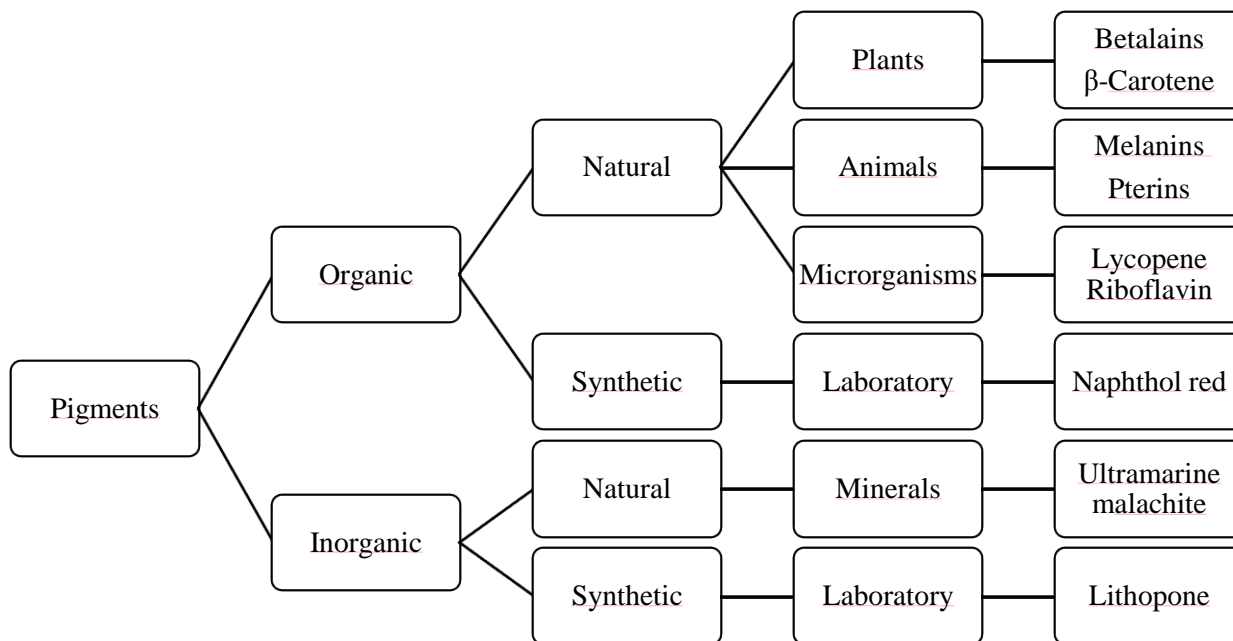
Figure 1.3- *Classification and examples of pigments* (Buxbaum 1998; Fremout and Saverwyns 2012; H. M. 2002; McGraw 2005; Sarvamangala and Aparna 2016; Yoshikazu Tanaka, Nobuhiro Sasaki 2008)*.*

Natural organic and inorganic pigments were used by humans since the dawn of civilization. However, when synthetic pigments were introduced into the industry, several cheap synthetic options have started to appear. The arising of these compounds led to a decrease in the use of natural pigments, because these are generally more expensive to obtain from the natural sources (Venil, Zakariab, and Ahmada 2013). But, environmental issues, sustainability and green economy policies have increased the trend towards the replacement of synthetic dyes by natural pigments. Nowadays, both are used in everyday life, for example in the food production and textile industries, paper production and in agricultural practices (Malik, Tokkas, and Goyal 2012). Besides increasing the market for the trade of natural products, some naturally obtained pigments can also have advantageous biological properties such as antioxidant, anti-inflammatory, anticancer and antimicrobial activity (Tuli, Chaudhary, and Beniwal 2014). On the other hand, these pigments have higher costs than the synthetic ones with similar color shades but this barrier can be overcome by the mass production of the natural pigments, bringing the cost down (Singh Nee Nigam and Pandey 2009). Despite the low costs of production of synthetic pigments, they can contain potential carcinogenic (Tuli et al. 2014; Velmurugan, Kamala-Kannan, et al. 2010) and adverse toxicological effects (Sopandi *et al.* 2012) and their use becomes risky, endangering human health, not also neglecting that they are a source of

environmental pollution. Thus, there is a growing search for the use of ecological and non-toxic pigments such as the natural ones.

As described before, organic natural pigments can be produced by plants, animals and some kinds of microorganisms. Plant pigments are the most used in the industry but despite the availability of various pigments from fruit and vegetables, these are generally dependent on the seasons and geographical conditions (Singh Nee Nigam and Pandey 2009). Regarding animal pigments, they are not very used since there are several ethical, religion and lifestyle (vegetarianism) issues, limiting their use in the industry (Edited by Reinhold Carle n.d.). In contrast, microbial pigments have a growing interest in industrial application because unlike the ones from plants they are independent of season and geographical conditions and can present higher production yields.

1.4. Microbial Pigments

The presence of pigments has been reported in the microbial world (Figure 1.4) including bacteria, fungi, yeasts, algae and protozoa, making microorganisms potential natural sources of these compounds (Table 1.1).

Figure 1.4- *Representation of various color producing microorganisms in a Petri plate* (Tuli *et al.* 2014).

Microorganisms come up with some vantages over plants as pigments producers namely their higher availability in nature (they are spread in various environmental sources such as water, plants, insects and animals) and their easy handling. Furthermore, microbial pigments generally present more advantages due to their stability, cost efficiency, high productivity and yield and solubility in water comparing to the other natural pigments obtained from plants or animals (Méndez *et al.* 2011; Tuli *et al.* 2014).

Table 1.1- *Example of microbial pigments and their color* (Sarvamangala and Aparna 2016; Singh Nee Nigam and Pandey 2009).

Microorganism	Pigment	Color
Fungus		
Phycomyces blakesleeanus	β-carotene	Yellow
Penicillium oxalicum	Arpink red	Red
Monascus spp.	Rubropunctatin	Orange
Penicillium candidum	Anthroquinone	Red
Fusarium sporotrichioides	Lycopene	Red
Bacteria		
Vibrio psychroerythrus	Prodigiosin	Red
Chromobacterium violaceum	Violacein	Violet
Bradirhizobium spp.	Canthaxanthin	Dark Red
Flavobacterium spp.	Zeaxanthin	Yellow
Yeast		
Phaffia rhodozyma	Astaxanthin	Red
Saccharomyces neoformis	Melanin	Black
Algae		
Rhodophyta	Phycoerythrins	Blue
Cyanophyta	Phycocyanins	Red

1.4.1. Fungal Pigments

Among all the microorganisms, fungi are reported as potent pigments' producers. Recent works have shown that this compounds hold important functions on hosts organisms and particularly on human well-being (Venil *et al.* 2013). Pigments produced by fungi are considered secondary metabolites generally synthetized by the mycelium when the supply of essential nutrients decreases, and the environmental conditions disfavor the fungal growth. These molecules can provide fungi protection against the harmful effect of sunlight, UV radiation and/or bacterial and insect attacks. Of all the fungal pigments studied, the most common are the melanins (dark brown pigments), carotenes (orange, red), lycopene (dark red) and xanthophylls (yellow), whose presence is crucial to improve fungal survival and spores' resistance. The most studied fungus for pigment production is *Monascus*

spp. whose compounds have been employed in the food and pharmaceutical industries due to its anticancer and antioxidant properties (Akilandeswari and Pradeep 2015). As a result of the scarcity of nutrients, which are essential for their metabolism, fungal mycelia produce these pigments to survive under the adverse conditions and to adapt to the new imposed environment (Gupta, Sreenivasaprasad, and Mach 2015).

There are several factors like pH, temperature or aeriation, that in biotechnological processes can affect the biosynthesis of metabolites like pigments (Méndez *et al.* 2011). Studies showed that the production of *Penicillium* spp. pigments is mainly affected by temperature and pH. These parameters influence the transport of certain media constituents, such as carbon and nitrogen sources, and can also limit the activity of enzymes involved in the biosynthetic pathways (Afshari, Shahidi, and Mortazavi 2015). The type of carbon source and aeriation rate can also affect the pigment production. Cho *et al.* concluded that the production of a red pigment by *Paecilomyces sinclairii* is greater for high rates of aeriation. Furthermore, the use of a sucrose-rich medium was more favorable for the mycelial growth, but the best pigment production was obtained in a starch-rich medium (Cho *et al.* 2002). The use of different salts and concentrations can also affect pigment production. For *Penicillium purpurogenum*, the intensity of the pigment color can be increased when NaCl or Na_2SO_4 is used in the fermentation broth (Santos-Ebinuma *et al.* 2013).

1.4.2. Fungal Mycotoxins

The production of secondary metabolite such as pigments could also lead to the production of other metabolites such as mycotoxins at the same time, in certain conditions (Dufossé *et al.* 2005). Several species of *Penicillium* are able to produce these toxic metabolites, being a great problem for the commercial application of this microorganism (Gmoser *et al.* 2017).

The term mycotoxin was first used by Bennett *et al.* in the 1960s to describe the toxin associated with contaminated food in animal feed (Bennett 1987). Mycotoxins are secondary metabolites produced by filamentous fungi, that can be phytopathogenic and/or mycotoxigenic, presenting toxic effects on human and animal consumers, although not showing immunogenicity. These compounds may contaminate agricultural products and animal feeds causing various food and feed borne mycotoxicosis through the consumption of these contaminated products (N. Magan 2004; Nguyen *et al.* 2017; De Ruyck *et al.* 2015).

Mycotoxins have no apparent function in the normal metabolism of fungi. They can be produced mostly, although not exclusively, when the fungus reaches the maturity. Their molecular structures can vary from simple heterocyclic rings with molecular weights up to 50 Da, to irregular arranged heterocyclic rings with a total molecular weight over 500 Da. Over the years, at least 400 different mycotoxins were identified but not all were found to be hazardous to human health (Rocha *et al.* 2014).

1.4.2.1. Principal Mycotoxins

a) Aflatoxins (AF)

The term aflatoxin was created based on the name of its main agent producer: *Aspergillus flavus* and has been recognized as the most important and widely studied mycotoxin (Nguyen *et al.* 2017; Rocha *et al.* 2014).

Aflatoxins are composed by 4 major groups (AFB1, AFB2, AFG1 and AFG2), and the differences between them are based on their fluorescence under ultraviolet light and their mobility during thin layer chromatography (TLC). These mycotoxins are mainly produced by *A. flavus* and *Aspergillus parasiticus*. However, more recently, the species *Aspergillus nomius*, *Aspergillus bombycis*, *Aspergillus pseudotamari* and *Aspergillus ochraceoroseus* were shown to be aflatoxin producers (Nguyen *et al.* 2017; Rocha *et al.* 2014; De Ruyck *et al.* 2015).

Among all the groups, aflatoxin B1 (AFB1, figure 1.5) was classified by IARC (International Agency for Research on Cancer) the most potent naturally occurring carcinogenic type, mutagenic and extremely toxic (IARC 2012; Ismaiel and Papenbrock 2015). They also have oncogenic and immunosuppressive properties, inducing diseases in people contaminated with these substances (Rocha *et al.* 2014).
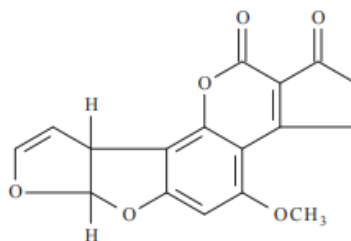
Figure 1.5- Chemical structure of Aflatoxin B1 (AFB1) ($C_{17}H_{12}O_6$) (IARC 2012).

Lactating animals contaminated with AFB1 can transform this type into aflatoxin M1 (AFM1) through their metabolism and digestion. The toxin can resist to the technological process like heat, cold-storage, freezing or fermentation (Duarte *et al.* 2013; Martins, Guerra, and Bernardo 2005; Nguyen *et al.* 2017).

b) Fumonisins (F)

Initially described and characterized in 1988, fumonisins are secondary metabolites produced by several species of the genus *Fusarium*, particularly by *Fusarium verticillioides* (previously classified as *Fusarium moniliforme*), *Fusarium proliferatum* and *Fusarium nygamai*, although other *Fusarium* and at least one *Aspergillus* species (*Aspergillus niger*) were found also to be producers of this mycotoxin (Rocha *et al.* 2014; Voss *et al.* 2017). Fumonisins are commonly found on maize but they can also be found in barley, millet, oats, wheat and corn (Bryła *et al.* 2016; De Ruyck *et al.* 2015; Smith 2018).

This group of mycotoxins is subdivided in 4 groups: A (FA1, FA2, FA3, FAK1), B (FB1, FB2, FB3, FB4, FB5 and FB6), C (FC1, FC3, FC4) and P (FP1, FP2, FP3, FPH1a and FPH1b) and by the year of 2010, over 28 fumonisins were identified (Nguyen *et al.* 2017; Rocha *et al.* 2014; Smith 2018; Voss *et al.* 2017).

Fumonisins have fatal effects on domestic animals and several studies showed that they also have hepatotoxic and nephrotoxic effect on rodents, and IARC has included fumonisin B1 (Figure 1.6) in Group 2B meaning that this toxin might be "possibly carcinogenic to humans." (IARC 2002; Nguyen *et al.* 2017; Smith 2018). It was also proven that a long last exposure to high doses of fumonisin B1 may result in an increased risk of developing esophagus cancer (Bryła *et al.* 2016). Experimentally, fumonisin has been shown to be the cause of liver damage in multiple species including pigs, horses, cattle, rabbits, and primates as well as leukoencephalomalacia in horses; pulmonary oedema and hydrothorax in pigs and liver cancer in rats (Ismaiel and Papenbrock 2015; Smith 2018).
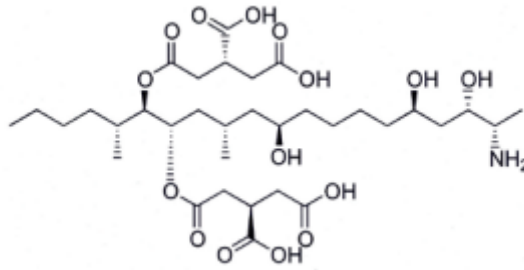
Figure 1.6- Chemical structure of Fumonisin B1 (FB1) ($C_{34}H_{59}NO_{15}$) (IARC 2002).

Fumonisin B1 has been showed to have co-occurrence with other fusariotoxins such as Deoxynivalenol (DON), Zearalenone (ZEA) and Ochratoxin A (Nguyen *et al.* 2017).

c) Zearalenone (ZEA)

Zearalenone (ZEA, figure 1.7) is a phenolic resorcyclic acid lactone (Mally, Solfrizzo, and Degen 2016) mycotoxin produced by various *Fusarium* species, detected in 90% of sampled cereal crops grown in European countries such as Austria, France and Germany (De Ruyck *et al.* 2015).
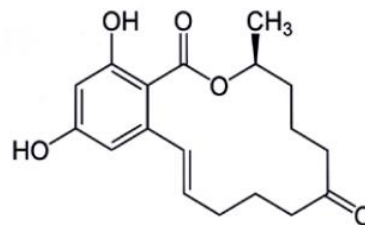
Figure 1.7- Chemical structure of ZEA ($C_{18}H_{22}O_5$) (IARC n.d.).

It is mainly synthetized by *F. oxysporum*, *F. tricintum*, *F. sporotrichioides*, *F. laterum*, *F. gramniearum*, *F. culmorum*, *F. crookwellense* and *F. equiseti* (Nguyen *et al.* 2017), and is commonly found in grains such as maize, wheat, barley, sorghum, rye and others (Mally *et al.* 2016).

Being a nonsteroidal estrogenic compound, ZEA has major effects on females reproduction (as evidenced by hyperestrogenism), but it affects the male reproductive system as well (Gupta, Mostrom, and Evans 2018), being classified by IARC in Group 3 (Not classifiable as to its carcinogenicity to

humans) (De Ruyck *et al.* 2015). But, although this mycotoxin exhibits low acute toxicity, at long-term exposure ZEA may present a health risk due to its high estrogenic activity (Mally *et al.* 2016).

Zearalenone was also found to co-occur with other mycotoxins like Deoxynivalenol (DON), AFB1 and Ochratoxin A (OTA) (Nguyen *et al.* 2017), but was reported to have antagonistic effects on the toxicity of some co-exposed mycotoxins.

d) Deoxynivalenol (DON)

Deoxynivalenol (DON, figure 1.8), also known as vomitoxin because of the emetic effects in pigs (Pestka 2010), is a polar organic compound, belonging to type B of trichothecenes mycotoxin group. It is mainly produced by *Fusarium* species, particularly by *F. graminearum* and can be found in human food as flour, bread, noodles, eggs, beer and malt (Liao *et al.* 2018; Sobrova *et al.* 2010). This mycotoxin can be found also in wheat, barley, maize, oats, rice, rye, sorghum and triticale and is related with damages in the gastrointestinal and immune systems, leading to symptoms like diarrhea, vomiting, leukopenia, hemorrhage and shock (Liao *et al.* 2018).
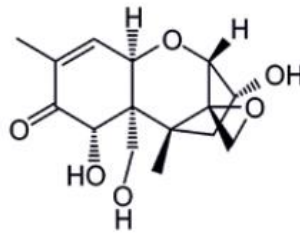
Figure 1.8- *Ch*emical structure of DON ($C_{15}H_{20}O_6$) (IARC n.d.).

DON is not classified as carcinogenic to humans, being placed in group 3 by IARC (Sobrova *et al.* 2010). Nevertheless, acute exposure to DON can cause gastroenteritis, vomiting, diarrhea while chronic exposure has shown immunotoxic effects in animals (Pagkali *et al.* 2018; Sobrova *et al.* 2010).

Once ingested, it starts to take effect when released from the food matrix and absorbed by the intestines into the bloodstream, where it can affect the morphometric properties of intestinal mucosa, serum hematological and other biochemical parameters (Gajęcka *et al.* 2018).

In a temperature range from 170–350 °C, DON can be very stable showing no reduction in its concentration after 30 minutes incubation indicating that this mycotoxin is very hazardous for human

and animal health because it cannot be eliminated by heat processes (Liao *et al.* 2018; Sobrova *et al.* 2010).

e) Ochratoxin A (OTA)

Ochratoxin A (OTA, figure 1.9) was discovered half century ago as a metabolite produced by *Aspergillus ochraceus*. Later, *Penicillium* species like *Penicillium verrucosum* (as *Penicillium veridicatum*), *P. nordicum* and *P. thymicola* were also reported as OTA producers (Cabañes and Bragulat 2018; Geisen *et al.* 2018). Usually, *P. nordicum*, *A. carbonarius* and *A. niger* are considered the main OTA producers (Geisen *et al.* 2018; Paterson *et al.* 2017).
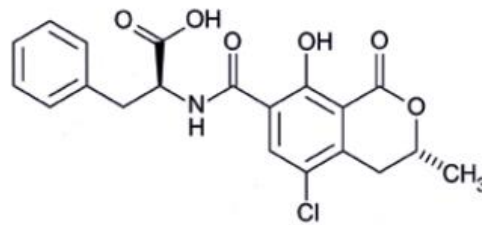
Figure 1.9- *Ch*emical structure of OTA ($C_{20}H_{18}ClNO_6$) (IARC n.d.).

OTA is classified in group 2B by the IARC being considered as a possible carcinogen agent in humans, that may cause urothelial tumors, testicular cancer and chronic interstitial nephropathy (Paterson *et al.* 2017). OTA is considered a cumulative toxin (Abrunhosa, Paterson, and Venâncio 2010) which can be quickly absorbed in the gastrointestinal tract, after oral administration. When it enters into the bloodstream, it binds to serum proteins, so it is subsequently absorbed through the liver, kidneys, stomach and the small intestine being hardly eliminated through urinary and fecal routs (Abrunhosa *et al.* 2010; Calado *et al.* 2018). OTA was also found in human blood, urine and milk (Calado *et al.* 2018).

From the ochratoxins family, OTA is the most predominant and toxic, exhibiting nephrotoxic, immunosuppressive, teratogenic, hepatotoxic , neurotoxic, embryotoxic, genotoxic and carcinogenic properties (Calado *et al.* 2018; Ostry, Malir, and Ruprich 2013; Paterson *et al.* 2017).

This mycotoxin can be found in meat products like raw pork meat, pork blood products, cured meats, salami or dry-cured ham (Ostry *et al.* 2013)**.** It is also found in cereal products, beer, cocoa,

coffee beans, spices, vegetables, tea, figs, raisins, grape juice, wine, liquorice and chestnuts as well as in dried fruits. Dairy products for humans consumption may also be contaminated with OTA like milk and cheese as a result of the presence of molded spots of the mycotoxigenic fungi (Calado *et al.* 2018; Ostry *et al.* 2013).

Recent studies found out that this mycotoxin could possibly be biodegraded by several microorganisms like bacteria's, filamentous fungi and protozoa (Abrunhosa *et al.* 2010).

1.4.3. Fungi pigments as bioactive compounds

As mentioned before, synthetic pigments can be associated to chronic diseases (cancer, diabetes, cardiovascular, autoimmune and respiratory disorders) making the use of natural pigments, (namely those with bioactive features) in the industry a world request (Nigam and Luke 2016; Wani and Tirumale 2018).

The presence of free radicals as products of cellular mechanisms of living organisms turns antioxidants a group of compounds with an important role in the prevention or removal of oxidative damages of spoiled cells (Carocho and Ferreira 2013). These compounds can trap free radicals which in excess cause serious damages such as neurodegenerative diseases like Parkinson's (Gonçalves Tavares *et al.* 2018).

There are two types of antioxidants acting in the human system: endogenous and exogenous. The group of endogenous antioxidants is composed by enzymes (glutathione, catalase and superoxide dismutase) that prevent or neutralize the formation of free radicals. Despite the efficiency of endogenous antioxidants, these are not produced in enough quantity by the organism, being necessary the presence of exogenous antioxidants in daily diet to maintain the concentration of free radicals at low levels. So, exogenous antioxidants (carotenoids, flavonoids, phenolic and polyphenolic compounds and vitamins) are obtained from different sources like plants, fruits, vegetables and microorganisms, specially fungi (Carocho and Ferreira 2013; Gülçin 2012).

Fungi may be able to provide a wide variety of secondary metabolites with bioactivity such as alkaloids, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones and xanthones (Arora and Chandra 2011; Wani and Tirumale 2018).

Secondary metabolites with antimicrobial activity are also a demand in industry as there are concerns in fields like pharmaceutical, textile and food industry. This need and search for novel products with bioactivity aims to substitute synthetic pigments and avoid their hazardous effects on health or even to found out other sources of antibiotics since nowadays there is a crisis related with antibiotics resistance (Tuli *et al.* 2014).

It has been reported that microorganisms have potential to synthetize antioxidants and antimicrobial compounds as bioactive metabolites (Dufossé 2017; Hameed *et al.* 2017), being considered as a promising source of them. Several microbial pigments were shown to be able to help preventing several diseases (Table 1.2) and have been used as food additives, in cosmetics and pharmaceuticals (Gonçalves Tavares *et al.* 2018).

Table 1.2- *Bioactive activities of microbial pigments* (Nigam and Luke 2016).

Microbial Pigment	Bioactive activity
Carotenoids	Prevents cardiovascular diseases (CVD), protection against sun-burns and inhibit the development of few cancers.
Lycopene	Prevention of arteriosclerosis and coronary diseases and aids in oxidation of low-density lipoprotein cholesterol (LDL)
Prodigiosin	Anticancer
Astaxanthin	Boosts the immune system
Zeaxanthin and Xanthophylls	Prevention of age-related macular degeneration (cause of human retina blindness)
Violacein	Protection against oxidative damage in gastric ulceration

1.4.4. Fungal Pigments in industry

Natural pigments have been used to replace synthetic pigments in industrial applications. In the food industry, some natural pigments used are generally obtained through fermentation. The pigments benefit and improve the appearance of the product and confer additional medicinal and nutritional

values due to their antibiotic and antioxidant characteristics (Benjamin K. Simpson, Soottawat Benjakul 2012). In addition to antioxidant, anticancer and antimicrobial features, pigments may also contain other properties that help fight certain health issues such as cholesterol (Kumar *et al.* 2015). Table 1.3 shows natural pigments authorized in the food industry by the European Union.

Table 1.3- *Fungal pigments used as natural food colorants* (Sarvamangala and Aparna 2016).

Color	Pigment	Microorganisms
Red	Anthroquinone	*Pencilllium candidum*
	Monascorubramine	*Monascus* spp.
Yellow	Ankaflavin	*Monascus* spp.
	Riboflavin	*Ashbya gossypi*
Orange	Rubropanctatin	*Monascus* spp.
Yellow-orange	β-carotene	*Blakeslea trispora*

The pharmaceutical industry uses a very wide variety of pigments in its products. Many of these pigments are secondary metabolites of microorganisms that have great clinical potential of application. They are also used in research studies to find treatments for diseases such as cancer, leukaemia, diabetes mellitus, among others. Many of these pigments act as antibiotics, anticancer, antiproliferative and immunosuppressive agents. Some examples of these pigments are presented in Table 1.4.

Table 1.4- *Antimicrobial activity of some fungal pigments.*

Microorganism	Pigment	Color	Activity	Reference
Fusarium Sporotrichioides *Blakeslea trispora*	Lycopene	Red	Anticancer	(Giovannucci *et al.* 2002; Di Mascio, Kaiser, and Sies 1989)
Penicillium oxalicum	Anthroquinone		Antifungal, virucidal	(Agarwal *et al.* 2000)
Monascus spp	Monascorubramin		Antimicrobial, anticancer	(BLANC *et al.* 1994)
	Ankaflavin	Yellow	Antitumor, anti-inflammatory	(Hsu *et al.* 2011)
Ashbya gossypi	Riboflavin		Anticancer, protection against cardiovascular diseases	(Unagul *et al.* 2005)
Monascus spp.	Rubropunctatin	Orange	Anticancer	(Zheng *et al.* 2016)
Monascus roseus	Canthaxanthin		Anticancer	(Chew *et al.* n.d.)
Cordyceps unilateralis	Naphtoquinone	Deep blood red	Anticancer, antibacterial	(Nematollahi, Aminimoghadamfarouj, and Wiart 2012)

Textile industry uses a large amount of pigments in the dyeing of clothes, many of them are synthetic. But these pigments, considered as dyes by the industry, are frequently associated with skin allergies and other reactions harmful to the human body. During their synthesis, other undesirable toxic and dangerous chemical hazards are produced in conjunction with pigments. For the successful commercial use of natural dyes applied to any textile fibers, suitable and standardized dyeing techniques need to be adopted. Accordingly, to obtain a new shade with acceptable color resistance behavior and reproducible color yield, appropriate scientific dyeing techniques/procedures should be derived (Kumar *et al.* 2015; Venil *et al.* 2013). Hamlyn (1995) reported the importance of pigments

such as anthraquinone, anthraquinone carboxylic acids and pre-anthraquinones extracted from filamentous fungi in dyeing of cotton, silk and wool (Hamlyn 1995).

1.5. Pigments Extraction

After the production of pigments, it is necessary to extract them from the culture medium. The method of extraction is selected according to the pigment's nature (extra or intracellular) and production conditions (Velmurugan, Lee, Venil, *et al.* 2010).

1.5.1. Conventional Methods

One of the most common methodologies for pigment recovery is the organic solvent extraction using several solvents (Robinson *et al.* 2014). This method consists in the addition of a solvent, or a mixture of solvents, to the sample containing the pigment. Depending on the type of solvent and sample, the pigment can be directly extracted to the solvent or a biphasic system can be formed, with the pigment preferably concentrated in one equilibrium phase (Sachindra, Bhaskar, and Mahendrakar 2006). The ideal solvent for fungal pigments' extraction must meet certain criteria like low toxicity, show more affinity towards pigments, be able to evaporate at room temperature and not degrade or react with the pigment (Robinson *et al.* 2014).

Some solvents commonly applied in pigment extraction are: acetone, chloroform, ethanol, hexane, methanol and ethyl acetate. To measure the concentration of the pigment extracted, it is used the UV-VIS spectrometer (Velmurugan, Lee, Venil, *et al.* 2010).

1.5.2. Alternative Methods

Over the last few years, researches have been working in different ways to extract pigments because the common way to extract them has a lot of problems, such as low selectivity, long process time and extraction efficiency (Santos-Ebinuma *et al.* 2015). Also, the use of organic solvents may cause the denaturation of the biomolecules recovered (Ebrahimi and Shahriari 2016).

Aqueous Two-Phase Systems (ATPSs) were found accidently by Martinus Willem Beijerinck in 1896, while he was mixing an aqueous solution of starch with gelatin. But only a few years after this

discovery, Per-Âke Albertsson discovered its real application potential (Albertsson 1986; Iqbal *et al.* 2016). Since then, ATPSs have been used for a wide range of biotechnological applications, mainly in recovering and purifying biological products including proteins, genetic material, bio nanoparticles, cells and organelles (de Albuquerque Wanderley *et al.* 2017).

ATPSs are obtained by mixing two aqueous solutions of different constituents that become immiscible above certain critical conditions, like temperature, concentration, etc. Both phases are composed mainly by water (>70%) and each one is enriched in a different component. The predominant component in each phase may vary according to the type of ATPS prepared (Kaul 2000).

There are several types of ATPSs but the most common are formed by two polymers (usually Polyethylene glycol (PEG) and Dextran) or a polymer and a salt (e.g., Phosphate, Sulphate or Citrate). Another types of ATPSs include biphasic systems composed by ionic liquids, short-chain alcohols or surfactants have been already reported (Santos-Ebinuma *et al.* 2015). Additionally, ionic liquids and/or surfactants are used for the formation of a novel type of biphasic systems called micellar and reverse micellar ATPSs.

For separation approaches aiming the recovery and purification of solutes which are sensitive to the ionic environment, polymer-polymer ATPSs are desirable due to the low ionic composition of the system (Iqbal *et al.* 2016). ATPSs had made the opportunity to applicate liquid-liquid extraction concepts from classical chemical engineering to the purification of biological products. The classical liquid-liquid extraction (or organic-liquid extraction) cannot be used for biological products' recovery due to the presence of organic solvents which can damage these products. However, the use of components that are biodegradable and biocompatible, like polymers and salts, in a gentle environment, makes ATPSs a suitable approach to this end. To preserve the biological molecules' activity during the purification process, the most important features of ATPSs are its low interfacial tension and high water content (Grilo, Aires-Barros, and Azevedo 2014).

To ensure the success of solute recovery by ATPSs, it is necessary to set the suitable conditions in which the process will be carried out. Solute partition in ATPSs can be influenced by several factors, including the salt and/or polymer concentrations, molecular weight of the polymer, temperature, pH and the concentration of additives such as NaCl, KCl or $NaClO_4$ (Zaslavsky 1995)**.**

The partition coefficient (K) of a specific solute quantifies its distribution between the equilibrium phases. As mentioned before, K is defined as the ratio between solute concentration in the top and bottom phases (Equation 1).

$$K = \frac{Concentration\ (top\ phase)}{Concentration\ (bottom\ phase)} \qquad \textbf{Equation 1}$$

In ATPSs, partition coefficients are generally used to evaluate the extension of the separation process. If the target molecule presents a relatively high (or low) K-value, -its recovery to the top (or bottom) phase will be effective. K-values lower than unity, indicate that the partition occurred preferably towards the bottom phase. On the other hand, K values higher the unity denote that the partition occurred preferably towards the top phase. Finally, K-values close to unity indicate that the partitioning between the equilibrium phases is similar, so the separation in such ATPS will not be effective. To obtain high purity levels in extraction processes it is important to ensure that the K-value for the target molecule and the K-value for the contaminants are considerably different (selectivity) (Silvério *et al.* 2013).

1.5.2.1. *ATPSs application in pigments recovery*

The recovery of pigments, dyes or colorants by ATPSs have been successfully reported in the literature. According with the type of pigment and its source, some systems were already applied for its extraction (Table 1.5.1).

Table 1.5.1- *ATPSs used for the extraction/recovery of different types of pigments.*

	Pigment	Source	System	Ref.
Natural	Red colorants	Fermentation broth of *Penicillium purpurogenum*	**Polymer-Polymer** PEG6000 – NaPA8000 – NaCl 0.1 M PEG10000 – NaPA8000 – Na_2SO_4 0.5 M	(Santos-Ebinuma *et al.* 2015)
			Ionic Liquid-Salt $[N_{2,2,2,2}]$Br (Tetraethylammonium bromide) – Potassium citrate buffer	(Vale 2013)

Table 1.5.1 - *Continuation.*

Natural	Rubropunctamin and Monascorubramin	Whole culture medium of *Monascus purpureus* CCT3802	**Polymer-Salt** PEG6000 – Potassium phosphate	(Esmanhoto and Kilikian 2004)
	β-carotene and Lutein	Cyanobacteria *Synechocystis sp.* PCC 6803 and/or commercial compound	**Polymer-Polymer** PEG3350 – Dextran 66900, pH 7.0 PEG8000 – Dextran 66900, pH 7.0	(Chavez-Santoscoy *et al.* 2010)
			Ionic Liquid-Sugar 1-butyl-3-methyl-imidazolium trifluoromethanesulfonate [C_4mim][CF_3SO_3] – Carbohydrate	(Freire *et al.* 2011)
			Ionic Liquid-Salt [Pi(444)4][Tos] – K3PO4	(Louros *et al.* 2010)
	Anthraquinones	*Aloe vera* L. peel powder crude extract	**Ionic Liquid-Salt** [C_4mim]BF_4 – Na_2SO_4	(Tan, Li, and Xu 2012)
Synthetic	Sudan I-IV	Candies	**Ionic Liquid-Salt** C_4[MIM]BF_4 – Sodium dodecyl benzene sulphonate – $(NH_4)_2SO_4$	(Yu *et al.* 2015)
	Carmoisine and Brilliant blue FCF	Food samples (strawberry jelly, fruity candy, etc)	**Polymer-Salt** PEG6000 – Sodium carbonate – 0.05M Borate buffer pH 9.0	(Avazpour *et al.* 2014)

1.6. Penicillium brevicompactum

Among all fungal microorganisms that produce pigments, the genera *Monascus*, *Aspergillus* and *Penicillium* have been widely studied as potential producers of natural pigments. Also, the production of *Monascus*-like pigments from *Penicillium* strains has been reported, showing that these strains and their pigments have potential for industrial applications (Méndez *et al.* 2011).

Penicillium brevicompactum is a fungus commonly found in the form of mould mostly isolated from wood (Nielsen 2003). Considered one of the most existing xerophilic *penicillia* (Ndagijimana *et al.* 2008), this specie is able to grow in environments with low quantity of water (Duckworth and International Union of Food Science and Technology. 1975). This fungus has the following taxonomy: *Eukaryota, Fungi, Dikarya, Ascomycota, Pezizomycotina, Eurotiomycetes, Eurotiomycetidae, Eurotiales, Aspergillaceae, Penicillium, Penicillium brevicompactum*.

This well-characterized specie is known to produce mycophenolic acids (MPA), which are an immunosuppressive agent (Mansfield, Jones, and Kuldau 2008; Sørensen, Mogensen, and Nielsen 2010) with antiviral, antifungal, antibacterial, antitumor and antipsoriasis properties (Regueira *et al.* 2011) that is used as specific therapeutic agents (Ndagijimana *et al.* 2008), but if exposed to this toxin, it may not present an obvious deterioration of health, but its immunosuppressive action may lead to the immune system becoming more vulnerable to infectious diseases, leading the infected host to be more susceptible when exposed to other potentially mycotoxins that could be hazardous to health (Mansfield *et al.* 2008). In addition to the production of mycophenolic acids, *Penicillium brevicompactum* is known to produce some valuable products such as brevianamide A, asperphenamate and ergosterol (Ardestani 2011). The most toxic metabolite produced by *P. brevicompactum* is the mutagenic compound botryodiploidin (Nielsen 2003).

Until today, there are low reports about the ability of *P. brevicompactum* to produce pigments, but this fungus showed a high potential to produce these types of compounds from a yellow to a red color range, depending on the growth conditions.

2. Materials and methods

2.1. Fungal Strain

Penicillium brevicompactum (MUM 02.07) was obtained from MUM (Mycology collection of University of Minho). Stock cultures were stored at room temperature (around 25°C) in suspensions of semi solid agar medium 0.2% (w/v), and then propagated on Petri plates containing MEA (Malt Extract Agar (% w/v): malt extract (2), glucose (2), peptone (0.1) and agar (2)).

2.2. Pigment production

Inoculum suspensions for submerged fermentation were prepared using saline solution 0.85% (w/v) NaCl containing 0.01% (w/v) Tween 80 (Cardoso *et al.* 2017). Conidia density was adjusted to 10^6 conidia/mL using a Neubauer counting chamber. All fermentations were performed in triplicate using 250 mL flask each containing 50mL of culture medium. Several fermentation conditions were studied for 12 days, as detailed as follows (Table 2.1).

Table 2.1- Fermentation conditions tested.

Assay	Conditions
Culture Media	NAGY (A), CZAPEC (B), MEA (C)
Orbital shake (rpm)	200, 150, 100, 0
Sugars (20 g/L)	Glucose, Lactose, Sucrose
Temperature (°C)	23, 28, 33
Supplements: Peptone – Yeast Extract (P-YE) (g/L)	(0-8), (0-16), (8-0), (2-2), (4-4), (2-6), (6-2), (8-8)
Lactose Concentration (g/L)	10, 20, 30
Natural light	With or without
pH	4.5, 7, 9.5

2.2.1. Culture media

Three different culture media (A, B and C) were evaluated for pigment production. Synthetic medium (A) comprised (% w/v): lactose (2), peptone (0.4), yeast extract (0.4) and salts (KH_2PO_4 (0.2), $Na_2HPO_4.12H_2O$ (0.8) and $MgSO_4.7H_2O$ (0.025) (Nagy *et al.* 2001); Synthetic medium (B) comprised (% w/v): lactose (2), $NaNO_3$ (0.3), K_2HPO_4 (0.1), $MgSO_4 \cdot 7H_2O$ (0.05), KCl (0.05), $FeSO_4 \cdot 7H_2O$ (0.01) (Méndez *et al.* 2011); Synthetic medium (C) comprised (% w/v): lactose (2), malt extract (2) and peptone (0.1). All the fermentations were performed at 200 rpm and 28 °C.

2.2.2. Orbital shake

Four different rotations were tested for pigment production: 200 rpm *(Méndez et al.* 2011), 150 rpm (Silvério *et al.* 2018), 100 rpm and 0 rpm (Velmurugan, Lee, Venil, *et al.* 2010). All the fermentations were performed with medium A at 28 °C (Silvério *et al.* 2018).

2.2.3. Sugars

Regarding the influence of the carbon source for pigment production, three sugars were assessed: lactose, glucose and sucrose. All the fermentations were performed with medium A at 28 °C and 150 rpm with 20 g/L of each sugar.

2.2.4. Temperature

Three different temperatures were tested to evaluate pigment production: 23, 28 and 33 °C. All the fermentations were performed with medium A at 150 rpm.

2.2.5. Supplements: Peptone-Yeast Extract (P-YE)

Eight different combinations of concentrations (g/L) of supplements were evaluated for pigment production: (0-8), (0-16), (8-0), (2-2), (4-4), (2-6), (6-2), (8-8). All the fermentations were performed with medium A at 150 rpm and 23 °C.

2.2.6. Lactose Concentration

Three different lactose concentrations were tested to evaluate pigment production: 10, 20 and 30 g/L. All the fermentations were performed with medium A at 150 rpm, 23 °C and with (8-8) g/L of supplements.

2.2.7. Natural light

Two different conditions were studied, namely with and without natural light (Velmurugan, Lee, Venil, *et al.* 2010) using medium A at 150 rpm, 23 °C, with (8-8) g/L of supplements and 20 g/L of lactose.

2.2.8. pH

To evaluate the effect of the pH during the fermentation, three different pH were tested: 4.5 for acid, 7.0 for neutral and 9.5 for alkaline region. These fermentations were performed using medium A at 150 rpm, 23 °C, with (8-8) g/L of supplements, 20 g/L of lactose and natural light.

2.3. Biomass dry weight determination

For the biomass dry weight determination, all culture broth was filtered through pre-weighed filter papers; biomass was conveniently washed with distilled water; the mycelium retained by the filter papers was dried to a constant weight.

2.4. Pigment production monitoring

The pigment production was spectrophotometrically monitored after 3, 5, 7, 10 and 12 days of fermentation, using a microplate reader (Cytation3,). A sample of 1 mL of culture medium was collected in aseptic conditions and centrifuged prior the absorbance read. Pigment production was followed by checking the absorbance values at 400 nm, 470 nm and 500 nm (corresponding to the yellow, orange and red regions of the visible spectrum, respectively) (Kantifedaki *et al.* 2018; Srianta *et al.* 2016). To determine of the best conditions for pigment production, the sum of the three wavelengths was considered. Also, Tukey's range test was performed to estimate significant differences ($p < 0.05$) among samples.

2.5. Quantification of sugars

The sugars quantification 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 (Cardoso *et al.* 2017). A calibration curve was previously prepared with standards of lactose, glucose and sucrose in the range 0.5–20 g/L.

2.6. Quantification of total protein content

Total protein concentration was determined using the O-phthaldialdehyde (OPA) fluorometric method. The calibration curve was obtained using standard stock solutions of bovine serum albumin (BSA) in the concentration range from 0 to 2.5 mg/mL. A volume of 250 μL of fresh OPA reagent was added to 100 μL of sample (containing 30 μL of filtrated fermentation broth and 70 μL of deionized water). The fluorescence from the reaction was read using a Cytation 3, 96 microplate fluorescence reader (Biotek) following moderate shaking for 45 min at 37 °C with a 360 nm excitation filter and a 460 nm emission filter, with a sensitivity setting of 50 (Silva *et al.* 2015). The data were collected from the bottom with a 7 mm probe using static sampling with a 1 millisecond delay, 50 measurements per well.

2.7. Statistical analysis

All the experiments were performed in triplicate and the values obtained are presented with the means and respective standard deviations. Tukey's range test was considered to estimate significant differences ($p < 0.05$) among samples with a confidence interval of 95%.

2.8. Pigment Extraction

2.8.1. Ethanol extraction

The mycelium plugs collected from the agar plates (7-10 days growth) were weighted and transferred to a sterile 1L flask. Ethanol 95% (v/v) was added to the mycelium using a ratio of 5 mL ethanol per gram of plugs. The content was mixed on a rotary shaker at 100 rpm overnight and then filtered (0.45 μm). The process was repeated twice in order to increase the amount of pigment extracted from the mycelium (Johns R. and Stuart M. 1991). The same protocol was also used to extract pigment from the biomass obtained through submerged fermentation performed for 12 days.

2.8.2. Aqueous Two-Phases Systems (ATPSs)

All systems were prepared with a final composition of 15% (w/w) polymer(s) and 15% (w/w) salt. Known masses of PEG 8000 – DEX75, PEG 8000 – Na$_2$SO$_4$, PEG 10000 – Na$_2$SO$_4$, PEG 8000 – (NH$_4$)$_2$SO$_4$ and PEG 8000 – Na$_3$C$_6$H$_5$O$_7$ were weighted for the respective assay tubes. Known amounts of water were added to the tube in order to obtain a blank with 2g total system and the same was done with the liquid fermentation broth. Next, all test tubes were homogenized in a vortex and centrifuged at 4000 rpm for 1h. Formerly, the tubes were visually analyzed regarding phase formation. After resting, the samples of the top and bottom phases were carefully collected. Each partition experiment was made in duplicate and the respective standard deviations and confidence intervals calculated. The experiments were performed at 25 °C with an uncertainty of ± 0.01 °C (Santos-Ebinuma *et al.* 2015; Silvério *et al.* 2008).

The recovery was analyzed in terms of partition coefficient of colorants and protein (K$_{pig}$, K$_{prot.}$) (Equation 1). The recovery in the top phase (%η$_{TOP}$) and volumetric ratio (R), were also determined accordingly to the following equations:

$$\%\eta_{TOP} = \frac{Abs_{TOP} \times V_{TOP}}{Abs_{INI} \times V_{INI}} \times 100 \qquad \qquad \textbf{Equation 2}$$

$$R = \frac{V_{TOP}}{V_{BOT}} \qquad \qquad \textbf{Equation 3}$$

where Abs$_{TOP}$, Abs$_{BOT}$, and Abs$_{INI}$ are the pigment absorbance at 400, 470 and 500 nm in the top and bottom phase, and the initial fermentation broth, respectively. V$_{TOP}$, V$_{BOT}$ and V$_{INI}$ are the volume of top and bottom phase and the initial volume of the fermented broth.

Selectivity of pigments separation from the remaining proteins present in the fermentation broth (Se) was also calculated according Equation 5.

$$Se = \frac{K_{Pig.}}{K_{Prot.}} \qquad \qquad \textbf{Equation 4}$$

2.9. Pigment Analysis

The fermentation broth and the ethanolic extracts obtained from MEA agar plates (7-10 days growth) and from the biomass (12 days of fermentation) were qualitatively analyzed by Thin Layer Chromatography (TLC). 20 µL of each extract were loaded on a silica plate (Macherey-Nagel, DC-Fertigfolien ALUGRAM SIL G/UV$_{254}$, Macherey-Nagel GmbH & Co., Germany) and the pigments were allowed to separate with different mobile phases.

Different mixtures were used as eluent: Chloroform:methanol:acetic acid (285:21:9); Chloroform:methanol:water (90:25:4); Chloroform:acetone (9:1); CH$_2$Cl$_2$:acetone (99:1); Ethyl:acetate:formic acid:acetic acid:water (100:11:11:26); Chloroform:acetone:water (89:1:10); Methanol:water (50:50); Butanol:water:ethanol (25:12.5:12.5); Water:ethanol (50:50); Water:ethanol (75:25); Water:ethanol (25:75); Water:ethanol (60:40); Water:ethanol (40:60) (Feng, Shao, and Chen 2012). The Retention factor (Rf) of the bands were recorded after the plates were exposed to UV light.

2.10. Pigments Stability

All stability tests were performed by mixing the fermentationed broth containing the pigments and the buffer in the desired pH (pigments:buffer = 1:1). The mixture was then homogenized in a vortex. The first set of experiments was performed to evaluate the effects of pH (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0) on the stability of the pigments color at room temperature. In the second set of experiments, pigment's stability at different temperatures was determined by incubation at 5, 15, 25, 35, 45, 55, 65, 75 and 85 °C for 24h at pH 8.0. The effects of the salts NaCl, Na2SO4, (NH$_4$)$_2$SO$_4$ and Na$_3$C$_6$H$_5$O$_7$ at concentrations from 0.1 up to 1.5 M, on pigments color change, were also evaluated at room temperature. Finally, the effects of different concentration of polymers (5 to 50% (w/w)) PEG 8000, PEG 10000 and DEX75 were evaluated on pigments color change at room temperature. For all sets of experiments, the absorbance was read (400, 470 and 500 nm) after 24h. All experiments were performed in triplicate and the standard deviations and confidence intervals were calculated (Santos-Ebinuma *et al.* 2013).

2.11. Functional properties

For the quantification of phenolic compounds and flavonoids, as well as the analysis of the antioxidant activity of the three extracts, solutions with final concentration of 60 mg/mL were prepared in ultrapure water or a 10% (v/v) ethanol solution (depending on the extract). Subsequently, the samples were vortexed for 1 min, centrifuged and filtered through 0.22 μm membranes. Tukey's range test was considered to estimate significant differences ($p < 0.05$) among samples.

2.11.1. Phenolic compounds

The total content of phenolic compounds was determined by using the Folin-Ciocalteu reagent according to the colorimetric method described in the literature (Singleton and Rossi 1965), adapted to a 96-well microplate. For the reaction, 5 μL of sample was mixed with 60 μL of sodium carbonate solution at 7.5% (w/v) and 15 μL of Folin–Ciocalteu reagent. Subsequently, 200 μL of distilled water were added and the solution was mixed. Thereafter, the sample was heated at 60 °C for 5 min and allowed to cool at room temperature (around 23 °C). The absorbance was then measured by means of a spectrophotometric microplate reader (Cytation3, Biotek) set at 700 nm. A calibration curve was made from gallic acid standard solutions (5, 50, 100, 150, 200, 300, 350, 400, 450, 500 mg/L) and the blank was prepared with distilled water. The total content of phenolic compounds was expressed as milligram of gallic acid equivalent per gram of dry material in the extracts (mg GAE/g extract) (Singleton and Rossi 1965).

2.11.2. Flavonoids

The total content of flavonoids was estimated by colorimetric assay as described by Chang *et al.* (2002) with some modifications. A volume of 30 μL of the extract was sequentially added to 90 μL methanol, 6 μL aluminum chloride at 10% (w/v), 6 μL potassium acetate (1M), and 170 μL distilled water, in a 96-well microplate. The mixtures were maintained during 30 min in the dark at room temperature, and the absorbance was then measured at 415 nm against a blank of distilled water using a spectrophotometric microplate reader (Cytation3, Biotek). A calibration curve was prepared with a

standard solution of quercetin (25, 50, 100, 150, 200 mg/L). The content of total flavonoids was expressed as milligram quercetin equivalent per gram of dry material in the extract (mg QE/g extract) (Chang, Yang, and Wen 2002).

2.11.3. Antioxidant activity

2.11.3.1. Ferric Reducing Antioxidant Power assay (FRAP assay)

The antioxidant activity by using the ferric reducing antioxidant power (FRAP) assay was determined according to the method described in the literature (Benzie and Strain 1996) with some modifications. A 10 µL aliquot of sample was mixed with 290 µL of FRAP reagent in a 96-well microplate, and incubated at 37 °C for 15 min. After that, the absorbance was determined at 593 nm against a blank of distilled water using a spectrophotometric microplate reader (Cytation3, Biotek). FRAP reagent was freshly prepared by mixing a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with a 20 mM $FeCl_3$ solution and 0.3 M acetate buffer (pH 3.6) in a proportion 1:1:10 (v/v/v). A calibration curve was constructed using an aqueous solution of ferrous sulfate ($FeSO_4.7H_2O$ at 200, 400, 600, 800 and 1000 µM). The FRAP values were expressed as micromoles of ferrous equivalent of ferrous equivalent per gram of dry material (µmol Fe(II)/g extract) (Benzie and Strain 1996).

2.11.3.2. Free radical scavenging activity (DPPH assay)

The DPPH radical scavenging activity was determined using the method described by Ballesteros *et al.* (2015) with some modifications. For each sample, a dilution series were prepared and vortexed. The reaction was carried out in a 96-well microplate containing 25 µL of sample and 200 µL of 150 µM DPPH solution (dissolved in 80% methanol to an absorbance value of 0.700 at 515 nm). The produced solutions were left for 1 h in the dark at room temperature (around 23 °C), and then the absorbance was measured at 515 nm in a spectrophotometric microplate reader (Cytation3, Biotek). A control solution consisted in using methanol instead of the sample and a blank solution consisted in mixing the sample with methanol instead of the DPPH solution. The radical scavenging activity was calculated by

using the Equation (5), where A_c, A_b and A_s represent the absorbances of the control, blank and sample, respectively. A calibration curve was prepared with a standard solution of trolox diluted in methanol (50, 100, 200, 250, 300, 400 and 500 µM). DDPH percent inhibition data were plotted as a function of antioxidant concentration to obtain DPPH inhibition concentration at 50% (IC_{50}). The IC_{50} values were expressed as micromoles of trolox equivalent per gram of dry material (µmol TE/g extract) (Jesus *et al.* 2017).

$$\% \; inhibition \; = \; (1 - (As - Ab)/Ac) * 100 \hspace{3cm} \textbf{Equation 5}$$

2.11.3.3. Radical Cation Decolorization Assay (ABTS assay)

The ABTS radical cation decolorization assay was determined as described by Ballesteros (Ballesteros *et al.* 2015) with some modifications. Each sample was diluted to four different concentrations such that the percent inhibition was between 20-80%. Assays were conducted by combining 130 µL of sample with 3 mL of ABTS radical cation solution. The resulting solutions were maintained during 30 min in darkness at room temperature, and the absorbance was then measured at 734 nm using a spectrophotometer V-560 (Jasco, Japan) being distilled water used as control solution instead of the sample. A blank solution consisted in using the sample with methanol instead of the ABTS radical cation solution. ABTS radical cation was prepared by mixing 7 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) dissolved in water with a 2.45 mM potassium persulfate solution. This mixture was vortexed for 2 min, set in ultrasonic bath during 20 min and then, left in the dark at 4 °C between 12-16 h for achieving a stable oxidative state. After this time, ABTS radical cation solution was diluted in a 20 mM acetate buffer (pH 4.5) solution to an absorbance of 0.70 ± 0.01 at 734 nm. A calibration curve was constructed using a standard solution of trolox diluted in ethanol (50, 100, 200, 250, 300, 400, 500 and 600 µM). The percent inhibition of ABTS radical cation was calculated using the same equation employed in the DPPH radical scavenging. The IC_{50} values were expressed as micromoles of trolox equivalent per gram of dry material (µmol TE/g extract) (Jesus *et al.* 2017).

In all antioxidant assays, curcumin (a natural commercial pigment produced by some plants) was used as standard to compare with the antioxidant capacity of the extracts.

2.12. Antimicrobial Activity

To evaluate the potential antimicrobial activity of the pigments produced, two tests were performed: the agar diffusion method and the Minimal Inhibitory Concentration method (MIC). Three extracts were tested: FEL (Fermentative Extract Lyophilized), MEE (Mycelium Ethanolic Extract) and FBEE (Fermentative Biomass Ethanolic Extract).

2.12.1. Bacterial strains

For antibacterial assays, six bacteria were selected for the experiment based on their spoilage factors in foods and human health (Table 2.2). The maintenance of the microorganisms was done following the respective culture medium conditions (Table 2) and subsequently stored at 5°C. All the microorganisms were provided by CEB collection.

Table 2.2- Bacteria and yeast growth conditions for antimicrobial assay.

Bacteria	Temperature (°C)	Medium	Time (h)	REF
Escherichia coli/ CECT 736	37	MHB	overnight	(Ng *et al.* 2018)
Pseudomonas fluorescens DSMZ 50090	29	MHB	overnight	(Gutiérrez-Larraínzar *et al.* 2013; Tyagi and Malik 2010)
Paenibacillus larvae/ LMG 16252	37	MYPGP	24	(Boligon *et al.* 2013)
Lactobacillus fructivorans CECT 8288	29	MRS	24	(Inglin *et al.* 2015)
Zymomonas mobilis ATCC 29191	29	SMZ	48	(Zoltán Nagy, Tünde Kiss, Attila Szentirma 2001)
Wickerhamomyces anomalus	29	SMW	24	(Souza *et al.* 2017)

*MHB: Mueller-Hinton Broth; SMW: Specific Medium *Wickerhamomyces* ((% w/v) Glucose (2); Mueller-Hinton Broth (2.3)), SMZ: Specific Medium *Zimomonas* ((% w/v): Glucose (2); Yeast extract (0.5))

2.12.2. Fungal strains

The fungi tested in the antimicrobial activity assays were obtained from collection of (MUM Mycology Laboratory of the Minho University, Portugal) and DIA-UAC (Food Research Department, Autonomous University of Coahuila, Mexico). Antimicrobial evaluation was performed against six food spoilage fungi: *Alternaria* sp. (MUM 00.23), *Mucor* sp. (DIA-UAC 3P), *Penicillium expansum* (MUM 02.14), *Aspergillus niger* (01UAs83), *Aspergillus carbonarius* (01UAs130) and *Aspergillus flavus* (MUM 00.06). All strains were grown into malt extract agar medium (MEA) at 25 ± 2 °C during 7 days before antimicrobial test.

2.12.3. Antimicrobial test using the agar diffusion method

The antimicrobial activity of the extracts was screened using the agar diffusion test, according to the recommendations of the Clinical and Laboratory Standards Institute (Franklin R. Cockerill, III *et al.* 2012), with some modifications. Cells were standardized to a cell density of 0.5 McFarland (1.5×10^8 colony forming unit/mL for bacteria and $(0.4–5) \times 10^6$ conidia/mL for fungi) (Balouiri, Sadiki, and Ibnsouda 2016) in the appropriate medium. The three extracts LFE, MEE and FBEE were dissolved in distilled water with final concentrations ranging from 200 mg/mL to 1 mg/mL. Then, 100 μL inoculum of the test microorganism was poured onto a petri plate with the respective medium. After that, 4 mL of top agar were poured above the inoculum and allowed to dry (Soković and Van Griensven 2006). Then, a 5 μL drop of the extract was pipetted to the agar and was allowed to dry (Hili, Evans, and Veness 1997). The diameter of the zone of inhibition was measured in millimeters after 24 h for bacteria and yeast and after 48h for fungi and was compared with the positive and negative control (antimicrobial agents and water respectively (Table 2.3).

Table 2.3- *Antimicrobial agents used as control in the agar diffusion assay.*

Antimicrobial Agent	Microrganism
Ampicillin	*E. coli*, *P. larvae* and *L. fructivorans*
Tetracycline	*P. fluorescens* and *Z. mobilis*
Anphotericin B	*Alternaria* sp.
Ciclohexamide	*W. anomalus* and all the remain fungi

2.12.4. Minimal inhibitory concentration

The determination of MICs was performed using the micro-dilution methodology for filamentous fungi (CLSI, 2002) and bacteria that grow aerobically (CLSI, 2012) described by the Clinical and Laboratory Standards Institute. Briefly, 200 mg of MEE was dissolved in 1 mL of sterile ultrapure water and filtered through a 0.22 μm cellulose membrane. The resulting mixture was serially two-fold diluted in the respective medium for each microorganism (Table 2.4). The cell suspension of each microorganism was also adjusted to achieve a final concentration between 0.4×10^4 and 5×10^4 conidia/mL for fungi and 2×10^5 and 8×10^5 CFU (Colony forming unity)/mL for bacteria and yeast, when mixed with the extract concentrations. Experiments were carried out in a sterile 96-well microplate, in which 100 μL of inoculum suspension were added to 100 μL of extract. The microplate was incubated at the temperatures mentioned on Table 4, during 96 h for fungi and 48h for bacteria and yeast. The absorbance was measured at 530 nm for fungi and 600 nm for bacteria and yeast, using a spectrophotometric microplate reader (Cytation 3, 96 microplate reader, Biotek) to evaluate the behavior of the samples against growth and sterility controls, which consisted in using 100 μL of medium, 100 μL of inoculum suspension as microbial growth control and 200 μL of medium as sterility control. Moreover, antimicrobial agents' solutions were used as standard control. MIC values were determined as being the lowest sample concentration that prevents visible fungal and bacterial growth (Ballesteros *et al.* 2015), through visual analyses as MIC (visual) and through data treatment using the final absorbance after 24h or 96h, depending on the microorganism. Minimal Bactericide Concentration (MBC) and Minimal Fungicide Concentration (MFC) were determined. Bacteria and fungi were sub-cultured on the respective agar plates, for concentrations above the visual MIC (inclusive) determined. If the sub-cultured microorganisms cannot grow, the MIC and MBC and/or MFC concentrations will be equal (Rostami, Hamedi, and Yolmeh 2016).

Table 2.4- *Experimental conditions used for MIC assay.*

Microorganism	Medium	Dilutions range (mg/mL)	Antibiotic	Dilutions range (mg/mL)	Incubation Temperature (°C)
E. coli	MHB				37
L. fructivorans	MRS		Ampicillin		29
P. larvae	MYPGP	10 – 0.02		10 – 0.02	37
P. fluorescens	MHB		Tetracycline		29
A. carbonarius	MHB		Cycloheximide	7 – 0.01	25

2.13. Mycotoxins Analysis

To investigate the production of mycotoxins by *P. brevicompactum*, high performance liquid chromatography (HPLC) was used to verify the possible presence of 5 mycotoxins: OTA, DON, AFB1 and AFB2, ZEA and FB1. Using the method described by Abrunhosa et. al (2007) with some modifications, 0.5 mL of the three extracts (Fermentation (FE), Plate (PE) and Biomass (BE)) were mixed with 0.5 mL of HPLC mobile phase constituted by acetonitrile/methanol/acetic acid (78/20/2, v/v/v) and filtered. Using a Varian Postar 2010 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector (λex = 333 nm and λem = 460 nm) and a reverse phase C18 column YMC-Pack ODS-AQ (250 mm x 4.6 mm, 5 μm) fitted with a pre-column using the same stationary phase. The mobile phase was pumped at 1.0 mL/min and the injection volume was defined as 50 μL (Abrunhosa, Santos, and Venâncio 2006).

It was also used the immunoaffinity clean-up test for OTA, that consists of passing the extract sample diluted with a solution constituted with 0.5% (w/v) $NaHCO_3$ and 1% (w/v) PEG 8000 through the immunoaffinity column (IAC) and washing the IAC with 5 mL of solution B (2.5% NaCl, 0.5% $NaHCO_3$), followed by 5 mL of deionized water. OTA was eluted with 2 mL of methanol, completely evaporated with a gentle stream of nitrogen and redissolved in 1 mL of mobile phase (Serra *et al.* 2004).

2.13.1. Mycophenolic acid (MPA) analysis

Ultra-high-performance liquid chromatography (UHPLC) was used to analyse, identified and quantified the MPA present in the extracts as reported by Jesus *et al.* (Jesus *et al.* 2017). Briefly, a Shimatzu Nexpera X2 UHPLC chromatograph equipped with a Diode Array detector (Shimadzu, SPD-M20A) and a reversed-phase Acquity UHPLC BEH C18 column (2.1 × 100 mm and 1.7 μm particle size, obtained from Waters, Portugal) at 40 °C were used to separate the compounds. The mobile phase consisted of 0.1% formic acid in water (v/v) as the solvent A and acetonitrile as the solvent B under the following gradient profile: 95% A and 5% B (0 to 5.5 min), a linear increase to 60% B (5.5 to 17 min), a linear increase to 100% B (17 to 18.5 min) and finally, 95% A and 5% B (18.5 to 30.0 min) for the column equilibration. The flow rate of mobile phase and the injection volume of the samples were adjusted to 0.4 mL/min and 1 μL, respectively. Different HPLC grade phenolic compounds were used to prepare standard curves to identify and quantify the compounds present in the extracts, which were expressed as the ratio between mass of each compound in the extracts with respect to the mass of extracted material (dry weight). The responses of the UV detector were integrated using the LabSolutions software (Shimadzu) (Ballesteros *et al.* 2015).

An isocratic method was also evaluated where the eluent was a mixture of acetonitrile and water (80/20) at a flow rate of 0.5 mL/min, temperature 25 °C and 20 μL of sample injection volume.

3. Results and Discussion

3.1. Pigment production by submerged fermentation

Since previous studies demonstrated the potential of *P. brevicompactum* for pigment production and it is poorly reported in the literature as a pigment producer, the present work aims to optimize the pigment production, its extraction and further characterization.

3.1.1. Effect of the culture media

As previously mentioned, pigment production was observed by the fungus studied in this work using a medium rich in salts and nitrogen sources (medium A) (Nagy *et al.* 2001). In this medium it was observed that throughout the fermentation time for production of enzymes using lactose as source of carbon (Silvério *et al.* 2018), the fermentation broth became reddish, intensifying its color with time. Thus, the present work had as one of the main objectives, the optimization of pigment production by this fungus.

After a careful search in the current literature, it was verified that one of the most used media in the production of fungal pigments is medium B, rich in salts and poor in nitrogen sources, using simple sources of carbon (Méndez *et al.* 2011; Morales-Oyervides *et al.* 2017; Narendrababu and Shishupala 2017; Pandey *et al.* 2018; Pisareva and Kujumdzieva 2010; Pradeep *et al.* 2013).

Medium C was used once it was observed a great amount of pigment produced on the mycelium in solid agar. This medium was also tested in pigments production using *Monascus* by submerged fermentation to be compared with other media too (Neera, Ramana, and Sharma 2017).

It was also analyzed that the growth medium of the fungus has great influence both in the growth of the microorganism as well as in the production of secondary metabolites (Gmoser *et al.* 2017; Pisareva and Kujumdzieva 2010).

So, based on what was written before, the production of the pigment was studied for the three media described above.

For the media under study pigment production was found to occur after 5 days of fermentation for medium B (culture medium color turned pink) and A (color turned brownish) (Table 1). The fungus was

not able to produce pigment in medium C. The maximum pigment production was observed for medium A in the end of the 12 days of fermentation for all wavelengths (400 nm, 470 nm and 500 nm), being 400 nm the wavelength that showed the biggest production in the 3 media (Figure 3.1). Also, in medium A, the sum of the wavelengths was the highest.
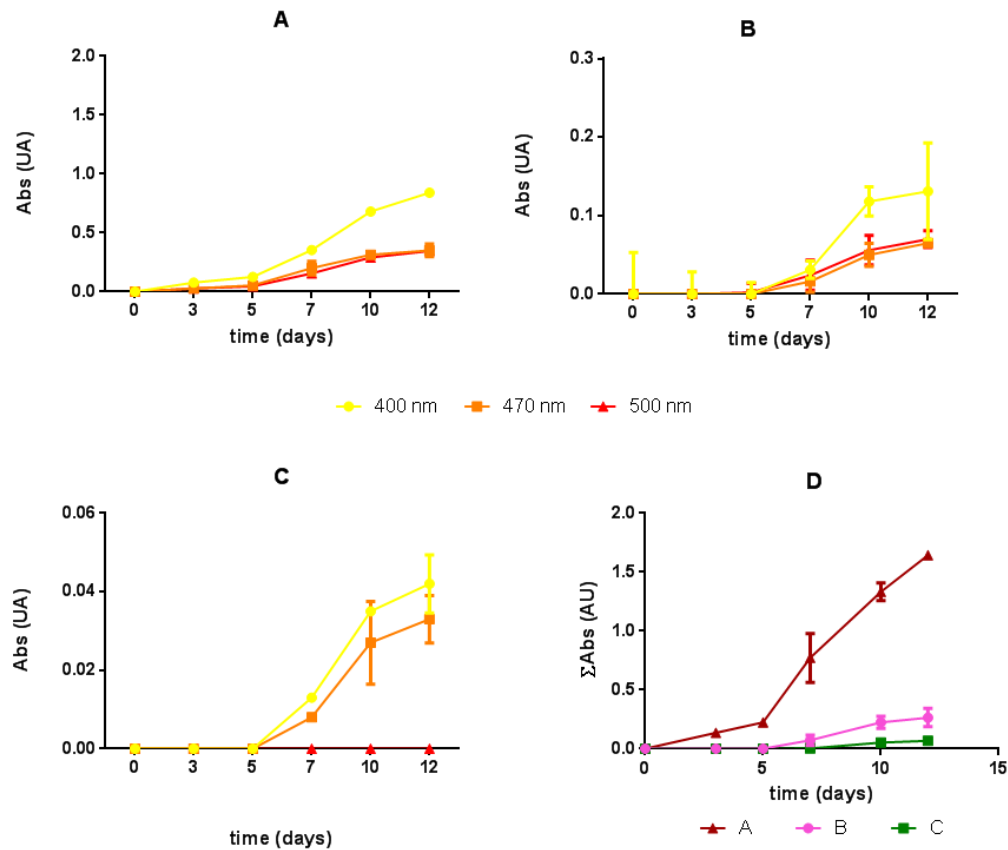
Figure 3.1- *Pigment production during 12 days of fermentation, for the 3 culture media tested. (A) culture medium A; (B) medium B and (C) medium C and the last figure (D) represents the sum of the wavelengths for each medium.*

It has been reported that for other fungi of the *Penicillium* species, medium B, using xylose as carbon source, was suitable for pigment production, where the maximum production occur after 4 days of submerged fermentation (Méndez *et al.* 2011). Kaur *et al.* that also studied the effects of different media in the production of pigments with other microorganisms found out that in comparison with media with supplements on it, like peptone or yeast extract, medium B was not as efficient as them, most probably because of the lack of nutrients coming from the supplements (Kaur, Chakraborty, and Kaur 2009). So, this as fundament for the results obtained between the two media that produced pigment. In contradiction with the results obtained for medium C in this work, there are studies that showed pigment production in this medium although with low yields, compared with a potato dextrose

broth (PDB) and with a synthetic medium (composition gL^{-1}: glucose, 30; MSG, 1.5; KH$_2$PO$_4$, 2.5; MgSO4 .7H2O, 0.5 and FeSO4. 7H$_2$O, 14) (Neera *et al.* 2017).

It was verified in the end of the fermentation that the fungus consumed all the carbon source available in the medium A. However, in B and C media the sugar concentration did not vary, indicating that there was no lactose consumption by the fungus.

Biomass obtained after 12 days of fermentation was higher for medium A (0.4245 ± 0.01 g). In medium B it was observed the lowest amount of biomass (0.0421 ± 0.01 g) while in medium C the value was 0.3253 ± 0.01 g. The biomass color also varied in all media. For medium A the biomass was yellow, in medium B the biomass was black and in C was greenish (spore color). This fact does not contract was is found in the literature, where an increase in the biomass yield is connected to the richness of nutrients in the medium, while pigment production is related to the lack of nutrient conditions and/or external stresses, as a way of protection (Lebeau *et al.* 2017).

The quantification of protein (Figure 3.2) showed that for media A and C, in the first five days there was a consumption of protein, which could be explained by the supplements contained in both media, and between 5th and 12th days they remained stable.
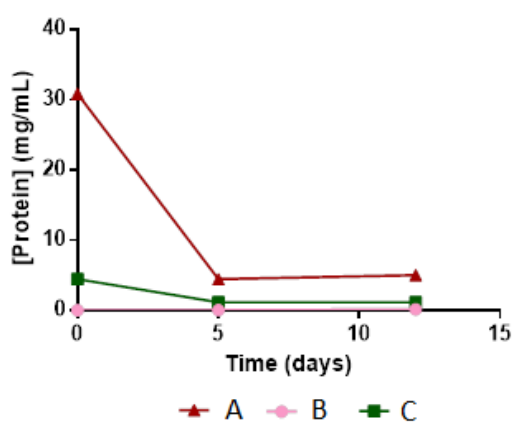
Figure 3.2- *Concentration of protein during fermentation.*

After carrying out the test of significance to the results of the three media, it was verified that the difference between medium A and media B and C is significant for the three wavelengths studied (p< 0.05). Thus, medium A was chosen as the best pigment producing medium.

For effects of analysis, the future studies were evaluated using de sum of absorbances for all the wavelengths.

3.1.2. Effect of rotation

It has been reported in the literature that optimal pigment production in fungi can be achieved at different stirring rates namely: 0, 100, 150 and 200 rpm (Méndez *et al.* 2011; Santos-Ebinuma *et al.* 2013; Velmurugan, Lee, Venil, *et al.* 2010). Thus, these were the rotations chosen for the study of agitation effect in pigment production.

With static state fermentation (0 rpm), no pigment production was observed, contrary to some studies found in the literature where it was seen that under this condition interesting production yields was obtained (Pandey *et al.* 2018; Velmurugan, Lee, Venil, *et al.* 2010).

For all the other rotations tested there was pigment production after 5 days of fermentation. At the end of the fermentation, those performed at 100 rpm had a red coloration and 150 rpm presented a dark red coloration while the fermentation at 200 rpm turned to a red-brownish medium (Figure 3.3). For this last one this color could be due to the fact that high agitation speed, could cause more aeration and so this could lead to the oxidation of the pigment (Mantzouridou, Roukas, and Kotzekidou 2002).
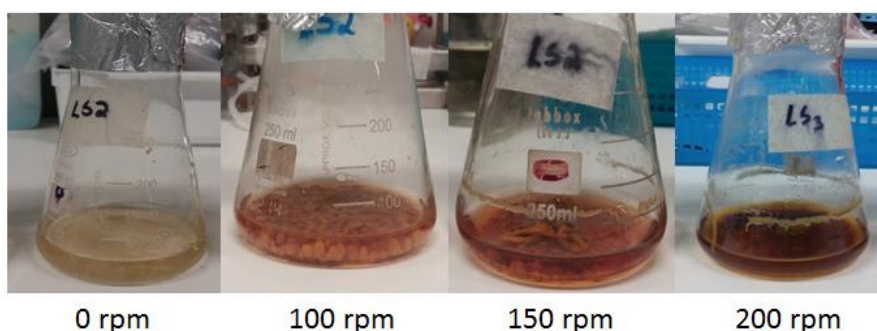
Figure 3.3- *Color observed in the end of the fermentation performed at different agitations.*

Figure 3.4 shows the pigment production profile during the fermentation and it is verified that the maximum production was obtained for 200 rpm, and then the highest value was verified for 150 rpm and then 100 rpm (Appendix A.2). These results are shown in accordance with the literature since the two rotations that obtained the highest yields of production are the most used in the production of fungal pigments (Afshari *et al.* 2015). But due to the red-brownish color obtained and as explained above, the pigments in the medium could be oxidizing due to the fact that the agitation rate is very high, and that is not intended here.
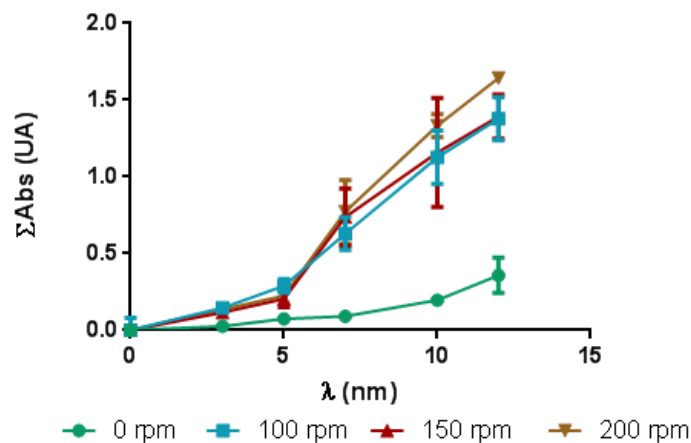
Figure 3.4- *Profile of pigment production throughout the fermentation.*

The carbon source was completely consumed by the fungus after 10 days at 100 rpm and after 12 days at 150 rpm and 200 rpm. In the static fermentation there was no consumption of lactose during the 12 days of fermentation.

Regarding the biomass, in all the conditions tested it was seen different pellet sizes but always presenting an orange coloration, exception for static fermentation where a white biomass was observed. Table 3.1 shows the biomass values obtained for each fermentation. It can be verified that with 100 rpm the biomass obtained was the largest, followed by the fermentation at 150 rpm. This result seems to suggest that the high production of pigment is not directly associated to a high biomass growth (Chatterjee *et al.* 2009; Méndez *et al.* 2011).

Table 3.1- *Biomass obtained for the all rotation conditions studied.*

Rotation	Biomass (g)
0	0.074 ± 0.02
100	0.462 ± 0.01
150	0.453 ± 0.01
200	0.425 ± 0.01

During the first five days there was a noticeable consumption of protein but at the end of the fermentation there was a slight increase in protein concentration showing that the fungus could possibly

produce some proteins and release them in the culture medium or it is just remaining proteins from the medium components (Figure 3.6).
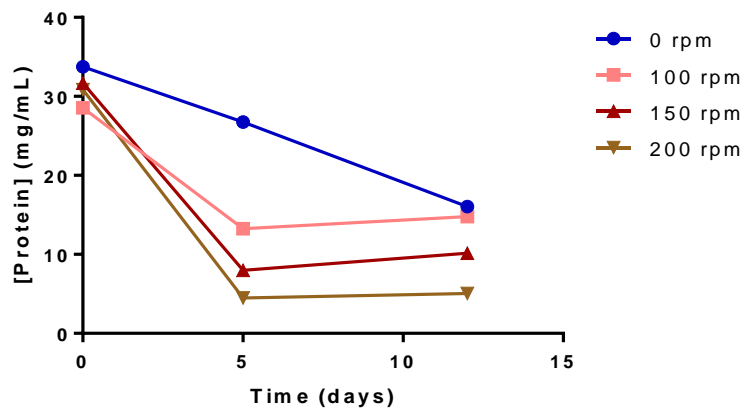
Figure 3.5- *Protein concentration during fermentation.*

At the end, the best condition obtained to produce pigments was 200 rpm, but once obtained a pronounced brownish color and with a medium quite turbid and biomass being discarded, most probably because of the strong rotation, this was not chosen. Thus, after statistical tests performed for all rotations ($p < 0.05$), and with further analyze, it was seen that 150 rpm was significantly different from 0 rpm and for the other two rotations not. So, since 200 rpm showed a possibility to oxidize the produced pigments and 100 rpm had less production, the rotation selected for further studies was 150 rpm.

3.1.3. **Effect of the carbon source**

In addition to lactose, glucose and sucrose were found in the literature as sources of carbon to be used in the production of microbial pigments (Akilandeswari and Pradeep 2017; Celestino *et al.* 2014; Santos-Ebinuma *et al.* 2013). Thus, these two sugars were tested, as well as lactose to verify which one would be the best option to be applied.

After 12 days of production, it was noticed that the carbon source has influence in the color of the pigment produced. This fact can also be explained by the difference in the final pH of the media. For the medium with glucose a final acid pH was obtained while for the media containing lactose and

sucrose, the pH measured were alkaline or neutral. It was reported in general that the pH influenced the production of pigments by submerged fermentation and some studies also reported the influence of pH on pigment color (Gunasekaran, S.; Poorniammal 2008; Méndez *et al.* 2011). Figure 3.6 shows that for glucose, the final color of the fermentation broth was different (orange) from that obtained for lactose and sucrose which provided approximately the same medium color (dark red). Another fact noticed was that the medium with glucose has become turbid probably because of some biomass disintegration.
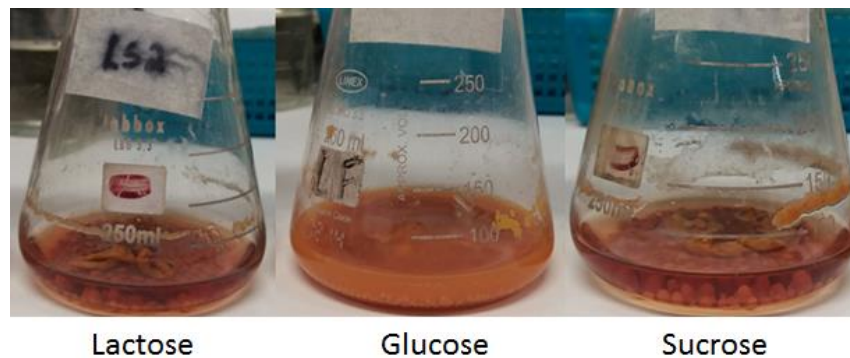
Figure 3.6- *Color obtained after 12 days of production with different carbon sources.*

Regarding pigment production, of the three sugars tested, lactose was the one that has presented the best result, showing pigment production after 5 days of fermentation (Figure 3.7). These results contradict most of the works reported in the literature, that identify glucose as the main sugar that allow to achieve the best production yields of fungi pigments (Akilandeswari and Pradeep 2017; Chatterjee *et al.* 2009; Neera *et al.* 2017). Sucrose had the lowest production of pigments and in literature is also referred as that, not being generally chosen as main carbon source (Boonyapranai *et al.* 2008; Geweely 2011). Although lactose is not the most used carbon source for the production of pigments, recently this sugar was reported as a good carbon source for the synthesis of secondary metabolites as pigments (Afshari *et al.* 2015).
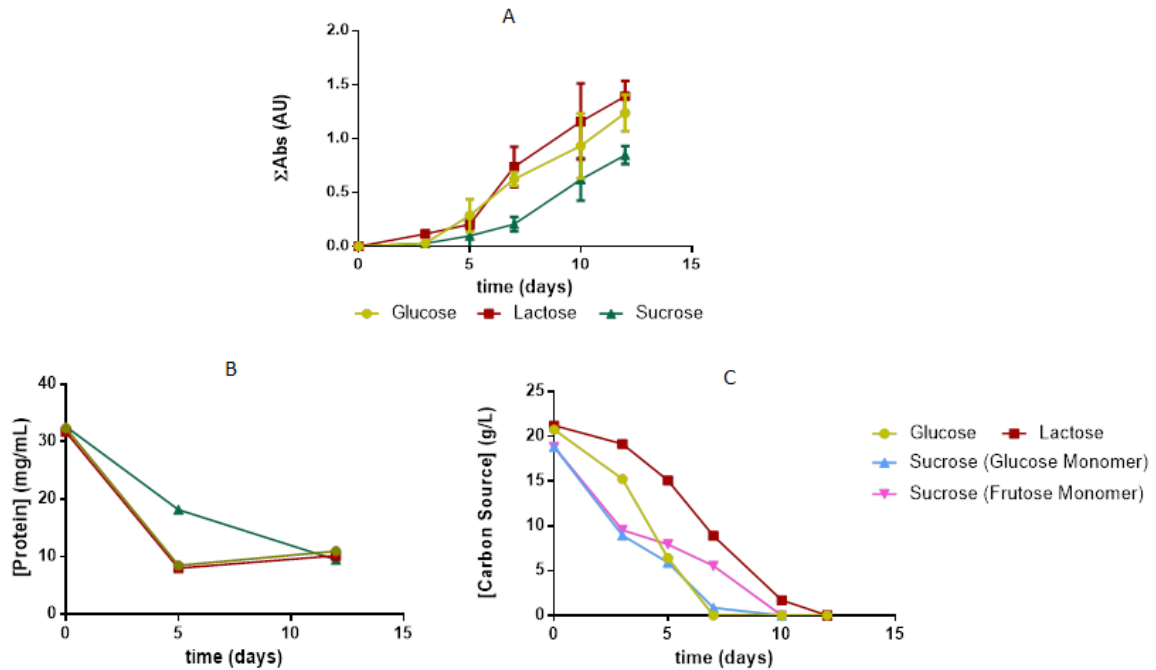
Figure 3.7- *Profiles of pigment production (A), of protein (B) and of carbon source consumption (C) for 12 days.*

Lactose was the carbon source that produced the most amount of biomass (0.4532 ± 0.01 g). Glucose produced (0.3699 ± 0.05) g and sucrose produced (0.3828 ± 0.13) g. In this case, the sugar that produced the higher amount of pigment was also the one with the highest quantity of biomass.

Concerning the consumption of the 3 carbon sources tested (Figure 3.9) throughout the fermentation, it was seen that glucose was the one consumed the quickly (after 7 days). Sucrose was hydrolyzed within 3 days and their respective monomers (glucose and fructose) were both consumed after 10 days. Lactose was also completely consumed after 12 days. Since these last two sugars are disaccharides, this may be the reason why they were slowly consumed by the fungus.

Throughout the fermentation, the protein concentration (Figure 3.10) always decreased using sucrose as carbon source. That was not the case for glucose and lactose, since the protein content decreased during the first 5 days of fermentation and then slightly increased, like what happened in previous studies

In the end and after statistical analysis of all variables ($p < 0.05$), pigment production using lactose was found to be significantly different from sucrose, thus eliminating this sugar. Since the difference between glucose and lactose was not significant and since lactose was the sugar with the highest

production of pigment, which is the major factor, it was then considered the sugar with optimal production condition.

3.1.4. Effect of the temperature

One of the main parameters found in the literature that strongly influences the production of pigments is the temperature. Fungus reacts differently by synthesizing various secondary metabolites when exposed to temperature variations, which could affect cellular growth and metabolite production by enzymes that could be related to pigment production (Chatterjee *et al.* 2009; Gmoser *et al.* 2017; Gunasekaran, S.; Poorniammal 2008)

It is reported that optimum temperature conditions for the production of fungal pigments is usually 30 °C (Afshari *et al.* 2015; Chatterjee *et al.* 2009; Dikshit and Tallapragada 2011; Mukherjee and Singh 2011). So, it was decided to assess if a higher (33 °C) or a lower (23 °C) temperature would have any impact in the production of pigment. Although the color of the medium has become more reddish in the fermentation performed at 33 °C (Figure 3.8), the fungus only began to produce pigment after 10 days while at 23 °C the pigment began to be produced at day 5. It was found that the final pH of the two fermentations was different. At 23 °C the medium was acidic, hence the orange color and at 33 °C the pH was alkaline (Appendix A.4).

23 °C   33 °C
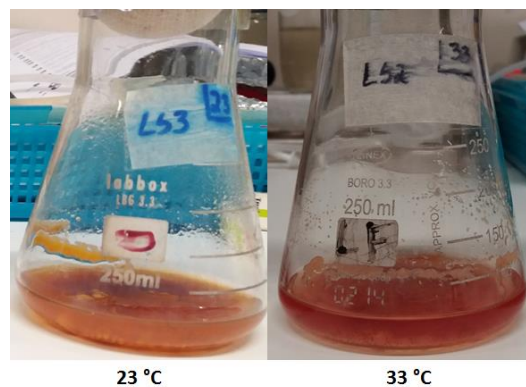
Figure 3.8 *- Color obtained in the last day of fermentation.*

During the fermentation time it was observed a higher production of pigment at 23 °C (Figure 3.9), being in agreement with what is reported in the literature by some authors which concluded that for example *P. purpurogenum* shown a high production of pigment in fermentations at 24 °C and

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Monascus sp. had better production at 25 °C for red pigments (Méndez *et al.* 2011; Neera *et al.* 2017). It was also verified that, at 33 °C the fungus was also capable of producing pigment. Although in small quantities, this result is also in agreement with the research of other authors as described before. Bhosale (2004) described that for carotenoid production, low temperatures could decrease the rate of nutrient uptake from the environment, and thus metabolic processes, such as protein synthesis, slow down. So the increase of these pigments is a response due to the environmental changes in order to compensate the decrease of functionality of the biological pathways (Bhosale 2004).
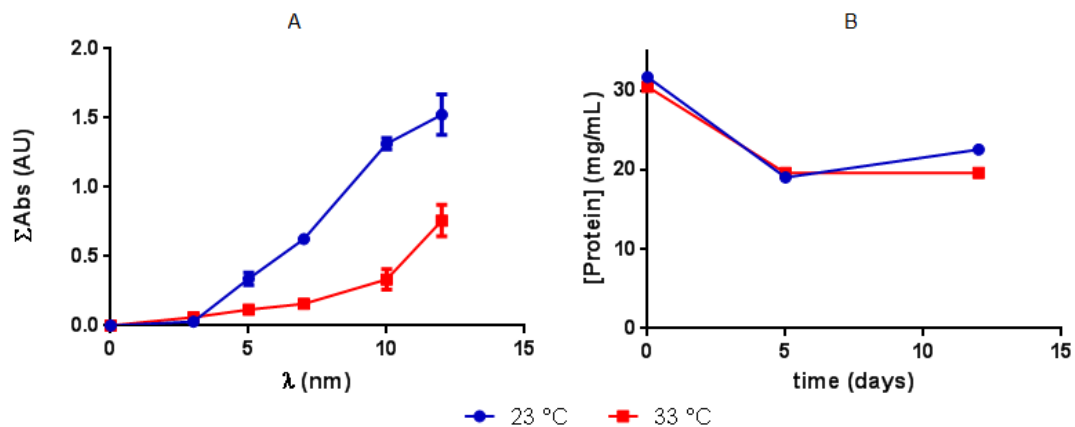
Figure 3.9- *Profile of pigment production (A), and protein concentration (B) during fermentation at different temperature.*

The biomass obtained in both fermentations (0.4284 ± 0.02) g at 23 °C and (0.2662 ± 0.02) g at 33 °C were both orange. Analyzing these results, it is possible to conclude that the condition with best pigment production was also the one with the highest amount of biomass. The complete consumption of lactose by the fungus was seen after 10 days of fermentation at 23 °C while at 33°C, the sugar was not totally consumed. Afshari *et al.* reported that the consumption of lactose, as carbon source, is increased by rising the temperature from 25 to 30 °C and reducing from 35 to 30 °C (Afshari *et al.* 2015).

During the first 5 days of production, the protein concentration (Figure 3.13) dropped abruptly for both cases under study. In the remaining 7 days, there was an increase in protein concentration at 23 ° C, but at 33 ° C it remained constant.

Using a significance test ($p < 0.05$), it was concluded that the best temperature for the pigment production was 23 °C.

3.1.5. Effect of the medium supplements

It's found in the literature that the use of nitrogen sources may increase the yield of production of secondary metabolites derived from microorganisms. These sources regulate the expression of genes of interest and activate important pathways in the production of these compounds (Celestino *et al.* 2014). In this study the combined effect of different concentrations of two distinct supplements, peptone and yeast extract were analyzed.

Pigment production was confirmed in all fermentation's conditions after 5 days, except the one with (0 – 16) g/L which has presented production only after 7 days. The media colors varied between orange and dark red (Figure 3.10). It was found that when the concentration of peptone was higher than the yeast extract, the color became more orange, so the pH became more acidic. When the media contained more yeast extract, they became more reddish and the pH was more alkaline. Shi *et al.* reported that the utilization of nitrogen sources as peptone lead to the production of orange or yellow pigments and the utilization of yeast extract leads to the production of red pigments with *Monascus anka* under submerged fermentation (Shi *et al.* 2015). The more acidic the media more turbid was seen, probably due to the disaggregation of the biomass.

| (2 − 6) g/L | (8 − 0) g/L | (2 − 2) g/L | (0 − 8) g/L |
| (8 − 8) g/L | (4 − 4) g/L | (6 − 2) g/L | (0 − 16) g/L |
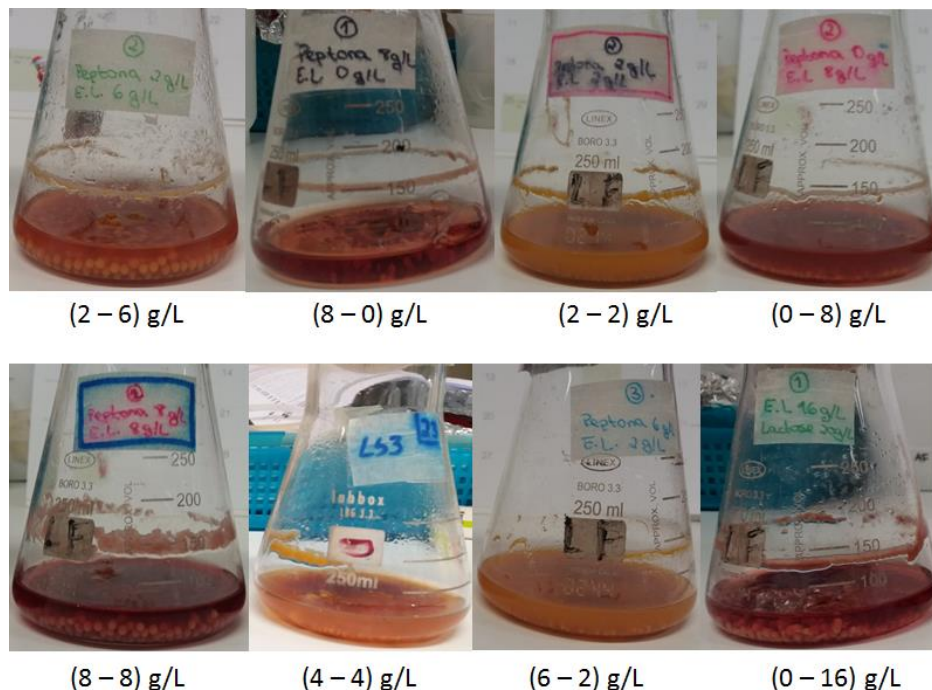
Figure 3.10- *Color obtained in the end of the fermentation with the different (P − E) concentrations used.*

Of all the compositions studied, the best was the one that contained (8 - 8) g/L of the supplements studied (Figure 3.11). It should also be noticed that when the medium only had peptone, the production was the lowest of all, which contradicts with a study carried out by Geweely (2011) with *P. purpurogenum*, *A. nidulans*, *Fusarium moniliforme* and *Phoma herbarium* where peptone, as the only source of nitrogen, compared with other sources, was the one which had the greater pigment production yield, followed by yeast extract (Geweely 2011). Akilandeswari and Pradeep also concluded that for the production of pigments from *Aspergillus terreus*, peptone was the nitrogen source that had the highest yield of pigment production, also followed by yeast extract (Akilandeswari and Pradeep 2017). The results show that when the concentrations of each one of the supplements is equal, the production of the pigment is very high, except for (2 – 2) g/L. Celestino *et al.* reported that the production of a red pigment by *Penicillium* and *Aspergillus* species was increased with the combined use of peptone and yeast extract. Because fungi have the ability to use peptone during their metabolism, this leads to an increase of pigments production, and yeast extract provides vitamins and coenzymes that also favor the growth of these microorganisms (Celestino *et al.* 2014).
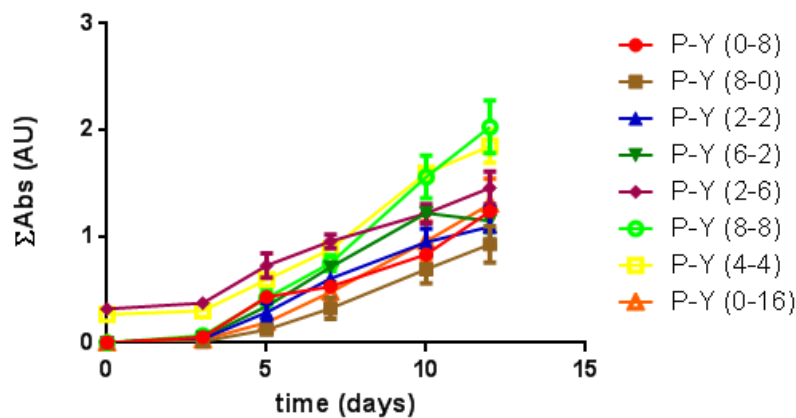
Figure 3.11- *Profile of pigment production.*

During the fermentation time, lactose was entirely consumed after 10 days for four supplemental concentrations (Table 3.2). At the end of the fermentation, 12 days, the fermentation media with (0 – 16) g/L and (4 – 4) g/L were also lactose free. The two media that did not obtain the best pigment production values were also the ones where there was no lactose consumption.

Table 3.2- Day of the occurrence of total lactose consumption.

(P – Y) g/L	Total Lactose consumption (Day)
0 – 8	10
8 – 0	-*
2 – 2	-*
6 – 2	10
2 – 6	10
8 – 8	10
4 – 4	12
0 – 16	12

*Lactose was not totally consumed at the end of the fermentation.

Considering the biomass, in this study, the medium containing the highest amount of biomass was not the one with the best pigment production (table 3.3). The reason why the medium containing the highest amount of yeast extract (0 – 16) g/L was the one showing the highest amount of biomass, may be the fact that this medium had more available nutrients that help the fungus growth.

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Table 3.3- *Quantity of dry biomass for each condition of (P-Y) concentration.*

(P – Y) g/L	Biomass (g)
0 – 8	0.428 ± 0.02
8 – 0	0.220 ± 0.05
2 – 2	0.357 ± 0.01
6 – 2	0.425 ± 0.02
2 – 6	0.445 ± 0.02
8 – 8	0.488 ± 0.06
4 – 4	0.428 ± 0.02
0 – 16	0.621 ± 0.02

The protein concentration in the fermentation medium (Figure 3.12), for all the conditions tested, decreases during the first 5 days of fermentation. After that, the concentration continues to decrease for the conditions (0 – 16) g/L and slightly for (2 – 2) g / L and (8 – 8) g/L. For all the other conditions there was a slight increase in protein concentration in the fermentation medium for the same period of time.
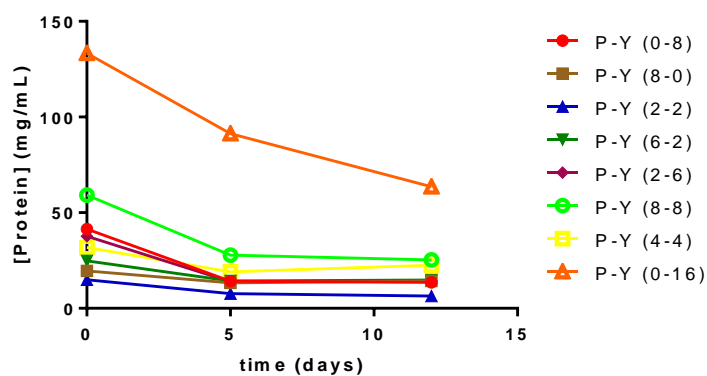
Figure 3.12- *Concentration of protein during fermentation.*

In conclusion, it was found that the best supplement condition for pigment production was for the concentrations (8 – 8) g/L, being significantly different ($p < 0.05$) from the other conditions studied.

3.1.6. Effect of lactose concentration

The variation of the concentration of the carbon source used may also influence the production of fungal pigments. In this step of the study, the behavior of the pigment production was investigated using 3 different concentrations of lactose.

At the end of the 12 days of fermentation, the color of the media with 10 g/L and 20 g/L was dark red while for 30 g/L the color seen was red-orange (Figure 3.13). The first two concentrations mentioned above have promoted pigment production at the end of 5 days of fermentation while for 30 g/L, it was seen production only after 10 days. For higher initial lactose concentrations, the final medium pH became more acidic.

10 g/L	20 g/L	30 g/L
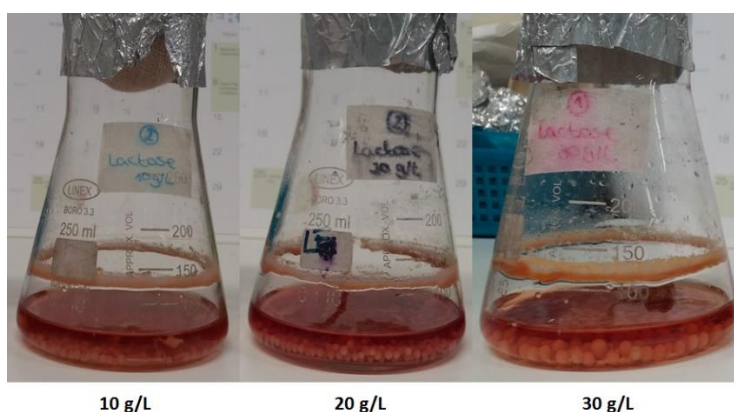
Figure 3.13- *Color obtained in the end of the fermentation using different concentrations of lactose.*

The condition that allowed to obtain the highest amount of pigment was 20 g/L of lactose, followed by 30 g/L and then 10 g/L (Figure 3.14). This result may be in agreement with the literature, since it was reported by several authors that the C-N ratio influences pigment production (Pandey *et al.* 2018). When there is a small amount of carbon in the medium, its rapid consumption may lead to the accumulation of a higher amount of nitrogen, which is not used for pigment synthesis (Hamdiyati, Kusnadi, and Yuliani 2016). Santos – Ebinuma *et al.* reported that high concentrations of carbon and nitrogen sources negatively affected pigment production, most likely due to the catabolic repression of the microorganism under these conditions (Santos-Ebinuma *et al.* 2013).
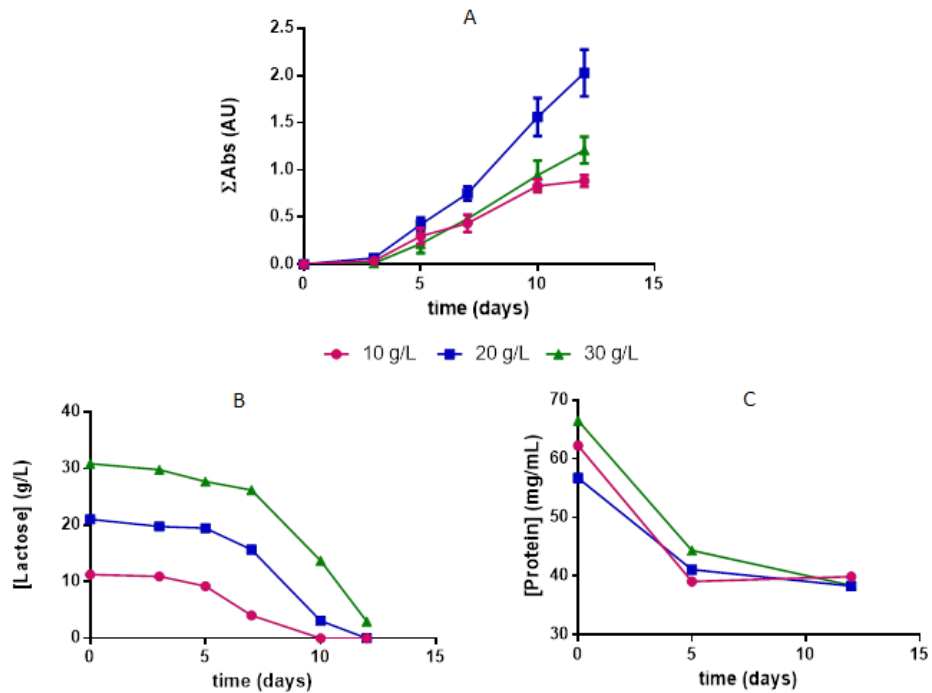
Figure 3.14 - *Pigment production profile for fermentation with the 3 concentrations of lactose under evaluation.*

After having followed up the variation of lactose concentration in the three media studied, it was seen that all the sugar was consumed after 10 days of fermentation using the lowest concentration (10 g/L). After 12 days lactose in the medium containing an initial concentration of 20 g/L was also completely metabolized. However, in the medium with the highest concentration (30 g/L) the sugar was not totally consumed, (Figure 3.19).

The medium presenting more biomass was the one with 30 g/L of lactose, and the amount of biomass declined as the lactose concentration decreased. This corroborates the general idea that lactose, as a source of energy, when added in great amounts to the medium, may contribute to the microorganism to continue its growth.

During the fermentation, the protein available in the fermentation medium was consumed in the 2 conditions with higher lactose concentrations, while for 10 g/L, the protein was consumed up to 5 days of fermentation and then its concentration slightly increased (Figure 3.20).

In conclusion, the best lactose concentration for pigment production was found to be 20 g/L, which was statistically significant in comparison with the other two concentrations studied ($p < 0.05$).

3.1.7. Effect of the light

The effect of light on the production of pigments is well reported in the literature. Velmurugan *et al.* described that the absence or the presence of light influences the production of pigment (Velmurugan, Lee, Venil, *et al.* 2010). Typically, it is said that fungi need to be growth in total darkness in order to achieve better pigment production yields (Mapari, Meyer, and Thrane 2006; Velmurugan, Lee, Venil, *et al.* 2010), but a few studies were found showing that they can also produce pigments when grown with natural light. Thus, this stage was about the analysis of the effect of light on the production of the pigment.

The pigment production started after 5 days for both fermentations (with and without natural light) and the final pH of both was alkaline, explaining the dark red color of the media (Figure 3.15).

Light   Darkness
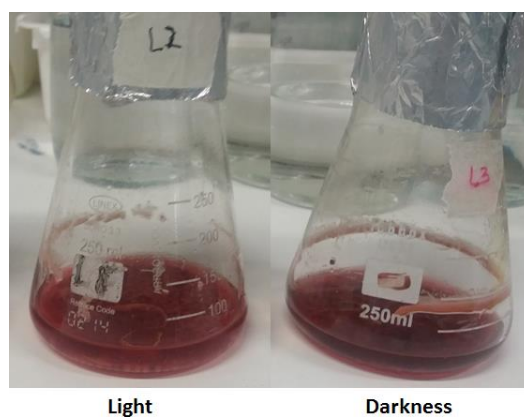
Figure 3.15- *Color obtained in the end of fermentation with natural light and without.*

Fig. 3.16 shows that for both fermentation conditions, the pigment production was the same, contrary to what is described in the literature that the condition of total darkness is the one that favors the most pigment production (Velmurugan, Lee, Venil, *et al.* 2010). In this case, the production profile over time is very similar in both situations showing that the natural light has no effect on the pigment production by *P. brevicompactum*, contrary to what is reported by Velmurugan *et al.* that total illumination of the medium during the fermentation of *Isaria farinosa*, *Aspergillus nidulans*, *Fusarium verticillioides* and *P. purpurogenum*, resulted in a low production which does not happened here (Velmurugan, Lee, Venil, *et al.* 2010).
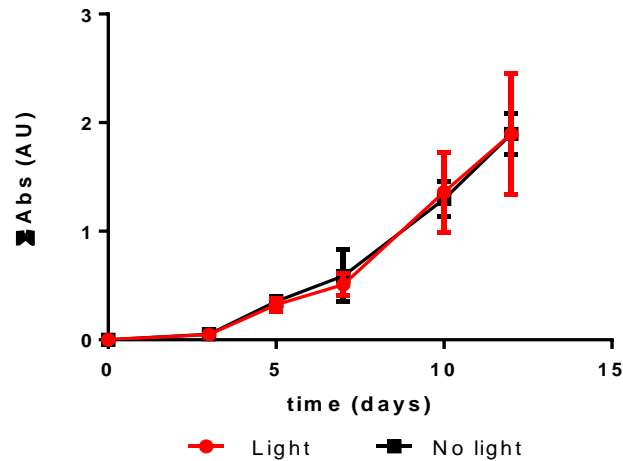
Figure 3.16- *Pigment production during fermentation with and without natural light.*

Regarding the consumption of lactose during the fermentation, it also had a very similar consumption profile in both situations, lactose was totally consumed after 10 days (Appendix A.8). Again, it is found here that light has no effect on the consumption of the carbon source.

The biomass grown in the two conditions was also partially equivalent, (0.5330 ± 0.04) g of biomass and (0.5188 ± 0.06) g of biomass obtained in presence and absence of light respectively. This also shows that light has no effect on the biomass growth. Proteic content available in the fermentative broth, was decreased up to 5 days of fermentation and then remained constant (Appendix A.8).

It was seen that there is no significant difference (p< 0.05) between the two conditions analyzed in this stage. So, the fermentations performed in the next steps of this work were in the presence of natural light.

3.1.8. Effect of the initial pH

The pH is one of the most studied parameters during the production of pigments by fermentation. Mendez *et al.* identified the effect of pH and temperature in *P. purpurogenum* submerged fermentations and Lv *et al.* and Chatterjee *et al.* showed the effect of pH on the production of pigments by *Monascus* (Chatterjee *et al.* 2009; Lv *et al.* 2017).

All fermentation media began to present some coloration from the 5 days of fermentation. At the end of the fermentation the pH of the media initially starting at 4.5 and 7.0 became neutral (colors

ranging from orange to red). The medium whose initial pH was 9.5 lowered the pH at the end of the fermentation to 8.3 showing a dark red color (Figure 3.17). The influence of the nitrogen source on the pH change is reported in the literature for pigments produced by *Monascus*. It is described that the pigments react with the ammonium ion or with free amino groups under neutral conditions (Chatterjee *et al.* 2009; Pisareva and Kujumdzieva 2010; Shi *et al.* 2015), resulting in the transformation of orange pigments into red pigments. Other authors also reported that when the initial pH is acid the tendency is for the production of yellow and orange pigments and when the initial pH is alkaline, the trend is for the production of red pigments (Chatterjee *et al.* 2009; Méndez *et al.* 2011; Mukherjee and Singh 2011).

| 4.5 | 7.0 | 9.5 |
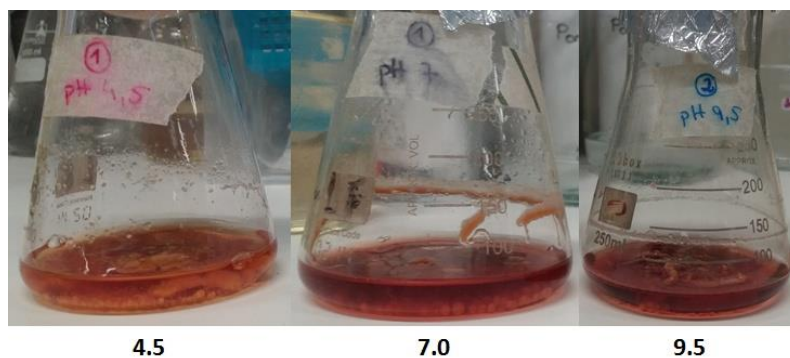
Figure 3.17- *Color obtained at the end of fermentation with the 3 pH tested (acid, neutral and alkaline respectively).*

The production of the pigment during fermentation (Figure 3.18) shows that the condition with the highest production was at an initial pH of 4.5, followed by neutral pH production. These results are in agreement with Mendez *et al.* who concluded that the highest level of production of red pigment by *P. purpurogenum* was reached with an initial pH 5.0 at 24 °C (Méndez *et al.* 2011). In contradiction, Gunasekaran and Poorniammal (2008) described pH 9.0 as the best value for red pigments synthesis for a *Penicillium* sp. (Gunasekaran, S.; Poorniammal 2008). Afshari *et al.* demonstrated that the best pH for production of yellow pigments is 6.5 (almost neutral pH) at 25 °C for *P. aculeatum* (Afshari *et al.* 2015). It is reported that fungi produce a great amount of pigment under acidic conditions during submerged culture and that low pH inhibits the conidia development and enhances pigment production, indicating that pH values influence the uptake of certain media constituents and enzyme activity involved in the biosynthesis of pigments (Pandey *et al.* 2018)
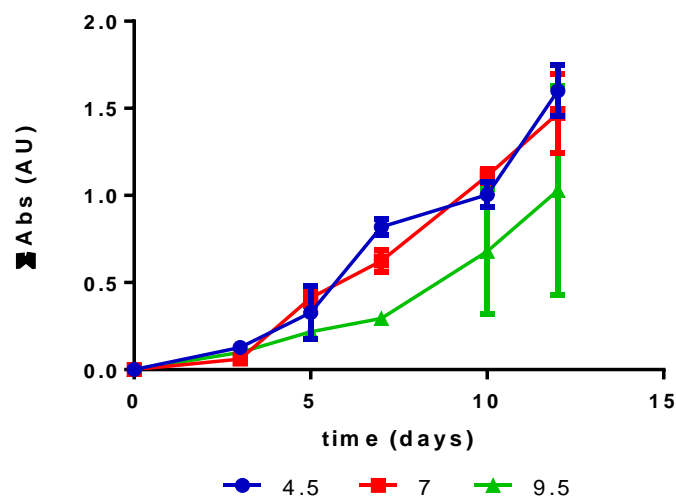
Figure 3.18- *Pigment production during fermentation for the 3 pH evaluated.*

The lactose consumption was complete after 10 days for the acidic and neutral pH media and only after 12 days of fermentation for the alkaline medium. Biomass increased with the increase of pH and protein was extensively consumed through the first 5 days and then remained constant. It can be suggested that pigment production is not related to the biomass amount as it was reported by other authors (Afshari *et al.* 2015; Méndez *et al.* 2011).

To conclude, and because this work is focused on the production of red pigments, the statistical tests were performed to compare pH 7.0 with the other ones. After the test, it was seen that the results between pH 7.0 and 9.5 were significantly different (p <0.05), but no significant difference was found between pH 7.0 and 4.5. As it is reported in literature (Chatterjee *et al.* 2009; Méndez *et al.* 2011) that the production of red pigments is better for neutral to basic pH, pH 7.0 was selected.

3.1.9. Characterization of the fermentation extract

3.1.9.1.   Thin Layer Chromatography (TLC)

After the study of a set of conditions aiming to improve pigment production, a fermentative extract (FE) was obtained through the separation of the fermentation broth from the biomass.

This extract was then analyzed by TLC to evaluate its content and check if it was composed by a single type or by a mixture of pigments. From the mobile phases tested, first it was evaluated the mixtures with 4 and 3 solvents, which didn't present good results since the pigment fractions were not well separated or just not left the application point. Next it was evaluated the eluents with only two solvents. Here, the separation of the fractions was better, being the mobile phase composed by 50:50 water/ethanol (Figure 3.19) the better mobile phase for separation. To test the proportions of this type of mobile phase, it was tested more water or more ethanol, and it was concluded that more water did not show a good separation, where the samples showed trailing signs at the end of the separation. With more ethanol in the composition, the samples took too long to begin the separation and the fractions were very close to each other. In the end, the best mobile phase for the separation of the pigments was water:ethanol (50:50).

According to some authors, the most used mobile phases to separate pigments from *Monascus sp.* by TLC are composed by chloroform, methanol and water with different proportions depending on the author (Babitha, Soccol, and Pandey 2006; Martinkova, L.; Juzlova, P.; Vesely 1995; Srianta *et al.* 2016; Zhong, Zhang, and Wang 2015). Hailei *et al.* used a different composition of 1-butanol:ethanol:water (3:5:2) to analyze a red pigment produced from *Penicillium sp.* in co-culture with *Candida tropicalis* having a good separation between the fractions (Hailei *et al.* 2011).

Observing figure 3.19, it is verified that FE presents 3 fractions of red color, which may indicate a possible mixture of red pigments on the extract, showing a great separation.

Figure 3.19- TLC plate with FE eluted with 50:50 water:ethanol.

3.1.9.2. Stability of pigments

To be used at the industrial level, it is important that pigments remain stable during the processes to which they will be exposed. The pigments present in the fermentation extract (FE) were characterized according to their color stability (for 24 hours) at different conditions of pH and temperature, and also at different salts and polymers concentrations.
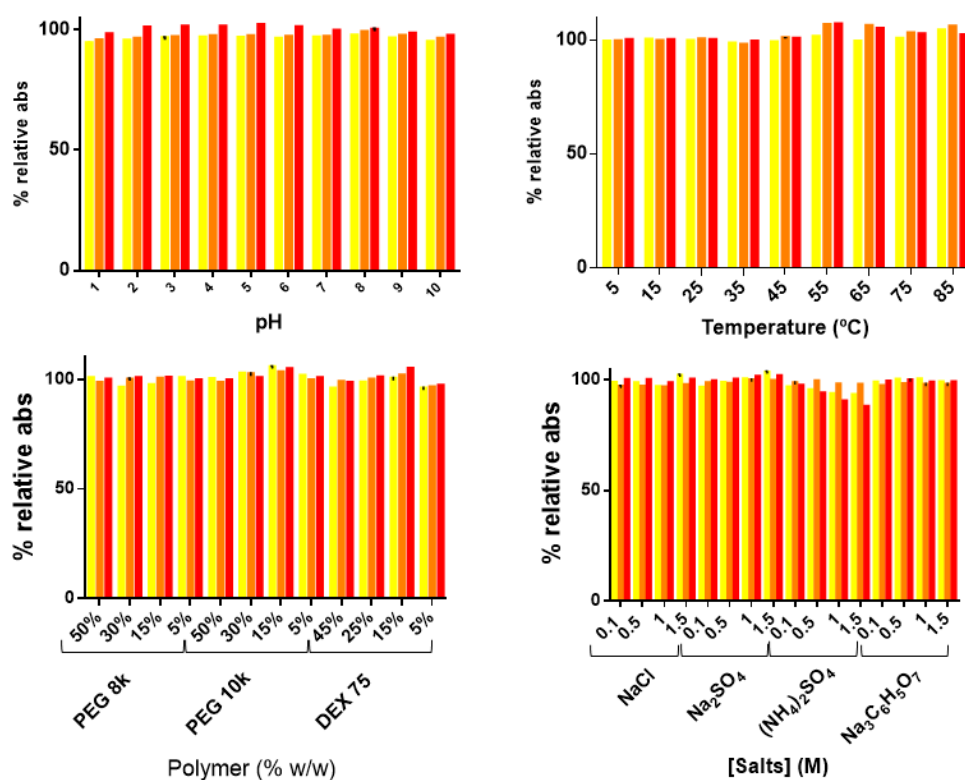
Figure 3.20- *Relative abs (%) for all stability tests after 24h incubation of the extract with different conditions of pH, temperature, polymers and salts. Yellow range (400 nm) of the spectrum is represented in the yellow bar), the orange range (470 nm) is represented by the orange bar and red range (500 nm) is represented by the red bar.*

Overall, the fermentation extract color is stable for all the pH range studied but it can be slightly more stable for alkaline pH values. Although FE have showed to be stable, it has the particularity of changing its color when is mixed with acid or base (Figure 3.21). In an acidic environment the color of the extract turns yellow while in alkaline conditions its red coloration is intensified.

Figure 3.21- *Color of FE after HCl 1M and NaOH 1M addition for pH stability test performance. Acid pH range (1.0 – 6.0), 7.0 as neutral pH and alkaline pH range (8.0 – 10.0).*

Regarding the effect of the temperature over color it was seen that FE is only stable for temperatures up to 45 °C and there is some concentration of the pigment with increasing temperature.

After incubation of EF with crescent concentrations of 2 different polymers, it was found out that high stability levels were achieved with PEG (similar values despite the molecular weight). Concerning the effect of salts, it was verified a decreasing in the stability of the extract when the concentration of $(NH_4)_2SO_4$ was increased. It has been reported in the literature that pigments produced by *Monascus* and *Penicillium* are more stable at alkaline or neutral pH and at temperatures below 60 °C (Santos-Ebinuma *et al.* 2013; Silveira *et al.* 2013; Velmurugan *et al.* 2011).

Perumal *et al.* studied the stability of a pink pigment from *Sclerotinia* sp. and found out that this pigment was stable for a temperature range of 40 °C to 70 °C and was also stable for alkaline pH (Perumal *et al.* 2009). Velmurugan *et al.* evaluated the stability of a red pigment produced from *Isaria farinose* and they concluded that it was stable for a pH range of 4.0 to 7.0 and temperatures below 60 °C. The authors also concluded that the pigment was more stable in the presence of NaCl (Velmurugan, Lee, Nanthakumar, *et al.* 2010).

Santos – Ebinuma *et al.* studied the stability of red colorants produced by *Penicillium purpurogenum* and concluded that the pigments were more stable at alkaline pH. This can be due to the acid-catalyzed degradation of red colorants be stronger than the alkaline one. It was also concluded by them that the red colorants were more stable below 50 °C. Above these temperatures there was an increasing degradation of the pigments. In terms of stability with the addition of salts (NaCl and Na2SO₄) and polymers (PEG 1000, PEG 6000, PEG 10,000 and NaPA), these authors found that neither one of them affected the stability of the pigment, which are in agreement with the results obtained for the present study (Santos-Ebinuma *et al.* 2013).

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These results have great interest, because pH and temperature variations during an industrial process are frequent. Since it was going to apply ATPSs tests to evaluate the efficiency of pigment recovery of this method, this was a good result since it could be expected the test to be relied without any problem towards the stability of the pigment in the phases.

3.1.9.3.   Aqueous Two – Phase Systems (ATPSs)

In recent years ATPSs have been studied as a more effective way of separating biomacromolecules, like pigments, from the fermentation culture medium, being an alternative to the usual methods, using organic solvents. In the ATPS process, it could be possible to obtain the pigment separation in few steps unlike the solvent extraction processes where several steps are required (Santos-Ebinuma *et al.* 2015). This extraction technique is also more environmentally friendly because extracting pigments with organic solvents requests a great amount of them to have a considerably yield of pigment recuperation. Moreover, the use of organic solvents may present some risks to human health and contribute to the denaturation of the biomolecules (Khanra *et al.* 2018).

For these reasons, the partial purification of the pigments contained in FE were attempted by ATPSs extraction.
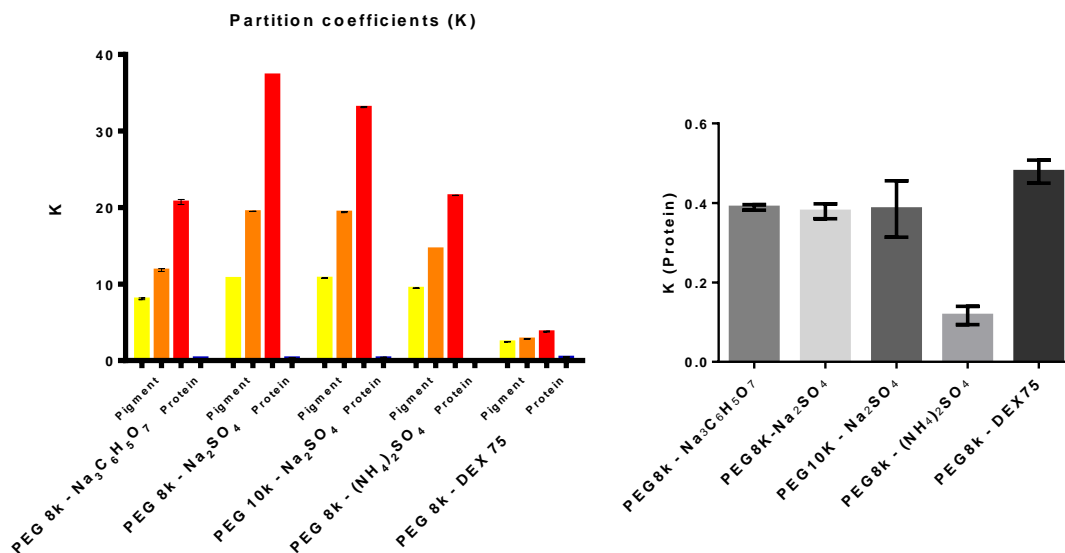
Figure 3.22- *Partition coefficients (K) for Pigment (yellow bar – 400 nm, orange bar – 470 nm, red bar – 500 nm) and partition coefficients of total protein in all the ATPSs tested (grey graphic).*

In all polymer-salt systems tested, the pigment was found to have more affinity for the polymer-rich phase (top phase) than the salt-rich phase, once K-values>1 were obtained. In the polymer-polymer system, the pigment also had more affinity for the PEG-rich phase (top phase). In general, the system which presented the best partition coefficient for the pigment was the one composed by PEG 8000 - Na_2SO_4, being sodium sulfate described in the literature as the most commonly used salt in ATPSs. Lower molecular weight polymer were also better, which could be due to the fact that, by increasing the polymer chain length and using the same top phase volume, the lower becomes the volume available for the pigment at that stage, forcing part of the pigment to migrate to the bottom phase (Ebrahimi and Shahriari 2016; Santos-Ebinuma *et al.* 2015).

Since the system PEG 8000-DEX75 uses two polymers, the low Kpig. could be explained due to the similarity of the constituents of the system (Chavez-Santoscoy *et al.* 2010), showing that in this case the pigments had affinity for both phases (DEX75 rich phase).

As for the protein partition coefficient, it was found that the best biphasic system (i.e., the one the highest difference between $K_{pig.}$ And K_{prot}) was the PEG 8000-$(NH_4)_2SO_4$ system, with a K_{pig} of 9.45 ± 0.01 for yellow, 14.63 ± 0.01 for orange and 21.60 ± 0.02 for red range, and a K_{prot} of 0.12 ± 0.02. This can also be explained by the fact that the ionic strength of the salt is associated with the migration of the proteins in the bottom phase (Ebrahimi and Shahriari 2016).

Regarding the selectivity and volume ratio of the systems (Appendix B), PEG 8000-$(NH_4)_2SO_4$ revealed the best values for all the wavelengths evaluated. This shows that this system was the one with better partition of the pigment, since the pigment has preference for the PEG rich-phase and the protein has preference for the Salt rich-phase. These results corroborate the ones described by Santos-Ebinuma *et al.* for the extraction of red colorants from *Penicillium brevicompactum* with ATPSs, where systems constituted by polymer-salt had also high selectivity and volume ratio (Santos-Ebinuma *et al.* 2015).

3.2. Ethanolic Extracts

It is described in the literature that fungi can produce extracellular pigments as well as intracellular pigments that can be release in the medium by excretion (Morales-Oyervides *et al.* 2017)

To test what it is already described, the yellowish biomass collected in the end of the submerged fermentation (12 days) and greenish fungal mycelial recovered from agar plates (7-10 days of growth)

were subjected to organic solvent extraction using ethanol (95% v/v). The extracts obtained were then analyzed by TLC.

All the mobile phases tested for FE were also tested in this step being the mobile phase constituted by ethanol:water (50:50) the best to separate the pigments extracted.

Both extracts showed several fractions of pigments, FBEE with three orange and three red bands, which may indicate that this extract can be a mixture of intracellular pigments, while MEE with one big yellow fraction followed by four orange and three red fractions. The fact that the pigments fractions are different in the two extracts may be explained by the fact of different media had been used, which is a factor described in the literature, that have impact on pigments production (Gmoser *et al.* 2017).

FBEE   MEE
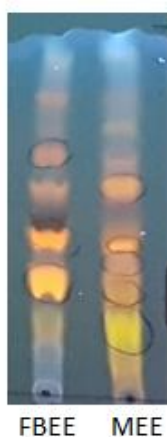
Figure 3.23*- TLC plate of* FBEE *and* MEE*.*

3.3. Functional Characterization

3.3.1. Bioactivity of extracts

Pigments can reach great values in the market specially if they own antioxidant characteristics, if they are of phenolic or polyphenolic nature and/or belong to the flavonoids group. Various biocompounds such as carotenoids, anthraquinones, among others are reported as pigments with high antioxidant activity and have been already marketed (Narsing Rao, Xiao, and Li 2017).
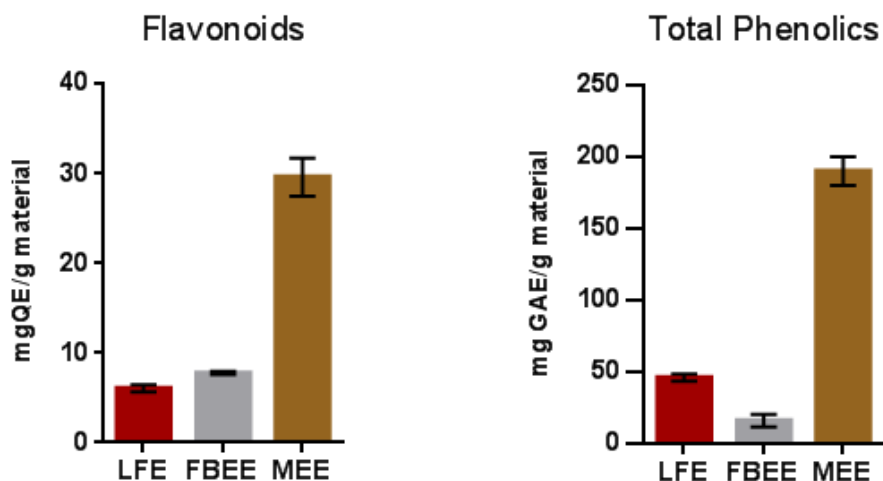
Figure 3.24- *Total phenolics and flavonoids of LFE and* MEE, FBEE *extracts.*

Observing figure 3.24, it is possible to conclude that there is a presence of phenolic and flavonoid compounds in all extracts analyzed. This fact suggest that possibly the extracts may exhibit some antioxidant potential since normally these group of compounds are responsible for this type of bioactivity (Chen and Xu 2018; Smith, Doyle, and Murphy 2015). MEE was the extract that showed the highest concentration of these compounds. This can be due to the fact that this extract has been obtained from fungi grown in agar plates under different experimental conditions (MEA medium, 25 °C, static and without light). The differences concerning sugar and nitrogen sources in the culture media influence the production of compounds produced by the fungus (Pisareva and Kujumdzieva 2010), as also the type of medium (solid vs liquid), light (presence or not) and agitation (static or agitated). Based on the results obtained for total phenolics and flavonoids, it was performed the antioxidant activity assays to verify if the existence of these compounds in the extracts conferred some antioxidant capacity to the extracts.
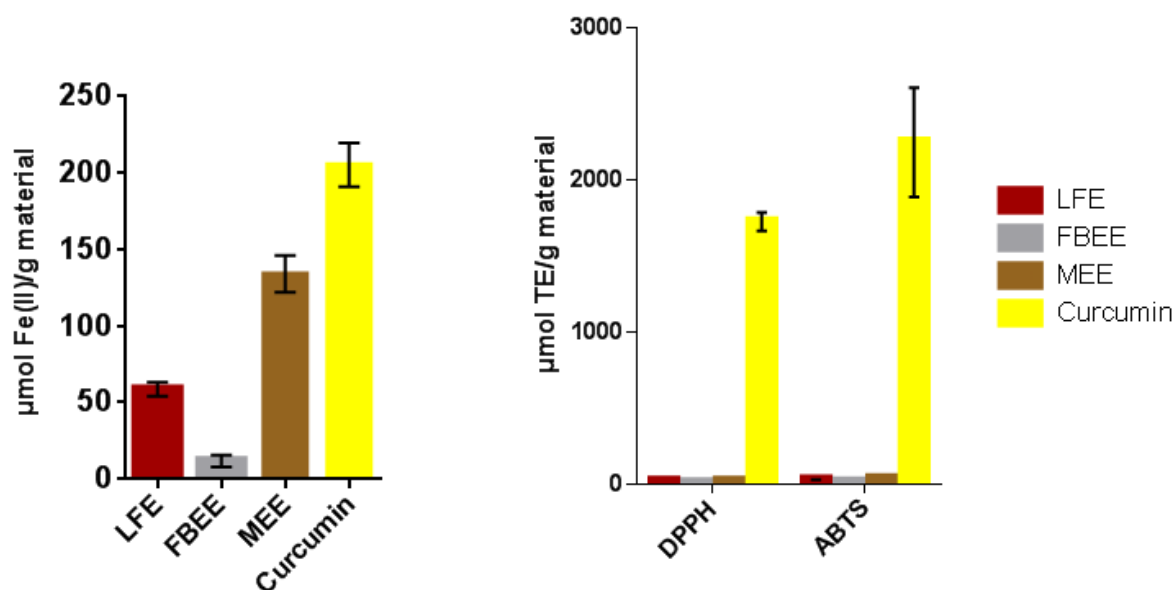
Figure 3.25- *Results of FRAP, DPPH and ABTS assays.*

In terms of evaluation of the antioxidant activity (Figure 3.25), it is recommended in literature the analysis of the samples by different methods, once each one is based on different reactional principles (Ballesteros *et al.* 2015).

For FRAP assay, MEE (267.7 ± 23.80 µmol $FeSO_4$/mL with 2 mg/mL of pigment) was the one with the highest activity but comparing with curcumin (used as a commercial standard pigment with antioxidant potential) the extract is still less strong than the curcumin. Zhang *et al.* found that with 10 µg/mL of lutein and lycopene, 12.78 and 9.14 µmol $FeSO_4$/mL were obtained respectively for FRAP activity (Zhang *et al.* 2014).

For DPPH and ABTS assays, MEE was also the extract that exhibited higher antioxidant activity with an IC_{50} of 11.54 ± 0.60 mg/mL and 9.86 ± 0.11 mg/mL respectively (Table 3.4). Though, once again the antioxidant potential of the extract is lower than that presented by curcumin. The results obtained suggest that these extracts are not comparable to pigment extracts obtained by Srianta *et al.* with *Monascus* sp. in a culture medium composed of rice, corn and sorghum that reaches the IC_{50} value in a range of 1.79 to 10.6 mg/mL with DPPH scavenging activity (Srianta *et al.* 2017) and by Yang *et al.* with *Monascus*-fermented rice (IC_{50} of 1.79 mg/mL) (Yang *et al.* 2006). MEE DPPH- scavenging activity is comparable to *Monascus*-fermented adlay (IC_{50} of 12.45 mg/mL) using DPPH scavenging (Tseng *et al.* 2006). Rostami *et al.* concluded that pigments extracted from *Micrococcus roseus* (PTCC 1411) and *Rhodotorula glutinis* (PTCC 5257) had an IC_{50} of 0.573 ± 0.15 mg/mL and 0.555 ± 0.05 mg/mL

respectively, also using DPPH scavenging activity, showing a higher potential antioxidant activity than all the extracts tested in this work (Rostami *et al.* 2016). A study carried out by Mani *et al.* found that a fungal methanolic extract produced by a *Fusarium* sp. presented an IC_{50} value of 1.62 mg/mL using DPPH scavenging activity, having also a better antioxidant activity potential than all extracts used in this work, because it needs less amount of extract to obtain the same percentage of inhibition (50%) (Mani *et al.* 2015).

Table 3.4- *IC_{50} value for all extracts and for curcumin (used as a reference) obtained from DPPH and ABTS assays.*

Extract	DPPH (mg/mL)	ABTS (mg/mL)
LFE	12.30 ± 0.20	11.94 ± 1.60
MEE	11.54 ± 0.60	9.86 ± 0.11
FBEE	27.30 ± 1.20	22.09 ± 0.15
Curcumin	0.136 ± 0.004	0.15 ± 0.02

In conclusion, it is seen that the extracts analyzed do not have a high antioxidant activity compared to other pigments-rich extracts.

3.3.2. Antimicrobial Activity

3.3.2.1. Agar diffusion method

Natural antimicrobial compounds present an enormous therapeutic potential, since they can be used mostly without any side effects that are usually associated with synthetic antimicrobials.

Since all extracts were shown to have in their composition compounds that are associated to antimicrobial capacity (flavonoids and phenolic compounds), these were tested to verify if any antimicrobial activity could be detected.

An attempt to use Whatman filter paper disks, according to the CLSI norm, was taken. According to the norm, filter paper disks were disposed on the agar plates and a 5 μL was poured into the disks. The results obtained in these tests were not conclusive regarding the effect of the extracts on the microorganisms, since there was little or no diffusion of the extract samples into the agar. So, these results are not presented in this work.

LFE and FBEE did not show any antimicrobial capacity against all the microorganisms selected for the agar diffusion test (Figure 3.29). Only MEE was the one presenting activity against all the microorganisms (Figure 3.32). *Z. mobilis* was the bacteria inhibited the most (higher halo of inhibition) for almost all the concentrations (Appendix C), *P. larvae* was the one with more inhibition at the concentration of 1 mg/mL of extract. *E. coli* and *P. fluorescens* weren't inhibited by MEE at 1 mg/mL. Regarding the fungi, *P. expansum* was the one that showed more susceptibility to the extract for all concentrations tested. On the other hand, *Mucor* sp. was not inhibited at 1 mg/mL, being the only fungus to show resistance ate this concentration (Appendix C).

Vendruscolo *et al.* reported the antimicrobial activity of red pigments produced by *Monascus* sp. by submerged fermentation, against gram-negative bacteria (Vendruscolo *et al.* 2014) and other authors reported the effectiveness of pigments against bacteria's and molds (Vendruscolo *et al.* 2016; Yolmeh and Khomeiri 2017). Pandey *et al.* reported the antimicrobial activity of a *Penicillium* sp. against gram-negative bacteria and fungi (Pandey *et al.* 2018).
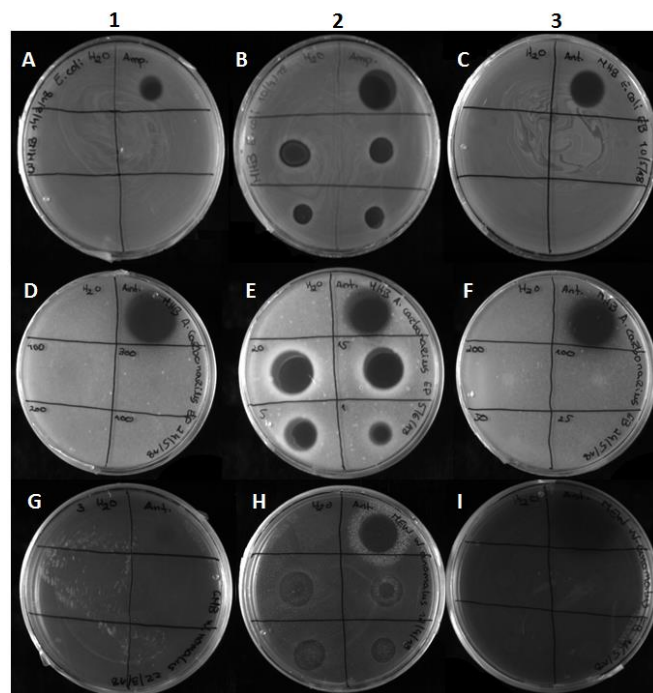
Figure 3.26- *Results of agar diffusion method for E. coli (A, B, C), A. carbonarius (D, E, F) and W. anomalus (G, H, I). Column 1- LFE, column 2- MEE and column 3- FBEE. The plate was divided by: Negative control (water), Positive control (antibiotic) and extract tested (from the highest concentration – 200 mg/mL dry extract, to the lowest concentration – 1 mg/mL).*

3.3.2.2. Minimal inhibitory concentration

Since MEE was the only extract that showed some antimicrobial capacity against all the microorganisms tested, this extract was used in further antimicrobial studies.

For bacteria's, MIC's were taken after 24h and for fungi was taken after 96h. Two types of values were taken, MIC (abs) which was calculated through measured absorbance taken in the end of the assay. MIC (visual) is the value recorded by visual analysis in the end of the assay. Next, minimal bactericide concentration and minimal fungicidal concentration (MBC/MFC) were evaluated in a way to study the real toxic effect of the extract in the microorganisms.

Of all the microorganisms, *L. fructivorans* was the only one which had 99.9% of inhibition with 0.04 mg/mL of MEE, having the same MBC as well. For the rest of the bacteria tested, *E. coli* only had 85.8% of inhibition with 0.31 mg/mL extract, and at higher concentrations the %inhibition was lower. This could be due to the possible contribution of other compounds that could favor the growth of the microorganism in relation to the compound that gives the antimicrobial activity. For *P. larvae* the highest %inhibition was obtained at 0.08 mg/mL of extract. Regarding to *P. fluorescens*, it had 86.2 % of inhibition at 0.62 mg/mL extract. *A. carbonarius* presented 98.8% of inhibition with 0.16 mg/mL extract, showing the great effect of the extract towards the fungal. All visual MICs are in agreement with the absorbance MICs calculated, and only *P.* larvae and *A. carbonarius* MICs values are in agreement with MBC/MFC obtained.
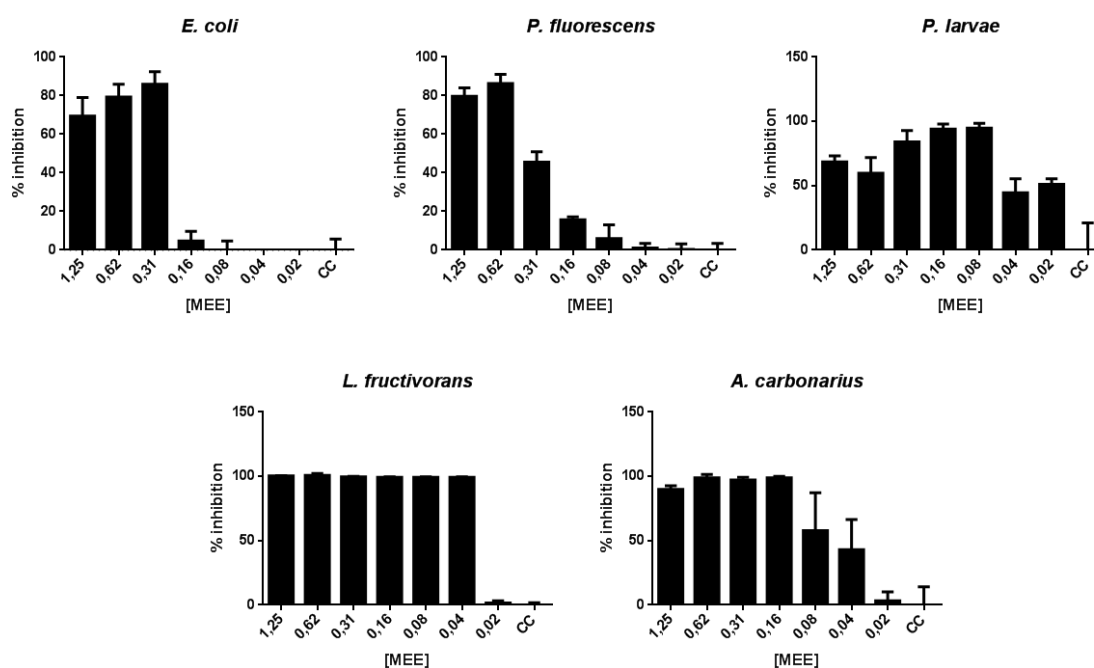
Figure 3.27- *Percentage of inhibition of the microorganisms used to find the MIC value for MEE.*

The results obtained in Table 3.5 shows that the extract had more antibacterial effect on gram-positive bacteria compared to gram-negative bacteria. This could be due to the presence of lipopolysaccharide in cell wall of gram-negative bacteria (Rostami *et al.* 2016; Yolmeh and Khomeiri 2017). McKeegan *et al.* described that lipopolysaccharides of cell wall can prevent influx of active compounds to cytoplasmic membrane of this type of bacteria (Mckeegan, Borges-walmsley, and Walmsley 2002).

MEE shows to be more effective than other pigments extracts from other microorganisms like *Micrococcus roseus* (MIC 64 mg/mL) and *Rhodotorula glutinis* (MIC 32 mg/mL) against bacteria and also presents a MIC of 128 mg/mL for *Aspergillus citri* (Rostami *et al.* 2016). These extracts had also better antimicrobial activity against *E. coli* than pigments extracted from *Penicillium melinni DPUA 1391* and *Penicillium simplicissimum DPUA 1379*, with a MIC of 5.0 mg/mL and 2.5 mg/mL respectively (Teixeira *et al.* 2012).

Table 3.5- *MIC values (mg/mL) for the microorganisms tested. MIC (abs) is the value found for treated data from absorbance in the end of the assay; MIC (visual) is the value found from visual analyses in the end of the assay.*

Microorganism	MIC (abs)	MIC (visual)	MBC/MFC
E. coli	_____*	0.31	0.62
P.fluorescens	_____*	0.62	1.25
P. larvae	_____#	0.08	0.08
L. fructivorans	0.04	0.04	0.04
A. carbonarius	_____*	0.16	0.16

*No concentration obtained 99.9% of inhibition.

#Other compounds could favor the growth of the fungal compared to the compound with activity.

3.4. Mycotoxins screening

During the production of pigments and other compounds, mycotoxins production may also be associated, since these are also secondary metabolites (Patel *et al.* 2018). Once the extract showed antimicrobial activity and the fungus *P. brevicompactum* is reported in the literature as the main producer of mycophenolic acid (MPA), considered a low acute mycotoxin (Fontaine *et al.* 2016), it was performed some mycotoxin analysis to mislead their production by the fungus, and to ensure that the antimicrobial activity to the extract was not due to their presence. Even though only MPA is associated

with this fungus, the production of other mycotoxins during the fermentation, such as (OTA, DON, AFB1 and AFB2, ZEA, FB) was also investigated. The result was negative for all the mycotoxins mentioned before and also for the presence of MPA. However, due to the complexity of the extracts, it is necessary to optimize the conditions for MPA analysis by UHPLC to ensure that this mycotoxin is not present. The results obtained have great interest, since these mycotoxins are considered many of them carcinogenic and that cause serious diseases. These results also suggest that the antimicrobial activity of the extract is not related to the presence of mycotoxins.

3.5. Pigments separation through chromatographic column

Since MEE was the only extract that showed antioxidant and antimicrobial activity, it was eluted with water:ethanol (50:50) through a silica column attempting to separate and recover different fractions (yellow, orange and red) for further characterization and additional studies.

Figure 3.28 shows 2 rows of the several fractions recovered from the column.
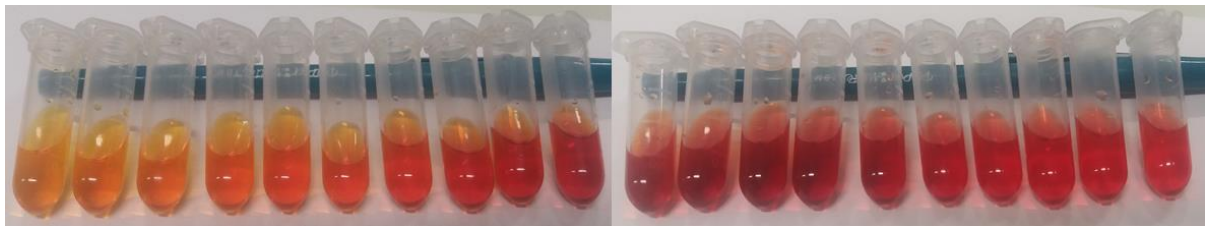
Figure 3.28- Column fractions recovered from the column. Fractions from yellow range to red.

4. Conclusion

Filamentous fungi are capable of produce pigments as valuable bioactive compounds with great market demands in order to replace synthetic pigments. It was demonstrated in this work that *P. brevicompactum* is a good producer of pigments in the color range from yellow to red.

After optimization of the production conditions, it was verified that the fungus produces more quantity of pigments with the synthetic medium A, at 23 °C, 150 rpm, with 20g/L of lactose, 8 g/L of supplements, and initial pH=7.0. Light was shown to be a factor that did not make a significant difference in the production yield of the pigments.

The pigments were recovered from the fermentation broth using two extraction methodologies: pigment from fermentation broth was recovered with an alternative extraction approach based on ATPSs composed of two polymers or a polymer and a salt, and pigments from MEE and FBEE were extracted with ethanol. PEG-salt ATPSs proved to be suitable to separate the pigments (top phase) from other components (proteins) present in the fermentation broth (bottom phase), being PEG 8000 – $(NH_4)_2SO_4$ the best biphasic system for pigment recovery and partial purification.

After performing a TLC with the best mobile phase water:ethanol (50:50), it was seen that all the extracts had a mixture of pigments.

In terms of functional characterization, all three extracts showed potential antioxidant activity with phenolic compounds and flavonoids in their composition. Only MEE showed antimicrobial activity against bacteria, fungi and yeast. In terms of MIC, this extract also showed good inhibition against bacterial and fungal microorganisms, being most effective against *L. fructivorans* with 99.9% of inhibition at 0.04 mg/mL of dry extract.

All the extracts were analyzed by UHPL and the presence of MPA was not seen, but this method must be optimized for better conclusions.

5. Future perspectives

For the future it is suggested the follow ideas:

- Test more mobile phases for the separation recovery of the pigments using a liquid chromatography;
- Purification and structural characterization of the fractions recovered from the column;
- Perform detailed antioxidant and antimicrobial activity tests for the purified fractions;
- Optimization of the UHPLC method for MPA analysis;
- Test the pigments as a dye in textile fabrics.

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Appendixes

In this section are all results not shown on the section of Results and Discussion. The appendixes are organized in three subsections, taking the results of pigment production by submerged fermentation, ATPSs and antimicrobial activity results with tables and graphics for a better understanding of the results explained in the section mentioned before.

A. Pigment production by submerged fermentation

This section presents all the data not shown on section 3. Results and Discussion. In this section it is represented the sum of absorbances and carbon source consumption during the fermentation time and the initial and final pH of the fermentative broth.

A.1. Effect of the media

Table A.1 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm).

Table A.1- Sum of the absorbances taken through 12 of fermentation for the effect of media on pigment production. A (Nagy medium), B (Czapec medium) and C (MEA medium).

Time (days)	A	B	C
0	0.000	0.000	0.000
3	0.136 ± 0.01	0.000	0.000
5	0.224 ± 0.02	0.000	0.000
7	0.772 ± 0.21	0.072 ± 0.05	0.072 ± 0.04
10	1.333 ± 0.08	0.224 ± 0.03	0.224 ± 0.05
12	1.644 ± 0.023	0.265 ± 0.02	0.265 ± 0.08

Table A.2 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for all the media.

Table A.2- Concentration of lactose during fermentation for the 3 media studied. A (Nagy medium), B (Czapec medium) and C (MEA medium).

Time (days)	A	B	C
0	15.65 ± 0.01	14.26 ± 0.01	34.34 ± 0.01
3	16.61 ± 0.01	15.62 ± 0.01	20.88 ± 0.01
5	11.90 ± 0.01	14.96 ± 0.01	21.01 ± 0.01
7	8.14 ± 0.01	14.40 ± 0.01	21.45 ± 0.01
10	2.70 ± 0.01	13.11 ± 0.01	22.15 ± 0.01
12	0.00	13.75 ± 0.01	23.53 ± 0.01

Table A.3 shows the initial and final pH of the fermentation broth. All the results were measured with pH meter in the beginning of the fermentation and at the end with pH stripes.

Table A.3- Initial and final pH of the 3 media studied. A (Nagy medium), B (Czapec medium) and C (MEA medium).

pH	A	B	C
Initial	8.0 ± 0.0	8.0 ± 0.0	6.3 ± 0.6
Final	[7-8]	8.0 ± 0.0	5.0 ± 0.0

*Measured with pH stripes.

A.2. Effect of the rotation

Table A.4 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for the 4 rotations studied.

Table A.4 - *Sum of the absorbances taken through 12 of fermentation for the effect of rotation on pigment production.*

Time (days)	0 rpm	100 rpm	150 rpm	200 rpm
0	0.000	0.000	0.000	0.000
3	0.026 ± 0.02	0.146 ± 0.02	0.115 ± 0.02	0.136 ± 0.01
5	0.074 ± 0.03	0.289 ± 0.04	0.204 ± 0.05	0.224 ± 0.02
7	0.091 ± 0.01	0.630 ± 0.11	0.738 ± 0.19	0.772 ± 0.21
10	0.196 ± 0.03	1.126 ± 0.17	1.158 ± 0.35	1.333 ± 0.08
12	0.358 ± 0.12	1.379 ± 0.14	1.392 ± 0.02	1.644 ± 0.02

Table A.5 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for all the media. At 0 rpm the samples were only measured for the initial day, day 3 and five because since there was no pigment production, it was assumed that there would be no lactose consumption also, which was the result obtained.

Table A.5- *Concentration of lactose during fermentation for the 4 rotation studied.*

Time (days)	0 rpm	100 rpm	150 rpm	200 rpm
0	19.6 ± 0.04	2.0 ± 0.04	21.24 ± 0.01	34.34 ± 0.01
3	-	21.2 ± 0.04	19.18 ± 0.01	20.88 ± 0.01
5	21.7 ± 0.04	15.8 ± 0.04	15.09 ± 0.01	21.01 ± 0.01
7	-	11.0 ± 0.04	8.91 ± 0.01	21.45 ± 0.01
10	-	0.0 ± 0.04	1.71 ± 0.01	22.15 ± 0.01
12	21.3 ± 0.04	0.0 ± 0.04	0.00 ± 0.01	23.53 ± 0.01

Table A.6 shows the initial and final pH of the fermentation broth. All the results were measured with pH meter in the beginning and at the end of the fermentation.

Table A.6- *Initial and final pH of the 4 rotations studied.*

pH	0 rpm	100 rpm	150 rpm	200 rpm
Initial	7.0 ± 0.2	7.0 ± 0.2	8.0 ± 0.0	6.3 ± 0.6
Final	8.0 ± 0.6	7.0 ± 0.2	8.0 ± 0.0	5.0 ± 0.0

A.3. Effect of the carbon source

Table A.7 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for the 3 carbon sources studied.

Table A.7- Sum of the absorbances taken through 12 of fermentation for the effect of carbon source on pigment production.

Time (days)	Lactose	Glucose	Sucrose
0	0.000	0.000	0.000
3	0.115 ± 0.02	0.029 ± 0.01	0.028 ± 0.01
5	0.204 ± 0.05	0.287 ± 0.15	0.097 ± 0.14
7	0.738 ± 0.19	0.624 ± 0.06	0.206 ± 0.07
10	1.158 ± 0.35	0.932 ± 0.30	0.621 ± 0.20
12	1.392 ± 0.14	1.237 ± 0.17	0.847 ± 0.08

Table A.8 compiles the HPLC results of all carbon sources consumption along the 12 days of the fermentation for all the media. Sucrose monomers (glucose and fructose) are also represented.

Table A.8- Concentration of all carbon sources during fermentation. Sucrose monomers (glucose and fructose) are also represented.

Time (days)	Lactose	Glucose	Sucrose	Glucose (Sucrose monomer)	Fructose (Sucrose monomer)
0	21.24 ± 0.01	20.78 ± 0.01	18.85 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
3	19.18 ± 0.01	15.27 ± 0.01	0.00 ± 0.01	8.95 ± 0.01	9.56 ± 0.01
5	15.09 ± 0.01	6.42 ± 0.01	0.00 ± 0.01	5.93 ± 0.01	7.95 ± 0.01
7	8.91 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.86 ± 0.01	5.55 ± 0.01
10	1.71 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
12	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01

Table A.9 shows the initial and final pH of the fermentation broth. All the results were measured with pH stripes in the beginning and at the end of the fermentation.

Table A.9- Initial and final pH for all carbon sources studied.

pH	Lactose	Glucose	Sucrose
Initial	[7-8]	8.0	[7-8]
Final	[7-8]	[7-8]	[7-8]

A.4. Effect of the temperature

Table A.10 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for the temperatures studied.

Table A.10- Sum of the absorbances taken through 12 of fermentation for the effect of the temperature on pigment production.

Time (days)	23 ºC	33ºC
0	0.000	0.000
3	0.029 ± 0.002	0.061 ± 0.01
5	0.339 ± 0.04	0.115 ± 0.01
7	0.626 ± 0.01	0.157 ± 0.01
10	1.314 ± 0.04	0.334 ± 0.07
12	1.524 ± 0.15	0.759 ± 0.11

Table A.11 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for the temperatures studied.

Table A.11- Consumption of lactose for all temperatures during fermentation.

Time (days)	23 ºC	33ºC
0	22.27 ± 0.04	20.63 ± 0.04
3	19.32 ± 0.04	22.39 ± 0.04
5	16.34 ± 0.04	22.90 ± 0.04
7	8.88 ± 0.04	22.33 ± 0.04
10	0.64 ± 0.04	15.29 ± 0.04
12	0.00 ± 0.04	11.07 ± 0.04

Table A.12 shows the initial and final pH of the fermentation broth. All the results were measured with pH stripes in the beginning and with the pH meter at the end of the fermentation.

Table A.12- Initial and final pH for all temperatures studied.

pH	23 ºC	33 ºC
Initial	[7-8]	[7-8]
Final	6.74 ± 0.02	7.12 ± 0.03

A.5. Effect of the supplements (P – Y)

Table A.13 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for all supplements combination studied.

Table A.13- Sum of the absorbances taken through 12 of fermentation for the effect of the supplements combination on pigment production.

Time (days)	(P-Y) (0-8)	(P-Y) (8-0)	(P-Y) (2-2)	(P-Y) (6-2)	(P-Y) (2-6)	(P-Y) (8-8)	(P-Y) (4-4)	(P-Y) (0-16)
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.054 ± 0.01	0.014 ± 0.01	0.036 ± 0.01	0.048 ± 0.01	0.374 ± 0.02	0.068 ± 0.01	0.301 ± 0.01	0.029 ± 0.01
5	0.430 ± 0.01	0.124 ± 0.05	0.286 ± 0.10	0.343 ± 0.04	0.726 ± 0.12	0.422 ± 0.07	0.594 ± 0.04	0.192 ± 0.04
7	0.529 ± 0.04	0.325 ± 0.10	0.604 ± 0.05	0.711 ± 0.02	0.954 ± 0.07	0.749 ± 0.07	0.878 ± 0.02	0.476 ± 0.10
10	0.831 ± 0.02	0.691 ± 0.13	0.946 ± 0.13	1.218 ± 0.03	1.215 ± 0.09	1.560 ± 0.20	1.593 ± 0.05	0.955 ± 0.19
12	1.236 ± 0.03	0.926 ± 0.17	1.092 ± 0.18	1.147 ± 0.10	1.457 ± 0.15	2.028 ± 0.25	1.848 ± 0.16	1.304 ± 0.24

Table A.14 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for all the supplements combinations studied.

Table A.14- *Consumption of lactose for all supplement's combinations during fermentation.*

Time (days)	(P-Y) (0-8)	(P-Y) (8-0)	(P-Y) (2-2)	(P-Y) (6-2)	(P-Y) (2-6)	(P-Y) (8-8)	(P-Y) (4-4)	(P-Y) (0-16)
0	21.61 ± 0.02	21.26 ± 0.02	22.01 ± 0.02	20.30 ± 0.02	20.19 ± 0.02	22.04 ± 0.02	22.27 ± 0.04	21.69 ± 0.02
3	19.89 ± 0.02	19.89 ± 0.02	19.08 ± 0.02	19.03 ± 0.02	20.08 ± 0.02	22.22 ± 0.02	19.32 ± 0.04	21.35 ± 0.02
5	12.34 ± 0.02	19.10 ± 0.02	16.01 ± 0.02	10.05 ± 0.02	15.99 ± 0.02	15.22 ± 0.02	16.34 ± 0.04	18.74 ± 0.02
7	2.67 ± 0.02	18.24 ± 0.02	9.39 ± 0.02	5.78 ± 0.02	5.99 ± 0.02	4.46 ± 0.02	8.88 ± 0.04	16.48 ± 0.02
10	0.00 ± 0.02	14.35 ± 0.02	4.52 ± 0.02	0.00 ± 0.02	0.00 ± 0.02	0.00 ± 0.02	0.64 ± 0.04	3.34 ± 0.02
12	0.00 ± 0.02	13.09 ± 0.02	1.99 ± 0.02	0.00 ± 0.02	0.00 ± 0.02	0.00 ± 0.02	0.00 ± 0.04	0.00 ± 0.02

Table A.15 shows the initial and final pH of the fermentation broth. All the results were measured with a pH meter in the beginning and at the end of the fermentation.

Table A.15- *Initial and final pH for all supplement's combinations studied.*

pH	(P-Y) (0-8)	(P-Y) (8-0)	(P-Y) (2-2)	(P-Y) (6-2)	(P-Y) (2-6)	(P-Y) (8-8)	(P-Y) (4-4)	(P-Y) (0-16)
Initial	7.02 ± 0.00	7.01 ± 0.00	7.06 ± 0.00	7.02 ± 0.00	7.03 ± 0.00	7.03 ± 0.00	[7-8]*	7.05 ± 0.00
Final	7.72 ± 0.07	6.98 ± 0.38	6.89 ± 0.06	6.76 ± 0.03	7.10 ± 0.17	8.43 ± 0.07	6.74 ± 0.02	7.97 ± 0.07

*Was measured with pH stripes.

A.6. Effect of the lactose concentration

Table A.16 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for the 3 lactose concentrations studied.

Table A.16- Sum of the absorbances taken through 12 of fermentation for the effect of lactose concentration on pigment production.

Time (days)	10 g/L	20 g/L	30 g/L
0	0.000	0.000	0.000
3	0.037 ± 0.01	0.068 ± 0.01	0.011 ± 0.004
5	0.299 ± 0.08	0.422 ± 0.07	0.218 ± 0.10
7	0.434 ± 0.09	0.749 ± 0.07	0.482 ± 0.04
10	0.831 ± 0.07	1.560 ± 0.20	0.944 ± 0.15
12	0.883 ± 0.06	2.028 ± 0.25	1.210 ± 0.14

Table A.17 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for the 3 lactose concentrations studied.

Table A.17- Consumption of lactose for all concentrations during fermentation.

Time (days)	10 g/L	20 g/L	30 g/L
0	11.29 ± 0.02	21.06 ± 0.01	30.90 ± 0.02
3	10.96 ± 0.02	19.78 ± 0.01	29.81 ± 0.02
5	9.23 ± 0.02	19.48 ± 0.01	27.72 ± 0.02
7	4.05 ± 0.02	15.67 ± 0.01	26.21 ± 0.02
10	0.00 ± 0.02	3.07 ± 0.02	13.73 ± 0.02
12	0.00 ± 0.02	0.00 ± 0.02	2.98 ± 0.02

Table A.18 shows the initial and final pH of the fermentation broth. All the results were measured with a pH meter in the beginning and at the end of the fermentation.

Table A.18- *Initial and final pH for all lactose concentrations studied.*

pH	10 g/L	20 g/L	30 g/L
Initial	7.03 ± 0.00	7.03 ± 0.00	7.03 ± 0.00
Final	8.45 ± 0.01	7.60 ± 0.29	6.86 ± 0.30

A.8. Effect of the natural light

Table A.19 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) with natural light and darkness.

Table A.19- *Sum of the absorbances taken through 12 of fermentation for the effect of natural light on pigment production.*

Time (days)	Light	Dark
0	0.000	0.000
3	0.049 ± 0.06	0.051 ± 0.04
5	0.324 ± 0.10	0.355 ± 0.06
7	0.510 ± 0.07	0.588 ± 0.24
10	1.360 ± 0.37	1.296 ± 0.16
12	1.897 ± 0.56	1.897 ± 0.19

Table A.20 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for natural light and darkness.

Table A.20- *Consumption of lactose with the use of natural light and darkness during fermentation.*

Time (days)	Light	Dark
0	20.80 ± 0.02	21.40 ± 0.02
3	20.79 ± 0.02	21.55 ± 0.02
5	17.89 ± 0.02	19.59 ± 0.02
7	9.34 ± 0.02	8.11 ± 0.02
10	0.00 ± 0.02	0.00 ± 0.02
12	0.00 ± 0.02	0.00 ± 0.02

Table A.21 shows the initial and final pH of the fermentation broth. All the results were measured with a pH meter in the beginning and at the end of the fermentation.

Table A.21- Initial and final pH with natural light and darkness.

pH	Light	Darkness
Initial	7.03 ± 0.00	7.03 ± 0.00
Final	8.05 ± 0.17	8.03 ± 0.44

A.8. Effect of the pH

Table A.22 contain all the results for the sum of the absorbances taken through the 12 days of fermentation at the three wavelengths measured (400, 470 and 500 nm) for the 3 pH (acid, neutral and alkaline) studied.

Table A.22- Sum of the absorbances taken through 12 of fermentation for the effect of pH on pigment production.

Time (days)	4.5	7.0	9.5
0	0,000	0,000	0,000
3	0.127 ± 0.02	0.061 ± 0.01	0.099 ± 0.004
5	0.328 ± 0.15	0.412 ± 0.05	0.218 ± 0.02
7	0.820 ± 0.05	0.624 ± 0.06	0.294 ± 0.02
10	1.005 ± 0.07	1.113 ± 0.04	0.679 ± 0.36
12	1.601 ± 0.15	1.471 ± 0.23	1.030 ± 0.60

Table A.23 compiles the HPLC results of lactose consumption along the 12 days of the fermentation for the three values of pH studied.

Table A.23- *Consumption of lactose for the 3 pH used during fermentation.*

Time (days)	4.5	7.0	9.5
0	21.39 ± 0.02	23.04 ± 0.02	19.25 ± 0.01
3	17.75 ± 0.01	20.89 ± 0.01	20.00 ± 0.01
5	16.41 ± 0.01	18.35 ± 0.01	19.66 ± 0.01
7	12.03 ± 0.01	8.74 ± 0.01	15.71 ± 0.01
10	0.00 ± 0.01	0.00 ± 0.01	8.10 ± 0.01
12	0.00 ± 0.01	0.00 ± 0.01	1.05 ± 0.01

Table A.24 shows the initial and final pH of the fermentation broth. All the results were measured with a pH meter in the beginning and at the end of the fermentation.

Table A.24- *Initial and final pH with natural light and darkness.*

pH	4.5	7.0	9.5
Initial	4.50 ± 0.00	7.00 ± 0.00	9.50 ± 0.00
Final	7.17 ± 0.10	7.65 ± 0.13	8.31 ± 0.07

B. Aqueous Two-Phase Systems (ATPSs)

In this subsection are concentrated all the results obtained for ATPSs that are treated in the section Results and Discussion. Table B.1 is regarded to the results of the selectivity of all systems studied.

Table B.1- *Results obtained for the selectivity of all ATPSs performed in the present work.*

System	400 nm	470 nm	500 nm
PEG 8000- $Na_3C_6H_5O_7$	20.77	30.45	53.31
PEG 8000- Na_2SO_4	28.36	51.36	98.41
PEG 10000- Na_2SO_4	28.05	50.47	86.11
PEG 8000-$(NH_4)_2SO_4$	80.89	125.0	184.4
PEG 8000- DEX75	5.13	5.92	7.92

Table B.2 displays the results obtained for the top phase yields ($\%\eta_{TOP}$). The higher the yield the best was the pigment partition in the system.

Table B.2- *Results obtained for the ($\%\eta_{TOP}$) of all ATPSs performed in the present work.*

System	400 nm	470 nm	500 nm
PEG 8000-$Na_3C_6H_5O_7$	111	100	110
PEG 8000- Na_2SO_4	120	110	120
PEG 10000- Na_2SO_4	127	123	133
PEG 8000-$(NH_4)_2SO_4$	108	112	117
PEG 8000- DEX75	66.6	55.0	59.5

Table B.3 presents the results for the volume ratio of the systems. Volume ratio above 1, shows that the volume of the Top phase was much higher that the volume of the bottom phase.

Table B.3- *Results obtained for the volume ratio of all ATPSs performed in the present work.*

System	Ratio
PEG 8000-Na$_3$C$_6$H$_5$O$_7$	0,773
PEG 8000- Na$_2$SO$_4$	0,796
PEG 10000- Na$_2$SO$_4$	0,884
PEG 8000-(NH$_4$)$_2$SO$_4$	1,918
PEG 8000- DEX75	1,738

Table B.3- *Results obtained for the volume ratio of all ATPSs performed in the present work.*

C. Antimicrobial Activity

In this section are all values obtained for the antimicrobial assays. First there is the tables with all the inhibition halos for the agar diffusion assays for all the microorganisms tested. Table C.1 displays the results for the bacteria assay and table C.2 shows the results for fungi and yeast.

Table C.1- *Inhibition halos for all bacteria used for agar diffusion assay. Here are all the concentrations used in the test. The weight (µg) is the value of mass of extract used in a drop of 5 µL.*

Bacteria	200 weight (µg)	200 halo (cm)	100 weight (µg)	100 halo (cm)	50 weight (µg)	50 halo (cm)	25 weight (µg)	25 halo (cm)	20 weight (µg)	20 halo (cm)	15 weight (µg)	15 halo (cm)	5 weight (µg)	5 halo (cm)	1 weight (µg)	1 halo (cm)
E. coli		1,20		0,97		0,87		0,87		1		0,85		0,85		0
P. fluorescens	1000,00	1,57	500,00	1,3	250,00	1,13	125,00	0,87	100	0,9	75	0,85	25	0,75	5	0
P. larvae		2,50		2,23		2		1,77		1,63		1,55		1,30		0,8
Z. mobilis		2,90		2,60		2,4		1,97		2		1,73		1,43		0,73
L. fructivorans		2,20		1,857		1,83		1,57		1,6		1,33		1,03		0,73

Table C.2- *Inhibition halos for all fungi and yeast used for agar diffusion assay. Here are all the concentrations used in the test. The weight (µg) is the value of mass of extract used in a drop of 5 µL.*

Fungus	[Extract] (mg/mL)															
	200		100		50		25		20		15		5		1	
	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)	weight (µg)	halo (cm)
A. flavus	1000,00	1,83	500,00	1,57	250,00	1,27	125,00	1,10	100	1,27	75	1,13	25	0,93	5	0,6
A. carbonarius		2,43		2,2		1,80		1,63		1,87		1,67		1,40		0,93
A. niger		2,52		2,30		1,77		1,53		1,87		1,50		1,47		0,9
P. expansum		2,88		2,65		2,5		2,33		2,37		2,2		1,63		1,13
Mucor sp.		1,67		1,6		1,8		1,53		1,7		1,3		0,60		0
Alternaria sp.		2,13		1,97		1,5		1,40		1,10		1,00		0,73		0,6
W. anomalus		1,83		1,9		1,63		1,6		1,6		1,5		1,45		1