Universidade do Minho
Escola de Engenharia

Marta Alexandra Lages Ferreira

Production of lipids and polyunsaturated fatty acids by solid-state fermentation

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Doutor José Manuel Salgado

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Professora Doutora Isabel Belo

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DECLARAÇÃO

Nome: Marta Alexandra Lages Ferreira

Endereço eletrónico: pg33056@alunos.uminho.pt Telefone: 932841214

Cartão do Cidadão: 14532028 6 ZY7

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Orientadores:

Doutor José Manuel Salgado

Professora Doutora Isabel Belo

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Resumo

A fermentação em estado sólido (SSF) é um processo biotecnológico promissor qu permite o uso de suportes inertes para o crescimento de microrganismos de forma a obter produtos de valor acrescentado como lípidos. O uso deste tipo de suportes para a realização de SSF, como espuma de poliuretano (PUF), oferece melhores processos de extração e permite que este material plástico residual seja reaproveitado.

Os ácidos gordos polinsaturados (PUFAs) são essenciais em dietas saudáveis e a sua produção é extremamente importante, uma vez que os mamíferos não conseguem sintetiza-los, por isso precisam de ingeri-los de fontes alimentares. Existem em produtos de origem animal como o óleo de peixe marinho, no entanto existem problemas associados, como a sobrepesca dos oceanos. Assim, o óleo microbiano é uma fonte preferível de PUFAs. Além disso, outros lípidos microbianos com elevado interesse industrial podem ser produzidos utilizando SSF em suporte inerte, como é o caso do ácido esteárico, linoleico, oleico e palmítico. Por este motivo foi avaliada a produção de lípidos em SSF usando dois microrganismos: um fungo filamentoso (*Mortierella alpina* Peyronel 9412) e uma levedura (*Yarrowia lipolytica W29*).

Num primeiro estudo foi otimizado o meio de cultura para a produção de PUFAs por *M. alpina* Peyronel 9412 em SSF utilizando um suporte inerte (PUF). Verificou-se que a maior quantidade de PUFAs totais (535.41 ± 24.12 mg/g PUF), ácido linoleico (129.66 ± 5.84 mg/g PUF) e ácido α-linoleico (401.93 ± 18.10 mg/g PUF) foi produzida quando o meio continha 20 g/L glucose, 10% (p/v) de óleo de linhaça, o rácio C/N era ajustado a 25 e a temperatura de incubação era de 25 ºC durante 3 dias descendo para 16 ºC nos restantes 4 dias de fermentação. Para além disso, foi também utilizado um hidrolisado hemicelulósico absorvido ao PUF, como meio de cultura de baixo custo em SSF, verificando-se o crescimento e produção de PUFAs por parte do fungo, ainda que em menor quantidade do que com o meio sintético.

Por outro lado, num segundo estudo foi testado o crescimento da levedura *Y. lipolytica W29* e a produção de lípidos em SSF utilizando PUF e a sua reutilização em vários ciclos de fermentação. Verificou-se que a maior quantidade de lípidos foi obtida ao sexto dia de fermentação. O perfil de ácidos gordos produzidos por esta levedura indica uma grande quantidade de ácido esteárico (31.77 ± 1.59%), seguido de ácido palmítico (30.05 ± 1.50%) e linoleico (19.52 ± 0.98%). Foi ainda possível verificar que quando a PUF foi espremida, as

células saíram para o meio de cultura, não sendo, portanto, útil extrair lípidos do suporte, já que a biomassa que fica unida a ele é reduzida. Por fim, foram realizados 3 ciclos de fermentação de 6 dias cada um e só foi adicionado inóculo no início da primeira fermentação, verificando-se que, quando a PUF é espremida após cada ciclo de fermentação, algumas células permanecem unidas a ela, conseguindo multiplicar-se com a adição de meio fresco.

Através deste estudo foi possível concluir que a SSF utilizando um suporte inerte como PUF é um processo biotecnológico adequado a produção de compostos de valor acrescentado como lípidos e, mais concretamente, PUFAs. A otimização dos parâmetros como a temperatura, ratio C/N e a adição de indutores permitirá definir as condições de fermentação de outros substratos naturais como os resíduos agroindustriais.

Abstract

Solid-state fermentation (SSF) is a promising biotechnological process that allows the use of inert supports to the growth of microorganisms to obtain value-added products such as lipids. The use of this type of supports for the realization of SSF, such as polyurethane foam (PUF), offers better extraction processes and allows this residual plastic material to be reused.

Polyunsaturated fatty acids (PUFAs) are essential in healthy diets and their production is extremely important, since mammals cannot synthesize them, so they need to ingest them from food sources. They are present in animal products such as marine fish oil, however there are associated problems such as overfishing of the oceans. Thus, microbial oil is a preferable source of PUFAs. In addition, other microbial lipids of high industrial interest can be produced using SSF in an inert support, such as stearic, oleic and palmitic acid. For this reason, the lipid production in SSF was evaluated using two microorganisms: a filamentous fungus (*Mortierella alpina* Peyronel 9412) and a yeast (*Yarrowia lipolytica W29*).

In a first study, the culture medium for the production of PUFAs by *M. alpina* Peyronel 9412 in SSF was optimized using an inert support (PUF). The highest amount of total PUFAs (535.41 ± 24.12 mg/g PUF), linoleic acid (129.66 ± 5.84 mg/g PUF) and α-linoleic acid (401.93 ± 18.10 mg/g PUF) were produced when the culture medium contained 20 g/L glucose, 10% (w/v) linseed oil, the C/N ratio was adjusted to 25 and the incubation temperature was 25 °C for 3 days decreasing to 16 °C on the remaining 4 days of fermentation. In addition, a hemicellulosic hydrolysate absorbed to PUF was also used as a low-cost culture medium in SSF, with the growth and production of PUFAs being observed by the fungus, although in a smaller amount than with the synthetic medium.

On the other hand, in a second study was tested the growth of yeast *Y. lipolytica W29* and the production of lipids in SSF using PUF as well as its reuse in several fermentation cycles. It was found that the highest amount of lipids was obtained on the sixth day of fermentation. The fatty acid profile produced by this yeast showed a large amount of stearic acid (31.77 ± 1.59%), followed by palmitic acid (30.05 ± 1.50%) and linoleic acid (19.52 ± 0.98%). It was also possible to verify that when the PUF was squeezed, the cells were released to the culture medium, therefore, it is not useful to extract lipids from the support, since the biomass that is attached to it is reduced. Finally, 3 cycles of fermentation of 6 days each were carried out and only inoculum was added at the beginning of the first cycle. It was verified that when the

PUF is squeezed after each fermentation cycle, some cells remain attached to it, being able to multiply with the addition of fresh medium.

Through this study it was possible to conclude that SSF using an inert support such as PUF is a suitable biotechnological process for the production of value-added compounds such as lipids and, more specifically, PUFAs. The optimization of parameters such as temperature, C/N ratio and the addition of inductors will allow to define the fermentation conditions of other natural substrates such as agroindustrial residues.

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List of abbreviations

ALA α-linolenic-acid
ANOVA analysis of variance
ARA arachidonic acid
a_w water activity
BSG Brewer's spent grains
CLA conjugated linolenic acid
DHA docosahexaenoic acid
DNS dinitrosalicylic acid
EDS energy-dispersive X-ray spectroscopy
EPA eicosapentaenoic acid
FAMES fatty acid methyl esters
FID flame ionization detector
GC gas chromatography
GLA γ-linolenic acid
GRAS generally regarded as safe
H Humidity
HMF 5-hydroxymethylfurfural
HPLC High Performance Liquid Chromatography
L:S liquid:solid
LA linoleic acid
MUM Micoteca da Universidade do Minho
OD optical density
P *p-value*
PDA potato dextrose agar
PUF Polyurethane foam
PUFAs Polyunsaturated fatty acids
SCO single cell oil
SD standard deviation
SEM scanning electron microscope
SmF submerged fermentation
SSF solid-state fermentation
w/v weight/volume
w/w weight/weight

CONTEXT AND MOTIVATION

Microbial oil, known as single cell oil (SCO), has long been recognized as a good source of lipids, such as polyunsaturated fatty acids (PUFAs) (Fakas *et al*., 2009a). These essential fatty acids also exist in plants, being present in some seeds in the form of C18 but they cannot produce longer chains without genetic manipulation. They also exist in animal products like marine fish oil, but beyond the problem with oceans overfishing, fish oil can contain contaminants such as heavy metals or dioxins, reason why it is not a reliable source of PUFAs (Asadi *et al*., 2013). It is well established that some fungi, such as *Mortierella alpina,* possess the ability of lipid accumulation and are well known for their tendency to produce essential fatty acids on various substrates (Béligon *et al*., 2016). However, there is few information on the cultivation of this fungus in SSF, in particular using an inert support, such as polyurethane foam (PUF). The study of growth conditions, including pH, temperature, carbon source, nitrogen source and supplementation of the growth medium is of the utmost importance, because these factors affect mycelial growth, the fatty acid composition of the fungus and these conditions could be different between submerged and solid-state fermentation.

Currently, there are more studies on submerged fermentation than solid-state fermentation using yeasts, such as *Yarrowia lipolytica*, and even less using inert supports. Additionally, the utilization of PUF as an inert support is a way to reuse and valorize this type of material, since millions of tons of this plastic residue are generated per year worldwide.

RESEARCH AND AIMS

The main goal of this thesis is the production of polyunsaturated fatty acids and lipids by solid-state fermentation on inert support (polyurethane foam). In this sense, several partial objectives were planned:

- Optimization of several fermentation parameters for PUFAs production by *M. alpina* Peyronel 9412 in SSF;
- Evaluation of incubation time for lipids production by *Y. lipolytica* W29 in SSF;

- Evaluation of reuse of PUF in several cycles of fermentation for lipids production by *Y. lipolytica* W29 in SSF.

1. INTRODUCTION

1.1. Solid-state fermentation

Solid-state fermentation (SSF) is a bioprocess that has potential applications in the production of biologically active secondary metabolites, such as antibiotics, plant growth factors, enzymes, alkaloids, biopesticides and organic acids (Singhania *et al*. 2009). It is a fermentation process that occurs in the absence or near absence of free water, however the substrate must contain sufficient moisture to allow the microorganism growth and metabolism (Thomas *et al*., 2013). The solid matrix may be the source of carbon (and other nutrients) or it may be an inert material whose function is to support the growth of microorganisms in it (with impregnated growth solution) (Thomas *et al*., 2013). Sometimes it is necessary to apply a pretreatment (chemical or mechanical) or add supplementation of some nutrients to substrates before being used in the SSF process (Pandey *et al*., 1999).

The main differences between SSF and submerged fermentation (SmF) are the microbial growth, product formation and energy requirements. In general, SSF has many advantages regarding SmF. It has lower energy requirement, it generates lower amounts of wastewater and with less risk of bacterial contamination, it has the possibility to use low cost media, the products have higher yield and the degradation of the enzymes by undesirable proteases is minimized (Couto and Sanromán, 2006; Hölker and Lenz, 2005; López-Pérez and Viniegra-González, 2015). There are several studies in the literature comparing both type of fermentations. For example, the filamentous fungus *Aspergillus caespitosus* was used to produce intracellular and extracellular invertases under SmF and SSF on wheat bran as carbon source and the production of extracellular invertase in SSF was 2.73 times superior (Alegre *et al*., 2009). Seesuriyachan *et al*., 2010 compared the production of extracellular polysaccharides using *Lactobacillus confusus* with coconut water and sugarcane juice as natural wastes. In SSF they obtained high concentrations of the compounds (62 and 18 g/L with sugarcane juice and coconut water, respectively). Maldonado and Strasser de Saad, 1998 reported the production of pectinesterase and polygalacturonase by *Aspergillus niger* and concluded that, with pectin as a sole carbon source, it was four and six times higher respectively in a solid-state system than in a submerged fermentation and required a shorter time for enzyme production.

To gain advantages using SSF, it is also necessary to provide the microorganisms an environment as close as possible to natural environment where usually they exist and from where they are isolated. This could be the principal factor why microorganisms give higher products yields in SSF when compared with SmF carried out in a closed bioreactor, even if with optimal conditions for growth were used (Thomas *et al*., 2013).

Generally, fungal and yeast cultures are the most suitable microorganisms for SSF processes, because they have lower water activity requirements, typically around 0,5–0,6 a_w. This contrasts with bacterial cultures that have higher water activity requirement (around 0,8–0,9 a_w), which make them not suitable for SSF processes (Thomas *et al*., 2013). Some examples of microorganisms that can grow and produce several compounds in SSF are *A. niger*, that has been employed to produce citric acid from kumara, a starch-containing root crop (Lu *et al*., 1998); *Saccharomyces cerevisiae* produced ethanol on sorghum bagasse (Yu *et al*., 2007); *Aureobasidium pullulans* for cellulase and hemicellulase production on wheat bran (Leite *et al*., 2007) and *Kluyveromyces marxianus* produced fruity aroma compounds using cassava bagasse and giant palm bran as substrates (Medeiros *et al*., 2000).

Although, SSF has also some disadvantages, such as difficulties in monitoring the process parameters like heat buildup, moisture level of the substrate and aeration, difficulty in rapid determination of microbial growth and the existence of limited types of microorganisms that can grow at low moisture levels (Durand and Chereau, 1988). However, this can be an advantage too, since decrease the risk of contamination.

1.1.1. Inert supports

The cultivation in SSF can use natural materials that serve as a carbon source (and other nutrients) and support, such as potato, cassava, or beans (Pandey, 1992; Thomas *et al*., 2013), or it can use an inert material whose function is only to support the growth of microorganisms in it, serving as an anchor and avoiding the presence of possible inhibitors that can be in natural supports (Ooijkaas *et al*., 2000). The materials used are porous, biologically inactive and absorb the defined culture media and the inoculum. Thus, there is not interaction between the microorganism and the support and its characteristics remain unchanged (Chen, 2013). The materials utilized can be diverse, such as perlite (Weber *et al*., 1999), vermiculite (Silman *et al*., 1991) and polyurethane foam (PUF) (Murado *et al*., 1997; Zhu *et al*., 1996). This last one has been very used because of its low density, relatively high

water absorption and high porosity with the adequate pore size, which supplies a good environment for fungal growth (Chen, 2013), readily available, low cost and can be used at industrial scale (Cui *et al*., 2013). For example, Zhu *et al*., 1994 used PUF as an inert support which, mixed with medium, simulated the chemical composition of wheat bran, to produce nuclease P1 from *Penicillium citrinum*; John *et al*., 2007 produced L-lactic acid in SSF using PUF as an inert carrier moisturized with cassava bagasse starch hydrolysate; López *et al*., 2009 used PUF for the cultivation of *Pichia pastoris* to produce a heterologous laccase. Furthermore, renewable resources can be used to perform reactions in order to produce PUF, such as soybean oil (Sonnenschein and Wendt, 2013), castor oil (Mosiewicki *et al*., 2009) and palm oil (Tanaka *et al*., 2008).

PUF materials have a broad range of applications, since automobile seating, furniture or carpets, which inevitably leads to many PUF wastes production. For example, approximately 1.3 million tons of waste polyurethane are generated each year in the United States of America. The utilization of this material as an inert support is a way to reuse and valorize this type of residue (Nikje *et al*., 2011).

The use of natural substrates could have a major disadvantage: the carbon source and the support are the same material, therefore the solid medium is degraded during the growth of the microorganism. This could lead to a change of the physical characteristics of the solid, decrease its porosity and reduction of the heat and mass transfer (Barrios-González and Mejía, 1996). For example, according to Weber *et al*., 1999, the evaporation of water causes shrinkage in oats which prevents homogeneous aeration and adequate heat removal.

SSF on inert supports also offers better downstream processes, because it is easier to extract products from the support, and they can be obtained with fewer impurities compared with the natural substrates. Also, the support can be reused (Ooijkaas *et al*., 2000). For example, according to Ramadas *et al*., 2012 one of the major problems associated with the use of SSF to produce poly-3-hydroxybutyrates is the difficulty in recovering the bacterial cells from the substrate after fermentation. This can be solved by using an inert support in SSF, such as PUF. For the extraction of cells, they made some washing steps with distilled water and scanning electron microscope (SEM) images revealed that most of the biomass could be recovered in this way.

The fermentation process on inert materials also allows the use of a synthetic medium, where the components are defined. This is a very important parameter, since the

nutritional component types and their proportions leads to differences in microbial growth and metabolism. Otherwise, it is not easy to change the composition of natural substrates that are used as a carbon source and evaluate which component affects the most the fermentation process (Chen, 2013).

Finally, the fact that inert supports have almost constant physical structure during the fermentation process, facilitates reproducible and detailed physiological and kinetic studies in SSF, which will be necessary for efficient process development, control strategies and reactor design (Lareo *et al*., 2006; Ooijkaas *et al*., 2000).

1.2. Lipids

Lipids has been recognized as versatile molecules that are involved in various cellular processes. They represent an important compound in energy storage, are present in cell membranes such as plasma and nuclear membrane and in the endoplasmic reticulum, the Golgi apparatus, in vesicles like endosomes and lysosomes and are also important in cell signaling as steroid hormones or messenger molecules that carry signals of cell surface receptors to target molecules inside the cell (Cooper, 2000; Klose *et al*., 2013).

The lipid production may be accomplished by oleaginous microorganisms who can accumulate microbial lipids in their cells and can use agro-industrial wastes as carbon sources, which makes the process more cost effective (Liu *et al*., 2013). Lipids may be accumulated via two different pathways in oleaginous microorganisms: *de novo* synthesis, which involves the production, under defined conditions, of fatty acid precursors and their integration into the storage lipids biosynthetic pathway; and the *ex novo* accumulation pathway which involves the uptake of fatty acids, oils and triacylglycerols from the culture medium and their accumulation in an unchanged or modified form within the cell (Beopoulos *et al*., 2009).

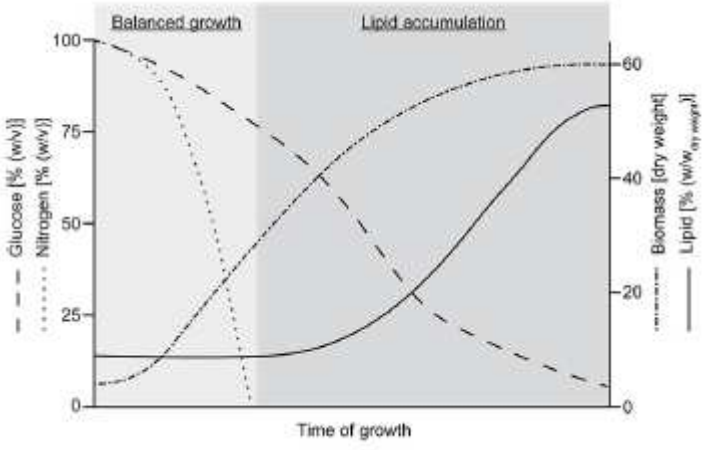
1.2.1. Production of lipids by microorganisms

There are some oleaginous yeasts that are a promising source of microbial oil, some strains are able to accumulate up to 70% of their dry weight in lipids (Angerbauer *et al*., 2008). These are accumulated as discrete fat globular deposits and can also be associated in minor quantities with different cell organelles (Ageitos *et al*., 2011). Yeasts exhibit advantages for lipid production over other microbial sources. They have the capacity to generate lipids from

various carbon sources, including lipids that are in the culture medium. They can also vary their lipid composition through replacing the fatty acids in the triglycerides with those present in the media (Iassonova *et al*., 2008). Yeasts have also a short duplication time (normally lower than 1 h), their cultures are easily scaled up (more than those of microalgae) and are less affected than plants by climate conditions. Some examples of oleaginous yeasts include *Rhodotorula glutinis*, *Yarrowia lipolytica*, *Rhodosporidium toruloides* and *Lipomyces starkeyi* (Ageitos *et al*., 2011).

Y. lipolytica is a dimorphic, non-pathogenic and aerobic yeast that has the ability to metabolize hydrophobic substrates such as fatty acids, lipids and alkanes and can be found in marine environments, soils contaminated with oils and in wastewaters (Ledesma-Amaro and Nicaud, 2016; Moftah *et al*., 2013; Rakicka *et al*., 2015). Several processes in which this yeast participated, they were considered as GRAS (generally regarded as safe) (Amaral *et al*., 2006). The SCO produced by *Y. lipolytica* is composed by stearic, linoleic, oleic and palmitic acid (Papanikolaou *et al*., 2002). According to Karatay and Dönmez, 2010 the lipid accumulation of this yeast in molasses medium included fatty acids C16 (palmitic and palmitoleic acid) and C18 (stearic, oleic and linoleic acid) which means these crude lipids can be a potential feedstock for the production of biofuel. Also, according to Katre *et al*., 2012 the SCO from *Y. lipolytica* 3589 appears to be a promising feedstock for biodiesel production since it has higher quantities of saturated and monounsaturated C16 and C18 fatty acids and lower concentration of long chain PUFAs.

There are some conditions that enhance lipid accumulation in oleaginous microorganisms: when the carbon source is present in excess and a compound of the growth medium becomes limiting. The most common is nitrogen limitation, because is the most efficient for inducing lipid accumulation (Béligon *et al*., 2016). While all nutrients are in excess,



occurs biomass production and consumption of the carbon and nitrogen source. When the nitrogen is exhausted, the microorganisms continues to assimilate the carbon source that is channeled into lipid synthesis, that leads to the emerge of triacylglycerols within the cell as oil droplets (**Figure 1**) (Ochsenreither *et al*., 2016; Ratledge, 2004).

Figure 1 - Typical development of lipid accumulation by oleaginous microorganisms (Ochsenreither *et al*., 2016).

1.2.2. Production of lipids by SSF

The lipids production evolves associated costs and SSF can use inexpensive substrates as agro-industrial wastes in this process (Liu *et al*., 2013). For example, Zhang and Hu, 2011 used soybean hull as solid substrate for *Mortierella isabellina*, achieving 47.9 mg lipid from 1 g soybean hull, 30% of total lipids consisted in fatty acids and 80.4% of total fatty acid was C16 and C18. Cheirsilp and Kitcha, 2015 utilized *Aspergillus tubingensis* TSIP9 to produce 39.5 ± 2.2 mg of lipids per gram of substrate (palm empty fruit bunches). Hui *et al*., 2010 concluded that using pure cellulose in SmF, *Aspergillus oryzae* A-4 yielded lipid 39.08 mg/g substrate. In SSF, using wheat straw and bran mixture, the same microorganism accumulated lipid of 62.87 mg/g dry substrate, under the optimized conditions from Plackett–Burman design. Vong *et al*., 2016 proceeded to okara (soybean residue) fermentation by *Y. lipolytica* and observed that the fat content increased from 7.41 to 8.73 g/100 g dry matter after fermentation. Also, the lipases produced by the microorganism could have hydrolyzed the lipid content of okara and that could lead to a triglyceride accumulation in *Y. lipolytica,* which can then be stored or consumed for energy.

1.2.3. Polyunsaturated fatty acids (PUFAs)

Polyunsaturated fatty acids (PUFAs) of the ω-3 class such as eicosapentaenoic acid (EPA or C20:5*n*-3), α-linolenic acid (ALA or C18:3*n*-3) and docosahexaenoic acid (DHA or C22:6*n*-3) and of the ω-6 class, like arachidonic acid (ARA or C20:4*n*-6), γ - linoleic acid (GLA or C18:3*n*-6), linoleic acid (LA or C18:2*n*-6) and conjugated linolenic acid (CLA) are essential due to affecting various physiological functions in the human body (Béligon *et al*., 2016). They are structural components of the membrane phospholipids and precursors of the eicosanoids (hormone-like substances that influence the immune, cardiovascular and central nervous systems, such as prostaglandins, thromboxanes and leukotrienes) (Dyal and Narine, 2005;

Ochsenreither *et al*., 2016). Furthermore, PUFAs are present in the cerebral cortex, retina and are components of immunocompetent cells like neutrophils and monocytes. Mammals cannot synthesize these essential fatty acids, so they should ingest from food sources (**Table 1**).

Table 1 – Food sources of PUFAs (Adapted from Béligon *et al*., 2016).

Family	PUFA	Food sources
ω-3 class	ALA	Vegetable oil (flaxseed oil, canola oil, chia seed oil, egg, meat, walnut, hazelnut); Fatty fish oil
	EPA	Fatty fish oil
	DHA	Fatty fish oil, breast milk
ω-6 class	LA	Vegetable oil, nuts
	GLA	Evening primrose oil, borage oil
	ARA	Egg yolk, animal fat, breast milk

ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; LA: linoleic acid; GLA: γ - linoleic acid; ARA: arachidonic acid.

PUFAs exist in plants, being present in some seeds in the form of C18 but they cannot produce longer chains without genetic manipulation. They also exist in animal products like marine fish oil, but there are problems with oceans overfishing and fish oil should also not be used as a source of supplements because it can contain heavy metals or dioxins, which are contaminants (Asadi *et al*., 2013).

On the other hand, there are oleaginous microorganisms that can accumulate SCO recognized as a good source of lipids, such as PUFAs (Béligon *et al*., 2016).

1.2.4. Production of PUFAs by microorganisms

The accumulation of PUFAs by microorganisms has various advantages compared with animal or plant sources, because it is independent from location, climate and season, does not require the use of arable land and microorganisms can be cultivated in various types of substrates, even on industrial wastes or renewable carbon sources (Béligon *et al*., 2016; Ochsenreither *et al*., 2016). The choice of carbon source has an increased significance because it can constitute about 60% of the total production cost in fermentation processes, so low-cost carbon sources should be considered (Béligon *et al*., 2016). Furthermore, microorganisms

are preferable to produce PUFAs to be used as food additives, dietary supplements or in feedstock unlike animal sources because of problems like cholesterol supply, bad taste and odor (Asadi *et al*., 2013; Ghobadi *et al*., 2011).

Several microorganisms were used to produce PUFAs, such as mycobacteria (*Mycobacterium phlei* and *Mycobacterium smegmatis*) (Fernandes *et al*., 2017), fungi as *Cunninghamella elegans* (Conti *et al*., 2001), *Cunninghamella echinulata* (Gema *et al.,* 2002*),* and various of *Mortierella* species like *M. alpina* (Jang *et al*., 2000), *M. isabellina* (Papanikolaou *et al*., 2004) and *M. alliacea* (Aki *et al*., 2001). In fact, there are various advantages that makes filamentous fungi, such as *M. alpina*, convenient microbial candidates for SSF processes. The fungal mycelia rapidly cover the surface of materials, the hyphae penetrate quickly in the substrates which causes it to be efficiently consumed, the fungi produce enzymes necessary for hydrolysis of substrates biopolymers, they also decrease the amount of anti-nutrient compounds (such as phytic acid) and can grow at reduced water activity, which avoids bacterial pollution (Certik *et al*., 2012).

M. alpina can produce a high amount of lipids (up to 50% of its dry weight) which are mainly composed by triacylglycerol with high quantities of ARA (Ratledge and Wynn, 2002; Ratledge, 2004). These lipids are accumulated inside the hyphae and, with time, form droplets on the mycelia (**Figure 2**) (Wang *et al*., 2011a). Jang *et al*., 2000 used *M. alpina* ATCC 3222 to produce 122.2 mg of PUFAs per gram of substrate (rice bran), including 54.5 mg of ARA, 47.8 mg of LA, 12.8 mg of EPA and 7.1 mg of ALA. Jang and Yang, 2008 also used *M. alpina* ATCC 3222 on rice bran and had a production of 127 mg of total PUFAs per gram of substrate, 117 mg of LA, 12 mg of EPA, 6 mg of ARA and 5 mg of ALA.

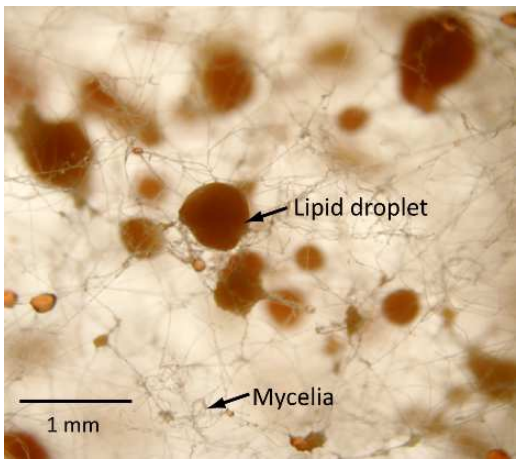


Figure 2 – Fungal culture on PDA plate stained with 0,5% triphenoltetrazolium chloride. The lipid droplets are stained brown (Wang *et al*., 2011a).

1.2.5. Production of PUFAs by SSF

Microbial production of PUFAs can be managed in both types of fermentation, however, in general, SSF has many advantages regarding SmF (Certik and Shimizu, 1999). According to Jang and Yang, 2008 the production of PUFAs per gram of substrate was higher in SSF than in SmF, and it was produced more PUFAs of the ω-3 class. Furthermore, the product obtained by SSF might be dried and directly consumed without any extraction treatment.

The production of PUFAs is also affected by the cost of the raw material, whereby the selection of growth media is very important. The most economic efficient substrates are the agro-industrial wastes, mainly when the oil is destined for human consumption. After appropriate pretreatment, the substrate could be fermented and with favorable conditions and sufficient quantities of inoculum, it can produce biomass rich in oil with PUFAs (Certik and Shimizu, 1999).

The utilization of agro-industrial wastes has another advantage since the fungi used could also decrease anti-nutrient compounds in the substrates (such as phytic acid) and partially hydrolyze substrate biopolymers, therefore prefermented mass with high content of PUFAs could be used as inexpensive food and feed supplement (Certik and Shimizu, 1999; Certik *et al*., 2012).

1.2.6. Use of inductors for PUFAs production

Oil supplements can be added to the medium since they can contribute to increase lipid accumulation and, additionally, influence the composition of the final product since the fatty acids present in the oils may function as precursors of PUFAs and thus stimulate their production (Asadi *et al*., 2013; Koritala *et al*., 1987). Filamentous fungi that possess an active oil-biotransforming system can utilize exogenous oils with fatty acid precursors and convert them to PUFAs (Certik and Shimizu, 1999). In order to increase the ω-3 PUFA production on substrates by oleaginous microorganisms, it could be added an oil rich in the ω-3 precursor to the substrate before fermentation (Dyal and Narine, 2005; Jang *et al*., 2000). For example,

linseed oil, which contains ~57% of the ω-3 precursor ALA, could be used as a supplement (Jacobs *et al*., 2009).

For instance, Jang *et al*., 2000 tested the effect of adding 1% soybean oil and 1% linseed oil to the solid substrate (rice bran) for production of PUFAs by *M. alpina* ATCC 32222 and verified that it could increase the production of LA by 84.9 and 36%, of EPA by 130.6 and 92.1%, and of ARA by 72 and 42.1%, respectively. They also concluded that the fatty acids present in the oils serve as precursors, thereby increasing the production of PUFAs, and that soybean oil and linseed oil are rich in oleic acid, LA and ALA.

Furthermore, Jacobs *et al*., 2010 utilized sunflower press cake as solid substrate and it was supplemented with 10% w/w linseed oil, which contains the ω -3 precursor ALA. The results showed the production of EPA was increased due to metabolization of ALA to EPA and *M. alpina Mo 46* and *M. basiparvispora* produced the highest EPA content, within the eight strains of the genus *Mortierella* studied.

Additionally, Certik *et al*., 2013 tested the addition of three types of oils at four concentrations to the substrate (cereals): sunflower oil (67% of linoleic acid), olive oil (68% oleic acid) and linseed oil (50% α-linolenic acid). The results show that, after growth of M. *circinelloides*, the maximal yield of GLA was achieved when sunflower oil was added to the substrate at 30% concentration, which represents the increase in 7.1 times compared to the fermentation without oil. Olive oil improves GLA accumulation by 6.3 times and linseed oil enhances GLA yield up to 3.5 times.

1.2.7. Downstream processing

The objective of lipid extraction is to separate efficiently the desired lipids from the other cellular components, such as proteins, polysaccharides, and other small molecules. The characteristics of the microorganisms like cell wall structure, chemical structure, and lipids content, characteristics and location should be considered (Mustafa and Turner, 2011). Furthermore, the application of the lipids is also a parameter to take in account to choose the best extraction method and solvents, for example, when the lipids are necessary for food application, it is preferable to use non-toxic and non-harmful solvents, easy to remove and recover (Ochsenreither *et al*., 2016). The polarity of lipids also influences the choose of solvents, since storage lipids, such as triacylglycerols, are neutral lipids, so it can be extracted

with non-polar solvents like chloroform and hexane. Lipids with high polarity, such as phospholipids and glycolipids are therefore extracted with more polar solvents, like alcohols. The mixture of the two types can improve the extraction efficiency since the polar solvent improves the release of lipids from protein lipid complexes and they dissolve in non-polar solvents (Li *et al*., 2014).

There are classical extraction methods, such as Soxhlet extraction in which the sample is dried, reduced to a fine powder, placed on a porous thimble inside the extraction chamber and there are made some washing rounds with an organic solvent (originally petroleum ether) under reflux. After extraction, the solvent is evaporated and the lipids are weighed (Ochsenreither *et al*., 2016). There's also the Folch extraction method to recover total lipids, that uses the chloroform: methanol (2:1) solvent system and the addition of salts to the crude extract. Through washing the crude extract with water or a salt solution, a biphasic system is formed, the lipid fraction stays in the lower phase and the non-lipid fraction in the upper (watery) phase (Folch *et al*., 1957). This is considered a standard method for the extraction of microbial lipids, however, other solvent systems have been studied to exchange toxic chloroform and methanol mixtures. Hexane has been applied as unpolar substitute and has good results when extracting triacylglycerols (Ochsenreither *et al*., 2016).

1.2.8. Application of PUFAS

In the food industry, lipids rich in PUFAs are highly demanded and used as food additives to improve and/or supplement the fatty acid composition of specific foods such as infant food. The microbial lipid rich in PUFAs can be added as pure oil or in the form of stable emulsions (Bellou *et al*., 2016). Otherwise, several agricultural products, like cereals and agro-industrial wastes such as rice bran, apple or pear pomace and sweet sorghum can be enriched in PUFAs using solid or semi-solid state fermentation with microorganisms that can produce PUFAs and directly used as food and/or feed supplements (Economou *et al*., 2010; Fakas *et al*., 2009b; Jang *et al*., 2000).

In the biomedical field, it has been studied the improvement of therapeutic efficacy of anticancer drugs using PUFA–drug conjugates with potent antitumor activities. Due to their lipophilic nature, PUFAs could cross the lipid bilayer of tumor cells, therefore, they can be used as a carrier to increase the therapeutic efficacy of anticancer drugs (Wang *et al*., 2011b). Also, the supplementation with ω-3 PUFA is being studied to work as a potential treatment

or preventive agent for several diseases, such as hypertension, heart failure or atherosclerosis (Cicero *et al*., 2012).

1.3. Lignocellulosic hydrolysate

Agro-industry, specially the food industry, generates large amounts of liquid, solid and gaseous wastes, which come from the processing operations, their treatment and disposal (Vandamme, 2009). They can be so diverse as straw, bran (rice and wheat), cassava, fruits, potato, beet, bagasse, pomace, press cake (sunflower, palm kernel soybean and olive), cocoa pod, peanut meal and brewer's spent grain (BSG) (Asadi *et al*., 2013). Most of these are used as animal feed or burned for elimination. However, these wastes are mainly composed by sugars, fibres, proteins and minerals, which are compounds of industrial interest. It is important to reuse them to take advantage of their composition rich in compounds that could be used in other processes and their large availability. Also, there are economic benefits since such wastes may be used as low-cost raw materials to produce other value-added compounds and with the expectation of reducing the production costs, and environmental benefits, because most of the agro-industrial wastes contain phenolic compounds and/or other compounds of toxic potential, which could cause environmental problems when the waste is landfilled (Mussatto *et al*., 2012).

The brewery industry generates a relatively large number of by-products and wastes, such as spent hops, yeast and BSG. This last one is the most abundant by-product, corresponding to about 85% of total generated (Mussatto *et al*., 2006). In Portugal, during 2013 were produced 4.15 x 10^9 kg of residues from this industry (INE, 2014). The BSG is considered as a lignocellulosic material rich in protein and fibre, which account for around 20 and 70% of its composition, respectively. Lignocellulosic materials are composed by cellulose, hemicellulose and lignin (Mussatto *et al*., 2006).

The cellulose structure is a linear homopolymer composed by glucose units, that presents a degree of polymerization that varies between 1000 and 10000 units. The breaking of this molecule requires the presence of catalysts (acids or enzymes) and it performs a supporting function in the plant, acting as a skeleton (Fan *et al*., 1982; Salgado, 2009).

On the contrary of the cellulose, hemicellulose is a heterogeneous polymer composed by different monosaccharides: arabinose, galactose, glucose, mannose and xylose. Its mission in plants is to facilitate the chemical bonds of cellulose with lignin, acting as a binding agent between the fibers. Hemicelluloses can be hydrolyzed to sugars more easily than cellulose, although the reaction must also be catalyzed by acids or enzymes (Mussatto *et al*., 2012; Salgado, 2009).

The lignin structure is formed by phenylpropane units linked in a large and very complex three-dimensional structure. It is closely bound to cellulose and hemicellulose and its function is to provide rigidity and cohesion to the material cell wall and to form a physic–chemical barrier against microbial attack. Due to these functions, lignin shows resistance to chemical and enzymatic degradation (Fan *et al*., 1982; Mussatto *et al*., 2012).

There are some chemical procedures used for the isolation of the hemicellulosic fraction. The use of this fraction requires its previous passage to oligo or monosaccharides, which can be done with dilute acids and elevated temperatures. The objective is the almost total solubilization of the hemicelluloses and part of the lignin (lignin soluble in acid), leaving a solid residue composed of cellulose and most of the lignin (Salgado, 2009). The acid more utilized for acid pre-hydrolysis is sulfuric acid (H_2SO_4). This method has several advantages, since it allows to recover a high portion of hemicellulosic sugars, it achieve high reaction rates and the price of sulfuric acid is much lower than the enzymes used in enzymatic hydrolysis (Taherzadeh and Karimi, 2007). Besides, the cellulosic fraction can be isolated in a second stage, being able to take advantage of the cellulosic fraction as the hemicellulosic (Moldes *et al*., 2002). However, this method has also some disadvantages, it can be formed potential inhibitory compounds, such as furfural, 5-hydroxymethylfurfural (HMF), levulinic acid, acetic acid and formic acid and it is necessary a neutralization of pH to use the hydrolysate (Carvalheiro *et al*., 2004; Taherzadeh and Karimi, 2007).

The BSG hydrolysate has been study in order to produce value-added compounds. For example, Mussatto and Roberto, 2005 used the hemicellulosic fraction of BSG hydrolysed with diluted acid under different conditions of liquid/solid ratio (8–12 g g^{-1}), sulfuric acid concentration (100–140 mg g^{-1} dry matter) and reaction time (17–37 minutes) to produce a liquor with a large amount of xylose and good fermentability to produce xylitol by *Candida guilliermondii*. In other study, Pejin *et al*., 2017 used BSG hydrolysate for lactic acid fermentation by *Lactobacillus rhamnosus* ATCC 7469.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Inert support

Polyurethane foam (PUF) was used as an inert support. PUF was provided by building materials supply store in the form of foam panels (1 m^2). The apparent density of the material was 25 kg/m^3 and it was cut into 1 cm^3 cubes before use. The PUF was stored at room temperature in bags hermetically sealed.

2.1.2. Raw material

Brewer's spent grain (BSG) was obtained from a brewery industry in the north of Portugal (Cerveja Letra, Vila Verde) during the season 2017/2018. The residue was dried at 55 ºC during 48 h. Then, the residue was stored at room temperature and dry conditions.

2.1.3. Reagents

Table 2 - List of reagents used during the study.

Reagents	Company
Activated charcoal	Sigma-Aldrich
Agar	Labkem
Ammonium sulfate	Sigma-Aldrich

Boron trifluoride-methanol solution	Sigma-Aldrich
Calcium carbonate	AppliChem Panreac ITW Companies
Calcium chloride dihydrate	AppliChem Panreac ITW Companies
Chloroform	Fisher Chemical
Dipotassium phosphate	Merck
Fructose	Acros-Organics
Glucose	Acros-Organics
Glycerol	Fisher Chemical
Hexane	Fisher Chemical
Lactose	DIFCO Laboratories
Magnesium Sulfate Heptahydrate	AppliChem Panreac ITW Companies
Methanol	Fisher Chemical
PDA	Sigma-Aldrich
Pentadecanoic acid	Sigma-Aldrich
Peptone	VWR Chemicals Prolab
Potassium hydroxide	AppliChem Panreac ITW Companies
Sucrose	Sigma-Aldrich
Sulfuric Acid	Fisher Chemical
Xylose	Sigma-Aldrich
Yeast extract	Labkem

2.1.4. Microorganisms

Two microorganisms were used in this study, the filamentous fungi *Mortierella alpina* Peyronel 9412 and the yeast *Yarrowia lipolytica* W29 *(ATCC 20460*). *M. alpina* Peyronel 9412 was obtained from MUM (Micoteca of University of Minho, Braga, Portugal), where it was preserved in glycerol stocks stored at - 80 °C. It was revived on potato dextrose agar (PDA) petri dishes (4 g/L potato extract, 20 g/L dextrose and 15 g/L agar). To obtain inoculum for SSF, the fungus was incubated in PDA medium at 25 ºC for 6 days*. Y. lipolytica* W29 was grown in petri dishes with yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) and agar (30 g/L) and incubated at 28 ºC for 1 day. During the experimental period, strains were preserved at 4 ºC and cultured monthly on fresh medium.

2.2. Solid-state fermentation on polyurethane foam with *M. alpina* Peyronel 9412

The fermentations were performed in Erlenmeyer flasks of 500 mL where it was weighed 2 g of PUF in cubes (1 cm^3). The synthetic medium was prepared with 40 mL of 50 g/L glucose, 5 g/L yeast extract, 4 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2 \cdot 2H_2O$ and the pH was adjusted to 6.5. The flasks with medium and PUF were sterilized separately at 121 ºC, 15 min. Then, in the laminar flow hood the Erlenmeyer flasks with PUF were inoculated with 15 mg of fungus mycelium (which corresponds to half of a petri dish), it was added the medium and incubated at 22 ºC for 7 days. Once a day the Erlenmeyer flasks were shaken. Cultivations were made in duplicate.

After each SSF on inert support, the PUF was recovered from the Erlenmyer flasks with the aid of tweezers, introduced into a syringe and squeezed, in order to recover the liquid phase. A sample of this phase (10 mL) was stored at -20 ºC. The PUF was then placed in a container and dried at 55 ºC (24 h).

Several studies about carbon sources, inductors addition, ratio C/N, temperature and concentration of inductor were performed. In all studies, the sterilization and inoculation processes were carried out in the same conditions described previously on these fermentations.

In order to evaluate the growth capacity of *M. alpina* Peyronel 9412 with different carbon sources, the glucose previously utilized in the basal medium was replaced by 50 g/L glycerol, 50 g/L sucrose, 50 g/L xylose, 50 g/L fructose and 50 g/L lactose.

Then, to test if the addition of inductor oils increased the lipid and PUFAs production, the basal medium with a lower concentration of glucose (20 g/L) was supplemented with commercial 10% (w/v) linseed oil, olive oil, sunflower oil and castor oil. These oils were added to the flasks with medium and sterilized.

After that, the effect of C/N ratio was studied by adjusting it to 15, 25 and 40 (w/w) altering the amount of yeast extract added to the medium.

Then, to study the temperature effect, fermentation assays were realized by incubating at 14 ºC, 16 ºC, 18 ºC, 22 ºC, 25 ºC for 7 days and also at a higher temperature for 3 days (22 ºC and 25 ºC) lowering in the following 4 days (14ºC, 16 ºC and 18 ºC). In these experiments the concentration of glucose, linseed oil and the C/N ratio were kept constant.

Finally, different concentrations of commercial linseed oil were tested in the medium (2% and 5%) (w/v) to verify if a diminished amount of oil would cause a similar accumulation of lipids and PUFAs. In these experiments the concentration of glucose, C/N ratio and temperature were kept constant.

Control fermentations were also performed using the synthetic culture medium initially described supplemented with linseed oil and without addition of fungus.

2.3. Solid-state fermentation on polyurethane foam with *Y. lipolytica* W29

To obtain the inoculum of *Y. lipolytica* W29, a portion of cells was removed from a Petri dish with a sterile loop and inoculated into an Erlenmeyer flask of 500 mL with 100 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) at 28 ºC for 24 h.

The fermentations were performed in Erlenmeyer flasks of 500 mL where it was weighed 2 g of PUF in cubes (1 cm^3). The medium used was proposed by Canonico *et al*., 2016, with 40 mL of 30 g/L glycerol, 0.5 g/L ammonium sulfate and yeast extract with C/N ratio 76 and a pH of 6.0. The flasks with medium and PUF were sterilized separately at 121 ºC, 15 min. The optical density (OD) of the inoculum was measured (600 nm) and the volume containing the amount of cells corresponding to an OD of 1 was centrifuged (8000 g, 5 minutes). In the laminar flow hood, the supernatant was discarded, the cells were resuspended in fermentation medium, added to the Erlenmeyer flask with PUF and incubated at 28 ºC for 1, 3, 6 and 7 days. Once a day the Erlenmeyer flasks were shaken.

After each SSF on inert support, the PUF was recovered from the Erlenmeyer flasks with the aid of tweezers, introduced into a syringe and squeezed to a falcon tube previously weighed. The falcon tubes were centrifuged (8000g, 5 minutes) and the supernatant was moved to another falcon tube. Both samples (supernatant and cells) were stored at -20 ºC. The squeezed PUF was placed in a container and dried at 55 ºC (24 h).

2.3.1. Reuse of PUF in several cycles of fermentation

Reused of PUF in several fermentations with *Y. lipolytica* W29 was evaluated. After 6 days of fermentation (the preparation was described in section 2.3), the liquid phase was extracted from PUF with a sterile syringe and fresh culture medium was added to the

Erlenmeyer flaks with fermented PUF. This procedure was performed in 3 cycles of 6 days each cycle.

2.3.2. Lipids extraction from cells

The cells of *Y. lipolytica* W29 that were stored at -20 ºC were dried at 60 ºC overnight. After that, the falcon tube with the cells was weighed and it was transferred 0.4 g of cells to another flask, added 1.6 mL of distilled water, 4 mL of chloroform and 2 mL of methanol and stirred for 1 hour. Then, the mixture was passed through a Pasteur pipette with glass wool. The flasks were washed with 2 mL of chloroform and 2 mL of distilled water and the mixture was also passed through the Pasteur pipette with glass wool. After that, the glass wool was also washed with 2 mL of chloroform and the different phases rested on a separating funnel. The lower phase (chloroform with lipids) was recovered and the chloroform was evaporated using nitrogen injection. The difference in weight between the empty flask and after evaporation of chloroform corresponds to the lipids mass.

2.4. Preparation of lignocellulosic hydrolysate as alternative culture medium

2.4.1. Humidity determination

A known quantity of waste, about 0.5 g, was added in a container with a known weight (previously dried in hot air oven until constant weight). The vessel with the waste was placed in the hot air oven at 105 ºC for about 24 h (until constant weight). After cooling in desiccator containing silica gel it was weighed. This determination was performed in duplicate. Humidity (H) percentage (grams of water per grams of humid waste) was calculated according to equation 1:

$$\mathrm{H}\ (\%) = \frac{\mathrm{WCHS} - \mathrm{WCDS}}{\mathrm{WCHS} - \mathrm{WC}} \times 100 \qquad \textbf{(eq.1)}$$

wherein WCHS is the weight of container with humid waste in grams, WCDS is the weight of container with dry waste in grams and WC is the weight of container in grams.

2.4.2. Acid hydrolysis of brewer's spent grain

The preparation of lignocellulosic hydrolysate followed the method described by Carvalheiro *et al*., 2007 with minor modifications. Brewer's spent grain, 25 g, was mixed with 3% (w/w) H_2SO_4 to obtain a liquid-to-solid ratio of 8 and held at 121 ºC for 30 min. After sterilization and cooling, the solution was vacuum filtered using a Büchner funnel, the pH was adjusted to 5.5 with solid $CaCO_3$ and the resulting precipitate was removed by filtration. A part of the hydrolysate obtained was detoxified, using activated charcoal in a liquid-to-solid ratio of 10 (w/w). After stirring for 1 h, the charcoal was removed by vacuum filtration using a Büchner funnel.

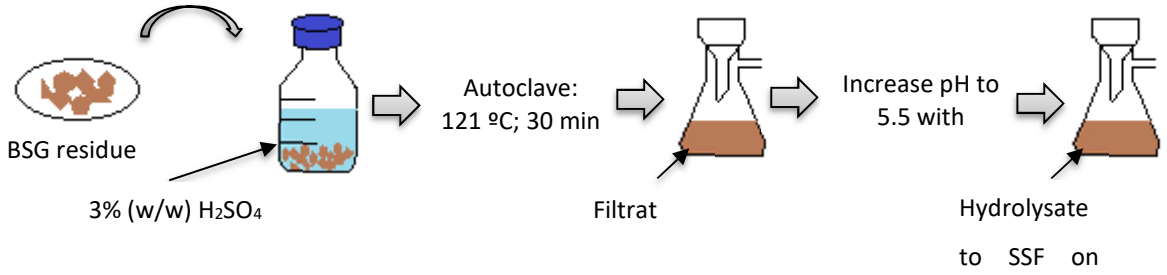


Figure 3 - Diagram which represents acid hydrolysis of BSG.

For fermentation assays, both hydrolysate (with and without being detoxified) were sterilized by filtration using 0.22 µm filters and supplemented with yeast extract (C/N ratio 25), 4 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.1 g/L $CaCl_2 \cdot 2H_2O$. The Erlenmeyer flasks with 2 g of PUF were sterilized at 121 ºC, 15 min, were inoculated with 15 mg of fungus mycelium (which corresponds to half of a petri dish), the corresponding hydrolysate was added to each flask and then incubated at 25 ºC for 3 days and at 16 ºC in the following 4 days. Cultivations were made in duplicate.

After that, it was realized fermentation assays with hydrolysate supplemented with 10% (w/v) commercial linseed oil in the same conditions described before.

After each SSF on inert support, the PUF was recovered from the Erlenmyer flasks with the aid of tweezers, introduced into a syringe and squeezed, in order to recover the liquid phase. A sample of this phase (10 mL) was stored at -20 ºC. The PUF was then placed in a container and dried at 55 ºC (24 h).

2.5. Biomass determination

Biomass (in grams) of *M. alpina* Peyronel 9412 and *Y. lipolytica* W29 that was attached to PUF was calculated according to **equation 2**:

$$\text{Biomass (g)} = \text{WPAF} - \text{WPBF} \quad \textbf{(eq.2)}$$

wherein WPBF is the weight of dry PUF before fermentation and WPAF is the weight of PUF after fermentation and dried at 55 ºC during 24 h.

Biomass (in grams) of *Y. lipolytica* W29 that was recovered from the liquid phase was calculated according to **equation 3**:

$$\text{Biomass (g)} = \text{WFTC} - \text{WEFT} \quad \textbf{(eq.3)}$$

wherein WEFT is the weight of the empty falcon tube and WFTC is the weight of the same falcon tube with dry cells.

2.6. Lipids extraction and fatty acid analysis

The lipids extraction and fatty acid methyl esters (FAMES) production were developed following the method proposed by Araujo *et al*., 2008 with minor modifications. The fermented PUF (0.25 g) was mixed with 10 mL of chloroform and was exposed to ultrasound waves in an ultrasonic bath for 2 hours to optimize the lipid extraction. Then, the chloroform was recovered by pressing with a syringe and evaporated using nitrogen injection. The difference in weight between the empty flask and after evaporation of chloroform corresponds to the lipids mass.

For derivatization of lipids to their FAMES, the residue of extracted lipid was dissolved in 1 mL of 0.5 M KOH–MeOH solution, vortex-mixed and heated for 10 min at 40 °C. It was added 100 µL of a solution with 1 g/L pentadecanoic acid as internal standard. After cooling the mixture in water, 2 ml of BF_3/CH_3OH (14%) was added, vortex-mixed and heated for 10 min at 40 °C. The mixture was cooled and subsequent portions of 1 ml of hexane and 2 ml of water added, vortex-mixed for 15 seconds, placed in a centrifuge, allowed to reach a speed of 3000 rpm, and then stopped immediately. After collecting the hexane phase (1 ml), an additional aliquot of 1 ml of hexane was added to the mixture, vortex-mixed and centrifuged. After that the hexane phase was collected.

2.7. Chemical analysis

The long chain fatty acids (LCFA) were analyzed by gas chromatography (GC). The analysis was carried out in a GC system (VARIAN 3800) equipped with a flame ionization detector (FID). LCFA were separated using an TRB-WAX 30 m x 0.25 mm x 0.25 μm column (TR140232, Teknokroma, Tr-wax), with helium as the carrier gas at 1.0 mL/min. The air flow was set at 250 mL/min, nitrogen and hydrogen flow of 30 mL/min. Temperatures of the injection port and detector was 250 and 280 ºC, respectively. Initial oven temperature was 40 ºC for 2 minutes, with a 30 ºC/ min ramp to 150 ºC, and a 3 ºC/ min final ramp to 250 ºC.

Glycerol, sucrose, lactose and BSG hydrolysate were analyzed by High Performance Liquid Chromatography (HPLC) system using a Jasco830-IR intelligent refractive-index detector and a Varian MetaCarb 87H column. The column was eluted with 0.005 M H_2SO_4 and the flux was 0.6 mL/min at 60 ºC.

Free reducing sugars (glucose, xylose and fructose) were measured by the 3,5-Dinitrosalicylic acid (DNS) method (Miller, 1959). To each tube was added 0.5 mL of DNS reagent to 0.5 mL of sample (0.5 mL of distilled water for blank) in triplicate. Tubes were placed in a bath at 100 ºC during 5 min. After cooling, it was added 5 mL of distilled water to stop the reaction. The absorbance was measured at 540 nm. Calibration curve was constructed with glucose standard solutions between 0 g/L and 2 g/L.

2.8. Scanning electron microscope (SEM)

The unfermented and fermented PUF were characterized using a desktop scanning electron microscope (SEM) coupled with energy-dispersive X-ray spectroscopy (EDS) analysis (Phenom ProX with EDS detector (Phenom-World BV, Netherlands)). All results were acquired using the ProSuite software. The samples were added to aluminum pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™), with the excess removed using compressed air. Samples were imaged without coating. The aluminum pin stub was then placed inside a Phenom Charge Reduction Sample Holder.

2.9. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of two replicates. The analysis was carried out using Microsoft Office Excel software. Statistically significant differences of the several assays were evaluated by a one-way ANOVA. A significant difference was considered if $p < 0.05$ applying the Tukey multiple-comparisons test. Statistical analyses were performed using GraphPad Prism 6 (San Diego, USA) software.

3. RESULTS AND DISCUSSION

3.1. Solid-state fermentation on polyurethane foam with *M. alpina* Peyronel 9412

3.1.1. Effect of carbon source on biomass and lipids accumulation

In order to evaluate the effect of substrate composition on the PUFAs production by *M. alpina* Peyronel 9412 under SSF conditions using PUF as inert support, several compounds were altered in the synthetic medium, such as different carbon source, supplementation with several commercial oils and C/N ratio. Besides that, the temperature incubation was also a factor studied.

Firstly, the effect of six carbon sources – glucose, glycerol, sucrose, xylose, fructose and lactose - in the growth and lipid accumulation by *M. alpina* Peyronel 9412 was studied. These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. **Figure 4A** shows the fungal biomass, where it was possible to notice that it grew well on glucose (0.73 ± 0.05 g biomass/g carbon source) and fructose (0.78 ± 0.08 g biomass/g carbon source), where there were no statistically significant differences, followed by glycerol (0.42 ± 0.06 g biomass/g carbon source). A poor growth it was observed in the medium containing sucrose (0.26 ± 0.03 g biomass/g carbon source), xylose (0.25 ± 0.02 g biomass/g carbon source) and lactose (0.30 ± 0.03 g biomass/g carbon source). According to Kendrick, 2001, *M. alpina* effectively uses simple sugars, which may be because generally fungi that belong to *Mortierellales* are saprophytes whereby they prefer to grow rapidly and proliferate extensively on simple sugars compared to complex molecules. In this fermentation assays, *M. alpina* Peyronel 9412 consumed about half of glucose, fructose and xylose of the initial sugar concentration (50 g/L) during the seven days of experiment, verifying that the initial sugar concentration was too high.

These results are in agreement with that of Yuan *et al*., 2002, with *M. alpina* I_{49}-N_{18} in SmF, since this strain also grew very slowly in the medium with sucrose and glycerol and very well with glucose, reaching approximately 5 g/L, 2.5 g/L and 21 g/L of biomass, respectively. In this study, the biomass in SSF with the medium containing glucose reached 14.66 ± 0.98 g/L while with sucrose and glycerol achieved 5.15 ± 0.60 g/L and 8.40 ± 1.29 g/L, respectively. Chen *et al*., 1997 also verified a poor biomass yield of *M. alpina* Wuji-H4 in SmF, with lactose

and glycerol (approximately 7 g/L and 2 g/L, respectively). In this case, the biomass of *M. alpina* Peyronel 9412 in SSF with medium containing lactose reached 5.97 ± 0.64 g/L. Shinmen *et al*., 1989 observed that in the SmF medium with fructose, *M. alpina* 1S-4 had almost the same mycelial yields and ARA contents to that with glucose. This was also verified in this study, since the biomass produced in SSF with medium containing fructose achieved 15.67 ± 1.68 g/L, very similar to the value of glucose.

In relation to the accumulation of lipids (**Figure 4B**), it was possible to observe that the fungus produced significantly more when grew in the medium with glucose (0.17 ± 0.04 g lipids/g carbon source) than with the other five carbon sources (p-value=0.0107). Glucose has been described as the most suitable carbon source for the fungal lipid production (Yamada *et al*., 1987; Shinmen *et al*., 1989). The efficient conversion of glucose into lipids can be explained by an assumption that glucose is metabolized exclusively through the glycolysis pathway to pyruvate (Stredanská and Šajbidor, 1993). Therefore, glucose was chosen as the carbon source for the rest of experimental work, since *M. alpina* Peyronel 9412 grew well in the medium containing this compound, it gave the most quantity of lipids, it is the most important sugar in nature and can be extracted from lignocellulosic materials.

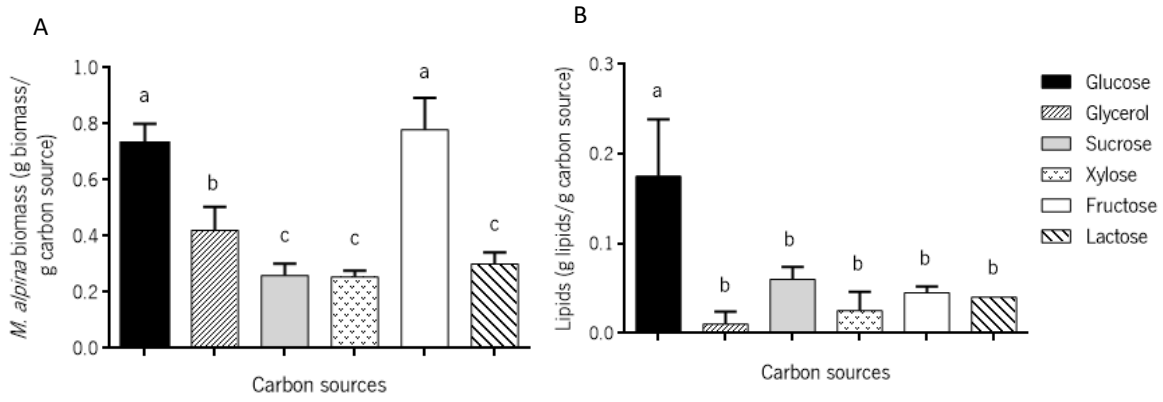


Figure 4 - Effects of different carbon sources (glucose, glycerol, sucrose, xylose, fructose and lactose) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g biomass/g carbon source) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

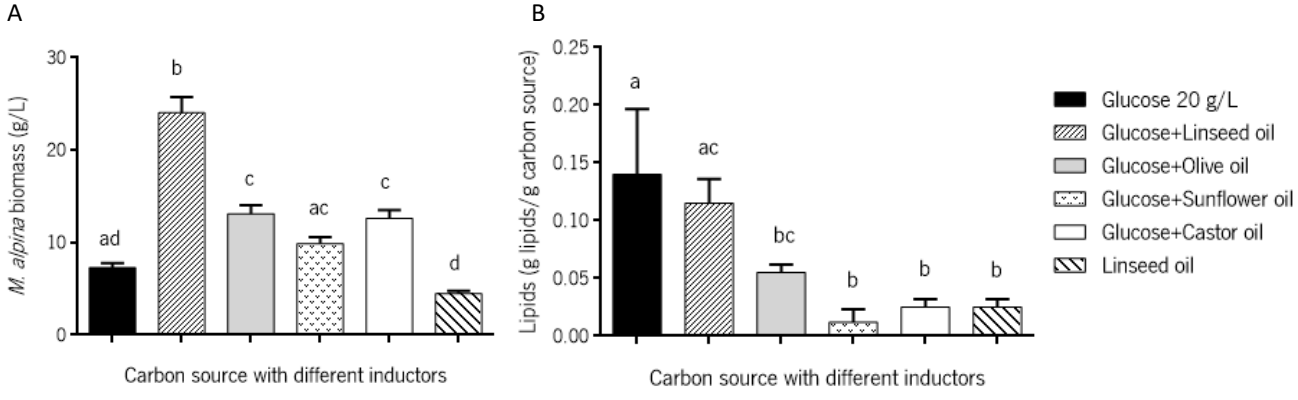
3.1.2. Effect of inductors on biomass, lipids and PUFAs accumulation

As *M. alpina* Peyronel 9412 did not consume entirely the glucose (50 g/L) in 7 days of fermentation, to choose the best carbon source, it was decided to lower its concentration to 20 g/L in the next experiments. In order to test if the supplementation with inductor oils

increases the lipid and PUFAs production, four vegetal oils were added to the basal medium: 10% (w/v) of linseed oil, olive oil, sunflower oil and castor oil. It was also made a fermentation just with linseed oil as carbon source, without glucose. These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. **Figure 5A** shows that, in general, the supplementation with oils increased the production of *M. alpina* Peyronel 9412 biomass, and the supplementation with linseed oil gave the highest biomass, reaching a value of 24.03 ± 1.20 g/L, which was statistically superior compared the other cultures (p-value < 0.0001). Jang *et al*., 2005 had similar results, where supplementation of the basal medium (which contains 2% of soluble starch) with 1% of oil increased the biomass production of *M. alpina* ATCC 32222 in SmF, and linseed oil gave the highest biomass (17.5 ± 0.9 g/L). When the soluble starch was replaced by glucose, the biomass achieved 17.0 ± 0.2 g/L.

In case of lipids (**Figure 5B**), *M. alpina* Peyronel 9412 in SSF with medium containing just glucose achieved 1.08 ± 0.30 g/L while with the medium supplemented with 10% (w/v) of linseed oil reached 9.96 ± 1.27 g/L. However, taking into account the initial carbon source, there was no difference statistically significant between the quantity of lipids produced in the fermentation just with glucose and glucose supplemented with 10 % (w/v) of linseed oil (0.14 ± 0.04 g lipids/g carbon source and 0.11 ± 0.01 g lipids/g carbon source, respectively). This is due to the amount of carbon source is 10 times lower in fermentations just with glucose, than the ones with supplementation. Therefore, the quantity of lipids per gram of carbon source had a higher value.

In this study, the SSF on PUF with medium without supplementation obtained a lipid content of 14.86 ± 4.18% g lipids/g biomass and the addition of 10% (w/v) of linseed oil increased 23.75%. Jacobs *et al*., 2009 tested SSF on BSG without additional linseed oil as substrate and reached the highest lipid content with *Mortierella sarnyensis* Mo 064 (8.2%,



w/w). On the other hand, the lipid content of cultures of *Mortierella sclerotiella* Mo 031 was found to be 11.3% more than cultures of this strain growing on BSG without the linseed oil supplement.

Figure 5 – Effects of oil supplementation (linseed, olive, sunflower and castor oil) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g/L) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

The fermentation just with glucose was the worst to produce PUFAs, obtaining the minor value of total PUFAs (**Table 3**). *Mortierella* isolates can incorporate supplemented oil such as linseed oil (Ratledge, 1997) or simultaneously utilize, incorporate and modify them in the fungal cells (Certik *et al*., 1998). The supplementation with the four oils increased the content of PUFAs compared to cultures that only had glucose and with the addition of linseed oil to glucose, differences statistically significant were verified in the amounts of linoleic acid (LA) (p-value=0.0007), α-linolenic acid (ALA) (p-value=0.0002) and total PUFAs (p-value=0.0003). Besides, the linseed oil induced the production of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which was not achieved with the other oils. Linseed oil is rich in the ω-3 precursor ALA, which can be metabolized for fungal EPA and DHA production (Certik and Shimizu, 1999), and that was verified in these experiments. These results are in agreement with the literature, since it has been shown that more EPA is produced with the addition of ω -3 precursor to brewer's spent grain (with *Mortierella antarctica* Mo 67 and *Mortierella epicladia* Mo 101) and sunflower press cake (with *M. alpina* Mo 46 and *Mortierella basiparvispora* Mo 88) (Jacobs *et al*., 2009; Jacobs *et al*., 2010).

Table 3 – Effect of oils supplementation (linseed, olive, sunflower and castor oil) on PUFAs production (mg PUFA/g PUF).

Carbon source	LA (mg/g support)	ALA (mg/g support)	EPA (mg/g support)	DHA (mg/g support)	Others (mg/g support)	Total PUFAs (mg/g support)
Glucose	1.02 ± 0.01[a]	0.18 ± 0.01[a]	ND	ND	ND	1.21 ± 0.02[a]
Glucose + Linseed oil	51.25 ± 9.10[b]	139.38 ± 23.58[b]	1.89 ± 0.77[a]	0.12 ± 0.07	0.30 ± 0.08	192.94 ± 33.60[b]
Glucose + Olive oil	9.87 ± 0.92 [a]	0.18 ± 0.01[a]	0.35 ± 0.11[a]	ND	ND	10.40 ± 1.04[a]
Glucose +	6.08 ±	0.15 ± 0.01[a]	0.22 ± 0.03[a]	ND	ND	6.45 ± 0.48[a]

Sunflower oil	0.45 [a]					
Glucose + Castor oil	4.01 ± 0.11 [a]	0.23 ± 0.06[a]	ND	ND	ND	4.24 ± 0.17[a]
Linseed oil	5.58 ± 2.47 [a]	1.50 ± 1.06[a]	ND	ND	ND	7.09 ± 3.54[a]

Mean±SD. n=2. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Tukey's test.
ND: not detectable; PUFAs: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic-acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; others: ARA - arachidonic acid; GLA - γ-linolenic acid).

The composition of olive oil is abundant in monounsaturated fatty acids (68,2%), such as oleic acid (66,4%) (Orsavova *et al*., 2015) and this one can be desaturated to yield LA (Certik and Shimizu, 1999), which corresponds to the most PUFA produced in the fermentations with this oil (95% of total PUFAs produced).

In case of sunflower oil, Orsavova *et al*., 2015 referred that PUFAs are the predominant part of its fatty acid composition (62,4%), however it was not observed a high content of this molecules in cultures with this oil. In this case, the fungus probably consumed the oil as carbon source to produce biomass (9.89 ± 0.49 g/L) and not for the production of lipids (0.86 ± 0.51 g/L).

The last oil to be tested was castor oil and it was possible to observe that as in the previous two oils, the quantity of PUFAs produced was low, there being only LA and ALA. This oil has a high content of unsaturated fatty acids (97.6%) mainly composed by ricinoleic acid (84.2%) and a small part of PUFAs composed by ALA (7.5%) and LA (0.5%) (Khaliq *et al*., 2017).

In fermentations where the culture medium had linseed oil (without glucose), LA and ALA were also the only PUFAs present in small quantities. In these cultures, there was low production of biomass (4.48 ± 0.22 g/L) and lipids (1.63 ± 0.08 g/L) because the quantity of carbon source was lower than in the other fermentations.

In general, the supplementation with oils increased the PUFAs production, although a statistically significant increase was only achieved with the addition of 10% (w/v) linseed oil. Furthermore, only in the medium with glucose 20 g/L supplemented with linseed oil it was observed the production of EPA and DHA. Therefore, in the next optimization stages, it will always be used as carbon source glucose with a supplementation of 10% (w/v) linseed oil.

3.1.3. Optimization of C/N ratio

Concentrations of carbon and nitrogen in SSF and the ratio of these nutrients had a significant impact on the quality and quantity of lipids produced by *Mortierella* (Sajbidor *et al*., 1990). The effect of C/N ratio in the growth, lipid and PUFAs accumulation by *M. alpina* Peyronel 9412 was studied by adjusting it to 15, 25 and 40 (w/w). The ratio C/N was changed by the addition of yeast extract in different quantities. These results were analysed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test

Until this point, the C/N ratio used in the previous fermentations was 50 and, as it is possible to observe in **Figure 6A**, it was not the best (0.28 ± 0.01 g biomass/g carbon source), since with the ratio of 15 (0.76 ± 0.04 g biomass/g carbon source), 25 (0.76 ± 0.04 g biomass/g carbon source) and 40 (0.71 ± 0.06 g biomass/g carbon source) the biomass produced was significantly higher (p-value=0.0030). Jang *et al*., 2005 used *M. alpina* ATCC 32222 in SmF and tested several C/N ratios (2.5, 5.2, 9.0, 12.8, 24.6 and 48.3), obtained a high cell yield with C/N ratio of 9 (7.3 ± 0.2 g/L). In this study, the SSF on PUF with *M. alpina* Peyronel 9412 and a synthetic medium with C/N ratio of 25 achieved 66.09 ± 3.30 g/L of biomass. In case of lipids it happened the same (**Figure 6B**), with the ratio of 50 it was achieved 0.13 ± 0.03 g lipids/g carbon source while with lower ratios of 15 (0.28 ± 0.04 g lipids/g carbon source) and 25 (0.28 ± 0.02 g lipids/g carbon source) higher amounts of lipids were obtained.

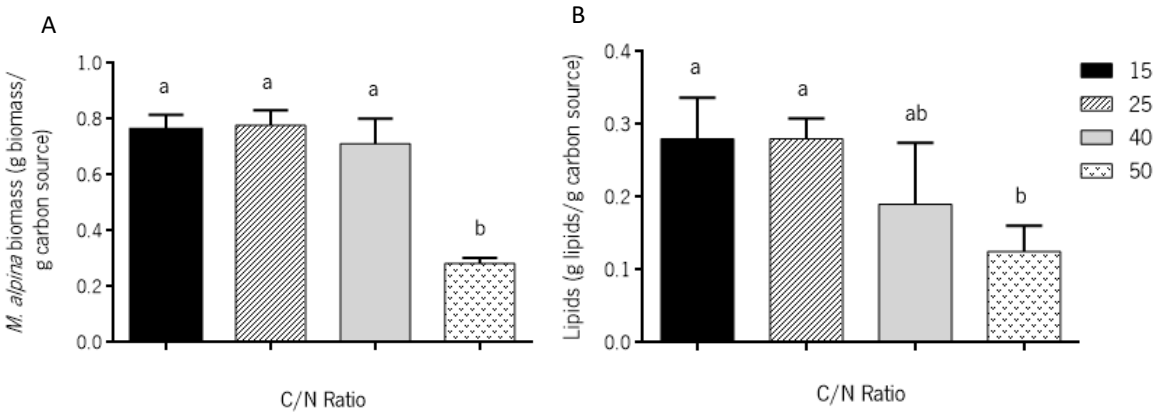


Figure 6 – Effects of different C/N ratios (15, 25, 40 and 50) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g biomass/g carbon source) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

The amount of PUFAs produced by *M. alpina* Peyronel 9412 was also higher with lower C/N ratios and there were no differences statistically significant between total PUFAs produced with C/N ratios of 15, 25 and 40 (**Table 4**). Jang *et al*., 2005 obtained the maximum

value of total PUFAs in SmF at the ratio of 5.2 (1409.7 ± 13.8 mg/L), followed by 24.6 (1090.7 ± 3.1 mg/L), 9.0 (1086.3 ± 2.9 mg/L) and 12.8 (1051.2 ± 2.4 mg/L). In this study, the total PUFAs produced expressed in g/L with the ratio of 25 were 23.79 ± 1.64 g/L, which is 22 times higher that it was achieved in SmF with the ratio of 24.6. Jang *et al.,* 2000 also referred that with C/N ratios between 14.5 and 18.5 favored EPA and LA production, while C/N ratios between 19.8 and 21 stimulated ARA and total PUFAs production in SSF.

According to Koike *et al*., 2001, when the C/N ratio is high the rate of the desaturation reaction becomes low and the saturated fatty acids that were accumulated are not converted to PUFAs. Therefore, the concentrations of carbon and nitrogen sources should be well-adjusted. According to the same source, the optimum C/N ratio for lipid biosynthesis by *Mortierella* was 20 to 23. For the following optimization stages the C/N ratio was adjusted to 25.

Table 4 - Effect of different C/N ratios (15, 25, 40 and 50) on PUFAs production (mg PUFA/g PUF).

C/N Ratio	LA (mg/g support)	ALA (mg/g support)	EPA (mg/g support)	DHA (mg/g support)	Others (mg/g support)	Total PUFAs (mg/g support)
15	98.03 ± 2.48^{a}	302.64 ± 11.24^{ab}	1.98 ± 0.02^{a}	0.46 ± 0.08^{a}	0.45 ± 0.01^{ab}	403.56 ± 13.83^{ab}
25	120.06 ± 7.02^{a}	351.82 ± 25.54^{a}	2.64 ± 0.03^{a}	0.76 ± 0.17^{a}	0.61 ± 0.02^{a}	475.89 ± 32.78^{a}
40	114.74 ± 10.69^{a}	320.87 ± 57.65^{ab}	3.03 ± 0.02^{a}	0.54 ± 0.44^{a}	0.66 ± 0.05^{a}	439.84 ± 69.04^{a}
50	51.25 ± 9.10^{a}	139.38 ± 23.58^{b}	1.89 ± 0.77^{a}	0.12 ± 0.07^{a}	0.30 ± 0.08^{b}	192.94 ± 33.60^{b}
Control	86.61 ± 24.36^{a}	308.68 ± 22.25^{ab}	ND	ND	ND	396.58 ± 47.29^{ab}

Mean±SD. n=2. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Tukey's test.

ND: not detectable; PUFAs: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic-acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; others: ARA - arachidonic acid; GLA - γ-linolenic acid).

3.1.4. Evaluation of incubation temperature to increase the PUFA production

One of the most important environmental aspects affecting the growth of microorganisms is the cultivation temperature. In order to study this parameter on growth,

lipids accumulation and PUFAs production by *M. alpina* Peyronel 9412, fermentation assays were realized by incubating at 14 ºC, 16 ºC, 18 ºC, 22 ºC, 25 ºC for 7 days and also at a higher temperature for 3 days (22 ºC and 25 ºC) lowering in the following 4 days (14ºC, 16 ºC and 18 ºC). These results were analyzed by one-way ANOVA followed by a multiple comparison test, Tukey's Multiple Comparison Test. **Figure 7A** shows the effects of these different incubation temperatures on growth and it was possible to observe that intermediate temperatures (7 days at 18 and 22 ºC) and a higher temperature for 3 days lowering in the next 4 days (22 – 16; 25 – 14; 25 – 16 and 25 – 18 ºC) had a positive effect on biomass production. On the other hand, with an incubation temperature of 25 ºC it was verified the lowest biomass production (p-value=0.0007). Jang *et al*., 2005 also studied this parameter and concluded that with *M. alpina* ATCC 32222 in SmF, the biomass production was higher at 20 ºC (7.2 ± 0.3 g/L), followed by 15 and 12 ºC, and 25 ºC (6.03 ± 0.3 g/L) was the lowest. Yang and Zhang., 2016 also studied the growth of *M. alpina* in SSF on distiller's dried grains with solubles at 10, 12, 15, 20 and 25 ºC and concluded that microbial growth was better at 20 ºC. **Figure 7B** shows that the production of lipids was very similar in all incubation temperatures, not highlighting any. It is noteworthy that in some fermentations that had a high biomass production, such as 25 – 14 and 25 – 18 ºC, the quantity of lipids produced was lower (0.20 ± 0.01 and 0.17 ± 0.04 g lipids/g carbon source, respectively) than in all other temperatures tested.

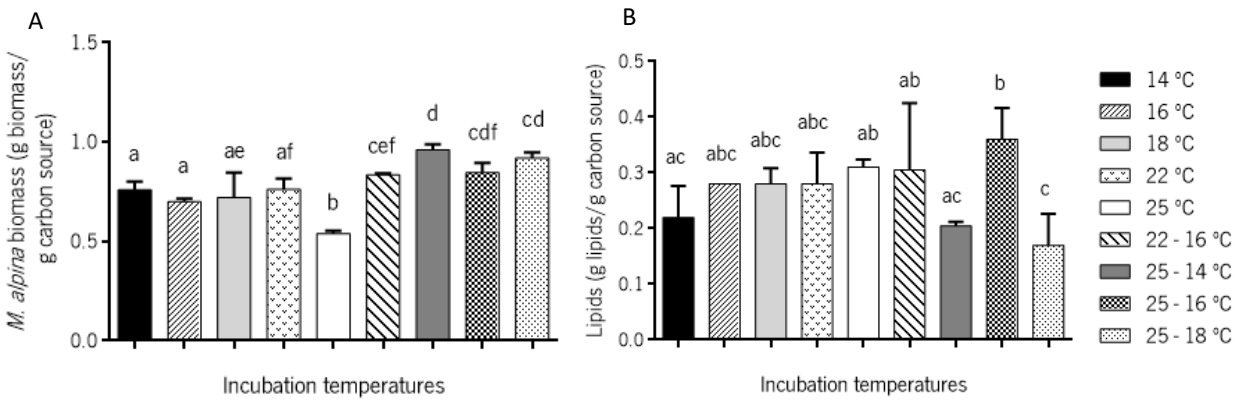


Figure 7 – Effects of different incubation temperatures (14 ºC, 16 ºC, 18 ºC, 22 ºC, 25 ºC for 7 days and 22 ºC and 25 ºC for 3 days lowering in the following 4 days to 14ºC, 16 ºC and 18 ºC) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g biomass/g carbon source) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

The quantity of PUFAs produced by *M. alpina* Peyronel 9412 varied with different incubation temperatures tested (**Table 5**). The highest values of total PUFAs obtained were at 16 ºC, followed by 25 – 16 ºC, 22 ºC and 18 ºC. Several authors refer that low incubation temperatures generally favored the production of PUFAs and increased the degree of unsaturation of PUFAs in *Mortierella* (Dyal and Narine., 2005; Shimizu *et al*., 1989). In this case, the lower temperature tested (7 days at 14 ºC) did not enhance the PUFAs production, comparing to almost all other temperatures tested (except with 25 – 18 ºC). However, in fermentations at 16 ºC for 7 days, the total PUFAs increased 12.82% comparing to fermentations at 22 ºC for 7 days (used in the previous experiment).

Jang *et al*., 2000 referred that it can be used a higher temperature for mycelial growth and a lower temperature for PUFAs production in SSF. The same authors were able to increase the production of total PUFAs and EPA by 12.0% and 84.4%, respectively, by decreasing the temperature from 20 to 12 ºC on the fifth day with SSF cultivation of *M. alpina* ATCC 32222. In this study, comparing the total PUFAs obtained in fermentation at 22 ºC for 7 days with the other incubation temperatures in which there was a decrease of temperature after 3 days, it was possible to verify that only when the fungus grew at 25 ºC for 3 days and then at 16 ºC for the next 4 days, the total PUFAs produced were higher (12.50%).

Therefore, for the following optimization stage the incubation temperature will be at 25 ºC for 3 days lowering to 16 ºC in the next 4 days.

Table 5 - Effect of different incubation temperatures (14 ºC, 16 ºC, 18 ºC, 22 ºC, 25 ºC for 7 days and 22 ºC and 25 ºC for 3 days lowering in the following 4 days to 14ºC, 16 ºC and 18 ºC) on PUFAs production (mg PUFA/g PUF).

Incubation temperature (° C)	LA (mg/g support)	ALA (mg/g support)	EPA (mg/g support)	DHA (mg/g support)	Others (mg/g support)	Total PUFAs (mg/g support)
14	72.59 ± 20.51^{a}	110.62 ± 30.08^{a}	1.45 ± 0.37^{a,b}	0.62 ± 0.15^{a}	0.33 ± 0.09^{a,b}	185.61 ± 51.19^{a,e}
16	129.05 ± 15.30^{a}	403.16 ± 46.82^{b}	2.56 ± 0.28^{a}	1.51 ± 0.52^{a}	0.59 ± 0.08^{a}	536.88 ± 63.00^{b,c}
18	104.65 ± 14.09^{a}	301.24 ± 24.04^{b,c}	1.95 ± 0.10^{a,b}	0.96 ± 0.09^{a}	0.39 ± 0.03^{a,b}	409.18 ± 38.34^{a,c,d}
22	120.06 ± 7.02^{a}	351.82 ± 25.54^{b,d}	2.64 ± 0.03^{a}	0.76 ± 0.17^{a}	0.61 ± 0.02^{a}	475.89 ± 32.78^{b,d,f}

25	82.34 ± 28.14[a]	128.04 ± 43.62[a]	1.63 ± 0.62[a,b]	0.90 ± 0.37[a]	0.36 ± 0.12[a,b]	213.27 ± 72.88[a,e]
22 - 16	131.33 ± 7.66[a]	198.54 ± 12.67[a,c,d]	2.94 ± 0.14[a]	1.11 ± 0.09[a]	0.60 ± 0.02[a]	334.52 ± 20.58[a,c,e,f,g]
25 - 14	111.48 ± 6.28[a]	164.85 ± 10.32[a,c]	2.39 ± 0.14[a,b]	0.68 ± 0.08[a]	0.52 ± 0.04[a,b]	279.92 ± 16.85[a,e,f]
25 - 16	129.66 ± 5.84[a]	401.93 ± 18.10[b]	2.56 ± 0.12[a]	0.67 ± 0.03[a]	0.59 ± 0.03[a]	535.41 ± 24.12[b,d,g]
25 - 18	61.56 ± 3.08[a]	87.62 ± 4.38[a]	1.20 ± 0.06[b]	0.30 ± 0.02[a]	0.24 ± 0.01[b]	150.92 ± 7.55[e]
Control	86.61 ± 24.36[a]	308.68 ± 22.25[b,c]	ND	ND	ND	396.58 ± 47.29[a,b]

Mean±SD. n=2. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Tukey's test.

ND: not detectable; PUFAs: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic-acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; others: ARA - arachidonic acid; GLA - γ-linolenic acid).

3.1.5. Optimization of the addition of linseed oil

As can be observed in the section 3.1.2, the addition of 10% (w/v) linseed oil to the basal medium had a positive effect in PUFAs accumulation by *M. alpina* Peyronel 9412. In order to see if lowering this percentage had the same effect, the addition of 2% and 5% was tested. **Figure 8** shows that although the biomass decreased with the use of 10% linseed oil, there are no significant differences between the amount of lipids produced with any percentage of oil added.

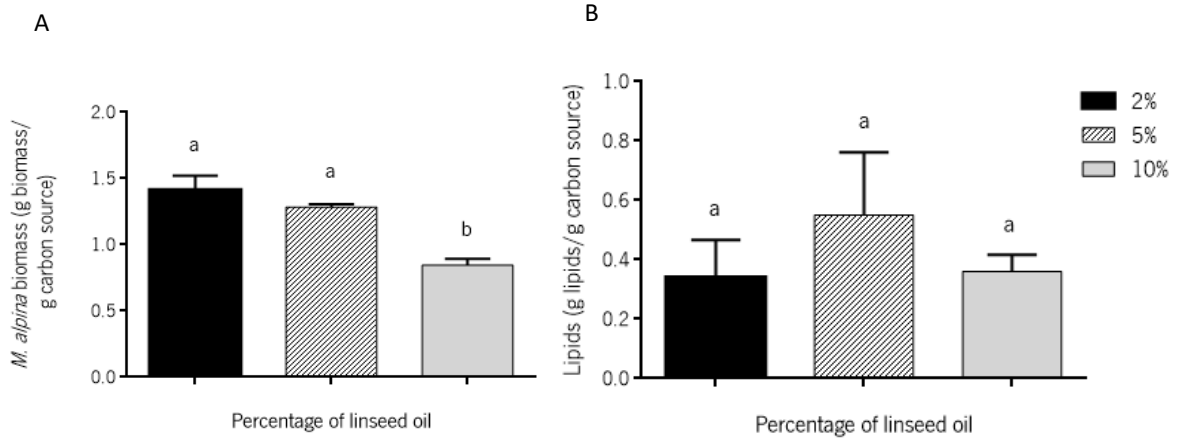


Figure 8 – Effects of supplementation with different percentages of linseed oil (2, 5 and 10%) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g biomass/g carbon source) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

In case of PUFAs, it was possible to verify that the higher quantity of oil added led to the higher amount of LA, ALA, EPA and other PUFAs (**Table 6**). In terms of total PUFAs there was differences statistically significant between all conditions tested (p-value=0.0038), the supplementation with 10% (w/v) linseed oil reached 3.9 times higher than 2% and 0.8 times higher than 5%. Therefore, the addition of 10% (w/v) linseed oil has shown to be the best supplementation for PUFAs production.

Table 6 - Effect of supplementation with different percentages of linseed oil (2, 5 and 10%) on PUFAs production (mg PUFA/g PUF).

Linseed oil (%)	LA (mg/g support)	ALA (mg/g support)	EPA (mg/g support)	DHA (mg/g support)	Others (mg/g support)	Total PUFAs (mg/g support)
2	32.36 ± 11.67[a]	74.95 ± 29.08[a]	0.71 ± 0.28[a]	0.34 ± 0.14[a]	0.10 ± 0.01[a]	108.46 ± 41.9[a]
5	104.12 ± 22.24[a,b]	185.82 ± 17.69[a]	2.39 ± 0.53[a]	0.17 ± 0.04[a]	0.36 ± 0.17[a]	292.86 ± 40.67[b]
10	129.66 ± 5.84[b]	401.93 ± 18.10[b]	2.56 ± 0.12[a]	0.67 ± 0.03[a]	0.59 ± 0.03[a]	535.41 ± 24.12[c]

Mean±SD. n=2. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Tukey's test.
PUFAs: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic-acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; others: ARA - arachidonic acid; GLA - γ-linolenic acid).

3.1.6. PUF-SSF using lignocellulosic hydrolysate as low-cost culture medium

BSG is a hemicellulose-rich agroindustrial residue that may be used to produce value-added products through different processes, such as a fermentation. For that, it is necessary a complete polysaccharide hydrolysis to ensure an efficient fermentation process and one pre-treatment that can be done is acid hydrolysis (Carvalheiro *et al*., 2004). During this procedure, pentose sugars (produced from xylan and arabinan) are the main compounds formed, therefore, xylose and arabinose were the monosaccharides present in highest quantities in

BSG hydrolysates (Mussatto and Roberto., 2005). Acid hydrolysis of BSG with dilute sulfuric acid produced a sugar rich hydrolysate, which it was already used as culture medium for the production of xylitol by the yeast *Debaryomyces hansenii* (Carvalheiro *et al*., 2004).

However, the chemical composition of the hydrolysate obtained in the present experiment after acid hydrolysis showed a high content in glucose (60%), and low content in xylose (30%) and arabinose (10%). During the brewing process, the cereal starch is hydrolyzed into glucose monosaccharides, however if some starch remains intact it can be hydrolyzed during acid hydrolysis, increasing the glucose content of BSG. Besides that, the residues of BSG can bring attached to it free sugars released during hydrolytic processes occurred in the brewing industry. In fact, this residue used had 65.02 mg free sugars/g dry waste, which contributes to a higher percentage of glucose than it was expected.

During hydrolysis some potential inhibitory compounds are cogenerated, which lead to a decrease in yield and productivity. To overcome this problem, a detoxification treatment with activated charcoal can be done (Carvalheiro *et al*., 2004). **Figure 9** shows that the detoxification of the hydrolysate with activated charcoal did not improve biomass or the quantity of lipids produced. Similar results were obtained by Carvalheiro *et al*., 2005. Therefore, a previous detoxification step before fermentation seems to be unnecessary. The supplementation with 10% (w/v) of linseed oil not cause an increase statistically significant of biomass and lipids production comparing to the other mediums. Therefore, hydrolysate without detoxification and linseed oil supplementation is a suitable medium for biomass growth and lipids accumulation by *M. alpina* Peyronel 9412.

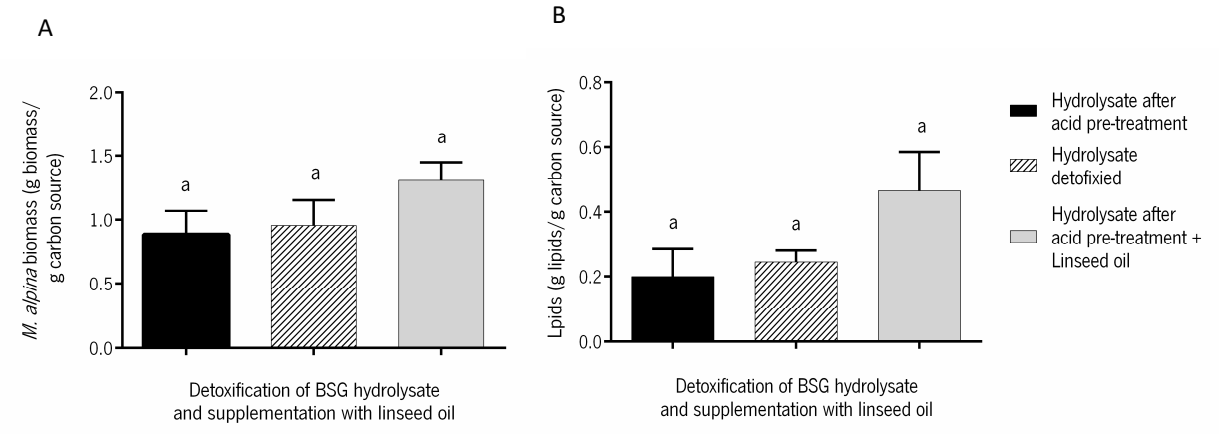


Figure 9 – Effects of detoxification of BSG hydrolysate with activated charcoal and supplementation with linseed oil (10%, w/v) on growth of *M. alpina* Peyronel 9412 in SSF on PUF (g biomass/g carbon source) (A) and lipid accumulation (g lipids/g carbon source) (B). The results represent the average of two independent experiments

and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

There were no statistically differences between the amount of LA, ALA and total PUFAs accumulated in the fermentations with and without detoxification (**Table 7**). The supplementation with 10% (w/v) of linseed oil to the hydrolysate without detoxification increase significantly the accumulation of LA, EPA and total PUFAs (p-value=0.0154). However, comparing the quantity of LA, ALA, EPA and total PUFAs accumulated in the previous fermentation with a synthetic medium also supplemented with 10% (w/v) of linseed oil, it was possible to verify that in cultures with BSG hydrolysate the accumulation of PUFAs was lower.

Table 7 - Effects of detoxification of BSG hydrolysate with activated charcoal and supplementation with linseed oil (10%, w/v) on PUFAs production (mg PUFA/g PUF).

Pre-treatments	LA (mg/g support)	ALA (mg/g support)	EPA (mg/g support)	DHA (mg/g support)	Others (mg/g support)	Total PUFAs (mg/g support)
Control	0.04 ± 0.01^{a}	0.55 ± 0.05^{a}	ND	ND	ND	0.59 ± 0.06^{a}
Hydrolysate after acid pre-treatment	1.67 ± 0.25^{a}	0.41 ± 0.04^{a}	0.26 ± 0.02^{a}	ND	ND	2.34 ± 0.31^{a}
Hydrolysate detoxified	1.75 ± 0.44^{a}	0.40 ± 0.08^{a}	0.06 ± 0.01^{b}	ND	ND	2.20 ± 0.53^{a}
Hydrolysate after acid pre-treatment + Linseed oil	10.68 ± 1.00^{b}	9.09 ± 4.45^{a}	1.61 ± 0.04^{c}	ND	ND	21.38 ± 5.49^{b}

Mean±SD. n=2. Means in the same column that do not share the same alphabetic superscript show significant difference at 5% level according to Tukey's test. ND: not detectable; PUFAs: polyunsaturated fatty acids; LA: linoleic acid; ALA: α-linolenic-acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; others: ARA - arachidonic acid; GLA - γ-linolenic acid).

3.1.7. SEM of unfermented and fermented PUF

The growth of *M. alpina* Peyronel 9412 on PUF was observed by scanning electron microscope (SEM). This analysis allowed to characterize the morphology of the fungus and its distribution over the PUF. In **Figure 10** can be compared the PUF before and after SSF. **Figure 10A** and **10B** shows this material without fungus growth and it was possible to verify that is highly porous with some range of sizes. Fariza *et al*., 2010 also evaluated the microstructure of PUF and concluded that had high porosity, pores ranging from 100-900 µm and they are uniformly distributed in the sample with enormous interconnection between them.

As can be observed in **Figure 10C** and **10D**, which are after SSF with basal medium containing 20 g/L glucose, the fungus colonized dispersedly the PUF by hyphae. Also, it seems

that the hyphae grew attached to the external surface of the PUF, not penetrating to the interior. Furthermore, *M. alpina* Peyronel 9412 does not appear to degrade the structure of this material with his growth.

In case of **Figure 10E** and **10F**, which are after SSF with basal medium containing 20 g/L glucose and 10% (w/v) linseed oil, it was possible to verify what was discussed in the section 3.1.2, with the supplementation of linseed oil the fungus produced more biomass. This is visible in these SEM images where a massive colonization is observed compared to the images **10C** and **10D**. However, it seems that the hyphae remain without penetrating into the porous.

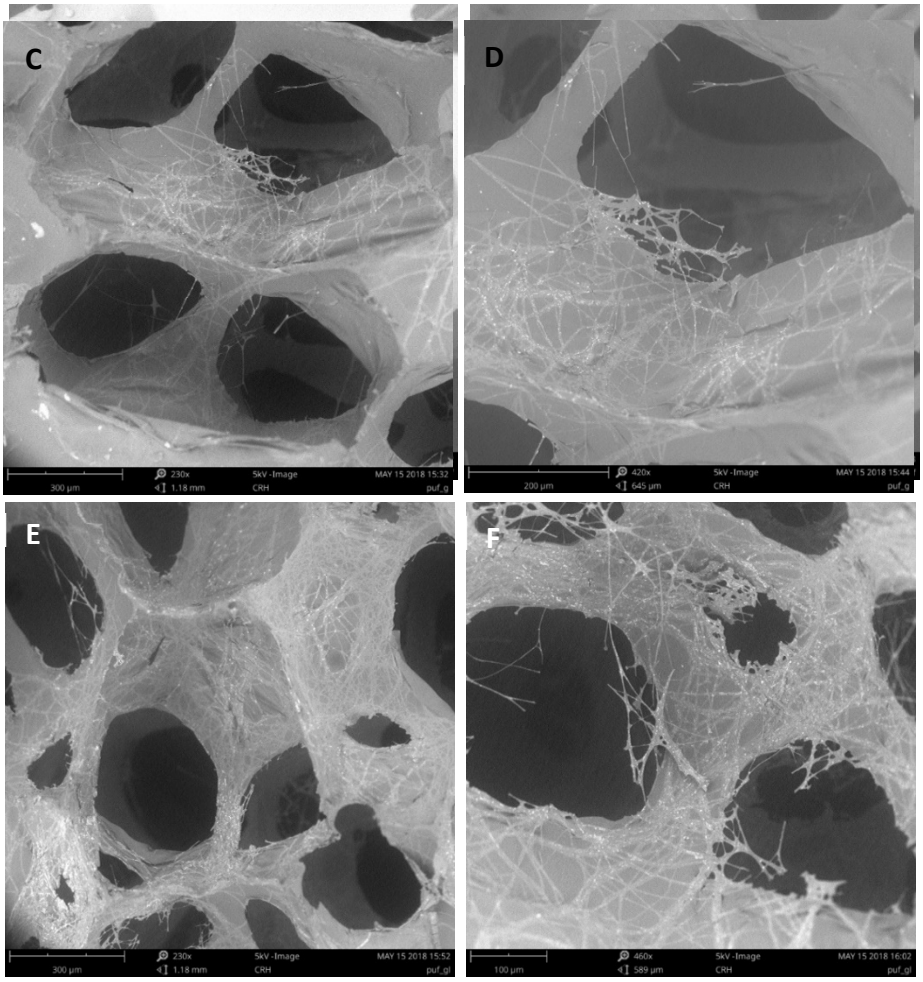


Figure 10 - SEM images of unfermented and fermented PUF used as inert support in SSF for the production of PUFAs. (A-B) Structure of unfermented PUF at different scales. (C-D) View of *M. alpina* Peyronel 9412 growth on PUF with 20 g/L glucose at different scales. (E-F) View of *M. alpina* Peyronel 9412 growth on PUF with 20 g/L glucose and 10% (w/v) linseed oil at different scales.

3.2. Solid-state fermentation on polyurethane foam with *Y. lipolytica* W29

3.2.1. Evaluation of incubation time for lipids production

Different carbon sources have been considered as substrates for the *de novo* lipid biosynthesis from oleaginous microorganisms, including glycerol (Chatzifragkou *et al*., 2011; Makri *et al*., 2010). Therefore, in order to study the lipids accumulation by *Y. lipolytica* W29 using SSF on PUF with glycerol, several days of incubation were tested – 1, 3, 6 and 7. In the case of yeast, it was observed that after separation of liquid medium from PUF by pressure, an important quantity of yeast biomass was released in liquid fraction and low quantity of biomass remained on PUF. However, when filamentous fungus *M. alpina* was growth on PUF, at the end of fermentation all fungal biomass remained on PUF.

In **Figure 11A** shows the amount of *Y. lipolytica* W29 biomass that was in the culture medium and the biomass that was attached to the PUF over time, after liquid extraction from PUF. It was found that the biomass in the culture medium increased over the fermentation time while the biomass remaining in the PUF did not undergo major changes and was much lower than in the culture medium. Kautola *et al*., 1991 studied different cell immobilization methods on citric acid production with *Y. lipolytica* A-101, including PUF cubes of 1 cm^3, and verified that during the first 6-day batch the productivities were only about 20% of that reached with free cells. On the other hand, Canonico *et al*., 2016 verified that the biomass production of *Y. lipolytica* 347 in SmF was maximized at C/N 118, time 144 h, with 90 g/L glycerol (11.5 ± 0.00 g/L). In this study, the biomass in culture medium achieved the maximum at seventh day with less glycerol (12.87 ± 0.64 g/L).

In terms of lipids production (**Figure 11B**), it reached the maximum on the sixth day of fermentation, both in the cells that were attached to PUF (21.12 ± 1.71 mg lipids/g PUF) and in the cells in the culture medium (215.70 ± 1.70 mg lipids/g biomass). It was possible to verify that when the PUF was squeezed, the cells went out into the culture medium, therefore it is not worthwhile to extract lipids from the foam since the biomass therein is reduced. Canonico *et al*., 2016 achieved the maximum value of lipids with the same conditions described before (2.60 ± 0.18 g/L), while in this study the biomass in culture medium reached 1.31 ± 0.07 g/L with less glycerol (30 g/L). However, the maximum lipid content obtained was 10.22 ± 1.02% w/w also on the sixth day, while the same authors achieved 32.6% w/w with a glycerol concentration of 30 g/L, C/N 63 and time 96 h.

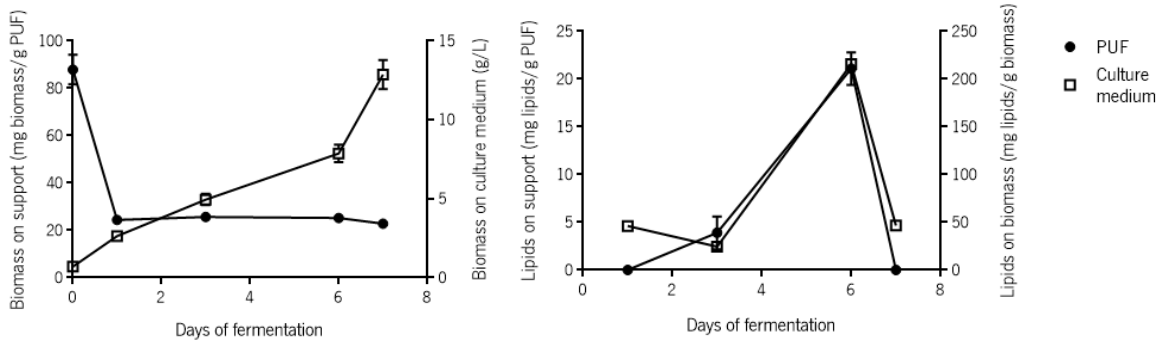


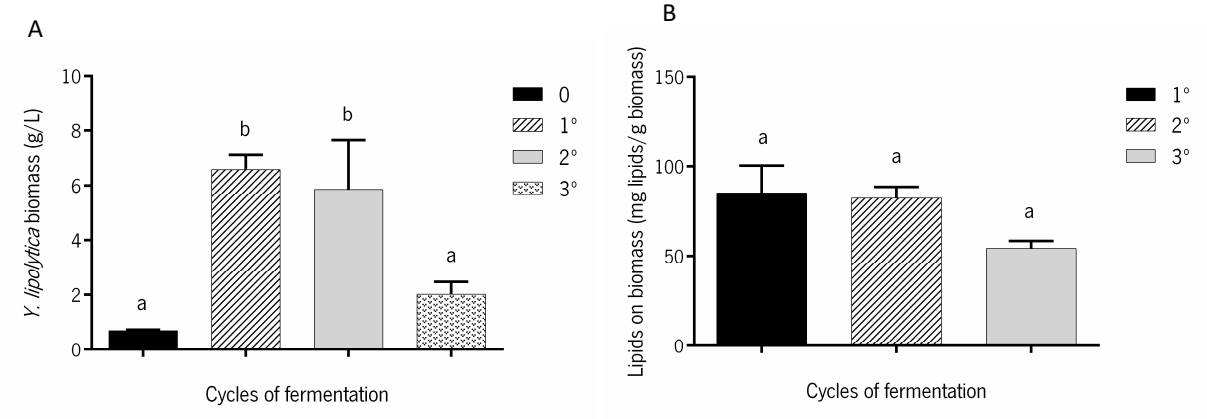
Figure 11 - *Y. lipolytica* W29 biomass produced on PUF (mg biomass/g PUF) and in the culture medium (g/L) for 0, 1, 3, 6 and 7 days of fermentation (A) and lipids accumulation on biomass in the support (mg lipids/g PUF) and on biomass in the culture medium (mg lipids/g biomass) (B). The results represent the average of two independent experiments and error bars represent SD.

3.2.2. Reuse of PUF in several cycles of fermentation

In order to evaluate if it is viable to perform several cycles of SSF maintaining the same PUF and without the addition of cells after the first cycle, 3 cycles of 6 days each were performed.

When analyzing the biomass production of *Y. lipolytica* W29 throughout the assay (**Figure 12A**) it was possible to observe that there was an increase in the first cycle (until the sixth day), decreasing in the second (from the sixth day to the twelfth) and third (from the twelfth to the eighteenth day) cycles. However, it was notable that the inoculum was only added at the beginning of the fermentation, so it was possible to conclude that even when the PUF is squeezed after each cycle of fermentation, some cells remain attached to it, being able to multiply with the addition of fresh medium.

As can be seen in **Figure 12B**, the cells could also produce lipids in this experiment, however a decrease was observed throughout the fermentation cycles, such as in biomass



production. Although the culture medium was replaced at each fermentation cycle, dead cells or excretory products of the cell metabolism may remain attached to PUF or in the Erlenmeyer flask, so that as cycles are added, biomass and lipid production is not as high as in the beginning. In the literature the immobilization on PUF is used for lipase production. For example, Elibol and Özer., 2000 immobilized cells of *Rhizopus arrhizus* on PUF and verified that they might be kept viable and could produce lipase for a 120 h period. Adamczak and Bednarski., 2004 used PUF as the carrier for immobilizing intracellular lipases of *Rhizomucor miehei* and *Y. lipolytica* and verified that lipolytic activity of immobilized lipases was 2.1–4.3 times higher than the activity of lipases obtained from free biomass.

Figure 12 - *Y. lipolytica* W29 biomass (g/L) produced during reuse of PUF in several cycles of fermentation (6, 12 and 18 days) (A) and the quantity of lipids produced in each cycle (mg lipids/g biomass) (B). The results represent the average of two independent experiments and error bars represent SD. Letters above each bar indicate the results of Tukey's test ($P < 0.05$); values with shared letters in the same graph are not significantly different.

The fatty acids profile of SCO produced by *Y. lipolytica* W29 after 6 days of fermentation are present in **Table 8**. It was possible to verify that 74.65 ± 3.73% corresponded to saturated fatty acids while 5.83 ± 0.29% was monounsaturated fatty acids and 19.52 ± 0.98% was polyunsaturated fatty acids. Katre *et al*., 2012 had similar results using *Y. lipolytica* NCIM 3229 in SmF with glucose, achieving 70.6% of saturated, 0.6% of monounsaturated and 28.8% of polyunsaturated fatty acids. Papanikolaou and Aggelis., 2002 used *Y. lipolytica* LGAM S(7)1 in continuous cultures and obtained high percentages of oleic acid (47 ± 4% w/w) followed by 21 ± 3% of linoleic acid, 13 ± 2% of stearic acid and 15 ± 3% of palmitic acid. In this study, oleic acid was the less produced (0.29 ± 0.01%) and it was observed higher amounts of linoleic acid (19.52 ± 0.98%), palmitic acid (30.05 ± 1.50%) and stearic acid (31.77 ± 1.59%). The mainly produced fatty acids were C16 (35.61 ± 1.78%) and C18 (51.56 ± 2.58%) long-chain fatty acids, which goes according to literature (Karatay and Dönmez., 2010; Sara *et al*., 2016).

Table 8 – Fatty acids profile (mg/L) produced by *Y. lipolytica* W29 after 6 days of fermentation.

Fatty acids	Quantity (mg/L)
Hexanoic acid (C6:0)	17.16 ± 0.86
Capric acid (C10:0)	16.60 ± 0.83
Lauric acid (C12:0)	21.83 ± 1.09

Myristic acid (C14:0)	7.35 ± 0.37
Pentadecanoic acid (C15:0)	116.04 ± 5.80
Palmitic acid (C16:0)	441.07 ± 22.05
Palmitoleic acid (C16:1)	81.57 ± 4.08
Heptadecanoic acid (C17:0)	9.24 ± 0.46
Stearic acid (C18:0)	466.31 ± 23.32
Oleic acid (C18:1n9c)	4.03 ± 0.20
Linoleic acid (C18:2n6c)	286.41 ± 14.32

4. CONCLUSION AND FUTURE PERSPECTIVES

SSF using PUF as inert support and synthetic medium as source of carbon, nitrogen and nutrients allowed to produce lipids by *Y. lipolytica* W29 (ATCC 20460) and PUFAs by *M. alpina* Peyronel.

Through the optimization of culture composition and fermentative parameters, it was increased PUFAs production by *M. alpina* Peyronel 9412. It was possible to conclude that glucose was the best carbon source, the supplementation with linseed oil and lower C/N ratios also contributed to a higher production of these fatty acids. Additionally, an incubation temperature higher for the first 3 days lowering in the next 4 days of fermentation also favored PUFAs production.

It was demonstrated that only the supplementation with linseed oil induced the production of EPA and DHA, due to the fact that this oil is rich in ω-3 precursor ALA, that can be metabolized for fungal EPA and DHA production.

Hemicellulosic hydrolysate of BSG can be used as culture medium in SSF using PUF as inert support to produce PUFAs, although in a smaller amount than with the synthetic

medium. The detoxification of the hydrolysate with activated charcoal did not improve biomass or the quantity of lipids produced.

The lipids production by *Y. lipolytica* W29 in SSF on PUF achieved the maximum at sixth day of fermentation, both in the cells that were attached to the PUF (21.12 ± 1.71 mg lipids/g PUF) and in the cells in the culture medium (215.70 ± 1.70 mg lipids/g biomass). It was verified that after separation of liquid medium from PUF by pressure, an important quantity of yeast biomass was released in liquid fraction and low quantity of biomass remained on PUF. Thus, the extraction of lipids from cell biomass was performed only with biomass without the presence of PUF.

The reutilization of PUF in several cycles of fermentation (3 cycles of 6 days each) without the addition of cells after the first cycle was evaluated and it was verified that the low quantity of biomass that remained on PUF after this material be squeezed can multiply with the addition of fresh medium.

The fatty acid profile produced by *Y. lipolytica* W29 in SSF on PUF indicates a large amount of stearic acid (31.77 ± 1.59%), followed by palmitic acid (30.05 ± 1.50%) and linoleic acid (19.52 ± 0.98%).

In future research, it would be interesting to analyze other parameters to continue to improve PUFAs production by *M. alpina* Peyronel 9412 in SSF on PUF, such as pH of culture medium or the size of PUF cubes, since through the SEM images it was possible to observe that the fungus does not penetrate into de PUF. It would be also interesting to scale-up the process to a column or packed-bed bioreactor.

Other future study could be perform SEM on PUF after fermentation by *Y. lipolytica* W29 to understand how this yeast connects to inert support.

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