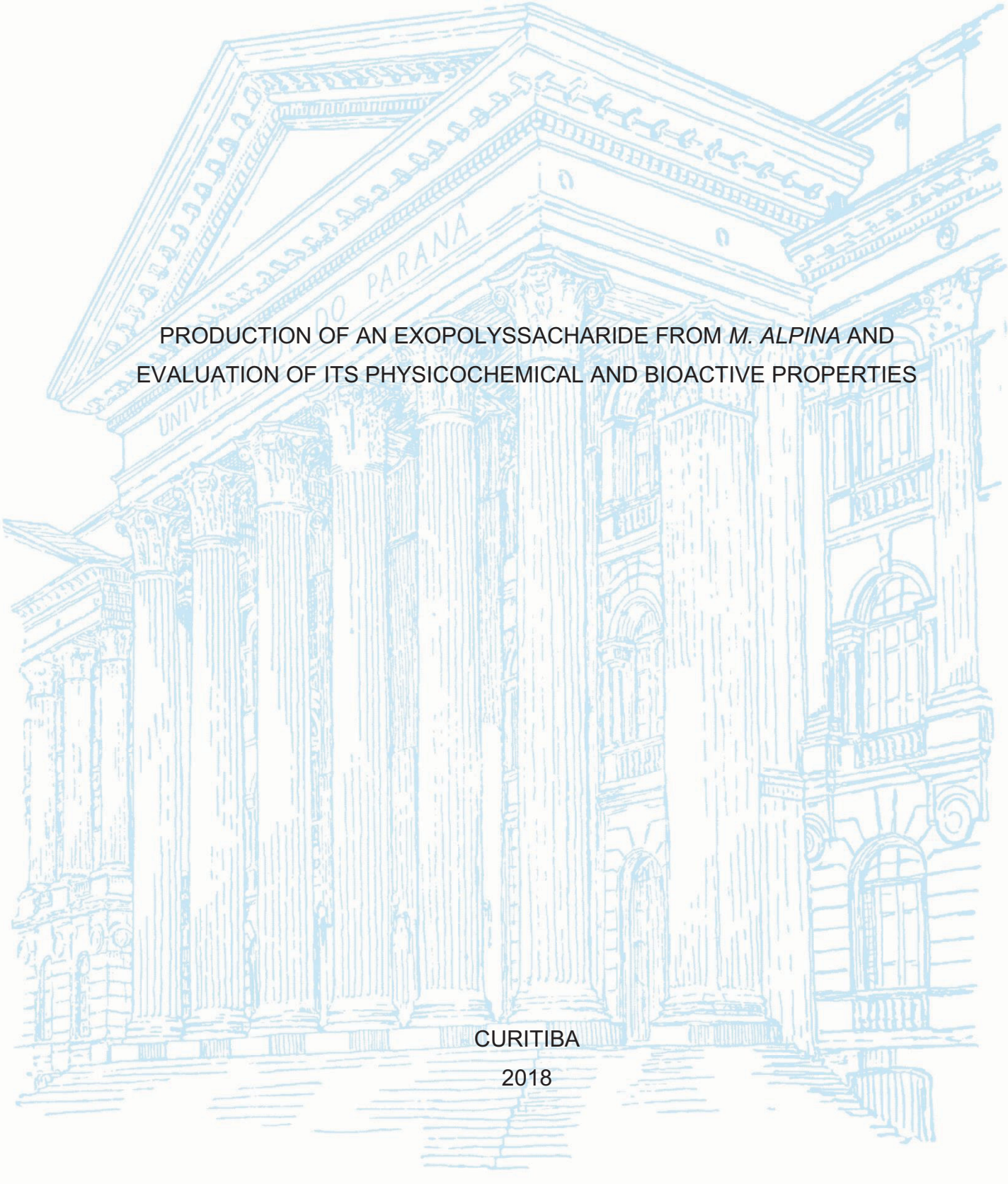
UNIVERSIDADE FEDERAL DO PARANÁ

LUIS DANIEL GOYZUETA MAMANI



PRODUCTION OF AN EXOPOLYSSACHARIDE FROM *M. ALPINA* AND EVALUATION OF ITS PHYSICOCHEMICAL AND BIOACTIVE PROPERTIES

CURITIBA

2018

LUIS DANIEL GOYZUETA MAMANI

PRODUCTION OF AN EXOPOLYSSACHARIDE FROM *M. ALPINA* AND EVALUATION OF ITS PHYSICOCHEMICAL AND BIOACTIVE PROPERTIES

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Programa de Pós-graduação e Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Júlio Cesar de Carvalho

Co Orientador: Prof. Dr. Carlos Ricardo Soccol

Curitiba

2018

Catalogação na Fonte: Sistema de Bibliotecas,
UFPR Biblioteca de Ciência e Tecnologia

M263p Mamani, Luis Daniel Goyzueta
Production of an exopolyssacharide from M. alpina and evaluation of its physicochemical and bioactive properties [recurso eletrônico] / Luis Daniel Goyzueta Mamani. – Curitiba, 2018.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientador: Júlio Cesar de Carvalho – Coorientador: Carlos Ricardo Soccol.

1. Quitina. 2. Mortierellaceae. 3. Exopolissacarídeo. 4. Antioxidantes. I. Universidade Federal do Paraná. II. Carvalho, Júlio Cesar de. III. Soccol, Carlos Ricardo. IV. Título.

CDD: 573.774

Bibliotecário: Elias Barbosa da Silva CRB-9/1894

MINISTÉRIO DA EDUCAÇÃO
SETOR SETOR DE TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA - 40001016036P8

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LUIS DANIEL GOYZUETA MAMANI** intitulada: **Production of an exopolyssacharide from *M. alpina* and evaluation of its physicochemical and bioactive properties**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 11 de Dezembro de 2018.

JULIO CESAR DE CARVALHO
Presidente da Banca Examinadora

CRISTINE RODRIGUES
Avaliador Externo (UFPR)

RILTON ALVES DE FREITAS
Avaliador Externo (UFPR)

SANDRO JOSE RIBEIRO BONATTO
Avaliador Externo (IPPP)

MIGUEL DANIEL NOSEDA
Avaliador Interno (UFPR)

I dedicate this work of doctoral conclusion to my family, for infinite support and unconditional love, and especially to my mother, who thanks to her I am who I am and I am where I am.

AGRADECIMENTOS

Agradeço infinitamente a mina mãe, Bertha, pela forca, sapiência e amor incondicional; agradeço o seu total sacrifício, educação e integridade. Para você mãe minha eterna gratidão e admiração.

A minha vovó, por me incluir nas suas orações e ser um porto seguro.

Meu pai, e irmãos pelos conselhos a sempre seguir em frente, mesmo em momentos difíceis.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa de estudo;

A Universidade Federal de Paraná e ao Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, pela grande oportunidade outorgada.

Ao meu orientador Prof. Dr. Júlio Cesar de Carvalho, pela colaboração, orientação, amizade e autonomia no trabalho de doutorado.

Ao meu co-orientador Prof. Dr. Carlos Ricardo Soccol, pela oportunidade de desenvolver a pesquisa e oportunidade de trabalhar com um grupo bem respeitado.

Ao Prof. Dr. Daniel Miguel Noseda, Prof. Dr. Sandro Bonatto e ao Prof. Dr. Rilton Alves de Freitas pelo seu apoio, colaboração e orientação na pesquisa desta tese de doutorado.

Ao centro de Pesquisa Pelé Pequeno Príncipe, e seu staff pela ajuda e momentos de descontração.

Aos técnicos de laboratório e professores do Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia,

E finalmente, aos amigos verdadeiros que me acompanharam nesta jornada, tornando-a um capitulo maravilhoso na minha vida.

A todos vocês, muito obrigado.

RESUMO

A contínua procura por substâncias com potencial biológico tem sido objetivo de numerosas pesquisas no último século, especificamente na prospecção de substâncias que apresentem estruturas estáveis que possam ser utilizadas em formulações de produtos. Por esses motivos a finalidade deste trabalho foi de produzir, otimizar, caracterizar e avaliar o potencial de atividades biológicas do exopolissacarideo (EPS) produzido pelo fungo filamentoso *Mortierella alpina*. O meio de cultura para produção de EPS pelo fungo filamentoso foi primeiramente otimizado mediante técnicas de desenho experimental (desenho fatorial e delineamento composto central rotacional), conseguindo um aumento do 50% da produção final quando comparado com o meio convencional. Na sequência, o EPS foi caracterizado por técnicas de ressonância magnética nuclear (RMN), infravermelho por transformada de Fourier (FTIR), espalhamento dinâmico de luz (DLS), cromatografia de alta pressão com exclusão por tamanho (HPSEC) e cromatografia gasosa acoplada a espectrometria de massas (GC MS). O exopolissacarideo foi caracterizado como quitina. A quitina é o segundo polissacarídeo mais abundante na natureza, produzido convencionalmente a partir de fontes animais, e comumente comercializada devido as suas propriedades reológicas e múltiplas atividades biológicas. Mas pouco é pesquisado sobre fontes alternativas de quitina, tais como fungos filamentosos, estudada nesse trabalho. A produção de quitinas e seus derivados aminados geram uma grande quantidade de resíduos tóxicos para o meio ambiente no seu processo de extração, além de ser alergênicos para pessoas com intolerância a frutos do mar.
O potencial em atividades biológicas e funcionais deste EPS, também foi analisado mostrando ação bacteriostática frente a organismos Gram positivos e negativos, um efeito antioxidante, uma leve capacidade quelante de íons Fe^{2+} e finalmente um grande potencial como agente antitumoral frente a linhas celulares tumorais de câncer de mama, cólon e de carcinoma adrenocortical, resultando inócuas frente aos seus respectivos controles não tumorais. O carcinoma adrenocortical por ser um câncer incomum, mas com frequentes casos na região sul do país, foi selecionado para posteriores estudos do potencial anti-tumoral e pro-apoptótico do EPS, mostrando resultados promissórios na etapa *in vitro.* Finalmente, um estudo físico-químico das

propriedades reológicas do EPS foi realizado devido á particularidade e comportamento deste carboidrato com respeito à temperatura, solubilização e formação de géis.

Os resultados obtidos nesse trabalho demonstraram que o exopolissacarideo (quitina) produzido por *Mortierella alpina* pode ser utilizado como um agente para posteriores formulações de biomateriais, como fonte de quitina “vegana” na formulação de alimentos e outros, e indubitavelmente como agente antitumoral que pode ser posteriormente avaliado para formulação de drogas quimioterapeuticas, adjuvante em formulações afins.

Palavras-chave: *Mortierella alpina*, exopolissacarídeo, antioxidante, bacteriostático, antitumoral, quitina.

ABSTRACT

The continuous search for substances with biological potential has been the object of intensive research in the last century, specifically in the prospection of substances that have stable structures that can be used in product formulations. For these reasons the purpose of this work was to produce, optimize, characterize and evaluate the potential biological activity of exopolysaccharides (EPS) produced extracellularly by the filamentous fungus *Mortierella alpina*. The culture medium for the production of EPS by the fungus was firstly optimized using experimental design techniques (factorial design and central rotational compound design), leading to a 50% increase in final production when compared to the conventional medium. Then, the EPS was characterized by nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), dynamic light scattering (DLS), high pressure size exclusion chromatography (HPSEC) and gas chromatography coupled to mass spectrometry (GC MS). The exopolysaccharide was characterized as chitin. Chitin is the second most abundant polysaccharide in nature, conventionally produced from animal sources, produced and marketed because of its rheological properties and multiple biological activities. But little is researched on alternative sources of chitin, such as filamentous fungi, studied in this work. The production of chitins and their amino derivatives generate a large amount of toxic waste for the environment in its extraction process, besides being allergenic for people with intolerance to various seafoods.

The potential biological and functional activities of this EPS were observed a bacteriostatic action against Gram-positive and Gram-negative organisms, an antioxidant effect, a slight Fe^{2+} ion chelating capacity and finally a great potential as antitumor agent against cancer tumor cell lines of breast, colon and adrenocortical carcinoma.

Adrenocortical carcinoma, 1 is an uncommon cancer, but with frequent cases in the southern region of the country, for which it was selected for further studies of the anti-tumor and pro-apoptotic potential of the EPS, showing promising results in the *in vitro* stage. Finally, a physicohemical study of the rheological properties of EPS was performed due to the particularity and behavior of this carbohydrate with respect to temperature, solubilization and gel formation.

The results obtained in this work demonstrated that the exopolysaccharide (chitin) produced by *Mortierella alpina* can be used as an agent for subsequent formulations of biomaterials, as a source of "vegan" chitin in food and other formulation, and undoubtedly as an antitumor agent that can be subsequently evaluated for formulation of chemotherapeutic drugs, adjuvant in related formulations.

Key words: *Mortierella alpina*, exopolysaccharide, antioxidant, bacteriostatic, antitumor, chitin

SUMMARY

INTRODUCAO

Mortierella alpina é um fungo filamentoso que possui grau GRAS (Generally Recognized as Safe), e reconhecido como a maior fonte de ácido araquidônico (ARA) (Chand, Rajesh, Tomar, & Kumar, 2016). Dietas suplementadas com esse ácido graxo poli-insaturado são altamente recomendadas pela FAO (Food and Agriculture Organization) e WHO (World Health Organization). Por isso, os estudos sobre otimização de meios de cultura de fermentação, produção de biomassa, produção de ácidos graxos, entre outros tem aumentado significativamente nas duas últimas décadas

Por outro lado, poucas são as pesquisas sobre outras substâncias bioativas produzidas por esses microrganismos. Nas últimas décadas, inúmeros polissacarídeos têm sido isolados de várias fontes com a intenção de determinar potenciais atividades e aplicações para diferentes indústrias como biofarmacêuticas, de biomateriais ou de alimentos (Ooi & Liu, 2000). Polissacarídeos de fontes como plantas, fungos (especificamente basidiomicetos) e animais têm demonstrado uma ampla variedade de atividades, dentre elas a capacidade antitumoral, imunomoduladora, antioxidante e anti-inflamatória (Y. Yu, Shen, Song, & Xie, 2018). Porém, a busca de novas fontes naturais eficientes com ação antitumoral com baixa toxicidade tem tido bastante atenção nos últimos anos (Choromanska et al., 2015).

Levando-se em consideração esses aspectos, o presente trabalho tem como finalidade a produção e obtenção do exopolissacarideo produzido por *Mortierella alpina* como uma alternativa de produto bioativo, assim como a elucidação da suas características físico-químicas, a avaliação das suas propriedades reológicas e bioativas. Para nosso conhecimento, este é o primeiro trabalho sobre a produção, caracterização e avaliação de potencial antitumoral e adjuvante de um exopolissacarideo produzido por *M. alpina* assim como das suas propriedades reológicas.

.

OBJETIVO GERAL

O principal objetivo desse trabalho foi a caracterização do exopolissacarideo produzido por *Mortierella alpina*, assim como a avaliação de suas atividades biológicas e físico-químicas.

OBJETIVOS ESPECIFICOS

- Otimização do meio de cultura para produção do exopolissacarideo.
- Recuperar o exopolissacarideo resultado de fermentação submersa.
- Avaliar a pureza e homogeneidade do exopolissacarideo.
- Elucidar por diferentes técnicas de RMN e espectroscopia a estrutura química do exopolissacarídeo.
- Avaliar o grau de acetilação do exopolissacarídeo.
- Avaliar o potencial antioxidante, bacteriostático e antitumoral do exopolissacarídeo.
- Avaliar o efeito antitumoral e pro-apoptotico do exopolissacarideo em células de carcinoma adrenocortical.
- Avaliar as características reológicas do exopolissacarídeo.

ESTRUTURA DO DOCUMENTO

Esta tese foi redigida na forma de capítulos, em língua inglesa, refletindo as publicações preparadas em paralelo com este documento. O primeiro capítulo descreve a revisão bibliográfica baseada especificamente nas características na produção de ácidos graxos poli-insaturados por *Mortierella alpina*, descreve também generalidades do microrganismo assim como um estudo de análise das tendências do mercado. Esta revisão bibliográfica está complementada com um levantamento de patentes e revisão sobre trabalhos de pesquisa relacionados à produção de polissacarídeos produzidos por *Mortierella alpina.* O segundo capitulo descreve a otimização em escala laboratorial, produção e investigação da estrutura do

polissacarídeo produzido por este fungo filamentoso, estudos de caracterização físico-química e de avaliação de propriedade bioativas foram efetuadas para avaliação do seu potencial como agente bioativo. No terceiro capitulo, estudou-se *in vitro* o potencial deste EPS em um tipo especifico de câncer, o carcinoma adrenocortical, que como mencionado antes, é um câncer pouco comum no mundo mas que apresentam frequentes casos específicos nesta região sul do Brasil, especificamente na população infanto-juvenil, e finalmente no quarto capitulo descreve-se propriedades reológicas do EPS, devido às particularidades observadas deste carboidrato no decorrer dos estudos feitos. Na última seção, são apresentadas as conclusões gerais e referências.

1. CHAPTER I (LITERATURE REVIEW) - TRENDS IN THE MARKET, INDUSTRIAL ARACHIDONIC ACID-RICH OIL AND POLYSACCHARIDE PRODUCTION BY *MORTIERELLA ALPINA*

1.1. ABSTRACT

Polyunsaturated fatty acids are essential, health-promoting nutrients that are widely used in medicine, pharmaceutics, cosmetics, nutrition, and other fields. Suitable plant and animal sources for these lipids are limited, but alternatives have been actively researched and developed in the last three decades, especially those produced microbiologically. Arachidonic acid (ARA) is one of the most valuable among these nutraceutical lipids, being associated with good development of the nervous central system and enhancement of immune response. Currently, microbial sources of ARA are used for industrial production due to their rapid and controllable production, as compared to animal (fish) and plant sources. Microbial sources are also eco-friendly and reduce the pressure on marine life. The fungus *Mortierella alpina* is one of the most important microbial lipid sources, but there are few accounts of industrial and market information and other bioactive metabolites. The demand for the essential fatty acid ARA will likely continue to grow, with a production forecast of 287,500 tons/year by 2020. This will keep stimulating research for intensified production, which can be optimized in terms of concentration and yields (through bioprospecting and metabolic and biomolecular engineering), productivity and economics (through media optimization and byproduct use), and formulation. This work reviews aspects such as the important considerations for arachidonic rich oil production, its industrial production by *Mortierella alpina*, based on patents and studies, a global market analysis, the forecast for the arachidonic acid market and a patent research related to another metabolites produced of this filamentous fungi.

Keywords: ARA; *Mortierella alpina*; PUFA; downstream; industrial production; market.

1.2. INTRODUCTION

The inclusion of essential fatty acids (EFA) in human diets, especially for infants, has become popular in recent decades and this is fueling their production around the world. More specifically, polyunsaturated fatty acids (PUFA) are of great interest because of their beneficial properties to human health. There are several well-studied PUFA, such as arachidonic acid (ARA) and docosahexaenoic acid (DHA), which have been biotechnologically produced from the oleaginous fungus *Mortierella alpina* since 2001, after having been granted the generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA). Nowadays, some industries use PUFA as additives resulting in different products. ARA can be used such as infant and geriatric formulas, food supplements, cosmetics, and drugs.

ARA is a long-chain (20:4 Δ5,8,11,14 n-6) polyunsaturated fatty acid of the ω-6 class. This fatty acid can be found in the blood, brain, glandular organs, and liver. ARA plays a role in maintaining hippocampal neuron membrane fluidity (Kenichi Higashiyama, Fujikawa, Park, & Shimizu, 2002) and is synthesized in animals and humans by the desaturation and elongation of linoleic acid (Oregon State University, 2014). It is a precursor of several mediators of essential processes, acting in the modulation of inflammation, cytokine release, platelet aggregation, immune response, allergic phenomena, and thrombosis (Uauy, Mena, & Rojas, 2000). It is also important as a second messenger in the central nervous system and plays a role in the expression of long-term potentiation (Nishizaki, Nomura, Matsuoka, & Tsujishita, 1999).

ARA-rich oil is commonly used in food formulation to enhance the development and health of the brain, vascular systems, and nervous system. ARA and DHA comprise over 90% of the brain essential fatty acids (A Nisha & Venkateswaran, 2011). ARA is used in formulas to reach erythrocytes and blood lipid fatty acid profiles that are equivalent to those in breast-fed infants (Hoffman et al., 2000). Its mixture with DHA helps to improve visual function, cognitive development, and normal blood pressure (J. S. Forsyth et al., 2003). In 2014, about 120 thousand tonnes of ARA were produced (QY Research center, 2015). Based on the market's growth by 2020, ARA

production might reach 290 thousand tonnes per year (as oil with 40% of ARA), and sales of up to US$50 million may be expected.

Industrial production of ARA is a relatively new technology that relies on the cultivation of lipid-rich biomass in submerged media as well as the extraction and purification of the oil. The aim of this research is to present the main steps of the industrial processing of ARA by *M. alpina*, as well as the innovations and world market trends. This manuscript describes the industrial aspects of productivity, details of ARA and the growth conditions, the processing of biomass into ARA-rich oil, and an overview of commercial products, the market for this valuable fatty acid and promising paths for further research and development.

1.3. ARACHIDONIC ACID PRODUCED BY *MORTIERELLA ALPINA*

The genus *Mortierella* (family *Mortierellaceae*, order *Mortierellales*, class *Zygomycetes*) was first described in the 19th century and the first mention of the species *M. alpina* dates back to 1913 (Saccardo, Traverso, & Trotter, n.d.). The species *alpina* is the most important industrially. Several wild and genetically modified strains of *M. alpina* have been evaluated to optimize industrial processes for PUFA-rich oil production. Table 1 presents literature productivity data for several strains of *M. alpina* and shows significant differences between various producers, with final ARA concentrations ranging from 1 to 20 g L^{-1} and productivities ranging from 0.2 to 1.8 g L^{-1} day^{-1}.

Table 1. Arachidonic acid and total lipid production using *M. alpina* in multiliter or pilot-scales.

Strain	Assay volume (L)	ARA (g L^{-1})	Time (days)	Productivity (g L^{-1} day^{-1})	Reference
Submerged culture - Flask					
ME-1	0.250	11.4	-	-	(Ji et al., 2014)
ATC 32222	0.250	11	11	1	(Singh & Ward, 1997)
Wuji-H4	0.250	3.9	5	0.8	(Ho & Chen, 2008)
Submerged culture - Fermenter					
ME-1	3	19.8	11	1.8	(Jin et al., 2008)
	3.5	9.2	7	1.3	(Peng et al., 2010)
1S-4	10	13	10	1.3	(Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawa, & Shimizu, 1998)
	5	3	8	0.4	(Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawa, et al., 1998)
ATCC 32221	500	11	16	0.7	(Nagao Totani, Someya, & Oba, 1992)
ATCC 16266	45	6.5	11	0.6	(Fischer et al., 2013)
UW-1	20	5.5	8	0.7	(Li, Lu, Yadwad, & Ward, 1995)
LPM 301	30	4.5	8	0.6	(Eroshin, Satroutdinov, Dedyukhina, & Chistyakova, 2000)
	6	4.5	8	0.6	(Eroshin et al., 2000)
ATCC 42430	20	4.1	6	0.7	(David J Kyle, 2001)
DSA-12	500	3.3	6	0.6	(E. Y. Park, Koike, Higashiyama, Fujikawa, & Okabe, 1999)
M18	6	1.41	7	0.2	(L. J. Yu, Qin, Lan, Zhou, & Zhu, 2003)
CBS 528,72	-	1.39	7	0.2	(Alpettiyil Nisha, Rastogi, & Venkateswaran, 2011)
CBS 343.66	5	1.0	6	0.2	(Lindberg & Molin, 1993)
Solid culture - Flask					
IFO 8568	-	13[a]	20	0.65	(N. Totani, Watanabe, & Oba, 1987)
CCF 185	0.300	36[a]	21	1.42	(Stredanská & Šajbidor, 1993)

The biosynthesis and accumulation of lipids in oleaginous microorganisms depend on the ability to produce acetyl-CoA (precursor for fatty acid synthetase – FAS) and NADPH. Nitrogen depletion induces the formation of acetyl-CoA through the cleavage of citrate in the cytosol by the ATP:citrate:lyase (ACL) (Colin Ratledge, 2004).

The unsaturated fatty acids synthesis starts from a C-16 backbone produced by FAS. This backbone is elongated and unsaturated in successive enzymatic steps to form two important families of PUFAs: ω3 and ω6. For the formation of arachidonic acid, a ω6-PUFA, palmitic acid (C16:0) is elongated forming stearic acid (C18:0), which is then sequentially unsaturated at the Δ9, Δ12 and Δ6 positions, forming gamma linolenic acid. This is further elongated, and unsaturated by Δ5-desaturase to form ARA (Certik & Shimizu, 1999). These desaturases are oxygen-dependent and require cytochrome b5 as a co-factor (Michaelson, Lazarus, Griffiths, Napier, & Stobart, 1998). A variety of mutants with enhanced desaturase (Δ9, Δ12, Δ6, Δ5 and ω-3) and elongase (EL1) activity have been derived from *M. alpina* 1S-4 (Saeree Jareonkitmongkol, Kawashima, Shimizu, & Yamada, 1992). Several mutants derived from 1S-4 and their characteristics are summarized in Table 2. *M. alpina* strains ME-1 and 1S-4 have been used for industrial production as well as a model for lipogenesis studies due to their high oleaginous capacity, lipogenesis regulation, PUFA production, and capacity of absorption and transformation of exogenous fatty acids (S Shimizu & Jareonkitmongkol, 1995).

Table 2. Derivations of mutants from *M. alpina* 1S-4.

Mutant	Characteristic	Reference
Δ5 desaturase-defective	High dihomo-gamma-linolenic (DHGLA) and low ARA level production. Does not need an inhibitor for DHGLA production; high yields of ARA (4.1 g/L, 42% in oil). Commercial strain.	(S Jareonkitmongkol, Kawashima, & Shimizu, 1993)
Δ12 desaturase-defective	n-6, n-3, and n-9 PUFAs absent from their mycelia. Produces an oil rich in mead acid. Produces ARA and eicosapentaenoic acid (EPA) when n-6 or n-3 fatty acids are added (i.e., J180, M-209-7). Used for production of EPA rich-oil with low ARA quantities	(S Jareonkitmongkol et al., 1993)
Double defective in both Δ12 and Δ6	Accumulation of 20:2 n-9 in large quantities and α-linolenic acid inhibits the conversion of oleic acid to 20:2 n-9 which is converted in 20:4 n-3	(Kamada et al., 1999; Kawashima et al., 1998)
Δ6 desaturase-defective	High linoleic acid synthesis and low concentration of GLA, DHGLA, and arachidonic acid. Characterized by production of eicosadienoic acid (20:2n-6) and eicosatrienoic acid (20:3n-6 (Δ5)). Capable of producing methylene-interrupted n-3 eicosatrienoic acid (20:4n-3 (Δ5)) from α-linolenic.	(S Jareonkitmongkol et al., 1993)
n-3 desaturase-defective	Unable to synthesize n-3 PUFAs at low temperature (<20º). High quantities of ARA at 20 ºC, but part of it is converted to EPA (i.e., Y11, Y135, Y61).	(Eiji Sakuradani et al., 2004)
Δ9 desaturase-defective	Stearic acid is the main fatty acid produced (up to 40%). This enzyme is not completely blocked, since a minimum activity is needed for cell viability (introduction of the first double bond)	(S Jareonkitmongkol, Sakuradani, & Shimizu, 1994)
Elongase (EL1 for the conversion of 16:0 to 18:0)-defective mutants	Produces high levels of palmitic acid (16:0) and palmitoleic acid (16:1 n-7), with low amounts of n-7 and n-4	(Eiji Sakuradani et al., 2004)
Mutants with enhanced desaturase activities	The mutant 209-7 with 1.4-fold activity of elevated Δ6 desaturase activity. JT-180 is another mutant with elevated Δ5 desaturase activity, which produces a large quantity of mead acid (49% in oil).	(E Sakuradani et al., 2002; Eiji Sakuradani, Ando, Shimizu, & Ogawa, 2013)
Diacylglycerol-accumulating	The percentage of triacylglycerols is about 90% of the total lipids in the mutant *M. alpina* 1S-4. KY1 accumulates 30% of diacylglycerol and is expected to be used to produce diacylglycerols rich in C20 PUFAs.	(Eiji Sakuradani et al., 2004)

Besides the selection of good producers, the composition of fatty acids in *M. alpina* can be manipulated by varying fermentation conditions (e.g., agitation, oxygenation, pH). The lipid concentration in the fungus may reach 50% of the biomass (Singh & Ward, 1997), with levels of ARA between 30% and 70% of the total fatty acids (Amano et al., 1992).

Two types of cultivation are used for industrial-scale production: submerged and solid-state. Submerged cultivation is preferred because of its easier scale-up, biomass recovery, higher ARA production yield, and fermentation time (solid-state fermentation needs a cultivation period of over 20 days). High yields of arachidonic acid have been reported in over 10 g L^{-1} of liquid culture (Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawa, et al., 1998; Jin et al., 2008; Singh & Ward, 1997), with a productivity of approximately 1 g L^{-1} per day. The final ARA concentration and the productivity of multiliter or pilot-scale cultures of *M. alpina* presented in Table 1 shows that most of the best-producing strains are mutants, which have distinctive features in their fatty acid synthesis pathway. Therefore, understanding the biosynthesis of fatty acids in *M. alpina* is the key to enhancing lipid production.

1.4. INFLUENCES ON THE FERMENTATION PROCESS

The parameters that influence the biotechnological production of ARA are essential to achieve a high productivity and yield. The suitable conditions for an improved ARA production according to several authors are: glucose as a carbon source; yeast extract as a nitrogen source; supplementation with calcium, magnesium, manganese, iron, copper, zinc, and potassium; at 25º C, pH 6.00, and 15 ppm of oxygen. Most literature investigating *Mortierella* cultures describe its production at a small scale. In this section, we describe the main factors that can affect the production of ARA by *M. alpina*.

1.4.1. Conservation and inoculation of *Mortierella alpina*

In industrial processes, it is essential that the strains maintain viability and lipid production capacity, especially the ARA yield. Most species of *M. alpina* do not sporulate in laboratory conditions. The mutant strain 1S-4u (uridine-requiring auxotrophic mutant) is an exception (Lounds, Eagles, Carter, MacKenzie, & Archer, 2007). Since 1S-4u is mostly used in industrial fermentations, its preservation is important for maintaining reproducible ARA yields.

A punctual example is the worked carried out by (Higashiyama,2005), in which the author developed a conservation technique based on the spores produced by this mutant strain grown in Czapeck medium (with pH adjusted to 4–7). Spores were harvested with sterilized water (10^6 spores mL^{-1}). Addition of 10% cryoprotectant, usually glycerin (Morris, Smith, & Coulson, 1988), and the storage of the solution in ultra-low temperatures (between -85 and -50 ºC) to guaranteed viability. In this study, the microorganism conserved produced ARA reliably for 5 years. The average ARA yield with inocules produced from spore suspensions were reduced by 3%, a result better than the 7% reduction observed when refrigerated slants with mycelium are used. For short periods (< 3 months), a preparation of spore/mycelia suspension from slants of PDA and Czapeck agar can be stored for use at 5 ºC (Zhu, Yu, Liu, & Xu, 2004).

Due to the poor sporulation of *M. alpina*, a mycelial suspension may be used as an inoculum in fermentation scale-up after partial mechanical disruption using a blade mill (Rocky-salimi, Hamidi-esfahani, & Abbasi, 2011).

1.4.2. Medium components

The strain, carbon sources, and fermentation conditions must be selected based on the fatty acid of interest. For arachidonic acid production, the most frequently used carbon source is glucose (Jang, Lin, & Yang, 2005), probably because it directly feeds the glycolytic pathway (Wynn, Hamid, Li, & Ratledge, 2001). (Shinmen & Shimizu, 1989) tested glucose and other carbon sources – fructose, maltose, soluble starch and corn starch – using *M. alpina* 1S-4. These authors obtained the same amount of biomass among all carbon sources assays but higher ARA yields when glucose was used as the carbon source. Stredanská & Šajbidor (1993) also affirmed that glucose was the most suitable source of carbon for biomass and total lipid production, compared to dextrins. Alpettiyil Nisha et al. (2011) achieved 40.41% of total PUFA using rhamnose. However, this is an expensive sugar and is possibly not feasible for use in producing ARA on an industrial scale. These authors ranked the carbon sources in the following order: rhamnose > glucose > mannose > fructose > lactose > raffinose > starch.

Koike, Jie Cai, Higashiyama, Fujikawa, & Park (2001) affirms that when glucose concentration increases from 2.0% to 12.0% in *M. alpina* production media, the fatty acid content tends to increase. However, the ARA yield shows the opposite behavior. More than 20% of glucose (w/v) in culture media increases the osmotic pressure, hindering lipid and biomass production (Nagao Totani, Someya, et al., 1992). Starch can be an option to use instead of high glucose concentrations. In a study conducted by (Jang et al., 2005), arachidonic acid comprised 57.3–64.8% of the total PUFA, with soluble corn starch as the carbon source. Alkane hydrocarbons such as decane and alcohols such as hexadecanol were also tested in the search for alternative carbon sources. A total of 14 g L^{-1} of biomass and 5 g L^{-1} of total fatty acids was obtained using decane (Yokochi, Kamisaka, Nakahara, & Suzuki, 1995), showing an increment of the biosynthesis of linolenic acid (47% of the total fatty acids) (Xian et al., 2001).

Nitrogen is essential for protein synthesis, while the C/N ratio affects the cellular composition. In general, low C/N ratios – i.e., nitrogen-rich media – favor the production of biomass, while high C/N ratios favor the synthesis of substances for energy reserve, such as carbohydrates and lipids. The depletion of nitrogen during cultivation is a prerequisite for fatty acid accumulation, but it is not favorable for biomass growth. Koike et al., (2001) evaluated different C/N ratios and showed that the optimum ratio was in the range of 15 to 20 (weight basis). ARA production in a medium with a C/N ratio below 15 is constant; above that it increases proportionally to the C/N ratio, up to 20. Šajbidor, Dobroňová, & Certik, (1990) reported the same range as being optimum for ARA production in *Mortierella* sp. S-17. Packter, (1981); Nagao Totani, Someya, et al., (1992); Wynn et al. (2001) affirmed that organic nitrogen sources (amino acids and peptides) are preferable for *M. alpina* growth. Yeast extract was found to be the most suitable nitrogen source for biomass, lipid, and ARA production (due to the presence of micronutrients). Organic sources of nitrogen affect lipogenesis regulation and increases acetyl-CoA carboxylase (ACC) activity. Oleaginicity depends on ACC, which synthesizes malonyl-CoA – the key building block for lipid synthesis. Malic enzyme (ME) activity also increases when organic nitrogen sources are present (Certik, Megova, & Horenitzky, 1999). After studying the use of yeast extract and soybean meal, Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawa, et al., (1998) concluded that yeast extract is effective as a source of nitrogen. E. Y. Park et al. (1999) investigated

several nitrogen sources (yeast extract, corn steep liquor, pharmamedia, fishmeal, and gluten meal) and obtained better results with yeast extract than with other sources. These authors also established a relationship between ARA production and morphology, affirming the efficacy of feather-like pellets.

Nitrogen sources also affect the mycelia morphology and ARA production of *Mortierella* (E. Y. Park et al., 1999). This is important because the morphology affects oxygen transfer and nutrient diffusion due to the availability of a greater transfer area (Y. Park, Tamura, Koike, Toriyama, & Okabe, 1997). Koike et al. (2001) noted that the C/N ratio influenced the ARA production and pellet morphology of *M. alpina*. Natural sources of nitrogen may also change the morphology of *M. alpina* in submerged fermentation. A Nisha & Venkateswaran (2011) reported morphology variations using yeast extract, which resulted in fluffy circular pellets (feather-like pellets), while other nitrogen sources resulted in filamentous mycelia growth. (Nagao Totani, Hyodo, & Ueda, 2000) tested salts as nitrogen sources, including ammonium nitrate, sodium nitrate, ammonium acetate, and ammonium sulfate, and obtained low biomass production yield. Yeast extract at 1% (w/v) concentration was considered optimum for ARA production.

Micronutrients, such as manganese, calcium, iron, copper and zinc, are also important in the medium composition for ARA fermentation. Sajbidor, Kozelouhova, & Certik (1992) investigated the effect of selected metal ions for better ARA production in *Mortierella* sp. S-17. They found that biomass production was not affected, but ARA yields were maximized with the addition of 2 mg L^{-1} manganese. There are metal ions with positive effects on mycelial growth but negative effects in fatty acid production. (Nagao Totani et al., 2000) found that manganese, calcium chloride, and iron are essential for lipid production. (Kyle,1997) concluded that iron, copper, and zinc stimulate ARA production in *M. alpina* while (Nagamuna, Uzuka, & Tanaka, 1985) observed the beneficial effect of calcium (100 mg L^{-1}) on lipid overproduction. The use of bivalent cations is important because of their role as a cofactor of acetyl-CoA carboxylase and other enzyme complexes that are essential in the first stage of fatty acid synthesis. (Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawaa, & Shimizu, 1998) studied the influence of minerals in morphology, showing that mycelia dispersion is possibly suppressed by an increase in the ionic strength, which induces pellet

formation. Pellets with 1–2 mm of diameter gave the best results with higher ARA concentrations, compared to pellets of different sizes (e.g., 0 – 1 mm and 2 – 4 mm). This could also explain the positive effect of NaCl reported by (Ho & Chen, 2008).

Since arachidonic acid is a secondary metabolite, its production may be enhanced using metabolic engineering techniques. The use of precursors, inducers, and other additives may drive the metabolism towards enhanced lipid and ARA content. Several oils have been tested as main carbon sources or culture media supplements. Presumably, when the culture media is supplemented with oils, the mold produces lipases, cleaves fats into fatty acids and glycerol, and incorporates these residues as lipid structures/skeletons (Akhtar, Mirza, Nawazish, & Chughtai, 1983). This could favorably affect the production of unsaturated fatty acids. Jang et al. (2005) tested different fats and oils (linseed, sunflower, soybean, lard, peanut and corn), and the best results, in terms of biomass production and ARA content, came from using 1% linseed oil. They also reported a positive influence of sunflower oil in DHA production. Their results showed an increment of eicosapentaenoic acid (EPA) and linoleic acid as well as a decrement in the degree of unsaturation. These fatty acids are added to long-chain fatty acids as precursors. (Shinmen & Shimizu, 1989) also tested different oils to increase the ARA production of *M. alpina* 1S-4. Their results showed an increase of 2.8 times when soybean oil was applied as a supplement. However, the use of oils as carbon sources, as tested by Nisha et al. A Nisha & Venkateswaran (2011), gave unsatisfactory results: the oils were not assimilated as easily as carbohydrates. The nature of the oil and the presence of minor components could have inhibition/repression power over desaturases and elongases. For example, the presence of sesamine in sesame oil inhibits Δ5 desaturase; therefore the conversion of dihomo-gamma-linolenic (DHGLA) into ARA is blocked, resulting in higher amounts of DHGLA (Wynn & Ratledge, 2005). There is no consensus among researchers about the most suitable oil for producing arachidonic acid due to the variable concentrations and yields obtained using different oils. Table 3 summarizes the effects of several additives tested for *M. alpina* cultivation.

Table 3. Additives for fatty acid production using *M. alpina* strains.

Additive	Effects	Reference
NaCl 2%	Added after 3 days of cultivation. A stimulation of diacylglycerol acyl transferase was reported, increasing total fatty acids (TFAs) and TAG content.	[12]
Octadecanol 2%	Directly transformed into oleic acid by desaturation.	[50]
Ethanol	Applied after 5 days of cultivation. It increases NADPH levels, which is available for desaturase activity. Ethanol is converted directly into acetyl-CoA avoiding the glycolytic way.	[62]
Glutamate (0.8 g L^{-1})	Activates Acetyl-CoA carboxylase, catalyzing the formation of malonyl-CoA (the substrate for fatty acid synthesis and elongation).	[22]
Soybean and linseed oil	Used by the microorganism as a backbone to formation of ARA. The linoleic, oleic, and linolenic acids present in these oils are converted into GLA by Δ6, Δ5 desaturase and EL2 to form DHGLA and finally ARA.	[63]
Citrates	They are cleaved for the lipid synthesis. It is cleaved by ACL (ATP:citrate:lyase) to give acetyl-CoA (principal precursor of fatty acid synthesis).	[64,65]
Glycerol	It its phosphorylated, oxidized, and converted into 3-P-glyceraldehyde. It enters the glycolytic pathway and results in acetyl-CoA.	[66]
Octadecanol, Hexadecanol	Alcohols are considered primary intermediates in the oxidation and dissimilation of alkanes towards aldehyde alcohol dehydrogenase (6-route).	[67]

1.5. FERMENTATION CONDITIONS

M. alpina growth was observed from 8 to 28 ºC, with an optimal range of 20 to 25 ºC (P Bajpai & Bajpai, 1992). (Hansson & Dostálek, 1988) evaluated several species of *Mortierella*, reporting that 25 ºC was the optimal temperature for biomass production. (Pratima Bajpai, Bajpai, & Ward, 1991) reported a decrease in arachidonic acid and biomass production of *M. alpina* ATCC 32222 when the temperature was shifted from 25 to 28 ºC, while (Sakayu Shimizu, Kawashima, Shinmen, Akimoto, & Yamada, 1988) observed a 26% decrease in ARA production when comparing cultures from 25 to 20 ºC. The fatty acid profile varies with temperature: the unsaturation level increases as the temperature decreases. In most species, the highest levels of ARA were observed at 25 ºC, while EPA prevailed at lower temperatures (Nishizaki T, Nomura T, Masuoka et al, 1999)(Huong LM, Slugen D, Sajbdor J; 1972). This may have been due to an adaptation of the PUFAs for membrane stabilization, because of the stress caused by colder conditions. However, although PUFA content increases in

lower temperatures, biomass production and ARA production are indeed highest in the range of 20º to 25ºC (Ratledge C, 1004) (Ono K, Aki T, Higashiyama K, 2011)

The pH generally shows a positive influence on the production of saturated and monounsaturated fatty acids when it is close to neutral. (Alpettiyil Nisha et al., 2011) reported an important effect of pH on biomass growth and ARA production: biomass growth ceased at pH levels outside of the range from 4.5 to 8.0. The optimum pH level for ARA production was at the range of 6–6.5 (46% of total lipids produced) (A Nisha & Venkateswaran, 2011). The initial pH influences the fungal mycelial morphology, which is a critical factor for metabolite formation (Shu & Lung, 2004) that allows the capability of cell aggregation due to the production of hydrophobic proteins to coordinate the adherence of hyphae (Feofilova, 2010).

Elongation and desaturation reactions give rise to PUFAs. These are aerobic reactions, so the dissolved oxygen concentration is an important factor (Davies, Holdsworth, & Reader, 1990). At lower temperatures, the microorganism adjusts its membrane fluidity by increasing the desaturation of its lipids (Cohen, Vonshak, & Richmond, 1987). Lower temperatures may increase the unsaturation of fatty acids because of the higher availability of oxygen for desaturase enzymes (Hunter & Rose, 1972). The oxygen concentration can also be controlled by increasing the pressure or oxygen concentration of the gas fed to the reactor. Kenichi Higashiyama, Yaguchi, Akimoto, Fujikawa, et al. (1998) studied the effect of oxygen in air under normal atmospheric pressure, with or without oxygen enrichment. They found that 15–20 ppm of oxygen concentration is an adequate level for biomass and ARA production (1.6-fold better than at 7 ppm). A morphological change was observed using an oxygen enrichment method from filaments to pellets (20–50 ppm), and a dramatic reduction of ARA yield was noted because of low transfer rates of oxygen into the pellets.

1.6. INDUSTRIAL PRODUCTION

The industrial production of bioproducts must ensure high productivity and reproducibility. Therefore, multiple variables must be evaluated when scaling up from a small laboratorial scale to maximize throughput and profit. Huong et al. (1998) compared solid and submerged cultivation at a small scale. Their solid medium was

composed of milled sesame seeds and resulted in 9-fold more DHGLA than submerged cultures provide. Nagao Totani, Someya, et al. (1992) assert that submerged fermentation is preferred for producing ARA-rich oils because it provides optimal growth conditions. As seen before, many aspects of the culture can affect the microorganism development and lipid production. However, optimized conditions at the laboratory scale are not necessarily replicated in the scaling-up, requiring further adaptation. Multiliter-scale data are valuable and rare in the literature. Table 4 shows the conditions for multiliter cultivation, as retrieved from patent literature.

Table 4. Compilation of industrial patents developed for pilot-scale production of ARA.

Strain	Company	Culture Medium	Conditions	ARA (g L^{-1})	Reference
M. alpina IFO 8568	Lion Corporation	Potato extract 1 kg L^{-1}; glucose 30 g L^{-1}	20 days; 25 ºC; pH 6.0; batch	5.7[a]	(Nagao Totani, Suzaki, & Kudo, 1992)
M. alpina 200012201-12-2-2	Wuan Polytechnic University	Glucose 80 g L^{-1}; corn flour 20 g L^{-1}; yeast extract 2 g L^{-1}; peptone 1 g L^{-1}; KH_2PO_4 1 g L^{-1}; $MgSO_4$ 0.1 g L^{-1}; cottonseed oil 10 g L^{-1}	6 days; 28 ºC, pH 6.5; 50 rpm, 1 vvm; 50 L; batch	15.1	(Dongping, Kai, Tao, & Hua, 2014)
M. alpina LU166	Xiamen University (UYXI-C)	Glucose 50 g L^{-1}; yeast extract 8 g L^{-1}; corn meal 3 g L^{-1}; KH_2PO_4 2 g L^{-1}; glutamic acid 1 g L^{-1}; $MgSO_4.7H_2O$ 0.1 g L^{-1}; $ZnSO_4.7H_2O$ 2 g L^{-1}; $CaCO_3$ 0.05 g L^{-1}; trace elements 1 mL L^{-1}; malic acid 0.0025%	5 days; 28° C; 150 rpm; pH 6; batch	6.2	(CN106244468-A.2017)
M. alpina ATCC42430	Martek Corporation	Dextrose 80 g L^{-1}, soy flour 16 g L^{-1}; $FeCl_3.6H_2O$ 30 mg L^{-1}; $ZnSO_4.7H_2O$ 1.5 mg L^{-1}; $CuSO_4.5H_2O$ 0.1 mg L^{-1}; biotin 1 mg L^{-1}; thiamine 2 mg L^{-1}; pantothenic acid 2 mg L^{-1}	10 days; 28 ºC; pH > 7.3; 0.5 vvm; 11 psi; 3 m s^{-1} agitation; 20 L; feed batch	5.29	(David J Kyle, 1997)
M. alpina CBS 754.68	Suntory Holdings Limited	Glucose 2%; soybean oil 0.1%; soybean protein 1.5%	8 days; 24 ºC, pH 6.0; 200 rpm; 1.0 vvm; 200 kPa; 50 L; feed batch	7.32	(K. Higashiyama, Yaguchi, Akimoto, & Shimizu, 2005)

		Soybean powder 4%; soybean oil 0.1%, KH_2PO_4 0.3%; Na_2SO_4 0.1%; $CaCl_2.2H_2O$ 0.05%; $MgCl_2.6H_2O$ 0.05%; saccharified starch 30%	10 days, 25 ºC, pH 6, 160 rpm; 50 L; feed batch	4.3	(Ono, Aki, & Higashiyama, 2011b)
		Glucose 2%; soybean oil 0.1%; soybean protein 1.5%; KH_2PO_4 0.3%; $MgCl_2.6H_2O$ 0.05%; Na_2SO_4 0.1%; $CaCl_2.2H_2O$ 0.05%	8 days; 24 ºC, pH 6.0; 200 rpm; 1.0 vvm; 200 kPa; 50 L; feed batch	9.04	(K. Higashiyama et al., 2005)
M. alpina SAM 2241		Glucose 1.0%; KH_2PO_4 0.3%; $MgSO_4.7H_2O$ 0.02%; polypeptone 1.5%; NaCl 0.2%; yeast extract 0.1%	10 days; 24 ºC; pH 5.0; 1.0 vvm; 10 L; feed batch	6.4	(Akimoto, Kawashima, & Shimizu, 2011)
M. alpina SAM 2197		Glucose 1.8%; soybean powder 4%; soybean oil 0.1%; KH_2PO_4 0.3%; Na_2SO_4 0.1%; $CaCl_2.2H_2O$ 0.05%; $MgCl_2.6H_2O$ 0.05%	10 days; 24 ºC; 100 rpm; 50 L; feed batch	13.5	(Ono, Aki, & Higashiyama, 2011a)
M. alpina 1S-4		Glucose 2%; soybean powder 3.1%; glycerol 0.02%; soybean oil 0.1%; K_2HPO_4 0.3%; $MgSO_4.7H_2O$ 0.06%; $CaSO_4.2H_2O$ 0.06%	8 days; 26 ºC; 1 vvm; 300 rpm, pH 6.3; 50 L; feed batch	7.17	(Katano & Kawashima, 2015)
Mutant strain	Jiabi Yousheng Bioengineering W. Co.L.	Sucrose 35 g L^{-1}; yeast extract 12 g L^{-1}	7 days; 28 ºC; pH 7.0; 220 rpm, 1 vvm; 50 L; feed batch	44.97[b]	(Zhiming, Huan, & Xu, 2014)

[a]g kg-1; [b]%

The downstream process, represented in Figure 1, is similar among these patents listed in Table 4: Biomass is harvested after the fermentation process by solid-liquid separation (i.e., filtration or centrifugation). A prior pasteurization step is recommended (60 – 65 ºC) to deactivate lipases that could degrade lipids in the final stage of fermentation, avoiding alterations in quality, appearance, odor, and taste in the final product (Colin Ratledge & Hopkins, 2006). The filtered biomass is washed with water and is usually dried and grinded. The drying process can be done using conventional air-drying or advanced methods such as with a spray dryer. After this step, the biomass cake that is formed can be pressed, loosened (by extrusion), and/or crushed. The next step is to extract the lipids, which can be done using wet or dried biomass, using organic solvents such as hexane, chloroform, ethanol, methanol, and petroleum ether.

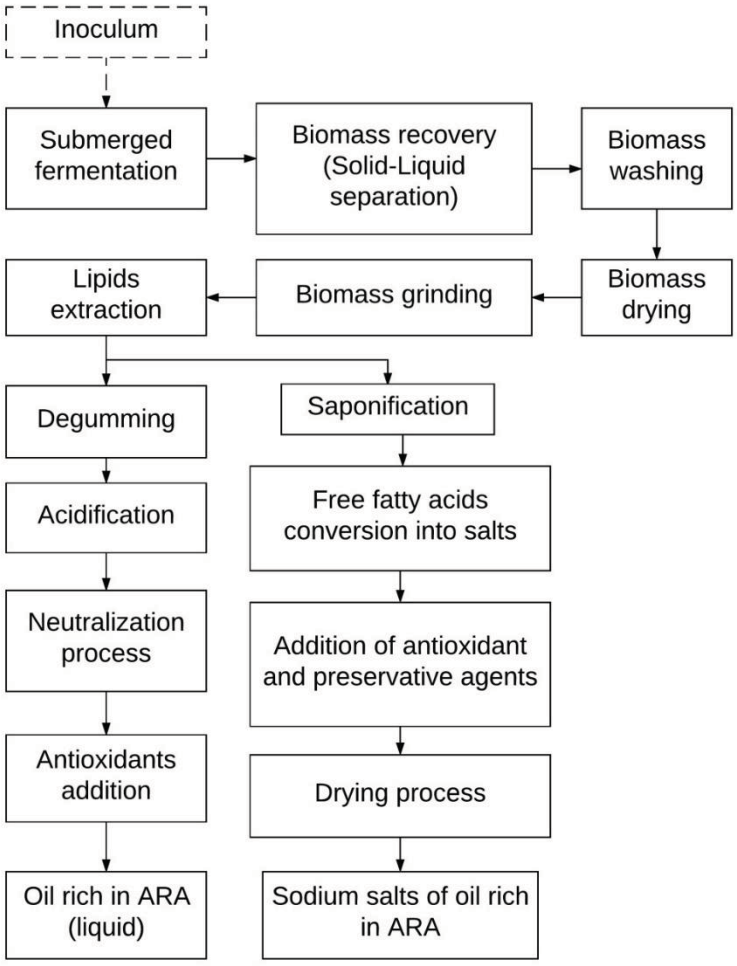


Figure 1. Process of ARA rich oil production of *M. alpina* for liquid and solid presentations.

The use of wet biomass may complicate the extraction because of the three phases that may form – solids, solvent and water micelles. The solvent must be of low polarity (Ono et al., 2011a). The most common solvent for lipid extraction in industry is hexane. Extraction may be improved with an extra step for the residual lipids in the biomass using a chloroform-methanol-water layer system, for example (Dueppen, Zeller, Diltz, & Driver, 2005). Although batch solvent extraction is the most common method reported, the lipids can also be obtained by less conventional methods, such as counter-current extraction of the dry biomass using commercial units such as an immersion extractor or a supercritical fluid extraction using CO_2 or N_2O (Akhtar MW, Mirza AQ, Nawazish MN et al., 1983). The industrial units used for SCO extraction and recovery are relatively small, compared with those used for vegetable oils. The solvent must be evaporated from the crude oil. This crude oil resulting from the extraction step can be cloudy due to the presence of biomass particles, lecithins, and free fatty acids (FFAs). Therefore, clarification is usually done, improving the aspect of the product. Lipid oxidation during the whole process can be avoided by using an inert atmosphere, e.g., nitrogen.

There are five steps in the refining process of edible oils when using the classical alkaline method (WO Patent No. 02/10322, 2002). These steps consist first of a degumming process (using membrane filtration and supercritical extraction, in which water is used to remove hydratable phospholipids and metals) (Yan et al., 2014). Then, 85% phosphoric acid is added in a proportion of 1.5 g of the acid solution per kg of oil to convert the remaining non-hydratable phospholipids into hydratable phospholipids (Patent WO No. 02/10322, 2002) . Third, the FFA is neutralized using a sodium hydroxide solution at 15% to wash out the soap and the remaining hydrated phospholipids (De Greyt, 2013). Next, there is a bleaching process with natural or acid-activated clay that absorbs colored components (Brooks, Berbesi, & Hodgson, 2011); another adsorbent option is silica gel (1% w/v). Finally, a deodorizing process is done at high temperatures (180 – 220 ºC) and low pressures (2 – 6 mbar) to remove volatile components such as ketones and aldehydes (De Greyt, 2013).

The intracellular lipids produced by *M. alpina* can be secreted to the medium by induction using surfactants or by mutation (Fukui, 1995). Akimoto et al. (Akimoto et al., 2011) patented a process to produce, recover, and encapsulate extracellular lipids

through a process in which the biomass is separated using a centrifuge at 1500x g and washed with sterile water to prepare the lipid vesicles. These vesicles were added to powdered milk to reach an ARA concentration similar to that of breast milk.

In the patent developed by Kyle (U.S Patent No. 5658767A,1997) for the formulation of the baby formula Similac®, a lipid-rich biomass was freeze-dried and then extracted using hexane in a 5:1 solvent to biomass proportion with continuous stirring for 2 h. After biomass separation, the miscella (filtrate) was evaporated to recover the oils. An additional extraction step was done with the residual biomass using ethanol in a proportion of (50:1) for 1 h, evaporating the solvent to recover it, yielding 26 g of crude oil from 100 g of biomass with an ARA concentration of 30 – 35%. This oil was added to the formula dropwise (1 g L^{-1}).

Sodium ARA salt is another product form of *M. alpina* oil that is approved for infant formula supplementation. This salt is a produced by Jost Chemical Company and is presented as a powder. The product received FDA-GRAS status in 2014; it contains a minimum of 15% of ARA, which is a lower concentration than those of known commercial oils available in the market (usually > 40% of ARA, such as ARASCO, SUNTGA, and RAO). The manufacturing process of Jost's Sodium ARA consists of three phases: first, oil that is rich in ARA is saponified into FFA using water and sodium hydroxide at 50 – 80 ºC in a nitrogen atmosphere and diluted sulfuric acid to separate the FFA, converted from triglyceride, in an organic phase. Second, FFA is converted back into sodium salts and sodium hydroxide is added, and finally, other ingredients are added to create a commercial blend (antioxidants, phosphates, maltodextrins, ascorbates, citrates, and caseinates) (Vandamme & Revuelta, 2016), which is dried. In the case of baby and geriatric milk formulas, a pool of vitamins is added to preserve the final product; some of the vitamins also work as stabilizer and antioxidant agents that protect the ARA structure from degradation. In the case of ARA, when it is not just a complement of a formulation (i.e., capsules of the microbial oil), 500 ppm of vitamin E is added (U.S Patent No. 20150064749 A1, 2015)

The presentation of the product, in Table 5 (i.e., oil SUN-TGA40S and sodium salts of *M. alpina* oil), defines some of the product is physicochemical characteristics, such as solubility and final ARA concentration. The compositional fatty acid concentrations vary between the commercial oils (ARASCO®, SUNTGA 40S and RAO) and salts (sodium ARA), but the PUFA content of these commercial products is

always superior to human milk and fish oil, which re both common sources of ARA (Table 5).

Table 5. Fatty acids compositional profile and specifications of commercial ARA oils (from GRAS-certified *M. alpina*) and conventional sources.

Fatty acid	Sodium ARA (sodium salt of the fatty acids)	ARASCO (GRN 41 and GRN 80)	AA-rich oil SUNTGA 40S (GRN94)	RAO (GRN 326)	Anchovy and sardine oil[1]	Human milk[2] %w/w (Germany)
Arachidonic acid (%)	Minimum 15	42.69 – 44.26	38.60 – 42.40	41.15 – 45.55	2.00	0.35
Myristic acid (%)	0.10 – 0.50	0.34 – 0.58	0.40 – 0.60	0.23 – 0.35	7.00	8.75
Palmitic acid (%)	4.30 – 8.10	7.17 – 9.59	11.30 – 15.10	5.43 – 6.87	16.00	24.06
Palmitoleic acid (%)	0.00 – 0.40	-	-	0.02 – 0.04	8.00	0.50
Stearic acid (%)	4.20 – 7.60	7.70 – 10.20	7.50 – 8.90	4.48 – 5.98	3.00	0.63
Oleic acid (%)	3.40 – 9.50	15.95 – 23.35	6.20 – 6.70	4.33 – 5.76	10.00	29.95
Linoleic acid (%)	3.80 – 15.20	5.56 – 7.62	-	6.92 – 8.78	1.00	5.59
γ-Linolenic acid (%)	1.70 – 2.70	2.45 – 2.99	1.35 – 2.70	-	1.00	0.55
Arachidic acid (%)	0.60 – 1.00	0.83 – 0.96	0.70 – 0.80	0.69 – 0.80	< 1.00	0.25
Homo-gamma-linolenic acid (%)	3.00 – 5.00	1.43 – 2.57	3.10 – 3.40	3.61 – 3.77	< 1.00	0.19
Behenic acid (%)	2.50 – 4.10	1.98 – 2.02	2.20 – 3.00	3.00 – 3.24	< 1.00	0.07
Lignoceric acid (%)	7.80 – 12.60	1.85 – 2.04	5.10 – 9.10	9.37 – 10.50	< 1.00	Trace
Trans-fatty acids (%)	≤ 0.50	< 1.00	-	0.04 – 0.51	-	-
Free fatty acids (%)	≤ 0.45	0.10 – 0.27	0.02 – 0.06	0.03 – 0.09	-	-
Solubility	Water soluble	Insoluble	Insoluble	Insoluble	Insoluble	-
Peroxide value (meq kg^{-1})	≤ 10.00	< 5.00	≤ 5.00	≤ 2.00	≤ 2.00	-
Anisidine value (AV)	≤ 30.00	-	≤ 20.00	≤ 20.00	≤ 20.00	-
Free fatty acids (% oleic acid)	-	< 0.40	≤ 0.20%	≤ 0.2%	-	-
Unsaponifiable matter (w/w %LOD = 0.3)	-	≤ 3.00	≤ 1.00	≤ 3.00	≤ 3.00	-
Lead (ppm)	≤ 0.10	≤ 0.10	≤ 0.10	≤ 0.10	≤ 0.10	-
Arsenic (ppm)	≤ 0.10	≤ 0.10	≤ 0.20	≤ 0.10	≤ 0.10	-
Cadmium (ppm)	≤ 0.10	≤ 0.20	≤ 0.10	≤ 0.10	≤ 0.10	-
Mercury (ppm)	≤ 0.05	≤ 0.20	≤ 0.10	≤ 0.05	≤ 0.10	-
Microbiological	Total plate count: < 1,000 cfu g^{-1}	Total plate count: < 250 cfu mL^{-1}	Bacteria ≤ 10 cell g^{-1}	Total plate count: < 10 cfu g^{-1}	-	-

[1] (Srigley & Rader, 2014); [2] (Koletzko, Mrotzek, & Bremer, 1988),

Arachidonic acid can be purified from the oil of *M. alpina* if required; several techniques with potential in industrial purification are available to do so, such as a selective esterification using microbial lipases (A. Yamauchi, Nagao, & Watanabe, 2005) consisting of three steps: a) a non-selective hydrolysis of the oil using lipase from *Alcaligenes* sp. (1,200 U g^{-1}) mixed with water (67%) at 40 ºC for 48 h at 500 rpm under nitrogen atmosphere. After the reaction, the FFAs are extracted with n-hexane; b) dissolving the FFA from the previous step in a solution of methanol, water, and urea, for elimination of long-chain, saturated fatty acids by urea adduct fractionation, and c) a selective esterification of the FFA with lauryl alcohol (Shimada, Sugihara, Nakano, & Kuramoto, 1997) (1:2 mol mol^{-1}), catalyzed by a lipase from *Burkholderia cepacia* (20 units U g^{-1} of mixture of lipase-PS) at 30 ºC for 16 h at 500 rpm. Ramjan et al. (Vali, Sheng, & Ju, 2003) proposed a technique using commercial oil from *M. alpina* that also follows three steps: i) first, commercial oil ARASCO® was saponified and fractionated by low-temperature solvent crystallization; ii) then, a selective esterification was done with LA (2:1 mol_{FA} mol_{LA}^{-1}) catalyzed by a lipase from *Candida rugosa*, at 50 ºC for 24 h and 400 rpm of agitation; and iii) finally, a solvent extraction was done using acetonitrile. The use of high-performance liquid chromatography (HPLC) was included in a methodology developed by Yuan, Wang, & Yu (2007), which combined the use of a C_{18} preparative column (methanol and water as mobile phases) after a urea-inclusion step performed in the FFA (optimum conditions: ratio of FFA/urea/methanol was 1:2:8 wt/wt, urea inclusion reaction was -10ºC) for ARA purification. The purity of the ARA reached when using these three methodologies were 97%, 95.5%, and 99%, respectively.

1.7. Arachidonic Acid Market Overview

Single-cell oils are highly valued products. The price of the oil can vary from $65/kg to over $500/kg or even more if it is composed of pure, specific, fatty acids (Gunstone, 2001). Because of the promise of sustainable and efficient production of SCO, the interest in research and development for the technology had significant contributions in the last 30 years (Ratledge, 2001). These fatty acids are currently in demand as dietary supplements for adults (in geriatric and body builder supplements) and infants (baby milk formulas).

DHA and ARA-rich oils from microorganisms are gradually substituting fish oils in infant formulas. This substitution is beneficial because 1) reduces the risk of extinction for different fish species, and 2) microbial oils are intrinsically safer; toxic substances such as dioxins and heavy metals, that may bioaccumulate in fish (Vadivelan & Venkateswaran, 2014), are absent in microbial oils. Some of the most common oils produced are oils that are rich in arachidonic acid (ARASCO™) and docosahexaenoic acid (DHASCO™) (Wynn & Ratledge, 2005). This mixture is commercialized in Europe, Asia, Australia, and North America as an ingredient of infant formulas. The FDA gave the GRAS status to DHA/ARA-SCO in May 2001 for its use in formulas in the USA. An important increase (over 50%) of formulas in the USA has happened since the first fortified formula was sold in February of 2002. Over 95% of global production is destined for formula use; in 2006, the production did not meet demand.

The first companies to produce ARA by fermentation were Gist-brocades Co. (now DSM) and Suntory Ltd., while DSM had the exclusive rights to the ARA-rich oil produced by Martek Inc.(Colin Ratledge & Hopkins, 2006). The ratio of the commercialized mixture is two parts of ARA to one of DHA (2:1 v/v). Until 2011, Martek Biosciences earned $450 million of oil sales annually before acquiring DSM acquisition (Colin Ratledge & Hopkins, 2006). Ratledge estimated in 2103 that 6000 tonnes of ARA-SCO was produced (Colin Ratledge, 2013). In 2003, the total production of ARA was around 560 tonnes, which was mostly used in infant formulas, much higher than the production in the period of 1985-2002 (690 tonnes total). At that time, 80% was used in formulas and the rest was used in other dietary supplements. Another player in this market is Suntory Ltd., which sells only the ARA-rich oil named SUNTGA40S in Japan. Since 2010, Cargill Inc. has become a rival of these two enterprises.

In 2006, the global production of infant milk formula was 1.8 million MT, including about 400,000 MT of oil and fat ingredients containing ARA and DHA (Euromonitor, 2006). By 2008, sales of supplemented milk formulas increased considerably, reaching 98% of the sales – only 2% of the formulas were unsupplemented. (Oliveira & Smallwood, 2011). According to the Global Arachidonic Acid Industry Report of 2015(QY Research center, 2015), the price of ARA has decreased by 0.89% since 2010. The expectation of industrial utilization capacity and

production of ARA by 2020 is approximately 287.5 and 262.5 thousand tonnes, respectively. The global arachidonic acid market is projected to reach $1.8 billion by 2019, and the market study predicts a big dynamic market growth in Asia (GostReports, 2016).

The demand of ARA might reach 1 million tons per year by 2025, based on the daily intake (reported by S. Forsyth, Gautier, & Salem Jr., 2016), and the growth rate of each country. (United Nations, Department of Economic and Social Affairs - Population Division - World Population Prospects, 2017) (Figure 2B). A considerably lower projection for production – 410 thousand tons in 2025 - can be made by extrapolating data from (QY Research center, 2015), which estimated the global production of ARA as 284 thousand tons in 2020, with a growth rate of 10%.

The market demand or deficit can also be based on nutritional recommendations. The minimum daily intake recommendation for ARA is 150mg/day per capita (S. Forsyth et al., 2017). Considering this requirement, a global deficit of 170 thousand tons of ARA will exist on top of the projected demand (Figure 2A), totaling 1.17 million tons/year of ARA needed by 2025, between ARA food sources and supplementation. Therefore, the global production will not meet the demand of ARA for 2025 without an increment of 35% in global production.

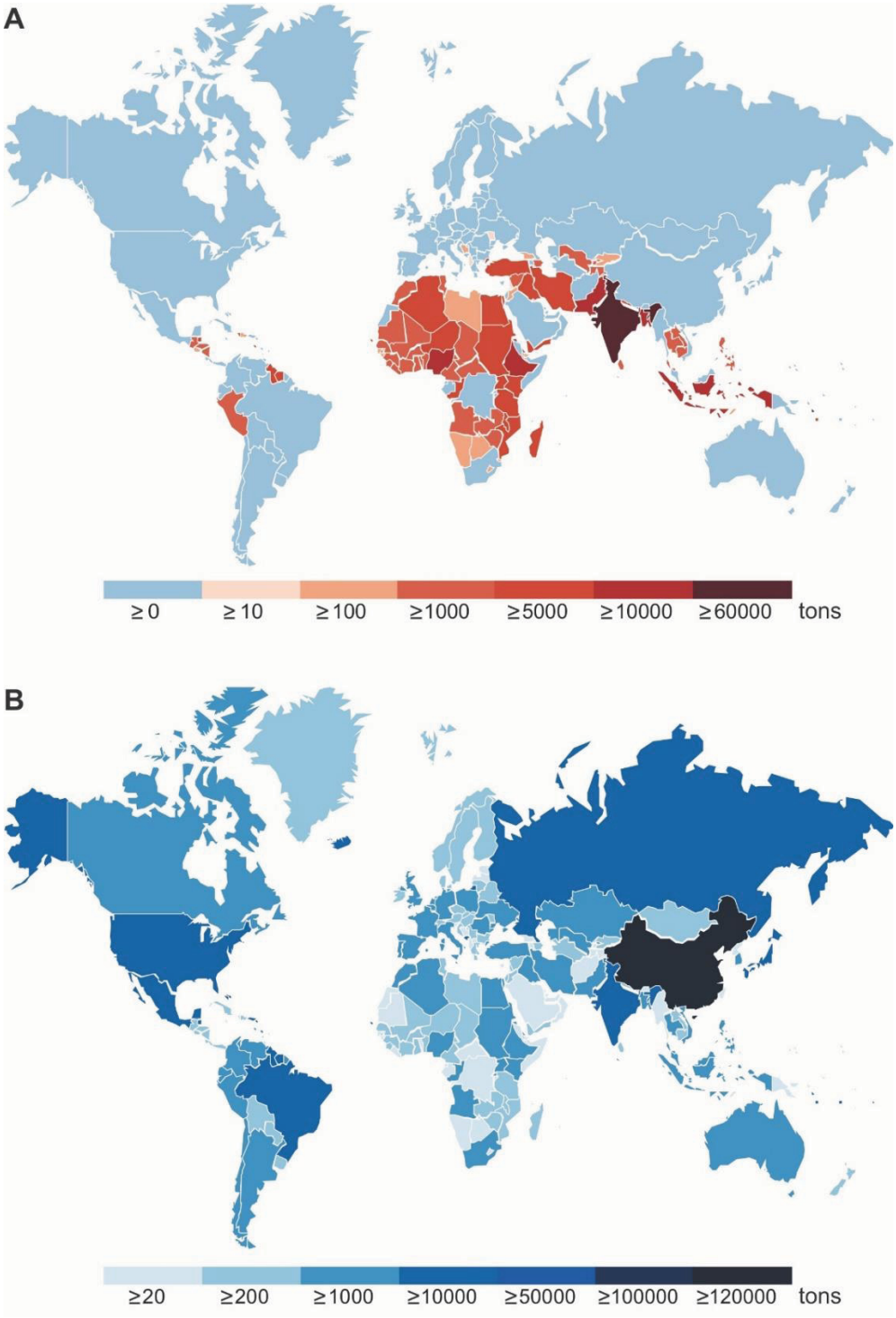


Figure 2. Global projection of ARA demand by 2025 expressed in tons of ARA per country, per year. A) In red-orange scale, ARA-deficient countries[a] and B) In blue scale, demand of ARA per country.

[a] Projection based on the minimum daily recommendation (150 mg/day per capita).

The use of traditional sources of ARA has decreased since the adoption of biotechnology for production using microbial resources. It is expected that the future production of ARA and other PUFAs will remain microbial-based, due to the process's feasibility and eco-friendliness.

1.8. CURRENT AND FUTURE DEVELOPMENTS

According to the World Intellectual Property Organization (WIPO), there are 277 patents under the *Mortierella* + arachidon* research key-words and more specifically 53 patents under the *M. alpina* + arachidon* + oil research key-words, the main topic of this review. This last group is divided mostly in the research of the fatty acids and formulation of food and beverage fields (Figure 3A), according to the IP code classification. The principal market players of this innovation are shown in the Figure 3B.

Due to the importance and potential of *Mortierella*, many studies have been elaborated since their first laboratory tests in 1978, approximately 6,993 studies figures when a research in the Derwent database is performed using *Mortierella* as key-word, in which 77% are patents and 23% are research articles among others.

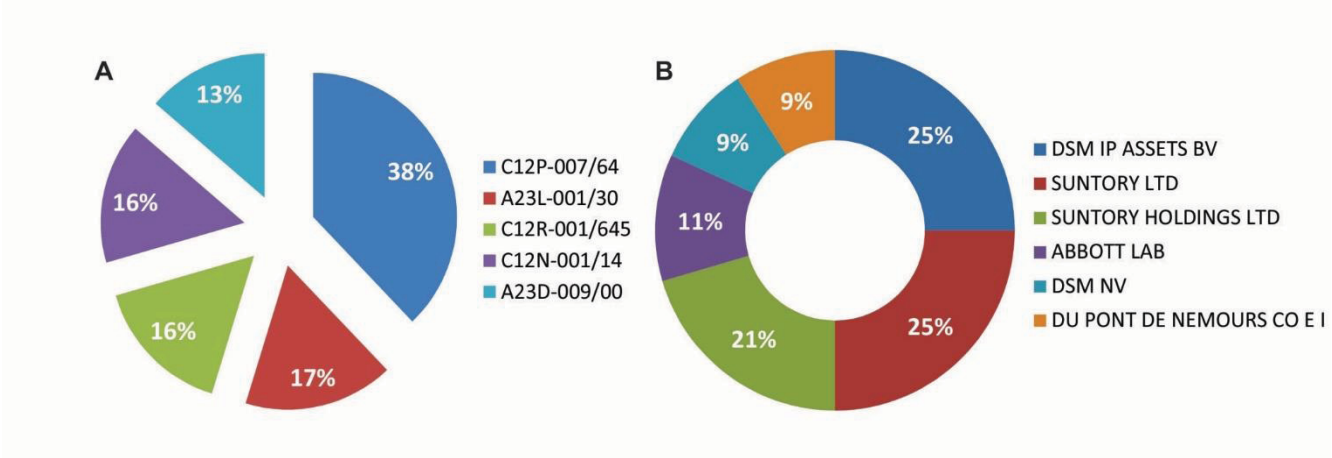


Figure 3. Chart of published patents: A) Codified patents according to the IP classification; B) Principal market players of innovation.

The production of patents until today (Figure 4) showed a non-uniform pattern in the last 30 years and it is a fact that the production remains a lucrative business. Thus, further studies and/or improvements – besides genetic and molecular techniques – can be performed at different steps of the production pipeline, i.e., in the fermentation step, the improvement of media culture and conditions using cheap

alternative substrates and the development of suitable morphology and oxygen transfer technologies. In the biomass production step, carbon sources, operational modes, and oxygen transfer can be improved. Although *M. alpina* prefers glucose, more complex carbon sources such as molasses could be used after a pretreatment of hydrolysis. The morphology of biomass in the reactor is linked to agitation, inoculation rate and type of inoculum, and further studies must be done to ensure that small pellets are produced, enhancing oxygen transfer to the cells.

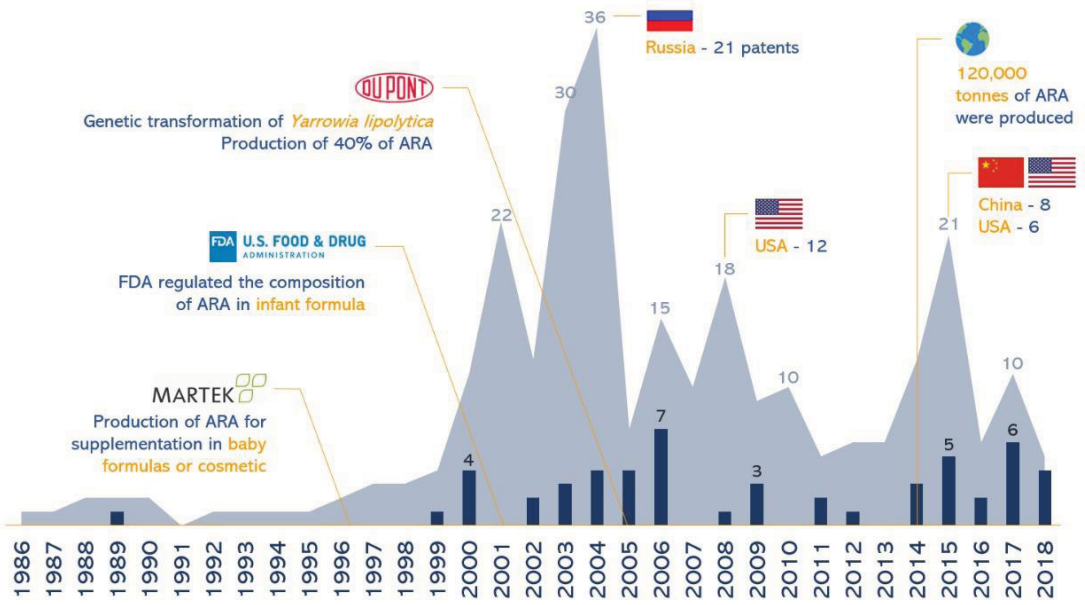


Figure 4. Time evolution in patent publications. In Bars, number of patents found at the Derwent database under the search terms *"Mortierella alpina + arachidon* + oil"*; in area, number of patents found at the Derwent database under the search terms *"Mortierella + arachidon*"*.

In the extraction step, the use of solvents is accepted by industry, but there is space for enhancing the yield. Mechanical pre-treatment of the biomass could boost the extraction efficiency, requiring less solvent, and resulting in an eco-friendly process. There are plenty of mechanical disruption pre-treatment options of the biomass, such as ultrasound, electric pulses, electroporation, sonication, among others, although some may not yet be cost-effective. As for the use of solvents, supercritical fluid extraction (SFE) and humid extraction can prove promising. SFE using solvents such as CO_2, is efficient and eco-friendly because the solvent is non-toxic. Despite SFE being uncommon for large-scale oil extraction, the price and quality

of the resulting oil may justify its use of SFE. Humid extraction, at the other side, uses common solvents but dispenses the biomass drying step. It is a more complex extraction because three phases (water, solids and solvent) may form, and additional studies must be performed to check the extraction yield and stability of PUFAs in this extraction. In case of incomplete disruption, additional alternatives could be applied such as the use of enzymes (e.g. lysozymes, snailase, proteases) to achieve a successful extraction.

In the oil refining step, the use of antioxidants and modified atmospheres is advised right after the fermentation step, to prevent the oxidation of PUFA. However, the oxidation and cleavage products of PUFAs may give undesirable organoleptic characteristics to the oil. This can be reduced through distillation and adsorption treatments and be avoided by careful formulation. New salts or microencapsulated forms of ARA-rich oil can have promising uses in the food and nutraceutical markets. Even the raw biomass, if properly formulated, may find use in human nutrition. Finally, the residual biomass from the extraction process can be used for animal feed, due to its nutritional content of proteins and remaining fatty acids – another possibility of product to be investigated.

1.9. POLYSACCHARIDES PRODUCED BY *MORTIERELLA ALPINA*

Polysaccharides are well distributed in the biosphere and they are synthetized by different living organisms at different stage of life. Cellulose and chitin are the most abundant carbohydrates in nature (Morganti, Del Ciotto, Carezzi, Nunziata, & Morganti, 2016). Chitin is extracted mostly from plants and animal sources.

Polysaccharides are synthesized in an intracellular way (being part of structures, such as membrane walls, cytoskeletons, etc.) as well as in an extracellular way (Giavasis, 2013). This last way of production has more process advantages when compared to the intracellular way, since no extraction and milder processing are required and the production can be easily controlled. It is important to consider that these extraction process generates a different toxic residues (Pal et al., 2014.).

According to their physicochemical properties, polysaccharides are used by different industries such as food, textile, biomaterials among others (Banerjee, Mr.

Mahapatra, & D Banerjee, 2013), and due to their biological activities, they are used also in medical and pharmacy industries (J. Chen & Seviour, 2007).

Research about the production of polysaccharides by *Mortierella alpina*, specially those produced extracellularly, is scarce. In the work of Mahapatra & Banerjee, 2013, a detailed review of existing literature about filamentous fungi producers of EPS is carried out, concerning production, biological properties, application, composition and perspectives of the necessity of more studies and applications.

Ruiter, Lugt, & Rombouts, 1993, identified a polysaccharide as characteristic of the *Mucor* family, a polymer formed by β(1-4)-D-glucuronic acid units, but no other polysaccharides, besides this one, were characterized in the work's author or in any other about *Mortierella* family.

Chitin, is the second most abundant polysaccharide found in nature as mentioned (Liu et al., 2013), formed by N-acetyl glucosamine units (2-acetamide 2-deoxy-β-D-glucose) ,β(1→4) linked (R. A. Muzzarelli, 1999). It is commonly extracted by alkaline treatments of exoskeletons of crustaceans (i.e shrimp and crab) (Rane & Hoover, 1993), and there are just few researches about alternative sources of production.

Chitin derivates, such as chitosan, have multiple uses. Chitin *per se* is hardly used *in natura* due to its particular physico-chemical properties and low solubility (Roberts, 1992a). A deacetylation process must be performed in order to obtain chitosan and N-acetylglucosamine (GlcNAc) derivates. This amino-sugar is found in joints, cartilages, and is commonly used in medicine to relief arthritis symptoms (Anderson, Nicolosi, & Borzelleca, 2005) and there are other 200 possible applications (Abdou, Nagy, & Elsabee, 2008; Ravi Kumar, 2000) in cosmetics, agriculture, food, biomedicine, textile (Rathke & Hudson, 1994) waste-waters remediation (N., Leela, & Prabhakara, 1988).

In the process of chitin extraction or derivatization, a considerable quantity of toxic residues are produced along the process (Bautista et al., 2001), chitin might be dangerous for persons allergic to products derivated from marine sources. For this reason, there is still room and necessity to research and evaluation of alternative "green" sources, with an eco-friendliness sense.

1.10. BIOACTIVE PROPERTIES OF POLYSACCHARIDES

1.10.1. Inmunological and antitumoral effects

Polysaccharides can trigger a non-specific reaction against tumor cells, this effect is dependent of the physicochemical characteristics (molecular weight, conformation, composition)(Ganeshpurkar, Rai, & Jain, 2010; Maeda, Watanabe, Chihara, & Rokutanda, 1988; Seviour, Stasinopoulos, Auer, & Gibbs, 1992; Thakur & Singh, 2013). Various EPSs have shown efficiency in the treatment of different types of cancer such as lung, breast, gastric, colon and even when combined or used as a coadjuvants of conventional drugs used as chemotherapeutics (Vannucci et al., 2013). Polysaccharides, i.e. glucans, show a reduction of metastasis processes (Taki et al., 1995) and induction of apoptosis in human prostate cancer, and proliferation suppression of colon and breast cancer cells (Yamamoto, Yamashita, & Tsubura, 1981; Min Zhang et al., 2005)

These substances are recognized as BRMs (biological response modifiers), innocuous to the body, and able to help the body to adapt to environmental changes and stress, stimulating the nervous, hormonal and immune system (Brekhman, 2013). The mechanisms of action of polysaccharides can be divided in three types according to Mei Zhang, Cui, Cheung, & Wang, (2007): 1) cancer-preventing activity – as part of a regular diet, in which the polysaccharide can reduce the development of tumors; 2) Immuno-enhancing activity – stimulant of the activity of immunologic cells, such as neutrophils, monocytes, natural killer, T-cells, and increasing levels of cytokines, tumor necrosis factor (TNF-a) (Hamuro, 1985), interleukins and interferons (Nozaki et al., 2008); and 3) Direct tumor activity – possibly arrest the cell life cycle and causing an apoptosis cell death (Song et al., 2011). This could explain the anti-proliferative process of cancer cells (Zaidman, Yassin, Mahajna, & Wasser, 2005). Chow, Lo, Loo, Hu, & Sham, (2003) and Kim, Choi, Lee, & Park, (2004) reported the proliferation reduction of different cancer cells when a polysaccharide-peptide complex was tested from *Trametes versicolor* and *Phellinus linteus*, respectively. These complexes increased the p21 and decreased the cyclin D1 expression (Chow et al., 2003). The cytotoxicity attributed to polysaccharides could be associated with the decrease in Bcl-2 and the reduction of cyclin B1 expression (Song et al., 2011), inducing apoptosis.

Mei Zhang et al., (2007). demonstrated the inhibition of proliferation of breast cancer (MCF-7) by cell cycle blocking and consequently an apoptosis.

1.11. CANCER

Cancer is a disease resulted by inherited environment induct mutations or by errors in the DNA replication (Tomasetti, Li, & Vogelstein, 2017). The most common types of human cancer are skin, colon, breast, prostate and lung which lead to million of deaths per year (Blanpain, 2013).

In Brazil, by 2016, a total of 596 thousand new cases of cancer were registered. Prostate, lung and colon were more expected in men, and breast, colon and lung in women (MS/INCA, 2016).

1.11.1. Adrenocortical carcinoma

The adrenocortical carcinoma (ACC) is a rare type of aggressive cancer (Dackiw, Lee, Gagel, & Evans, 2001), with an incidence of 0.5 – 2 cases per million people per year (Plager, 1984), its pathogenesis is still controversial, and its prognosis is poor (Wajchenberg et al., 2000). In most cases, this type of cancer can be diagnosed due to the increase of steroid hormone secretion by the tumor (cortisol – Cushing's syndrome). The tumor can be observed as not homogeneous-shaped in the imaging studies. In any case, to diagnose its stage of malignity, a histopathologic study must be performed (Allolio & Fassnacht, 2006; Flack & Chrousos, 1996; Vassilopoulou-Sellin & Schultz, 2001)

The clinical manifestations in patients with ACC are: weight gain, obesity, low muscle mass, hypertension, acne, virilization (Koschker, Fassnacht, Hahner, Weismann, & Allolio, 2006; Mantero et al., 2000)

According Steiner, (1954), this cancer represents 0.2% of deaths from cancers in United State of America.

The ACC, at early stages, can be removed, but its local recurrence is frequent, triggering a metastatic process (Ng & Libertino, 2003). At advanced stages, when surgery is not suitable, the treat with chemotherapeutics is required; mitotane, an organochloride drug, is the treatment of choice combine with some other cytotoxic

drugs such as etoposide, doxorubicin, cisplatin, streptozotocin (Berruti et al., 2005; Kasperlik-Zalułska, Migdalska, Zgliczyński, & Makowska, 1995). Unfortunately, mitotane can present some adverse effects, most of them related to gastrointestinal and neurological events (Terzolo et al., 2007). After 5 years of diagnosis only 16 to 38% of patients survives after treatment (Wajchenberg et al., 2000).

According to Letouzé et al., (2012) and Michalkiewicz et al., 2004, ACC is a consequence of a mutation in the suppressor gene TP53 (responsible for the production of the protein p53 – a tumoral suppressor),

In southern Brazil, Curitiba city, an extraordinarily high annual incidence of this cancer has been reported 3.4 – 4.2 per million of children, compared to the worldwide estimations of 0.3 per million of children younger than 15 years old, due to the mutations of the TP53 tumor suppressor gene (Pianovski et al., 2006). Cancer was the second cause of death in juvenile child between 0 to 15 years in 2013 (MS/INCA, 2016; Pianovski et al., 2006). Allolio & Fassnacht, (2006), affirms that women are more affected than man and the incidence is more common in the childhood and between the fourth and fifth decade.

For *in vitro* assays purposes, different authors use the cell strain H295R, a derivate of the cell strain NCL-H295, due to its adhesion capacity and monolayer growth (T. Wang & Rainey, 2012). This cell strain is suitable to evaluate the production of steroids hormones (Strajhar et al., 2017), effects of exogenous substances in the expression and enzymatic activity of steroidogenesis genes, cellular cycle, proliferative capacity and viability, being representative and derivate of the ACC (Lehmann, Wrzesiński, & Jagodziński, 2013; T. Wang & Rainey, 2012)

1.12. PATENT LANDSCAPE OF THE PRODUCTION OF POLYSACCHARIDES BY *MORTIERELLA SPP.*

A research in the Derwent Database was performed in order to find literature reference about the production of polysaccharides, chitin and/or its derivates produced in an extracellular way by *Mortierella alpina.*

The research method used was starting with generic keywords of search and finalizing with punctual keywords, according to this work.

When using the keywords in all fields of research “polysaccharide + *Mucor*”, a total of 42 patents were found, most of them applied in Chemistry and Biotechnology applied microbiology. These applications are more associated to wastewaters treatments, antigens, infections caused by molds, among others that not are related to any antitumoral or bacteriostatic activity.

When using the words “polysaccharide + *Mortierella*”, a total of 10 patents (Table. 6) were found in the Chemistry and Biotechnology applied microbiology subject area.

Table. 6 Patents related to application of *Mortierella* spp. registered at the Derwent data base.

Patent	Inventor	Cessionary	Application
CN101497902-A	ZHAO Z, WU S	CHINESE ACAD SCI DALIAN CHEM PHYS INST	Preparation of biomass rich in fatty acids.
RU2264733-C2	KVASENKOV O I, LOMACHNSKII V A, PROTUNKEVICH V A, SHURYGIN S V.	CANNING VEGETABLES DRYING IND RES INST	Use of polysaccharide produced by Mortierella as an emulsifier agent
CN108323618-A	LI X, XU Z, CAO Z	HEFEI MICRO BIOLOGICAL ENG CO LTD	Production of biomass for improving rooster reproductive performance.
WO2012124520-A1	ISHIHARA S	AMANI ENZYME INC	Production of alpha-glucosidase and use as an enzymatic agent.

When using the words “exopolysaccharide + *Mortierella”*, no patents were found.

Finally, when using the words “chitin + *Mortierella*”, as specific words, a total of 5 patents were found in this database, in which the patent KR2008103159-A was the only one involving both terms *Mortierella* and chitin, but for chitin deacetylase production and its use as a killing agent for insects and nematodes.

1.13. REFERENCES

Abdou, E. S., Nagy, K. S. A., & Elsabee, M. Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology*, *99*(5), 1359–1367. https://doi.org/https://doi.org/10.1016/j.biortech.2007.01.051

Akhtar, M. W., Mirza, A. Q., Nawazish, M. N., & Chughtai, M. I. D. (1983). Effect of triglycerides on the production of lipids and lipase by Mucor hiemalis. *Canadian Journal of Microbiology*, *29*(6), 664–669. https://doi.org/10.1139/m83-108

Akimoto, K., Kawashima, H., & Shimizu, S. (2011). *Microorganisms that extracellularly secrete lipids and methods of producing lipid and lipid vesicles encapsulating lipids using said microorganisms.* (No. U.S. Patent No. 7,972,855). Washington, DC: U.S.

Allolio, B., & Fassnacht, M. (2006). Adrenocortical carcinoma: clinical update. *The Journal of Clinical Endocrinology & Metabolism*, *91*(6), 2027–2037.

Amano, H., Shinmen, Y., Akimoto, K., Kawashima, H., Amachi, T., Shimizu, S., & Yamada, H. (1992). Chemotaxonomic significance of fatty acid composition in the genus Mortierella. *Mycotaxon*, *45*, 257–265.

Anderson, J. W., Nicolosi, R. J., & Borzelleca, J. F. (2005). Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food and Chemical Toxicology*, *43*(2), 187–201. https://doi.org/https://doi.org/10.1016/j.fct.2004.11.006

Bajpai, P., & Bajpai, P. K. (1992). Arachidonic acid production by microorganisms. *Biotechnology and Applied Biochemistry*, *15*(1), 1–10. https://doi.org/10.1111/j.1470-8744.1992.tb00194.x

Bajpai, P., Bajpai, P. K., & Ward, O. P. (1991). Eicosapentaenoic acid (EPA) production by Mortierella alpina ATCC 32222. *Applied Biochemistry and Biotechnology*, *31*, 267–272. https://doi.org/10.1007/BF02921753

Banerjee, Mr. Mahapatra, & D Banerjee. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, 1. https://doi.org/10.4137/MBI.S10957

Bautista, J., Jover, M., Gutierrez, J. F., Corpas, R., Cremades, O., Fontiveros, E., … Vega, J. (2001). Preparation of crayfish chitin by in situ lactic acid production. *Process Biochemistry*, *37*(3), 229–234. https://doi.org/https://doi.org/10.1016/S0032-9592(01)00202-3

Berruti, A., Terzolo, M., Sperone, P., Pia, A., Della Casa, S., Gross, D. J., … Mantero, F. (2005). Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocrine-Related Cancer*,

12(3), 657–666.

Blanpain, C. (2013). Tracing the cellular origin of cancer. *Nature Cell Biology*, *15*(2), 126.

Brekhman, I. I. (2013). *Man and biologically active substances: the effect of drugs, diet and pollution on health*. Elsevier.

Brooks, D. D., Berbesi, R., & Hodgson, A. S. (2011). Optimization of bleaching process. Retrieved March 28, 2016, from http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40321

Certik, M., Megova, J., & Horenitzky, R. (1999). Effect of nitrogen sources on the activities of lipogenic enzymes in oleaginous fungus *Cunninghamella echinulata*. *The Journal of General and Applied Microbiology*, *45*(6), 289–293. https://doi.org/10.2323/jgam.45.289

Certik, M., & Shimizu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Bioscience and Bioengineering*, *87*(1), 1–14.

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3),(1→ 6)-glucans. *Mycological Research*, *111*(6), 635–652.

Chow, L. W. C., Lo, C. S. Y., Loo, W. T. Y., Hu, X., & Sham, J. S. T. (2003). Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D 1 gene. *The American Journal of Chinese Medicine*, *31*(01), 1–9.

Cohen, Z., Vonshak, A., & Richmond, A. (1987). Fatty acid composition of Spirulina strains grown under various environmental conditions. *Phytochemistry*, *26*(8), 2255–2258. https://doi.org/10.1016/S0031-9422(00)84694-4

Dackiw, A. P. B., Lee, J. E., Gagel, R. F., & Evans, D. B. (2001). Adrenal cortical carcinoma. *World Journal of Surgery*, *25*(7), 914.

Davies, R. J., Holdsworth, J. E., & Reader, S. L. (1990). The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast Apiotrichum curvatum. https://doi.org/10.1007/BF00172553

De Greyt, W. (2013). Edible oil refining: Current and future technologies. In W. Hamm, R. J. Hamilton, & G. Calliauw (Eds.), *Edible Oil Processing* (2nd ed, pp. 127–152). Chichester, UK: John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118535202.ch5

Dongping, E., Kai, C., Tao, C., & Hua, T. (2014). *Mortierella alpina mutant strain capable of producing high-yield arachidonic acid, and fermentation method and application thereof* (No. CN103484385 A). Retrieved from http://www.google.com/patents/CN103484385A?cl=en

Dueppen, D. G., Zeller, S. G., Diltz, S. I., & Driver, R. H. (2005). *Extraction and winterization of lipids from oilseed and microbial sources* (No. U.S. Patent n. 7,695,626). Washington, DC: US: Extraction and winterization of lipids from oilseed and microbial sources.

Eroshin, V. K., Satroutdinov, A. D., Dedyukhina, E. G., & Chistyakova, T. I. (2000). Arachidonic

acid production by Mortierella alpina with growth-coupled lipid synthesis. *Process Biochemistry*, *35*(10), 1171–1175. https://doi.org/http://dx.doi.org/10.1016/S0032-9592(00)00151-5

Euromonitor, a market research provider. (2006). Infant milk formula industry. Retrieved February 24, 2016, from http://www.euromonitor.com/global-baby-food-overview-key-categories-countries-and-trends/report

Feofilova, E. P. (2010). The fungal cell Wall : modern concepts of its composition. *Microbiology*, *79*(6), 711–720. https://doi.org/10.1134/S0026261710060019

Fischer, L., Stressler, T., Eisele, T., Rost, J., Haunschild, E., & Kuhn, A. (2013). Production of polyunsaturated fatty acids by Mortierella alpina using submerse and solid state fermentation. *Chemie Ingenieur Technik*, *85*(3), 318–322. https://doi.org/10.1002/cite.201200094

Flack, M. R., & Chrousos, G. P. (1996). Neoplasms of the adrenal cortex. *Cancer Medicine*, *1*, 1567.

Forsyth, J. S., Willatts, P., Agostoni, C., Bissenden, J., Casaer, P., & Boehm, G. (2003). Long chain polyunsaturated fatty acid supplementation in infant formula and blood pressure in later childhood: follow up of a randomised controlled trial. *BMJ : British Medical Journal*, *326*(7396), 953. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC153849/

Fukui, S. (1995). Selection and breeding of microorganisms secreting and producing fuel lipids. *Bio Industry*, *12*(3), 36–46.

Ganeshpurkar, A., Rai, G., & Jain, A. P. (2010). Medicinal mushrooms: Towards a new horizon. *Pharmacognosy Reviews*, *4*(8), 127.

Giavasis, I. (2013). 16 - Production of microbial polysaccharides for use in food. In B. McNeil, D. Archer, I. Giavasis, & L. B. T.-M. P. of F. I. Harvey Enzymes and Nutraceuticals (Eds.), *Woodhead Publishing Series in Food Science, Technology and Nutrition* (pp. 413–468). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9780857093547.2.413

GostReports. (2016). Global arachidonic acid market research report 2016. Retrieved September 5, 2016, from http://www.gosreports.com/global-arachidonic-acid-market-worth-1-18-billion-by-2019/

Gunstone, F. D. (2001). Oilseed crops with modified fatty acid composition. *Journal of Oleo Science*, *50*(5), 269–279.

Hamuro, J. t. (1985). Lentinan, a T-cell oriented immunopotentiator: its experimental and clinical applications and possible mechanism of immune modulation. *Immunomodulation Agents and Their Mechanisms*.

Hansson, L., & Dostálek, M. (1988). Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus Mortierella ramanniana. *Applied Microbiology*

and Biotechnology, *28*(3), 240–246. https://doi.org/10.1007/BF00250448

Higashiyama, K., Fujikawa, S., Park, E. Y., & Shimizu, S. (2002). Production of arachidonic acid by Mortierella fungi. *Biotechnology and Bioprocess Engineering*, *7*(5), 252–262. https://doi.org/10.1007/BF02932833

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S., & Shimizu, S. (1998). Enhancement of arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1501–1505. https://doi.org/10.1007/s11746-998-0085-9

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawaa, S., & Shimizu, S. (1998). Effects of mineral addition on the growth morphology of and arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(12), 1815–1819. https://doi.org/10.1007/s11746-998-0336-9

Higashiyama, K., Yaguchi, T., Akimoto, K., & Shimizu, S. (2005). *Media for culturing microorganisms and process for producing unsaturated fatty acids or lipids containing the same* (No. U.S. Patent No. 6,746,857). Washington, DC: U.S.

Ho, S.-Y., & Chen, F. (2008). Lipid characterization of Mortierella alpina grown at different NaCl concentrations. *Journal of Agricultural and Food Chemistry*, *56*(17), 7903–7909. https://doi.org/10.1021/jf801404y

Hoffman, D. R., Birch, E. E., Birch, D. G., Uauy, R., Castañeda, Y. S., Lapus, M. G., & Wheaton, D. H. (2000). Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. *Journal of Pediatric Gastroenterology and Nutrition*, *31*(5). Retrieved from http://journals.lww.com/jpgn/Fulltext/2000/11000/Impact_of_Early_Dietary_Intake_and_Blood_Lipid.16.aspx

Hunter, K., & Rose, A. H. (1972). Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. *Biochimica et Biophysica Acta (BBA) Lipids and Lipid Metabolism*, *260*(4), 639–653. https://doi.org/http://dx.doi.org/10.1016/0005-2760(72)90013-6

Huong, L. M., Slugen, D., & Sajbidor, J. (1998). Production of dihomo-y-linolenic acid by Mortierella alpina. *Biologia Bratislava*, *53*(6), 801–806.

Ishihara. S, Satoru, I. (2012). New Modified alpha-glucosidase useful as enzymatic agent, and in pharmaceutical composition, quasi-drug composition, cosmetics composition, foodstuff composition, and feed composition. (n° WO2012124520-A1). China.

Jang, H.-D., Lin, Y.-Y., & Yang, S.-S. (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by Mortierella alpina. *Bioresource Technology*, *96*(15), 1633–1644. https://doi.org/http://dx.doi.org/10.1016/j.biortech.2004.12.027

Jareonkitmongkol, S., Kawashima, H., & Shimizu, S. (1993). Inhibitory effects of lignan compounds on the formation of arachidonic acid in a delta-5-desaturase-defective mutant

of Mortierella alpina 1s-4. *Journal of Fermentation and Bioengineering*, *76*(5), 406–407. https://doi.org/Doi 10.1016/0922-338x(93)90033-5

Jareonkitmongkol, S., Kawashima, H., Shimizu, S., & Yamada, H. (1992). Production of 5,8,11-cis-Eicosatrienoic acid by a Δ12-desaturase defective mutant of Mortierella alpina 1S-4. *J. Am. Oil Chem. Soc.*, *69*(9), 939–944.

Jareonkitmongkol, S., Sakuradani, E., & Shimizu, S. (1994). Isolation and characterization of an omega-3-desaturation-defective mutant of an arachidonic acid-producing fungus, Mortierella alpina 1s-4. *Archives of Microbiology*, *161*(4), 316–319. https://doi.org/Doi 10.1007/Bf00303586

Ji, X.-J., Zhang, A.-H., Nie, Z.-K., Wu, W.-J., Ren, L.-J., & Huang, H. (2014). Efficient arachidonic acid-rich oil production by Mortierella alpina through a repeated fed-batch fermentation strategy. *Bioresource Technology*, *170*(5), 356–360. https://doi.org/10.1016/j.biortech.2014.07.098

Jin, M.-J., Huang, H., Xiao, A.-H., Zhang, K., Liu, X., Li, S., & Peng, C. (2008). A novel two-step fermentation process for improved arachidonic acid production by Mortierella alpina. *Biotechnology Letters*, *30*(6), 1087–1091. https://doi.org/10.1007/s10529-008-9661-1

Kamada, N., Kawashima, H., Sakuradani, E., Akimoto, K., Ogawa, U., & Shimizu, S. (1999). Production of 8,11-cis-eicosadienoic acid by a delta 5 and delta 12 desaturase-defective mutant derived from the arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists Society*, *76*(11), 1269–1274. https://doi.org/Doi 10.1007/S11746-999-0138-8

Kasperlik-Zalułska, A. A., Migdalska, B. M., Zgliczyński, S., & Makowska, A. M. (1995). Adrenocortical carcinoma. A clinical study and treatment results of 52 patients. *Cancer*, *75*(10), 2587–2591.

Katano, K., & Kawashima, H. (2015). *Methods for producing polyunsaturated fatty acid and lipid containing polyunsaturated fatty acid* (No. US20150064749 A1). Suntory Ltd, Kenichi Higashiyama.

Kawashima, H., Sakuradani, E., Kamada, N., Akimoto, K., Konishi, K., Ogawa, J., & Shimizu, S. (1998). Production of 8,11,14,17-cis-eicosatetraenoic acid (20:4ω-3) by a Δ5 and Δ12 desaturase-defective mutant of an arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1495–1500. https://doi.org/10.1007/s11746-998-0084-x.

Kim, G.-Y., Choi, G.-S., Lee, S.-H., & Park, Y.-M. (2004). Acidic polysaccharide isolated from Phellinus linteus enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. *Journal of Ethnopharmacology*, *95*(1), 69–76.

Kim, Y.,OH. K, and Park, R. (2008). Use of *Motierella* strain DY-52 for producing chitin

deacetylase used for killing insects or nematode (no. KR2008103159-A). China.

Koike, Y., Jie Cai, H., Higashiyama, K., Fujikawa, S., & Park, E. Y. (2001). Effect of consumed carbon to nitrogen ratio of mycelial morphology and arachidonic acid production in cultures of mortierella alpina. *Journal of Bioscience and Bioengineering*, *91*(4), 382–389. https://doi.org/10.1016/S1389-1723(01)80156-0

Koletzko, B., Mrotzek, M., & Bremer, H. J. (1988). Fatty acid composition of mature human milk in Germany. *The American Journal of Clinical Nutrition*, *47*(6), 954–959.

Koschker, A.-C., Fassnacht, M., Hahner, S., Weismann, D., & Allolio, B. (2006). Adrenocortical carcinoma-improving patient care by establishing new structures. *Experimental and Clinical Endocrinology & Diabetes*, *114*(02), 45–51.

Kyle, D. J. (1997). *Arachidonic acid and methods for the production and use thereof* (No. 5658767). US.

Kyle, D. J. (1997). *Plant extract; vegetable oil; infant's formula.* (No. U.S. Patent No. 5,658,767). Washington, DC: U.S.

Kyle, D. J. (2001). The large-scale production and use of a single-cell oil highly enriched in docosahexaenoic acid. In F. Shahidi & J. W. Finley (Eds.), *Omega-3 Fatty Acids* (pp. 92–107). Washington, DC: ACS symposium series. https://doi.org/10.1021/bk-2001-0788.ch008

Lehmann, T. P., Wrzesiński, T., & Jagodziński, P. P. (2013). The effect of mitotane on viability, steroidogenesis and gene expression in NCI-H295R adrenocortical cells. *Molecular Medicine Reports*, *7*(3), 893–900.

Letouzé, E., Rosati, R., Komechen, H., Doghman, M., Marisa, L., Flück, C., … Pianovski, M. A. D. (2012). SNP array profiling of childhood adrenocortical tumors reveals distinct pathways of tumorigenesis and highlights candidate driver genes. *The Journal of Clinical Endocrinology & Metabolism*, *97*(7), E1284–E1293.

Li, Z.-Y., Lu, Y., Yadwad, V. B., & Ward, O. P. (1995). Process for production of arachidonic acid concentrate by a strain of mortierella alpina. *The Canadian Journal of Chemical Engineering*, *73*(1), 135–139. https://doi.org/10.1002/cjce.5450730115

Lindberg, A. M., & Molin, G. (1993). Effect of temperature and glucose supply on the production of polyunsaturated fatty acids by the fungus Mortierella alpina CBS 343.66 in fermentor cultures. *Applied Microbiology and Biotechnology*, *39*(4–5), 450–455. https://doi.org/10.1007/BF00205031

Liu, L., Liu, Y., Shin, H. D., Chen, R., Li, J., Du, G., & Chen, J. (2013). Microbial production of glucosamine and N-acetylglucosamine: Advances and perspectives. *Applied Microbiology and Biotechnology*, *97*(14), 6149–6158. https://doi.org/10.1007/s00253-013-4995-6

Lounds, C., Eagles, J., Carter, A. T., MacKenzie, D. A., & Archer, D. B. (2007). Spore germination in Mortierella alpina is associated with a transient depletion of arachidonic acid and induction of fatty acid desaturase gene expression. *Archives of Microbiology*, *188*(4), 299–305. https://doi.org/10.1007/s00203-007-0248-3

Maeda, Y. Y., Watanabe, S. T., Chihara, C., & Rokutanda, M. (1988). Denaturation and renaturation of a β-1, 6; 1, 3-glucan, lentinan, associated with expression of T-cell-mediated responses. *Cancer Research*, *48*(3), 671–675.

Mahapatra, S., & Banerjee, D. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, *6*, MBI.S10957. https://doi.org/10.4137/MBI.S10957

Mantero, F., Terzolo, M., Arnaldi, G., Osella, G., Masini, A. M., Alì, A., … Endocrinology, S. G. on A. T. of the I. S. of. (2000). A survey on adrenal incidentaloma in Italy. *The Journal of Clinical Endocrinology & Metabolism*, *85*(2), 637–644.

Michaelson, L. V, Lazarus, C. M., Griffiths, G., Napier, J. A., & Stobart, A. K. (1998). Isolation of a ⌬5 -Fatty Acid desaturase gene from Mortierella alpina. *Journal of Biological Chemistry*, *273*(30), 19055–19059.

Michalkiewicz, E., Sandrini, R., Figueiredo, B., Miranda, E. C. M., Caran, E., Oliveira-Filho, A. G., … Cristofani, L. M. (2004). Clinical and outcome characteristics of children with adrenocortical tumors: a report from the International Pediatric Adrenocortical Tumor Registry. *Journal of Clinical Oncology*, *22*(5), 838–845.

Morganti, P., Del Ciotto, P., Carezzi, F., Nunziata, M. L., & Morganti, G. (2016). A Chitin Nanofibril-based non-woven tissue as medical dressing. The role of bionanotechnology. *Nanomaterials & Regenerative Medicine, Zagreb, Criatia, IAPC Publishing*, 123–142.

Morris, G. J., Smith, D., & Coulson, G. E. (1988). A comparative study of the changes in the morphology of hyphae during freezing and viability upon thawing for twenty species of fungi. *Microbiology*, *134*(11), 2897–2906. https://doi.org/10.1099/00221287-134-11-2897

MS/INCA. (2016). Estimativa de Câncer no Brasil. Retrieved from www.inca.gov.br/dncc

Muzzarelli, R. A. (1999). Native, industrial and fossil chitins. *EXS*, *87*, 1—6. Retrieved from http://europepmc.org/abstract/MED/10906948

N., J. I., Leela, I., & Prabhakara, R. A. V. S. (1988). Removal of Cadmium Using Chitosan. *Journal of Environmental Engineering*, *114*(4), 962–974. https://doi.org/10.1061/(ASCE)0733-9372(1988)114:4(962)

Nagamuna, T., Uzuka, Y., & Tanaka, K. (1985). Physiological factors affecting total cell number and lipid content of the yeast Lipomyces starkeyi. *The Journal of General and Applied Microbiology*, *31*(1), 29–37. https://doi.org/10.2323/jgam.31.29

Ng, L., & Libertino, J. M. (2003). Adrenocortical carcinoma: diagnosis, evaluation and

treatment. *The Journal of Urology*, *169*(1), 5–11.

Nisha, A., Rastogi, N. K., & Venkateswaran, G. (2011). Optimization of media components for enhanced arachidonic acid production by Mortierella alpina under submerged cultivation. *Biotechnology and Bioprocess Engineering*, *16*(2), 229–237. https://doi.org/10.1007/s12257-010-0294-6

Nisha, A., & Venkateswaran, G. (2011). Effect of culture variables on mycelial arachidonic acid production by Mortierella alpina. *Food and Bioprocess Technology*, *4*(2), 232–240. https://doi.org/10.1007/s11947-008-0146-y

Nishizaki, T., Nomura, T., Matsuoka, T., & Tsujishita, Y. (1999). Arachidonic acid as a messenger for the expression of long-term potentiation. *Biochemical and Biophysical Research Communications*, *254*(2), 446–449. https://doi.org/http://dx.doi.org/10.1006/bbrc.1998.9961

Nozaki, H., Itonori, S., Sugita, M., Nakamura, K., Ohba, K., Suzuki, A., & Kushi, Y. (2008). Mushroom acidic glycosphingolipid induction of cytokine secretion from murine T cells and proliferation of NK1. 1 α/β TCR-double positive cells in vitro. *Biochemical and Biophysical Research Communications*, *373*(3), 435–439.

Oliveira, V., & Smallwood, D. (2011). *The infant formula market: consequences of a change in the WIC contract brand* (No. ERR-124). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011a). *Culturing mortierella in nutrient broth containing saccharified starch and recovering gamma-linolenic, dihomo-gamma-linolenic, arachidonic, eicosapentaenoic, mead, 6, 9-octadecadienoic and 8, 11-eicosadienoic acids* (No. U.S. Patent No. 7,863,024). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011b). *Process for producing highly unsaturated fatty acid-containing lipid* (No. EP1498488 B1). Suntory Ltd, Kenichi Higashiyama.

Oregon State University. (2014). Essential fatty acids. Retrieved March 3, 2016, from http://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids

Packter, N. M. (1981). Lipid biochemistry of fungi and other organisms. *Biochemical Education*, *9*(4), 156. https://doi.org/http://dx.doi.org/10.1016/0307-4412(81)90142-4

Pal, J., Verma, H. O., Munka, V. K., Maurya, S. K., Roy, D., & Kumar, J. (n.d.). Biological method of chitin extraction from shrimp waste an eco-friendly low cost technology and its advanced application.

Park, E. Y., Koike, Y., Higashiyama, K., Fujikawa, S., & Okabe, M. (1999). Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of Mortierella alpina. *Journal of Bioscience and Bioengineering*, *88*(1), 61–67. https://doi.org/10.1016/S1389-1723(99)80177-7

Park, Y., Tamura, S., Koike, Y., Toriyama, M., & Okabe, M. (1997). Mycelial pellet

intrastructure visualization and viability prediction in a culture of Streptomyces fradiae using confocal scanning laser microscopy. *Journal of Fermentation and Bioengineering*, *84*(5), 483–486. https://doi.org/http://dx.doi.org/10.1016/S0922-338X(97)82014-4

Peng, C., Huang, H., Ji, X., Liu, X., You, J., Lu, J., … Ouyang, P. (2010). A temperature-shift strategy for efficient arachidonic acid fermentation by Mortierella alpina in batch culture. *Biochemical Engineering Journal*, *53*(1), 92–96. https://doi.org/10.1016/j.bej.2010.09.014

Pianovski, M. A. D., Maluf, E. M. C. P., de Carvalho, D. S., Ribeiro, R. C., Rodriguez-Galindo, C., Boffetta, P., … Figueiredo, B. C. (2006). Mortality rate of adrenocortical tumors in children under 15 years of age in Curitiba, Brazil. *Pediatric Blood & Cancer*, *47*(1), 56–60.

Plager, J. E. (1984). Carcinoma of the adrenal cortex: clinical description, diagnosis, and treatment. *International Advances in Surgical Oncology*, *7*, 329–353.

QY Research center. (2015). *Global arachidonic acid industry Report 2015*. Retrieved from http://www.qyresearch.com/english/goods.php?id=45013

Rane, K. D., & Hoover, D. G. (1993). An evaluation of alkali and acid treatments for chitosan extraction from fungi. *Process Biochemistry*, *28*(2), 115–118. https://doi.org/https://doi.org/10.1016/0032-9592(93)80016-A

Rathke, T. D., & Hudson, S. M. (1994). Review of Chitin and Chitosan as Fiber and Film Formers. *Journal of Macromolecular Science, Part C*, *34*(3), 375–437. https://doi.org/10.1080/15321799408014163

Ratledge, C. (2001). Microorganisms as sourcs of polyunsaturated fatty acids. *Structured and Modified Lipids*, 351–399.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, *86*, 807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Ratledge, C. (2013). Microbial oils: an introductory overview of current status and future prospects. *OCL*, *20*(6), D602. https://doi.org/10.1051/ocl/2013029

Ratledge, C., & Hopkins, S. (2006). Lipids from microbial sources. In F. D. Gunstone (Ed.), *Modyfing lipids for use in food.* (pp. 80–113). Department of Biological Sciences, University of Hull, UK.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1–27. https://doi.org/10.1016/S1381-5148(00)00038-9

Roberts, G. A. F. (1992). *Chitin chemistry*. Macmillan International Higher Education.

Rocky-salimi, K., Hamidi-esfahani, Z., & Abbasi, S. (2011). Statistical optimization of arachidonic acid production by Mortierella alpina CBS 754 . 68 in submerged fermentation. *Iranian Journal of Biotechnology*, *9*(2), 87–93.

Ruiter, G. A. D. E., Lugt, A. W. V. A. N. B. D. E. R., & Rombouts, F. M. (1993). Approaches to

the classification of the Mortievella isabellina group : antigenic extracellular polysaccharides. *Mycological Research (An International Journal of Fungal Biology)*, *97*(6), 690–696. https://doi.org/10.1016/S0953-7562(09)80149-4

Saccardo, P. A., Traverso, G. B., & Trotter, A. (n.d.). *Sylloge fungorum omnium hucusque cognitorum. Digessit P. A. Saccardo.* (Vol. v.1). Patavii,sumptibus auctoris,. Retrieved from http://www.biodiversitylibrary.org/item/102968

Šajbidor, J., Dobroňová, S., & Čert\'ik, M. (1990). Arachidonic acid production byMortierella sp. S-17 influence of C/N ratio. https://doi.org/10.1007/BF01024404

Sajbidor, J., Kozelouhova, D., & Certik, M. (1992). Influence of some metal-ions on the lipid-content and arachidonic-acid production by Mortierella sp. *Folia Microbiologica*, *37*(6), 404–406.

Sakuradani, E., Ando, A., Shimizu, S., & Ogawa, J. (2013). Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus Mortierella alpina 1S-4. *Journal of Bioscience and Bioengineering*, *116*(4), 417–422. https://doi.org/10.1016/j.jbiosc.2013.04.008

Sakuradani, E., Hirano, Y., Kamada, N., Nojiri, M., Ogawa, J., & Shimizu, S. (2004). Improvement of arachidonic acid production by mutants with lower n-3 desaturation activity derived from Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *66*(3), 243–248. https://doi.org/10.1007/s00253-004-1682-7

Sakuradani, E., Kamada, N., Hirano, Y., Nishihara, M., Kawashima, H., Akimoto, K., … Shimizu, S. (2002). Production of 5,8,11-eicosatrienoic acid by a Δ5 and Δ6 desaturation activity-enhanced mutant derived from a Δ12 desaturation activity-defective mutant of Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *60*(3), 281–287. https://doi.org/10.1007/s00253-002-1128-z

Seviour, R. J., Stasinopoulos, S. J., Auer, D. P. F., & Gibbs, P. A. (1992). Production of pullulan and other exopolysaccharides by filamentous fungi. *Critical Reviews in Biotechnology*, *12*(3), 279–298.

Shimada, Y., Sugihara, A., Nakano, H., & Kuramoto, T. (1997). Purification of docosahexaenoic acid by selective esterification of fatty acids from Tuna oil with Rhizopus delemar lipase. *Journal of the American Oil Chemists Society*, *74*(2), 97–101.

Shimizu, S., & Jareonkitmongkol, S. (1995). Mortierella Species (Fungi): Production of C20 Polyunsaturated Fatty Acids. In Y. P. S. Bajaj (Ed.), *Medicinal and Aromatic Plants* (pp. 308–325). Berlin: Springer. https://doi.org/10.1007/978-3-662-08612-4_17

Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., & Yamada, H. (1988). Production of eicosapentaenoic acid by Mortierella fungi. *Journal of the American Oil Chemists' Society*, *65*(9), 1455–1459. https://doi.org/10.1007/BF02898307

Shinmen, Y., & Shimizu, S. (1989). Production of arachidonic acid by Mortierella fungi. *Applied Microbiology*, *31*(1), 11–16. https://doi.org/10.1007/BF00252518

Shu, C.-H., & Lung, M.-Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by Antrodia camphorata in batch cultures. *Process Biochemistry*, *39*(8), 931–937. https://doi.org/10.1016/S0032-9592(03)00220-6

Singh, A., & Ward, P. O. (1997). Production of high yields of arachidonic acid in a fed-batch system by Mortierella alpina ATCC 32222. *Applied Microbiology and Biotechnology*, *48*(1), 1–5. https://doi.org/10.1007/s002530051005

Song, K.-S., Li, G., Kim, J.-S., Jing, K., Kim, T.-D., Kim, J.-P., … Hwang, B.-D. (2011). Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/β-catenin in SW480 human colon cancer cells. *BMC Cancer*, *11*(1), 307.

Srigley, C. T., & Rader, J. I. (2014). Content and composition of fatty acids in marine oil omega-3 supplements. *Journal of Agricultural and Food Chemistry*, *62*(29), 7268–7278. https://doi.org/10.1021/jf5016973

Steiner, P. E. (1954). *Cancer: Race and geography: some etiological, environmental, ethnological, epidemiological, and statistical aspects in Caucasoids, Mongoloids, Negroids, and Mexicans*. Williams & Wilkins Company.

Strajhar, P., Tonoli, D., Jeanneret, F., Imhof, R. M., Malagnino, V., Patt, M., … Odermatt, A. (2017). Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids. *Toxicology*, *381*, 51–63.

Stredanská, S., & Šajbidor, J. (1993). Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by Mortierella alpina. *Acta Biotechnologica*, *13*(2), 185–191. https://doi.org/10.1002/abio.370130218

Taki, H., Ohishi, K., Okano, A., Suga, T., Akiyama, Y., & Yamashita, A. (1995). Antimetastatic effect of lentinan on liver metastasis of colon carcinoma (colon26): possible role of activated kupffer cells. *International Journal of Immunotherapy*, *11*(1), 29–38.

Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., … Grossrubatscher, E. (2007). Adjuvant mitotane treatment for adrenocortical carcinoma. *New England Journal of Medicine*, *356*(23), 2372–2380.

Thakur, M. P., & Singh, H. K. (2013). Mushrooms, their bioactive compounds and medicinal uses: A review. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, *5*(1), 1–20.

Tomasetti, C., Li, L., & Vogelstein, B. (2017). Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science*, *355*(6331), 1330–1334.

Totani, N., Hyodo, K., & Ueda, T. (2000). Minerals Essential for Growth of the Filamentous

Fungus, Mortierella alpina. *Journal of Japan Oil Chemists' Society*, *49*(5), 487–493,515. https://doi.org/10.5650/jos1996.49.487

Totani, N., Someya, K., & Oba, K. (1992). Industrial production of arachidonic acid by Mortierella. In D. J. Kyle & C. Ratledge (Eds.), *Industrial Applications of Single Cell Oils* (pp. 52–60). Illinois: AOCS Publishing.

Totani, N., Suzaki, K., & Kudo, T. (1992). *Process for the production of arachidonic acid-containing lipids* (No. EP0223960 B1). Lion Corporation.

Totani, N., Watanabe, A., & Oba, K. (1987). An improved method of arachidonic acid production by Mortierella alpina. *Yukagaku*, *36*(5), 328–331. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=8169393

Uauy, R., Mena, P., & Rojas, C. (2000). Essential fatty acids in early life : structural and functional role. *Proceedings of the Nutrition Society*, *59*, 3–15. https://doi.org/10.1017/S0029665100000021

Vadivelan, G., & Venkateswaran, G. (2014). Production and enhancement of omega-3 fatty acid from Mortierella alpina CFR-GV15: its food and therapeutic application. *BioMed Research International*, *2014*, 657414. https://doi.org/10.1155/2014/657414

Vali, S. R., Sheng, H. Y., & Ju, Y. (2003). An efficient method for the purification of arachidonic acid from fungal single-cell oil (ARASCO). *Journal of the American Oil Chemists Society*, *80*(7), 725–730.

Vandamme, E., & Revuelta, J. L. (2016). Vitamins, biopigments, antioxidants and related compounds: a historical, physiological and (Bio) technological perspective. In E. Vandamme & J. L. Revuelta (Eds.), *Industrial biotechnology of vitamins, biopigments, and antioxidants* (pp. 1–14). Weinheim, Germany: WILEY-VCH Verlag.

Vannucci, L., Krizan, J., Sima, P., Stakheev, D., Caja, F., Rajsiglova, L., … Saieh, M. (2013). Immunostimulatory properties and antitumor activities of glucans. *International Journal of Oncology*, *43*(2), 357–364.

Vassilopoulou-Sellin, R., & Schultz, P. N. (2001). Adrenocortical carcinoma: clinical outcome at the end of the 20th century. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *92*(5), 1113–1121.

Wajchenberg, B. L., Albergaria Pereira, M. A., Medonca, B. B., Latronico, A. C., Carneiro, P. C., Ferreira Alves, V. A., … Kirschner, M. A. (2000). Adrenocortical carcinoma: clinical and laboratory observations. *Cancer*, *88*(4), 711–736.

Wang, T., & Rainey, W. E. (2012). Human adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *351*(1), 58–65.

Wynn, J. P., Hamid, A. A., Li, Y., & Ratledge, C. (2001). Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi Mucor circinelloides and

Mortierella alpina. *Microbiology*, *147*(10), 2857–2864.

Wynn, J. P., & Ratledge, C. (2005). Microbial production of oils and fats. In K. Shetty, G. Paliyath, A. Pometto, & E. Levin (Eds.), *Food Biotechnology* (2nd ed., pp. 443–472). NY: CRC Press.

Xian, M., Yan, J., Kang, Y., Liu, J., Bi, Y., & Zhen, K. (2001). Production of c-linolenic acid by Mortierella isabellina grown on hexadecanol. *Letters in Applied Microbiology*, *33*, 367–370. https://doi.org/10.1046/j.1472-765X.2001.00924.x

Yamamoto, T., Yamashita, T., & Tsubura, E. (1981). Inhibition of pulmonary metastasis of Lewis lung carcinoma by a glucan, Schizophyllan. *Invasion & Metastasis*, *1*(1), 71–84.

Yamauchi, A., Nagao, T., & Watanabe, Y. (2005). Purification of arachidonic acid from Mortierella single-cell oil by selective esterification with Burkholderia cepacia lipase. *Journal of the American Oil Chemists Society*, *82*(11), 833–837.

Yan, Y., Li, X., Wang, G., Gui, X., Li, G., Su, F., … Liu, T. (2014). Biotechnological preparation of biodiesel and its high-valued derivatives: a review. *Applied Energy*, *113*, 1614–1631. https://doi.org/10.1016/j.apenergy.2013.09.029

Yokochi, T., Kamisaka, Y., Nakahara, T., & Suzuki, O. (1995). Production of γ-Linolenic acid containing phospholipids by Mortierella ramanniana in a fed-batch culture of decane. *Journal of Japan Oil Chemists' Society*, *44*(1), 9–15. https://doi.org/10.5650/jos1956.44.9

Yu, L. J., Qin, W. M., Lan, W. Z., Zhou, P. P., & Zhu, M. (2003). Improved arachidonic acids production from the fungus Mortierella alpina by glutamate supplementation. *Bioresource Technology*, *88*(3), 265–268. https://doi.org/http://dx.doi.org/10.1016/S0960-8524(02)00312-7

Yuan, C., Wang, X., & Yu, Z. (2007). Separation and preparative purification of arachidonic acid from microbial lipids by urea inclusion reaction and HPLC. *Preparative Biochemistry and Biotechnology*, *37*(2), 149–159. https://doi.org/10.1080/10826060701199098

Zaidman, B.-Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*, *67*(4), 453–468.

Zhang, M., Chen, H., Huang, J., Li, Z., Zhu, C., & Zhang, S. (2005). Effect of lycium barbarum polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sciences*, *76*(18), 2115–2124.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*(1), 4–19.

Zhiming, W., Huan, L., & Xu, J. (2014). *Method for producing arachidonic acid grease by utilizing mortierella alpina mutant strain, and arachidonic acid grease produced by method*

(No. CN 103571896 A).

Zhu, M., Yu, L. J., Liu, Z., & Xu, H. B. (2004). Isolating Mortierella alpina strains of high yield of arachidonic acid. *Letters in Applied Microbiology*, *39*(4), 332–335. https://doi.org/10.1111/j.1472-765X.2004.01581.x

2. CHAPTER II (RESEARCH RESULTS) – CULTURE MEDIUM OPTIMIZATION, PHYSICOCHEMICAL CHARACTERIZATION AND BIOACTIVE PROPERTIES EVALUATION OF AN EXOPOLYSACCHARIDE PRODUCED BY *MORTIERELLA ALPINA* UNDER SUBMERGED FERMENTATION.

2.1. ABSTRACT

In this work, the production of an exopolysaccharide was optimized through experimental design methods, evaluating the influence of urea, phosphate, and glucose at 5 days of fermentation at 25°C with continuous agitation of 120 rpm. Production under optimized conditions gave a total of 1.51 g/L of exopolysaccharide. The exopolysaccharide was purified and its chemical characteristics were evaluated by hydrolysis and monosaccharide composition chromatography, NMR and FTIR spectroscopy. The results obtained showed a homogeneous polymer composed by chitin, a backbone of repeated units of N-acetyl-D-glucosamine. The acetylation degree was also determined to evaluate further applications due to its potential related to biological activities. The potential of the biological activities of this exopolysaccharide such as the antioxidant effect, the ferrous-ion chelating activity, the antimicrobial activity, and the antitumor activity was also evaluated. The exopolysaccharide showed an antioxidant activity of 85%, and a bacteriostatic effect for bacteria as *Salmonella tiphy* and *Staphylococcus aureus*; an citotoxic effect for breast cancer cell (MCF7, MDA-MB 231 and MDA-MB 468), colorectal tumor cell (CACO-2), and no cytotoxicity for non-tumoral cell lines (MCF 10A and VERO). To the best of our knowledge, this is the first study about the production of a new alternative of green chitin with promising bioactive properties by the filamentous fungus *M. alpina,* extracellularly produced.

Keywords: *Mortierella alpina*, chemical characterization, exopolysaccharide, chitin, antitumoral.

2.2. INTRODUCTION

The fungus *Mortierella alpina* is well-known as a producer of arachidonic acid, a polyunsaturated fatty acid commonly used by different industries, such as food, medical, cosmetics and others, due to its nutraceutical properties (Ratledge, 2013). However, there is a lack of research about other bioactive substances produced by this fungus, such as polysaccharides. In the work of Ruiter, Lugt, & Rombouts, (1993), an polysaccharide of *Mortierella isabellina* was characterized, constituted by 4-linked β-D-glucuronic acid residues, and also chitin was recovered after cell walls disruption. These biopolymers, with high molar mass, have shown important potential in the biochemical and medical areas due to its biological activities such as antioxidant, antitumor, antibacterial, among others.

There are several studies about the involvement of free radicals and reactive oxygen species (ROS) in aging (Finkel & Holbrook, 2000) and its influence in the development of diseases such as cancer and neurodegenerative disorders (Emerit, Edeas, & Bricaire, 2004). The use of polysaccharides as antioxidants agents and considered in the formulation of effective non-toxic drugs (Carocho & Ferreira, 2013) when compared to common antioxidants used in food industry is promising (i.e butylated hydroxytoluene, butylated hydroxyanisole, etc)(Ye, Liu, Wang, Wang, & Zhang, 2012). These polysaccharides can act increasing cells natural defenses or by scavenging the free radical species (Sun, Wang, Fang, Gao, & Tan, 2004).

The food industry also faced other important issues such as food spoilage and food poisoning (Sokmen et al., 2004). Microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella tiphy* are common pathogens in food (Walker, 2018) and the research of novel alternatives of ubiquitous antibacterial agents became important to this industry (Alekshun & Levy, 2007).

The antitumor potential of these natural substances is also another factor important to consider, especially becausethe search for non-aggressive drugs has gained significant attention (Gutierrez & Ramirez, 2012), introducing an alternative to the conventional treatments such as chemotherapy, radiotherapy.

The aims of this work were to introduce an eco-friendly approach for obtaining an exopolysaccharide from *Mortierella alpina* as well as elucidate its chemical structure

and to measure the *in vitro* bacteriostatic activity, antioxidant activity (ABTS free radical scavenging), and antitumoral effect against breast, colo-rectal and adrenocortical carcinoma tumor cell strains.

2.3. MATERIAL AND METHODS

2.3.1. Materials and microorganism

The monosaccharide standards (D-glucosamine, D-glucose, D-mannose, L-fucose, D-fructose, D-galactose and D-glucuronic acid), chloramphenicol, 2,2'-azinobis-3-etilbenzothiazoline-6-sulfonic acid, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (Ferrozine), Iron(II) chloride, Disodium ethylenediaminetetraacetate dihydrate (EDTA-Na_2), 3-(4,5-dhimethylthiazol-2-yr)-2,5- diphenyltetrazolium bromide (MTT) and a Collagenase Activity Colorimetric Assay Kit were all from Sigma-Aldrich, St. Louis, MO, USA.

The fungal strain *M. alpina* CBS 528.72 was purchased from the Centraalbureau voor Schimmelcultures (CBS, Netherlands). The culture was maintained on potato dextrose agar (PDA; glucose 20 $g.L^{-1}$, potato extract 4 $g.L^{-1}$, and agar 17 $g.L^{-1}$) slants at 5 ±1ºC and subcultured every 2 months.

2.3.2. Culture medium and optimization of EPS production

The fixed culture medium composition for EPS production was in ($g.L^{-1}$): KNO_3 1.0; $MgSO_4 \cdot 7H_2O$ 0.3; and in ($mg.L^{-1}$): $CaCl_2 \cdot 2H_2O$ 0.62; $FeCl_3 \cdot 6H_2O$ 1.5; $ZnSO_4 \cdot 7H_2O$ 1.0; $CuSO_4 \cdot 5H_2O$ 0.1; $MnCl_2 \cdot 4H_2O$ 1.0. Concentrations of glucose, urea, and phosphate at diverse pH were tested, to evaluate their influence on EPS production (Banerjee et al., 2013). In this first phase of the optimization process, a 2^4 full factorial experimental design was used (16 experimental runs are required and 3 central points were added to measure intrinsical error). Experiments were performed randomly and the results were analyzed with 95% confidence intervals using the Statistica 7.0 (StatSoft, Tulsa, OK, USA) software.

Table 7 shows the four independent variables, their concentrations at the different coded levels.

Table 7. Variables and their coded levels, 2^4 full factorial experimental design.

Independent Variables	Code levels		
	-1	0	+1
X_1 : Glucose ($g.L^{-1}$)	40.0	60.0	80.0
X_2: Urea ($g.L^{-1}$)	2.0	4.0	6.0
X_3: pH	5.0	6.0	7.0
X_4: Phosphate (as KH_2PO_4) ($mmol.L^{-1}$)	1.5	3.8	6.1

Run	X_1	X_2	X_3	X_4	Biomass ($g.L^{-1}$)	Viscosity (cP)
1	-1	-1	-1	-1	15.10	1.01
2	1	-1	-1	-1	17.00	1.07
3	-1	1	-1	-1	14.90	1.77
4	1	1	-1	-1	18.40	1.77
5	-1	-1	1	-1	14.50	0.96
6	1	-1	1	-1	19.30	1.10
7	-1	1	1	-1	14.20	1.76
8	1	1	1	-1	17.60	1.34
9	-1	-1	-1	1	17.20	1.02
10	1	-1	-1	1	23.90	1.17
11	-1	1	-1	1	16.50	2.50
12	1	1	-1	1	23.60	2.26
13	-1	-1	1	1	14.30	0.96
14	1	-1	1	1	23.20	1.37
15	-1	1	1	1	15.90	2.77
16	1	1	1	1	25.20	2.49

The batch fermentation tests were carried out in 500 mL Erlenmeyer flasks at 25°C for 5 days at 120 RPM; the fermentation volume was 100 mL and was inoculated with 10 % (V/V) of a mycelial suspension. After the fermentation process, the biomass mass was separated through vacuum filtration to obtain the filtrate broth. To compare the concentration of EPS produced, a indirect evaluation by the measurement of viscosity was performed using an Ostwald viscometer. The viscosity variable, which was calculated using the following equation:

$$\eta_L = \frac{\eta_W \,.\, t_L\, \rho_L}{\rho_W \,.\, t_W}$$

Where η_W = Absolute viscosity of water; t_W = Time of flow of water; ρ_W = Density of water;

η_L = Absolute viscosity of liquid; t_L = Time of flow of liquid; ρ_L = Density of liquid.

Finally, a relation between the viscosity (cP) and EPS (g.L-1) produced (data not shown) was determined ($R^2 = 0.98$) as the following equation:

$$EPS\ (g.L-1)\ =\ 0.3254 * (X) - 0.0204\ *\ V$$

Where X = Viscosity of the filtrate (cP); and V = Total volume of the filtrate broth.

The second phase of the optimization process was using a central-composite design, to elucidate a response surface in the optimal region, with 3 variables at 5 coded levels. Two axial points were chosen -1.68179 and 1.68179 to make the design orthogonal. The software Statistica 7.0 (StatSoft, Tulsa, OK, USA) was used to analyze results at a 95% confidence interval.

Table 8. Variables and their coded levels, Central-composite experimental design.

Independent Variables	Coded levels				
	-1.681	-1	0	+1	+1.681
Glucose ($g.L^{-1}$)	26.36	40.0	60.0	80.0	93.64
Urea ($g.L^{-1}$)	2.63	4.0	6.0	8.0	9.36
Phosphate (as KH_2PO_4) ($mmol.L^{-1}$)	1.95	6.11	12.22	18.32	22.48

Run	X_1 ($g.L^{-1}$)	X_2 ($g.L^{-1}$)	X_4 ($mmol.L^{-1}$)	Biomass ($g.L^{-1}$)	Viscosity (cP)
1	-1	-1	-1	17.1	3.7
2	-1	-1	1	19.9	1.5
3	-1	1	-1	17.9	3.8
4	-1	1	1	19.0	3.9
5	1	-1	-1	22.5	2.0
6	1	-1	1	29.8	1.5
7	1	1	-1	23.9	2.1
8	1	1	1	26.5	1.6
9	-1.68179	0	0	13.1	3.1
10	1.68179	0	0	25.8	2.0

11	0	-1.68179	0	24.7	1.5
12	0	1.68179	0	26.3	4.1
13	0	0	-1.68179	19.5	1.5
14	0	0	1.68179	26.4	1.5
15	0	0	0	26.3	4.4
16	0	0	0	27.0	4.3
17	0	0	0	26.4	4.3

2.3.3. Exopolysaccharide recovery

After the fermentation process, the biomass was removed by vacuum filtration, and ethanol was added to the filtrate (3:1, v/v) to precipitate the EPS. The solution was kept at 5°C overnight to ensure sedimentation. The exopolysaccharide was recovered by centrifugation, resuspended in water and dialyzed (cut-off 20 kDa) against distilled water (48 h) and ultrapure water (24 h). The suspension of exopolysaccharide was then freeze-dried.

2.3.4. Purity of the EPS

The purity of the EPS was evaluated by UV-VIS spectroscopy recorded using a SHIMADZU (VIS1601PC) spectrophotometer, between 200 and 800 nm, to determine the presence of proteins and nucleic acids, at 260 and 280 nm respectively. The EPS was dissolved in LiCl 0.28M.

2.3.5. Partial acid hydrolysis

Crude EPS (30.0 mg) was dissolved in 0.1 M TFA (10 mL) and then hydrolyzed at 100 °C for 1 h in a sealed glass tube (Wang et al., 2013). The hydrolyzed polysaccharide was dialyzed against ultrapure water until no more carbohydrates were detected by the anthrone-sulfuric acid method (cut off dialysis tube – 1 kDa). The diluted and retentate solutions were dried by freeze-drying. Total monosaccharide composition, in both fractions, were performed following the methodology mentioned in item 2.3.6.

2.3.6. Analytical methods

The total protein content was measured by the Bradford method (Bradford, 1976). Sulfates and uronic acids were determined by (Dodgson & Price, 1962) and (Carpita & Ci, 1991), respectively.

For total monosaccharide composition, 1-2 mg of EPS was totally hydrolyzed in 1 mL of 1 M TFA (100°C, 4 h). The solution was reduced by $NaBDH_4$ for 16 h at room temperature, and residual boric acid was co-distilled with methanol washings. The solution was acetylated with 1 mL of acetic anhydride (1 h, 100°C), and analyzed by GC-MS/MS QP2010 model coupled to TQ8040 tandem mass spectrometer Shimadzu Corporation (Kyoto, Japan) and a Combi Palm AOC-5000 autosampler,
SH-Rtx-5ms column (30 m x 0,25 mm x 0,25 µm). The chromatograph was programmed to run from 100 to 250°C at 8°C min^{-1}, using He 99.9999 % at 1,00 mL min^{-1} (constant flow) as a carrier gas. The alditol acetates were identified by their typical electron-impact fragmentation profiles and GC retention times

Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra were obtained using KBr pellets. The EPS sample (1 mg) was ground with KBr, and the FTIR spectra were recorded on a Bomen-Hartmann & Braun, MB-series spectrophotometer with a resolution of 4 cm^{-1} and 32 scans per minute, using transmittance technique. The range of wavenumber scanned was from 400cm^{-1} to 4,000 cm^{-1}. The data obtained were analyzed using the ACD/NMR processor, academic version.

The deacetylation degree was calculated using the spectrum obtained from the FTIR spectroscopy following the formula (Roberts, 1992b):

$$\%deacetylation = 100\% \text{ x } (1 - \frac{A_{1655}}{A_{3340}} \text{ x } \frac{1}{1.33})$$

Where *A* is absorbance. The absorbance at 1655 and 3340 cm^{-1} correspond to the amide and the primary amino groups of the EPS respectively. The factor 1.333 represents the value of the ratio of A_{1655}/A_{3340} for the fully N-acetylated chitin.

Nuclear magnetic resonance (NMR) spectroscopy

For liquid NMR analyses, the EPS samples were dissolved in 99.99% D_2O under ultrasonic treatment at 20% amplitude (12 $W.cm^{-3}$) for 10 min in an ice-water bath (Z. M. Wang, Cheung, Leung, & Wu, 2010). The concentrations used were 40 mg.mL-^{1} for ^{13}C and 15 mg.mL^{-1} for ^{1}H, and 2D NMR, and were recorded at 30°C using a spectrometer (Bruker Avance DRX400). The base frequency was 400.13 and 100.61 MHz for ^{1}H and ^{13}C nuclei, respectively. Chemical shifts were expressed relative to acetone (internal standard) at 31.45 and 2.225 ppm for ^{13}C and ^{1}H, respectively. Acquisition parameters were carried out using the pulse programs supplied with the Bruker manual.

For solid-state RMN, ^{13}C CP-MAS NMR spectrum was recorded on Bruker AVANCE 400 spectrometer at 293K, at 100.62 MHz on a (9.4 T), observing the ^{13}C core, equipped with a 4 mm multinuclear probe with magic angle spinning (MAS). ^{13}C (^{1}H) NMR spectra were acquired with the aid of a pulse sequence, 2.0 ms of contact time, acoustic 1K scans, 2k dots distributed in a spectral window of approximately 260 ppm, based on a spectral adjustment of 25.6 Hz, 5s recycle delays and magic angle spinning (54.74 °) at 5 kHz. The spectrum was processed by the exponential Lorentzian (LB) multiplication on the FID by a factor of 50 Hz, together with the Fourier transform with 4k points. The baseline was corrected manually. For more resolution of the spectrum, the sample was humidified for 3 days in a closed vessel containing water-saturated atmosphere (Paradossi & Lisi, 1996).

The degree of acetylation (DA) of the polysaccharide was determined by dividing the intensity of the methyl group carbon by the average intensity of the anomeric carbons, following the equation (Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991):

$$\%DA = \frac{I(CH_3)}{(I(C1) + I(C2) + I(C3) + I(C4) + I(C5) + I(C6))/6} \text{ X } 100$$

Where *I* represent the intensity of the corresponding particular resonance peak.

For semi-solid state NMR, HR-MAS, ^{1}H HR-MAS NMR spectra were recorded at 293 K on a Bruker AVANCE spectrometer operating at 9.4 T, observing 1H at 400.13 MHz, equipped with a 4 mm four channel (1H, 13C, 15N, and 2H) HR-MAS probe.

The samples were spun at the magic angle (θ = 54.74 °) at 5 KHz. 1H HRMAS-NMR spectra were acquired by using a water suppression pulse sequence, noesypr1d (Bruker library), using 64 K data points over a 5999 Hz spectral width averaged over 512 transients. A recycle delay of 1.0 s, power for pre-saturation of 43 dB and a mixing time of 100 ms were used, the later to help reduce the water signal through T1 relaxation and chemical exchange. The saturation of the water residual signal was achieved by irradiation during recycle and the NOE mixing time at the H_2O/HOD NMR frequency. The spectra were apodized via an exponential multiplication corresponding to a 0.3 Hz line broadening in the transformed spectrum and zero filled by a factor of 2. The magic angle was adjusted daily using the 79Br signal from a powdered KBr for reference. The samples were locked on the deuterium signal from D_2O, and the magnetic field homogeneity was optimized for each sample. The total experiment time was 60 min for each sample, including the time used for rotor preparation.

High-pressure size-exclusion chromatography (HPSEC)

The EPS (1.0 mg.mL^{-1}) was dissolved in 0.1 M $NaNO_2$ containing NaN_3 (0.2 g L^{-1}) at 25 °C. The samples were filtered using 0.2 mm cellulose acetate membranes. The biopolymer analysis was performed using a differential refractive index detector (Waters 2410) coupled with a multi-angle laser light scattering detector (Wyatt Technology Dawn DSP). The products were separated isocratically at 0.6 mL min^{-1} (Waters 515 peristaltic pump), and four size-exclusion columns with exclusion limits of 7.106, 4.105, 8.104, and 5.103 g mol^{-1} were placed in series (Waters, Massachusetts, USA). For $\frac{\partial n}{\partial c}$ determination, fractions were dissolved in five concentrations (0.2–1.0 mg.mL-1) using the same eluent and filtered through 0.2 mm cellulose membrane prior to injection. The HPSEC data were collected and analyzed with ASTRA (Wyatt Technology, Massachusetts, USA).

2.3.7. Antibacterial activity.

Minimal inhibitory concentration of the EPS

The minimal inhibitory concentrations (MICs) of the EPS was performed according to the (CLSI, 2017) protocol. 100µL of the EPS was tested at different concentrations (0.01, 0.1; 0.5; 1,0; 1,5; 2,0; 2,5; 3.0; 3.5 and 4.0 mg.L-^{1}). The test was performed adding 80 µL Muller-Hinton broth medium with 20 µL of an inoculum of 10^{7} CFU/mL of each microorganism suspension (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Salmonella tiphy* ATCC 14028, *Escherichia coli* ATCC 35218), pippeted in a 96-well-flat-bottomed plate. A Chloramphenicol positive control and EPS plus culture medium as negative control were used. The incubation was carried out for 24h at 37°C, and then the absorbance was measured at 600nm. The percentage of growth inhibition was calculated as shown in Equation 2:

$$\%\ Growth\ inhibition = \left[\left(1 - \frac{A_{eps}}{A_c}\right)\right] x\ 100$$

Where A_{eps} was the absorbance of the samples and A_c was the absorbance of the negative control.

Resazurin indication solution (0.1%) was used to observed the cell viability after 2h of incubation.

2.3.8. Ferrous ion-chelating assay

he ferrous ion-chelating activity was performed as described by (Wang, 2014). 100 µL of the EPS (at 5 ug.mL^{-1}; 10 ug.mL^{-1}, 50 ug.mL^{-1}, 100 ug.mL^{-1}, and 1 g.mL^{-1}; 5 g.mL^{-1}) was mixed with 135 µL of ultrapure water and 5 µL of 2mM $FeCL_3$ in each well of a 96-well flat bottom microplate the reaction was initiated by the addition of 10 µL of 5mM ferrozine, mixed and incubated for 10 minutes at room temperature. Absorbance was measured at 562 nm with a PoweWave XS Microplate Spectrophotometer. Distilled water was used as a control and EDTA-Na_2 as a positive control. The assays were performed in five replicates. The activity was determined following the equation:

$$\% \text{ ferrous ion-chelating activity} = \left[\left(\frac{Ao-(A1-A2}{A0}\right)\right] \text{ x } 100$$

Where A_0 was the absorbance of the control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.3.9. Antioxidant activity – ABTS radical scavenging

The analysis was performed on various concentrations of the EPS to determine the quenching of the $ABTS^+$ radical cation. The 2,2'-azinobis-3-etilbenzothiazoline-6-sulfonic acid (ABTS) Radical Scavenging assay was performed as described previously (Lee, Oh, Cho, & Ma, 2015). The ABTS reagent was prepared to a final concentration of 7 mM with potassium persulphate, and the mixture was incubated for 16 h at room temperature in the dark. After this time, the ABTS reagent was diluted to 1:45 with ultrapure water until reaching an absorbance of 0.700, which was measured in the spectrophotometer at 734 nm. Trolox (6-hydroxy-2,5,7,8- 23 tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, St. Louis, MO, USA) was prepared from a stock solution (1 mM) over the concentration range (200, 100, 50, 25, 10, 5, 2 µM). The EPS samples were dissolved in ultrapure water to the final concentrations of 0.5 – 5.0 $mg.L^{-1.}$

For determining the scavenging activity a 96-well-flat-bottomed plate was used where 100 µL of the samples or standard were mixed with 100 µL of ABTS reagent, in the dark at room temperature. All measures were performed in triplicate. The absorbance was measured by using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, USA) at 734 nm at 1 5 and 10 minutes of reaction.

The percentage of ABTS radical scavenging was calculated as shown in Equation 1:

$$\% \, ABTS \, radical \, scavenging = \left(\frac{A_0 - A_1}{A_0}\right) x \, 100$$

Where A_0 was the absorbance control and A_1 was the absorbance of the sample.

2.3.10. Tumor cell lines and culture condition

Breast cancer cell lines (MCF7, MDA-MB 231 and MDA-MB 468) and its corresponding control (MCF 10A) were purchased from the cells bank of Rio de Janeiro – Brazil (APABCAM – Associaao tecnico cientifica Paul Erlich). All the cells were cultivated in Dulbecco's Modified Eagle's Medium F12 (DMEM) supplemented with 10% fetal bovine serum, except MCF7 in which a concentration of 20% of fetal bovine serum was used. The MCF10A cell line, non-tumorigenic epithelial cell, was cultivated in DMEM F12 medium supplemented with 10 ug/mL human insulin, 0.5 ug/mL hydrocortisone, 10ng/mL EGF and 100 ng/mL cholera toxin horse serum 5% instead fetal bovine serum. This culture conditions were recommended by the cells bank.

Colorectal adenocarcinoma cell line CACO-2 was cultured in DMEM medium supplemented with 20% of fetal bovine serum.

Adrenocortical carcinoma H295R cell line (purchased from the ATCC bank) and the non-tumoral VERO cell line from kidney (purchased from the cells bank of Rio de Janeiro –Brazil) were also cultivated in DMEM F12 medium supplemented with 10% of fetal bovine serum. All cultivations contained an antibiotic solution of 10U.Ml-1 of streptomycin and 20 U.mL- 1 of penicillin.

The cells were collected in a logarithmic growth stage by using 0.6% trypsin and viability was evaluated using the trypan blue exclusion test. The concentration of cells used was $1x10^6$ cells/well, pipetted in a 96-well-flat-bottomed plate. The incubation process was carried out for 24h at 37°C in a CO_2 (5%) humidified incubator.

2.3.11. Effect on cell viability of the EPS

The evaluation of the effect on cell viability of the EPS was carried out in the Research Institute Pelé Pequeno Príncipe – Curitiba – Paraná - Brazil (IPPPP). The EPS was solubilized at suitable conditions in ultrapure water by ultrasonic treatment at 20% amplitude (12 $W.cm^{-3}$) for 10 minutes in an ice-water bath (Wang et al., 2010) , were added to each well of each cell line respectively.

The citotoxic effect was evaluated using the 3-(4,5-dhimethylthiazol-2-yr)-2,5-diphenyltetrazolium bromide (MTT) assay. 100 µL of the cell suspension was added

to each well and incubated for 24h at 37°C with 5% CO_2. After the incubation process, the culture medium was removed and 180 µL of fresh culture medium and 20 µL of the EPS at different concentration (0.5; 1.0; 1.5; 2.0; 2.5; 3.0.; 3.5 and 4.0 $mg.L^{-1}$). After 24 hours of incubation, 20 µl of the MTT solution were added to each well to a final concentration of 0.5 $mg.mL^{-1}$ was used. The final reaction time was 3 hours at 37°C and CO2 5% was performed, and finally, after a careful removal of the supernatant, 100 µL of DMSO was added and gently agitated for 5 min to dissolve the formazan crystals – formed as MTT product degradation. The readings were performed at 595 nm on an ELISA microplate reader (SHIMADZU). The viability of the untreated cell lines group was considered as 100%. All assays were performed in five replicates To calculate the percentage of viability the following equation was used:

$$\%\ cell\ viability = \frac{100\ x\ A_{595e}}{A_{595b}}$$

In which A_{595e} is the mean value of the treatments samples, and A_{595b} is the mean value of the blanks.

2.4. RESULT AND DISCUSSIONS

2.4.1. Culture medium and optimization of EPS production

The culture medium composition is an important factor for a suitable growth of biomass and EPS production, thus the importance of the optimization process. A previous work regarding culture medium optimization (data not shown) was carried out to establish the best conditions for a suitable biomass growth of *M. alpina* (Goyzueta, 2014). Using this culture medium as base, a new culture medium for EPS production was developed by evaluating the best conditions for production using viscosity as a proxy for EPS production, and using glucose ($g.L^{-1}$) as (X_1), urea ($g.L^{-1}$) (as $X_{2)}$, pH (as X_3) and phosphate ($mmol.L^{-1}$) (as X_4), as factors. In this first phase, the first-order polynomial equations from the statistical regression for the production of biomass and viscosity enhancement, with only significant effects, are the following:

$$\text{Viscosity (cP)} = 1.53 + 0.5*X_2 + 0.23*X_4 - 0.1*X_1X_2 + 0.2*X_2X_4 \quad (1)$$

$$\text{Biomass } (g.L^{-1}) = 18.17 + 2.85*X_1 + 1.8*X_4 + 1.15*X_1X_4 \quad (2)$$

Eq. 1, indicates that increasing X_2 and X_4 and quantities enhance the production. The factors X_1 and X_3 were not significant alone for the viscosity enhancement, as Eq.2 indicates, the factor X_1 was significant for the biomass production. Thus, X_1 X_2 and X_4 were used for the further optimization process. The factor X_3 was maintained at its minimum level.

In the second part of the optimization process, a central-composite design was formulated (Figure 5.), the experimental results are given in Table 8. The second-order polynomial equation from the statistical regression for viscosity (cP) and biomass (g.L-1) production are as follows:

$$\text{Viscosity (cP)} = - 7.83 - 0.002*X_1^2 + 0.162*X_1 - 0.117*X_2^2 + 1.795*X_2 - 0.025*X_4^2 + 0.354*X_4 \quad (3)$$

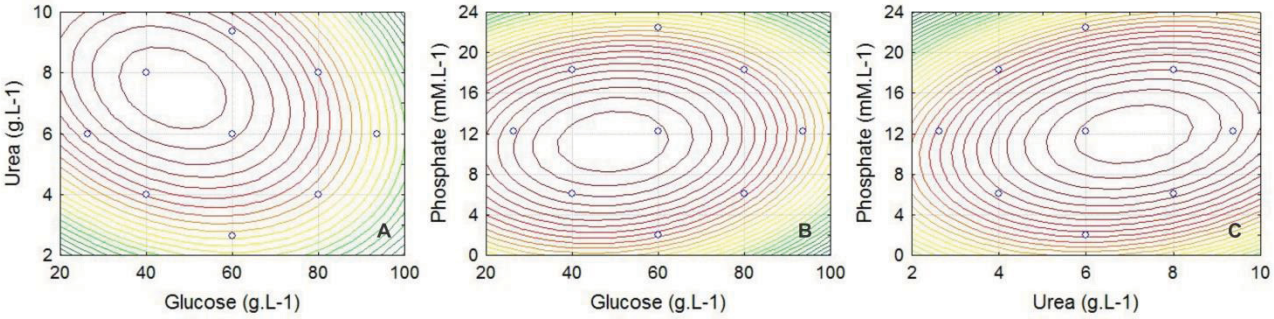


Figure 5. Contour plots for the optimization of viscosity (cP): A) Effect of Urea and glucose, B) effect of phosphate and glucose and c) Effect of phosphate and urea.

The analysis of the t-test (Table 9) showed that all the quadratic factors influenced in the viscosity of the fermentation ($p<0.05$), hence the EPS production. Using this mathematical model the optimal concentration of the factors for a maximum predicted production was: glucose 46.01 g.L-1; urea 7.48 $g.L^{-1}$ and phosphate 11.81 $mM.L^{-1}$ a production of 4.71 cP, equivalent to 1.51 $g.L^{-1}$ of EPS (at a 95% of confidence) was estimated. When applying this optimal concentration, the maximum production was 1.46 ± 0.11

Table 9. The result of Statistical Analysis and Regression Coefficient for viscosity (cP)

	SE	*t*(7)	ρ	Coefficient
Mean/interc.	-7.8316	4.0073	-1.9543	0.0915
X_1	0.1623	0.0701	2.31447	0.0538
X_1^2	-0.0013	0.0004	-2.8978	0.0230
X_2	1.7945	0.7015	2.5579	0.0376
X_2^2	-0.1175	0.0472	-2.4889	0.0416
X_4	0.3544	0.2010	1.7629	0.1212
X_4^2	-0.0248	0.0050	-4.9032	0.0017
X_1X_2	-0.0068	0.0056	-1.2177	0.2627
X_1X_4	0.0012	0.0018	0.6748	0.5214
X_2X_4	0.0235	0.0183	1.2790	0.2416

2.4.2. The purity of the EPS

The EPS was dissolved in a solution of LiCl 0.29 M due to poor solubility in organic solvents and its purity was evaluated.

The solution was transparent and no precipitation occurred after centrifugation. In the ultraviolet region, a higher absorbance characteristic of protein was observed at

260 nm (Figure 6), this was expected to be observed due to the presence of proteins as part of the composition of the EPS, characterized in the following items. No observations of any cell walls or detritus were observed in the visible range.

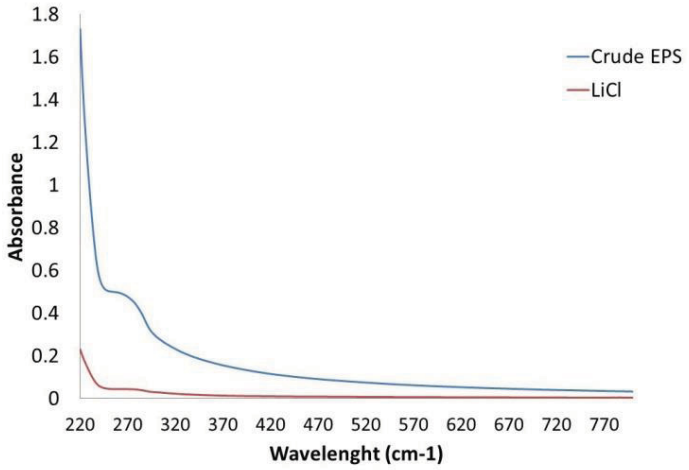


Figure 6. UV-VIS scan spectrum of the EPS.

2.4.3. EPS characterization

Physicochemical characteristics of the EPS

After the purification process, the EPS obtained remained with a white-cotton appearance, containing 0.5% of uronic acids, 4.5% of sulfate and 5.3% of proteins. The monosaccharide composition of the crude EPS was composed of 98% of D-glucosamine (product of N-acetylglucosamine after hydrolysis), 1% mannose and 1% glucose. The two fractions obtained after partial hydrolysis, eluted fraction (PF) and retained fraction (RF) are composed by 98% of N-acetyl-D-glucosamine, 1% D-mannose and 1% of D-glucose, and 99% of N-acetyl-D-glucosamine and 1% of D-glucose, respectively. The dispersity of the polymer was also evaluated; the HPSEC-RID elution profile showed a unique and symmetric peak (Figure 7) indicating that the EPS has a homogeneous molar mass distribution. The HPSEC-MALLS analysis revealed the molar mass of the EPS to be 4.9x10^5 g.mol^{-1} ($\frac{\partial n}{\partial c} = 0{,}121\ \mathrm{mL.g^{-1}}$).

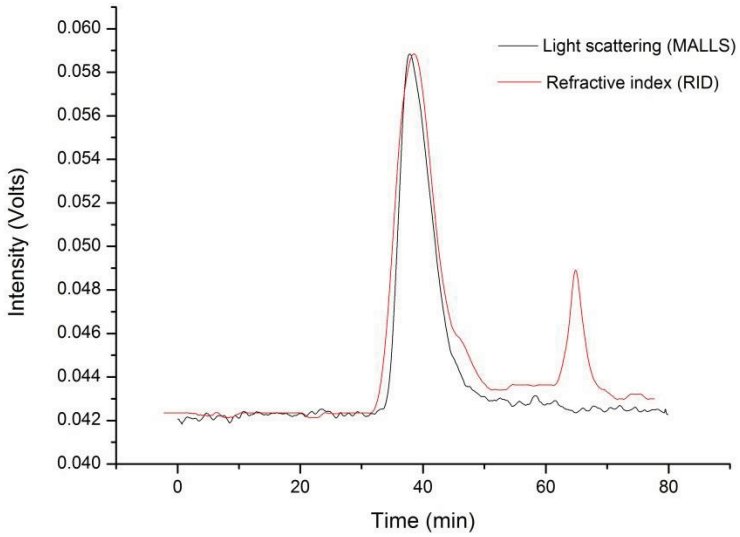


Figure 7. Elution profile (HPSEC-MALLS-RID) of the exopolysaccharide, eluted with 0.1M $NaNO_3$ at a flow rate of 0.6 $mL.min^{-1}$.

Structural analysis of the EPS

Solid state and semi-solid state NMR is a powerful and high-resolution tool, helpful to identify the structure and conformation of exopolysaccharides with low solubility capacity. First, an HR-MAS NMR was performed and EPS was partially solubilized in D_2O giving the spectrum of Figure 8-A. Wide signals were observed probably due to the homogeneity of the polymer. The groups of non-anomeric signals between 3.0 and 4.0 ppm were attributed to N-acetyl-D-glucosamine ring residues, and the signal at 1.9 ppm corresponds to CH_3 of the acetyl group.

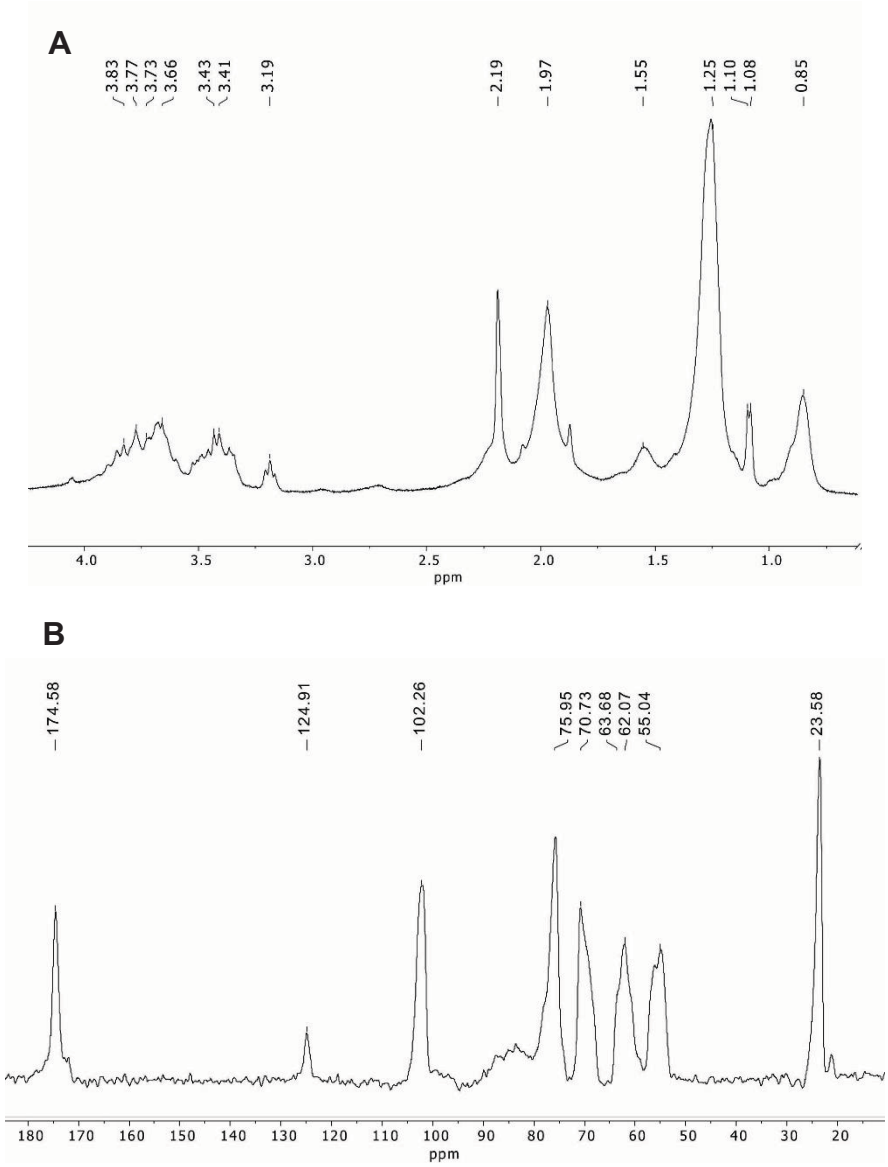


Figure 8. NMR spectra of EPS from *Mortierella alpina* CBS 528.72: A) ^{1}H HR-MAS and B) ^{13}C CP-MAS.

The other signals, background noise, may be due to the presence of some impurities and artifacts. To continue to identify the typical conformation of the EPS, a second NMR was performed, a 13C CP-MAS (Figure 8-B). This type of NMR is known for being very sensitive to any changes in the local structure of the polymer. In this second phase of identification, the ^{13}C spectrum (Figure 8-B) showed 6 signals which were attributed to the non-anomeric region of N-acetyl-D-glucosamine residues, as follows: C_2 (55.04 ppm)(characteristic of GlcNAc), C_3 and C_5 overlapped (75.95 ppm), C_4 (70.73 ppm) and C_6 (62.07 ppm) and anomeric carbon C_1 ressoned at 101.71 ppm, Additionally, the signal at 23.58 ppm corresponds to CH_3 of the acetyl group and the signal at 174.58 ppm was attributed to the carbonyl group. (Heux, Brugnerotto,

Desbrières, Versali, & Rinaudo, 2000; Kono, 2004; Poirier & Charlet, 2002; Saitô, Tabeta, & Hirano, 1981; Tanner, Chanzy, Vincendon, Roux, & Gaill, 1990; Younes & Rinaudo, 2015) . The high areas of the carbonyl and acetyl groups suggest a high acetylation degree of the polysaccharide, >90% of acetylation was calculated by the intensities of the ^{13}C CP-MAS NMR.

A 2D HSQC NMR was also carried out in order to evaluate both fractions of the partial acid hydrolysis, the RF and PF (spectrum was not shown). The spectrum of the RF showed the N-acetyl-D-glucosamine backbone of the polysaccharide (Figure 9) in which the letter “a” indicate the presence of the CH_3 group of the carbonyl group (2.01 ppm and 22.49 ppm), and the non-anomeric region (3-5 ppm and 55-101 ppm), some signals of the non-anomeric region were overlapped, suggesting a low resolution due to the low solubility nature of the biopolymer in D_2O.

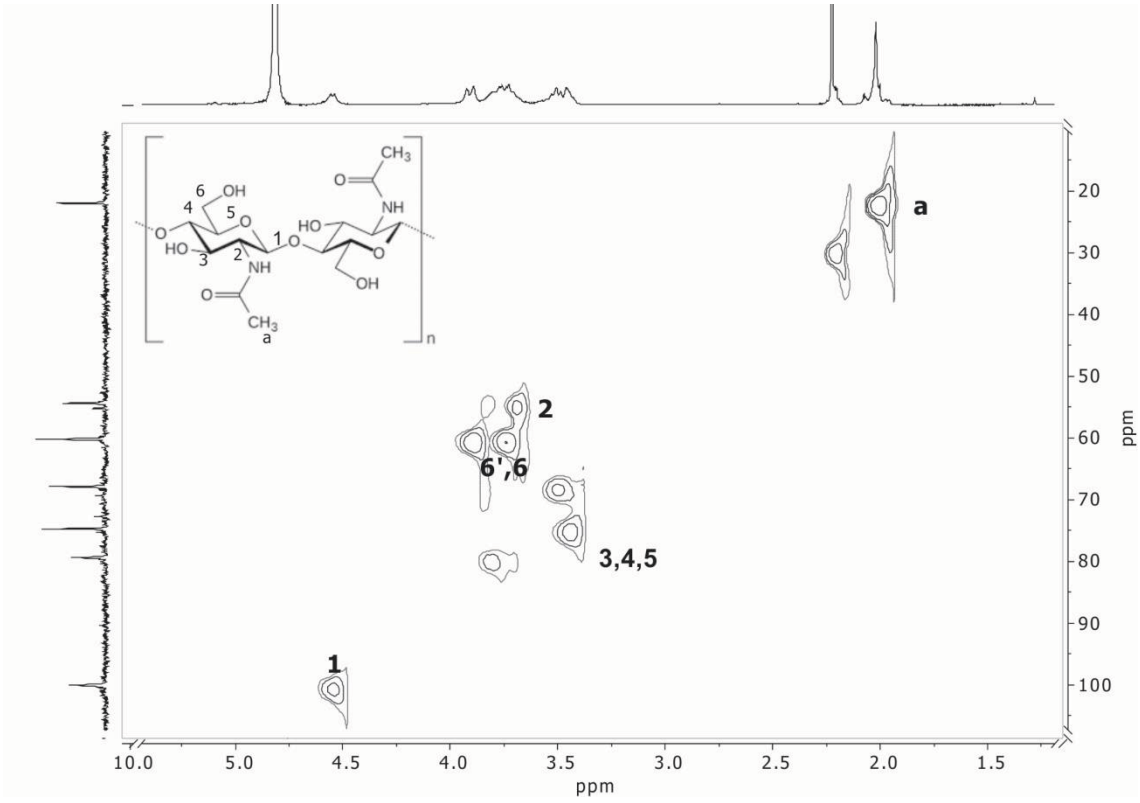


Figure 9. 2D NMR HSQC of the retentate fraction (backbone) of the EPS. Acetone was used as internal standard.

The FTIR spectrum of the EPS is shown in Figure 10, in which some characteristics bands were observed: 3489 cm^{-1} attributed to OH groups, typical in

polysaccharides (Duarte, Ferreira, Marvão, & Rocha, 2002), 3307 cm^{-1} attributed to NH_2 groups, 2899 cm^{-1} attributed to an aliphatic C-H stretching band, the main characteristic of chitins (C=O stretching) attributed to the vibration of the amide I band at 1682 cm^{-1} (Rumengan et al., 2014), 1574 cm^{-1} attributed to the N-H deformation of amide II, 1438 cm^{-1} attributed to the CH_3 group deformation (Schenzel & Fischer, 2001), and finally, the band showed at 1097 cm^{-1} was attributed to the C-O-C vibration glycosidic link (Puspawati & Simpen, 2010).

The identification of the exopolysaccharide as chitin can be confirmed after summarising all the results of the spectra obtained by NMR and FTIR

Chitin is a polysaccharide that is synthesized by multiple organisms such as crustraceans and fungi, consists of 2-cetoamido 2-deoxy-β-D-glucose through a β(1→4) linkage (R. A. Muzzarelli, 1999). Chitin is mostly extracted by alkaline or acid treatments from exoskeletons of crustaceans (shrimp and crabs)(Rane & Hoover, 1993), hence the lack of research of alternatives sources of production. Due to their physicochemical properties and low solubility of chitin (Roberts, 1992a), a deacetylation process is performed to obtain chitosan. This amino sugar can be found in cartilages, joint tissues and is used in medicine to relief arthritic symptoms (Anderson et al., 2005) among other 200 applications (Abdou et al., 2008; Ravi Kumar, 2000) distributed among cosmetics, agriculture, food, biomedical, textile (Rathke & Hudson, 1994) and effluents remediation (N. et al., 1988). It is important to consider that the extraction process of chitin and its derivatives (chitosan and GlcNAc) produces a reasonable level of contaminants for the environment (Bautista et al., 2001) and also could be dangerous for allergic people to shellfish

In order to improve the solubility of the EPS for potential applications, due to its similarity to known chitins, an alkali deacetylation process to remove the acetyl group was carried out to convert the chitin into chitosan. This chemical method is used for commercial chitosan preparation due to its low cost and industrial production (No & Meyers, 1995). Following the methodology of Chen, Wang, & Ou, 2004, a deacetylation of 70% was reached, estimated from the FTIR spectrum (Figure 10). The deacetylated EPS was soluble in dilute acid solution, such as 0.1 M acetic acid but still not water-soluble possibly due to the formation of copolymers of N-acetyl-D-glucosamine and D-glucosamine units, giving random different structures of the

products (amorphous crystals), changing its solubility (Kurita, Sannan, & Iwakura, 1977).

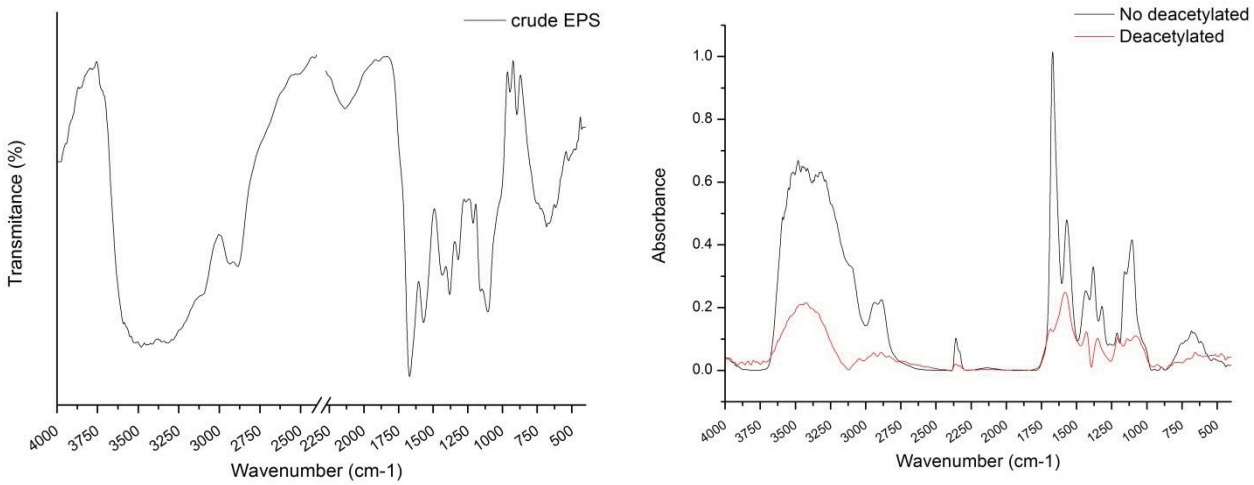


Figure 10. FTIR spectra of the EPS, as a wavenumber function. A) EPS (transmittance%) and B) deacetylated and no deacetylated EPS (absorbance).

A 2D NMR was also performed of the deacetylated chitin, in order to confirm the production of chitosan from this new alternative source (Figure 11). The correlation for the anomeric proton and carbon H1/C1 was observed at 4.8/98, for H3-H6/C3-C6 (marked in a circle) were observed at 3.7-4 ppm/59-76 ppm and the methyl hydrogen atoms H/CH_3 appeared at 2.1/21ppm (marked as “a”). The C2 signal, characteristic in deacetylated chitin, was observed at C2/H2 56.3/3.2 ppm.

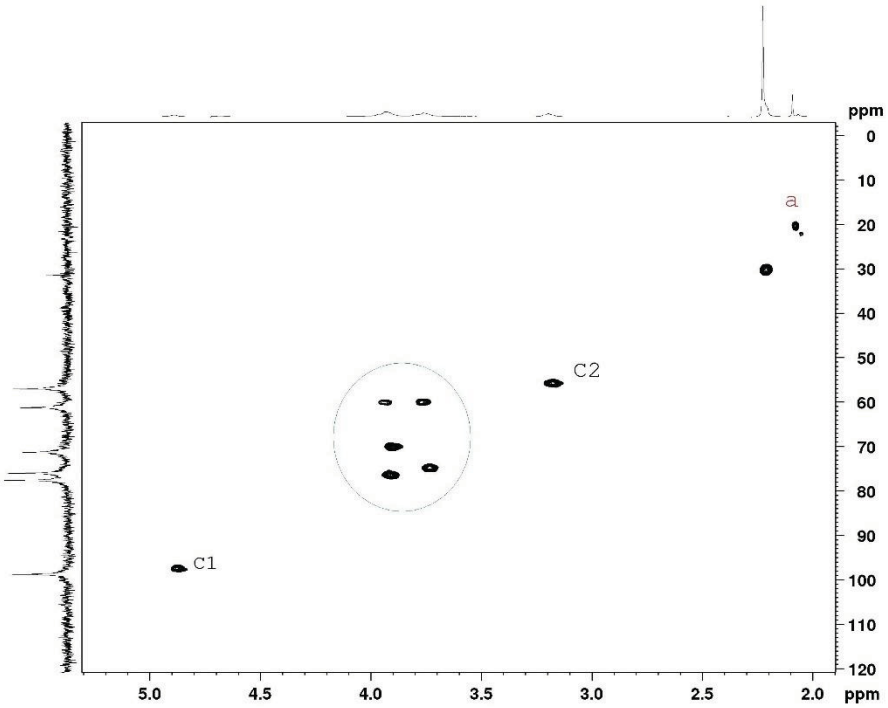


Figure 11. 2D NMR HSQC of the deacetylated exopolysaccharide under alkaline conditions.

2.4.4. Minimal inhibitory concentration (MIC) of the EPS

In the evaluation of MICs of the EPS against the bacteria *S. tiphy*, *S. aureus* and *E. coli* (3.5; 3.0, and 3.5 mg.mL^{-1} respectively), it was observed that the EPS was most effective for the gram-negative bacteria *S. tiphy* and *S. aureus*.

On the other hand, *Bacillus subtilis* was resistant to the EPS activity possibly because of its high molar mass and viscosity. According to Raafat & Sahl, (2009), the antibacterial activity against *Bacillus* sp. depends on its viscosity. Benhabiles et al., (2012), speculates that this fact is because of smaller units of chitin can be able to interact in a suitable way with microbial cells. The results obtained in this work showed an effect of chitin as a bacteriostatic agent rather than bactericidal because a growth inhibition over 80% was not observed after 24h.

The mechanism of action of chitin as an antimicrobial agent could be attributed to its acetylation degree (Tsai, Su, Chen, & Pan, 2002), being over 90% in the case of the EPS produced by *M. alpina* (determinated of the FTIR spectra). According to

Muzzarelli, (1977), an alternative mechanism is also the inhibition of enzymes linking glucans from the celullar wall to chitin.

Comparing the results of this work to those of Benhabiles et al. (2012), a low inhibitory effect in *S. tiphy* was noted while alternative sources of chitin as *Aspergillus niger* and *Mucor rouxi* from cell walls reduced the cell counts in 50% during 4 days incubation (Wu, Zivanovic, Draughon, Conway, & Sams, 2005).

2.4.5. Ferrous-ion chelating assay

The results showed in Figure 12 indicated a low chelating effect of the EPS compared with EDTA; in which a ion chelated effect of 18% was observed at 1 and 5 $mg.mL^{-1}$. The EPS characterized showed a chitin biopolymer and these results showed the same low activity as chemically sulfated from animal sources reported by Xing et al., (2005). It is important to consider ferrous ions as one of the most effective pro-oxidants present in food (Yamauchi, Tatsumi, Asano, Kato, & Ueno, 1988), the low chelating effect of EPS can even be considered when a biomaterial formulation for preserving food could be proposed. Another metal that could also must be tested as copper (unbounded form), which causes oxidant damage due to the generation of reactive oxygen species.

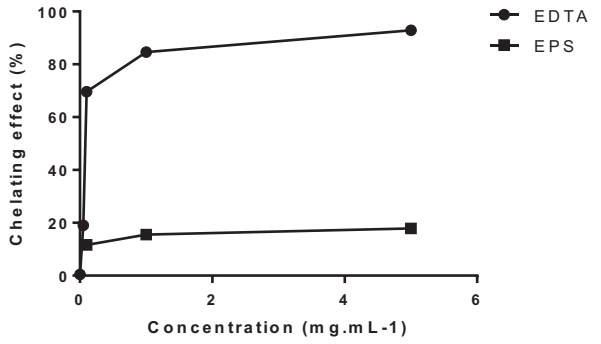


FIGURE 12. Chelating effect (%) of crude EPS compared to EDTA-Na_2.

2.4.6. Antioxidant activity – ABTS radical scavenging

There are numerous studies about the antioxidant properties of chitin and its derivates (chitosan and N-acetylglucosamines) (Harish Prashanth & Tharanathan, 2007; No & Meyers, 1995; Ravi Kumar, 2000; Younes & Rinaudo, 2015), but few references are available about microbial chitin or chitosan, specially produced as an extracellular product.

The antioxidant capacity of the EPS produced by *Mortierella alpina* was evaluated by the ABTS radical scavenging, different reaction times (1 min, 5 min, and 10 min) were evaluated in despite to determine the most suitable time response (Figure 13). No significant differences were observed when more than 5 minutes of reaction times was tested (data not shown).

The IC_{50} calculated in 5 min of the assay was 2.08 $mg.mL^{-1}$ in which a final scavenging activity of 85% was reached compared to 1 minute of the assay where at 2.5 $mg.mL^{-1}$ a 56% of activity was attained. The Trolox Equivalent Antioxidant Capacity (TEAC) was calculated as 989.0 µmol equivalent/g of EPS (247 mg of Trolox/g of EPS). It was observed that when the concentration of EPS was an increase, the scavenging activity was increased in shorter time. This phenomenon can be explained by the interaction of free radicals with hydroxyl or amine groups of the chitin, forming stable macromolecule radicals (Xie, Xu, & Liu, 2001).

The EPS, chitin, from *M. alpina* showed a promising antioxidant effect when compared to another sources of chitin such as insecta ($IC_{50:}$ 10.91 $mg.mL^{-1}$ with an scavaging activity of 40%) (Kaya et al., 2015) or marine ($IC_{50:}$ 5 $mg.mL^{-1}$ with an scavaging activity of 85%) (Vinsova & Vavrikova, 2011),

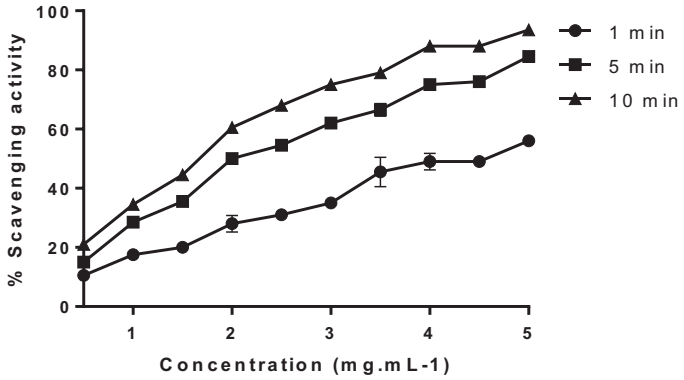


Figure 13. % scavenging activity of EPS evaluated at 1, 5 and 10 min of reaction time.

2.4.7. Antitumoral effect of the EPS

In the research for new biomolecules with active antitumoral effects, polysaccharides have been of particular interest (Ooi & Liu, 2000). There are several studies about the anticancer potential of polysaccharides from fungi, but just a few about chitin excreted (Lenardon, Munro, & Gow, 2010).

In recent years the study about the use of chitosan has increased significatively due to their multiples bioactivities such as antioxidant, antitumoral, flocculant, immunologic, among others that are used by multiples industries (agriculture, food, and beverages, cosmetics and biopharmaceutics. On the other hand, studies about the potential antitumoral effect of chitin as itself are escarse. Thus, this is the main objective of this part of the work.

In order to evaluate the potential of this new alternative of chitin, a total of seven cell lines were tested, in which 2 of them were non-tumoral controls (VERO and MFC10A). The MTT assay showed a significant inhibitory effect of cellular proliferation in all the tumoral cell lines after 24 h (Figure 14.) and the half maximal inhibitory concentration (IC50) compared to the untreated controls. Significant tumoral cell growth inhibitions >50% of the cell were determined at 1.37 $mg.mL^{-1}$ (for H295R); 2.1 $mg.mL^{-1}$ (for CACO-2); 1.5 $mg.mL^{-1}$ (for MDA MB 231); 1.4 $mg.mL^{-1}$ (for MDA MB 468)

and 1.3 mg.mL-^{1} (for MCF 07), which means that increasing concentration of the EPS resulted in dose-dependent cell proliferation inhibition. In the case of non-tumoral cell lines, no cytotoxicity over 50% was observed at a higher concentration of EPS.

The cytotoxicity of the EPS in the tumoral cell lines was also evaluated observing morphological changes of cell during the assay, however, it was noted that when the concentration of the EPS was increased, the change of the morphological characteristics of the cells also increased, this phenomena can be explained by apoptosis cellular death due to the all membrane damage (shrinkage or collapse) (Chen et al., 2013).

The *in vitro* researches about the chitin mechanism on healthy cell demonstrated that the charge properties are important for the anticancer activity (Karagozlu, Karadeniz, Kong, & Kim, 2012), highly charged chitin compounds or derivates were triggered to an apoptotic pathway (Rinaudo, 2006).

Adrenocortical carcinoma is a rare cancer with some drug approved option of its treatment such as mitotane (Fassnacht et al., 2012), the EPS tested showed a high potential for further studies to be performed and evaluated to analyze the exact mechanism of action on the tumor cells and also in breast cancer, colon cancer cells, that nowadays are considered as aggressive cancer types when diagnosed in an advanced phase. More option of chemotherapeutic agents can be researched and proposed by the use of exopolysaccharide, such as the one tested in this work.

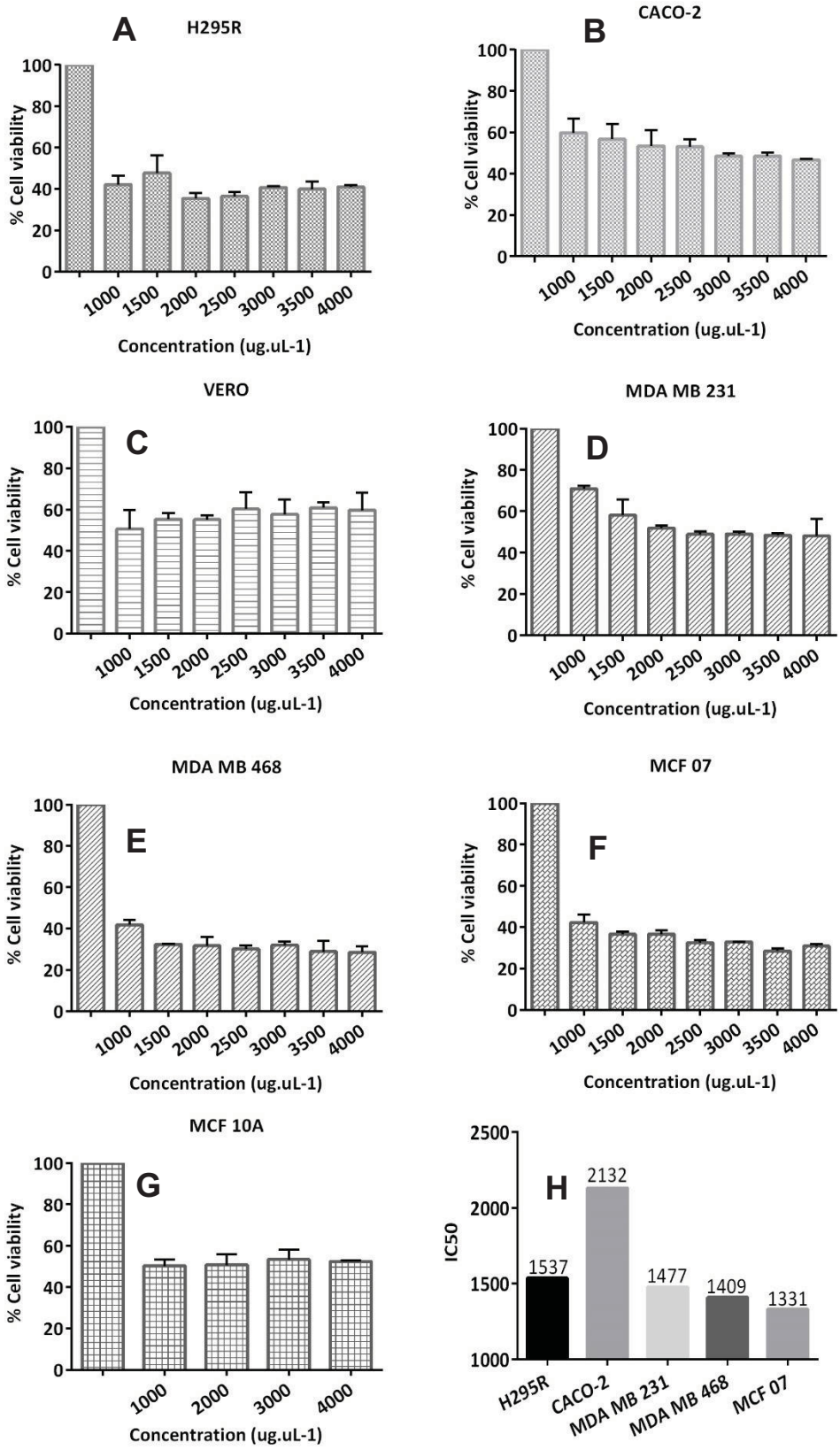


Figure 14. Cell viability (%) of the EPS produced by *M. alpina* on tumor cells: A)H25R, B)CACO-2, C) VERO, D) MDA MB 231, E) MDA MB 468, F) MCF07 and G) MCF10A determined by the MTT assay. Figure H shows the IC50 concentrations determined for tumor cells.

2.5. CONCLUSIONS

This work shows for the first time the characterization and bioactive potential of an alternative extracellular “green” chitin produced by *Mortierella alpina* as an antioxidant agent with a bacteriostatic effect and antitumor activity.

A new study about exopolysaccharide, from *Mortierella alpina* was carried out, a 50% of increment of EPS production was attained by the optimization process using a full factorial and central composite rotational design, the EPS was homogeneous with a molecular weight of 4.9x10^5 Da (estimated $\frac{\partial n}{\partial c} = 0{,}121\ \mathrm{mL.g^{-1}}$) , determined by HPSEC-MALLS. The EPS was a chitin biopolymer, elucidated by different NMR techniques, especially by HR-MAS NMR spectroscopy, which allows a direct analysis of the biopolymer providing access to structural and dynamic properties.

The evaluation of its toxicity against non-tumoral cells strains such as VERO and MCF10A provide the possibility of its safe utilization as adjuvant in chemotherapeutics and as a chemopreventive drug option for breast, colorectal cancer disease, and adrenocortical carcinoma. Further studies must be performed to know the mechanism of action of the antitumoral effect and applications as a biopolymer in biomaterials formulations.

ACKNOWLEDGMENTS

The authors thank the funding agencies CNPq and CAPES for their support.

2.6. REFERENCES

Abdou, E. S., Nagy, K. S. A., & Elsabee, M. Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology*, *99*(5), 1359–1367. https://doi.org/https://doi.org/10.1016/j.biortech.2007.01.051

Akhtar, M. W., Mirza, A. Q., Nawazish, M. N., & Chughtai, M. I. D. (1983). Effect of triglycerides on the production of lipids and lipase by Mucor hiemalis. *Canadian Journal of Microbiology*, *29*(6), 664–669. https://doi.org/10.1139/m83-108

Akimoto, K., Kawashima, H., & Shimizu, S. (2011). *Microorganisms that extracellularly secrete lipids and methods of producing lipid and lipid vesicles encapsulating lipids using said microorganisms.* (No. U.S. Patent No. 7,972,855). Washington, DC: U.S.

Alekshun, M. N., & Levy, S. B. (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell*, *128*(6), 1037–1050. https://doi.org/https://doi.org/10.1016/j.cell.2007.03.004

Allolio, B., & Fassnacht, M. (2006). Adrenocortical carcinoma: clinical update. *The Journal of Clinical Endocrinology & Metabolism*, *91*(6), 2027–2037.

Amano, H., Shinmen, Y., Akimoto, K., Kawashima, H., Amachi, T., Shimizu, S., & Yamada, H. (1992). Chemotaxonomic significance of fatty acid composition in the genus Mortierella. *Mycotaxon*, *45*, 257–265.

Anderson, J. W., Nicolosi, R. J., & Borzelleca, J. F. (2005). Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food and Chemical Toxicology*, *43*(2), 187–201. https://doi.org/https://doi.org/10.1016/j.fct.2004.11.006

Bajpai, P., & Bajpai, P. K. (1992). Arachidonic acid production by microorganisms. *Biotechnology and Applied Biochemistry*, *15*(1), 1–10. https://doi.org/10.1111/j.1470-8744.1992.tb00194.x

Bajpai, P., Bajpai, P. K., & Ward, O. P. (1991). Eicosapentaenoic acid (EPA) production by Mortierella alpina ATCC 32222. *Applied Biochemistry and Biotechnology*, *31*, 267–272. https://doi.org/10.1007/BF02921753

Banerjee, Mr. Mahapatra, & D Banerjee. (2013). Fungal Exopolysaccharide: Production,

Composition and Applications. *Microbiology Insights*, 1. https://doi.org/10.4137/MBI.S10957

Bautista, J., Jover, M., Gutierrez, J. F., Corpas, R., Cremades, O., Fontiveros, E., … Vega, J. (2001). Preparation of crayfish chitin by in situ lactic acid production. *Process Biochemistry*, *37*(3), 229–234. https://doi.org/https://doi.org/10.1016/S0032-9592(01)00202-3

Benhabiles, M. S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., & Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloids*, *29*(1), 48–56. https://doi.org/https://doi.org/10.1016/j.foodhyd.2012.02.013

Berruti, A., Terzolo, M., Sperone, P., Pia, A., Della Casa, S., Gross, D. J., … Mantero, F. (2005). Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocrine-Related Cancer*, *12*(3), 657–666.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

Blanpain, C. (2013). Tracing the cellular origin of cancer. *Nature Cell Biology*, *15*(2), 126.

Bonatto, S. J. R., Folador, A., Aikawa, J., Yamazaki, R. K., Pizatto, N., Oliveira, H. H. P., … Fernandes, L. C. (2004). Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. *Cellular Immunology*, *231*(1–2), 56–62.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brekhman, I. I. (2013). *Man and biologically active substances: the effect of drugs, diet and pollution on health*. Elsevier.

Brooks, D. D., Berbesi, R., & Hodgson, A. S. (2011). Optimization of bleaching process. Retrieved March 28, 2016, from http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40321

Calder, P. C. (2012). Mechanisms of Action of (n-3) Fatty Acids, 2. *The Journal of Nutrition*, *142*(3), 592S–599S.

Carocho, M., & Ferreira, I. C. F. R. (2013). A review on antioxidants, prooxidants and related

controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, *51*, 15–25. https://doi.org/https://doi.org/10.1016/j.fct.2012.09.021

Carpita, C., & Ci, D. (1991). Measurement of Uranic Acids without from Neutral Sugars, *162*, 157–162.

Certik, M., Megova, J., & Horenitzky, R. (1999). Effect of nitrogen sources on the activities of lipogenic enzymes in oleaginous fungus *Cunninghamella echinulata*. *The Journal of General and Applied Microbiology*, *45*(6), 289–293. https://doi.org/10.2323/jgam.45.289

Certik, M., & Shimizu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Bioscience and Bioengineering*, *87*(1), 1–14.

Chand, A., Rajesh, R., Tomar, S., & Kumar, R. (2016). Arachidonic Acid Synthesis in Mortierella alpina : Origin , Evolution and Advancements. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*. https://doi.org/10.1007/s40011-016-0714-2

Chen, C. H., Wang, F. Y., & Ou, Z. P. (2004). Deacetylation of b-chitin. I. Influence of the deacetylation conditions. *Journal of Applied Polymer Science*, *93*(5), 2416–2422. https://doi.org/10.1002/app.20753

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3),(1→ 6)-glucans. *Mycological Research*, *111*(6), 635–652.

Chen, Y. T., Yuan, Q., Shan, L. T., Lin, M. A., Cheng, D. Q., & Li, C. Y. (2013). Antitumor activity of bacterial exopolysaccharides from the endophyte bacillus amyloliquefaciens sp.isolated from ophiopogon japonicus. *Oncology Letters*, *5*(6), 1787–1792. https://doi.org/10.3892/ol.2013.1284

Choromanska, A., Kulbacka, J., Rembialkowska, N., Pilat, J., Oledzki, R., Harasym, J., & Saczko, J. (2015). Anticancer properties of low molecular weight oat beta-glucan – An in vitro study. *International Journal of Biological Macromolecules*, *80*, 23–28. https://doi.org/https://doi.org/10.1016/j.ijbiomac.2015.05.035

Chow, L. W. C., Lo, C. S. Y., Loo, W. T. Y., Hu, X., & Sham, J. S. T. (2003). Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D 1 gene. *The American Journal of Chinese Medicine*, *31*(1), 1–9.

Chung, Y. C., Su, Y. P., Chen, C. C., Jia, G., Wang, H. L., Wu, J. C. G., & Lin, J. G. (2004).

Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. *Acta Pharmacol. Sinica, 25*, 932–936.

CLSI. (2017). *M100 Performance standards for antimicrobial susceptibility testing. 27th ed. Clinical and Laboratory Standards Institute*.

Cohen, Z., Vonshak, A., & Richmond, A. (1987). Fatty acid composition of Spirulina strains grown under various environmental conditions. *Phytochemistry*, *26*(8), 2255–2258. https://doi.org/10.1016/S0031-9422(00)84694-4

Dackiw, A. P. B., Lee, J. E., Gagel, R. F., & Evans, D. B. (2001). Adrenal cortical carcinoma. *World Journal of Surgery*, *25*(7), 914.

Davies, R. J., Holdsworth, J. E., & Reader, S. L. (1990). The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast Apiotrichum curvatum. https://doi.org/10.1007/BF00172553

De Angelis, A. A., Capitani, D., & Crescenzi, V. (1998). Synthesis and 13C CP-MAS NMR characterization of a new chitosan-based polymeric network. *Macromolecules*, *31*(5), 1595–1601.

De Greyt, W. (2013). Edible oil refining: Current and future technologies. In W. Hamm, R. J. Hamilton, & G. Calliauw (Eds.), *Edible Oil Processing* (2nd ed, pp. 127–152). Chichester, UK: John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118535202.ch5

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*(1), 106–110. https://doi.org/10.1042/bj0840106

Dongping, E., Kai, C., Tao, C., & Hua, T. (2014). *Mortierella alpina mutant strain capable of producing high-yield arachidonic acid, and fermentation method and application thereof* (No. CN103484385 A). Retrieved from http://www.google.com/patents/CN103484385A?cl=en

Duarte, M. L., Ferreira, M. C., Marvão, M. R., & Rocha, J. (2002). An optimised method to determine the degree of acetylation of chitin and chitosan by FTIR spectroscopy. *International Journal of Biological Macromolecules*, *31*(1), 1–8. https://doi.org/https://doi.org/10.1016/S0141-8130(02)00039-9

Dueppen, D. G., Zeller, S. G., Diltz, S. I., & Driver, R. H. (2005). *Extraction and winterization of lipids from oilseed and microbial sources* (No. U.S. Patent 7,695,626). Washington,

DC: US: Extraction and winterization of lipids from oilseed and microbial sources.

Emerit, J., Edeas, M., & Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomedicine and Pharmacotherapy*, *58*(1), 39–46. https://doi.org/10.1016/j.biopha.2003.11.004

Eroshin, V. K., Satroutdinov, A. D., Dedyukhina, E. G., & Chistyakova, T. I. (2000). Arachidonic acid production by Mortierella alpina with growth-coupled lipid synthesis. *Process Biochemistry*, *35*(10), 1171–1175. https://doi.org/http://dx.doi.org/10.1016/S0032-9592(00)00151-5

Euromonitor, a market research provider. (2006). Infant milk formula industry. Retrieved February 24, 2016, from http://www.euromonitor.com/global-baby-food-overview-key-categories-countries-and-trends/report

Fassnacht, M., Terzolo, M., Allolio, B., Baudin, E., Haak, H., Berruti, A., … Skogseid, B. (2012). Combination Chemotherapy in Advanced Adrenocortical Carcinoma. *New England Journal of Medicine*, *366*(23), 2189–2197. https://doi.org/10.1056/NEJMoa1200966

Feofilova, E. P. (2010). The fungal cell Wall : modern concepts of its composition. *Microbiology*, *79*(6), 711–720. https://doi.org/10.1134/S0026261710060019

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*, 239. Retrieved from http://dx.doi.org/10.1038/35041687

Fischer, L., Stressler, T., Eisele, T., Rost, J., Haunschild, E., & Kuhn, A. (2013). Production of polyunsaturated fatty acids by Mortierella alpina using submerse and solid state fermentation. *Chemie Ingenieur Technik*, *85*(3), 318–322. https://doi.org/10.1002/cite.201200094

Flack, M. R., & Chrousos, G. P. (1996). Neoplasms of the adrenal cortex. *Cancer Medicine*, *1*, 1567.

Forsyth, J. S., Willatts, P., Agostoni, C., Bissenden, J., Casaer, P., & Boehm, G. (2003). Long chain polyunsaturated fatty acid supplementation in infant formula and blood pressure in later childhood: follow up of a randomised controlled trial. *BMJ : British Medical Journal*, *326*(7396), 953. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC153849/

Forsyth, S., Gautier, S., & Salem Jr., N. (2016). Global Estimates of Dietary Intake of Docosahexaenoic Acid and Arachidonic Acid in Developing and Developed Countries. *Annals of Nutrition and Metabolism*, *68*(4), 258–267. https://doi.org/10.1159/000446855

Forsyth, S., Gautier, S., & Salem Jr., N. (2017). Dietary Intakes of Arachidonic Acid and Docosahexaenoic Acid in Early Life - With a Special Focus on Complementary Feeding in Developing Countries. *Annals of Nutrition and Metabolism*, *70*(3), 217–227. https://doi.org/10.1159/000463396

Fukui, S. (1995). Selection and breeding of microorganisms secreting and producing fuel lipids. *Bio Industry*, *12*(3), 36–46.

Ganeshpurkar, A., Rai, G., & Jain, A. P. (2010). Medicinal mushrooms: Towards a new horizon. *Pharmacognosy Reviews*, *4*(8), 127.

Gerentes, P., Vachoud, L., Doury, J., & Domard, A. (2002). Study of a chitin-based gel as injectable material in periodontal surgery. *Biomaterials*, *23*(5), 1295–1302.

Germano, A., Rapa, I., Volante, M., Buono, N. Lo, Carturan, S., Berruti, A., … Papotti, M. (2014). Cytotoxic activity of gemcitabine, alone or in combination with mitotane, in adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *382*(1), 1–7.

Giavasis, I. (2013). 16 - Production of microbial polysaccharides for use in food. In B. McNeil, D. Archer, I. Giavasis, & L. B. T.-M. P. of F. I. Harvey Enzymes and Nutraceuticals (Eds.), *Woodhead Publishing Series in Food Science, Technology and Nutrition* (pp. 413–468). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9780857093547.2.413

GostReports. (2016). Global arachidonic acid market research report 2016. Retrieved September 5, 2016, from http://www.gosreports.com/global-arachidonic-acid-market-worth-1-18-billion-by-2019/

Gunstone, F. D. (2001). Oilseed crops with modified fatty acid composition. *Journal of Oleo Science*, *50*(5), 269–279.

Gutierrez, R. M. P., & Ramirez, A. M. N. G. and A. M. (2012). Compounds Derived from Endophytes: A Review of Phytochemistry and Pharmacology. *Current Medicinal Chemistry*. https://doi.org/http://dx.doi.org/10.2174/092986712800672111

Hamuro, J. t. (1985). Lentinan, a T-cell oriented immunopotentiator: its experimental and clinical applications and possible mechanism of immune modulation. *Immunomodulation Agents and Their Mechanisms*.

Hansson, L., & Dostálek, M. (1988). Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus Mortierella ramanniana. *Applied Microbiology and Biotechnology*, *28*(3), 240–246. https://doi.org/10.1007/BF00250448

Harish Prashanth, K. V., & Tharanathan, R. N. (2007). Chitin/chitosan: modifications and their unlimited application potential-an overview. *Trends in Food Science and Technology*, *18*(3), 117–131. https://doi.org/10.1016/j.tifs.2006.10.022

Heux, L., Brugnerotto, J., Desbrières, J., Versali, M.-F., & Rinaudo, M. (2000). Solid State NMR for Determination of Degree of Acetylation of Chitin and Chitosan. *Biomacromolecules*, *1*(4), 746–751. https://doi.org/10.1021/bm000070y

Higashiyama, K., Fujikawa, S., Park, E. Y., & Shimizu, S. (2002). Production of arachidonic acid by Mortierella fungi. *Biotechnology and Bioprocess Engineering*, *7*(5), 252–262. https://doi.org/10.1007/BF02932833

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S., & Shimizu, S. (1998). Enhancement of arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1501–1505. https://doi.org/10.1007/s11746-998-0085-9

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawaa, S., & Shimizu, S. (1998). Effects of mineral addition on the growth morphology of and arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(12), 1815–1819. https://doi.org/10.1007/s11746-998-0336-9

Higashiyama, K., Yaguchi, T., Akimoto, K., & Shimizu, S. (2005). *Media for culturing microorganisms and process for producing unsaturated fatty acids or lipids containing the same* (No. U.S. Patent No. 6,746,857). Washington, DC: U.S.

Hirano, S., & Ohe, Y. (1975). Chitosan gels: a novel molecular aggregation of chitosan in acidic solutions on a facile acylation. *Agricultural and Biological Chemistry*, *39*(6), 1337–1338.

Ho, S.-Y., & Chen, F. (2008). Lipid characterization of Mortierella alpina grown at different NaCl concentrations. *Journal of Agricultural and Food Chemistry*, *56*(17), 7903–7909. https://doi.org/10.1021/jf801404y

Hoffman, D. R., Birch, E. E., Birch, D. G., Uauy, R., Castañeda, Y. S., Lapus, M. G., & Wheaton, D. H. (2000). Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. *Journal of Pediatric Gastroenterology and Nutrition*, *31*(5). Retrieved from http://journals.lww.com/jpgn/Fulltext/2000/11000/Impact_of_Early_Dietary_Intake_and_Blood_Lipid.16.aspx

Högel, H., Rantanen, K., Jokilehto, T., Grenman, R., & Jaakkola, P. M. (2011). Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma

cells. *PloS One*, *6*(11), e27112.

Hunter, K., & Rose, A. H. (1972). Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. *Biochimica et Biophysica Acta (BBA) Lipids and Lipid Metabolism*, *260*(4), 639–653. https://doi.org/http://dx.doi.org/10.1016/0005-2760(72)90013-6

Huong, L. M., Slugen, D., & Sajbidor, J. (1998). Production of dihomo-y-linolenic acid by Mortierella alpina. *Biologia Bratislava*, *53*(6), 801–806.

Jang, H.-D., Lin, Y.-Y., & Yang, S.-S. (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by Mortierella alpina. *Bioresource Technology*, *96*(15), 1633–1644. https://doi.org/http://dx.doi.org/10.1016/j.biortech.2004.12.027

Jareonkitmongkol, S., Kawashima, H., & Shimizu, S. (1993). Inhibitory effects of lignan compounds on the formation of arachidonic acid in a delta-5-desaturase-defective mutant of Mortierella alpina 1s-4. *Journal of Fermentation and Bioengineering*, *76*(5), 406–407. https://doi.org/Doi 10.1016/0922-338x(93)90033-5

Jareonkitmongkol, S., Kawashima, H., Shimizu, S., & Yamada, H. (1992). Production of 5,8,11-cis-Eicosatrienoic acid by a Δ12-desaturase defective mutant of Mortierella alpina 1S-4. *J. Am. Oil Chem. Soc.*, *69*(9), 939–944.

Jareonkitmongkol, S., Sakuradani, E., & Shimizu, S. (1994). Isolation and characterization of an omega-3-desaturation-defective mutant of an arachidonic acid-producing fungus, Mortierella alpina 1s-4. *Archives of Microbiology*, *161*(4), 316–319. https://doi.org/Doi 10.1007/Bf00303586

Ji, X.-J., Zhang, A.-H., Nie, Z.-K., Wu, W.-J., Ren, L.-J., & Huang, H. (2014). Efficient arachidonic acid-rich oil production by Mortierella alpina through a repeated fed-batch fermentation strategy. *Bioresource Technology*, *170*(5), 356–360. https://doi.org/10.1016/j.biortech.2014.07.098

Jin, M.-J., Huang, H., Xiao, A.-H., Zhang, K., Liu, X., Li, S., & Peng, C. (2008). A novel two-step fermentation process for improved arachidonic acid production by Mortierella alpina. *Biotechnology Letters*, *30*(6), 1087–1091. https://doi.org/10.1007/s10529-008-9661-1

Kamada, N., Kawashima, H., Sakuradani, E., Akimoto, K., Ogawa, U., & Shimizu, S. (1999). Production of 8,11-cis-eicosadienoic acid by a delta 5 and delta 12 desaturase-defective mutant derived from the arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists Society*, *76*(11), 1269–1274. https://doi.org/Doi 10.1007/S11746-999-0138-8

Karagozlu, M. Z., Karadeniz, F., Kong, C. S., & Kim, S. K. (2012). Aminoethylated chitooligomers and their apoptotic activity on AGS human cancer cells. *Carbohydrate Polymers*, *87*(2), 1383–1389. https://doi.org/10.1016/j.carbpol.2011.09.034

Kasperlik-Zalułska, A. A., Migdalska, B. M., Zgliczyński, S., & Makowska, A. M. (1995). Adrenocortical carcinoma. A clinical study and treatment results of 52 patients. *Cancer*, *75*(10), 2587–2591.

Katano, K., & Kawashima, H. (2015). *Methods for producing polyunsaturated fatty acid and lipid containing polyunsaturated fatty acid* (No. US20150064749 A1). Suntory Ltd, Kenichi Higashiyama.

Kawashima, H., Sakuradani, E., Kamada, N., Akimoto, K., Konishi, K., Ogawa, J., & Shimizu, S. (1998). Production of 8,11,14,17-cis-eicosatetraenoic acid (20:4ω-3) by a Δ5 and Δ12 desaturase-defective mutant of an arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1495–1500. https://doi.org/10.1007/s11746-998-0084-x

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., … Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and Bioprocess Engineering*, *20*(1), 168–179. https://doi.org/10.1007/s12257-014-0391-z

Kim, G.-Y., Choi, G.-S., Lee, S.-H., & Park, Y.-M. (2004). Acidic polysaccharide isolated from Phellinus linteus enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. *Journal of Ethnopharmacology*, *95*(1), 69–76.

Koike, Y., Jie Cai, H., Higashiyama, K., Fujikawa, S., & Park, E. Y. (2001). Effect of consumed carbon to nitrogen ratio of mycelial morphology and arachidonic acid production in cultures of mortierella alpina. *Journal of Bioscience and Bioengineering*, *91*(4), 382–389. https://doi.org/10.1016/S1389-1723(01)80156-0

Koletzko, B., Mrotzek, M., & Bremer, H. J. (1988). Fatty acid composition of mature human milk in Germany. *The American Journal of Clinical Nutrition*, *47*(6), 954–959.

Kono, H. (2004). Two-dimensional magic angle spinning NMR investigation of naturally occurring chitins: Precise 1H and 13C resonance assignment of α- and β-chitin. *Biopolymers*, *75*(3), 255–263. https://doi.org/10.1002/bip.20124

Koschker, A.-C., Fassnacht, M., Hahner, S., Weismann, D., & Allolio, B. (2006). Adrenocortical carcinoma-improving patient care by establishing new structures. *Experimental and*

Clinical Endocrinology & Diabetes, *114*(2), 45–51.

Krecic, A. M., & Swanson, M. S. (1999). hnRNP complexes: composition, structure, and function. *Current Opinion in Cell Biology*, *11*(3), 363–371.

Kroiss, M., Plonné, D., Kendl, S., Schirmer, D., Ronchi, C. L., Schirbel, A., … Fassnacht, M. (2016). Association of mitotane with chylomicrons and serum lipoproteins: practical implications for treatment of adrenocortical carcinoma. *European Journal of Endocrinology*, *174*(3), 343–353.

Kurita, K., Sannan, T., & Iwakura, Y. (1977). Studies on chitin, 4. Evidence for formation of block and random copolymers of N-acetyl-D-glucosamine and D-glucosamine by hetero- and homogeneous hydrolyses. *Die Makromolekulare Chemie*, *178*(12), 3197–3202. https://doi.org/10.1002/macp.1977.021781203

Kyle, D. J. (1997). *Arachidonic acid and methods for the production and use thereof* (No. 5658767). US.

Kyle, D. J. (1997). *Plant extract; vegetable oil; infant's formula.* (No. U.S. Patent No. 5,658,767). Washington, DC: U.S.

Kyle, D. J. (2001). The large-scale production and use of a single-cell oil highly enriched in docosahexaenoic acid. In F. Shahidi & J. W. Finley (Eds.), *Omega-3 Fatty Acids* (pp. 92–107). Washington, DC: ACS symposium series. https://doi.org/10.1021/bk-2001-0788.ch008

Lee, K. J., Oh, Y. C., Cho, W. K., & Ma, J. Y. (2015). Antioxidant and Anti-Inflammatory Activity Determination of One Hundred Kinds of Pure Chemical Compounds Using Offline and Online Screening HPLC Assay. *Evidence-Based Complementary and Alternative Medicine*, *2015*. https://doi.org/10.1155/2015/165457

Lehmann, T. P., Wrzesiński, T., & Jagodziński, P. P. (2013). The effect of mitotane on viability, steroidogenesis and gene expression in NCI-H295R adrenocortical cells. *Molecular Medicine Reports*, *7*(3), 893–900.

Lenardon, M. D., Munro, C. A., & Gow, N. A. R. (2010). Chitin synthesis and fungal pathogenesis. *Current Opinion in Microbiology*, *13*(4), 416–423. https://doi.org/10.1016/j.mib.2010.05.002

Letouzé, E., Rosati, R., Komechen, H., Doghman, M., Marisa, L., Flück, C., … Pianovski, M. A. D. (2012). SNP array profiling of childhood adrenocortical tumors reveals distinct

pathways of tumorigenesis and highlights candidate driver genes. *The Journal of Clinical Endocrinology & Metabolism*, *97*(7), E1284–E1293.

Li, Z.-Y., Lu, Y., Yadwad, V. B., & Ward, O. P. (1995). Process for production of arachidonic acid concentrate by a strain of mortierella alpina. *The Canadian Journal of Chemical Engineering*, *73*(1), 135–139. https://doi.org/10.1002/cjce.5450730115

Lindberg, A. M., & Molin, G. (1993). Effect of temperature and glucose supply on the production of polyunsaturated fatty acids by the fungus Mortierella alpina CBS 343.66 in fermentor cultures. *Applied Microbiology and Biotechnology*, *39*(4–5), 450–455. https://doi.org/10.1007/BF00205031

Liu, L., Liu, Y., Shin, H. D., Chen, R., Li, J., Du, G., & Chen, J. (2013). Microbial production of glucosamine and N-acetylglucosamine: Advances and perspectives. *Applied Microbiology and Biotechnology*, *97*(14), 6149–6158. https://doi.org/10.1007/s00253-013-4995-6

Lounds, C., Eagles, J., Carter, A. T., MacKenzie, D. A., & Archer, D. B. (2007). Spore germination in Mortierella alpina is associated with a transient depletion of arachidonic acid and induction of fatty acid desaturase gene expression. *Archives of Microbiology*, *188*(4), 299–305. https://doi.org/10.1007/s00203-007-0248-3

Maeda, Y. Y., Watanabe, S. T., Chihara, C., & Rokutanda, M. (1988). Denaturation and renaturation of a β-1, 6; 1, 3-glucan, lentinan, associated with expression of T-cell-mediated responses. *Cancer Research*, *48*(3), 671–675.

Mahapatra, S., & Banerjee, D. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, *6*, MBI.S10957. https://doi.org/10.4137/MBI.S10957

Mantero, F., Terzolo, M., Arnaldi, G., Osella, G., Masini, A. M., Alì, A., … Endocrinology, S. G. on A. T. of the I. S. of. (2000). A survey on adrenal incidentaloma in Italy. *The Journal of Clinical Endocrinology & Metabolism*, *85*(2), 637–644.

McDowell, R. E., Amsler, M. O., Li, Q., Lancaster Jr, J. R., & Amsler, C. D. (2015). The immediate wound-induced oxidative burst of Saccharina latissima depends on light via photosynthetic electron transport. *Journal of Phycology*, *51*(3), 431–441.

Michaelson, L. V, Lazarus, C. M., Griffiths, G., Napier, J. A., & Stobart, A. K. (1998). Isolation of a ⌬ 5 -Fatty Acid desaturase gene from Mortierella alpina. *Journal of Biological*

Chemistry, *273*(30), 19055–19059.

Michalkiewicz, E., Sandrini, R., Figueiredo, B., Miranda, E. C. M., Caran, E., Oliveira-Filho, A. G., … Cristofani, L. M. (2004). Clinical and outcome characteristics of children with adrenocortical tumors: a report from the International Pediatric Adrenocortical Tumor Registry. *Journal of Clinical Oncology*, *22*(5), 838–845.

Morganti, P., Del Ciotto, P., Carezzi, F., Nunziata, M. L., & Morganti, G. (2016). A Chitin Nanofibril-based non-woven tissue as medical dressing. The role of bionanotechnology. *Nanomaterials & Regenerative Medicine, Zagreb, Criatia, IAPC Publishing*, 123–142.

Morris, G. J., Smith, D., & Coulson, G. E. (1988). A comparative study of the changes in the morphology of hyphae during freezing and viability upon thawing for twenty species of fungi. *Microbiology*, *134*(11), 2897–2906. https://doi.org/10.1099/00221287-134-11-2897

MS/INCA. (2016). Estimativa de Câncer no Brasil. Retrieved from www.inca.gov.br/dncc

Muzzarelli, R. A. (1977). *Chitin*. Oxford, UK: Pergamon Press.

Muzzarelli, R. A. (1999). Native, industrial and fossil chitins. *EXS*, *87*, 1—6. Retrieved from http://europepmc.org/abstract/MED/10906948

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9.

N., J. I., Leela, I., & Prabhakara, R. A. V. S. (1988). Removal of Cadmium Using Chitosan. *Journal of Environmental Engineering*, *114*(4), 962–974. https://doi.org/10.1061/(ASCE)0733-9372(1988)114:4(962)

Nagamuna, T., Uzuka, Y., & Tanaka, K. (1985). Physiological factors affecting total cell number and lipid content of the yeast Lipomyces starkeyi. *The Journal of General and Applied Microbiology*, *31*(1), 29–37. https://doi.org/10.2323/jgam.31.29

Ng, L., & Libertino, J. M. (2003). Adrenocortical carcinoma: diagnosis, evaluation and treatment. *The Journal of Urology*, *169*(1), 5–11.

Nisha, A., Rastogi, N. K., & Venkateswaran, G. (2011). Optimization of media components for enhanced arachidonic acid production by Mortierella alpina under submerged cultivation. *Biotechnology and Bioprocess Engineering*, *16*(2), 229–237. https://doi.org/10.1007/s12257-010-0294-6

Nisha, A., & Venkateswaran, G. (2011). Effect of culture variables on mycelial arachidonic acid

production by Mortierella alpina. *Food and Bioprocess Technology*, *4*(2), 232–240. https://doi.org/10.1007/s11947-008-0146-y

Nishizaki, T., Nomura, T., Matsuoka, T., & Tsujishita, Y. (1999). Arachidonic acid as a messenger for the expression of long-term potentiation. *Biochemical and Biophysical Research Communications*, *254*(2), 446–449. https://doi.org/http://dx.doi.org/10.1006/bbrc.1998.9961

No, H. K., & Meyers, S. P. (1995). Preparation and Characterization of Chitin and Chitosan—A Review. *Journal of Aquatic Food Product Technology*, *4*(2), 27–52. https://doi.org/10.1300/J030v04n02_03

Nozaki, H., Itonori, S., Sugita, M., Nakamura, K., Ohba, K., Suzuki, A., & Kushi, Y. (2008). Mushroom acidic glycosphingolipid induction of cytokine secretion from murine T cells and proliferation of NK1. 1 α/β TCR-double positive cells in vitro. *Biochemical and Biophysical Research Communications*, *373*(3), 435–439.

Oliveira, V., & Smallwood, D. (2011). *The infant formula market: consequences of a change in the WIC contract brand* (No. ERR-124). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011a). *Culturing mortierella in nutrient broth containing saccharified starch and recovering gamma-linolenic, dihomo-gamma-linolenic, arachidonic, eicosapentaenoic, mead, 6, 9-octadecadienoic and 8, 11-eicosadienoic acids* (No. U.S. Patent No. 7,863,024). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011b). *Process for producing highly unsaturated fatty acid-containing lipid* (No. EP1498488 B1). Suntory Ltd, Kenichi Higashiyama.

Ooi, V. E. C., & Liu, F. (2000). Immunomodulation and Anti-Cancer Activity of Polysaccharide-Protein Complexes. *Current Medicinal Chemistry*, *7*, 715–729. https://doi.org/10.2174/0929867003374705

Oregon State University. (2014). Essential fatty acids. Retrieved March 3, 2016, from http://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids

Packter, N. M. (1981). Lipid biochemistry of fungi and other organisms. *Biochemical Education*, *9*(4), 156. https://doi.org/http://dx.doi.org/10.1016/0307-4412(81)90142-4

Pal, J., Verma, H. O., Munka, V. K., Maurya, S. K., Roy, D., & Kumar, J. (n.d.). Biological method of chitin extraction from shrimp waste an eco-friendly low cost technology and its advanced application.

Paradossi, G., & Lisi, R. (1996). New chemical hydrogels based on poly (vinyl alcohol). *Journal of Polymer …*, 3417–3425. https://doi.org/10.1002/pola.1996.874

Park, E. Y., Koike, Y., Higashiyama, K., Fujikawa, S., & Okabe, M. (1999). Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of Mortierella alpina. *Journal of Bioscience and Bioengineering*, *88*(1), 61–67. https://doi.org/10.1016/S1389-1723(99)80177-7

Park, Y., Tamura, S., Koike, Y., Toriyama, M., & Okabe, M. (1997). Mycelial pellet intrastructure visualization and viability prediction in a culture of Streptomyces fradiae using confocal scanning laser microscopy. *Journal of Fermentation and Bioengineering*, *84*(5), 483–486. https://doi.org/http://dx.doi.org/10.1016/S0922-338X(97)82014-4

Peng, C., Huang, H., Ji, X., Liu, X., You, J., Lu, J., … Ouyang, P. (2010). A temperature-shift strategy for efficient arachidonic acid fermentation by Mortierella alpina in batch culture. *Biochemical Engineering Journal*, *53*(1), 92–96. https://doi.org/10.1016/j.bej.2010.09.014

Pianovski, M. A. D., Maluf, E. M. C. P., de Carvalho, D. S., Ribeiro, R. C., Rodriguez-Galindo, C., Boffetta, P., … Figueiredo, B. C. (2006). Mortality rate of adrenocortical tumors in children under 15 years of age in Curitiba, Brazil. *Pediatric Blood & Cancer*, *47*(1), 56–60.

Pick, E., & Mizel, D. (1981). Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods*, *46*(2), 211–226.

Pipe, R. K., Coles, J. A., & Farley, S. R. (1995). Assays for measuring immune response in the mussel Mytilus edulis. *Tech Fish Immunol*, *4*, 93–100.

Plager, J. E. (1984). Carcinoma of the adrenal cortex: clinical description, diagnosis, and treatment. *International Advances in Surgical Oncology*, *7*, 329–353.

Poirier, M., & Charlet, G. (2002). Chitin fractionation and characterization in N,N-dimethylacetamide/lithium chloride solvent system. *Carbohydrate Polymers*, *50*(4), 363–370. https://doi.org/https://doi.org/10.1016/S0144-8617(02)00040-1

Poli, G., Guasti, D., Rapizzi, E., Fucci, R., Canu, L., Bandinelli, A., … Luconi, M. (2013). Morphofunctional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocrine-Related Cancer*, *20*(4), 537–550.

Puspawati, N. M., & Simpen, I. N. (2010). Optimasi deasetilasi khitin dari kulit udang dan

cangkang kepiting limbah restoran seafood menjadi khitosan melalui variasi konsentrasi NaOH. *Jurnal Kimia*, *4*(1). Retrieved from https://ojs.unud.ac.id/index.php/jchem/article/view/2760

QY Research center. (2015). *Global arachidonic acid industry Report 2015*. Retrieved from http://www.qyresearch.com/english/goods.php?id=45013

Rajamohan, S. B., Raghuraman, G., Prabhakar, N. R., & Kumar, G. K. (2012). NADPH oxidase-derived H2O2 contributes to angiotensin II-induced aldosterone synthesis in human and rat adrenal cortical cells. *Antioxidants & Redox Signaling*, *17*(3), 445–459.

Rane, K. D., & Hoover, D. G. (1993). An evaluation of alkali and acid treatments for chitosan extraction from fungi. *Process Biochemistry*, *28*(2), 115–118. https://doi.org/https://doi.org/10.1016/0032-9592(93)80016-A

Rathke, T. D., & Hudson, S. M. (1994). Review of Chitin and Chitosan as Fiber and Film Formers. *Journal of Macromolecular Science, Part C*, *34*(3), 375–437. https://doi.org/10.1080/15321799408014163

Ratledge, C. (2001). Microorganisms as sourcs of polyunsaturated fatty acids. *Structured and Modified Lipids*, 351–399.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, *86*, 807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Ratledge, C. (2013). Microbial oils: an introductory overview of current status and future prospects. *OCL*, *20*(6), D602. https://doi.org/10.1051/ocl/2013029

Ratledge, C., & Hopkins, S. (2006). Lipids from microbial sources. In F. D. Gunstone (Ed.), *Modyfing lipids for use in food.* (pp. 80–113). Department of Biological Sciences, University of Hull, UK.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1–27. https://doi.org/10.1016/S1381-5148(00)00038-9

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science (Oxford)*, *31*(7), 603–632. https://doi.org/10.1016/j.progpolymsci.2006.06.001

Rizk-Rabin, M., Assie, G., Rene-Corail, F., Perlemoine, K., Hamzaoui, H., Tissier, F., … Bouizar, Z. (2008). Differential Expression of Parathyroid Hormone–Related Protein in Adrenocortical Tumors: Autocrine/Paracrine Effects on the Growth and Signaling Pathways in H295R Cells. *Cancer Epidemiology and Prevention Biomarkers*, *17*(9),

2275–2285.

Roberg, K. (2001). Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. *Laboratory Investigation*, *81*(2), 149.

Roberts, G. A. F. (1992a). *Chitin chemistry*. Macmillan International Higher Education.

Roberts, G. A. F. (1992b). Solubility and Solution Behaviour of Chitin and Chitosan BT - Chitin Chemistry. In G. A. F. Roberts (Ed.) (pp. 274–329). London: Macmillan Education UK. https://doi.org/10.1007/978-1-349-11545-7_6

Rocky-salimi, K., Hamidi-esfahani, Z., & Abbasi, S. (2011). Statistical optimization of arachidonic acid production by Mortierella alpina CBS 754 . 68 in submerged fermentation. *Iranian Journal of Biotechnology*, *9*(2), 87–93.

Ruiter, G. A. D. E., Lugt, A. W. V. A. N. B. D. E. R., & Rombouts, F. M. (1993). Approaches to the classification of the Mortievella isabellina group : antigenic extracellular polysaccharides. *Mycological Research (An International Journal of Fungal Biology)*, *97*(6), 690–696. https://doi.org/10.1016/S0953-7562(09)80149-4

Rumengan, I. F. M., Suryanto, E., Modaso, R., Wullur, S., Tallei, T. E., & Limbong, D. (2014). Structural Characteristics of Chitin and Chitosan Isolated from the Biomass of Cultivated Rotifer, Brachionus rotundiformis. *International Journal of Fisheries and Aquatic Sciences*, *3*(1), 12–18. https://doi.org/10.1016/j.ejphar.2011.01.031

Saccardo, P. A., Traverso, G. B., & Trotter, A. (n.d.). *Sylloge fungorum omnium hucusque cognitorum. Digessit P. A. Saccardo.* (Vol. v.1). Patavii,sumptibus auctoris,. Retrieved from http://www.biodiversitylibrary.org/item/102968

Saitô, H., Tabeta, R., & Hirano, S. (1981). CONFORMATION OF CHITIN AND N-ACYL CHITOSANS IN SOLID STATE AS REVEALED BY 13C CROSS POLARIZATION/MAGIC ANGLE SPINNING (CP/MAS) NMR SPECTROSCOPY. *Chemistry Letters*, *10*(10), 1479–1482. https://doi.org/10.1246/cl.1981.1479

Šajbidor, J., Dobroňová, S., & Čert'ik, M. (1990). Arachidonic acid production byMortierella sp. S-17 influence of C/N ratio. https://doi.org/10.1007/BF01024404

Sajbidor, J., Kozelouhova, D., & Certik, M. (1992). Influence of some metal-ions on the lipid-content and arachidonic-acid production by Mortierella sp. *Folia Microbiologica*, *37*(6), 404–406.

Sakuradani, E., Ando, A., Shimizu, S., & Ogawa, J. (2013). Metabolic engineering for the

production of polyunsaturated fatty acids by oleaginous fungus Mortierella alpina 1S-4. *Journal of Bioscience and Bioengineering*, *116*(4), 417–422. https://doi.org/10.1016/j.jbiosc.2013.04.008

Sakuradani, E., Hirano, Y., Kamada, N., Nojiri, M., Ogawa, J., & Shimizu, S. (2004). Improvement of arachidonic acid production by mutants with lower n-3 desaturation activity derived from Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *66*(3), 243–248. https://doi.org/10.1007/s00253-004-1682-7

Sakuradani, E., Kamada, N., Hirano, Y., Nishihara, M., Kawashima, H., Akimoto, K., … Shimizu, S. (2002). Production of 5,8,11-eicosatrienoic acid by a Δ5 and Δ6 desaturation activity-enhanced mutant derived from a Δ12 desaturation activity-defective mutant of Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *60*(3), 281–287. https://doi.org/10.1007/s00253-002-1128-z

Schenzel, K., & Fischer, S. (2001). NIR FT Raman Spectroscopy–a Rapid Analytical Tool for Detecting the Transformation of Cellulose Polymorphs. *Cellulose*, *8*(1), 49–57. https://doi.org/10.1023/A:1016616920539

Seviour, R. J., Stasinopoulos, S. J., Auer, D. P. F., & Gibbs, P. A. (1992). Production of pullulan and other exopolysaccharides by filamentous fungi. *Critical Reviews in Biotechnology*, *12*(3), 279–298.

Shimada, Y., Sugihara, A., Nakano, H., & Kuramoto, T. (1997). Purification of docosahexaenoic acid by selective esterification of fatty acids from Tuna oil with Rhizopus delemar lipase. *Journal of the American Oil Chemists Society*, *74*(2), 97–101.

Shimizu, S., & Jareonkitmongkol, S. (1995). Mortierella Species (Fungi): Production of C20 Polyunsaturated Fatty Acids. In Y. P. S. Bajaj (Ed.), *Medicinal and Aromatic Plants* (pp. 308–325). Berlin: Springer. https://doi.org/10.1007/978-3-662-08612-4_17

Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., & Yamada, H. (1988). Production of eicosapentaenoic acid by Mortierella fungi. *Journal of the American Oil Chemists' Society*, *65*(9), 1455–1459. https://doi.org/10.1007/BF02898307

Shinmen, Y., & Shimizu, S. (1989). Production of arachidonic acid by Mortierella fungi. *Applied Microbiology*, *31*(1), 11–16. https://doi.org/10.1007/BF00252518

Shu, C.-H., & Lung, M.-Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by Antrodia camphorata in batch cultures. *Process Biochemistry*, *39*(8), 931–937. https://doi.org/10.1016/S0032-9592(03)00220-6

Sim Choi, H., Woo Kim, J., Cha, Y., & Kim, C. (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of Immunoassay and Immunochemistry*, *27*(1), 31–44.

Singh, A., & Ward, P. O. (1997). Production of high yields of arachidonic acid in a fed-batch system by Mortierella alpina ATCC 32222. *Applied Microbiology and Biotechnology*, *48*(1), 1–5. https://doi.org/10.1007/s002530051005

Sokmen, A., Gulluce, M., Askin Akpulat, H., Daferera, D., Tepe, B., Polissiou, M., … Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control*, *15*(8), 627–634. https://doi.org/https://doi.org/10.1016/j.foodcont.2003.10.005

Song, K.-S., Li, G., Kim, J.-S., Jing, K., Kim, T.-D., Kim, J.-P., … Hwang, B.-D. (2011). Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/β-catenin in SW480 human colon cancer cells. *BMC Cancer*, *11*(1), 307.

Srigley, C. T., & Rader, J. I. (2014). Content and composition of fatty acids in marine oil omega-3 supplements. *Journal of Agricultural and Food Chemistry*, *62*(29), 7268–7278. https://doi.org/10.1021/jf5016973

Steiner, P. E. (1954). *Cancer: Race and geography: some etiological, environmental, ethnological, epidemiological, and statistical aspects in Caucasoids, Mongoloids, Negroids, and Mexicans*. Williams & Wilkins Company.

Strajhar, P., Tonoli, D., Jeanneret, F., Imhof, R. M., Malagnino, V., Patt, M., … Odermatt, A. (2017). Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids. *Toxicology*, *381*, 51–63.

Stredanská, S., & Šajbidor, J. (1993). Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by Mortierella alpina. *Acta Biotechnologica*, *13*(2), 185–191. https://doi.org/10.1002/abio.370130218

Sun, C., Wang, J.-W., Fang, L., Gao, X.-D., & Tan, R.-X. (2004). Free radical scavenging and antioxidant activities of EPS2, an exopolysaccharide produced by a marine filamentous fungus Keissleriella sp. YS 4108. *Life Sciences*, *75*(9), 1063–1073. https://doi.org/https://doi.org/10.1016/j.lfs.2004.02.015

Taki, H., Ohishi, K., Okano, A., Suga, T., Akiyama, Y., & Yamashita, A. (1995). Antimetastatic effect of lentinan on liver metastasis of colon carcinoma (colon26): possible role of

activated kupffer cells. *International Journal of Immunotherapy*, *11*(1), 29–38.

Tanner, S. F., Chanzy, H., Vincendon, M., Roux, J. C., & Gaill, F. (1990). High-resolution solid-state carbon-13 nuclear magnetic resonance study of chitin. *Macromolecules*, *23*(15), 3576–3583. https://doi.org/10.1021/ma00217a008

Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., … Grossrubatscher, E. (2007). Adjuvant mitotane treatment for adrenocortical carcinoma. *New England Journal of Medicine*, *356*(23), 2372–2380.

Thakur, M. P., & Singh, H. K. (2013). Mushrooms, their bioactive compounds and medicinal uses: A review. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, *5*(1), 1–20.

Tomasetti, C., Li, L., & Vogelstein, B. (2017). Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science*, *355*(6331), 1330–1334.

Totani, N., Hyodo, K., & Ueda, T. (2000). Minerals Essential for Growth of the Filamentous Fungus, Mortierella alpina. *Journal of Japan Oil Chemists' Society*, *49*(5), 487–493,515. https://doi.org/10.5650/jos1996.49.487

Totani, N., Someya, K., & Oba, K. (1992). Industrial production of arachidonic acid by Mortierella. In D. J. Kyle & C. Ratledge (Eds.), *Industrial Applications of Single Cell Oils* (pp. 52–60). Illinois: AOCS Publishing.

Totani, N., Suzaki, K., & Kudo, T. (1992). *Process for the production of arachidonic acid-containing lipids* (No. EP0223960 B1). Lion Corporation.

Totani, N., Watanabe, A., & Oba, K. (1987). An improved method of arachidonic acid production by Mortierella alpina. *Yukagaku*, *36*(5), 328–331. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=8169393

Tsai, G. J., Su, W. H., Chen, H. C., & Pan, C. L. (2002). Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. *Fisheries Science*, *68*(1), 170–177. https://doi.org/10.1046/j.1444-2906.2002.00404.x

Uauy, R., Mena, P., & Rojas, C. (2000). Essential fatty acids in early life : structural and functional role. *Proceedings of the Nutrition Society*, *59*, 3–15. https://doi.org/10.1017/S0029665100000021

United Nations, Department of Economic and Social Affairs - Population Division - World Population Prospects. (2017). Retrieved August 23, 2018, from

https://esa.un.org/unpd/wpp/Download/Standard/Population/

Vadivelan, G., & Venkateswaran, G. (2014). Production and enhancement of omega-3 fatty acid from Mortierella alpina CFR-GV15: its food and therapeutic application. *BioMed Research International*, *2014*, 657414. https://doi.org/10.1155/2014/657414

Vali, S. R., Sheng, H. Y., & Ju, Y. (2003). An efficient method for the purification of arachidonic acid from fungal single-cell oil (ARASCO). *Journal of the American Oil Chemists Society*, *80*(7), 725–730.

Vandamme, E., & Revuelta, J. L. (2016). Vitamins, biopigments, antioxidants and related compounds: a historical, physiological and (Bio) technological perspective. In E. Vandamme & J. L. Revuelta (Eds.), *Industrial biotechnology of vitamins, biopigments, and antioxidants* (pp. 1–14). Weinheim, Germany: WILEY-VCH Verlag.

Vannucci, L., Krizan, J., Sima, P., Stakheev, D., Caja, F., Rajsiglova, L., … Saieh, M. (2013). Immunostimulatory properties and antitumor activities of glucans. *International Journal of Oncology*, *43*(2), 357–364.

Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991). 13C-N.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydrate Research*, *217*, 19–27. https://doi.org/https://doi.org/10.1016/0008-6215(91)84113-S

Vassilopoulou-Sellin, R., & Schultz, P. N. (2001). Adrenocortical carcinoma: clinical outcome at the end of the 20th century. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *92*(5), 1113–1121.

Vinsova, J., & Vavrikova, E. (2011). Chitosan Derivatives with Antimicrobial, Antitumour and Antioxidant Activities - a Review. *Current Pharmaceutical Design*, *17*(32), 3596–3607. https://doi.org/10.2174/138161211798194468

Wajchenberg, B. L., Albergaria Pereira, M. A., Medonca, B. B., Latronico, A. C., Carneiro, P. C., Ferreira Alves, V. A., … Kirschner, M. A. (2000). Adrenocortical carcinoma: clinical and laboratory observations. *Cancer*, *88*(4), 711–736.

Walker, S. J. (2018). Major spoilage micro-organisms in milk and dairy products. *International Journal of Dairy Technology*, *41*(4), 91–92. https://doi.org/10.1111/j.1471-0307.1988.tb00606.x

Wang, G. (2014). Human Antimicrobial Peptides and Proteins. *Pharmaceuticals* . https://doi.org/10.3390/ph7050545

Wang, T., & Rainey, W. E. (2012). Human adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *351*(1), 58–65.

Wang, Y., Zhao, H., Miao, X., Liu, D., Jiang, H., Liu, P., … Yin, H. (2013). Structural determination and antitumor activities of a water-soluble polysaccharide from Mortierella hepiali. *Fitoterapia*, *86*, 13–18. https://doi.org/10.1016/j.fitote.2013.01.001

Wang, Z. M., Cheung, Y. C., Leung, P. H., & Wu, J. Y. (2010). Ultrasonic treatment for improved solution properties of a high-molecular weight exopolysaccharide produced by a medicinal fungus. *Bioresource Technology*, *101*(14), 5517–5522. https://doi.org/10.1016/j.biortech.2010.01.134

Wilhelm, S., Wagner, H., & Häcker, G. (1998). Activation of caspase-3-like enzymes in non-apoptotic T cells. *European Journal of Immunology*, *28*(3), 891–900.

Wu, T., Zivanovic, S., Draughon, F. A., Conway, W. S., & Sams, C. E. (2005). Physicochemical Properties and Bioactivity of Fungal Chitin and Chitosan. *Journal of Agricultural and Food Chemistry*, *53*(10), 3888–3894. https://doi.org/10.1021/jf048202s

Wynn, J. P., Hamid, A. A., Li, Y., & Ratledge, C. (2001). Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi Mucor circinelloides and Mortierella alpina. *Microbiology*, *147*(10), 2857–2864.

Wynn, J. P., & Ratledge, C. (2005). Microbial production of oils and fats. In K. Shetty, G. Paliyath, A. Pometto, & E. Levin (Eds.), *Food Biotechnology* (2nd ed., pp. 443–472). NY: CRC Press.

Xian, M., Yan, J., Kang, Y., Liu, J., Bi, Y., & Zhen, K. (2001). Production of c-linolenic acid by Mortierella isabellina grown on hexadecanol. *Letters in Applied Microbiology*, *33*, 367–370. https://doi.org/10.1046/j.1472-765X.2001.00924.x

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters*, *11*(13), 1699–1701. https://doi.org/https://doi.org/10.1016/S0960-894X(01)00285-2

Xing, R., Yu, H., Liu, S., Zhang, W., Zhang, Q., Li, Z., & Li, P. (2005). Antioxidant activity of differently regioselective chitosan sulfates in vitro. *Bioorganic & Medicinal Chemistry*, *13*(4), 1387–1392. https://doi.org/https://doi.org/10.1016/j.bmc.2004.11.002

Yamamoto, T., Yamashita, T., & Tsubura, E. (1981). Inhibition of pulmonary metastasis of Lewis lung carcinoma by a glucan, Schizophyllan. *Invasion & Metastasis*, *1*(1), 71–84.

Yamauchi, A., Nagao, T., & Watanabe, Y. (2005). Purification of arachidonic acid from Mortierella single-cell oil by selective esterification with Burkholderia cepacia lipase. *Journal of the American Oil Chemists Society*, *82*(11), 833–837.

Yamauchi, R., Tatsumi, Y., Asano, M., Kato, K., & Ueno, Y. (1988). Effect of Metal Salts and Fructose on the Autoxidation of Methyl Linoleate in Emulsions. *Agricultural and Biological Chemistry*, *52*(3), 849–850. https://doi.org/10.1271/bbb1961.52.849

Yan, Y., Li, X., Wang, G., Gui, X., Li, G., Su, F., … Liu, T. (2014). Biotechnological preparation of biodiesel and its high-valued derivatives: a review. *Applied Energy*, *113*, 1614–1631. https://doi.org/10.1016/j.apenergy.2013.09.029

Ye, S., Liu, F., Wang, J., Wang, H., & Zhang, M. (2012). Antioxidant activities of an exopolysaccharide isolated and purified from marine Pseudomonas PF-6. *Carbohydrate Polymers*, *87*(1), 764–770. https://doi.org/https://doi.org/10.1016/j.carbpol.2011.08.057

Yokochi, T., Kamisaka, Y., Nakahara, T., & Suzuki, O. (1995). Production of γ-Linolenic acid containing phospholipids by Mortierella ramanniana in a fed-batch culture of decane. *Journal of Japan Oil Chemists' Society*, *44*(1), 9–15. https://doi.org/10.5650/jos1956.44.9

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*, *13*(3), 1133–1174. https://doi.org/10.3390/md13031133

Yu, L. J., Qin, W. M., Lan, W. Z., Zhou, P. P., & Zhu, M. (2003). Improved arachidonic acids production from the fungus Mortierella alpina by glutamate supplementation. *Bioresource Technology*, *88*(3), 265–268. https://doi.org/http://dx.doi.org/10.1016/S0960-8524(02)00312-7

Yu, Y., Shen, M., Song, Q., & Xie, J. (2018). Biological activities and pharmaceutical applications of polysaccharide from natural resources: A review. *Carbohydrate Polymers*, *183*, 91–101. https://doi.org/https://doi.org/10.1016/j.carbpol.2017.12.009

Yuan, C., Wang, X., & Yu, Z. (2007). Separation and preparative purification of arachidonic acid from microbial lipids by urea inclusion reaction and HPLC. *Preparative Biochemistry and Biotechnology*, *37*(2), 149–159. https://doi.org/10.1080/10826060701199098

Zaidman, B.-Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*, *67*(4), 453–468.

Zhang, M., Chen, H., Huang, J., Li, Z., Zhu, C., & Zhang, S. (2005). Effect of lycium barbarum polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sciences*, *76*(18), 2115–2124.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*(1), 4–19.

Zhiming, W., Huan, L., & Xu, J. (2014). *Method for producing arachidonic acid grease by utilizing mortierella alpina mutant strain, and arachidonic acid grease produced by method* (No. CN 103571896 A).

Zhu, M., Yu, L. J., Liu, Z., & Xu, H. B. (2004). Isolating Mortierella alpina strains of high yield of arachidonic acid. *Letters in Applied Microbiology*, *39*(4), 332–335. https://doi.org/10.1111/j.1472-765X.2004.01581.x

3. CHAPTER III. THE EFFECT OF CHITIN PRODUCED BY *MORTIERELLA ALPINA* ON VIABILITY AND CYTOTOXICITY, AND ITS USE AS MITOTANE ADJUVANT ON ADRENOCORTICAL CARCINOMA CELL H295R.

3.1. ABSTRACT

An evaluation of the antitumoral potential of chitin produced by *Mortierella alpina* on ACC (adrenocortical carcinoma) cells was evaluated in this research, comparing its effect to mitotane, a commercial chemotherapeutic drug used commonly for the treatment of ACC, but also known for its side effects in patients. Techniques for determination of the cellular viability such as MTT and fluorescence were used to measure the toxic effect of chitin and mitotane in tumoral cells (H295R) and non-tumoral cells (VERO) observing a high cytotoxicity of mitotane in cells and a pro-apoptotic effect of chitin on cells, 10% ($p<0.05$) superior to mitotane. The effect of the cytotoxicity of chitin in tumoral cells was similar to the effect of 50 µM ($p<0.05$) of mitotane on tumoral cells. This pro-apoptotic effect may be explained by the induction of formation of reactive oxygen species in tumoral cells as a response to the antioxidant effect of the polysaccharide. An increment of the lysosomal volume was also noted in tumoral cells treated with chitin. In an attempt to increase the antitumoral effect, a combination of mitotane with lower dosage and chitin (as adjuvant) was also tested showing a slight enhancement of the cytotoxicity effect on tumoral cells. Therefore, the *in vitro* results obtained suggest the potential and useful application of chitin produced by *Mortierella alpina* in biomedical formulations for adrenocortical carcinoma additional treatments.

Keywords: chitin, *Mortierella alpina*, antitumor effect, apoptosis, mitotane, adrenocortical carcinoma

3.2. INTRODUCTION

Mortierella alpina is a GRAS (Generally Recognized as Safe) filamentous fungi, known as a good producer of arachidonic acid. Little is known about the potential use of other metabolites of this microorganism, such as polysaccharides.

Polysaccharides can trigger a non-specific reaction against tumor cells, in fact, there are various polysaccharides that have shown efficiency in the treatment of different types of cancer such as lung, breast, gastric and colon. Their potential as adjuvants of conventional drugs (used as chemotherapeutics) has also been demonstrated (Vannucci et al., 2013). Polysaccharides, i.e. glucans, shown a reduction of metastasis processes (Taki et al., 1995) and induction of apoptosis in human prostate cancer and proliferation suppression of colon and breast cancer cells (Yamamoto et al., 1981; Min Zhang et al., 2005)

According to Mei Zhang et al (2007), one mechanism of action of polysaccharides is the direct activity in tumor – possibly arresting the cell life cycle and generating an apoptosis cell death (Song et al., 2011), this could explain the anti-proliferative process in cancer cells (Zaidman et al., 2005).

The adrenocortical carcinoma (ACC) is a rare type of malign cancer(Dackiw et al., 2001), with an incidence of 0.5 – 2 cases per million people per year (Plager, 1984), its pathogenesis is still controversial, and its prognosis is poor (Wajchenberg et al., 2000). In most cases, this type of cancer can be diagnosed due to the increase of steroid hormone secretion by the tumor (cortisol). In any case, to diagnose its stage of malignity, a histopathologic study must be performed (Allolio & Fassnacht, 2006; Flack & Chrousos, 1996; Vassilopoulou-Sellin & Schultz, 2001)

According to Steiner (1954), this cancer represents 0.2% of deaths from cancers in the United State of America. Allolio & Fassnacht (2006), affirms that women are more affected than man and the incidence is more common in children and between the fourth and fifth decade.

The ACC, at early stages, can be removed, but its local recurrence is frequent, triggering a metastatic process (Ng & Libertino, 2003). At advanced stages, when surgery is not suitable, the treatment with drugs is mandatory and mitotane is the treatment of choice combined with some other cytotoxic drugs such as etoposide, doxorubicin, cisplatin, streptozotocin (Berruti et al., 2005; Kasperlik-Zalułska et al.,

1995). Unfortunately, mitotane can present some side effects related to gastrointestinal and neurological events (Terzolo et al., 2007). After 5 years of diagnosis, only 16 to 38% of patients survive (Wajchenberg et al., 2000).

The aim of this work was to evaluate at *in vitro* level the antitumoral potential action of chitin produced extracellularly by *Mortierella alpina* on H295R cells (derivated from ACC) comparing its bioactivity to mitotane, the most common chemotherapeutic drug known for ACC treatment. Its application as a mitotane adjuvant was also evaluated.

3.3. MATERIAL AND METHODS

3.3.1. EPS production and recovery

The fixed culture medium composition for EPS production was in ($g.L^{-1}$): Glucose 46.01; urea 7.48 and KH_2PO_4 2.31; KNO_3 1.0; $MgSO_4 \cdot 7H_2O$ 0.3; and in ($mg.L^{-1}$): $CaCl_2 \cdot 2H_2O$ 0.62; $FeCl_3 \cdot 6H_2O$ 1.5; $ZnSO_4 \cdot 7\ H_2O$ 1.0; $CuSO_4 \cdot 5H_2O$ 0.1; $MnCl_2 \cdot 4H_2O$ 1.0. Fermentation was carried out at 25°C for 5 days at 120 rpm.

After the fermentation process, the biomass was removed by filtration, and ethanol was added to the filtrate (3:1, v/v) to precipitate the EPS. The solution was kept at 5° C overnight to improve the precipitation process. The exopolysaccharide was recovered by centrifugation resuspended in water and dialyzed (cut-off 20 kDa) against distilled water (48 h) and ultrapure water (24 h). The suspension of exopolysaccharide was then lyophilized. The exopolysaccharide was identified as chitin (previous work) and will be referred as EPS in this work.

3.3.2. Tumor cell lines and culture condition

Adrenocortical carcinoma H295R cell line (purchased from the ATCC bank) and the non-tumoral VERO cell line from kidney purchased from the cells bank of Rio de Janeiro –Brazil (APABCAM – Associacao tecnico cientifica Paul Erlich). Both cell strains were cultivated in Dulbecco's Modified Eagle's Medium F12 (DMEM F12)

(Sigma-Aldrich®) supplemented with 10% fetal bovine serum (Gibco®), and an antibiotic solution of 10U.mL^{-1} of streptomycin and 20 U.mL^{-1} of penicillin (Invitrogen®).

The confluent cells were collected using 0.25% trypsin (Invitrogen®) and viability was evaluated using the trypan blue exclusion test. The concentration of cells used was 1x10^6 cells/mL, pipetted in a 96-well-flat-bottomed plate. The incubation process was carried out for 24h at 37°C in a CO_2 (5%) humidified incubator.

3.3.3. Effect on cell viability of the EPS

The evaluation of the effect on the cell viability effect of the EPS was carried out in the Research Institute Pelé Pequeno Príncipe – Curitiba – Paraná - Brazil (IPPP).

The viability was evaluated using the 3-(4,5-dhimethylthiazol-2-yr)-2,5-diphenyltetrazolium bromide (MTT) assay. 100 µL of the cell suspension was plated to each well and incubated for 24 h at 37°C with 5% CO_2. After the incubation process, the culture medium was removed and 180 µL of fresh culture medium and 20 µL of the treatments were added.

After 24 h of incubation, 20 µl of the MTT solution to a final concentration of (0.5 mg.mL^{-1}) was added to each well, a final reaction time of 3 hours at 37°C and CO_2 5% was performed, and finally, after a careful removal of the supernatant, 100 µL of DMSO (dimethyl sulfoxide) (Panreac®), was added and gently agitated for 5 min to dissolve the formazan crystals. The readings were performed at 595 nm on an ELISA microplate reader (Biotek®). The viability of the untreated cell lines group was considered as 100%. All assays were performed in five replicates. The viability was calculated following the equation:

$$\%\ cell\ viability = \frac{100\ x\ A_{595e}}{A_{595b}}$$

In which A_{595e} is the mean value of the treatment' samples, and A_{595b} is the mean value of the blanks.

The treatments were: EPS at 1.5 and 5 mg.mL^{-1} solubilized in ultrapure water by ultrasonic treatment at 20% amplitude (12 W.cm^{-3}) for 10 min in an ice-water bath (Z. M. Wang et al., 2010), Mitotane at concentrations of 20; 31,3; 50 and 62,5 µM and finally a mixture of EPS and Mitotane at 1.5 mg.mL^{-1} and 31.3µM respectively. Cell

control, vehicle control and positive control (Mitotane at 1 mM) were also included for comparison. The IC50 concentration of the exopolysaccharide determined in our previous work was chosen as a concentration in this research.

3.3.4. Fluorescence viability assay (Live/dead fluorescence imaging)

H295R and VERO cells were plated at 8 x 10^4 cells/well in a black-walled 96-well plates with an optically clear-bottom surface, cells were incubated for 24 h of adhesion and then the medium was aspirated and replaced with DMEM supplemented with 10% FBS and the respective treatments were added, after 24 h the medium was aspirated and stained for 30 min in the dark with Hoechst 33342 (5 $\mu g.mL^{-1}$), ethidium bromide (0.6 µM) and calcein (0.3 µM) in phosphate-buffered saline (PBS) (pH 7.4). Fluorescence images from 4 fields within each well were taken on the IN Cell Analyzer 1000 (GE Healthcare, Chalfont St. Giles, United Kingdom) to give the number of dead cells (ethidium bromide, red) and the total number of cells (Hoechst-stained, blue) in each well as a function of the treatments. The number of dead cells was subtracted from the total number to obtain the number of live cells in each well, which is represented as a percentage of the total number (% viability). Assays were performed in five replicates. The treatments were: EPS at 1.5 and 4 $mg.mL^{-1}$ solubilized as mentioned before, Mitotane at concentrations of 20; 31.3; 50 and 62.5 µM and finally a mixture of EPS and Mitotane at 1.5 $mg.L^{-1}$ and 31.3 µM respectively. Cell control, vehicle control and positive control (Mitotane at 1 mM) were also included in the comparison.

For the production of short videos, cells were cultivated and treated as mentioned before but were only stained with Hoechst 33342 (20 µM) in PBS and were incubated for 48 h in the IN Cell Analyzer 1000 (GE Healthcare, Chalfont St. Giles, United Kingdom) shooting one picture every 20 min per field per well. Assays were performed in triplicate. The treatments evaluated were: EPS at 1.5 $mg.mL^{-1}$ solubilized as mentioned before, Mitotane at concentrations of 31.3 and 62.5 µM and finally a mixture of EPS and Mitotane at 1.5 mg.L-^{1} and 31.3 µM, respectively. Cell control was also included in the comparison.

3.3.5. Apoptosis flow cytometry analysis

For the apoptosis analysis, H295R cells were plated at a density of 1 x 10^6 cells per well into 6-wells microplates. After 24 h incubation, treatments were added and incubated for another 24 h. Controls wells, vehicle controls, and positive controls were also included for comparison. After treatment incubation process cells were trypsinized, a stained with Annexin V according to the instruction of manufacturer (*Annexin V Apoptosis Detection Kit I*, BD Bioscence®). In this assay Annexin V was used in conjunction with 7-Aminoactinomycin D (7-AAD) to discriminate apoptotic cells, dead cells and viable cells, and collected in 300 µL of a solution of human serum albumin 5% in PBS and measured with a FACS Canto II (BD Biosciences®) cytometer. Results were analyzed using the software BD FACSDiva™ (BD Bioscience®). The flow cytometer was calibrated with magnetic beads (*BD™ CS&T Beads*, BD Biosciences®) before analysis according to fabricant's instructions.

The treatments evaluated were: EPS at 1.5 mg.mL^{-1} solubilized as mentioned before, Mitotane at concentrations of 31.3 and 62.5 µM and finally a mixture of EPS and Mitotane at 1.5 mg.mL^{-1} and 31.3 µM, respectively. Cell control and vehicle control were also included in the comparison.

3.3.6. Lysosomal volume

The evaluation of lysosomal volume was following the methodology proposed by (Pipe, Coles, & Farley, 1995) modified by (Bonatto et al., 2004) In which a density of 1 x 10^6 H295R cells per well into 96-wells microplates. After 24 h incubation, 20 µL of treatments were added, finally, after 24 h 20 µL of 2% neutral red (Sigma-Aldrich®) was added and incubated for 30 min. After discarding the supernatant, neutral red was solubilized adding 100 µL of extraction solution (0.1 mL of 10% acetic acid plus 40% ethanol solution). The absorbance was read at 595 nm, and lysosomal volume was expressed as absorbance (per 1 x 10^6 cells.mL^{-1}). The treatments tested were: EPS at 1.5 and 4 mg.mL-^{1} solubilized as mentioned before, Mitotane at concentrations of 20; 31.3; 50 and 62.5 µM and finally a mixture of EPS and Mitotane at 1.5 mg.mL^{-1} and

31.3 µM respectively. Cell control, vehicle control and positive control (Mitotane at 1 mM) were also included in the comparison.

3.3.7. Superoxide anion production (O_2^-)

Superoxide anion production was estimated by the nitro blue tetrazolium (NBT – Sigma) reduction assay according (Sim Choi, Woo Kim, Cha, & Kim, 2006). After treatment incubation of 24 h, the culture medium was removed and replaced by 100 µL of DEMEM F12 and 100 µL of NBT (nitroblue tetrazolium 0,25%) (Amresco®) and Zymosan solution. After 30 minutes of incubation at 37° C, the supernatant was discarded, and the cells were fixed by adding 100 µL of methanol (50%) for 10 min. After, the supernatant was discarded, and the plate was dried. Then 120 µL of KOH (2M) and 140 µL of dimethyl sulfoxide were added to the wells. After 30 min the reduction of NBT resulted in the formation of blue formazan. The absorbance was read at 595 nm and the results were expressed as absorbance (per 1 x 10^6 cells.mL^{-1}).

The treatments tested were: EPS at 1.5 and 4 mg.mL^{-1} solubilized as mentioned before, Mitotane at concentrations of 20; 31.3; 50 and 62.5 µM and finally a mixture of EPS and Mitotane at 1.5 mg.L^{-1} and 31.3 µM respectively. Cell control, vehicle control and positive control (Mitotane at 1 mM) were also included in the comparison.

3.3.8. Hydrogen peroxide production (H_2O_2)

Hydrogen peroxide production was based on the horseradish peroxidase-dependent conversion of phenol red into a colored compound by H_2O_2 (Pick & Mizel, 1981). H295R cells (100 µL) were incubated in the presence of glucose (5 mM), phenol red solution (0.56 mM), and horseradish peroxidase (Sigma-Aldrich®) (8.5 U.mL^{-1}) and zymosan) (Sigma-Aldrich®) in the dark for 30 min at 37°C. Then, 10µL of 1M NaOH (Fluka Cehmika®) was added to the wells to stop the reaction. The absorbance was read at 620 nm and the results were expressed as absorbance (per 1 x 10^6 cells.mL^{-1}). The treatments evaluated were: EPS at 1.5 and 4 mg.mL^{-1} solubilized as mentioned before, Mitotane at concentrations of 20; 31.3; 50 and 62.5 µM and finally a mixture of

EPS and Mitotane at 1.5 mg.L^{-1} and 31.3 µM respectively. Cell control, vehicle control and positive control (Mitotane at 1 mM) were also included in the comparison.

3.3.9. Statistical analysis

Results (means ± SEM) from at least three independent experiments at three replicates were expressed as the fold change from a control value. Post-hoc comparisons were performed with ANOVA-Newman-Keuls test in GraphPad Prism 6 software. $P<0.05$ was considered to indicate a statistically significant difference.

3.4. RESULTS AND DISCUSSION

3.4.1. Effect on cell viability of the EPS

Mitotane is a potent chemotherapeutic drug used for ACC treatment. The tumoral cell strain H295R and no tumoral cell strain VERO were incubated under different treatment and concentration of Mitotane, Exopolysaccharide and a mixture of these two. Concentrations were: Mitotane 20; 31.3; 50; and 62.5 μM; EPS 1.5 $mg.mL^{-1}$ and a mixture of Mitotane 31.3 μM and EPS 1.5 $mg.mL^{-1}$. The mixture of Mitotane and the EPS was proposed to evaluate the potential use of the EPS as a mitotane adjuvant. The concentration of EPS was chosen according to the screening performed in our previous work.

In patients with ACC the windows of mitotane observed could vary between 14 (43.75 μM) to a maximum of 20 $\mu g.mL^{-1}$ (62.5 μM). There is not a final consensus about this concentration since every case presents their own particularities. The concentration of Mitotane between 31.3 and 62.5 μM showed a significant ($p<0.05$) decrease VERO cells viability by 2.7-fold while the EPS and the mixture of mitotane decrease viability by 1.6 and 1.7-fold respectively (Fig. 1). In the case of the H295R cells, a viability decrease of 10-fold was observed when mitotane was applied at 62.5 μM, lower concentration did not affect in the viability of this tumoral cell strain. EPS at 1.5 mg.mL-1 and the mixture of mitotane and EPS (31.3 μM + 1.5 mg.mL-1) decreased the viability by 2.5-fold.

These results were compared to the control and considering a final mitotane window of 62.5 μM reported by (Lehmann et al., 2013). The positive control used in this test was mitotane at 100 μM.

In this first screening by using MTT a considerable reduction of VERO cells population was observed among the assays; the damage was exponential according to the increment of concentrations of mitotane being less than 40% ($p<0.05$) of living VERO cells when the window concentration was tested. On the other hand, when EPS and EPS as an adjuvant of mitotane were evaluated, a population up of 58% ($p<0.05$) of living VERO cells was observed which indicates a lower cytotoxicity effect when compared to mitotane treatment at the window concentration.

Different results were observed when mitotane was used for H295R cells. Mitotane, at the window concentration, showed a high cytotoxicity, a cellular death of 84.2% of cells population was attained but no efficacy of mitotane was observed at lower concentrations. It must be considered that at the window concentration, mitotane reduced VERO cells population to a 34.5%.

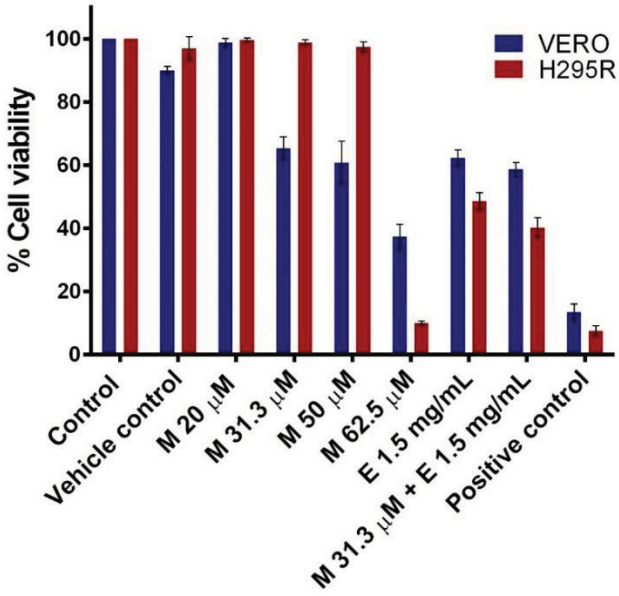


Figure 15. H295R cell viability in cells treated with different concentration of mitotane (M), EPS (E) and a mixture of mitotane + EPS after 24 h of incubation, followed by 3 hours of incubation with MTT. Data are represented as a percentage of the control (100%). Assays were performed five times; error bars are presented ± SEM.

The MTT method evaluates the viability of cells by its metabolism, but this method is limited when an accurate proportion of living and dead cells is aimed, for this reason, a test of Living/Dead dye exclusion assay was performed.

3.4.2. Fluorescence viability assay (Live/dead fluorescence imaging)

This method was used to analyze live/dead populations by dye exclusion using Hoechst 33342 to dye viable cells and ethidium bromide that dyes specifically nuclei of cells that have lost plasma membrane integrity. The use of mitotane at different concentration showed a cytotoxicity in VERO cells diminishing the viability in 1.35-fold ($p<0.05$) when low doses were used (20 and 31.3 μM), and 1.6 and 2.9-fold (50 and

62.5 µM) when high doses were tested, respectively. Only 34.5% of cells survived at the window concentration selected (Figure 16-A). The use of the EPS and the mixture of EPS and mitotane caused a 1.7 and -1.9-fold decrease in cells viability respectively. Figure 16-B showed and notable and significant reduction of viability in H295R when the concentration of 50 and 62.5 µM was used, in which a maximum decrement of viability was noted (6.3-fold). The use of EPS (1.5 mg.mL^{-1}) and a mixture of mitotane and EPS showed a decrement of 2.1-fold of total viability. In this assay, it was also observed the significant cytotoxicity effect of mitotane in VERO cells at the window concentration as observed in the MTT assay.

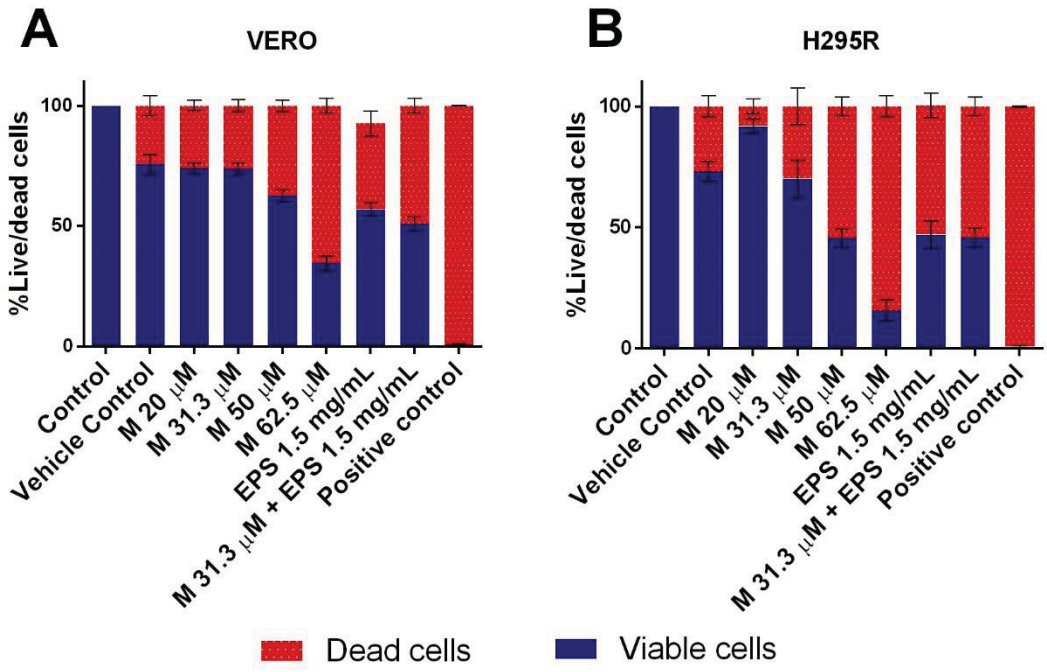


Figure 16. Live cell number after 24 hours of incubation at different treatments of mitotane (M), EPS (E) and combination of mitotane and EPS. A) VERO cells and B) H295R cells. In red, the percentage of dead cells and in blue, percentage of viable cells.

The viability of both cell strains was confirmed by the two tests with a similar sensitivity. In a parallel assay, an evaluation of the cellular morphology change was carried out for 48 h (Figure 17) in the IN Cell Analyzer 1000 (GE Health Sciences), showing a lysis of the plasmatic membrane when mitotane was used, and a reduction of the cancer cell motility when EPS and the combination of mitotane and EPS were used, compared to the control. Debris was observed at 48 h of treatment, especially at 62.5 µM.

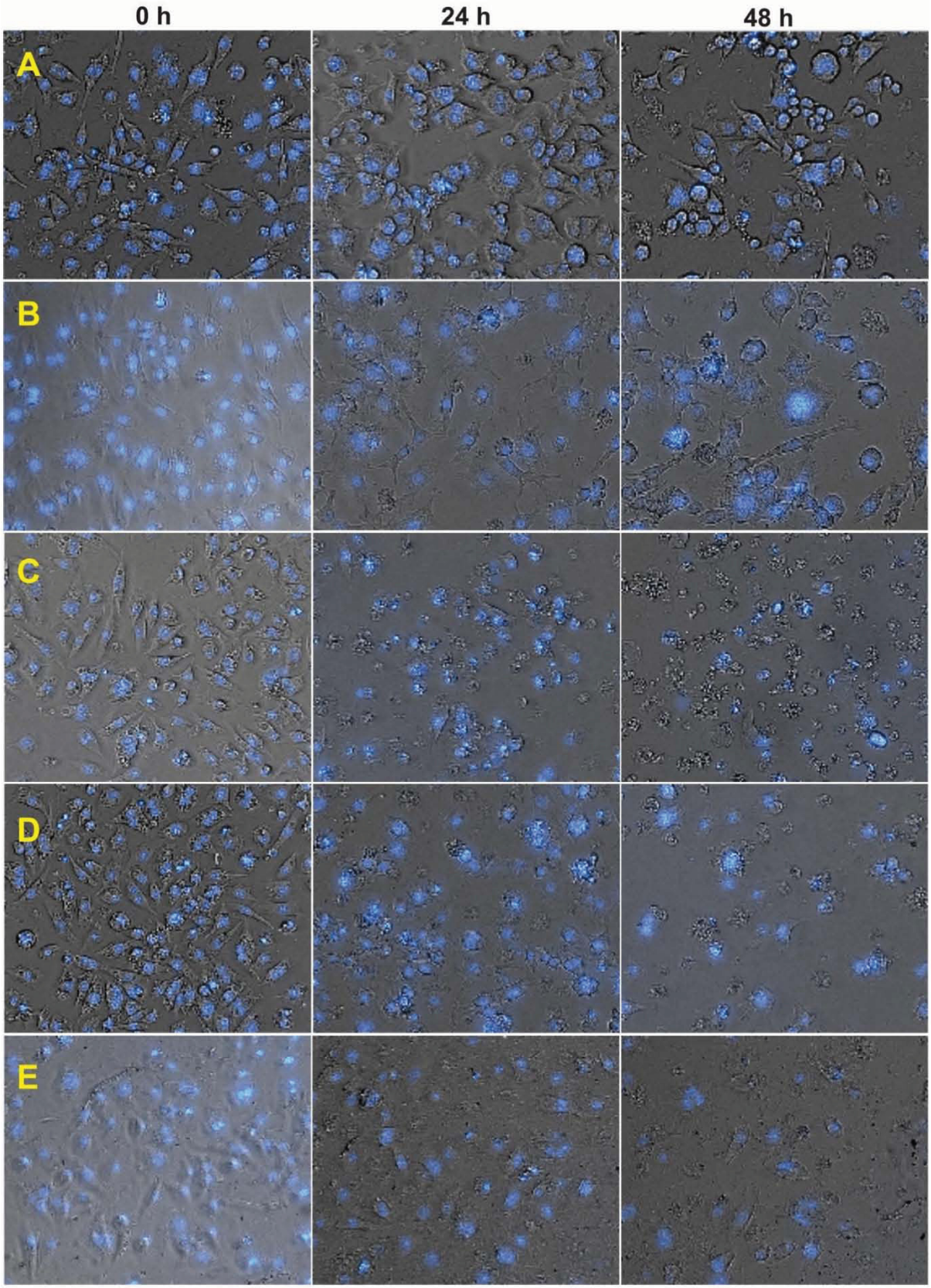


Figure 17. Time-lapse of H295R cells (Hoechst stained) under different treatments at 0, 12 and 24 hours. A) Control cells, B) Mitotane 31.3 μM, C) Mitotane 62.5 μM, D) EPS 1.5 mg.mL^{-1} and E) EPS 1.5 mg.mL^{-1} + Mitotane 31.3 μM.

The results of viability in this work are in agreement with results obtained by different authors (Fassnacht et al., 2012; Kroiss et al., 2016; Lehmann et al., 2013) in which the toxicity of mitotane on H295R cell is evident (even at different concentrations).

3.4.3. Apoptosis flow cytometry analysis

In order to know about the effect of the mitotane, EPS and their combination in cellular death, an evaluation of apoptosis by cytometry analysis were performed. In cancer treatment, the ideal treatments are immunotherapy - enhancing the immune system response, or treatments that can stimulate an apoptotic process.

7-AAD is membrane impermeant dye that is generally excluded from viable cells, and ANNEXIN V is a specific protein that has the ability to bind to phosphatidylserine (an apoptosis marker); for apoptosis determination only cells marked with ANNEXIN V were considered as apoptotic cells positive, cells marked by ANNEXIN V and 7-AAD were not considered as late apoptotic cells in comparison of criteria adopted by different authors (Germano et al., 2014; Poli et al., 2013).

The results of the treatments were expressed as events percentage and were distributed as showed in Figure 18. It was observed 13.5% of apoptotic cells with EPS, 2% with mitotane and 17% with a combination of EPS and mitotane, compared to the control at $p<0.05$.

No increment in apoptosis was observed when higher concentrations of EPS were tested (data not shown).

According to some researchers, the apoptotic effect of mitotane is inconsistent, and the determination of the cellular death whether by necrosis or apoptosis must be studied (Högel, Rantanen, Jokilehto, Grenman, & Jaakkola, 2011, and Wilhelm, Wagner, & Häcker, 1998 and Lehmann et al., 2013). The necrosis hypothesis could explain high quantity of cellular debris observed when cells were analyzed after mitotane 62.5 µM treatment at 24 h and 48 h (data not shown).

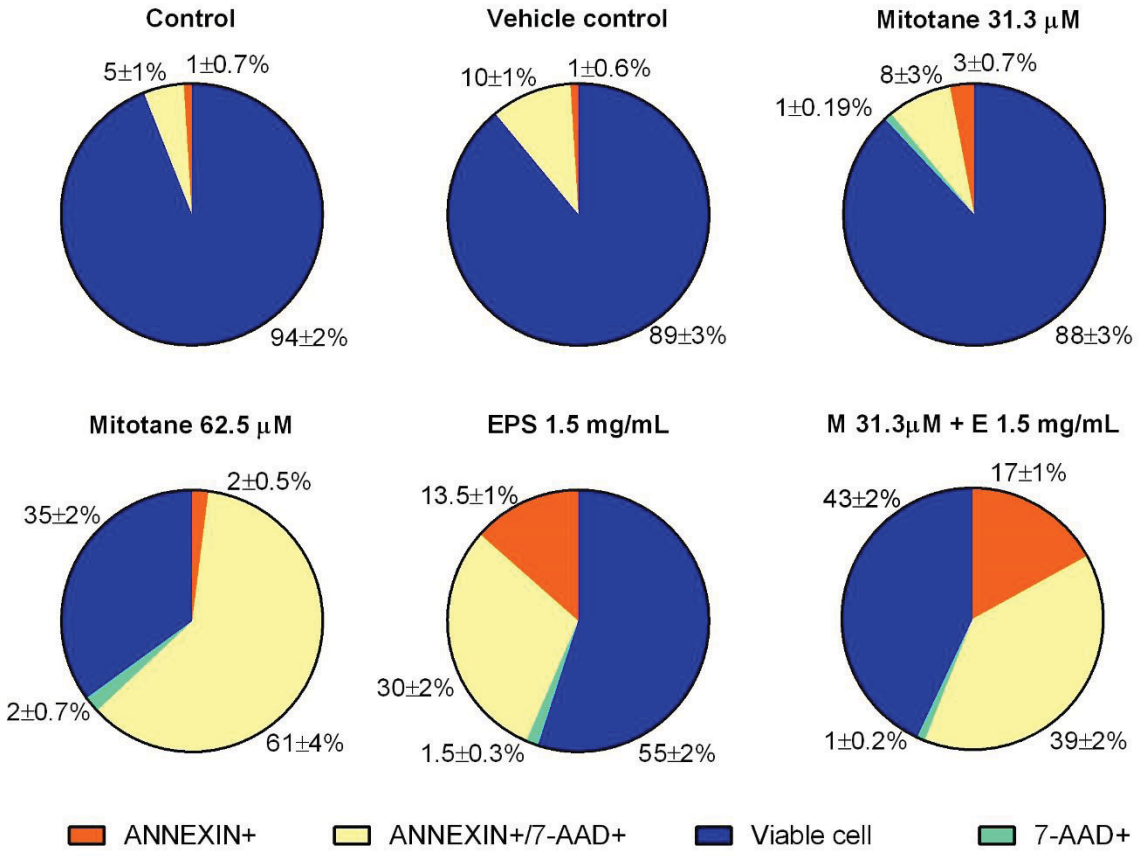


Figure 18. Distribution of apoptosis, live and dead analysis of H295R cells performed by flow cytometric analysis with ANNEXIN V. Treatments were using mitotane (M), EPS (E) and a combination of both substances. The experiment was performed in triplicate.

3.4.4. Lysosomal volume

The lysosomal volume is a relatively new method to evaluate the capability of the neutral red retention dye by lysosomes, indicating in a direct way the functionality of lysosomes.

H205R cells did not show an alteration of the lysosomal volume, when treated with concentrations of mitotane below 31.3 μM at 24h. At 50 μM a decrement of the 6% was noted. The window concentration (62.5 μM) caused a considerable negative alteration in the volume, 22% more when compared to the negative control and 48% less when compared to the positive control (Figure 19). Due to mitotane cytotoxicity, most of the cells population, at the positive control concentration, could have to be dead by the experiment time, which indicates a high toxicity on the cells, possibly acting

as an inducer of necrotic by mitochondrial oxidative injury as reported by Lehmann et al., 2013; Poli et al., 2013, and possibly lysosomes damaged since this organelle can act as cellular death inducers by a partial release of its content to the cell triggering an apoptotic process (Rizk-Rabin et al., 2008).

On the other hand, necrosis could be triggered when all the lysosomal content is released due to membrane disruption. Krecic & Swanson, 1999, reported the action of mitotane in the depletion of the protein hnRNP (protein implicated in mRNA metabolism and tumorigenic process) leading to a tumorigenesis, and also increasing levels of cathepsin D, a lysosomal protease involved in cellular death (Roberg, 2001).

The use of only EPS as an adjuvant of mitotane showed a slight increase in the lysosomal volume, an increment of 4 and 10% ($p<0.05$) respectively. This might explain the apoptotic cells observed in the cytometry assay previously described.

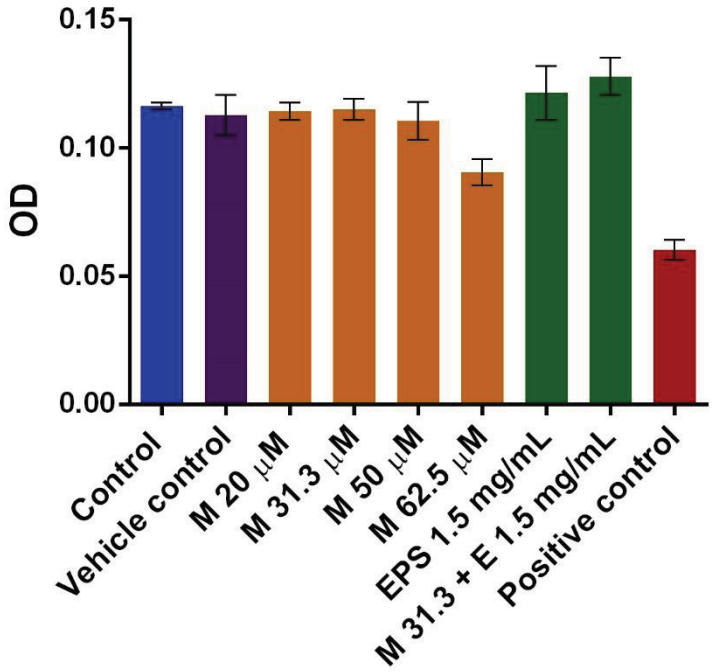


Figure 19. Lysosomal retention of H295R cells, treated with mitotane (M), EPS (E) and a mixture of mitotane and EPS. Results were presented ± SE of the mean. Experiments were carried out in five replicates.

3.4.5. Superoxide anion production (O_2)

The production of the superoxide anion (a reactive oxygen species - ROS) was not significant when mitotane at a concentration below 50 µM was tested. On the contrary, when 62.5 µM of concentration was used a significant decrement of 28% of production was noted, but this might be an answer to mitotane's toxicity, the same trend was observed in the positive control (Figure 20).

When EPS and EPS combined with mitotane as an adjuvant were tested a significant production ROS of 32% was obtained. The production could be a response by an oxidative burst (McDowell, Amsler, Li, Lancaster Jr, & Amsler, 2015) to the stimulation caused by the antioxidant effect of the EPS (as found in our previous work) which can be formed in the mitochondria or via activation of oxidases as reports by (Rajamohan, Raghuraman, Prabhakar, & Kumar, 2012). Generation of ROS is a survival alternative mechanism of tumoral cells, but it can also leads to death when high quantities are produced (Calder, 2012), some of the objectives of the action mechanism of chemotherapeutics are the induction of intracellular ROS synthesis (McDowell et al., 2015). In this work, the increment of ROS production might be related to the pro-apoptosis effect of the EPS.

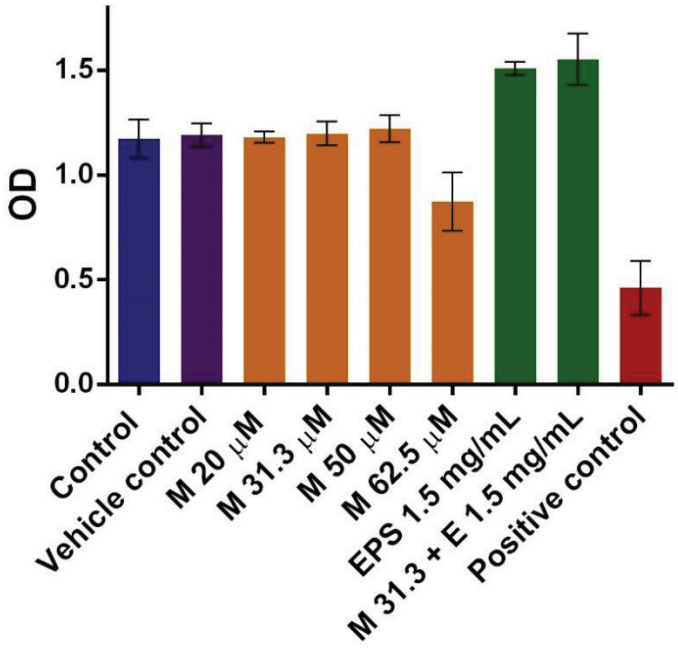


Figure 20. Superoxide anion production by H295R cells, treated with mitotane (M), EPS (E) and a mixture of mitotane and EPS. Results were presented ± SE of the mean. Experiments were carried out in five replicates.

3.4.6. Hydrogen peroxide production (H_2O_2)

The increment of the production of hydrogen peroxide was not significant among the treatments when compared to the control, as showed in Figure 21. In fact, a decrease in of production was noted when concentrations up to 50 µM of mitotane were tested, but this might be due to the cytotoxic effect on H295R cells. An apoptotic process induced by the increment of the hydrogen peroxide level was not observed in this study.

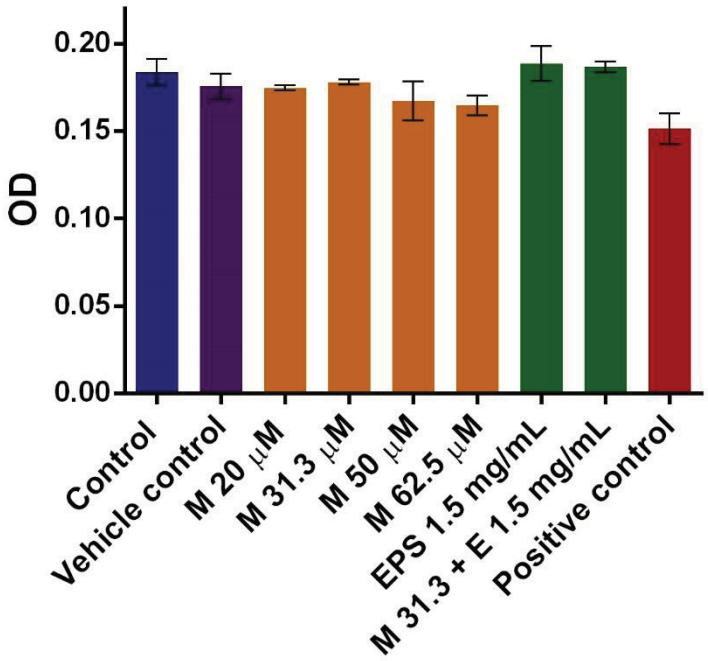


Figure 21. H295R hydrogen peroxide production by H295R cells treated with mitotane, EPS and a mixture of mitotane and EPS. Results were presented ± SE of the mean. Experiments were carried out in five replicates.

3.5. CONCLUSIONS

In this work, for the first time the antitumoral potential of an exopolysaccharide obtained from *Mortierella alpina,* chitin, was evaluated. Results obtained using viability techniques demonstrated the high cytotoxicity of mitotane on tumoral (H295R) and non-tumoral cells (VERO) suggesting more a necrotic effect than a pro-apoptotic effect, specifically at the concentration of treatment as a chemotherapeutic drug. On the other hand, the exopolysaccharide showed an antitumoral action by a 3.3-fold increment of the pro-apoptotic effect tumoral cells and low damage on non-tumoral cells was not significant when compared to mitotane. A slight enhancement of the pro-apoptotic effect was observed when the exopolysaccharide was used as mitotane adjuvant (4%), again the cell death was more at a pro-apoptotic induction instead necrosis. This pro-apoptotic effect, in cellular death, might be explained by the formation of ROS from tumoral cells as a response to the antioxidant effect of the exopolysaccharide and also a conservation of lysosomal volume.

In sum, this work may contribute to the development of bioproducts with innovative applications on ACC treatment.

ACKNOWLEDGMENTS

The authors thank the funding agencies CNPq and CAPES for their support.

3.6. REFERENCES

Allolio, B., & Fassnacht, M. (2006). Adrenocortical carcinoma: clinical update. *The Journal of Clinical Endocrinology & Metabolism*, *91*(6), 2027–2037.

Berruti, A., Terzolo, M., Sperone, P., Pia, A., Della Casa, S., Gross, D. J., … Mantero, F. (2005). Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocrine-Related Cancer*, *12*(3), 657–666.

Bonatto, S. J. R., Folador, A., Aikawa, J., Yamazaki, R. K., Pizatto, N., Oliveira, H. H. P., … Fernandes, L. C. (2004). Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. *Cellular Immunology*, *231*(1–2), 56–62.

Calder, P. C. (2012). Mechanisms of Action of (n-3) Fatty Acids, 2. *The Journal of Nutrition*, *142*(3), 592S–599S.

Dackiw, A. P. B., Lee, J. E., Gagel, R. F., & Evans, D. B. (2001). Adrenal cortical carcinoma. *World Journal of Surgery*, *25*(7), 914.

Fassnacht, M., Terzolo, M., Allolio, B., Baudin, E., Haak, H., Berruti, A., … Skogseid, B. (2012). Combination Chemotherapy in Advanced Adrenocortical Carcinoma. *New England Journal of Medicine*, *366*(23), 2189–2197. https://doi.org/10.1056/NEJMoa1200966

Flack, M. R., & Chrousos, G. P. (1996). Neoplasms of the adrenal cortex. *Cancer Medicine*, *1*, 1567.

Germano, A., Rapa, I., Volante, M., Buono, N. Lo, Carturan, S., Berruti, A., … Papotti, M. (2014). Cytotoxic activity of gemcitabine, alone or in combination with mitotane, in adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *382*(1), 1–7.

Högel, H., Rantanen, K., Jokilehto, T., Grenman, R., & Jaakkola, P. M. (2011). Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. *PloS One*, *6*(11), e27112.

Kasperlik-Zalułska, A. A., Migdalska, B. M., Zgliczyński, S., & Makowska, A. M. (1995). Adrenocortical carcinoma. A clinical study and treatment results of 52 patients. *Cancer*, *75*(10), 2587–2591.

Krecic, A. M., & Swanson, M. S. (1999). hnRNP complexes: composition, structure, and function. *Current Opinion in Cell Biology*, *11*(3), 363–371.

Kroiss, M., Plonné, D., Kendl, S., Schirmer, D., Ronchi, C. L., Schirbel, A., … Fassnacht, M. (2016). Association of mitotane with chylomicrons and serum lipoproteins: practical implications for treatment of adrenocortical carcinoma. *European Journal of Endocrinology*, *174*(3), 343–353.

Lehmann, T. P., Wrzesiński, T., & Jagodziński, P. P. (2013). The effect of mitotane on viability, steroidogenesis and gene expression in NCI-H295R adrenocortical cells. *Molecular Medicine Reports*, *7*(3), 893–900.

McDowell, R. E., Amsler, M. O., Li, Q., Lancaster Jr, J. R., & Amsler, C. D. (2015). The immediate wound-induced oxidative burst of Saccharina latissima depends on light via photosynthetic electron transport. *Journal of Phycology*, *51*(3), 431–441.

Ng, L., & Libertino, J. M. (2003). Adrenocortical carcinoma: diagnosis, evaluation and treatment. *The Journal of Urology*, *169*(1), 5–11.

Pick, E., & Mizel, D. (1981). Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods*, *46*(2), 211–226.

Pipe, R. K., Coles, J. A., & Farley, S. R. (1995). Assays for measuring immune response in the mussel Mytilus edulis. *Tech Fish Immunol*, *4*, 93–100.

Plager, J. E. (1984). Carcinoma of the adrenal cortex: clinical description, diagnosis, and treatment. *International Advances in Surgical Oncology*, *7*, 329–353.

Poli, G., Guasti, D., Rapizzi, E., Fucci, R., Canu, L., Bandinelli, A., … Luconi, M. (2013). Morphofunctional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocrine-Related Cancer*, *20*(4), 537–550.

Rajamohan, S. B., Raghuraman, G., Prabhakar, N. R., & Kumar, G. K. (2012). NADPH oxidase-derived H2O2 contributes to angiotensin II-induced aldosterone synthesis in human and rat adrenal cortical cells. *Antioxidants & Redox Signaling*, *17*(3), 445–459.

Rizk-Rabin, M., Assie, G., Rene-Corail, F., Perlemoine, K., Hamzaoui, H., Tissier, F., … Bouizar, Z. (2008). Differential Expression of Parathyroid Hormone–Related Protein in Adrenocortical Tumors: Autocrine/Paracrine Effects on the Growth and Signaling Pathways in H295R Cells. *Cancer Epidemiology and Prevention*

Biomarkers, *17*(9), 2275–2285.

Roberg, K. (2001). Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. *Laboratory Investigation*, *81*(2), 149.

Sim Choi, H., Woo Kim, J., Cha, Y., & Kim, C. (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of Immunoassay and Immunochemistry*, *27*(1), 31–44.

Song, K.-S., Li, G., Kim, J.-S., Jing, K., Kim, T.-D., Kim, J.-P., … Hwang, B.-D. (2011). Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/β-catenin in SW480 human colon cancer cells. *BMC Cancer*, *11*(1), 307.

Steiner, P. E. (1954). *Cancer: Race and geography: some etiological, environmental, ethnological, epidemiological, and statistical aspects in Caucasoids, Mongoloids, Negroids, and Mexicans*. Williams & Wilkins Company.

Taki, H., Ohishi, K., Okano, A., Suga, T., Akiyama, Y., & Yamashita, A. (1995). Antimetastatic effect of lentinan on liver metastasis of colon carcinoma (colon26): possible role of activated kupffer cells. *International Journal of Immunotherapy*, *11*(1), 29–38.

Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., … Grossrubatscher, E. (2007). Adjuvant mitotane treatment for adrenocortical carcinoma. *New England Journal of Medicine*, *356*(23), 2372–2380.

Vannucci, L., Krizan, J., Sima, P., Stakheev, D., Caja, F., Rajsiglova, L., … Saieh, M. (2013). Immunostimulatory properties and antitumor activities of glucans. *International Journal of Oncology*, *43*(2), 357–364.

Vassilopoulou-Sellin, R., & Schultz, P. N. (2001). Adrenocortical carcinoma: clinical outcome at the end of the 20th century. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *92*(5), 1113–1121.

Wajchenberg, B. L., Albergaria Pereira, M. A., Medonca, B. B., Latronico, A. C., Carneiro, P. C., Ferreira Alves, V. A., … Kirschner, M. A. (2000). Adrenocortical carcinoma: clinical and laboratory observations. *Cancer*, *88*(4), 711–736.

Wang, Z. M., Cheung, Y. C., Leung, P. H., & Wu, J. Y. (2010). Ultrasonic treatment for improved solution properties of a high-molecular weight exopolysaccharide produced by a medicinal fungus. *Bioresource Technology*, *101*(14), 5517–5522. https://doi.org/10.1016/j.biortech.2010.01.134

Wilhelm, S., Wagner, H., & Häcker, G. (1998). Activation of caspase-3-like enzymes in non-apoptotic T cells. *European Journal of Immunology*, *28*(3), 891–900.

Yamamoto, T., Yamashita, T., & Tsubura, E. (1981). Inhibition of pulmonary metastasis of Lewis lung carcinoma by a glucan, Schizophyllan. *Invasion & Metastasis*, *1*(1), 71–84.

Zaidman, B.-Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*, *67*(4), 453–468.

Zhang, M., Chen, H., Huang, J., Li, Z., Zhu, C., & Zhang, S. (2005). Effect of lycium barbarum polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sciences*, *76*(18), 2115–2124.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*(1), 4–19.

4. CHAPTER IV. A STUDY OF GELATION OF A SOLUTION BASED IN CHITIN PRODUCED BY *MORTIERELLA ALPINA*, AND EFFECT OF ITS CONCENTRATION.

4.1. ABSTRACT

A study of gelation and viscoelastic properties of a solution based in chitin produced by *Mortierella alpina* was carried out in this work, investigating the influence of temperature (10 - 60°C and concentration of chitin in the solution using LiCl 0.28 M and NaCl 0.154 M as solvents. It was noted a polymer gel dispersion at a concentration of 5 $mg.mL^{-1}$ of chitin over the range of temperature, and a gelation with transient and permanent networks formed as the effect of temperatures higher than 45-50°C at 10 and 15 $mg.mL^{-1}$ of chitin concentration in solutions. Gels formed did not show a depercolation effect as the response of a thermoreversible process performed. The solvents used in this study also showed an increase of participation of the chains fractions in the network formed

Keywords: Chitin, *Mortierella alpina*, hydrogel, rheology, depercolation, gel.

4.2. INTRODUCTION

Chitin is a polysaccharide abundant in nature, it is commonly extracted from marine sources (Rinaudo, 2006), and the application of this polysaccharide and its derivates (chitosan and N-acetylglucosamine) are innumerous among different industries such as textile, food and nutrition, pharmaceutics, medicine, due to their biological activity and physicochemical properties (Harish Prashanth & Tharanathan, 2007). This explains, the considerable number of studies about the formulation and development of biomaterials composed by chitin, such as matrixes, bio gels and hydrogels, which have strong interactive properties with biological media (Gerentes, Vachoud, Doury, & Domard, 2002).

There are two possible types of chitin-based gels: Chemical gels crosslinked by means of various bi-functional agents (i.e. glutaraldehyde, epichlorohydrin, etc) (Muzzarelli, 2009) and physical gels formed from low energy junction, due to the poor

solubility of chitin and derivates themselves in water. Gels crosslinked to agents are most used for multiple formulations (Bhattarai, Gunn, & Zhang, 2010; De Angelis, Capitani, & Crescenzi, 1998; Hirano & Ohe, 1975).

Most of the chitin-based biomaterials are made from conventional marine sources, thus the lack of research on alternative sources, especially those with more eco-friendliness concern.

Due to these reasons, it is important and necessary to produce, identify and study the gelation properties and concentration influence of chitin-based solutions obtained from alternative sources, in this case, obtained from *Mortierella alpina* produced in an extracellular way.

In this work, a physical solution produced with physiological compatibilities was produced in order to preserve the natural structure and properties of chitin and investigate the gelation and influence of the chitin concentration using oscillatory shear rheology.

4.3. MATERIAL AND METHODS

4.3.1. Sample preparation

Chitin was extracted from the culture medium fermentation of *Mortierella alpina* as described in or previous work. Chitin was solubilized at concentration of LiCl 0.28 M and NaCl 0.154 M, the solutions were left to stand overnight at 3°C without agitation for degassing. The molecular weight of extracellular chitin produced by *Mortierella alpina* reported by (ME) was $4.9x10^5$ $g.mol^{-1}$ ($\frac{\partial n}{\partial c} = 0{,}121\ \mathrm{mL.\,g^{-1}}$).The total characterization of the polysacharide was described in the previous chapter.

4.3.2. Gelation study

Dynamic mechanical rheological measurements were carried out by monitoring viscoelastic moduli changes in the chitosan solutions using a Thermo Scientific Haake Rheostress 1 (Karlsruhe, Germany), equipped with a cone and plate geometry sensor (40mm diameter, cone 2°), the gap between plates was 1mm. The loss (G'') and storage (G') shear moduli were at a wide range of frequency (Hz). The imposed stress was chosen within the linear response regime (σ = 0.1 Pa) unless otherwise specified. Measurements were performed at 10 - 60°C with an increment of 5°C±1°C and the sensor was covered with a layer of mineral oil to avoid evaporation of the chitin solutions.

4.4. RESULTS AND DISCUSSION

Effect of temperature on chitosan-based solutions

In this work, the evaluation of the gel formation as a response to a temperature increment was carried out in order to understand the behavior of the chitin-based solution at different concentration using as NaCl and LiCl as a solvent due to the poor solubility of chitin in water.

All the samples were monitored at a frequency range of 50 to 0.01 Hz observing the responses of the shear moduli. The frequency dependence of the storage and loss shear moduli at different temperatures (10 - 60°C) are shown in Figure 22. In which, in the case LiCl as solvent, at 5mg.L^{-1}, a predominant liquid-like behavior was observed - high variations of the loss moduli (G'') at low frequencies is characteristic of solutions (no formation of gel), different than the gelation observed at concentration of 10 and 15 mg/L when temperature was up to 55°C - high variations of the storage module (G') at high frequencies (Winter & Chambon, 1986).

Similar behavior was noted when NaCl solution was used as a solvent, at 5 mg.L^{-1} no gel formation was observed, with the opposite effect when 10 and 15 mg.L-1 were used at 50°C and 45°C, respectively. These results confirmed particularities that were distinguished when different solvents for chitin solubilization were tested at different temperatures, such as DMSO, acetone, urea solution (Data not shown).

Figure 22 also shows the increment of the G' module of the solutions in function of temperature. Low concentrations of chitin solubilized in LiCl, showed a polymeric dispersion due to angular coefficient calculated in the G' module. At a concentration of 10 and 15 mg.L^{-1}, a solid-like behavior was observed, possibly caused by the formation of a permanent network of chitin chains via cross-links with long life.

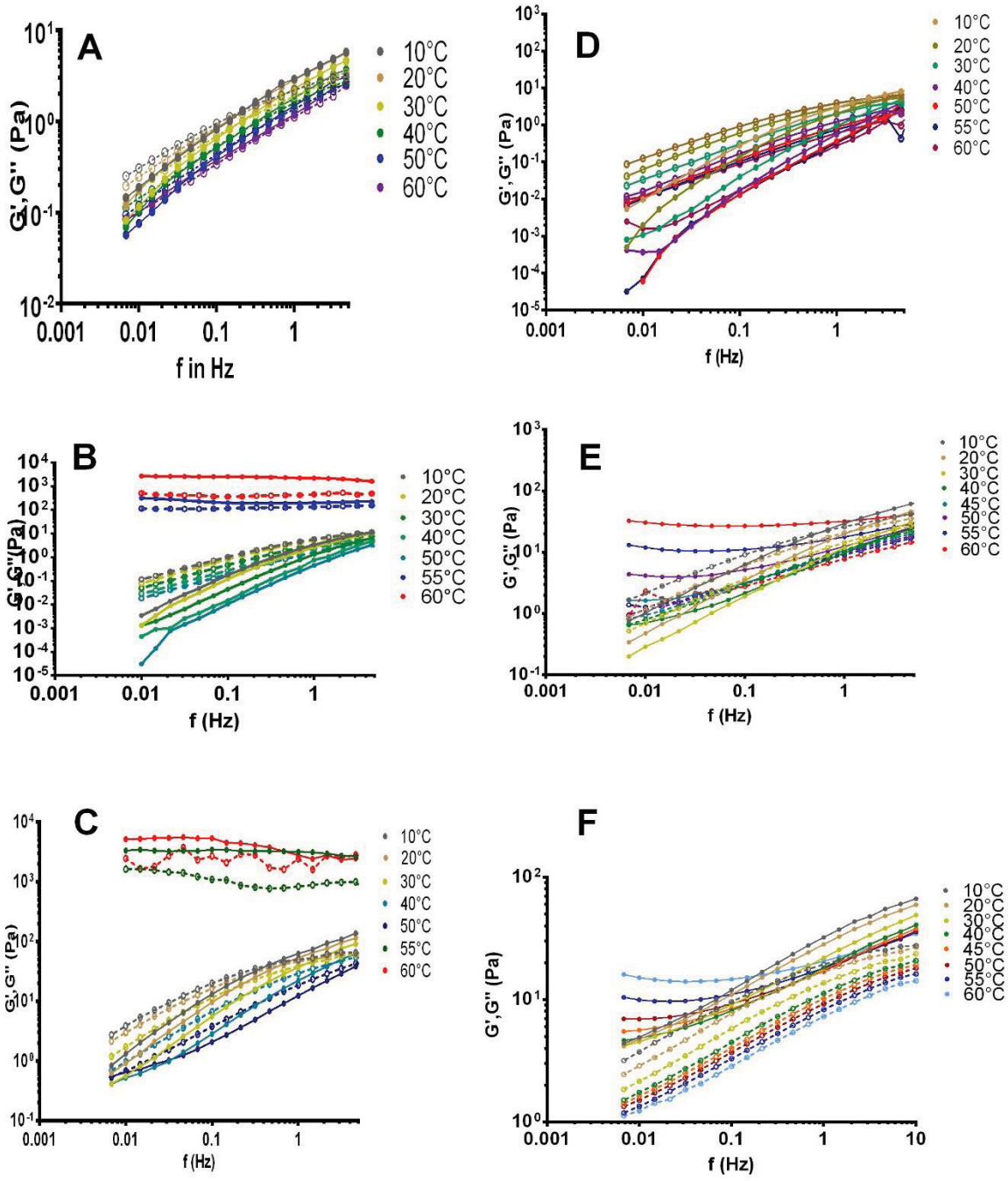


Figure 22. Frequency dependence of the storage and loss moduli of chitin-based solution at concentrations of A) 5 mg.mL^{-1}, B) 10 mg.mL^{-1} and C) 15mg.mL^{-1} of chitin in LiCL and D) 5 mg.mL^{-1}, E) 10 mg.mL^{-1} and F) 15 mg.mL^{-1} of chitin in NaCl over a wide range of temperature. Storage modulus is represented in filled symbols and Loss modulus in open symbols.

When NaCl was used as solvent same behavior was observed at a low concentration of chitin, but characteristic transitions phase between permanent and transient networks were observed at 45°C when 10 mg.L-1 was evaluated, and

possibly permanent networks at 15 mg.L^{-1}. Curves corresponding to 15, 25, 35, and in some cases 45°C were not shown due to the superposition of moduli.

As observed, the gelation mechanism of chitin was significantly dependent on the cross-linking conditions, the time to the onset of gelation was reduced as the temperature increase mostly from 50 to 60°C in the assays with a concentration above 10 mg.L^{-1} of chitin-based solutions.

The depercolation of gels formed were also evaluated in this work in order to know if a thermo-reversible effect could exist. Gels were cool down from 60°C to 10°C in a lapse of 1.5 hours and no depercolation effect (breakage of bonds) was observed (Figure 23), suggesting that long periods of time might be needed until attaining this effect.

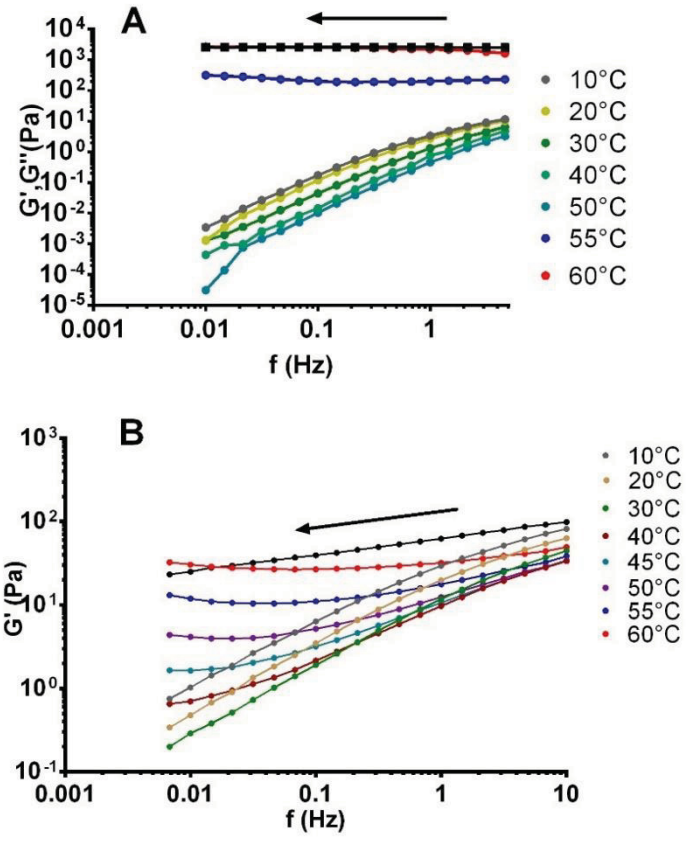


Figure 23. Temperature dependence at 10°C, thermo-reversion after 60°C of the shear moduli gels formed by chitin-based solutions at 10 g.mL^{-1}. A) in LiCl and B) in NaCl.

At higher temperatures, a formation of a high-density cross-links population increases for entangled polymer solutions (superior to the 10 $mg.L^{-1}$ of chitin) (Al-Muntasheri, Hussein, Nasr-El-Din, & Amin, 2007). This phenomenon can be observed in figure 24, as a function of the increment of the storage module (elastic module) with respect to the temperature, passing from a transient, quasi-transient to a permanent network. These cross-links might be formed by specific hydrophobic interaction between the chains (Wientjes, Duits, Jongschaap, & Mellema, 2000)

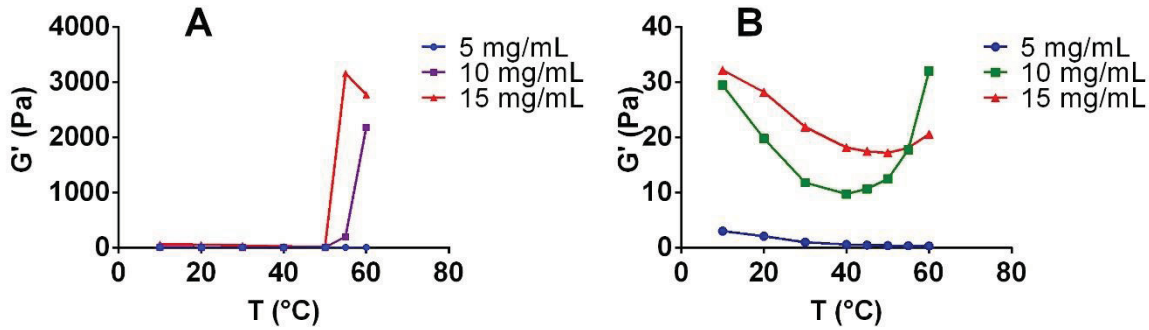


Figure 24. Frequency dependence of G' over a range of temperature of the shear moduli at f=1Hz. A) LiCl and B) NaCl as solvent respectively.

In this study we can speculate the formation of a permanent network at high concentration of chitin tested at high temperatures and a different behavior of the formation of networks when NaCl and LiCl were used as solvents, due to the decrement of the values of the moduli in NaCl compared to LiCl assays, that suggested a fewer participation and more participation of the chains fractions in the network formation, respectively (No & Meyers, 1995). In the work of Gérente et al., 2002, an evaluation of the gel formation on chitin-based hydrogels was performed, in order to determine its influence as injectable material in perinodal surgery due to stimulant effect of chitin on bone formation and cicatrization.

4.5. CONCLUSIONS

Gelation of the chitin-based solutions was induced by the influence of temperature and concentration of chitin, temperatures higher than 55°C incremented the elastic modulus creating a hydrogel when LiCl 0.28 M was used as a solvent, also forming a permanent network effect. The same behavior was observed when NaCl 0.154M was used as the solvent of chitin at a concentration of 10 $mg.mL^{-1}$ of chitin in temperatures higher than 50°C. At 45°C a gelation of the solution of 15 $mg.mL^{-1}$ of chitin showed a gelation composed possibly by a high density of permanent networks since no depercolation effect was observed when gels were cooled down from 60°C to 10°C due to the stiffness of the gel formed by higher temperatures. The formation of hydrogels of chitin-based solutions suggests multiple applications of chitin produces by *Mortierella alpina* as biomaterial due to the formation of a matrix composed of permanent cross-links networks.

ACKNOWLEDGMENTS

The authors thank the funding agencies CNPq and CAPES for their support.

4.6. REFERENCES

Al-Muntasheri, G. A., Hussein, I. A., Nasr-El-Din, H. A., & Amin, M. B. (2007). Viscoelastic properties of a high temperature cross-linked water shut-off polymeric gel. *Journal of Petroleum Science and Engineering*, *55*(1–2), 56–66.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

De Angelis, A. A., Capitani, D., & Crescenzi, V. (1998). Synthesis and 13C CP-MAS NMR characterization of a new chitosan-based polymeric network. *Macromolecules*, *31*(5), 1595–1601.

Gerentes, P., Vachoud, L., Doury, J., & Domard, A. (2002). Study of a chitin-based gel as injectable material in periodontal surgery. *Biomaterials*, *23*(5), 1295–1302.

Harish Prashanth, K. V., & Tharanathan, R. N. (2007). Chitin/chitosan: modifications and their unlimited application potential-an overview. *Trends in Food Science and Technology*, *18*(3), 117–131. https://doi.org/10.1016/j.tifs.2006.10.022

Hirano, S., & Ohe, Y. (1975). Chitosan gels: a novel molecular aggregation of chitosan in acidic solutions on a facile acylation. *Agricultural and Biological Chemistry*, *39*(6), 1337–1338.

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9.

No, H. K., & Meyers, S. P. (1995). Preparation and Characterization of Chitin and Chitosan—A Review. *Journal of Aquatic Food Product Technology*, *4*(2), 27–52. https://doi.org/10.1300/J030v04n02_03

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science (Oxford)*, *31*(7), 603–632. https://doi.org/10.1016/j.progpolymsci.2006.06.001

Wientjes, R. H. W., Duits, M. H. G., Jongschaap, R. J. J., & Mellema, J. (2000). Linear rheology of guar gum solutions. *Macromolecules*, *33*(26), 9594–9605.

Winter, H. H., & Chambon, F. (1986). Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. *Journal of Rheology*, *30*(2), 367–382.

5. CONCLUSÕES

Foi possível otimizar a produção do exopolissacarídeo produzido pelo fungo filamentoso *Mortierella alpina* assim como a sua caracterização e avaliação de propriedades bioativas. Esse exopolissacarídeo foi identificado como uma quitina produzida extracelularmente que demonstrou pureza e homogeneidade, contendo um alto nível de acetilação (>90%).

O exopolissacarideo avaliado apresentou efeitos biológicos:

- um efeito bacteriostático para células Gram negativas e Gram positivas ($p<0,05$), inibindo o crescimento celular de até 60% em 24 h de bactérias responsáveis de intoxicação alimentar, como *Salmonella tiphy;*

- um efeito antitumoral ($p<0,05$) de diversas linhas celulares tumorais testadas: câncer de mama, colorretal e carcinoma adrenocortical, inibindo a proliferação de células em até 60% quando comparado com o controle. Essa citotoxicidade não foi observada nas linhagens celulares não-tumorais avaliadas;

- efeito promissor como alternativa adicional no tratamento de carcinoma adrenocortical, devido ao seu efeito pro-apoptotico nas células tumorais e baixa citotoxicidade ($p<0,05$), em comparação com o mitotano, quimioterápico utilizado comumente no tratamento deste tipo de câncer.

O exopolissacarideo, quando dissolvido em NaCl e LiCl, produz hidrogéis com possível formações de redes permanentes em temperaturas superiores a 45°C, sendo influenciados pela concentração da solução. Não foi observado um efeito de depercolação dos géis em função da temperatura.

Esse exopolissacarideo pode ser considerado como um potencial insumo para diversas aplicações de interesses industriais, tais como em biomedicina e farmácia, devido a suas propriedades antioxidantes e antitumorais, cabe ressaltar que este polissacarídeo é produzido de uma forma amigável com o meio ambiente e que alternativo ao uso de quitina de origem animal.

6. CONSIDERACOES FINAIS PARA TRABALHOS FUTUROS

- Realizar avaliações do potencial do exopolisacarídeo em ensaios *in vivo,* para as mesmas atividades.

- Avaliar o potencial biológico do exopolissacarideo desacetilado.

- Avaliar a resposta imune do exopolissacarideo parcialmente hidrolisado ou despolimerizado.

- Avaliar o efeito da quitina na formulação de biomateriais devido a suas propriedades reológicas, assim como a avaliação como um agente floculante.

- Otimizar a produção do exopolissacarideo por técnicas de engenharia metabólica.

- Estudar a eficiência da produção do exopolissacarideo em uma escala industrial.

- Realizar mais estudos reológicos do exopolissacarideo para maior entendimento para a sua aplicação na formulação de biomaterias.

REFERENCIAS

Abdou, E. S., Nagy, K. S. A., & Elsabee, M. Z. (2008). Extraction and characterization of chitin and chitosan from local sources. *Bioresource Technology*, *99*(5), 1359–1367. https://doi.org/https://doi.org/10.1016/j.biortech.2007.01.051

Akhtar, M. W., Mirza, A. Q., Nawazish, M. N., & Chughtai, M. I. D. (1983). Effect of triglycerides on the production of lipids and lipase by Mucor hiemalis. *Canadian Journal of Microbiology*, *29*(6), 664–669. https://doi.org/10.1139/m83-108

Akimoto, K., Kawashima, H., & Shimizu, S. (2011). *Microorganisms that extracellularly secrete lipids and methods of producing lipid and lipid vesicles encapsulating lipids using said microorganisms.* (No. U.S. Patent No. 7,972,855). Washington, DC: U.S.

Al-Muntasheri, G. A., Hussein, I. A., Nasr-El-Din, H. A., & Amin, M. B. (2007). Viscoelastic properties of a high temperature cross-linked water shut-off polymeric gel. *Journal of Petroleum Science and Engineering*, *55*(1–2), 56–66.

Alekshun, M. N., & Levy, S. B. (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell*, *128*(6), 1037–1050. https://doi.org/https://doi.org/10.1016/j.cell.2007.03.004

Allolio, B., & Fassnacht, M. (2006). Adrenocortical carcinoma: clinical update. *The Journal of Clinical Endocrinology & Metabolism*, *91*(6), 2027–2037.

Amano, H., Shinmen, Y., Akimoto, K., Kawashima, H., Amachi, T., Shimizu, S., & Yamada, H. (1992). Chemotaxonomic significance of fatty acid composition in the genus Mortierella. *Mycotaxon*, *45*, 257–265.

Anderson, J. W., Nicolosi, R. J., & Borzelleca, J. F. (2005). Glucosamine effects in humans: a review of effects on glucose metabolism, side effects, safety considerations and efficacy. *Food and Chemical Toxicology*, *43*(2), 187–201. https://doi.org/https://doi.org/10.1016/j.fct.2004.11.006

Bajpai, P., & Bajpai, P. K. (1992). Arachidonic acid production by microorganisms. *Biotechnology and Applied Biochemistry*, *15*(1), 1–10. https://doi.org/10.1111/j.1470-8744.1992.tb00194.x

Bajpai, P., Bajpai, P. K., & Ward, O. P. (1991). Eicosapentaenoic acid (EPA) production by Mortierella alpina ATCC 32222. *Applied Biochemistry and Biotechnology*, *31*, 267–272. https://doi.org/10.1007/BF02921753

Banerjee, Mr. Mahapatra, & D Banerjee. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, 1. https://doi.org/10.4137/MBI.S10957

Bautista, J., Jover, M., Gutierrez, J. F., Corpas, R., Cremades, O., Fontiveros, E., … Vega, J. (2001). Preparation of crayfish chitin by in situ lactic acid production. *Process Biochemistry*, *37*(3), 229–234. https://doi.org/https://doi.org/10.1016/S0032-9592(01)00202-3

Benhabiles, M. S., Salah, R., Lounici, H., Drouiche, N., Goosen, M. F. A., & Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. *Food Hydrocolloids*, *29*(1), 48–56. https://doi.org/https://doi.org/10.1016/j.foodhyd.2012.02.013

Berruti, A., Terzolo, M., Sperone, P., Pia, A., Della Casa, S., Gross, D. J., … Mantero, F. (2005). Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocrine-Related Cancer*, *12*(3), 657–666.

Bhattarai, N., Gunn, J., & Zhang, M. (2010). Chitosan-based hydrogels for controlled, localized drug delivery. *Advanced Drug Delivery Reviews*, *62*(1), 83–99.

Blanpain, C. (2013). Tracing the cellular origin of cancer. *Nature Cell Biology*, *15*(2), 126.

Bonatto, S. J. R., Folador, A., Aikawa, J., Yamazaki, R. K., Pizatto, N., Oliveira, H. H. P., … Fernandes, L. C. (2004). Lifelong exposure to dietary fish oil alters macrophage responses in Walker 256 tumor-bearing rats. *Cellular Immunology*, *231*(1–2), 56–62.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brekhman, I. I. (2013). *Man and biologically active substances: the effect of drugs, diet and pollution on health*. Elsevier.

Brooks, D. D., Berbesi, R., & Hodgson, A. S. (2011). Optimization of bleaching process. Retrieved March 28, 2016, from http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40321

Calder, P. C. (2012). Mechanisms of Action of (n-3) Fatty Acids, 2. *The Journal of Nutrition*, *142*(3), 592S–599S.

Carocho, M., & Ferreira, I. C. F. R. (2013). A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, *51*, 15–25. https://doi.org/https://doi.org/10.1016/j.fct.2012.09.021

Carpita, C., & Ci, D. (1991). Measurement of Uranic Acids without from Neutral Sugars, *162*, 157–162.

Certik, M., Megova, J., & Horenitzky, R. (1999). Effect of nitrogen sources on the activities of lipogenic enzymes in oleaginous fungus *Cunninghamella echinulata*. *The Journal of General and Applied Microbiology*, *45*(6), 289–293. https://doi.org/10.2323/jgam.45.289

Certik, M., & Shimizu, S. (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Bioscience and Bioengineering*, *87*(1), 1–14.

Chen, C. H., Wang, F. Y., & Ou, Z. P. (2004). Deacetylation of b-chitin. I. Influence of the deacetylation conditions. *Journal of Applied Polymer Science*, *93*(5), 2416–2422. https://doi.org/10.1002/app.20753

Chen, J., & Seviour, R. (2007). Medicinal importance of fungal β-(1→ 3),(1→ 6)-glucans. *Mycological Research*, *111*(6), 635–652.

Chen, Y. T., Yuan, Q., Shan, L. T., Lin, M. A., Cheng, D. Q., & Li, C. Y. (2013). Antitumor activity of bacterial exopolysaccharides from the endophyte bacillus amyloliquefaciens sp.isolated from ophiopogon japonicus. *Oncology Letters*, *5*(6), 1787–1792. https://doi.org/10.3892/ol.2013.1284

Chow, L. W. C., Lo, C. S. Y., Loo, W. T. Y., Hu, X., & Sham, J. S. T. (2003). Polysaccharide peptide mediates apoptosis by up-regulating p21 gene and down-regulating cyclin D 1 gene. *The American Journal of Chinese Medicine*, *31*(01), 1–9.

CLSI. (2017). *M100 Performance standards for antimicrobial susceptibility testing. 27th ed. Clinical and Laboratory Standards Institute*.

Cohen, Z., Vonshak, A., & Richmond, A. (1987). Fatty acid composition of Spirulina strains grown under various environmental conditions. *Phytochemistry*, *26*(8), 2255–2258. https://doi.org/10.1016/S0031-9422(00)84694-4

Dackiw, A. P. B., Lee, J. E., Gagel, R. F., & Evans, D. B. (2001). Adrenal cortical carcinoma. *World Journal of Surgery*, *25*(7), 914.

Davies, R. J., Holdsworth, J. E., & Reader, S. L. (1990). The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast Apiotrichum curvatum. https://doi.org/10.1007/BF00172553

De Angelis, A. A., Capitani, D., & Crescenzi, V. (1998). Synthesis and 13C CP-MAS NMR characterization of a new chitosan-based polymeric network. *Macromolecules*, *31*(5), 1595–1601.

De Greyt, W. (2013). Edible oil refining: Current and future technologies. In W. Hamm, R. J. Hamilton, & G. Calliauw (Eds.), *Edible Oil Processing* (2nd ed, pp. 127–152). Chichester, UK: John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118535202.ch5

Dodgson, K. S., & Price, R. G. (1962). A note on the determination of the ester sulphate content of sulphated polysaccharides. *Biochemical Journal*, *84*(1), 106–110. https://doi.org/10.1042/bj0840106

Dongping, E., Kai, C., Tao, C., & Hua, T. (2014). *Mortierella alpina mutant strain capable of producing high-yield arachidonic acid, and fermentation method and application thereof* (No. CN103484385 A). Retrieved from http://www.google.com/patents/CN103484385A?cl=en

Duarte, M. L., Ferreira, M. C., Marvão, M. R., & Rocha, J. (2002). An optimised method to determine the degree of acetylation of chitin and chitosan by FTIR spectroscopy. *International Journal of Biological Macromolecules*, *31*(1), 1–8. https://doi.org/https://doi.org/10.1016/S0141-8130(02)00039-9

Dueppen, D. G., Zeller, S. G., Diltz, S. I., & Driver, R. H. (2005). *Extraction and winterization of lipids from oilseed and microbial sources* (No. U.S. Patent n. 7,695,626). Washington, DC: US: Extraction and winterization of lipids from oilseed and microbial sources.

Emerit, J., Edeas, M., & Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomedicine and Pharmacotherapy*, *58*(1), 39–46. https://doi.org/10.1016/j.biopha.2003.11.004

Eroshin, V. K., Satroutdinov, A. D., Dedyukhina, E. G., & Chistyakova, T. I. (2000). Arachidonic acid production by Mortierella alpina with growth-coupled lipid synthesis. *Process Biochemistry*, *35*(10), 1171–1175. https://doi.org/http://dx.doi.org/10.1016/S0032-9592(00)00151-5

Euromonitor, a market research provider. (2006). Infant milk formula industry.

Retrieved February 24, 2016, from http://www.euromonitor.com/global-baby-food-overview-key-categories-countries-and-trends/report

Fassnacht, M., Terzolo, M., Allolio, B., Baudin, E., Haak, H., Berruti, A., … Skogseid, B. (2012). Combination Chemotherapy in Advanced Adrenocortical Carcinoma. *New England Journal of Medicine*, *366*(23), 2189–2197. https://doi.org/10.1056/NEJMoa1200966

Feofilova, E. P. (2010). The fungal cell Wall : modern concepts of its composition. *Microbiology*, *79*(6), 711–720. https://doi.org/10.1134/S0026261710060019

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*, 239. Retrieved from http://dx.doi.org/10.1038/35041687

Fischer, L., Stressler, T., Eisele, T., Rost, J., Haunschild, E., & Kuhn, A. (2013). Production of polyunsaturated fatty acids by Mortierella alpina using submerse and solid state fermentation. *Chemie Ingenieur Technik*, *85*(3), 318–322. https://doi.org/10.1002/cite.201200094

Flack, M. R., & Chrousos, G. P. (1996). Neoplasms of the adrenal cortex. *Cancer Medicine*, *1*, 1567.

Forsyth, J. S., Willatts, P., Agostoni, C., Bissenden, J., Casaer, P., & Boehm, G. (2003). Long chain polyunsaturated fatty acid supplementation in infant formula and blood pressure in later childhood: follow up of a randomised controlled trial. *BMJ : British Medical Journal*, *326*(7396), 953. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC153849/

Fukui, S. (1995). Selection and breeding of microorganisms secreting and producing fuel lipids. *Bio Industry*, *12*(3), 36–46.

Ganeshpurkar, A., Rai, G., & Jain, A. P. (2010). Medicinal mushrooms: Towards a new horizon. *Pharmacognosy Reviews*, *4*(8), 127.

Gerentes, P., Vachoud, L., Doury, J., & Domard, A. (2002). Study of a chitin-based gel as injectable material in periodontal surgery. *Biomaterials*, *23*(5), 1295–1302.

Germano, A., Rapa, I., Volante, M., Buono, N. Lo, Carturan, S., Berruti, A., … Papotti, M. (2014). Cytotoxic activity of gemcitabine, alone or in combination with mitotane, in adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *382*(1), 1–7.

Giavasis, I. (2013). 16 - Production of microbial polysaccharides for use in food. In B. McNeil, D. Archer, I. Giavasis, & L. B. T.-M. P. of F. I. Harvey Enzymes and

Nutraceuticals (Eds.), *Woodhead Publishing Series in Food Science, Technology and Nutrition* (pp. 413–468). Woodhead Publishing. https://doi.org/https://doi.org/10.1533/9780857093547.2.413

GostReports. (2016). Global arachidonic acid market research report 2016. Retrieved September 5, 2016, from http://www.gosreports.com/global-arachidonic-acid-market-worth-1-18-billion-by-2019/

Gunstone, F. D. (2001). Oilseed crops with modified fatty acid composition. *Journal of Oleo Science*, *50*(5), 269–279.

Gutierrez, R. M. P., & Ramirez, A. M. N. G. and A. M. (2012). Compounds Derived from Endophytes: A Review of Phytochemistry and Pharmacology. *Current Medicinal Chemistry*. https://doi.org/http://dx.doi.org/10.2174/092986712800672111

Hamuro, J. t. (1985). Lentinan, a T-cell oriented immunopotentiator: its experimental and clinical applications and possible mechanism of immune modulation. *Immunomodulation Agents and Their Mechanisms*.

Hansson, L., & Dostálek, M. (1988). Effect of culture conditions on mycelial growth and production of γ-linolenic acid by the fungus Mortierella ramanniana. *Applied Microbiology and Biotechnology*, *28*(3), 240–246. https://doi.org/10.1007/BF00250448

Harish Prashanth, K. V., & Tharanathan, R. N. (2007). Chitin/chitosan: modifications and their unlimited application potential-an overview. *Trends in Food Science and Technology*, *18*(3), 117–131. https://doi.org/10.1016/j.tifs.2006.10.022

Heux, L., Brugnerotto, J., Desbrières, J., Versali, M.-F., & Rinaudo, M. (2000). Solid State NMR for Determination of Degree of Acetylation of Chitin and Chitosan. *Biomacromolecules*, *1*(4), 746–751. https://doi.org/10.1021/bm000070y

Higashiyama, K., Fujikawa, S., Park, E. Y., & Shimizu, S. (2002). Production of arachidonic acid by Mortierella fungi. *Biotechnology and Bioprocess Engineering*, *7*(5), 252–262. https://doi.org/10.1007/BF02932833

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawa, S., & Shimizu, S. (1998). Enhancement of arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1501–1505. https://doi.org/10.1007/s11746-998-0085-9

Higashiyama, K., Yaguchi, T., Akimoto, K., Fujikawaa, S., & Shimizu, S. (1998). Effects

of mineral addition on the growth morphology of and arachidonic acid production by Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(12), 1815–1819. https://doi.org/10.1007/s11746-998-0336-9

Higashiyama, K., Yaguchi, T., Akimoto, K., & Shimizu, S. (2005). *Media for culturing microorganisms and process for producing unsaturated fatty acids or lipids containing the same* (No. U.S. Patent No. 6,746,857). Washington, DC: U.S.

Hirano, S., & Ohe, Y. (1975). Chitosan gels: a novel molecular aggregation of chitosan in acidic solutions on a facile acylation. *Agricultural and Biological Chemistry*, *39*(6), 1337–1338.

Ho, S.-Y., & Chen, F. (2008). Lipid characterization of Mortierella alpina grown at different NaCl concentrations. *Journal of Agricultural and Food Chemistry*, *56*(17), 7903–7909. https://doi.org/10.1021/jf801404y

Hoffman, D. R., Birch, E. E., Birch, D. G., Uauy, R., Castañeda, Y. S., Lapus, M. G., & Wheaton, D. H. (2000). Impact of early dietary intake and blood lipid composition of long-chain polyunsaturated fatty acids on later visual development. *Journal of Pediatric Gastroenterology and Nutrition*, *31*(5). Retrieved from http://journals.lww.com/jpgn/Fulltext/2000/11000/Impact_of_Early_Dietary_Intake_and_Blood_Lipid.16.aspx

Högel, H., Rantanen, K., Jokilehto, T., Grenman, R., & Jaakkola, P. M. (2011). Prolyl hydroxylase PHD3 enhances the hypoxic survival and G1 to S transition of carcinoma cells. *PloS One*, *6*(11), e27112.

Hunter, K., & Rose, A. H. (1972). Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. *Biochimica et Biophysica Acta (BBA) Lipids and Lipid Metabolism*, *260*(4), 639–653. https://doi.org/http://dx.doi.org/10.1016/0005-2760(72)90013-6

Huong, L. M., Slugen, D., & Sajbidor, J. (1998). Production of dihomo-y-linolenic acid by Mortierella alpina. *Biologia Bratislava*, *53*(6), 801–806.

Ishihara. S, Satoru, I. (2012). New Modified alpha-glucosidase useful as enzymatic agent, and in pharmaceutical composition, quasi-drug composition, cosmetics composition, foodstuff composition, and feed composition. (n° WO2012124520-A1). China.

Jang, H.-D., Lin, Y.-Y., & Yang, S.-S. (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by Mortierella alpina. *Bioresource Technology*, *96*(15), 1633–1644. https://doi.org/http://dx.doi.org/10.1016/j.biortech.2004.12.027

Jareonkitmongkol, S., Kawashima, H., & Shimizu, S. (1993). Inhibitory effects of lignan compounds on the formation of arachidonic acid in a delta-5-desaturase-defective mutant of Mortierella alpina 1s-4. *Journal of Fermentation and Bioengineering*, *76*(5), 406–407. https://doi.org/Doi 10.1016/0922-338x(93)90033-5

Jareonkitmongkol, S., Kawashima, H., Shimizu, S., & Yamada, H. (1992). Production of 5,8,11-cis-Eicosatrienoic acid by a Δ12-desaturase defective mutant of Mortierella alpina 1S-4. *J. Am. Oil Chem. Soc.*, *69*(9), 939–944.

Jareonkitmongkol, S., Sakuradani, E., & Shimizu, S. (1994). Isolation and characterization of an omega-3-desaturation-defective mutant of an arachidonic acid-producing fungus, Mortierella alpina 1s-4. *Archives of Microbiology*, *161*(4), 316–319. https://doi.org/Doi 10.1007/Bf00303586

Ji, X.-J., Zhang, A.-H., Nie, Z.-K., Wu, W.-J., Ren, L.-J., & Huang, H. (2014). Efficient arachidonic acid-rich oil production by Mortierella alpina through a repeated fed-batch fermentation strategy. *Bioresource Technology*, *170*(5), 356–360. https://doi.org/10.1016/j.biortech.2014.07.098

Jin, M.-J., Huang, H., Xiao, A.-H., Zhang, K., Liu, X., Li, S., & Peng, C. (2008). A novel two-step fermentation process for improved arachidonic acid production by Mortierella alpina. *Biotechnology Letters*, *30*(6), 1087–1091. https://doi.org/10.1007/s10529-008-9661-1

Kamada, N., Kawashima, H., Sakuradani, E., Akimoto, K., Ogawa, U., & Shimizu, S. (1999). Production of 8,11-cis-eicosadienoic acid by a delta 5 and delta 12 desaturase-defective mutant derived from the arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists Society*, *76*(11), 1269–1274. https://doi.org/Doi 10.1007/S11746-999-0138-8

Karagozlu, M. Z., Karadeniz, F., Kong, C. S., & Kim, S. K. (2012). Aminoethylated chitooligomers and their apoptotic activity on AGS human cancer cells. *Carbohydrate Polymers*, *87*(2), 1383–1389. https://doi.org/10.1016/j.carbpol.2011.09.034

Kasperlik-Zalułska, A. A., Migdalska, B. M., Zgliczyński, S., & Makowska, A. M. (1995). Adrenocortical carcinoma. A clinical study and treatment results of 52 patients. *Cancer*, *75*(10), 2587–2591.

Katano, K., & Kawashima, H. (2015). *Methods for producing polyunsaturated fatty acid and lipid containing polyunsaturated fatty acid* (No. US20150064749 A1). Suntory

Ltd, Kenichi Higashiyama.

Kawashima, H., Sakuradani, E., Kamada, N., Akimoto, K., Konishi, K., Ogawa, J., & Shimizu, S. (1998). Production of 8,11,14,17-cis-eicosatetraenoic acid (20:4ω-3) by a Δ5 and Δ12 desaturase-defective mutant of an arachidonic acid-producing fungus Mortierella alpina 1S-4. *Journal of the American Oil Chemists' Society*, *75*(11), 1495–1500. https://doi.org/10.1007/s11746-998-0084-x

Kaya, M., Baran, T., Asan-Ozusaglam, M., Cakmak, Y. S., Tozak, K. O., Mol, A., … Sezen, G. (2015). Extraction and characterization of chitin and chitosan with antimicrobial and antioxidant activities from cosmopolitan Orthoptera species (Insecta). *Biotechnology and Bioprocess Engineering*, *20*(1), 168–179. https://doi.org/10.1007/s12257-014-0391-z

Kim, G.-Y., Choi, G.-S., Lee, S.-H., & Park, Y.-M. (2004). Acidic polysaccharide isolated from Phellinus linteus enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. *Journal of Ethnopharmacology*, *95*(1), 69–76.

Kim, Y.,OH. K, and Park, R. (2008). Use of *Motierella* strain DY-52 for producing chitin deacetylase used for killing insects or nematode (no. KR2008103159-A). China.

Koike, Y., Jie Cai, H., Higashiyama, K., Fujikawa, S., & Park, E. Y. (2001). Effect of consumed carbon to nitrogen ratio of mycelial morphology and arachidonic acid production in cultures of mortierella alpina. *Journal of Bioscience and Bioengineering*, *91*(4), 382–389. https://doi.org/10.1016/S1389-1723(01)80156-0

Koletzko, B., Mrotzek, M., & Bremer, H. J. (1988). Fatty acid composition of mature human milk in Germany. *The American Journal of Clinical Nutrition*, *47*(6), 954–959.

Kono, H. (2004). Two-dimensional magic angle spinning NMR investigation of naturally occurring chitins: Precise 1H and 13C resonance assignment of α- and β-chitin. *Biopolymers*, *75*(3), 255–263. https://doi.org/10.1002/bip.20124

Koschker, A.-C., Fassnacht, M., Hahner, S., Weismann, D., & Allolio, B. (2006). Adrenocortical carcinoma-improving patient care by establishing new structures. *Experimental and Clinical Endocrinology & Diabetes*, *114*(02), 45–51.

Krecic, A. M., & Swanson, M. S. (1999). hnRNP complexes: composition, structure, and function. *Current Opinion in Cell Biology*, *11*(3), 363–371.

Kroiss, M., Plonné, D., Kendl, S., Schirmer, D., Ronchi, C. L., Schirbel, A., … Fassnacht, M. (2016). Association of mitotane with chylomicrons and serum

lipoproteins: practical implications for treatment of adrenocortical carcinoma. *European Journal of Endocrinology*, *174*(3), 343–353.

Kurita, K., Sannan, T., & Iwakura, Y. (1977). Studies on chitin, 4. Evidence for formation of block and random copolymers of N-acetyl-D-glucosamine and D-glucosamine by hetero- and homogeneous hydrolyses. *Die Makromolekulare Chemie*, *178*(12), 3197–3202. https://doi.org/10.1002/macp.1977.021781203

Kyle, D. J. (1997). *Arachidonic acid and methods for the production and use thereof* (No. 5658767). US.

Kyle, D. J. (1997). *Plant extract; vegetable oil; infant's formula.* (No. U.S. Patent No. 5,658,767). Washington, DC: U.S.

Kyle, D. J. (2001). The large-scale production and use of a single-cell oil highly enriched in docosahexaenoic acid. In F. Shahidi & J. W. Finley (Eds.), *Omega-3 Fatty Acids* (pp. 92–107). Washington, DC: ACS symposium series. https://doi.org/10.1021/bk-2001-0788.ch008

Lee, K. J., Oh, Y. C., Cho, W. K., & Ma, J. Y. (2015). Antioxidant and Anti-Inflammatory Activity Determination of One Hundred Kinds of Pure Chemical Compounds Using Offline and Online Screening HPLC Assay. *Evidence-Based Complementary and Alternative Medicine*, *2015*. https://doi.org/10.1155/2015/165457

Lehmann, T. P., Wrzesiński, T., & Jagodziński, P. P. (2013). The effect of mitotane on viability, steroidogenesis and gene expression in NCI-H295R adrenocortical cells. *Molecular Medicine Reports*, *7*(3), 893–900.

Lenardon, M. D., Munro, C. A., & Gow, N. A. R. (2010). Chitin synthesis and fungal pathogenesis. *Current Opinion in Microbiology*, *13*(4), 416–423. https://doi.org/10.1016/j.mib.2010.05.002

Letouzé, E., Rosati, R., Komechen, H., Doghman, M., Marisa, L., Flück, C., … Pianovski, M. A. D. (2012). SNP array profiling of childhood adrenocortical tumors reveals distinct pathways of tumorigenesis and highlights candidate driver genes. *The Journal of Clinical Endocrinology & Metabolism*, *97*(7), E1284–E1293.

Li, Z.-Y., Lu, Y., Yadwad, V. B., & Ward, O. P. (1995). Process for production of arachidonic acid concentrate by a strain of mortierella alpina. *The Canadian Journal of Chemical Engineering*, *73*(1), 135–139. https://doi.org/10.1002/cjce.5450730115

Lindberg, A. M., & Molin, G. (1993). Effect of temperature and glucose supply on the

production of polyunsaturated fatty acids by the fungus Mortierella alpina CBS 343.66 in fermentor cultures. *Applied Microbiology and Biotechnology*, *39*(4–5), 450–455. https://doi.org/10.1007/BF00205031

Liu, L., Liu, Y., Shin, H. D., Chen, R., Li, J., Du, G., & Chen, J. (2013). Microbial production of glucosamine and N-acetylglucosamine: Advances and perspectives. *Applied Microbiology and Biotechnology*, *97*(14), 6149–6158. https://doi.org/10.1007/s00253-013-4995-6

Lounds, C., Eagles, J., Carter, A. T., MacKenzie, D. A., & Archer, D. B. (2007). Spore germination in Mortierella alpina is associated with a transient depletion of arachidonic acid and induction of fatty acid desaturase gene expression. *Archives of Microbiology*, *188*(4), 299–305. https://doi.org/10.1007/s00203-007-0248-3

Maeda, Y. Y., Watanabe, S. T., Chihara, C., & Rokutanda, M. (1988). Denaturation and renaturation of a β-1, 6; 1, 3-glucan, lentinan, associated with expression of T-cell-mediated responses. *Cancer Research*, *48*(3), 671–675.

Mahapatra, S., & Banerjee, D. (2013). Fungal Exopolysaccharide: Production, Composition and Applications. *Microbiology Insights*, *6*, MBI.S10957. https://doi.org/10.4137/MBI.S10957

Mantero, F., Terzolo, M., Arnaldi, G., Osella, G., Masini, A. M., Alì, A., … Endocrinology, S. G. on A. T. of the I. S. of. (2000). A survey on adrenal incidentaloma in Italy. *The Journal of Clinical Endocrinology & Metabolism*, *85*(2), 637–644.

McDowell, R. E., Amsler, M. O., Li, Q., Lancaster Jr, J. R., & Amsler, C. D. (2015). The immediate wound-induced oxidative burst of Saccharina latissima depends on light via photosynthetic electron transport. *Journal of Phycology*, *51*(3), 431–441.

Michaelson, L. V, Lazarus, C. M., Griffiths, G., Napier, J. A., & Stobart, A. K. (1998). Isolation of a ⌬5 -Fatty Acid desaturase gene from Mortierella alpina. *Journal of Biological Chemistry*, *273*(30), 19055–19059.

Michalkiewicz, E., Sandrini, R., Figueiredo, B., Miranda, E. C. M., Caran, E., Oliveira-Filho, A. G., … Cristofani, L. M. (2004). Clinical and outcome characteristics of children with adrenocortical tumors: a report from the International Pediatric Adrenocortical Tumor Registry. *Journal of Clinical Oncology*, *22*(5), 838–845.

Morganti, P., Del Ciotto, P., Carezzi, F., Nunziata, M. L., & Morganti, G. (2016). A Chitin Nanofibril-based non-woven tissue as medical dressing. The role of

bionanotechnology. *Nanomaterials & Regenerative Medicine, Zagreb, Criatia, IAPC Publishing*, 123–142.

Morris, G. J., Smith, D., & Coulson, G. E. (1988). A comparative study of the changes in the morphology of hyphae during freezing and viability upon thawing for twenty species of fungi. *Microbiology*, *134*(11), 2897–2906. https://doi.org/10.1099/00221287-134-11-2897

MS/INCA. (2016). Estimativa de Câncer no Brasil. Retrieved from www.inca.gov.br/dncc

Muzzarelli, R. A. (1977). *Chitin*. Oxford, UK: Pergamon Press.

Muzzarelli, R. A. (1999). Native, industrial and fossil chitins. *EXS*, *87*, 1—6. Retrieved from http://europepmc.org/abstract/MED/10906948

Muzzarelli, R. A. A. (2009). Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*(1), 1–9.

N., J. I., Leela, I., & Prabhakara, R. A. V. S. (1988). Removal of Cadmium Using Chitosan. *Journal of Environmental Engineering*, *114*(4), 962–974. https://doi.org/10.1061/(ASCE)0733-9372(1988)114:4(962)

Nagamuna, T., Uzuka, Y., & Tanaka, K. (1985). Physiological factors affecting total cell number and lipid content of the yeast Lipomyces starkeyi. *The Journal of General and Applied Microbiology*, *31*(1), 29–37. https://doi.org/10.2323/jgam.31.29

Ng, L., & Libertino, J. M. (2003). Adrenocortical carcinoma: diagnosis, evaluation and treatment. *The Journal of Urology*, *169*(1), 5–11.

Nisha, A., Rastogi, N. K., & Venkateswaran, G. (2011). Optimization of media components for enhanced arachidonic acid production by Mortierella alpina under submerged cultivation. *Biotechnology and Bioprocess Engineering*, *16*(2), 229–237. https://doi.org/10.1007/s12257-010-0294-6

Nisha, A., & Venkateswaran, G. (2011). Effect of culture variables on mycelial arachidonic acid production by Mortierella alpina. *Food and Bioprocess Technology*, *4*(2), 232–240. https://doi.org/10.1007/s11947-008-0146-y

Nishizaki, T., Nomura, T., Matsuoka, T., & Tsujishita, Y. (1999). Arachidonic acid as a messenger for the expression of long-term potentiation. *Biochemical and Biophysical Research Communications*, *254*(2), 446–449. https://doi.org/http://dx.doi.org/10.1006/bbrc.1998.9961

No, H. K., & Meyers, S. P. (1995). Preparation and Characterization of Chitin and

Chitosan—A Review. *Journal of Aquatic Food Product Technology*, *4*(2), 27–52. https://doi.org/10.1300/J030v04n02_03

Nozaki, H., Itonori, S., Sugita, M., Nakamura, K., Ohba, K., Suzuki, A., & Kushi, Y. (2008). Mushroom acidic glycosphingolipid induction of cytokine secretion from murine T cells and proliferation of NK1. 1 α/β TCR-double positive cells in vitro. *Biochemical and Biophysical Research Communications*, *373*(3), 435–439.

Oliveira, V., & Smallwood, D. (2011). *The infant formula market: consequences of a change in the WIC contract brand* (No. ERR-124). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011a). *Culturing mortierella in nutrient broth containing saccharified starch and recovering gamma-linolenic, dihomo-gamma-linolenic, arachidonic, eicosapentaenoic, mead, 6, 9-octadecadienoic and 8, 11-eicosadienoic acids* (No. U.S. Patent No. 7,863,024). U.S.

Ono, K., Aki, T., & Higashiyama, K. (2011b). *Process for producing highly unsaturated fatty acid-containing lipid* (No. EP1498488 B1). Suntory Ltd, Kenichi Higashiyama.

Oregon State University. (2014). Essential fatty acids. Retrieved March 3, 2016, from http://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids

Packter, N. M. (1981). Lipid biochemistry of fungi and other organisms. *Biochemical Education*, *9*(4), 156. https://doi.org/http://dx.doi.org/10.1016/0307-4412(81)90142-4

Pal, J., Verma, H. O., Munka, V. K., Maurya, S. K., Roy, D., & Kumar, J. (n.d.). Biological method of chitin extraction from shrimp waste an eco-friendly low cost technology and its advanced application.

Paradossi, G., & Lisi, R. (1996). New chemical hydrogels based on poly (vinyl alcohol). *Journal of Polymer …*, 3417–3425. https://doi.org/10.1002/pola.1996.874

Park, E. Y., Koike, Y., Higashiyama, K., Fujikawa, S., & Okabe, M. (1999). Effect of nitrogen source on mycelial morphology and arachidonic acid production in cultures of Mortierella alpina. *Journal of Bioscience and Bioengineering*, *88*(1), 61–67. https://doi.org/10.1016/S1389-1723(99)80177-7

Park, Y., Tamura, S., Koike, Y., Toriyama, M., & Okabe, M. (1997). Mycelial pellet intrastructure visualization and viability prediction in a culture of Streptomyces fradiae using confocal scanning laser microscopy. *Journal of Fermentation and Bioengineering*, *84*(5), 483–486. https://doi.org/http://dx.doi.org/10.1016/S0922-338X(97)82014-4

Peng, C., Huang, H., Ji, X., Liu, X., You, J., Lu, J., … Ouyang, P. (2010). A temperature-shift strategy for efficient arachidonic acid fermentation by Mortierella alpina in batch culture. *Biochemical Engineering Journal*, *53*(1), 92–96. https://doi.org/10.1016/j.bej.2010.09.014

Pianovski, M. A. D., Maluf, E. M. C. P., de Carvalho, D. S., Ribeiro, R. C., Rodriguez-Galindo, C., Boffetta, P., … Figueiredo, B. C. (2006). Mortality rate of adrenocortical tumors in children under 15 years of age in Curitiba, Brazil. *Pediatric Blood & Cancer*, *47*(1), 56–60.

Pick, E., & Mizel, D. (1981). Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods*, *46*(2), 211–226.

Pipe, R. K., Coles, J. A., & Farley, S. R. (1995). Assays for measuring immune response in the mussel Mytilus edulis. *Tech Fish Immunol*, *4*, 93–100.

Plager, J. E. (1984). Carcinoma of the adrenal cortex: clinical description, diagnosis, and treatment. *International Advances in Surgical Oncology*, *7*, 329–353.

Poirier, M., & Charlet, G. (2002). Chitin fractionation and characterization in N,N-dimethylacetamide/lithium chloride solvent system. *Carbohydrate Polymers*, *50*(4), 363–370. https://doi.org/https://doi.org/10.1016/S0144-8617(02)00040-1

Poli, G., Guasti, D., Rapizzi, E., Fucci, R., Canu, L., Bandinelli, A., … Luconi, M. (2013). Morphofunctional effects of mitotane on mitochondria in human adrenocortical cancer cells. *Endocrine-Related Cancer*, *20*(4), 537–550.

Puspawati, N. M., & Simpen, I. N. (2010). Optimasi deasetilasi khitin dari kulit udang dan cangkang kepiting limbah restoran seafood menjadi khitosan melalui variasi konsentrasi NaOH. *Jurnal Kimia*, *4*(1). Retrieved from https://ojs.unud.ac.id/index.php/jchem/article/view/2760

QY Research center. (2015). *Global arachidonic acid industry Report 2015*. Retrieved from http://www.qyresearch.com/english/goods.php?id=45013

Rajamohan, S. B., Raghuraman, G., Prabhakar, N. R., & Kumar, G. K. (2012). NADPH oxidase-derived H2O2 contributes to angiotensin II-induced aldosterone synthesis in human and rat adrenal cortical cells. *Antioxidants & Redox Signaling*, *17*(3), 445–459.

Rane, K. D., & Hoover, D. G. (1993). An evaluation of alkali and acid treatments for

chitosan extraction from fungi. *Process Biochemistry*, *28*(2), 115–118. https://doi.org/https://doi.org/10.1016/0032-9592(93)80016-A

Rathke, T. D., & Hudson, S. M. (1994). Review of Chitin and Chitosan as Fiber and Film Formers. *Journal of Macromolecular Science, Part C*, *34*(3), 375–437. https://doi.org/10.1080/15321799408014163

Ratledge, C. (2001). Microorganisms as sourcs of polyunsaturated fatty acids. *Structured and Modified Lipids*, 351–399.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie*, *86*, 807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Ratledge, C. (2013). Microbial oils: an introductory overview of current status and future prospects. *OCL*, *20*(6), D602. https://doi.org/10.1051/ocl/2013029

Ratledge, C., & Hopkins, S. (2006). Lipids from microbial sources. In F. D. Gunstone (Ed.), *Modyfing lipids for use in food.* (pp. 80–113). Department of Biological Sciences, University of Hull, UK.

Ravi Kumar, M. N. V. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymers*, *46*(1), 1–27. https://doi.org/10.1016/S1381-5148(00)00038-9

Rinaudo, M. (2006). Chitin and chitosan: Properties and applications. *Progress in Polymer Science (Oxford)*, *31*(7), 603–632. https://doi.org/10.1016/j.progpolymsci.2006.06.001

Rizk-Rabin, M., Assie, G., Rene-Corail, F., Perlemoine, K., Hamzaoui, H., Tissier, F., … Bouizar, Z. (2008). Differential Expression of Parathyroid Hormone–Related Protein in Adrenocortical Tumors: Autocrine/Paracrine Effects on the Growth and Signaling Pathways in H295R Cells. *Cancer Epidemiology and Prevention Biomarkers*, *17*(9), 2275–2285.

Roberg, K. (2001). Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. *Laboratory Investigation*, *81*(2), 149.

Roberts, G. A. F. (1992a). *Chitin chemistry*. Macmillan International Higher Education.

Roberts, G. A. F. (1992b). Solubility and Solution Behaviour of Chitin and Chitosan BT - Chitin Chemistry. In G. A. F. Roberts (Ed.) (pp. 274–329). London: Macmillan Education UK. https://doi.org/10.1007/978-1-349-11545-7_6

Rocky-salimi, K., Hamidi-esfahani, Z., & Abbasi, S. (2011). Statistical optimization of

arachidonic acid production by Mortierella alpina CBS 754 . 68 in submerged fermentation. *Iranian Journal of Biotechnology*, *9*(2), 87–93.

Ruiter, G. A. D. E., Lugt, A. W. V. A. N. B. D. E. R., & Rombouts, F. M. (1993). Approaches to the classification of the Mortievella isabellina group : antigenic extracellular polysaccharides. *Mycological Research (An International Journal of Fungal Biology)*, *97*(6), 690–696. https://doi.org/10.1016/S0953-7562(09)80149-4

Rumengan, I. F. M., Suryanto, E., Modaso, R., Wullur, S., Tallei, T. E., & Limbong, D. (2014). Structural Characteristics of Chitin and Chitosan Isolated from the Biomass of Cultivated Rotifer, Brachionus rotundiformis. *International Journal of Fisheries and Aquatic Sciences*, *3*(1), 12–18. https://doi.org/10.1016/j.ejphar.2011.01.031

Saccardo, P. A., Traverso, G. B., & Trotter, A. (n.d.). *Sylloge fungorum omnium hucusque cognitorum. Digessit P. A. Saccardo.* (Vol. v.1). Patavii,sumptibus auctoris,. Retrieved from http://www.biodiversitylibrary.org/item/102968

Saitô, H., Tabeta, R., & Hirano, S. (1981). CONFORMATION OF CHITIN AND N-ACYL CHITOSANS IN SOLID STATE AS REVEALED BY 13C CROSS POLARIZATION/MAGIC ANGLE SPINNING (CP/MAS) NMR SPECTROSCOPY. *Chemistry Letters*, *10*(10), 1479–1482. https://doi.org/10.1246/cl.1981.1479

Šajbidor, J., Dobroňová, S., & Čert\'ik, M. (1990). Arachidonic acid production byMortierella sp. S-17 influence of C/N ratio. https://doi.org/10.1007/BF01024404

Sajbidor, J., Kozelouhova, D., & Certik, M. (1992). Influence of some metal-ions on the lipid-content and arachidonic-acid production by Mortierella sp. *Folia Microbiologica*, *37*(6), 404–406.

Sakuradani, E., Ando, A., Shimizu, S., & Ogawa, J. (2013). Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus Mortierella alpina 1S-4. *Journal of Bioscience and Bioengineering*, *116*(4), 417–422. https://doi.org/10.1016/j.jbiosc.2013.04.008

Sakuradani, E., Hirano, Y., Kamada, N., Nojiri, M., Ogawa, J., & Shimizu, S. (2004). Improvement of arachidonic acid production by mutants with lower n-3 desaturation activity derived from Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *66*(3), 243–248. https://doi.org/10.1007/s00253-004-1682-7

Sakuradani, E., Kamada, N., Hirano, Y., Nishihara, M., Kawashima, H., Akimoto, K., … Shimizu, S. (2002). Production of 5,8,11-eicosatrienoic acid by a Δ5 and Δ6

desaturation activity-enhanced mutant derived from a Δ12 desaturation activity-defective mutant of Mortierella alpina 1S-4. *Applied Microbiology and Biotechnology*, *60*(3), 281–287. https://doi.org/10.1007/s00253-002-1128-z

Schenzel, K., & Fischer, S. (2001). NIR FT Raman Spectroscopy–a Rapid Analytical Tool for Detecting the Transformation of Cellulose Polymorphs. *Cellulose*, *8*(1), 49–57. https://doi.org/10.1023/A:1016616920539

Seviour, R. J., Stasinopoulos, S. J., Auer, D. P. F., & Gibbs, P. A. (1992). Production of pullulan and other exopolysaccharides by filamentous fungi. *Critical Reviews in Biotechnology*, *12*(3), 279–298.

Shimada, Y., Sugihara, A., Nakano, H., & Kuramoto, T. (1997). Purification of docosahexaenoic acid by selective esterification of fatty acids from Tuna oil with Rhizopus delemar lipase. *Journal of the American Oil Chemists Society*, *74*(2), 97–101.

Shimizu, S., & Jareonkitmongkol, S. (1995). Mortierella Species (Fungi): Production of C20 Polyunsaturated Fatty Acids. In Y. P. S. Bajaj (Ed.), *Medicinal and Aromatic Plants* (pp. 308–325). Berlin: Springer. https://doi.org/10.1007/978-3-662-08612-4_17

Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., & Yamada, H. (1988). Production of eicosapentaenoic acid by Mortierella fungi. *Journal of the American Oil Chemists' Society*, *65*(9), 1455–1459. https://doi.org/10.1007/BF02898307

Shinmen, Y., & Shimizu, S. (1989). Production of arachidonic acid by Mortierella fungi. *Applied Microbiology*, *31*(1), 11–16. https://doi.org/10.1007/BF00252518

Shu, C.-H., & Lung, M.-Y. (2004). Effect of pH on the production and molecular weight distribution of exopolysaccharide by Antrodia camphorata in batch cultures. *Process Biochemistry*, *39*(8), 931–937. https://doi.org/10.1016/S0032-9592(03)00220-6

Sim Choi, H., Woo Kim, J., Cha, Y., & Kim, C. (2006). A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of Immunoassay and Immunochemistry*, *27*(1), 31–44.

Singh, A., & Ward, P. O. (1997). Production of high yields of arachidonic acid in a fed-batch system by Mortierella alpina ATCC 32222. *Applied Microbiology and Biotechnology*, *48*(1), 1–5. https://doi.org/10.1007/s002530051005

Sokmen, A., Gulluce, M., Askin Akpulat, H., Daferera, D., Tepe, B., Polissiou, M., …

Sahin, F. (2004). The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control*, *15*(8), 627–634. https://doi.org/https://doi.org/10.1016/j.foodcont.2003.10.005

Song, K.-S., Li, G., Kim, J.-S., Jing, K., Kim, T.-D., Kim, J.-P., … Hwang, B.-D. (2011). Protein-bound polysaccharide from Phellinus linteus inhibits tumor growth, invasion, and angiogenesis and alters Wnt/β-catenin in SW480 human colon cancer cells. *BMC Cancer*, *11*(1), 307.

Srigley, C. T., & Rader, J. I. (2014). Content and composition of fatty acids in marine oil omega-3 supplements. *Journal of Agricultural and Food Chemistry*, *62*(29), 7268–7278. https://doi.org/10.1021/jf5016973

Steiner, P. E. (1954). *Cancer: Race and geography: some etiological, environmental, ethnological, epidemiological, and statistical aspects in Caucasoids, Mongoloids, Negroids, and Mexicans*. Williams & Wilkins Company.

Strajhar, P., Tonoli, D., Jeanneret, F., Imhof, R. M., Malagnino, V., Patt, M., … Odermatt, A. (2017). Steroid profiling in H295R cells to identify chemicals potentially disrupting the production of adrenal steroids. *Toxicology*, *381*, 51–63.

Stredanská, S., & Šajbidor, J. (1993). Influence of carbon and nitrogen sources on the lipid accumulation and arachidonic acid production by Mortierella alpina. *Acta Biotechnologica*, *13*(2), 185–191. https://doi.org/10.1002/abio.370130218

Sun, C., Wang, J.-W., Fang, L., Gao, X.-D., & Tan, R.-X. (2004). Free radical scavenging and antioxidant activities of EPS2, an exopolysaccharide produced by a marine filamentous fungus Keissleriella sp. YS 4108. *Life Sciences*, *75*(9), 1063–1073. https://doi.org/https://doi.org/10.1016/j.lfs.2004.02.015

Taki, H., Ohishi, K., Okano, A., Suga, T., Akiyama, Y., & Yamashita, A. (1995). Antimetastatic effect of lentinan on liver metastasis of colon carcinoma (colon26): possible role of activated kupffer cells. *International Journal of Immunotherapy*, *11*(1), 29–38.

Tanner, S. F., Chanzy, H., Vincendon, M., Roux, J. C., & Gaill, F. (1990). High-resolution solid-state carbon-13 nuclear magnetic resonance study of chitin. *Macromolecules*, *23*(15), 3576–3583. https://doi.org/10.1021/ma00217a008

Terzolo, M., Angeli, A., Fassnacht, M., Daffara, F., Tauchmanova, L., Conton, P. A., … Grossrubatscher, E. (2007). Adjuvant mitotane treatment for adrenocortical

carcinoma. *New England Journal of Medicine*, *356*(23), 2372–2380.

Thakur, M. P., & Singh, H. K. (2013). Mushrooms, their bioactive compounds and medicinal uses: A review. *Medicinal Plants-International Journal of Phytomedicines and Related Industries*, *5*(1), 1–20.

Tomasetti, C., Li, L., & Vogelstein, B. (2017). Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science*, *355*(6331), 1330–1334.

Totani, N., Hyodo, K., & Ueda, T. (2000). Minerals Essential for Growth of the Filamentous Fungus, Mortierella alpina. *Journal of Japan Oil Chemists' Society*, *49*(5), 487–493,515. https://doi.org/10.5650/jos1996.49.487

Totani, N., Someya, K., & Oba, K. (1992). Industrial production of arachidonic acid by Mortierella. In D. J. Kyle & C. Ratledge (Eds.), *Industrial Applications of Single Cell Oils* (pp. 52–60). Illinois: AOCS Publishing.

Totani, N., Suzaki, K., & Kudo, T. (1992). *Process for the production of arachidonic acid-containing lipids* (No. EP0223960 B1). Lion Corporation.

Totani, N., Watanabe, A., & Oba, K. (1987). An improved method of arachidonic acid production by Mortierella alpina. *Yukagaku*, *36*(5), 328–331. Retrieved from http://cat.inist.fr/?aModele=afficheN&cpsidt=8169393

Tsai, G. J., Su, W. H., Chen, H. C., & Pan, C. L. (2002). Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. *Fisheries Science*, *68*(1), 170–177. https://doi.org/10.1046/j.1444-2906.2002.00404.x

Uauy, R., Mena, P., & Rojas, C. (2000). Essential fatty acids in early life : structural and functional role. *Proceedings of the Nutrition Society*, *59*, 3–15. https://doi.org/10.1017/S0029665100000021

Vadivelan, G., & Venkateswaran, G. (2014). Production and enhancement of omega-3 fatty acid from Mortierella alpina CFR-GV15: its food and therapeutic application. *BioMed Research International*, *2014*, 657414. https://doi.org/10.1155/2014/657414

Vali, S. R., Sheng, H. Y., & Ju, Y. (2003). An efficient method for the purification of arachidonic acid from fungal single-cell oil (ARASCO). *Journal of the American Oil Chemists Society*, *80*(7), 725–730.

Vandamme, E., & Revuelta, J. L. (2016). Vitamins, biopigments, antioxidants and related compounds: a historical, physiological and (Bio) technological perspective.

In E. Vandamme & J. L. Revuelta (Eds.), *Industrial biotechnology of vitamins, biopigments, and antioxidants* (pp. 1–14). Weinheim, Germany: WILEY-VCH Verlag.

Vannucci, L., Krizan, J., Sima, P., Stakheev, D., Caja, F., Rajsiglova, L., … Saieh, M. (2013). Immunostimulatory properties and antitumor activities of glucans. *International Journal of Oncology*, *43*(2), 357–364.

Vårum, K. M., Anthonsen, M. W., Grasdalen, H., & Smidsrød, O. (1991). 13C-N.m.r. studies of the acetylation sequences in partially N-deacetylated chitins (chitosans). *Carbohydrate Research*, *217*, 19–27. https://doi.org/https://doi.org/10.1016/0008-6215(91)84113-S

Vassilopoulou-Sellin, R., & Schultz, P. N. (2001). Adrenocortical carcinoma: clinical outcome at the end of the 20th century. *Cancer: Interdisciplinary International Journal of the American Cancer Society*, *92*(5), 1113–1121.

Vinsova, J., & Vavrikova, E. (2011). Chitosan Derivatives with Antimicrobial, Antitumour and Antioxidant Activities - a Review. *Current Pharmaceutical Design*, *17*(32), 3596–3607. https://doi.org/10.2174/138161211798194468

Wajchenberg, B. L., Albergaria Pereira, M. A., Medonca, B. B., Latronico, A. C., Carneiro, P. C., Ferreira Alves, V. A., … Kirschner, M. A. (2000). Adrenocortical carcinoma: clinical and laboratory observations. *Cancer*, *88*(4), 711–736.

Walker, S. J. (2018). Major spoilage micro-organisms in milk and dairy products. *International Journal of Dairy Technology*, *41*(4), 91–92. https://doi.org/10.1111/j.1471-0307.1988.tb00606.x

Wang, G. (2014). Human Antimicrobial Peptides and Proteins. *Pharmaceuticals* . https://doi.org/10.3390/ph7050545

Wang, T., & Rainey, W. E. (2012). Human adrenocortical carcinoma cell lines. *Molecular and Cellular Endocrinology*, *351*(1), 58–65.

Wang, Y., Zhao, H., Miao, X., Liu, D., Jiang, H., Liu, P., … Yin, H. (2013). Structural determination and antitumor activities of a water-soluble polysaccharide from Mortierella hepiali. *Fitoterapia*, *86*, 13–18. https://doi.org/10.1016/j.fitote.2013.01.001

Wang, Z. M., Cheung, Y. C., Leung, P. H., & Wu, J. Y. (2010). Ultrasonic treatment for improved solution properties of a high-molecular weight exopolysaccharide produced by a medicinal fungus. *Bioresource Technology*, *101*(14), 5517–5522.

https://doi.org/10.1016/j.biortech.2010.01.134

Wientjes, R. H. W., Duits, M. H. G., Jongschaap, R. J. J., & Mellema, J. (2000). Linear rheology of guar gum solutions. *Macromolecules*, *33*(26), 9594–9605.

Wilhelm, S., Wagner, H., & Häcker, G. (1998). Activation of caspase-3-like enzymes in non-apoptotic T cells. *European Journal of Immunology*, *28*(3), 891–900.

Winter, H. H., & Chambon, F. (1986). Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. *Journal of Rheology*, *30*(2), 367–382.

Wu, T., Zivanovic, S., Draughon, F. A., Conway, W. S., & Sams, C. E. (2005). Physicochemical Properties and Bioactivity of Fungal Chitin and Chitosan. *Journal of Agricultural and Food Chemistry*, *53*(10), 3888–3894. https://doi.org/10.1021/jf048202s

Wynn, J. P., Hamid, A. A., Li, Y., & Ratledge, C. (2001). Biochemical events leading to the diversion of carbon into storage lipids in the oleaginous fungi Mucor circinelloides and Mortierella alpina. *Microbiology*, *147*(10), 2857–2864.

Wynn, J. P., & Ratledge, C. (2005). Microbial production of oils and fats. In K. Shetty, G. Paliyath, A. Pometto, & E. Levin (Eds.), *Food Biotechnology* (2nd ed., pp. 443–472). NY: CRC Press.

Xian, M., Yan, J., Kang, Y., Liu, J., Bi, Y., & Zhen, K. (2001). Production of c-linolenic acid by Mortierella isabellina grown on hexadecanol. *Letters in Applied Microbiology*, *33*, 367–370. https://doi.org/10.1046/j.1472-765X.2001.00924.x

Xie, W., Xu, P., & Liu, Q. (2001). Antioxidant activity of water-soluble chitosan derivatives. *Bioorganic & Medicinal Chemistry Letters*, *11*(13), 1699–1701. https://doi.org/https://doi.org/10.1016/S0960-894X(01)00285-2

Xing, R., Yu, H., Liu, S., Zhang, W., Zhang, Q., Li, Z., & Li, P. (2005). Antioxidant activity of differently regioselective chitosan sulfates in vitro. *Bioorganic & Medicinal Chemistry*, *13*(4), 1387–1392. https://doi.org/https://doi.org/10.1016/j.bmc.2004.11.002

Yamamoto, T., Yamashita, T., & Tsubura, E. (1981). Inhibition of pulmonary metastasis of Lewis lung carcinoma by a glucan, Schizophyllan. *Invasion & Metastasis*, *1*(1), 71–84.

Yamauchi, A., Nagao, T., & Watanabe, Y. (2005). Purification of arachidonic acid from Mortierella single-cell oil by selective esterification with Burkholderia cepacia lipase. *Journal of the American Oil Chemists Society*, *82*(11), 833–837.

Yamauchi, R., Tatsumi, Y., Asano, M., Kato, K., & Ueno, Y. (1988). Effect of Metal Salts and Fructose on the Autoxidation of Methyl Linoleate in Emulsions. *Agricultural and Biological Chemistry*, *52*(3), 849–850. https://doi.org/10.1271/bbb1961.52.849

Yan, Y., Li, X., Wang, G., Gui, X., Li, G., Su, F., … Liu, T. (2014). Biotechnological preparation of biodiesel and its high-valued derivatives: a review. *Applied Energy*, *113*, 1614–1631. https://doi.org/10.1016/j.apenergy.2013.09.029

Ye, S., Liu, F., Wang, J., Wang, H., & Zhang, M. (2012). Antioxidant activities of an exopolysaccharide isolated and purified from marine Pseudomonas PF-6. *Carbohydrate Polymers*, *87*(1), 764–770. https://doi.org/https://doi.org/10.1016/j.carbpol.2011.08.057

Yokochi, T., Kamisaka, Y., Nakahara, T., & Suzuki, O. (1995). Production of γ-Linolenic acid containing phospholipids by Mortierella ramanniana in a fed-batch culture of decane. *Journal of Japan Oil Chemists' Society*, *44*(1), 9–15. https://doi.org/10.5650/jos1956.44.9

Younes, I., & Rinaudo, M. (2015). Chitin and chitosan preparation from marine sources. Structure, properties and applications. *Marine Drugs*, *13*(3), 1133–1174. https://doi.org/10.3390/md13031133

Yu, L. J., Qin, W. M., Lan, W. Z., Zhou, P. P., & Zhu, M. (2003). Improved arachidonic acids production from the fungus Mortierella alpina by glutamate supplementation. *Bioresource Technology*, *88*(3), 265–268. https://doi.org/http://dx.doi.org/10.1016/S0960-8524(02)00312-7

Yuan, C., Wang, X., & Yu, Z. (2007). Separation and preparative purification of arachidonic acid from microbial lipids by urea inclusion reaction and HPLC. *Preparative Biochemistry and Biotechnology*, *37*(2), 149–159. https://doi.org/10.1080/10826060701199098

Zaidman, B.-Z., Yassin, M., Mahajna, J., & Wasser, S. P. (2005). Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Applied Microbiology and Biotechnology*, *67*(4), 453–468.

Zhang, M., Chen, H., Huang, J., Li, Z., Zhu, C., & Zhang, S. (2005). Effect of lycium barbarum polysaccharide on human hepatoma QGY7703 cells: inhibition of proliferation and induction of apoptosis. *Life Sciences*, *76*(18), 2115–2124.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor

polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology*, *18*(1), 4–19.

Zhiming, W., Huan, L., & Xu, J. (2014). *Method for producing arachidonic acid grease by utilizing mortierella alpina mutant strain, and arachidonic acid grease produced by method* (No. CN 103571896 A).

Zhu, M., Yu, L. J., Liu, Z., & Xu, H. B. (2004). Isolating Mortierella alpina strains of high yield of arachidonic acid. *Letters in Applied Microbiology*, *39*(4), 332–335. https://doi.org/10.1111/j.1472-765X.2004.01581.x