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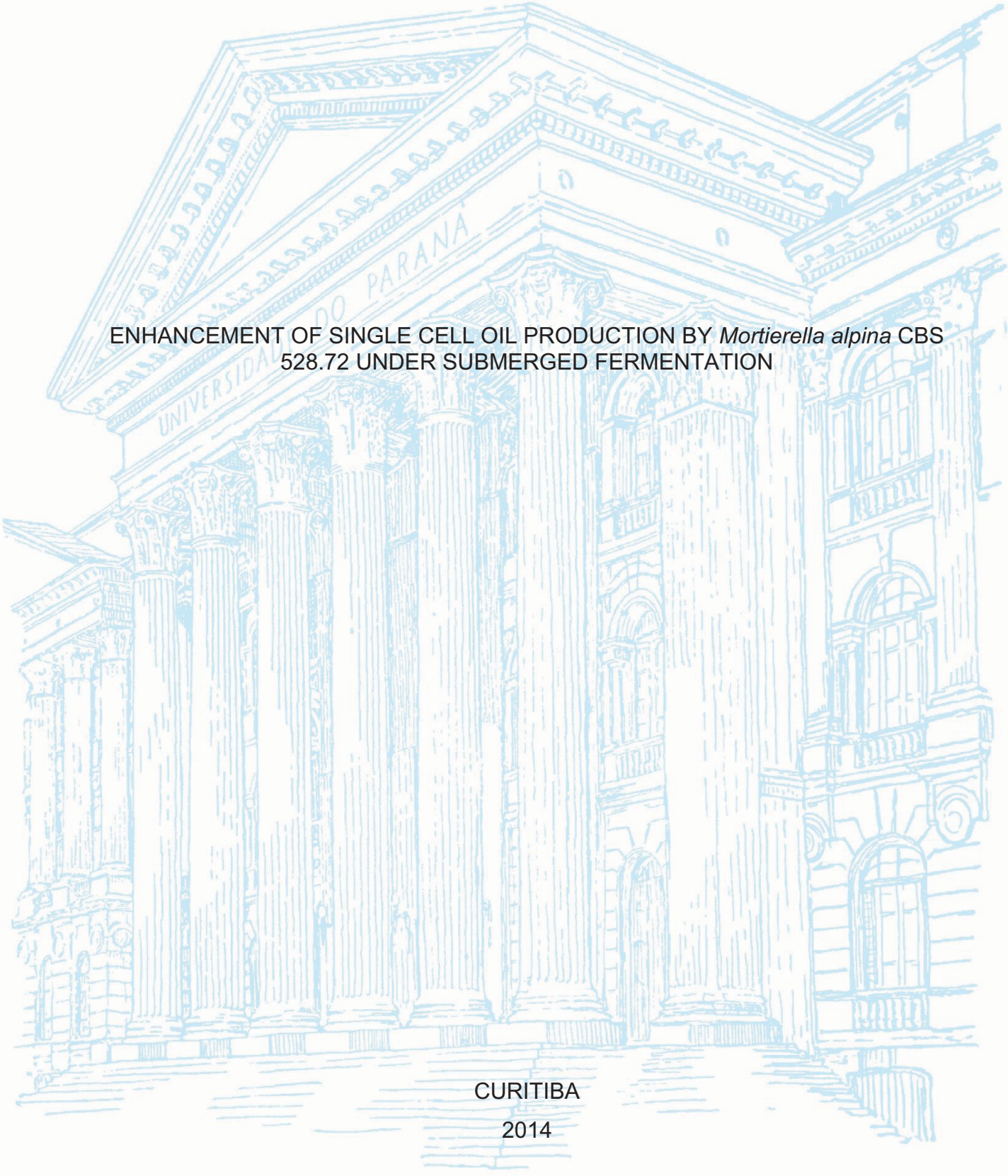

ENHANCEMENT OF SINGLE CELL OIL PRODUCTION BY *Mortierella alpina* CBS 528.72 UNDER SUBMERGED FERMENTATION

CURITIBA

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Dissertação apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Prof. Dr. Júlio Cesar de Carvalho.

Coorientador: Prof. Dr. Carlos Ricardo Soccol.

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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 Dissertação de Mestrado de **Luis Daniel Goyzueta Mamani** intitulada: " ENHANCEMENT OF SINGLE CELL OIL PRODUCTION BY *Mortierella alpina* CBS 528.72 UNDER SUBMERGED FERMENTATION", 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 mestre está sujeita à homologação do Colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e a pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 18 de Dezembro de 2014.

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To my family, especially to my parents for their unconditional faith, support and love.

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RESUMO

A *Mortierella alpina spp*. É conhecida como produtora de ácidos graxos poli-insaturados (PUFAS), particularmente do ácido araquidônico, porem as melhores condições para uma ótima produção ainda não estão bem definidas. Por esse motivo, a produção de grandes quantidades de biomassa ricas em lipídeos é o principal objetivo dessa pesquisa. Duas linhagens produtoras de lipídeos catalogadas na literatura de *M. alpina* (CBS 210.32 e CBS 528.72) foram avaliadas. A linhagem CBS 528.72 foi selecionada para futuros testes devido ao seu alto rendimento em lipídeos totais. Um método apropriado de inoculação foi desenvolvido utilizando um agitador mecânico. A influência de fontes de carbono, nitrogênio e parâmetros de fermentação foram estudados usando um desenho experimental de um-fator-de-cada-vez, determinando que entre as fontes de carbono e nitrogênio usadas, a glucose e o extrato de levedura foram os mais adequados. A temperatura de 25°C, pH 6 e uma taxa de inoculação de 10% (v/v) mostraram ser apropriadas para um correto desenvolvimento da biomassa e produção de lipídeos. Um meio base foi otimizado usando um desenho experimental de dois passos: a influência de nove sais, em um determinado rango de concentrações foram avaliadas usando um desenho Plackett-Burman; demonstrando a significância só do sal $CaCl_2$ e um modelo Composto Central Rotacional foi usado para otimizar as quantidades mínimas necessárias de $CaCl_2$ e extrato de levedura, atingindo uma concentração de 23.49 g/L de biomassa seca e 47.32& de lipídeos totais sob a condições antes mencionadas. Um pesquisa posterior a nível de biorreator foi realizadas usando 5L de volume útil de fermentação, obtendo 15.5 g/L de biomassa seca e 52.1% de lipídeos totais por batelada, e finalmente o uso de resíduos agroindustriais também foram avaliados como potenciais fontes de substrato, observando a vantagem do uso de polpa cítrica e resíduos do processa de batata chips.

Palavras-chave: *Mortierella alpina spp*. Ácidos graxos poli-insaturados. Plackett-Burman. Modelo composto central rotacional.

ABSTRACT

Mortierella alpina spp. is known as a producer of polyunsaturated fatty acids (PUFAs), arachidonic acid in particular, although the best conditions for production are not yet a consensus. For this reason, the production of high amounts of biomass with high lipids yield was the aim of this research. Two reportedly good lipid producer strains of *M. alpina* (CBS 210.32 and CBS 528.72) were evaluated. The strain CBS 528.72 was further selected because of its higher total lipids yield. A suitable inoculation method was developed using a mechanical mixer. The influence of carbon sources, nitrogen sources and fermentation parameters were studied using one-step-at-a-time experimental design, determining that among the common, industrial carbon and nitrogen sources used, glucose and yeast extract were the most suitable. A temperature of 25ºC, pH 6 and an inoculation rate of 10% (V/V) showed to be suitable conditions for biomass and lipids production. A base medium was optimized using a two-step experimental design: the influence of nine salts in a carefully chosen range was evaluated, using a Plackett-Burman design. It was demonstrated that only $CaCl_2$ needed to be further evaluated, and a rotational central composite design was used for the optimization of the $CaCl_2$ and yeast extract levels relative to glucose, reaching 23.49 g/L of dry cellular weight and 47.32% of total lipids under optimized conditions. A further bioreactor scale assay was performed using 5L of fermentation volume obtaining 15.5 g/L of biomass and 52.1% of total lipids per batch and finally the using of residual agroindustrial wasters as alternative substrate was evaluated, observing the convenience of using soy bean hulls and potato chips waste.

Keywords: *Mortierella alpina spp.* Polyunsaturated fatty acids. Plackett-Burman, Rotational central composite design.

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LIST OF ABREVIATIONS

ACC	- Acetyl CoA Carboxylase
ARA	- Arachidonic Acid
CBS	- Centraal Bureau voor Schimmelcultures
CFU	- Colony-Forming Unit
CCRD	- Central Composite Rotatable Design
DCW	- Dry Cell Weight
DGLA	- Dihomo-y-Linolenic Acid
DHA	- Docosahexaenoic Acid
EPA	- Eicosapentaenoic Acid
FAO	- Food and Agriculture Organization of the United Nations
FAME	- Fatty Acid Methyl Ester
FDA	- Food and Drug Administration
GLA	- Gamma-Linolenic Acid
GRAS	- Generally Recognized as Safe
GY	- Glucose Yeast extract medium
HD	- Hansson & Dostalek medium
ME	- Malic Enzyme
M. alpina	- *Mortierella alpina*
OFAT	- One Factor At a Time
PB	- Plackett-Burman design
PDA	- Potato Dextrose Agar
PUFA	- Polyunsaturated Fatty Acid
SCO	- Single Cell Oil
UFPR	- Universidade Federal do Paraná
USA	- United States of America
VVM	- Volume [of air] per Volume [of culture medium] per Minute
WHO	- World Health Organization

SUMMARY

1 INTRODUCTION

The use of single cell oil (SCO) as source of lipids has increased compared to that of animal and vegetables sources, because of its economic advantages (RATLEDGE, 1992). There are many different microorganisms that are capable to produce more than 40% (w/w) of total lipids, with an elevated proportion of polyunsaturated fatty acids (PUFAs).

Single cell oil is the term used for identifying lipids of single celled entities. This oil is suitable for utilization in foods or in other products where animal or plant oils are commonly used. It is necessary to remark that just some organisms can accumulate lipids; producers of about 20% of the dry cell biomass are considered oleaginous (mostly yeast and fungi), there are some microorganisms that accumulate up to 70% of lipids of the dry biomass (POMETTO *et al*., 2005).

SCO production a high valued material. The price of the oil can vary from USD 0.30/kg to over USD 100.00/kg, and even more if it is composed for individual fatty acids (GUSTONE *et al.*, 2001). Since the production of single cell oils and its interest in the commercial development, PUFAs have been intensely studied in the last twenty years (RATLEDGE, 2001). These fatty acids are currently on demand as dietary supplements, for adults and infants. PUFA's are now well known for their benefits in health: prevention of coronary heart disease, retinal and brain issues; as their use for biosynthesis of bioactive molecules (SERHAN, 2005).

PUFAs are divided in two main groups: the ω-6 and ω-3 fatty acids (depending on the position of the final double bound in the acyl chain). These two classes play an important role in brain and neural development. Docosahexaenoic acid (DHA) and Arachidonic acid (ARA) from microorganisms are preferable for addition in infant formulas instead animal sources, such as fish, due to the possibility of existence of high amounts of dioxins, heavy metals and other toxic substances from the ingest of the fishes (VADIVELAN & VENKATESWARAN, 2014).

Among the different PUFA's (GLA, ARA, EPA, DHA) all but GLA can be obtained from both animal and microbial sources; however, the production of oils from animal sources is extensive and expensive. For this reason, the microbial production is an alternative.

One of the common oils produced is the one rich in arachidonic acid (ARASCO™) and docosahexaenoic acid (DHASCO™) (WYNN AND RATLEDGE, 2006). This mixture is commercialized in Europe, Asia, Australia and North America as ingredient of infant formulae. FDA granted the GRAS status to DHA/ARA-SCO in May of 2001, for its use in formulae in the USA. An important increase (over 50%) of formulae in the USA happened since the first fortified formula was sold in February of 2002. Over 95% of global production is destined to formulae use; in 2006 the production did not supply the demand. The first company to produce ARA by fermentation was Martek (today part of DSM), in which Martek Inc. has the exclusivity of the ARA-rich oil produced by DSM (KYLE, 1996). The ratio of the commercialized mixture is two parts of ARA and one of DHA (2:1 v/v).

The company Martek Bioscience earned USD 317 million from oil sales. The selling price of the oil is commercially sensitive, and it is considered that 2000 tons of DHA-SCO are sold annually as minimum, and now have reached 3000 tons, which means that a total of 6000 tons of ARA-SCO was also produced (RATLEDGE, 2013). In 2003, the total production was around 560 tons being mostly used in infant formulae compared to the production in the period of 1985-2002 (690 tons), in which 80% was used in formulae and the rest in other dietary supplements. Another player in this market is Suntory Ltd. which sells just the ARA-rich oil named SUNTGA40S in Japan, and since 2010 Cargill Inc. became a rival of this last two.

The future perspectives of ARA and DHA production remains in the microbial production, but it is not the case for EPA-rich oils, the DuPont project is still studying and testing genetically modified yeast and different algae for better production. Plants production of oil rich in PUFA’s is still distant as ten years ago, but it is a challenge for geneticists who are researching and maybe positive surprising results will amaze the biotechnological community in the future.

1.1 OBJECTIVES

1.1.1 Main Objective

To study and optimize the *Mortierella alpina* CBS 528.72 biomass production

1.1.2 Specific objectives

To define an inoculation method.

To develop and optimize culture medium for biomass production.

To evaluate the use of residual agroindustrial substrates for biomass production.

To scale-up the biomass production and arachidonic acid production

2 LITERATURE REVIEW

2.1 ARACHIDONIC ACID

Arachidonic acid is a long chain (20:4 Δ5,8,11,14 n-6) polyunsaturated fatty acid (PUFA) of the ω-6 class. Its molecular formula is $C_{20}H_{32}O_2$ and the chemical structure is illustrated in the FIGURE 1. This fatty acid can be found in blood, brain, glandular organs and liver. ARA plays a role in the maintenance of the membrane fluidity in cells (Higashiyama *et al.,* 2002) and is synthesized in animal and humans by the desaturation and elongation of linoleic acid (JUMP *et al*, 2009). It is a precursor of mediators of essential processes, acting in the modulation of inflammation, cytokine release, platelet aggregation, immune response, allergic phenomena and thrombosis (UAUY *et al.,* 2000). It is also very important as a second messenger in the central nervous system, playing a role in expression of long-term potentiation (NISHIZAKI *et al.,* 1999).

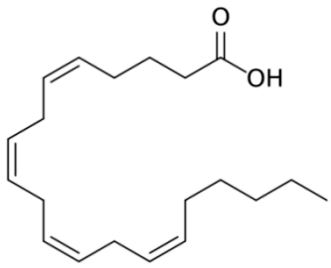


FIGURE 1. – ARACHIDONIC ACID CHEMICAL STRUCTURE

ARA-rich oil is used commonly in food formulation to ensure optimal conditions of brain, vascular systems and nervous development (NISHA & VENKATESWARAN, 2011); ARA and DHA (docosahexaenoic acid, another PUFA) comprises over 90% of brain essential fatty acids. ARA is supplemented during pregnancy through the placenta, and to infants through maternal milk.

The aim of the use of ARA in formulae is to reach erythrocytes and plasma levels equivalent to breast-fed infants (HOFFMAN *et al* 2000). Its mixture with DHA helps to improve the visual function, cognitive development and a good blood pressure (FORSYTH *et al* 2003). The Food and Agriculture Organization indicated that formulae

shall contain 60 mg of ARA and 40 mg of DHA per day per kilogram of infant weight (FAO/WHO Expert committee 1994).

2.2 OLEAGINOUS ORGANISMS AND SOURCES OF ARA

A wide variety of microorganisms which produce PUFA rich-oil are known and studied (TABLE 1), but just two fungi species are considered for commercial production: *Mortierella alpina* and *Pythium sp. M. alpina*, an ubiquitous filamentous fungi, is now the most productive specie, (KYLE, 1992)

Microorganisms could be used for the production of cocoa butter substitutes (the cocoa butter gives the desirable sensation of melting-in-mouth of the chocolate) and one alternative are the yeast lipids, especially from the genus *Cryptococcus* (BEAVAN *et al.*, 1992). Despite the existence of positive results in these investigations, the production on an industrial scale is not yet a fact due to the costs of production and refinement of the final product.

Algae species has been studied also: some species produce essential fatty acids of interest for human nutrition, as is the case of *Chlorella sp. (*MIUERA *et al*., 1997) which produces gamma linolenic acid (GLA) and the green alga *Parietochloris incisa* (TABLE 2) that produces ARA (up to 50% of the total fatty acids, more than 20% of total biomass as triacylglycerols) (BIGOGNO *et al*,. 2002).

2.2.1 MORTIERELLA ALPINA

The genus *Mortierella* (FIGURE.2) (member of the family Mortierellaceae, order Mortierellales class Zygomycetes) is one source of single cell oil (SCO) rich in n-6 PUFA and has been reported as a good producer of ARA (YAMADA *et al*., 1992). The species *alpina* is the most important industrially. Various strains of *M. alpina* have been studied for optimization purposes (TABLE 3)

TABLE 1 – BIOLOGICAL FUNCTIONS AND APLICATIONS OF SOURCES OF POLYUNSATURATED FATTY ACIDS

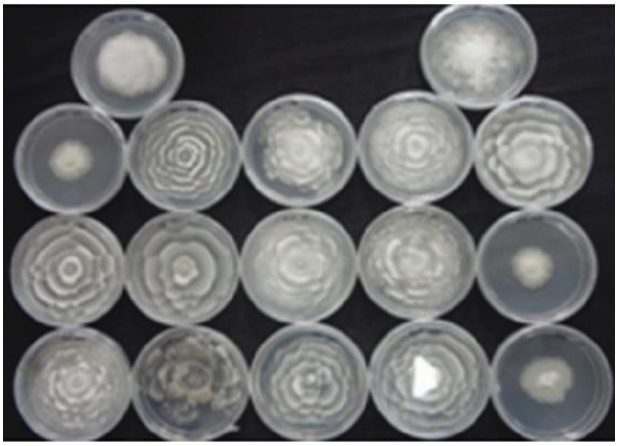
Sources	Gamma-Linolenic acid (GLA) (18:3;6.9.12)	Arachidonic acid (ARA) (20:4; 5.8.11.14)	Alpha-linolenic acid (ALA) (18:3;9.12.15)	Eicosapentaenoic acid (EPA) (20:5;5.8.11.14.17)	Docosahexaenoic acid (DHA) (20:6;4.7.10.13.19)
Plants and animals	Plants: *Oenothera, Borago, Ornithogamul spp.*	Fish: *Brevoortia, Clupea,Sardina spp.*	Plants:*Brassica,Glycine, Linum spp.*	*Minor component of tissues.*	Fish: *Brevoortia, Engraulis, Sardina, Scomber spp.*
Microorganisms	Fungi: *Mucor, Mortierella* Algae: *Chlorella and Spirulina spp.*	Fungi: *Pythium, Mortierella spp.* Algae: *Porphyridium spp.* Mosses: *Rhytidiadelphus, Brachythecium, Erthynchium spp.*	Algae: *Chlorella spp.*	Fungi: *Mortierella, Phytium spp.* Algae *Chlorella, Monodus, Porphyidium, Nannochloropsis, Crytoleura, Schizymenia, Navicula, spp.* Mosses: *Brachythecium, Eurhynchium, Scleropodium spp.* Bacteria: *Rhodopseudomonas, Shewanella spp, Photobacterium.*	Fungi: *Thraustochytrium, Entomophtora spp.* Algae: *Gonyaulax, Gyrodinium, Crypthecon idium* Bacteria: *Colwellia, Moritella (Vibrio) marinus*
Major dietary sources	Evening primrose seed oil, from linolenic acid	Liver, brain, egg, yolk lecithin	Vegetable oils (such as soy, lindseed, pumpkin seed oils), leafy vegetables.	Salmon, tuna, sardines, cod, shellfish, algae	Cold water fish, shellfish, algae
Biological functions	Intermediates in the biosynthesis of other fatty acids, precursor of prostaglandins PGE1	Major component of most membrane phospholipids, precursor of prostaglandin PGE2	Minor component of tissues	Precursor of prostaglandin PGE3, prevents thrombosis, inhibit the production of n-6 eicosanoids, and modulates immune function.	Present in the retina and the grey matter of brain of mammals, influences on the visual and neural development of infants
Nutrional values and applications	Dietary supplement intake for several disorders such as eczema, use in cosmetic	Ingredient in various infant formulae along with DHA	Beneficial effects on blood lipid profiles, to reduce the risk of coronary heart disease, arthritis, inflammation, hypertension, psoriasis, other autoimmune disorders and cancer, nutraceutical additives for processed food, DHA incorporated into infant formulae for improvement of vision and memory.		

SOURCE: Adapted from RATLEDGE and WYNN, 2002

TABLE 2 – FATTY ACIDS PROFILE OF VARIOUS FUNGI AND ALGAE BEING CONSIDERED FOR PUFA PRODUCTION.

	Major fatty acyl groups (relative % w/w)								
	16:0	16:1	18:0	18:1	18:2	18:3 (n-3)	18:3 (n-6)	20:3 (n-6)	20:4 (n-6)
Cunninghamella echinulata	13	1	2	46	16	-	19.5	-	-
Mortierella alpina	8	-	11	14	7	-	4	4	49
Mortierella isabellina	27	1	6	51	10	-	5	-	-
Mucor circinelloides	22	1	6	40	11	-	18	-	-
Pythion irregulare	17	7	2	14	18	-	-	-	11
Pythium ultimum	15	-	2	20	16	1	-	-	15
Syzgiles megalocarpus	14	-	1	12	10	-	62	-	-
Amphidinium carterae	12	1		2	1		3		20
Chlorellaminutissima	13	21		1	2		-		3
Cylindrotheca fusiformis	22	24		2	1				8
Isochrysis galbana	-	11		3	2		-		-
Monodus subterraneus	19	10		5	2		<1		14
Phaeodactylum tricormulum	10	21		1	4		1		1
Pinguiochrysis sp	1	1		0.6	1		-		3
Porphyridium cruentum	30	5		>1	5		1		16

SOURCE: Adapted from RATLEDGE (1997); RATLEDGE and HOPKINS (2006); WYNN and RATLEDGE (2006) and RATLEDGE (2006); LIANG *et al*., (2005); KAWACHI *et al*., (2002).



SOURCE: Vadivelan & Venkateswaran, 2014

FIGURE 2 – *MORTIERELLA* SP. FROM SOIL SOURCE OF ROSELLE PETAL GROWTH ON PDA PLATE.

2.2.2 CONSERVATION OF *M. ALPINA*

Since 1S-4u is mostly used in industrial fermentations, its preservation is very important. HIGASHIYAMA *et al* (2007) developed a conservation technique based in the spores produced by this mutant strain grown in Czapeck medium (pH adjusted 4-7) harvested with sterilized water, giving a stock solution. This solution may be diluted with sterilized water or with a solution containing surfactants or inorganic salts giving a final solution, to which 10% cryoprotectant is added, commonly glycerin. Finally, the final stock solution is stored under ultra-low temperatures between -85 and -50 ºC for long periods; no loss of reproducibility or ARA productivity was reported in 5 years test, confirming a high stability of this method.

For short periods (about 3 months) a preparation of spore/mycelial suspension by slants of PDA and Czapeck agar are used, stored at 5ºC.

2.2.3 LIPIDIC PRODUCTION CAPACITY OF MORTIERELLA STRAINS

Mortierella alpina strains ME-1 and 1S-4 have been used in particular for industrial production but also as a model for lipogenesis studies because of their high oleaginous capacity, simplicity and lipogenesis regulation, production of PUFA, capacity of incorporation and transformation of exogenous fatty acids, mutants availability and their disposition to molecular genetic studies (SHIMIZU and JAREONKITMONGKOL, 1995).

Mortierella alpina can contain levels between 20% and 70% of Arachidonic acid and 60% of total fatty acids (AMANO *et al*., 1992).

Amano *et al*., 1992 isolated and analyzed the fatty acids composition of over 50 subgenus of *Mortierella* confirming the *alpina* specie as the best producer of ARA.

TABLE 3 – ARA AND TOTAL LIPIDS PRODUCTION OF STRAIN OF *M. ALPINA* BY SUBMERGED FERMENTATION.

Strain of *M. alpina*	Assay volume	ARA yield/day	Fermentation time	Total lipids yield/day	Reference
ME-1	3-L	1.8 g/L	11d	2.25 g/L	JIN *et al*., 2008
ME-1	250 mL flask	1.14g/L	10d	2.66 g/L	NIE *et al*., 2013
ME-1	3.5-L	1.31 g/L	7d	2.65 g/L	PENG *et al*., 2010
ATCC 16266	45-L	0.59 g/L	11d	0.21 g/L	STRESSLER *et al*., 2013
LPM 301	6-L	0.56 g/L	8d	0.92 g/L	EROSHIN *et al*..2000
1S-4	5-L	0.37 g/L	8d	1.34 g/L	HIGASHIYAMA *et al*., 1998
M18	6-L	0.21 g/L	7d	1.31 g/L	YU *et al*., 2003
CBS 528.72	-	0.19 g/L	7d	1.21 g/L	NISHA *et al*., 2011

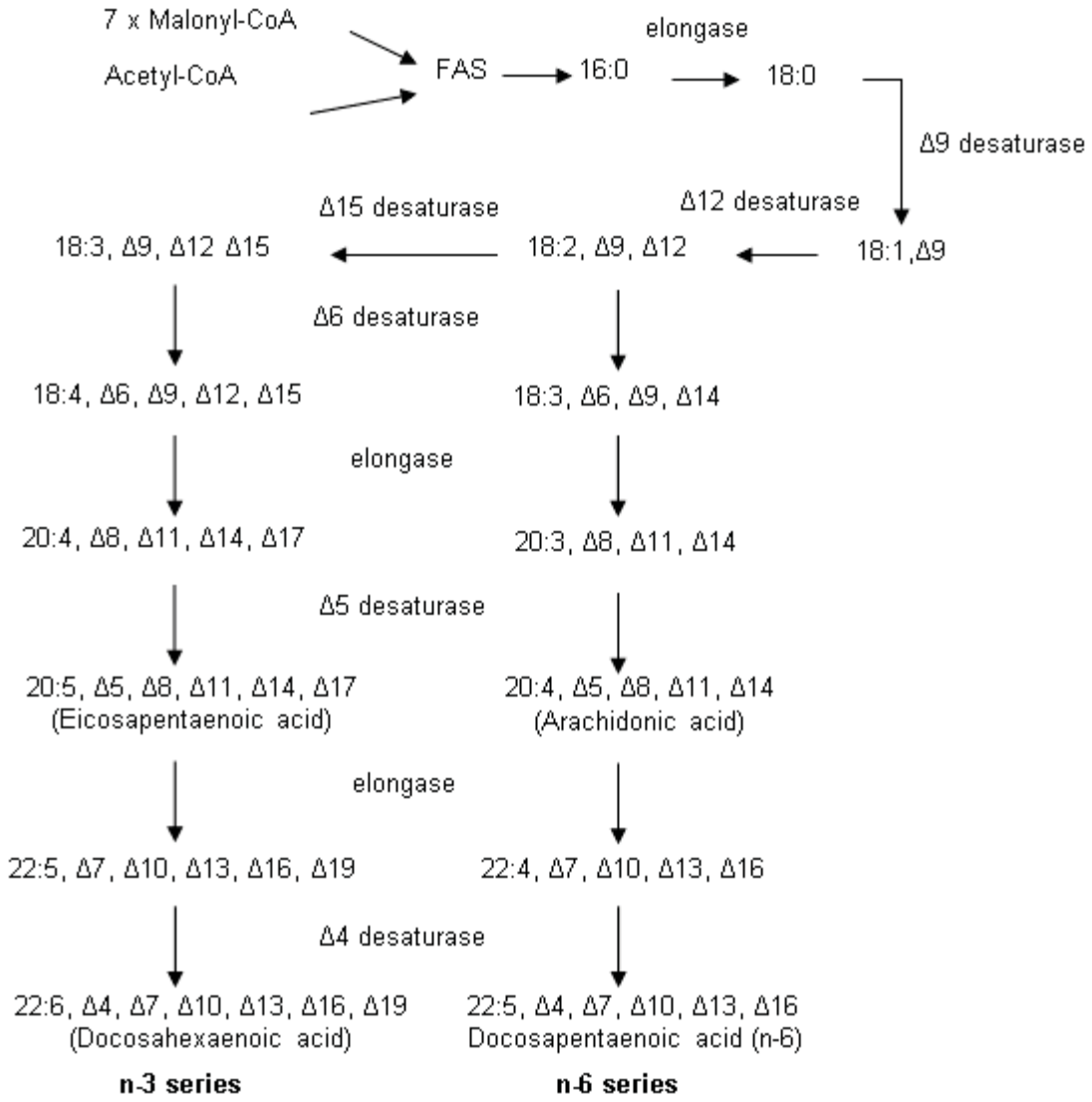
Two types of cultivation are used for industrial scale production: Submerged and solid-state. Submerged cultivation is preferred because of its scale-up capacity, biomass recovery, ARA production yield and fermentation time (solid-state fermentation needs a cultivation period over 20 days). High yields of Arachidonic acid have been reported in liquid culture, over 10 g/L (HIGASHIYAMA *et al*., 1998) with a productivity of 1 g/L per day approximately (TABLE 4).

TABLE 4 – ARA PRODUCTION OF *M. ALPINA* STRAINS BY SUBMERGED FERMENTATION AND SOLID-STATE CULTIVATION.

Microorganism	ARA yield/day	Fermentation time	Scale	Reference
Submerged culture				
1S-4	1.3 g/L	10d	10-kL fermenter	HIGASHIYAMA *et al* ., 1998
ATCC 32222	1.0 g/L	11d	250-mL flask	SINGH and WARD, 1997
ATCC 32221	0.7 g/L	16d	500-L fermenter	TOTANI *et al*., 1992
UW-1	0.7 g/L	8d	20-L fermenter	LI *et al*., 1995
LPM 301	0.6 g/L	8d	30-L fermenter	EROSHIN *et al*., 2000
ATCC 42430	0.7 g/L	6d	20-L fermenter	KYLE, 1996
Wuji-H4	0.8 g/L	5d	250-mL flask	CHEN *et al*., 1997
DSA-12	0.6 g/L	6d	500-L fermenter	PARK *et al*., 1999
CBS 343.66	0.2 g/L	6d	5-L fermenter	LINDBERG and MOLIN., 1993
Solid culture				
IFO 8568	0.7 g/kg-medium	20d		Totani *et al*., 1987
CCF 185	1.71 g/kg-medium	21d	300-mL flask	Stredanska *et al*., 1993

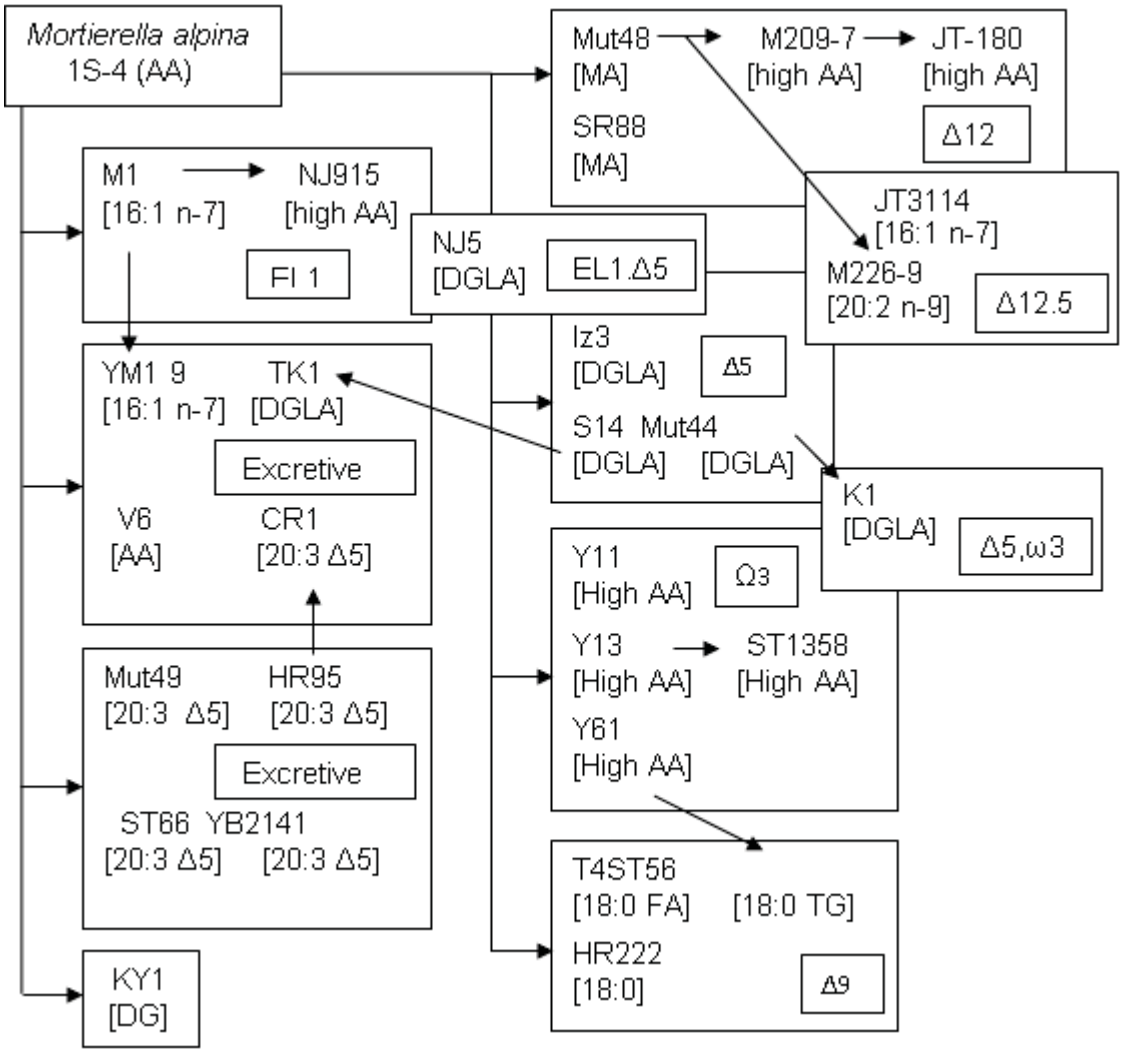
SOURCE: Adapted from HIGASHIYAMA, FUJIKAWA, PARK, and SHIMIZU, 2002.

ARA is synthesized by the n-6 series pathway (FIGURE 3) involving Δ12, Δ6 and Δ5 desaturase and elongase (EL2) therefore a variety of desaturases (Δ9, Δ12, Δ6, Δ5 and ω-3) and elongases (EL1) and also with desaturase enhanced activity (Δ5 and Δ6) have been derived from *M. alpina* 1S-4 (JAREONKITMONGKOL *et al*., 1992). The FIGURE.4 illustrates the main mutants of *M.alpina* 1S-4 and the biosynthesis of PUFA's is mentioned below, many mutants derivate from 1S-4 and their characteristics are resumed in TABLE 5.



SOURCE: Adapted from RATLEDGE and WYNN, 2002

FIGURE 3 – ROUTES OF DE NOVO BIOSYNTHESIS OF PUFA OF THE n-3 AND n-6 SERIES.



SOURCE: Adapted from SAKURADANI & SHIMIZU, 2009

FIGURE 4 – LIST OF THE MUTANTS DERIVED FROM *M. ALPINA* 1S-4 (IN SQUARE INDICATE APPARENT MUTATION SITES). FATTY ACIDS IN SQUARE BRACKETS ARE MAJOR FATTY ACIDS PRODUCED BY THE MUTANTS: DG, DIACYLGLYCEROL; FA, FREE FATTY ACID; TG, TRIACYLGLYCEROL.

TABLE 5 – DERIVATION OF MUTANTS FROM *M. ALPINA* 1S-4.

MUTANT	CHARACTERISTIC	REFERENCE
Δ5 desaturase-defective	High DGLA and low AA level Does not need inhibitor for DGLA production, high yields (4.1 g/L, 42% in oil) Commercially used	JAREONKITMONGKOL *et al*., 1993
Δ12 desaturase-defective	n-6 n-3 and n-9 PUFA's absence in their mycelia Produces oil-rich in mead acid Production of AA, EPA when n-6 or n-3 fatty acids is added Used for production of EPA rich-oil with low AA quantities	JAREONKITMONGKOL *et al*., 1993
Double defective in both Δ12 and Δ6	Accumulation of 20:2 n-9 in large quantities 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, DGLA and Arachidonic acid Characterized by eicosadienoic acid (20:2n-6) and eicosatrienoic acid (20:3n-6 (Δ5)) Capable to produce nomethylene-interrupted n-3 eicosatrienoic acid (20:4n-3 (Δ5)) from α-linolenic	JAREONKITMONGKOL *et al*., 1993
n-3 desaturase-defective	Unable to synthesize n-3 PUFA's at low temperature (<20º) The wild type produces high quantities of AA at 20ºC but a part is converted to EPA. This mutant produces higher content of AA	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)	JAREONKITMONGKOL *et al*., 2002
Elongase (EL1 for the conversion of palmitic acid to stearic acid)-defective mutants	Produces high levels of palmitic acid (16:0) and palmitoleic acid (16:1 n-7), with low quantities of n7 and n-4	SAKURADANI *et al*., 2004
Mutants with enhanced desaturase activities	The mutant 209-7 with 1.4-fold activity of Δ6 desaturase activity. JT-180 is another mutant with elevated Δ5 desaturase activity producing large quantity of mead acid (2.6 m MA/L, 49% in oil)	SHIMIZU *et al*., 2003 SAKURADANI *et al*., 2002
Diacylglycerol-accumulating	The percentage of triacylglycerols is about 90% in the mutant *M.alpina* 1S-4 of total lipids. KY1 accumulates 30% of diacylglycerol and is expected as a producer of diacylglycerol rich in C20 PUFA's	SAKURADANI *et al*., 2004

2.3 INDUSTRIAL ARACHIDONIC ACID PRODUCTION

The industrial production of arachidonic acid and any other essential fatty acids by fungal sources have to ensure the reproducibility, and multiple variables must be regulated and considered; the scaling up process, in an attempt to simulate industrial operations, is a factor that presents some difficulty.

The arachidonic acid is produced by solid-state cultivation (SSF) and submerged fermentations. The solid state process yields a good production of essential fatty acids, but submerged processes produces high ARA quantities and is easier to scale up, being more suitable for industrial production (SHINMEN *et al*., 1989). Researchers as Lai *et al*,. (1998) compared SSF and submerged cultivation: the solid-state culture was composed of milled sesame seeds, giving DHGLA (9-fold more than submerged cultures), but this study was not made on large scale. Yokochi *et al*,. (1995) found a problem when fungi were moved from flask to stirred-tank-fermenter because the carbon source (decane) got exhausted, but when decane was fed continuously the dry cell weight was almost 3-fold higher than shaker flask studies and total lipids about 4 times higher.

Totani *et al*., (1992) proved that ARA rich oils on an industrial scale is preferably produced by submerged fermentation since the yields may be low, therefore for industrial production a optimization of growth conditions is important to reach higher ARA quantities using a liquid medium since ARA is an intracellular product.

For ARA production, the following effects must be considered: adequate oxygenation therefore agitation, aeration and morphological control and effects media composition.

2.4 EFFECT OF GROWTH CONDITIONS

2.4.1 EFFECTS OF CARBON SOURCES

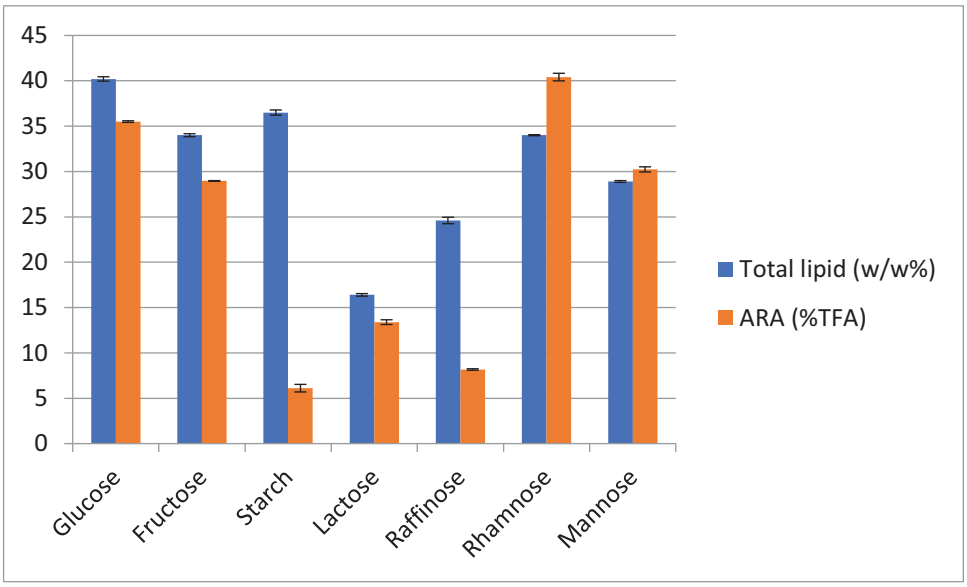
For arachidonic acid production the most frequently used carbon source is glucose (HIGASHIYAMA *et al*., 1998), and more than 20% of glucose in culture media induces the formation of filamentous pellets morphology (TOTANI *et al* 1992).

Buranova *et al*., (1990) studied the carbon influence in various strains from the *Mortierella* genus, concluding that some strains as *M. isabellina* could grow equally or

better in a malt extract medium than just glucose based medium, but in fatty acid composition matter this strain showed a low percentage of fatty acids compared with those grown in glucose. The strain and the carbon sources must be selected based in the fatty acid of interest, as well the fermentation conditions. For this reason in order to explore the influence of the medium composition Shinmen *et al*., (1989) tested different carbon sources using *M. alpina* 1S-4 (fructose, maltose, soluble starch and cornstarch) obtaining the same quantity of dry cellular weight among them, but was not the same for the ARA yield, in which glucose was the source with better yields. However, the use of hydrocarbons as n-octadecane or n-hexadecane as carbon sources increased the ARA yields until 1.5 times, but the biomass growth was poor. Stredankska and Sajbidor (1998) research results indicated that glucose was also the best source of carbon for biomass and lipids production compared to dextrin and starch.

In the study of Jang *et al*., (2005) arachidonic acid was 57.3-64.8% of the total PUFA's with soluble starch as carbon source, 78.05% with glycerol and 52.5% with glucose, in *M. alpina* ATCC 32222 (56%), in *M. alpina* ATCC 16266 (43%) and in *M. alpina* IS-4 (35%) and the value increased to 57% when glucose, at 6% of concentration, was consumed (LINDEBERG and MOLIN, 1998). Soluble starch and glucose are good for biomass production, total PUFA's and arachidonic acid. When glucose was used as sole carbon source it showed highest biomass production, arachidonic acid in total PUFA's, each carbon produced 101.1 mg of ARA. Koike *et al*., (2001) affirms that when glucose is increased from 2.0% to 12.0% in *Mortierella alpina*, the fatty acids content increased, but the yield of ARA production showed opposite behavior.

Nisha and Venkateswaran, (2011) tested different carbon sources achieving 40.41% of total PUFA's using rhamnose (FIGURE 5). The use of rhamnose is controversial since it is an expensive sugar and is not feasible to produce ARA on an industrial scale; these authors ranked the carbon sources in the following order: rhamnose > glucose > mannose > fructose > lactose > raffinose > starch. Glucose was the effective source and this because *Mortierellales* are saprophytes which prefer simple sugars consumption. Higher concentrations of glucose were not suitable for lipids production because of the increment of the osmotic potential, lower concentrations were also not effective.



SOURE: Adapted from NISHA and VENKATESWARAN (2011)

FIGURE 5 – EFFECT OF CARBON SOURCES ON TOTAL LIPID AND ARACHIDONIC ACID PRODUCTION BY *M. ALPINA* CBS 528.72.

All the studies indicate that the same carbon sources give different results for different strains and genera. Glucose appears to be the most efficient source, this, since glucose feeds directly into the glycolytic pathway.

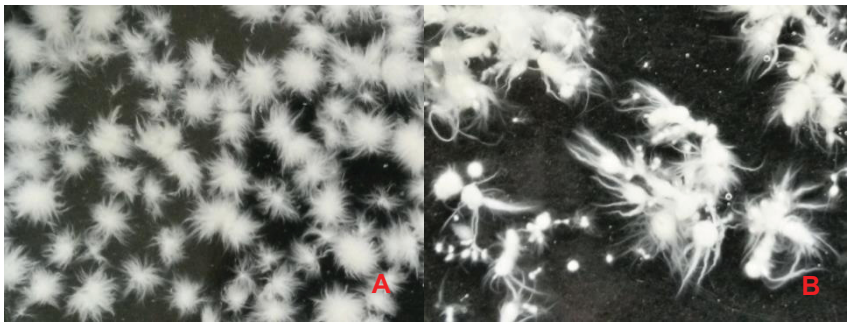
2.4.2 EFFECTS OF NITROGEN SOURCES AND C/N RATIO

The nitrogen sources affect the mycelia morphology and the arachidonic acid production (PARK *et al*., 1999). Depletion of nitrogen is a prerequisite for fatty acids accumulation, but it is not convenient for the biomass growth. *M. alpina* assimilates inorganic nitrogen sources with difficulty since the requirement of aminoacids and protein for higher biomass production come from organic sources. (WYNN *et al*,. 2001).

Weete (1980) affirms that organic nitrogen sources are more convenient for a better mold growth. Yeast extract was found to be the best nitrogen source for biomass, lipid and ARA production due the presence of micronutrients.

The nature of the sources of nitrogen is an important factor to consider because of the lipogenesis regulation (CERTIK *et al*,. 1999) since an increase of acetyl CoA carboxylase (ACC) occurs; the oleaginicity is dependent of ACC, in which CoA, once converted into malonyl CoA is used as the backbone for lipids synthesis. The Malic enzyme (ME) activity increase when the organic nitrogen sources are present.

Natural sources of nitrogen may change the morphology in submerged fermentation of *M. alpina*. Nisha and Venkateswaran (2011) reported the morphology variation using yeast extract, fluffy circular pellets (feather-like pellets), and filamentous mycelia growth using another nitrogen sources (FIGURE 6).



Source: The author

FIGURE 6 – *MORTIERELLA ALPINA* MORPHOLOGY IN SUBMERGED FERMENTATION: A) FEATHER-LIKE PELLETS AND B) FILAMENTOUS MYCELIA GROWTH.

Higashiyama *et al* (1998) studied the use of yeast extract and soybean meal concluding the effectiveness of yeast extract as nitrogen source. Park *et al*. (1999) investigated different nitrogen sources (yeast extract, corn steep liquor, pharmamedia, fish meal and gluten meal), obtaining better results with the use of yeast extract. These authors established a relation between the ARA production and the morphology, asserting the feather-like pellets efficacy. Totani *et al* (1992) suggested that *Mortierella* organic sources are more efficient than inorganic sources. Low biomass production was obtained testing inorganic salts as nitrogen sources (ammonium nitrate, sodium nitrate, ammonium acetate, ammonium sulfate).Yeast extract at 1% (w/v) concentration was defined as the optimum for ARA production, while higher quantities stimulated cellular growth.

Koike *et al*., (2001) noted that the C/N ratio influenced the ARA production and pellet morphology. Different C/N ratios were evaluated reporting that the optimum ratio was in the range of 15 to 20, the ARA production in a medium with C/N ratio number below this range is constant, and above it size increases proportionally to the C/N ratio. Sajbidor *et al*., (1990) reported the same range as optimum for ARA in *Mortierella* sp. S-17.

2.4.3 EFFECTS OF ADDITIVES AND SUPPLEMENTS

a) EFFECT OF SUPPLEMENTATION WITH OILS

There are many different oils tested as carbon sources or culture media supplement; Jang *et al*., (2005) tested different oils: Linseed, sunflower, soybean, lard, peanut and corn oil reporting better results with 1%, linseed oil gave the best results in biomass production and ARA content, it was also reported the positive influence of sunflower in DHA production. Their results also showed an increment of EPA and linoleic acid and a decrement in the degree of unsaturation. These fatty acids are added as precursors of long chain fatty acids; Shinmen *et al* (1989) concluded that olive and soybean oil increase the synthesis of arachidonic acid.

The use of oils as carbon sources were tested by Nisha and Venkateswaran (2011) with poor results: the oils were not assimilated as easily as carbohydrates. When culture media is supplemented with oils the mold produces lipases, cleaving fatty acids into residues from glycerol incorporating these residues as lipid structures/skeletons (AKTHAR *et al*., 1983)

The nature of the oil added could have an inhibitor/repressor power in desaturases and elongases, for example the presence of sesamine in sesame oil inhibits Δ5 desaturase and therefore the conversion of ARA from DGLA is blocked, giving higher amounts of DHGLA (RATLEDGE, 1989).

Shinmen *et al*,. (1989) also tested different oils to increase the ARA production using *M. alpina* 1S-4. Their results showed an increase of 2.8 times when soybean oil was applied as supplement.

TABLE 6 summarizes the effects of other additives used in fermentations by different authors.

TABLE 6 – ADDITIVES FOR FATTY ACIDS PRODUCTION USING *M. ALPINA* STRAINS.

ADDITIVES	EFFECTS	REFERENCE
NaCl 2%	Added after 3 days of cultivation, a stimulation of diacylglycerol acyltransferase was reported, increasing TFA and TAG content.	SZE-YUEN HO and FENG CHEN (2008)
Octadecanol 2%	Directly transformed in oleic acid by desaturation.	MO XIAN YIJAN KANG JICANG YAN (2001)
Ethanol	Applied after 5 days of cultivation increases NADPH levels necessary for desaturases activity, converted directly in Acetyl CoA avoiding the glycolytic way	SIJTSMA *et al* , (2005)
Glutamate (0.8 g/L)	It activates the Acetyl CoA carboxylase catalyzing the malonyl CoA formation (substrate for fatty acid synthesis and elongases)	L. J YU , W.M Qin *et al* (2002)

2.4.4 EFFECTS OF MICRO-NUTRIENTS ADDITION

Sajbidor *et al* (1992), investigated the effect of selected metal ions for a better ARA production in *Mortierella sp.* S-17, indicating a maximization in ARA yields with addition of 2 mg/L of manganese, while Hansson and Dostalek (1998) showed the importance of Copper and Zinc addition and their influence in the lipids production. There are metal ions with positive effects on mycelia growth but quite negative effects in fatty acid production, and vice versa. Manganese, for example, does not stimulate the biomass production, but it maximizes the lipid and ARA yield (SAJBIDOR *et al*., 1992).

Totani *et al* (1987) found that phosphorus, iron, manganese and potassium are necessary for fungal growth; especially manganese and iron are essential for lipid production. Results reported by Sajbidor et at (1992) also indicated that low concentrations of manganese (1 mg/L) influence positively in ARA production. Kyle *et al* (1992) found that also iron, copper and zinc stimulate the ARA production in *M. alpina.* Nagamuna *et al* (1985) observed the beneficial effect of calcium (100 mg /L) on lipid overproduction. The use of bivalent minerals is important because of its role as cofactor of acetyl-CoA carboxylase and others enzyme complexes essential in the first stage of fatty acids synthesis.

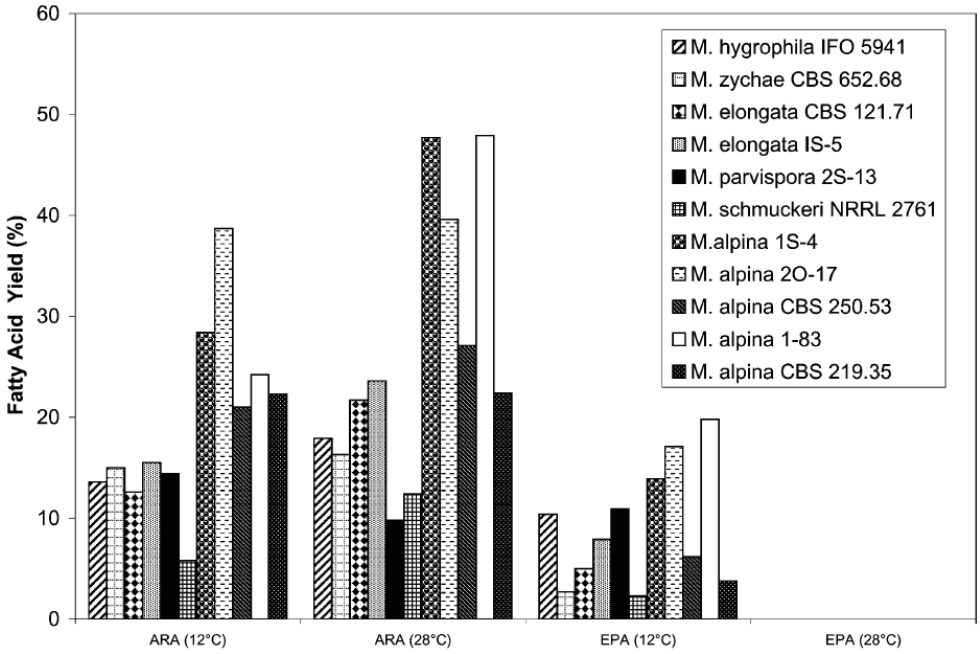
The influence of minerals in the morphology was studied by Higashiyama *et al*., (1998), showing that mycelia dispersion is possibly suppressed by an increase in ionic strength inducing a pellet formation. Pellets with 1-2 mm of diameter gave the

best results, with higher ARA concentrations than pellets with different sizes (e.g. 0-1 mm and 2-4 mm).

2.4.5 EFFECTS OF TEMPERATURE, pH AND DISSOLVED OXYGEN CONCENTRATION

Low temperature stimulates the unsaturation of fatty acids of long chain as ARA and EPA, temperatures between 12 and 15º C showed an increment of the unsaturation, but decreased the cellular growth. *M. alpina* had a high growth in the range 20 – 25 ºC, low at 8ºC and decreased significantly above 28ºC (BAJPAI and BAJPAI , 1992). Bajpai *et al*,. 1991, also reported a decreased of arachidonic acid and biomass production of *M. alpina* ATCC 32222 when temperature was shifted from 25ºC to 28ºC.

Nisha & Venkateswaran, 2011 reported that at lower temperatures *M. alpina* produced more EPA than ARA assuming the possibility of an adaptive process of PUFA's in membrane stabilization because of the stress caused by this temperatures according to the study of Shimizu *et al*., (1988)(FIGURE 7).



Adapted from SHIMIZU *et al*., 1988.

FIGURE 7 – ARA AND EPA YIELDS FOR DIFFERENT *MORTIERELLA* SPECIES GROWN AT DIFFERENT TEMPERATURES.

Another fact to consider is the oxygen concentration, at lower temperatures, more concentration is available for desaturase enzymes, occurring an homeoviscous adaptation, which is an adjust of the membrane fluidity for maintaining the cellular function (COHEN *et al*., 1987)

Hansson and Dostalek (1988) reported 25ºC as the optimum temperature for maximum biomass production; they evaluated the growth among different species of the genus *Mortierella* noting a variation of the fatty acid profile with respect to temperature, unsaturation level increased as temperature diminishing. In most species the highest level of ARA produced was at 28ºC while EPA production was at 12ºC.

Arachidonic acids take more time to be synthesized, alpha-linolenic acid and linoleic acid are the first fatty acids to be synthesized. Thus, an optimum temperature must be considered for essential fatty acids production based in the biomass and lipids yield obtained at lower temperatures.

The pH studies generally showed the influence in the production of saturated and monounsaturated fatty acids when pH level increased, Nisha & Venkateswaran (2011) reported an important effect of pH on biomass growth and ARA production, pH levels under 4.5 and superior to 8.0 ceased the biomass grow. The optimum level was at pH 6.5 for ARA production (46%) and 6.0 for lipids production (46%).

pH could reduce the carbon uptake efficiency of the strain and pH initial influences in the fungal mycelial morphology , a critical factor for metabolite formation (SHU and LUNG, 2004).

Reactions of elongation and desaturation give rise to PUFA’s, these are aerobic reaction so the dissolved oxygenation must be considered as an important factor (DAVIES *et al*., 1990).

Higashiyama *et al* (1998) studied the effect of oxygen under normal atmospheric pressure, with or without oxygen enrichment. They evaluated the influence in morphology *M. alpina* 1S-4 at different ranges of dissolved oxygen in a 50-L fermenter; the oxygen-enrichment methods used were: Oxygen enrichment method, feeding gas with 25-90% of oxygen content and also a pressurization method, in the180 – 380 kPa range. They found 15-20 ppm of DO concentration as an adequate level for biomass production and ARA production (1.6 fold better than at 7 ppm). Using oxygen enrichment method a morphological change was observed, from filaments to pellets, and a dramatic reduction of ARA yield because of low transfer rates of oxygen to the

pellet wall; using PR method the morphology did not changed and ARA yield decreased slowly.

Considering the above mentioned, there is no culture medium defined as optimum for *M. alpina*, since every strain has its own characteristics and requirements to consider, however many patents of media cultures were development with attractive yields of biomass production and arachidonic acid.

2.5 PRODUCTION PROCESSES

2.5.1 SUBMERGED FERMENTATION (SmF)

This fermentation type uses free flowing liquid substrate in tanks in diameter/height rate of 2:1 and 3:1; the SmF have the following advantages: operational facility, economy, small space are needed and an operational control facility (KUMAR and LONSANE, 1989).

2.5.2 TYPE OF REACTORS USED FOR BIOMASS AND ARACHIDONIC ACID PRODUCTION USING *M. ALPINA* strains

a) STIRRED TANK REACTOR

In stirred tank bioreactors (STR), the agitation is provided by internal impellers, and the aeration occurs through diffusers or perforated tubing; the heat exchange is made through internal coil or external jacket (DORAN, 1995).

Barclay, William R. (2002) conducted fermentations using *Mortierella* strains for ARA production in two 14-L fermenters, M-3 medium was the one reported with better yields of biomass yield and ARA production (22 g/L and 0.84 g/L/day respectively).The composition of the media was: 12 g/L of Nutrex 55 (Red Star Specialty Products, Milwaukee, WI) a spray dried inactive baker's yeast; 0.1 g/L $MgSO_4$-$7H_2O$; 0.1 g/L $CaCO_3$; 1 mL/L of PII Metals; 1 mL/L of vitamin mix; 2g/L of KH_2PO_4; 43.8 g/L glucose and 0.5mL/L of K60K antifoam. PII metals: (6.0 g Na_2EDTA; 0.24 g $FeCL_3$-$6H_2O$; 6.84 g H_3BO_3; 0.86 g $MnCl_2$-$4H_2O$; 0.133 g $ZnSO_4$-$7H_2O$; 0.026 g $CoCl_2$-$6H_2O$; 0.005 g $NaMoO_4$-$2H_2O$; 0.002 g $CuSO_4$-$5H_2O$ and 0.052 g $NiSO_4$-$6H_2O$

dissolved in 1L of water and pH adjusted to 8.0); Vitamin mix: (100 mg/L thiamin; 500 µg/L biotin and 500 µg/L vitamin B12)

Akimoto, Higashiyama and Shimizu, (2009) developed a patent for Suntory enterprise using *M. alpina* SAM2239 (a mutant strain from *M. alpina* IFO8568, ω-3 desaturase activity blocked). They used 2% of glucose, 1.5% soy flour, 0.3% KH_2PO_4, 0.05% $MgCl_2.6H_2O$, 0.05% $CaCl_2.2H_2O$, 0.1% Na_2SO_4, and 0.1% soybean oil as cultured media, 5 L was used in 10 L jar fermenters, sterilized at 120° C for 30 minutes. The fermentation conditions were an aeration of 1 vvm for 12 days at 24ºC and since the 3rd day the temperature was decreased to 12ºC. An addition of 1% of glucose was also fed in the first three days. They reported 75.1% of ARA of the total fatty acids, an amount of 4.1 g/L of ARA.

Ono, Aki and Higashiyama (2011) published a patent suggesting a culture media under fed-batch conditions for better yield of ARA production, they compared the efficiency using saccharified starch instead glucose using *M. alpina* CBS 754.68.The fermentation was performed in 50 L tank with culture medium composed by: soybean powder 4%, soybean oil 0.1%, KH_2PO_4 0.3%, Na_2SO_4 0.1%, $CaCl_2.2H_2O$ 0.05% and $MgCl_2.6H_2O$ 0.05%. The carbon sources were glucose and saccharified starch fed in different concentrations (TABLE 7), the sugar ratio of the saccharified starch (reducing sugar/total sugar) determinate was 30%. They showed the possibility of using starch avoiding initial high concentration of glucose which causes growth inhibition and the reduction of costs due the fewer feedings are needed.

TABLE 7 – CONCENTRATION OF CARBON SOURCES FEEDING DURING THE FERMENTATION PROCESS.

	Culture medium No		
	4A	**4B**	**4C**
	Carbon sources		
	Glucose	**Glucose**	**Saccharified starch**
Initial sugar concentration	1.8%	6%	6%
Flowing sugar concentration			
1 day flowing	4.1%	0%	0%
2 day flowing	4.1%	6%	6%
3 day flowing	3.6%	6%	6%
4 day flowing	2.7%	0%	0%
5 day flowing	1.7%	0%	0%
Total carbon source	18%	18%	18%
Amount of ARA produced (g/L)	13.50	7.00	13.30

Adapted from ONO, AKI and HIGASHIYAMA (2011)

Kyle (2011) developed a patent, applied for Martek Biosciences Co, for biomass production and for a better ARA production. They defined a culture media containing the following components: 80 g/L dextrose; 16 g/L soy flour; 30 mg/L $FeCl_3.6H_2O$; 1.5 mg/L $ZnSO_4.7H_2O$; 0.1 mg/L $CuSO_4.H_2O$; 1 mg/L biotin; 2 mg/L thiamine. HCl; 2 mg/L pantothenic acid (hemicalcium salt) . Adjusted to pH 4.8-5.0 pre-sterilization. The temperature was kept at 28ºC with an initial agitation of 162 cm/sec (23 rpm), the initial pressure in the vessel was 6 psi and 0.15 vvm as initial aeration. The oxygen level was maintained at D.O ≥40% increasing the pressure to 11 psi, agitation to 300 cm/sec and aeration to 0.5 vvm, finally the pH was maintained at 5.5 with 8N NaOH. This author also added 2g /L of KH_2PO_4 at 24-36 hours after inoculation. The yield of biomass reached was ≥ 24 g/L and about 51.2% of ARA content of total lipids produced.

b) AIR-LIFT COLUMN

These types of bioreactors have a simple structure and are stirred pneumatically, i.e. the system has no impellers and the stirring is performed by air injection. They are used industrially for the production of baker's yeast, beer and vinegar for the treatment of waste water, among others (DORAN, 1995; KANTARCIA, BORAK and ULGEN, 2005).

The air-lift column bioreactor has some advantages over the STR such as excellent heat transfer. Low cost of operation and conservation due to the absence of internal moving parts and has a longer durability of the material (KANTARCIA, BORAK and ULGEN, 2005). In this type of bioreactor hydrodynamics depends on the behavior of formed bubbles. Using low levels of aeration, bubbles are produced with a homogenous flow and using high rates of aeration, the air flow is named heterogeneous, wherein the bubbles have a chaotic flow movement (KAWAGOE *et al*, 1976;. DORAN, 1995).

Jiang and Xuan (2009) improved the arachidonic acid production by air lift type bioreactor of 10 000 L of volume using glucose, 20g/L; yeast extract, 10g/L; sodium, 7g/L; glutamic acid, 2g/L; KH_2PO_4, 1g/L ; $CaCl_2$. H_2O, 0.1 g/L; $MgSO_4$. $7H_20$, 0.5 g/L; $FeCl_3$. $6H_2O$, 15mg /L; $ZnSO_4$. $7H_20$, 7.5 mg/L and $CuSO_4$-$5H_2O$, 0.5mg/L. The fermentation process was performed at 31ºC with pH adjusted at 6.0, the inoculation rate was 3.5% and 0.5 vvm for ventilation. The author implied a fed-batch process

maintaining the glucose concentration at 40 g/L, after 5 days of cultivation the inventors stopped the feeding pulses and cooled the reaction to 23-24ºC and they also increased the ventilation rate to 2 vvm. Using this method they reached 37.5 g/L of DCW, 47.6% of oil production rate (of mycelia dry weight) and 52.8% of arachidonic acid of the total oil content.

3 MATERIALS AND METHODS

3.1 MICROORGANISMS

The microorganisms used in this study were two strains of *Mortierella alpina* purchased from the CBS-KNAW fungal biodiversity center; the strains CBS 528.72 (also denominated as ATCC 32222) and CBS 210.32.

The strains were maintained in PDA medium (potato dextrose agar) at 4ºC and were subcultured every 2 months.

These strains were activated and stocked for a posterior biomass, total lipids and arachidonic acid production evaluation under submerged fermentation conditions.

3.2 CULTIVATION AND INOCULUM PREPARATION

The seed cultures were prepared in a 250mLErlenmeyer flask containing 100mL of medium (g/L): 20, glucose and 10, yeast extract, pH was adjusted to 6.0 before autoclaving at 121ºC for 15 minutes and then inoculated with 3 discs of 6mm of diameter of 7 days old *Mortierella alpina* petri dish cultures.

The seed cultures were incubated at 25ºC and 120 rpm for 72h on an orbital shaker (Nova Técnica NT715).

After incubation the seed culture was aseptically processed using a hand-mixer (Eterny SW-108)to homogenization (pellet disruption). A 10% v/v of this suspension was inoculated in the assays. The concentration of biomass in the inoculum was about 6 to 8 mg/mL.

3.3 DETERMINATION OF INOCULATION METHOD

Three different inoculums techniques were tested:

a) Spores harvesting. Mortierella alpina strains CBS 578.32 and CBS 210.32 were cultivated in petri dishes with PDA medium and a medium with 5% glucose and 1% yeast extract, in three different incubation conditions to evaluate spore formation : a) 25 ºC, 7 days incubation ; b) 3 days at 25º C and then 4 days at

6ºC incubation and c) 7 days at 6ºC incubation. This temperature shifts were applied in order to stress the microorganism and stimulate the sporulation (BERGER, 1985).

b) Mycelial homogenization by vortexing. Mycelium of the seed culture were placed in a 5mL eppendorf tube containing half of its volume of glass beads (200 – 400 µm) a volume of 2 mL of 0.05% tween 80 solution was added and vortexed for 20 minutes, after this a cellular viability assay was performed.

c) Mycelial homogenization by mixer. The total seed culture was blended using a hand-mixer (Eterny SW-108), after this process a cellular viability assay was performed in three replicates and a 10% v/v mycelium suspension was inoculated in the modified HD medium, pH was adjusted to 6.00 and the culture was inoculated for 7 days in an orbital shaker at 120 rpm. After the fermentation period the biomass was separated by vacuum filtration and dried in an oven at 60ºC overnight and weighted.

3.4 CELLULAR VIABILITY

In order to evaluate the two homogenization methods, a cellular viability assay was done using 1mL mycelial suspension, added to 9 mL of saline solution (NaCl 0.85%). The resulting suspension was serially diluted until 10^{-3}. A 0.1mL of 1:10, 1:100 and 1:1000 dilutions were used to inoculate petri dishes with PDA medium in triplicate. These dishes were incubated for 5 days at 25ºC. After CFU counting the following equation was applied to obtain CFU/mL (Equation 1).

$$\text{CFU/mL} = \frac{\text{Colonies number x dilution factor}}{\text{Volume (mL)}}$$

3.5 CULTURE MEDIUM SELECTION

Four different media were selected for initial tests, from the literature, based in the following criteria: media tested with *Mortierella alpina sp.,* over 18 g/L biomass

production, bench-scale tested, components available (i.e. in common) and the media selected are free of supplements/additives.

1) Hansson and Dostalek medium (HANSSON, L. and M. DOSTALEK, 1988) which contained (per liter): glucose, 30.0 g; yeast extract, 5.0 g; KH_2PO_4, 2.4 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 15 mg; $ZnSO_4 \cdot 7\ H_2O$, 7.5 mg; and $CuSO_4 \cdot 5H_2O$, 0.5 mg. Inoculated with 10% v/v mycelia suspension with shaking at 120 rpm.
2) The medium used by (SINGH and WARD.1997) which contained (per liter): 50.0 g glucose, 5.0 g yeast extract, 3.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCl, 1.45 mg $FeCl_3$, 0.01 mg $CuSO_4$, 4.3 mg $MnCl_2 \cdot 4H_2O$, 0.13 mg $CoCl_2 \cdot 6H_2O$, and 0.3 mg $ZnCl_2$. Inoculated with 5% v/v mycelia suspension with shaking at 150 rpm
3) An improved medium (L.J. YU *et al*, 2003) which contained (per liter): glucose, 100.0 g; yeast extract, 5.0 g; beef extract, 3.0 g; $MgSO_4$, 1.5 g and 0.8 g glutamate. Inoculated with 10% v/v mycelia suspension with shaking at 130 rpm
4) An optimized medium (MIN ZHU *et al*, 2006) which contained (per liter): glucose, 60.0g ; Potassium nitrate, 3 g; Yeast extract, 4.5 g; $K_2HPO_4 \cdot 3H_2O$, 4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.5 g (pH 5.5). Inoculated with 10% v/v mycelia suspension with shaking at 150 rpm

The pH of these four media was adjusted at 6.0, autoclaved at 121ºC for 15 minutes and inoculated for 7 days at 25ºC .

3.6 ANALYTICAL METHODS

3.6.1 Dry cellular weight determination.

Mycelial suspensions were harvested from 50 mL of culture broth by vacuum filtration through qualitative filter paper, washed with distilled water until observation of a clear filtrate, removed from the filter using tweezers and placed into plastic cups to dry at 70ºC in an oven until constant weight.

3.6.2 Fermentable sugars analyses.

For reducing sugars determination, the dinitrosalicylic acid (DNS) method (Miller, 1959) was used: 1mL of the culture broth was filtered and incubated with 1mL of DNS in a boiling water bath for 5 minutes then cooled under running tap water adjusted to ambient temperature, 13 mL of distilled water was added, the tube was agitated and read in a spectrophotometer at 540 nm wavelength.

3.6.3 Lipid extraction

The extraction of lipids was performed according to a modified procedure based on the Blygh-Dyer method (1959): dried mycelium was grinded, giving a fine powder which was transferred to a 50mL falcon tube. For every 2 grams of dried mycelium10 mL of chloroform, 20 mL of methanol and 8 mL of water was used for the extraction. The mixture was then vortexed for 15 minutes, 10 mL of chloroform and 10 mL of sodium sulfate (1.5%) was added and centrifuged at 4000 rpm for 2 minutes. Finally, 5 mL of the lower phase was carefully pipetted and weighted in a falcon tube after filtering and evaporated at 60ºC for 3 hours to get constant weight.

3.6.4 Fatty acids analysis

A modified Direct methanolysis (LIU and ZHAO, 2007) was used for determination, 250mg of dried mycelium was put into a glass vial with 10mL of 0.2mol/L H_2SO_4 solution at 70 ◦C, for 20 h, well-sealed; the suspension was vigorously stirred. At a given time point, the suspension was cooled and diluted with 20 mL of n-hexane added, agitated, the supernatant phase was carefully pipetted and placed into a new vial and 20mL of n-hexane was newly added, the supernatant was pipetted and 1mL was stored for gas chromatography analysis (GC) in a Shimadzu GC-2010 instrument. The conditions for analysis were: Auto injector: AOC-20i at 130°C; split ratio 1:50, Column RTX-wax (30mm x 0.25mm x 0.25um); Hydrogen carrier gas, and temperature program: 150°C /3min; then heating at 10°C/min to 210°C; maintaining for 15 min; heating at 10°C/min to 240°C; maintaining for another 10 minutes. A FID detector was used, at 250°C. The peaks were identified by comparison of the retention

time of the unknown compounds with FAME (Sigma) standard compounds, and quantified based on their specific areas.

3.7 EFFECTS OF CULTURE CONDITIONS ON BIOMASS AND TOTAL LIPIDS PRODUCTION

a) Incubation temperature and pH. The modified HD medium was used for all culture conditions tests; temperatures of 20, 25, 28 and 30º C were tested adjusting pH at 6.00 and incubated at 120 rpm orbital shaker agitation for 7 days. Inoculated with 10% v/v of mycelial suspension.

   The pH levels (5.00, 6.00, 7.00 and 8.00) were tested using modified HD medium for 7 days incubation at 120 rpm orbital shaker incubator at 25ºC. Inoculated with 10% v/v of mycelial suspension.

b) Inoculation rate Concentrations of 5, 10, 15 and 20% v/v of mycelial suspension were tested in 50mL of modified HD medium in 250mL Erlenmeyer flasks. Before autoclaving, the pH was adjusted to 6.0, incubated at 25ºC in an orbital shaker at 120 rpm for 7 days. Inoculated with 10% v/v of mycelial suspension.

c) C/N ratio. The effect of C/N ratio was studied in the culture medium selection, having glucose as carbon source and yeast extract as a nitrogen source. The calculus was based in the quantity of glucose consumed divided between the nitrogen consumed (a total nitrogen consumption was assumed).

3.8 OPTIMIZATION OF MEDIUM CULTURE COMPOSITION.

In this phase of the study, the optimization of variables directly related to complementary addition of HD medium was involved, it means concentrations of regular salts presents in the formulated medium was optimized and also complementary salts collected from literature review of the last 25 years. The fermentations were performed for *M. alpina* CBS 578.72 and CBS 210.32 during 7 days at the best conditions obtained in the effect of culture “one-factor-at-a-time” (item 5.7) tests.

The total biomass of *Mortierella alpina spp*. depends of the carbon source and also on the morphology and total lipids production as well. Carbon sources as starch, sucrose, fructose and galactose were tested instead of glucose (60g/L); the nitrogen

source (yeast extract) of modified HD medium was replaced by (NH4)2SO4, KNO3, and urea, based in its total nitrogen molar masses; this carbon and nitrogen sources were based in literature reviews.

A salts composition study of the HD medium was made for each strain, using a Plackett & Burmann (PB) experimental design strategy with 11 factors, 12 runs and 3 central point repetition (TABLE 8).

TABLE 8 – FIRST EXPERIMENTAL DESIGN FOR CULTURE MEDIUM OPTIMIZATION, SALTS SCREENING, PLACKET & BURMANN (PB) TYPE, 12 RUNS WITH 3 CENTRAL POINT REPETITIONS FOR 11 FACTORS.

Run	KH_2PO_4	KNO_3	$CaCl_2$	$MgSO_4$	$FeCl_3$	$ZnSO_4$	$CuSO_4$	$MnCl_2$	$CoCl_2$
1	1	-1	1	-1	-1	-1	1	1	1
2	1	1	-1	1	-1	-1	-1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1
4	1	-1	1	1	-1	1	-1	-1	-1
5	1	1	-1	1	1	-1	1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1
7	-1	1	1	1	-1	1	1	-1	1
8	-1	-1	1	1	1	-1	1	1	-1
9	-1	-1	-1	1	1	1	-1	1	1
10	1	-1	-1	-1	1	1	1	-1	1
11	-1	1	-1	-1	-1	1	1	1	-1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0

The second experimental design utilized in this study was central composite rotatable design (CCRD) with 2 variables (TABLE 9), the variables were studied at 5 coded levels (-1.41; -1; 0; 1; +1.41) with 3 repetitions at the central point, 4 axial points. The variables studied were $CaCl_2.2H_2O$, for CBS 528.72 : 8 mg/L (-1.42), 24 mg/L (-1), 62 mg/L (0), 100 mg/L (+1) and 116 mg/L (+1.41); and KNO_3 for CBS 210.32 at the concentrations: 0.27 g/L (-1.41), 1.5 g/L (-1), 4.5 g/L (0), 7.5 g/L (+1) and 8.73 g/L (+1.41); yeast extract was also tested for both strains at the same levels : 0.9 g/L (-1.41), 5 g/L (-1), 15 g/L (0), 25 g/L (+1) and 29.1 g/L (+1.41).Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to superfluous factors.

TABLE 9 – SECOND EXPERIMENTAL DESIGN CCRD TYPE FOR *MORTIERELLA ALPINA* 528.72; 2 VARIABLES (22 + 4 AXIAL POINTS RUNS AND 3 CENTRAL POINT REPETITIONS).

Run	Yeast extract	$CaCl_2$
1	-1.000	-1.000
2	-1.000	1.000
3	1.000	-1.000
4	1.000	1.000
5 (axial)	-1.414	0.000
6 (axial)	1.414	0.000
7 (axial)	0.000	-1.414
8 (axial)	0.000	1.414
9	0.000	0.000
10	0.000	0.000
11	0.000	0.000

3.9 STATISTICAL ANALYSES

The effect of culture conditions was studied for the maximum biomass and total lipids production using “one-factor-at-a-time” (OFAT) design, performed on three replications. Experimental design experiments analyzes were analyzed using the software Statistica 7.0 (Statsoft, Tulsa, OK, USA)

One-way analysis of variance with Tukey post-test and t-student test was made using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Levels of significance were considered at $P \leq 0.05$ unless otherwise stated.

3.10 BIOREACTOR SCALE-UP

The bioreactor scale-up test was performed using a bioreactor BIOLAFITE, vessel 10L, with agitation and temperature controlled, pH was uncontrolled. The final medium culture optimized was used for each batch fermentation at 25ºC for 7 days of fermentation time at 120 rpm and 0.6 vvm. For fed-batch fermentation 25ºC;0.6 vvm at 120 rpm for 5 days and then the aeration was changed to 1 vvm at 25ºC at 80 rpm and a carbon pulse of 2% of glucose and 0.3% of NaNO3 of nitrogen pulse was added (JIN *et al*., 2008) Both fermentation types were autoclaved at 120ºC for 30 minutes at started with pH 6.00.

3.11 BRAZILIAN INDUSTRIAL WASTES AND BY-PRODUCTS AS NEW CULTURE MEDIA ALTERNATIVES

Brazilian industrial wastes were tested as new alternatives of culture media.

For this test residues passed under acid hydrolysis process to release sugars as carbon sources and get an extract and used it as culture medium. The industrial wastes treated were: Citrus pulp, soybean hulls, cassava wash water, potato chips waste, sugar cane juice and sugar cane molasses in a 10% (W/V) proportion,

a) Preparation of the extracts

The extracts were prepared hydrolyzing the residues (previously grounded and sifted to get a particle size inferior to 5 mm) in a proportion of 1g in 10 mL of water, with 2% of hydrochloric acid 37% P.A. The suspension was autoclaved for 15 min at 120ºC to help the sugar release, the pH was adjusted and then filtered.

For reducing sugars, 1mL of the extract was analyzed by the DNS method (MILLER, 1959) and a dilution was made when necessary.

Among the residues, total reducing sugars were standardized to 60 g/L, and glucose was added in those which total reducing sugars concentrations were lower than 60 g/L and a dilution in those which concentration was higher.

4 RESULTS AND DISCUSSION

4.1 DETERMINATION OF INOCULATIONMETHOD

In fermentation assays using molds there is a variety of different methods for inoculation: spores harvesting, mycelial suspension, mycelium harvesting, etc. Which of these methods should be used when the mold has a very low sporulation level?. This is why in this part of the study a inoculation method was studied and established.

4.1.1 Spores production and harvesting.

Mortierella alpina does not sporulate under laboratory conditions, the mutant strain 1S-4u being an exception (LOUNDS *et al*, 2007), and yet there are many authors from who used this method in their experiments. Three different ways were studied as commented in the item 6.3 of the Materials and Methods section.

a) **25 ºC at 7 days incubation:** Showed a regular hyphal radial grow of the fungus; the sporulation was poor, giving around 3-4 x 10^3 spores/mLas counted using a Neubauer chamber.

b) **3 days at 25º C and then 4 days at 6ºC incubation:** The hyphal radial growth of the mold was suitable for the first 3 days and when the temperature shift was applied an aerial hyphal growth was observed, and the sporulation kept poor (2.4x10^4 spores/mL)

c) **7 days at 6ºC incubation:** The best hyphal growth was observed at 6ºC (FIGURE.8), the radial grow was acceptable (the fungus covered the whole petri dish) and also the aerial growth was good, a number of 4-5X10^5 spores was counted. This condition showed the best results.

Different authors as (XIAN *et al*., 2001) used 2x10^6 spores/mL of *Mortierella sp*.and (YU, QIN, LAN, ZHOU, and ZHU, 2003) used a spore suspension as seed culture.

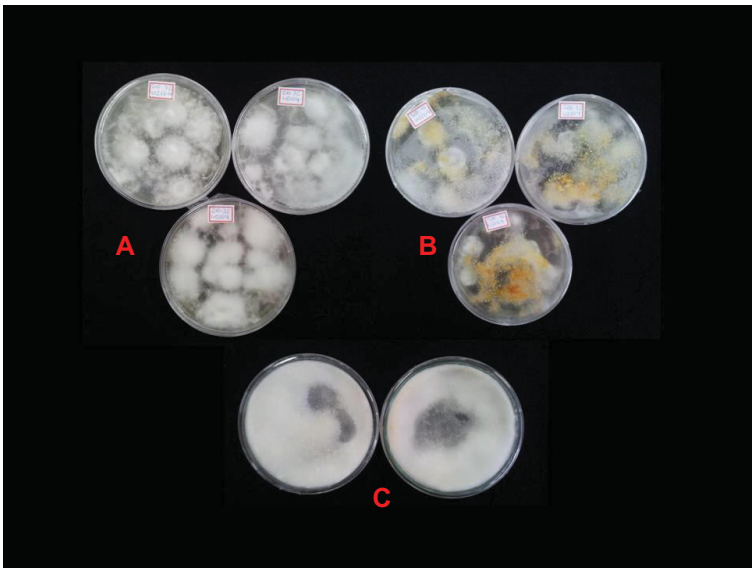


FIGURE 8 – SPORULATION ASSAY AT 6ºC FOR 7 DAYS INCUBATION. A) *MORTIERELLA ALPINA* 210.32 B) *MORTIERELLA ALPINA* 528.72 AND C) *MORTIERELLA ELONGATA* NRRL 5513 (for comparison).

4.1.2 Mycelium homogenization using vortexing process.

Since *Mortierella* sp. does not sporulate significantly (KHUNYOSHYENG *et al.*, 2002), another form of providing colony forming units was sought. A mycelial suspension was processed using a vortexing agitator, which should be able to break the biomass into shorter – and more numerous – hyphal segments (FIGURE 9). There is a contamination risk in this process, but it is of easy scale-up.

Best results for colony counting were obtained in 1:10 dilution, the colonies formed were between 30-300 CFU per Petri dish, these viable cells evaluation helped to determinate the exponential phase of the fungal growth in the seed culture, the figure. 9 showed that at 3 days (72h) of incubation the fungus came from a lag phase, suggesting that the seed culture could be incubating and used at the 3rd day, and not at 2nd days how some authors used in which the fungus is still adapting to the medium. After 3 days *Mortierella* was still growing but slowly, almost reaching a stationary phase.

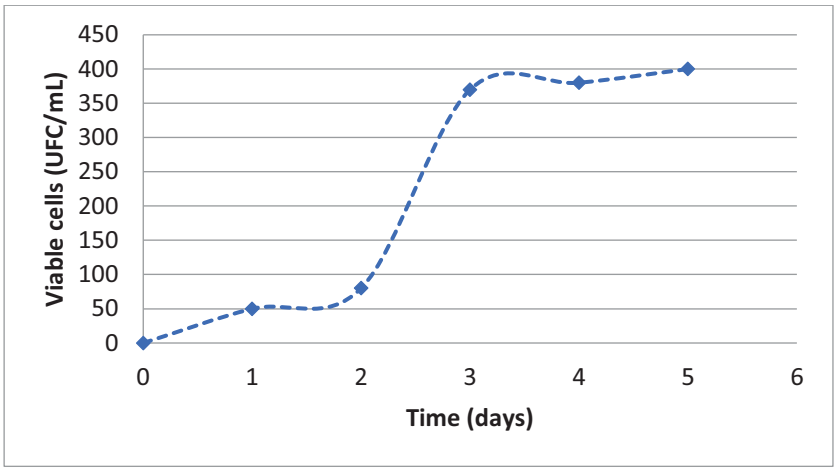


FIGURE 9 – KINETIC OF VIABLE CELLS IN SEED CULTURE MEDIUM USING A VORTEX DISRUPTION METHOD.

4.1.3 Mycelium homogenization using a hand-mixer process.

In the research of (JANG, LIN, and YANG, 2005) this technique was used as an inoculation method, but mixing process can be aggressive to the hyphae structure.

In a dilution of 10^{-1} used in the vortexing test, it was observed a viability of the mycelial suspension of 370 CFU/mL at the 3rd day, but using a hand-mixer a better result was reached, around 736 CFU/mL (FIGURE10) Hand-mixer disruption fractionates hyphae releasing tramped spores to the medium (FIGURE 11) the kinetic also confirm that at the 3rd day an exponential phase was reached, so a seed culture can be use used with 3 days of incubation using a hand-mixer as a disruption tool too.

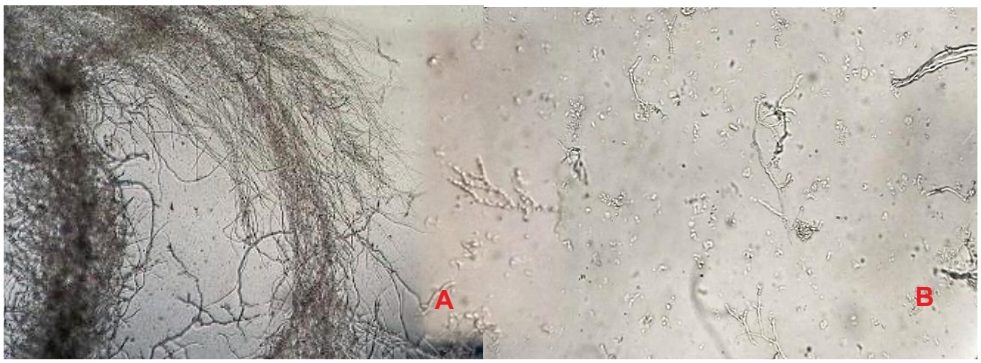


FIGURE 10 – HAND-MIXER DISRUPTION: A) BEFORE MIXING PROCESS, B) AFTER MIXING PROCESS. IMAGES WERE MAGNIFIED 10X.

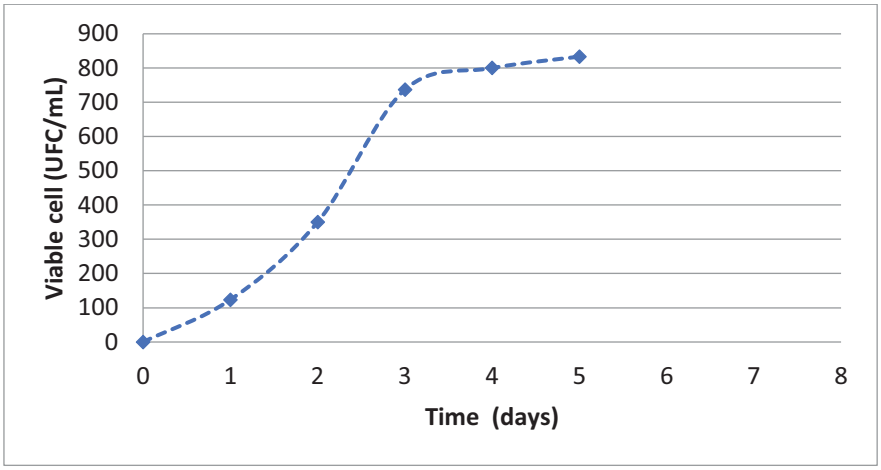


FIGURE 11 – KINETIC OF VIABLE CELL IN SEED CULTURE USING A HAND-MIXER DISRUPTION METHOD.

A confirmation test was made using 10% v/v of mycelial suspension and added to HD and GY medium (NISHA and VENKATESWARAN, 2011), getting 12.1 g/L and 10.33 g/L of total dry biomass weight respectively. This assay was performed in three replications to confirm any significant variation between every Erlenmeyer flask test inoculated, considering factors, such as: hyphae fragments or number of spores that would be pipetted for each replication.

The mixed seed broth suspension was weighted (g/mL), the quantity after 3 days obtained was around 6-9 mg/mL for every assay performed and about 3.72 x 10^6 mycelial residues/mL; after 7 days of fermentation process no significant statistical variation at 95% confidence level was found between the replications.

4.2 CULTURE MEDIUM SELECTION

To define a basal medium it was necessary to have more knowledge about *M.alpina sp.* and also select one medium as a base for a optimization process. In this part of the work different media were tested based in the following criteria: publications in which *Mortierella alpina* was used specifically, publications in which the biomass production was greater than 18 g/L, common culture media components available on the Laboratory of Bioprocess Engineering and Biotechnology – UFPR, and media in which no additives/supplements were used, but good results at a bench scale were obtained.

Four media were selected for testing both strains (CBS 528.72 and CBS 210.32) for the assays, evaluating its growth and total lipids production (TABLE 10).

The medium number 1 (HD medium) had a good yield of total lipids production (TABLE 10). *M. alpina* can tolerates a wide range of glucose concentration, lower glucose is exhausted quickly, and no carbon source for lipid synthesis remains for lipid accumulation (ZHU, YU, LI, ZHOU, and LI, 2006)

The results on TABLE. 10 show that the best medium was the number 4 (with 100 g/L as initial glucose), but the remaining sugar was too high and DCW concentration was not too acceptable for the initial quantity of glucose used.

Among the selected media, there is no glucose concentration fixed, concentrations from 30 g/L till 150 g/L can influence in the biomass production, arachidonic production and total lipids production.

Bajpai, Bajpaj, & Ward, (1991) affirmed that the best glucose concentration was around 60-80 g/L. For this reason, one duplication and triplication of the components of HD were also tested as the Bajpai *et al*., 1991 work.

TABLE 10 – MEDIA CULTURE SELECTION TEST.

Glucose (g/L)	C/N ratio	Strain	DCW [g/L]	Author's DCW [g/L] reached	Total lipids (%)	pH	Remaining sugar (g/L)	Yx/s (%)	Yp/s (%)
30[1]	22	CBS 528.72	13.11±0.4	17.5[1]	16.40±0.6	7.0	0.28±0.0	44.11	7.23
		CBS 210.32	12.58±0.6		15.53±0.2	7.8	0.29±0.4	42.34	6.57
50[2]	36	CBS 528.72	18.72±0.0	13.7[2]	16.62±0.6	8.3	0.49±0.4	37.80	6.28
		CBS 210.32	17.71±0.5		12.94±0.7	7.5	0.57±0.8	37.86	4.90
60[3]	40	CBS 528.72	19.77±0.1	22.5[3]	15.78±0.1	7.9	0.52±0.0	39.96	6.31
		CBS 210.32	17.84±0.8		18.68±0.8	8.0	0.47±0.9	40.07	7.48
100[4]	73	CBS 528.72	23.01±0.1	18.54[4]	20.24±0.9	6.8	52.92±0.1	48.88	9.89
		CBS 210.32	21.90±0.9		22.11±0.6	6.8	53.80±0.5	49.56	10.96

[1].(HANSSON, L. and M. DOSTALEK, 1988) medium culture formulation
[2].(WARD, 1997) medium culture formulation
[3] .(ZHU *et al*., 2006) medium culture formulation
[4] .(YU *et al*., 2003) medium culture formulation
Assays were performed in three replications, pH 6, 120 rpm at 25º for 7 days

A high C/N ratio is convenient for fungal growth and lipid production by *Mortierella* spp. (CHESTERS *et al*., 1965);in this study of culture medium selection, the best C/N ratio was 22 (FIGURE 12) because of the biomass and total lipids yields obtained. Sajbidor *et al*. (1990) reported that *Mortierella* sp. S-17 had high arachidonic acid concentration cultivated at C/N ratio 10, and had high arachidonic acid production at C/N ratio 20.

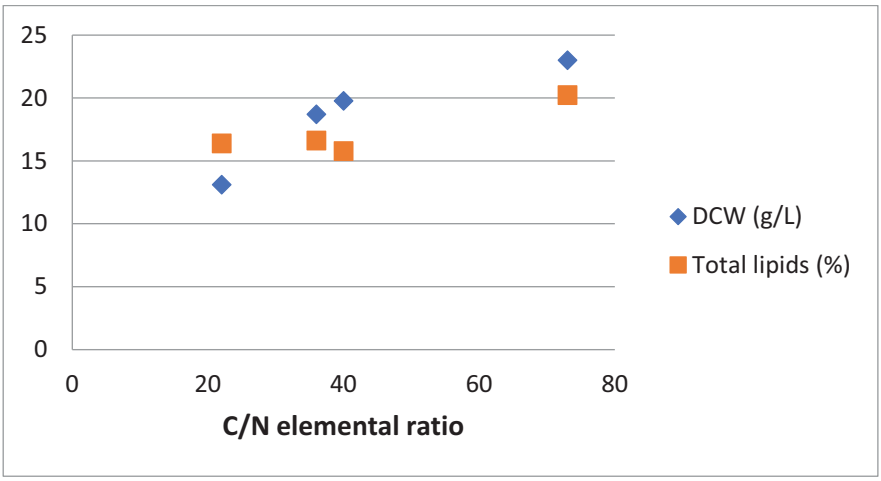


FIGURE 12 – C/N RATIO EVALUATION OF MEDIA CULTURE SELECTION

An assay duplicating the HD medium components, starting with 60 g/L of glucose to improve the mold development was also tested (TABLE11). A 23.76 g/L of DCW and 37.33% of total lipids was obtained, having low values of remaining sugar and maintaining the C/N elemental ratio (20), making it the best culture medium compared with the others, thus being selected for further test and for optimization process.

The strain CBS 528.72 produced a higher lipid and biomass amount. There is a variation of the parameter values ($Y_{x/s}$ and $Y_{p/s}$) between the strains and the medium tested, this is probably because of the inhibitor effect of the salts in major quantities and the slow uptake rate of glucose when higher concentrations are used, delaying the growth of the mold.

TABLE 11 – ADAPTATION OF (HANSSON, L. AND M. DOSTALEK, 1988) MEDIUM WITH HIGHER CONCENTRATION - STRAINS CBS 528.72 AND CBS 210.32.

Medium	Strain	pH	DCW (g/L)	Total lipids (%)	Remaining sugar (g/L)	Yx/s	Yp/s
A	CBS 528.72	7.32	12.60±0.5	28.42±0.4	0.28±0.1	42.37	10.99
	CBS 210.32	7.81	11.35±0.6	11.06±0.2	0.51±0.4	38.48	3.00
B	CBS 528.72	7.16	23.76±0.8	37.33±0.1	1.26±0.4	40.45	8.90
	CBS 210.32	6.40	6.83±0.8	4.50±0.5	5.01±0.5	14.19	0.42
C	CBS 528.72	5.39	15.06±0.1	20.88±0.9	71.90±0.7	85.71	14.18
	CBS 210.32	5.43	15.59±0.8	14.76±0.1	70.69±0.9	85.87	8.09

[A]. HD medium culture at original concentration (30 g/L of glucose)
[B.] HD medium culture at duplicated concentration (60 g/L of glucose)
[C] HD medium culture at triplicated concentration (90 g/L of glucose)
Assays were performed in three replications, pH 6, 120 rpm at 25º for 7 days

Koike *et al*., (2001) also reported that the optimal C/N ratio of the medium was around 15–20 for arachidonic acid production in a culture of *M. alpina* CBS754.68. This high C/N ratios (> 15) are important because a nitrogen depletion has to occur; the excess of carbon in the medium culture once that the nitrogen sources is depleted continues to be assimilated and converted directly into lipids, because of the inability to synthesize essential cell material (proteins).

At the end, two phases will exist: first, the one where a balanced growth happens and the second where, after a key nutrient exhaustion occurs, the lipid formation begins (RATLEDGE, 1997).

4.3 GROWTH AND LIPIDS PRODUCTION KINETIC WITH MODIFIED HANSSON AND DOSTALEK (HD) MEDIUM

To understand the correct development of the fungus in HD medium, in terms of growth and production level, a culture of 10 days was done in 250mL Erlenmeyer's flask using the strain CBS 528.72, as shown in the FIGURE 13.

The initial glucose concentration takes 192 h to be almost totally consumed (1 g/L remaining glucose). The mold showed a logarithmic phase starting at 96 h and reached a stationary phase at 168 h, the quantity of biomass formed at that time was 23.04 g/L; therefore 168h was selected as the final time of culture because after this time no statistical significantly rise was observed.

After 120 h a peak of total lipids (%) production was achieved, but the mold was still growing and accumulating lipids until glucose depletion.

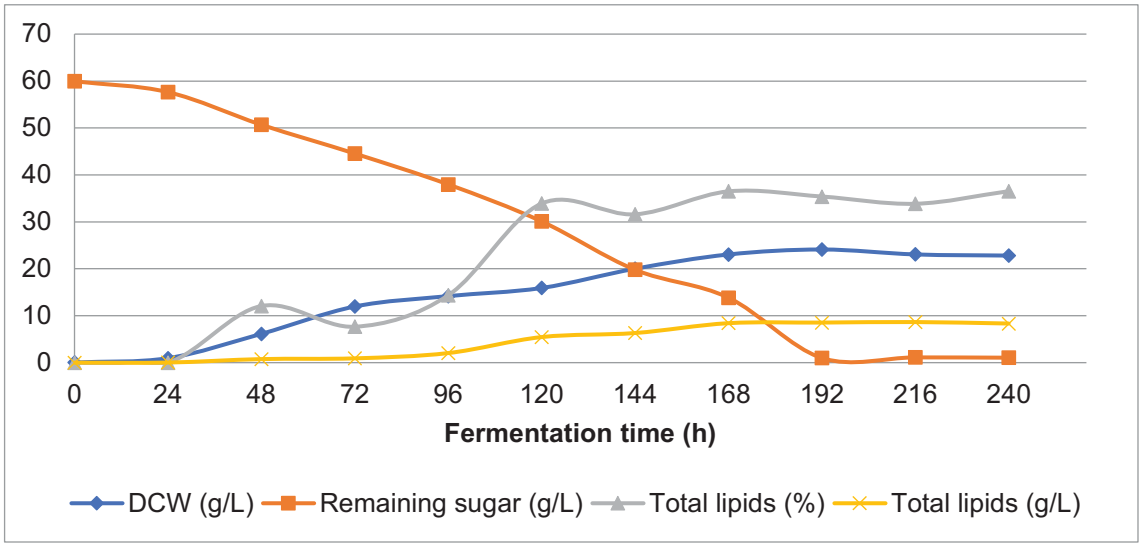


FIGURE 13 – GROWTH OF *MORTIERELLA ALPINA* CBS 528.72 IN HANSSON AND DOSTALEK MODIFIED MEDIUM.

As observed, when residual glucose was under 13 g/L, the mold stopped growing.

The pH variation in this kinetic study was from 6.00 to 7.00. The total lipids (%) concentration presented a behavior similar to that of biomass, initiating a plateau after168 h of culture.

A good biomass and lipid yield was reached at 168 h (50.04% and 18.26%, respectively). After this cultivation time the biomass yield tended to decrease (FIGURE 13), presumably because of cellular death. The product yield remained stationary. The highest DCW reached was about 24 g/L, and the total lipids were around 42 % at 168h (FIGURE 14)

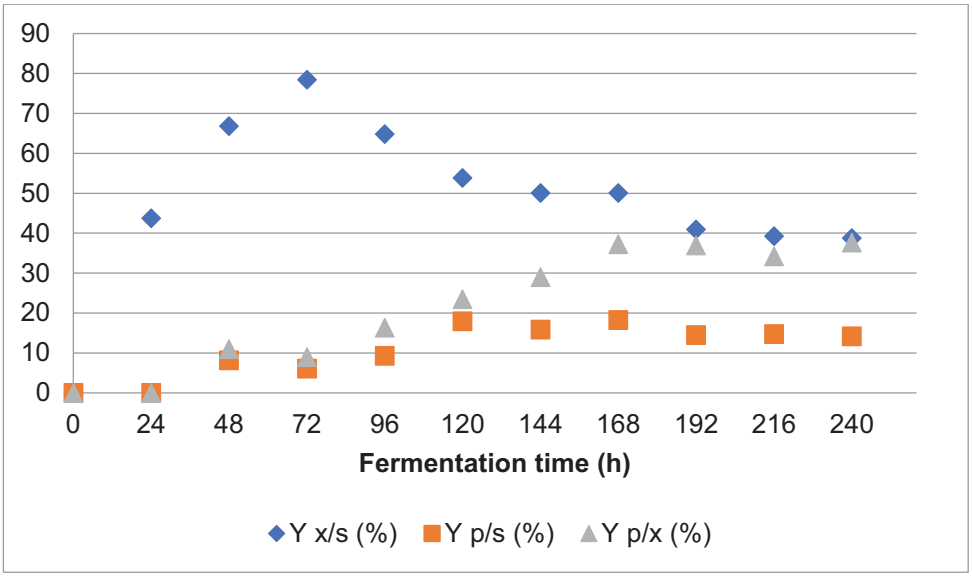


FIGURE 14 – BIOMASS AND PRODUCT YIELD OF *MORTIERELLA ALPINA* CBS 528.72 IN HANSSON AND DOSTALEK MEDIUM MODIFIED

4.4 EFFECT OF CULTURE CONDITION ON BIOMASS AND TOTAL LIPIDS PRODUCTION

a) *Incubation temperature and pH*

The influence of the temperature using a modified Hansson and Dostalek medium was tested showing that at 20º C a better production yield, in terms of lipids, was reached (18% approximately). The influence of temperature is important because at lower temperature more polyunsaturated lipids are formed (SAKURADANI & SHIMIZU, 2009). Biomass concentration was approximately constant in the range of temperatures tested, but the lipid content was considerably higher (up to 50% of lipids/DCW) at the lowest temperature tested. This result can be attributed to the proposed adaptive role of PUFAs in membrane stabilization under stress conditions of low temperatures, since lipids from membrane are also extracted.

Also, at lower temperatures more dissolved oxygen is available in the culture medium for desaturase enzymes – which are oxygen dependent (COHEN *et al.*, 1987) – thereby resulting in production of more total lipids.

The temperature of 25ºC was selected for this modified culture medium since no significant statistical difference at 95% confidence level was observed compared with 20ºC (FIGURE 15); and a temperature shift could be applied in a further study for total lipid yield increment.

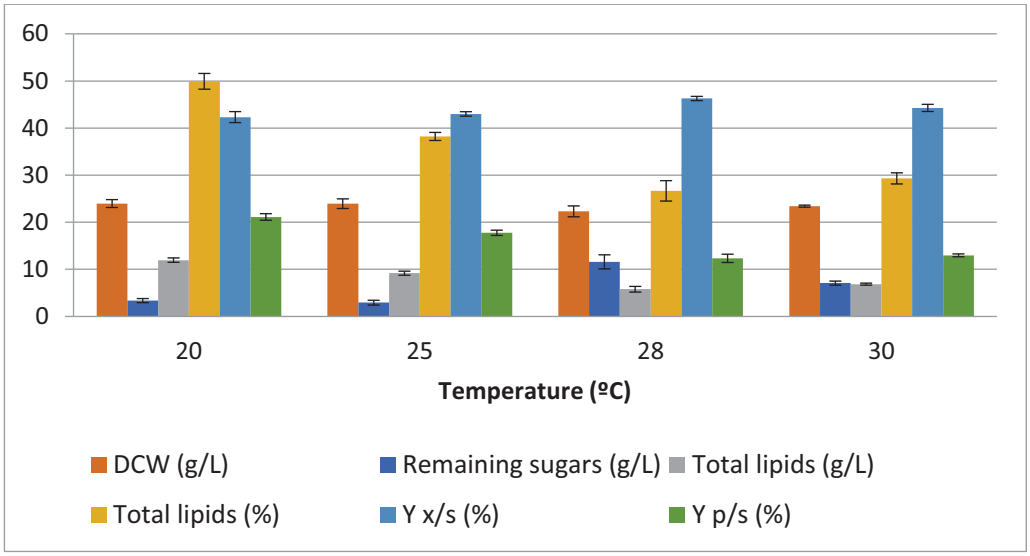


FIGURE 15 – EFFECT OF TEMPERATURE ON PRODUCT YIELD, BIOMASS AND TOTAL LIPID PRODUCTION BY *MORTIERELLA ALPINA* CBS 528.72

The pH value is very important in fungal growth and the metabolite production. In this part of the study an evaluation of pH (5-8) was made. The pH 6 was adequate for the development of *M. alpina* CBS 528.72, as illustrated in the FIGURE 16. The biomass concentration at this pH was 23 g/L and 36% of total lipids, the variation of the pH was one unit, from 6 to 7. This result agrees with Lindberg and Molin (1993), who affirm that the optimal pH is around 6.

The pH influences in the provided carbon uptake, reducing the efficiency when pH is very high or very low. The same happens with morphology of the mold, under a different initial pH value, there is a critical factor in biomass accumulation and metabolite formation (SHU and LUNG 2004; WANG and MC NEIL 1995).

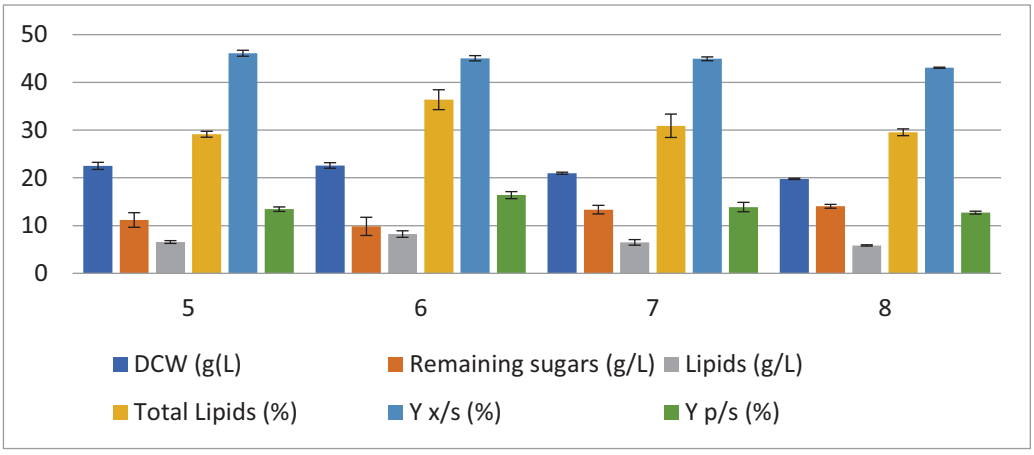


FIGURE 16 – EFFECT OF pH ON PRODUCT YIELD, BIOMASS AND TOTAL LIPID PRODUCTION BY *MORTIERELLA ALPINA* CBS 528.72

b) *Inoculation rate*

Since no standard inoculation method was found in literature for *Mortierella sp.* fermentation assays and also the influence of the concentration of the inoculum, a study (FIGURE 17) was performed to evaluate the effect in the biomass production and lipids production at 72h, 120h and 168h with 5,10,15 and 20% v/v of mycelial suspension having 9 mg of mycelia/mL.

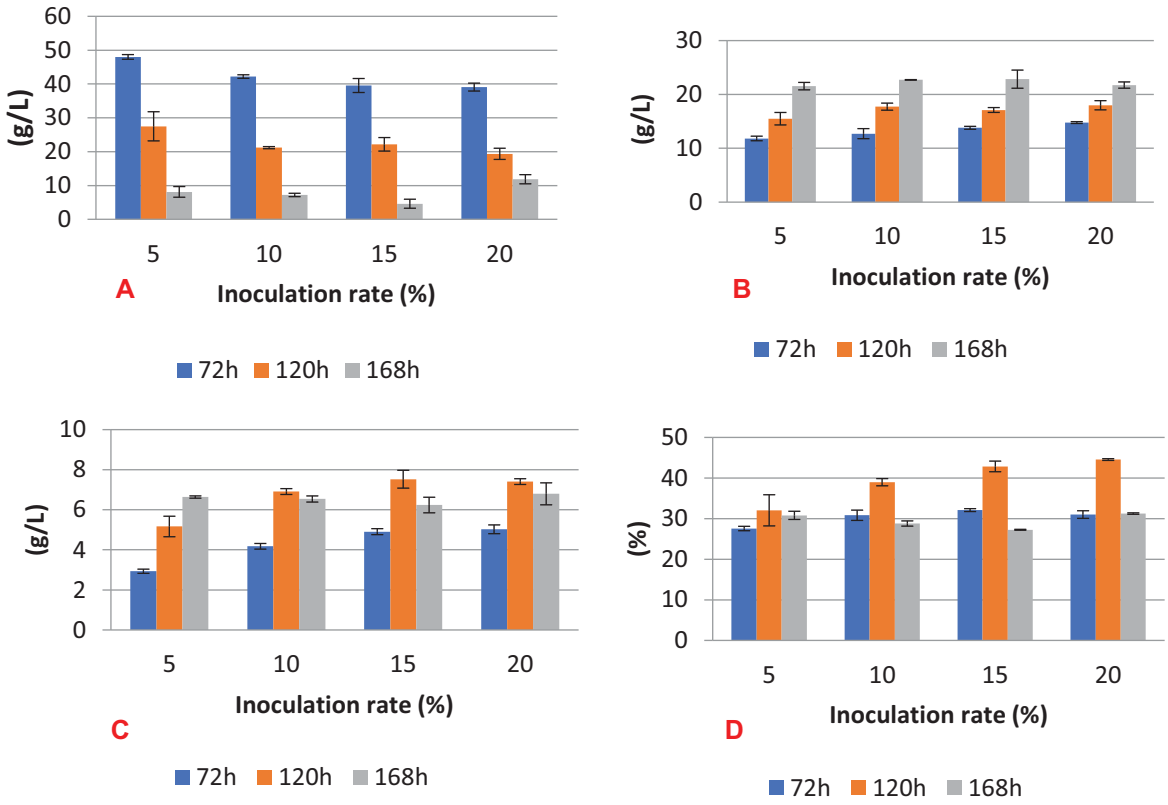


FIGURE 17 – EFFECT OF INOCULATION RATE, EVALUATION AT 72H, 120H AND 168H. A) REMAINING GLUCOSE (G/L), B) DCW (G/L), C) TOTAL LIPIDS (G/L) AND D) TOTAL LIPIDS (%)

In terms of remaining sugar (g/L) at 72h the glucose consumption was not high because of the mold lag phase, at this time about 30%; at 120h, 62% and at 168h, 85% of total sugar was consumed at the different inoculation rates.

Biomass concentration (g/L) tends to increment due to the inoculation rate and fermentation period, as show in the FIGURE 17 at 72h a statistically significant at 95% of confidence was observed between the rates of inoculation during the fermentation period, but not after 168h, which indicates that at this time any inoculation rate can be chose, even 5%, but 10% was chosen because of the high value reached (22.70 g/L).

A statistical significant difference was also observed between the fermentation times; being 168h the fermentation time in which more biomass was produced.

About total lipids (%) a better production was observed at 120h, 28% at 5% v/v, 40% at 10%, 42% at 15% and 44% of total lipids at 20% v/v of the suspension. At 168h of fermentation the percentage decreased in 10%, but biomass production continued, hence the total lipids (g/L) amount was increasing; for this reason 168h was selected as a fermentation time at the rate of 10% v/v of mycelial suspension. The aim of this part of the work is to obtain the largest possible amount of biomass to induce a greater production.

Another reason for choosing this fermentation time is because arachidonic acids content increases when glucose depletion happens; these results were observed by (EROSHIN *et al*., 2002), which showed that the ARA percentage increases as a result of other fatty acids conversion. Why glucose depletion stimulated fatty acids conversion to ARA and whether this conversion only occurred in *M. alpina* still remains unclear.

4.5 OPTIMIZATION OF MEDIUM CULTURE COMPOSITION

The modified Hansson and Dostalek (1988) medium was optimized, based in a review of papers from the last twenty years, testing both strains for comparison, the optimization was based in biomass production and keeping high levels of total lipids production (%).

For a better understanding of the carbon and nitrogen sources influence on biomass and lipids production, a study was done with “one-factor-at-the-time” experimental design to evaluate these sources individually, avoiding any interaction between them.

The carbon sources and concentrations (TABLE 12) were chosen based in the literature. For *Mortierella alpina* CBS 528.72, the best carbon source was glucose at 60 g/L, giving 20.9 g/L of DCW and about 48% of total lipids. As for the nature of the carbon sources, a relevant statistical difference was observed (at 95% of confidence); starch as a carbon source showed good results at 80 g/L of concentration, giving 21.3 g/L of DCW but not as good for total lipids (about 36%). *Mortierella* has no amylolytic activity, according to (HIGETA, NO, & UZUKI, 2002), but exo-type glucose-forming activity was detected implying that a glucose-forming enzyme could be responsible for

the digestion, with the formation of dextrin by the enzyme. In the case where sucrose was the carbon source, the results were not effective compared with other sources due to insufficient activity of invertase, namely β-galactosidase (PRASAD, 2006)

Since 60 g/L of glucose was adequate for an acceptable biomass and lipids production, an additional test was performed using *fructose and galactose* as carbon source. Generally fungi belonging to Mortierellales are saprophytes, which prefer to grow rapidly and proliferate extensively on simple sugars compared to complex molecules (Kendrick, 2001). Using fructose at 60 g/L a 23.6 g/L of DCW and 40.2% of total lipids was reached, being the best result among all the sources tested so far. At the other side, galactose is not an adequate carbon source, giving a low (7.6g/L) DCW.

The order of carbon sources consumption preference for this strain according to this study is:

Fructose > Glucose > Starch > Galactose > Sucrose.

TABLE 12 – EFFECT OF CARBON SOURCE IN BIOMASS AND TOTAL LIPIDS PRODUCTION FOR *MORTIERELLA ALPINA* CBS 528.72.

Carbon source	Concentration (g/L)	Dry cell weight (g/L)	Total lipids (g/L)	Total lipids (%)
Glucose	60	20.9±1.1	9.6±1.08	47.7±2.6
	80	21.1±0.3	8.4±1.5	35.6±5.4
	100	20.0±0.3	7.9±1.3	39.2±5.6
Starch	60	18.0±0.1	5.9±0.6	32.9±3.7
	80	21.3±1.8	7.6±0.1	35.8±3.7
	100	20.4±1.8	6.8±0.8	33.2±1.3
Sucrose	60	6.50±0.1	0.1±0.2	2.4±0.4
	80	7.2±0.2	0.4±0.7	5.2±1.1
	100	8.3±0.2	1.0±0.1	12.3±1.6
Fructose	60	23.6±0.3	9.0±0.6	40.2±2.3
Galactose	60	7.6±0.2	ND	ND

For the strain CBS 210.32 the results (TABLE 13) were similar, with the best carbon source being glucose at 60 g/L, giving 20.7 g/L of DCW and about 24% of total lipids. The variation of glucose concentration, showed no statistical difference for biomass production. As for the other carbon sources, starch and sucrose gave even poorer productions than that obtained with the other strain (CBS 528.72).

TABLE 13 – EFFECT OF CARBON SOURCE IN BIOMASS AND TOTAL LIPIDS PRODUCTION FOR *MORTIERELLA ALPINA* CBS 210.32.

Carbon source	Concentration (g/L)	Dry cell weight (g/L)	Total lipids (g/L)	Total lipids (%)
Glucose	60	20.7±0.5	4.8±0.5	23.4±1.7
	80	20.1±0.2	4.3±0.3	21.5±1.2
	100	20.0±0.6	4.2±0.2	21.1±0.2
Starch	60	15.8±0.2	3.1±0.1	19.4±0.1
	80	17.3±0.4	3.5±0.4	20.3±2.0
	100	18.2±0.1	4.0±0.1	21.8±0.7
Sucrose	60	2.4±0.1	ND	ND
	80	3.0±0.2	ND	ND
	100	2.9±0.1	ND	ND

DYAL *et al*., 2005, affirmed that fructose and glucose as carbon sources in culture medium yielded much higher biomass (48% compared with glycerol). In our experiments, a higher concentration of glucose was not convenient for lipid production.

In both strains it was observed that, at higher concentration of sugars, a biomass increment was not observed. This could be due to the osmotic potential of the medium, and also to different carbon sources having different effects of catabolic repression on the cellular secondary metabolism (NISHA & VENKATESWARAN, 2011).

Nitrogen source is a central factor in the *M. alpina* growth and secondary metabolites production, its depletion after growth phase is important for lipid accumulation (NISHA & VENKATESWARAN, 2011). In this work a preliminary evaluation was performed with different organic and inorganic N sources, evaluating both biomass and total lipids production.

The best results with *M. alpina* CBS. 528.72 in DCW (g/L) terms were obtained using organic sources of nitrogen (see in FIGURE 18). With yeast extract and peptone, the DCW reached 20.8 g/L and the total lipids (%) concentration was around 48%. Yeast extract is one of the most ubiquitous culture component, and has a relatively low cost. Weet (1980) studied the effect of nitrogen sources on biomass and lipid yield of various *Mortierella* strains. In all the cases, yeast extract was found to be the best nitrogen source for maximizing biomass, lipid, and ARA yields.

In this study, urea was also convenient, giving 18.5g/L of DCW but just around 34% of totals lipids. This may be due to the fact that urea acts as an inhibitor for delta 5 desaturase, a key enzyme which catalyzes the conversion of dihomo-γ-linolenic acid (DGLA) to ARA (OBUKOWICZ *et al*., 1999).

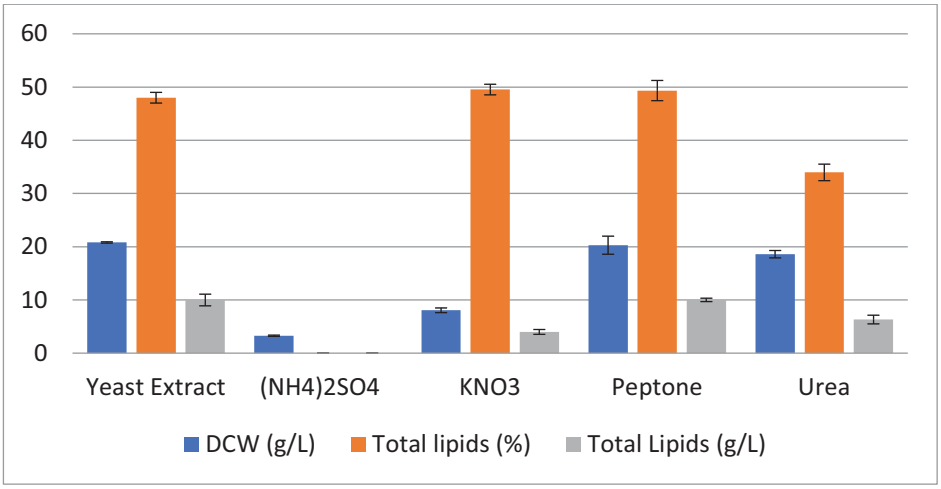


FIGURE 18 – EFFECT OF NITROGEN SOURCE IN BIOMASS AND TOTAL LIPIDS PRODUCTION FOR MORTIERELLA ALPINA CBS 528.72.

The inorganic salts were not adequate for the growth of the mold; 8g/L and 3 g/L of DCW were obtained using $KNO_3$and $(NH_4)_2SO_4$, respectively, with 50% and 3% of total lipids.

For *M. alpina* CBS 210.32, the results (FIGURE 19) show that nitrogen organic sources were adequate, and KNO_3 too – but $(NH_4)_2SO_4$ gave a poor growth. With yeast extract the value of DCW was 20.5 g/L, not statistically different of the production using peptone and urea, (18.6 and 20.8 g/L respectively), at the 95% confidence interval. The lowest values of DCW, were obtained with KNO_3 and $(NH_4)_2SO_4$ – 18.6g/L and 2.4 g/L respectively, but in terms of total lipids (%) the best nitrogen source was potassium nitrate (43% approximately).

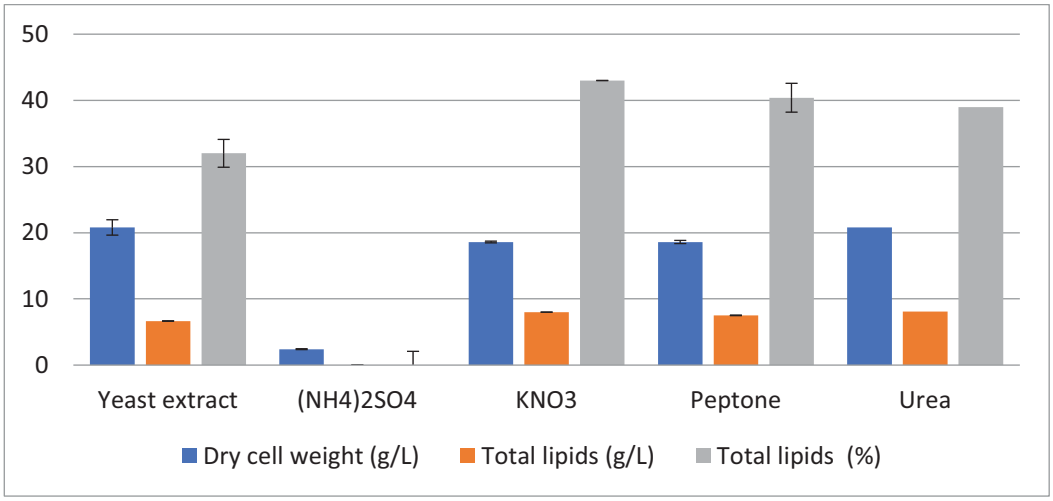


FIGURE 19 – EFFECT OF NITROGEN SOURCE IN BIOMASS AND TOTAL LIPIDS PRODUCTION FOR *MORTIERELLA ALPINA* CBS 210.32.

The second part of the optimization process consisted in the improvement of the biomass production based on the amount of media components other than carbon and nitrogen sources. A Plackett-Burman experimental design was done using the medium of Hansson and Dostalek as a base, but testing other salts and different concentrations used by different authors in their researches in the last twenty years: the common medium components Ca^{2+}, Mg^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}, and the not so common Co^{2+}.Both biomass production and lipids concentration were evaluated.

The influence of the salts was compared for both strains. Calcium chloride (FIGURE 20), was significant at 95% of confidence, for the development of *M.alpina* CBS 528.72 (R^2 = 0.983). No salt showed significant influence for *M. alpina* CBS 210.32 (FIGURE 22) (R^2 = 0.919). This is due to the range of concentrations selected, which means that the values selected between the maximum and minimum values did not influenced in the test. These values were obtained from a literature screening. Data was observed in the Pareto charts, a tendency of DCW to increase was observed when a high level of calcium chloride was used.

According to the results (TABLE 14) The maximum biomass and total lipids (%) reached testing *M. alpina* CBS 528.72 was 23.38 g/L and about 52% respectively; in this assay the concentration of glucose and yeast extract were 60 g/L and 10 g/L respectively, these results were higher than the ones obtained in the central point of the concentrations of the salts.

TABLE 14 – PLACKETT-BURMAN EXPERIMENTAL DESIGN FOR BIOMASS PRODUCTION OPTIMIZATION FOR *MORTIERELLA ALPINA* CBS 528.72

Run	KH_2PO_4 (g/L)	KNO_3 (g/L)	$CaCl_2$ (mg/L)	$MgSO_4$ (g/L)	$FeCl_3$ (mg/L)	$ZnSO_4$ (mg/L)	$CuSO_4$ (mg/L)	$MnCl_2$ (mg/L)	$CoCl_2$ (mg/L)	DCW (g/L)	Total lipids (g/L)	Total lipids (%)	Y x/s (%)	Y p/s (%)
1	7.00	1.00	100.00	0.30	1.50	1.00	0.50	4.30	0.13	22.19	9.78	44.09	42.83	26.22
2	7.00	7.50	12.00	1.50	1.50	1.00	0.10	4.30	0.13	20.08	7.24	36.07	43.75	18.54
3	1.00	7.50	100.00	0.30	15.00	1.00	0.10	1.00	0.13	23.01	11.23	48.80	38.98	28.40
4	7.00	1.00	100.00	1.50	1.50	7.50	0.10	1.00	-	22.03	10.13	45.97	42.39	31.42
5	7.00	7.50	12.00	1.50	15.00	1.00	0.50	1.00	-	20.78	8.76	42.13	42.82	22.70
6	7.00	7.50	100.00	0.30	15.00	7.50	0.10	4.30	-	21.03	9.15	43.49	40.48	20.93
7	1.00	7.50	100.00	1.50	1.50	7.50	0.50	1.00	0.13	21.86	9.19	42.02	40.37	20.47
8	1.00	1.00	100.00	1.50	15.00	1.00	0.50	4.30	-	23.38	12.22	52.25	39.84	27.77
9	1.00	1.00	12.00	1.50	15.00	7.50	0.10	4.30	0.13	21.40	9.68	45.25	42.62	21.43
10	7.00	1.00	12.00	0.30	15.00	7.50	0.50	1.00	0.13	19.74	7.25	36.73	39.10	16.29
11	1.00	7.50	12.00	0.30	1.50	7.50	0.50	4.30	-	17.66	6.69	37.88	39.68	15.12
12	1.00	1.00	12.00	0.30	1.50	1.00	0.10	1.00	-	20.94	9.60	45.82	38.73	16.00
13	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	19.45	8.89	39.89	38.78	14.82
14	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	19.90	9.29	40.20	39.90	15.48
15	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	19.61	9.45	40.65	39.02	15.75

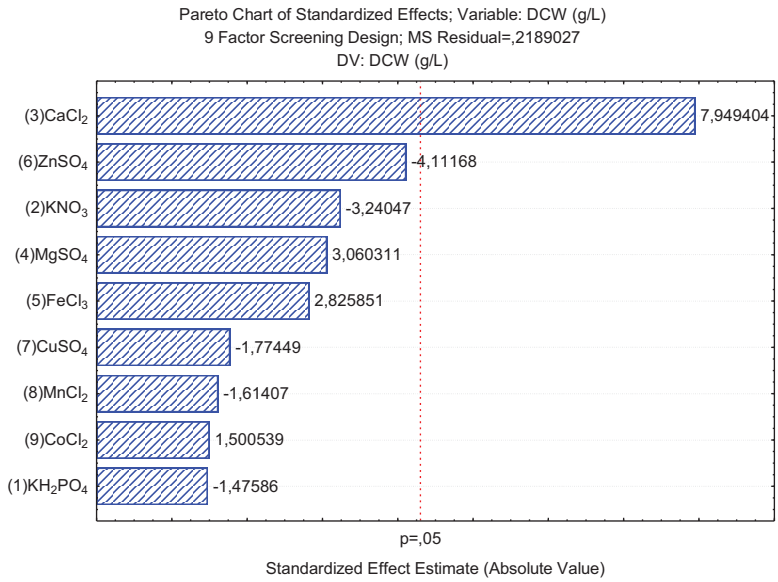


FIGURE 20 – PARETO CHART OF SALTS INFLUENCE IN BIOMASS (g/L) PRODUCTION IN *MORTIERELLA ALPINA* CBS 528.72.

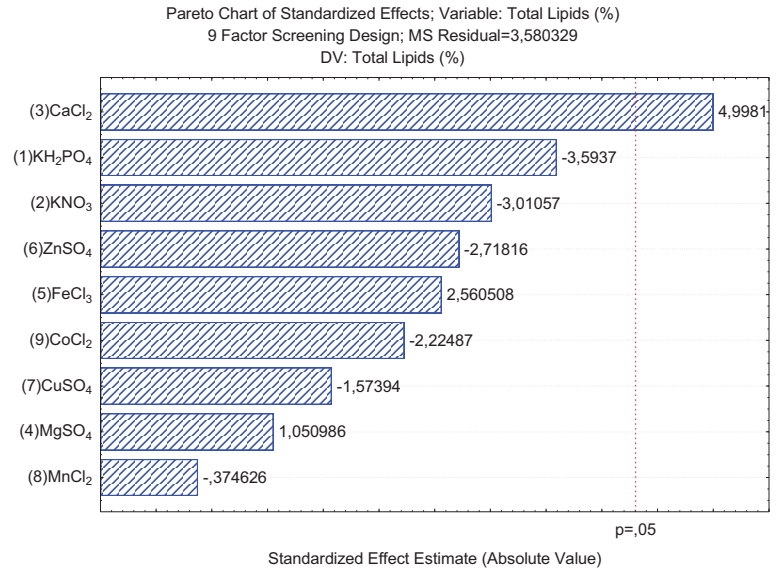


FIGURE 21 – PARETO CHART OF SALTS INFLUENCE IN TOTAL LIPIDS (%) PRODUCTION *MORTIERELLA ALPINA* CBS 528.72.

For the strain CBS 210.32 the total DCW was 21.61 g/L and 43% of total lipids (TABLE 15), being inferior to the strain CBS 528.72; also no salt was statistical significant at 95% of confidence for the lipids production (FIGURES. 21-23) (R-square = 0.83904).

Based in these initial results, CBS 528.72 was chosen as the strain for posterior studies and assays.

TABLE 15 – PLACKETT-BURMAN EXPERIMENTAL DESIGN FOR BIOMASS PRODUCTION OPTIMIZATION FOR MORTIERELLA ALPINA CBS 210.32.

Run	KH_2PO_4 (g/L)	KNO_3 (g/L)	$CaCl_2$ (mg/L)	$MgSO_4$ (g/L)	$FeCl_3$ (mg/L)	$ZnSO_4$ (mg/L)	$CuSO_4$ (mg/L)	$MnCl_2$ (mg/L)	$CoCl_2$ (mg/L)	DCW (g/L)	Total lipids (g/L)	Total lipids (%)	Y x/s (%)	Y p/s (%)
1	7.00	1.00	100.00	0.30	1.50	1.00	0.50	4.30	0.13	17.92	6.39	35.68	45.89	16.37
2	7.00	7.50	12.00	1.50	1.50	1.00	0.10	4.30	0.13	21.61	6.84	31.64	43.19	13.66
3	1.00	7.50	100.00	0.30	15.00	1.00	0.10	1.00	0.13	21.60	9.06	41.96	41.34	17.35
4	7.00	1.00	100.00	1.50	1.50	7.50	0.10	1.00	-	18.49	7.19	38.88	45.81	17.81
5	7.00	7.50	12.00	1.50	15.00	1.00	0.50	1.00	-	19.60	5.25	26.76	42.82	11.46
6	7.00	7.50	100.00	0.30	15.00	7.50	0.10	4.30	-	20.50	6.87	33.50	41.47	13.89
7	1.00	7.50	100.00	1.50	1.50	7.50	0.50	1.00	0.13	19.64	6.96	35.42	43.85	15.53
8	1.00	1.00	100.00	1.50	15.00	1.00	0.50	4.30	-	18.39	6.93	37.66	45.16	17.01
9	1.00	1.00	12.00	1.50	15.00	7.50	0.10	4.30	0.13	20.50	7.52	36.66	43.79	16.05
10	7.00	1.00	12.00	0.30	15.00	7.50	0.50	1.00	0.13	19.63	8.42	42.92	42.22	18.12
11	1.00	7.50	12.00	0.30	1.50	7.50	0.50	4.30	-	20.44	6.06	29.66	40.65	12.06
12	1.00	1.00	12.00	0.30	1.50	1.00	0.10	1.00	-	17.81	5.94	33.38	43.88	14.65
13	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	19.50	7.15	36.64	41.50	15.21
14	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	18.92	7.68	40.60	45.00	18.27
15	4.00	4.25	56.00	0.90	8.25	4.25	0.30	2.65	0.07	19.99	7.49	37.44	43.60	16.33

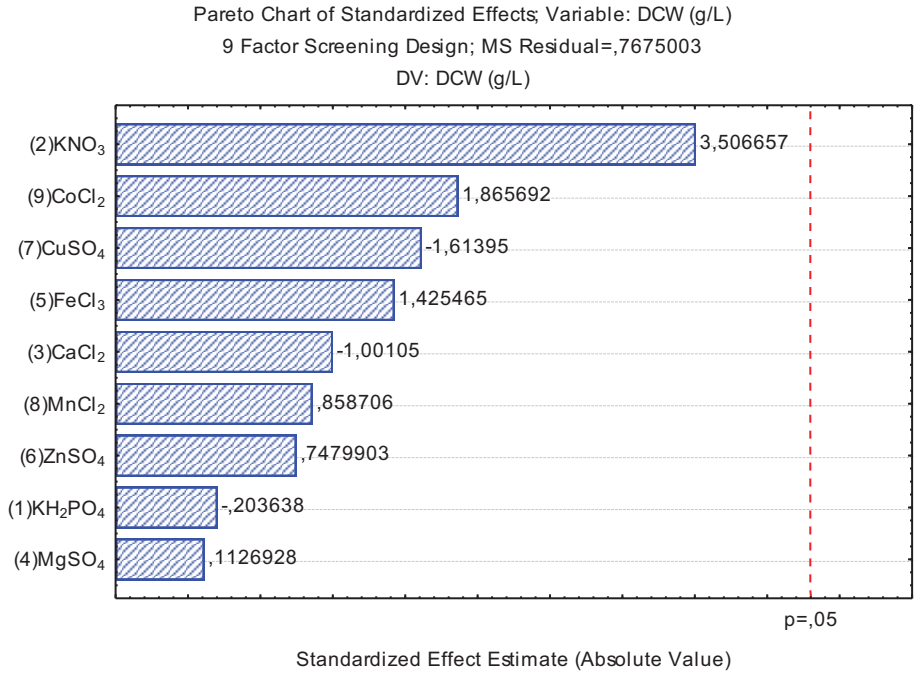


FIGURE 22 – PARETO CHART OF SALTS INFLUENCE IN BIOMASS (g/L) PRODUCTION FOR MORTIERELLA ALPINA CBS 210.32.

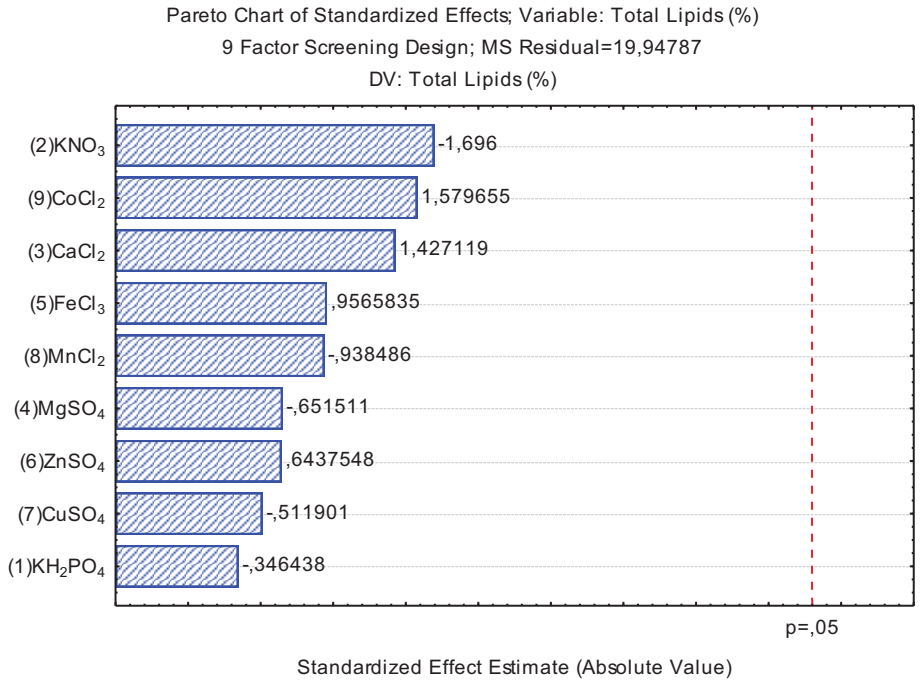


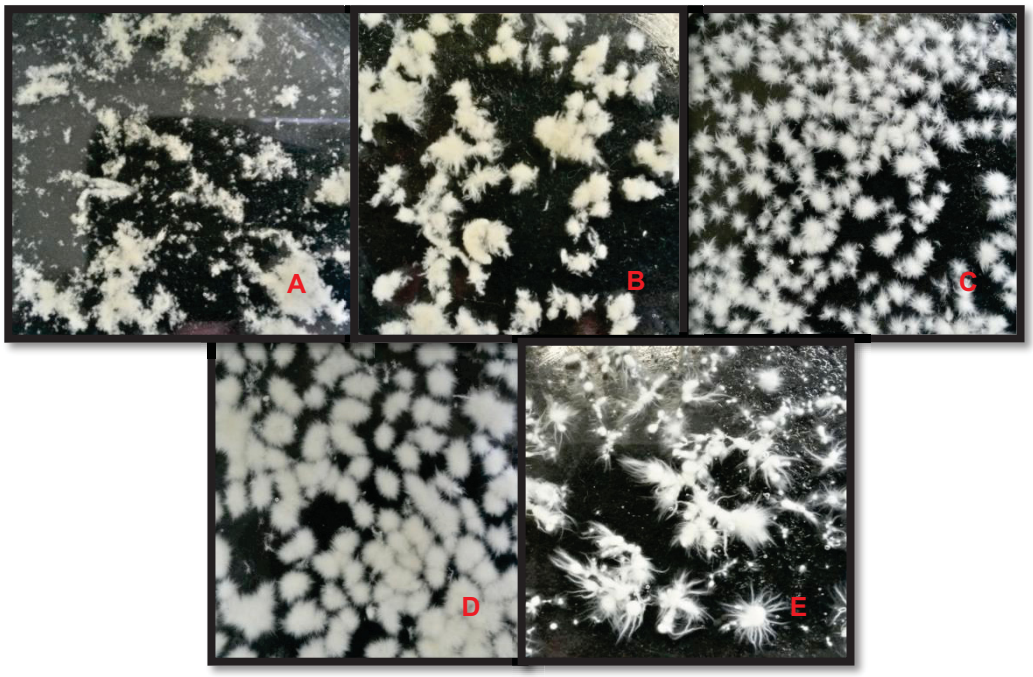
FIGURE 23 – PARETO CHART OF SALTS INFLUENCE IN TOTAL LIPIDS (%) PRODUCTION.

In the first part of the optimization test (PB design), calcium chloride showed a positive influence in the development of *M. alpina.* For this reason a posterior

experimental design was performed (CCRD) to optimize the adequate amount of $CaCl_2$, and also the amount of yeast extract as nitrogen source to reach a good biomass and lipid production for the strain CBS 528.72.

The results in the TABLE 16 show a trend when the concentration of yeast extract was incremented, the biomass was incrementing too until values over 25 g/L where tested, in which the biomass started to grow. The maximum biomass reached was 24.87 g/L, the opposite effect was observed for the production of lipids in which yeast extract in low quantities shown a positive effect (5 g/L) agreeing with the theory that for a better lipids production in biomass a depletion of nitrogen has to occur (LU, PENG, JI, and YOU, 2011). For this study glucose was kept at 60 g/L, pH 6, 120 rpm, 25ºC for 7 days of fermentation. The biomass yield was 45% while lipids 23%.

The calcium chloride also showed an influence in the mold morphology, as can be seen in FIGURE 24, which shows the development and shape of the mycelia formed at different calcium concentrations. Among the concentration the most adequate was 62 mg/L because of the fluffy pellet (feather-like pellet) formed with 2 mm diameter approximately according to Jang et al., (2005)



Source: This work

FIGURE 24– INFLUENCE OF $CaCl_2$ IN THE MORPHOLOGY OF *Mortierella alpina* CBS 528.72. A) 8.42 mg/L of $CaCl_2.2H_2O$ B) 24 mg/ of $CaCl_2.2H_2O$C) 62 mg/L of $CaCl_2.2H_2O$ D)100 mg/L of $CaCl_2.2H_2O$ E) 115.6 mg/L of $CaCl_2.2H_2O$

TABLE 16 – CCRD EXPERIMENTAL DESIGN OF CALCIUM CHLORIDE AND YEAST EXTRACT INFLUENCE IN THE BIOMASS, AND TOTAL LIPIDS PRODUCTION.

Run	Yeast Extract (g/L)	$CaCl_2$ (mg/L)	DCW (g/L)	Total Lipids (g/L)	Total Lipids (%)	Y x/s (%)	Y p/s (%)
1	5.00	24.00	16.43	8.43	51.32	39.75	20.40
2	5.00	100.00	16.53	10.11	61.18	37.96	23.22
3	25.00	24.00	24.18	11.11	45.94	55.33	25.42
4	25.00	100.00	24.57	7.16	29.14	55.67	16.22
5	0.90	62.00	9.22	4.70	51.00	34.98	17.84
6	29.10	62.00	22.68	8.13	35.84	57.14	20.48
7	15.00	84.20	24.87	8.12	32.64	45.12	14.73
8	15.00	115.60	24.17	10.95	45.32	45.74	20.73
9	15.00	62.00	23.27	10.47	44.98	48.94	22.01
10	15.00	62.00	23.50	11.07	47.12	46.19	21.77
11	15.00	62.00	23.49	11.12	47.32	47.20	19.56
12	15.00	62.00	23.39	10.90	46.60	47.09	21.94

The interaction between the variables can be observed in the TABLE 17. With a $R^2 = 0.981$, the ANOVA table shows that only the quadratic and linear effects of the yeast extract concentration were significant. This result may be observed in the response surface graphic (FIGURE 25) where there is a maximum biomass production (about 25g/L) at yeast extract concentrations around 20g/L. The fitted model for DCW (g/L) production obtained was z=23,912+4,3530607653021*x3,93225*x^2,062390133317053*y+,35275*y^2+,0729 99999999998*x*y

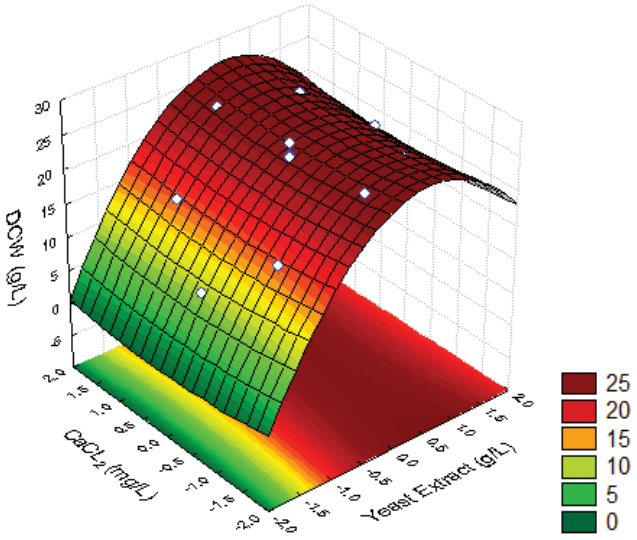


FIGURE 25 – EFFECT OF CALCIUM CHLORIDE AND YEAST EXTRACT IN BIOMASS YIELD.

TABLE 17 – ANOVA FOR CCRD EXPERIMENTAL DESIGN (YEAST EXTRACT AND CALCIUM CHLRORIDE) FOR BIOMASS PRODUCTION USING *MORTIERELLA ALPINA* CBS 528.72

Factor	SS	DF	MS	F	p
(1) Yeast extract (L)	151.5931	1	151.5931	181.6547	0.00001
Yeast extract (Q)	98.9606	1	98.9606	118.5849	0.000036
(2)$CaCl_2$(L)	0.0311	1	0.0311	0.0373	0.853197
$CaCl_2$(Q)	0.7964	1	0.7964	0.9543	0.366636
1L by 2L	0.0213	1	0.0213	0.0255	0.878266

R^2 = 0.98105

The ANOVA (TABLE 18) for total lipids (%) shows that overall effect is linear (and negative) of yeast extract (R^2 = 0.78) in lipid concentration. In fact, low levels of nitrogen are required for a better production. The interaction effect is secondary, that is possibly due to its effect on the pellet morphology and the effect of CaCl2 in the range tested is not significant. Higashiyama *et al*., 2002 affirms that this salt induces the formation of pellets with a fluffy texture, which is convenient for a contact of the mold with medium culture, thus nutrients and oxygen uptake.

TABLE 18 – ANOVA FOR CCRD EXPERIMENTAL DESIGN (YEAST EXTRACT AND CALCIUM CHLORIDE) FOR TOTAL LIPIDS (%) PRODUCTION USING *MORTIERELLA ALPINA* CBS 528.72

Factor	SS	df	MS	F	P
(1) Yeast extract (L)	433.0548	1	433.0548	14.63899	0.008699
Yeast extract (Q)	0.0903	1	0.0903	0.00305	0.957745
(2)$CaCl_2$(L)	15.1036	1	15.1036	0.51056	0.501736
$CaCl_2$(Q)	35.0064	1	35.0064	1.18336	0.318429
1L by 2L	177.6889	1	177.6889	6.0066	0.049734

R^2 = 0.78849

4.5.1 GROWTH KINETICS OF *M. ALPINA* CBS 528.72 IN THE OPTIMIZED CULTURE MEDIUM

In order to test the reproducibility of the results obtained in the experimental designs, a kinetic study was performed, in order to evaluate the biomass production and lipids yield.

The optimized culture medium for *Mortierella alpina* CBS 528.72, developed after PB and CCRD runs, is presented in TABLE 19:

TABLE 19 – OPTIMIZED CULTURE MEDIUM COMPOSITION FOR *M. ALPINA* CBS 528.72.

COMPOSITION	QUANTITY
Yeast extract (g/L)	15.0
Glucose (g/L)	60.0
KNO_3 (g/L)	1.0
KH_2PO_4 (g/L)	1.0
$CaCl_2 \cdot 2H_2O$ (mg/L)	62.0
$MgSO_4 \cdot 7H_2O$ (g/L)	0.3
$FeCL_3 \cdot 6H_2O$ (mg/L)	1.5
$ZnSO_4 \cdot 7\ H_2O$ (mg/L)	1.0
$CuSO_4 \cdot 5H_2O$ (mg/L)	0.1
$MnCL_2 \cdot 4H_2O$ (mg/L)	1.0

The growth kinetics (FIGURE 26) was done analyzing the batches of 250mL Erlenmeyer's cultures for a 10 days period.

The initial glucose concentration took 192 h to be almost completely consumed (11 g/L glucose remaining). The mold showed a logarithmic growth phase since 24 h and reached the stationary phase at 96 h, the quantity of biomass formed at this time was 23.13 g/L; this time was selected as the total time of fermentation because after this time no statistically significant rise was observed. The specific growth rate (μ) calculated in this work was 0,04 h^{-1}, under the batch conditions utilized, the productivity of total lipids obtained was of 43,52 mg L^{-1} h^{-1}, in 7 days of fermentation period, reaching a concentration of 43,3% of total lipids in relation to the dry cellular weight. While authors as Eroshin, Satroutdinov, Dedyukhina, & Chistyakova,(2000) reached at μ= 0,04 h^{-1} approximately 33,2 g L^{-1} h^{-1} of total lipids.

After 144 h a peak of total lipids (40%) production was achieved, but the mold was still growing and accumulating lipids until glucose depletion.

A pH variation was observed from 6.00 to 7.00. The total lipids (%) concentration presented a similar behavior as biomass, initiating a plateau from 144 h.

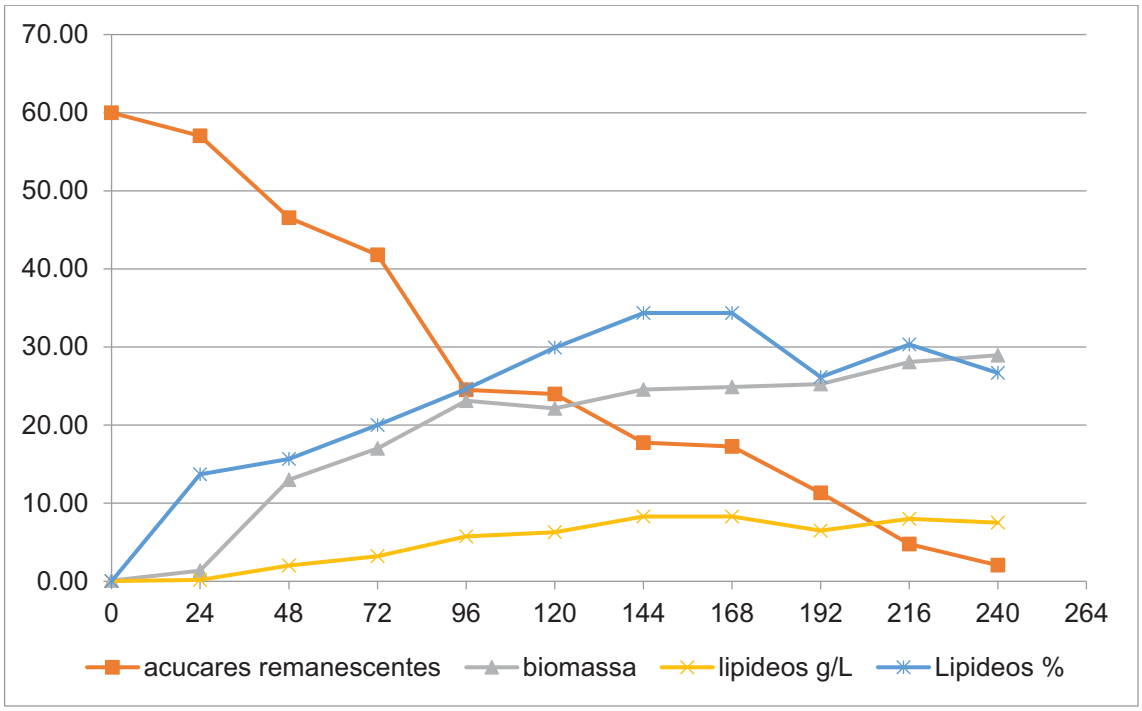


FIGURE 26 – KINETIC OF *M. ALPINA* CBS 528.72 IN THE OPTIMIZED MEDIA CULTURE.

A good biomass yield was reached at 168 h (58.17%) same as product yield (23.34%) in which after this fermentation time the biomass yield tended to diminish (FIGURE 27), because of cellular death and the product yield remained unchanged.

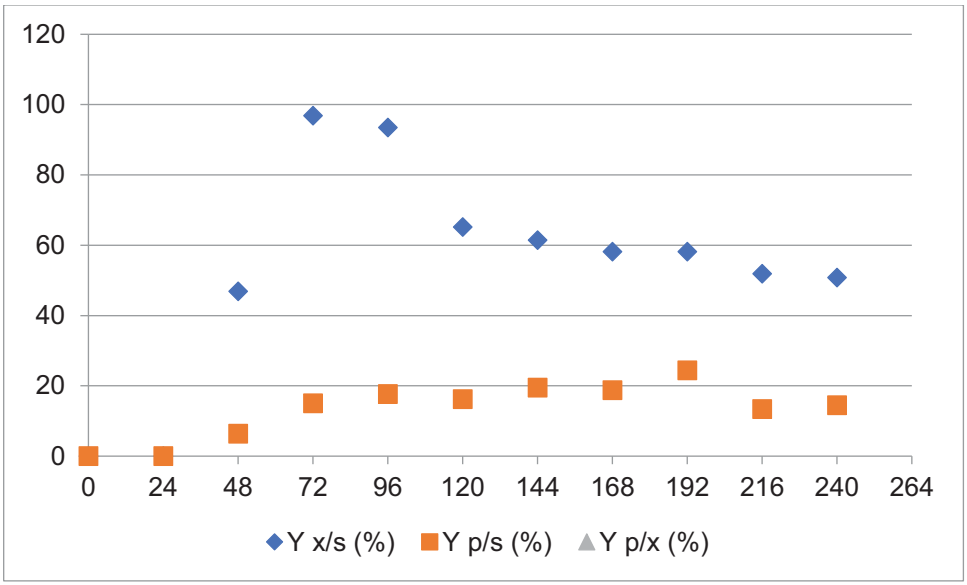


FIGURE 27– BIOMASS AND PRODUCT YIELD OF *MORTIERELLA ALPINA* CBS 528.72 IN OPTIMIZED CULTURE MEDIUM.

4.5.2 BIOREACTOR SCALE-UP

An optimization process is only applicable if its results can be replicated in higher scales, in order to prove the effectiveness of the culture medium developed.

A batch and fed-batch fermentation was performed in10-L pilot-scale fermenters using the optimized medium, using an inoculation rate of 10% (v/v) (FIGURE 28A). A formation of feather-like pellets with a diameter of 2 mm was observed (FIGURE 28B), and pH varied from 6.0 to 5.5 at the end of the fermentation. In both fermenters the agitation speed started at 180 rpm and after 5 days was reduced to 60 rpm; 0.6 vvm of aeration was injected at the beginning of the tests, and changing to 1 vvm after 5days. These modifications in agitation and aeration were done in order to avoid the shear stress and morphology deterioration. A pulse of 2% (w/v) of glucose and 0.3% (w/v) of $NaNO_3$ were injected in the Fed-batch fermentation after 5 days.

All these parameters were based in the study of Jin *et al*., 2008, as a novel procedure to produce high yields of biomass, total lipids and arachidonic acid.

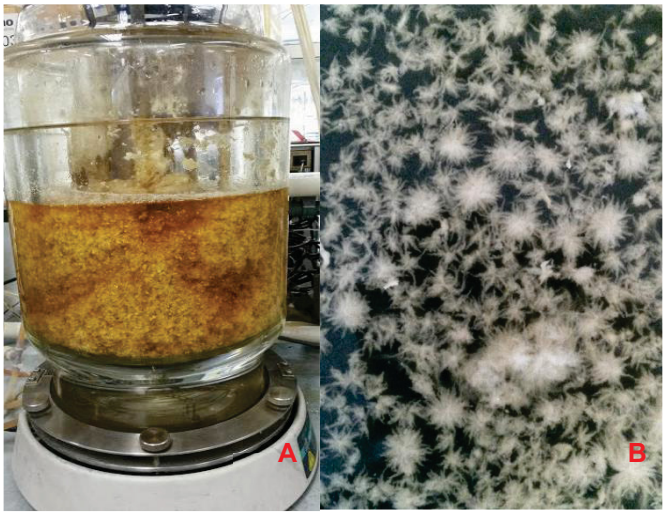


FIGURE 28 – SCALE-UP ASSAY OFTHE OPTIMIZED MEDIUM USING *M. ALPINA* CBS 528.72. A) BATCH FERMENTATION AND B) FEATHER-LIKE PELLETS OF M.ALPINA CBS 528.72.

The results comparing this study, after 7 days of fermentation (TABLE 20) and the reference were similar in terms of lipid production: Jin *et al*., 2008 reached 59% (w/w) and in this study the author reached 50%. The biomass production yield showed a difference of 50% due to fermentation control inconveniences and the strain of *Mortierella alpina* tested.

TABLE 20 – RESULTS OBTAINED FROM BATCH FERMENTATION AND FED-BATCH FERMENTATION PROCESS.

Fermentation type	DCW (g/L)	DCW (g/batch)	Total lipids (g/L)	Total lipids (%)	Total lipids (g/batch)	ARA (% of DCW)
Batch-fermentation	12,0	72,0	5,1	41,8	30,1	32
Fed-batch fermentation	15,5	77,0	7,7	49,9	38,4	ND
Fed-batch fermentation ^	14,3	71,5	7,3	51.1	36,5	ND
Fed-batch fermentation*	12,5	62,5	6,51	52,1	32,6	ND

^ : raw national yeast extract
* : 5 ppm oxygen dissolved

4.5.3 ECONOMICAL ANALYSIS OF CULTURE MEDIUM PRODUCTION

An economic analysis of the culture medium production was also calculated to compare the possible prices of the culture medium using different yeast extract sources (TABLE 21). Since the most expensive production costs coming from the components of the culture medium, in this case the yeast extract.

Brazil has enterprises that produces yeast extract for animal alimentation and fermentation, the kilogram of these yeast extract could vary between 5 to 7 USD per kilogram with a good quality, this yeast could be used substituting the conventional yeast extract used in laboratory (high purity) and turn the single cell oil even more rentable.

TABLE 21 – ECONOMICAL ANALYSIS OF THE CULTURE MEDIUM FOR *M. ALPINA* CBS 528,72 PRODUCTION.

COMPOSITION	ton/m3	Price (U$/ton)	Culture medium 1 (U$/m3)	Culture medium 2 (U$/m3)	Culture medium 3 (U$/m3)
Yeast extract (g/L)	0,0150	7692,30	115,385	2192,308	105
Glicose (g/L)	0,0600	320,00	19,200	19,200	19,200
KNO3 (g/L)	0,0010	960,00	0,960	0,960	0,960
KH2PO4 (g/L)	0,0010	1270,00	1,270	1,270	1,270
CaCl2·2H2O (mg/L)	0,0001	170,00	0,011	0,011	0,011
MgSO4·7H2O (g/L)	3,00E-07	150,00	0,000	0,000	0,000
FeCL3·6H2O (mg/L)	1,50E-06	400,00	0,001	0,001	0,001
ZnSO4·7 H2O (mg/L)	1,00E-06	600,00	0,001	0,001	0,001

CuSO4·5H2O (mg/L)	1,00E-07	1800,00	0,000	0,000	0,000
MnCL2·4H2O (mg/L)	1,00E-06	700,00	0,001	0,001	0,001
TOTAL			136,82	2213,75	126,44

1: Calculation based using national yeast extract
2: Calculation based using laboratorial yeast extra
3: Calculation based using imported bulk yeast extract

4.6 BRAZILIAN INDUSTRIAL WASTES AND BY-PRODUCTS AS NEW CULTURE MEDIA ALTERNATIVES

A variety of by-products are produced as a result of agroindustrial processing in the country, and most of them are also produced in Paraná. For this reason a test of the potential of selected “residues”, as well as two raw materials for fermentation (citrus pulp, soybean hulls, cassava wastewater, potato chips waste, sugar cane juice and sugar cane molasses) was performed.

In the FIGURE 29, it is shown that a first screening was done in order to evaluate which wastes will have a potential for the utilization as component in a formulated culture medium. Among the residues tested, a higher dry cellular weight production was observed using soybean hull (4.84 g/L), being chose for further testes. Soybean hull has 14% of protein and >80% of this protein is soluble (POLI-NUTRI, 2010). For this reason, this waste will be considered more as a nitrogen source than a carbon source per se.

The total reducing sugars of Potato chips waste were higher than the other residues (90%) being selected as a potential carbon source.

The other residues were discarded for further analyses.

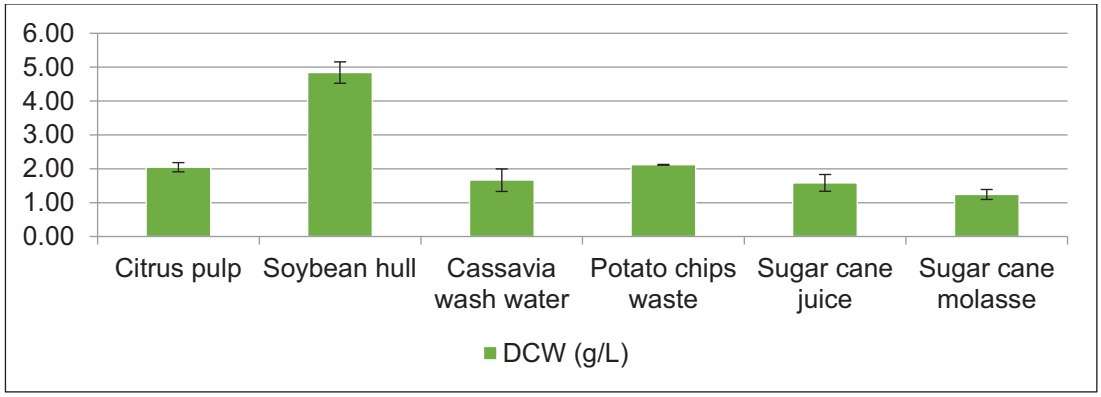
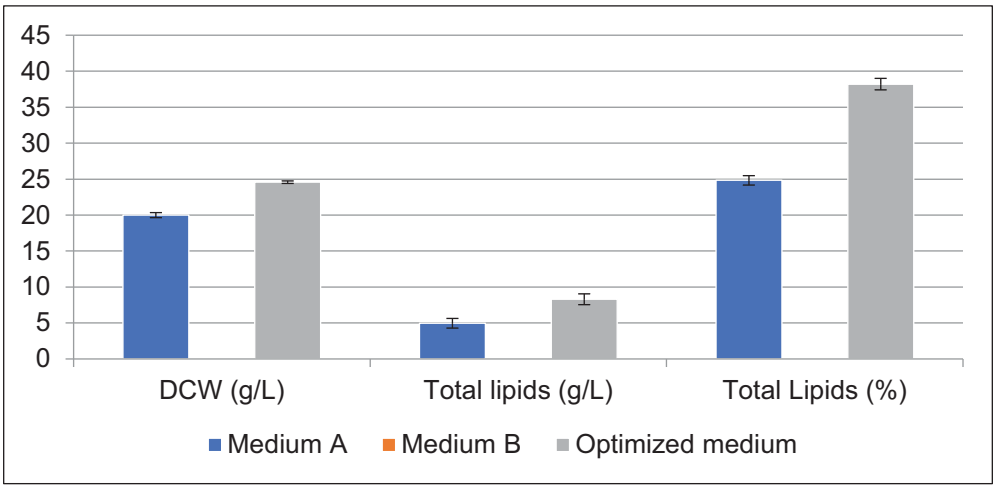


FIGURE 29 – SCREENING OF BRAZILIAN INDUSTRIAL WASTES AS CULTURE MEDIUM ALTERNATIVES.

A formulated medium A containing an extract of potato chips waste with a concentration of total reducing sugars of 60 g/L and 15 g/L of yeast extract; and a formulated medium B containing soybean hulls, standardized in a nitrogen basis (1.63 g of Nitrogen)and glucose (60 g/L) were prepared. This alternative culture media were compared with the optimized culture medium (FIGURE 30), *M. alpina* CBS 528.72 did not growth in the medium B, good results were obtained using medium A as a culture medium, 19.9 g/L DCW and 25% of total lipids. The optimized medium produced 24.5 g/L of DCW and about 39% of total lipids.



A: potato chips wastes + yeast Extract
B: Soybean hull + Glucose

FIGURE 30 – COMPARISION AMONG BRAZILIAN INDUSTRIAL WASTES BASED MEDIUM CULTURES AND OPTIMIZED CULTURE MEDIUM.

These results demonstrated that the use of Brazilian industrial wastes as media culture alternatives is promising, further researches could be carried out until get results as good as the ones obtained utilizing the optimized medium.

The use of wastes for biomass or essential fatty acids production must be well considered and analyzed since the product value is expensive, around 100 U$/kg.

5 CONCLUSIONS

- A culture medium was optimized for *M.alpina CBS 528.72,* obtaining 23,49 g/L of DCW and 47,32% of Total lipids
- The mycelial homogenization by mechanical mixer was chosen as the inoculation method for every test.
- The culture medium Hansson and Dostalek (1988) was used as a base for optimization.
- The effects of culture were evaluated concluding that: 25ºC, ph 6, 10 % inoculation rate were suitable for biomass and total lipids production
- In the optimization process the micronutrient CaCl2 showed influence in the total lipids production and the nitrogen sources yeast extract influenced in the biomass production.
- In the scaling-up process a 30% of the total production of biomass loss was observed, but a 6% in the total lipids production was gained

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