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EXTRAÇÃO DE ÁCIDOS GRAXOS PRODUZIDOS POR *MORTIERELLA ISABELLINA* UTILIZANDO FLUIDO SUPERCRÍTICO E ULTRASSOM

DISSERTAÇÃO DE MESTRADO

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Santa Maria, RS, Brasil
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EXTRAÇÃO DE ÁCIDOS GRAXOS PRODUZIDOS POR *MORTIERELLA ISABELLINA* UTILIZANDO FLUIDO SUPERCRÍTICO E ULTRASSOM

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Dissertação apresentada ao curso de Mestrado do Programa de Pós-Graduação em Engenharia Química, Área de Concentração em Desenvolvimento de Processos Industriais e Ambientais, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Engenharia Química.**

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elaborada por
Daniela Sallet

como requisito parcial para obtenção do grau de
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RESUMO

EXTRAÇÃO DE ÁCIDOS GRAXOS PRODUZIDOS POR *MORTIERELLA ISABELLINA* UTILIZANDO FLUIDO SUPERCRÍTICO E ULTRASSOM

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Os ácidos graxos poli-insaturados (PUFA) têm recebido considerável atenção nos últimos anos devido aos benefícios associados ao seu consumo. Dentre estes, destacam-se as suas múltiplas ações fisiológicas, desempenhando um papel crucial no metabolismo humano, bem como na manutenção de um estado saudável, estando também associados com a diminuição de alguns fatores relacionados a doença cardiovascular. A produção dos PUFA em processos fermentativos é intracelular e um processo eficiente de extração destes ácidos graxos é necessário para sua comercialização. Portanto, o objetivo principal deste trabalho foi definir uma metodologia para a extração de PUFA produzidos por fermentação submersa a partir do fungo *Mortierella isabellina* através de fluido supercrítico e ultrassom. Dois métodos de extração foram estudados: a extração com fluido supercrítico (EFS) e a extração assistida por ultrassom. Na extração supercrítica foram utilizados dióxido de carbono no estado supercrítico (EFS-CO_2) e gás liquefeito de petróleo pressurizado (GLP). Na extração com EFS-CO_2 a pressão variou na faixa de 150 a 250 bar e a temperatura entre 40 a 80 ºC, já para a extração com GLP pressurizado, estes parâmetros variaram na faixa de 10 a 20 bar e 20 a 40 ºC, respectivamente, sendo o rendimento de extração, os parâmetros cinéticos e o perfil dos ácidos graxos avaliados. De acordo com as curvas cinéticas, as taxas de transferência de massa, e os rendimentos de extrato, 80 ºC / 250 bar (EFS-CO_2) e 40 ºC / 20 bar (GLP) foram as condições mais favoráveis para extração dos lipídeos da biomassa, nestas condições os rendimentos foram 3,21 e 4,45%, respectivamente. De acordo com o perfil dos ácidos graxos, o EFS-CO_2 proporcionou um desempenho ligeiramente superior ao do GLP comprimido. Já na extração assistida por ultrassom dois solventes foram utilizados: o etanol e uma mistura de solventes composta por clorofórmio:metanol:água nas razões de 1:2:0,88. Através de um planejamento experimental foram avaliadas a intensidade do ultrassom (17 – 85 $W.cm^{-2}$) e o ciclo do pulso (0,5 – 1,0) na extração e perfil dos lipídeos da biomassa. Na melhor condição de extração, com uma intensidade de 75,11 $W.cm^{-2}$ e o fator de pulso de 0,93 o rendimento de lipídeos foi 14,47 % utilizando etanol e 19,49% utilizando a mistura de solventes. Em relação a composição de ácidos graxos, os principais ácidos graxos identificados foram os ácidos graxos esteárico, *cis*-10-pentadecenóico (C15:1), *cis*-10-heptadecenóico (C17:1), oléico (C18:1n9c), araquídico (C20:0), linoléico (C18:2n6c), α-linolênico (C18:3n3) e palmítico (C16:0). A extração com ultrassom utilizando etanol apresentou melhor perfil de ácidos graxos, e maior concentração de PUFA. Os principais resultados confirmaram a extração assistida por ultrassom como uma metodologia promissora na obtenção de ácidos graxos através de biomassa microbiana.

Palavras-chave: Ácidos graxos poli-insaturados. *Mortierella isabellina.* Extração supercrítica. Gás liquefeito de petróleo comprimido. Extração assistida por ultrassom.

ABSTRACT

EXTRACTION OF FATTY ACIDS PRODUCED BY *MORTIERELLA ISABELLINA* USING SUPERCRYTICAL FLUID AND ULTRASOUND

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Polyunsaturated fatty acids (PUFA) have received considerable attention in recent years due to the benefits associated with their consumption. Among these benefits, they emphasize their multiple physiological actions, playing a crucial role in human metabolism, as well as maintaining a healthy state, being also associated with the decrease of some factors related to cardiovascular disease. The production of PUFA in fermentative processes is intracellular and an efficient extraction process of these fatty acids is necessary for its commercialization. Therefore, the main objective of this work was to define a methodology for the extraction of PUFA produced by submerged fermentation from the fungus *Mortierella isabellina* through supercritical fluid and ultrasound. Two methods of extraction were studied: supercritical fluid extraction (SFE) and ultrasonic assisted extraction. In supercritical extraction were used supercritical carbon dioxide (SFE-CO_2) and compressed liquefied petroleum gas (LPG). In the extraction with SFE-CO_2 the pressure varied in the range of 150 to 250 bar and the temperature between 40 to 80 ºC, already for the extraction with compressed LPG, the pressure and the temperature were in the range of 10 to 20 bar and 20 to 40 ºC, respectively, extraction yield, the kinetic parameters and the fatty acid profile were evaluated. According to the kinetic curves, mass transfer rates, and extract yields, 80 °C / 250 bar (SFE-CO_2) and 40 °C / 20 bar (LPG) were the most favorable conditions for the extraction of lipids from biomass, in these conditions, the yields were 3.21 and 4.45%, respectively. According to the fatty acid profile, the SFE-CO_2 provided a slightly higher performance than the compressed LPG. In the ultrasonic assisted extraction two solvents were used: ethanol and a mixture of solvents composed of chloroform: methanol: water in ratios of 1:2:0.88. Ultrasound intensity (17-85 $W.cm^{-2}$) and pulse cycle (0.5 - 1.0) were evaluated through an experimental design in extraction and fatty acids profile. In the best extraction condition, with an intensity of 75.11 $W.cm^{-2}$ and the pulse factor of 0.93, the lipid yield was 14.47% using ethanol and 19.49% using the mixture of solvents. The main fatty acids identified were estearic (C18:0), cis-10-pentadecanoic (C15:1), cis-10-heptadecanoic (C17:1), oleic (C18:1n9c), arachidic (C20:0), linoleic (C18:2n6c), α-linolenic (C18:3n3) and palmitic (C16:0). The extraction with ultrasound using ethanol showed a better profile of fatty acids, and a higher concentration of PUFA. The main results confirmed that the ultrasonic assisted extraction is a promising methodology for obtaining fatty acids from microbial biomass.

Keywords: Polyunsaturated fatty acids. *Mortierella isabellina.* Supercritical extraction. Compressed liquefied petroleum gas. Ultrasound assisted extraction.

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SUMÁRIO

1. INTRODUÇÃO

Ao longo dos últimos anos, diversos estudos têm dado destaque para a utilização dos ácidos graxos poli-insaturados (PUFA - Poly Unsaturated Fatty Acids) numa dieta equilibrada e na manutenção de uma boa saúde, além de estarem relacionados a prevenção de diversas doenças. Estes PUFA estão presentes nas mais diversas formas de vida, desempenhando importantes funções na estrutura das membranas celulares e nos processos metabólicos (YOUDIM et al., 2000; YEHUDA et al., 2002). Dentre os benefícios associados ao seu consumo, têm recebido atenção os efeitos benéficos na saúde humana, como por exemplo, no coração, no cérebro, nos olhos, nas articulações, na pele, no humor e no comportamento (PELLICCIA et al., 2013). Além disso, estão envolvidos na prevenção da doença arterial coronariana, hipertensão, diabetes, artrite e câncer (TUR et al., 2012).

Os PUFA, assim chamados por conterem duas ou mais insaturações, são caracterizados pela localização das ligações duplas. Os PUFA que pertencem as famílias n-3 e n-6 são considerados essenciais, não sendo produzidos pelo organismo, necessitando assim serem ingeridos através da alimentação (DYAL e NARINE, 2005), podendo ser encontrados em óleos de peixes marinhos, frutos do mar (RUIZ-LOPEZ et al., 2012), linhaça, canola, soja, óleos de perila, óleo de cártamo. Além dos alimentos, algumas plantas e micro-organismos, bactérias marinhas, fungos, protistas e microalgas (GONG et al., 2014) são capazes de sintetizar os PUFA precursores da série n-3 e n-6.

As fontes de micro-organismos estão ganhando popularidade como fontes potenciais, pois os micro-organismos oferecem algumas vantagens para a produção de PUFA através de estratégia de engenharia metabólica, incluindo taxas de crescimento mais elevadas, exigência de entrada de nutrientes simples, condição de cultura controlável, composição de ácidos graxos simples, menor influência do meio ambiente, estação e clima; e a possibilidade de aumentar a sua escala de produção de acordo com a demanda do mercado (GONG et al., 2014; POLI et al., 2014). Dessa maneira, o fungo *Mortierella isabellina* vem recebendo destaque na produção de óleo microbiano, pois é capaz de acumular uma quantidade considerável de lipídeos (CHATZIFRAGKOU et al., 2010; FAKAS et al., 2009; GUCKERT et al., 1988; HALIM et al., 2012; HARDE et al., 2016; HUSSAIN et al., 2014; SALLET et al., 2017; XING et al., 2012; ZHOU et al., 2013).

Entretanto, os ácidos graxos quando produzidos por via biotecnológica são produzidos intracelularmente e um processo de extração destes lipídeos é necessário para o rompimento

celular. Dessa forma, métodos de extração, como a extração por solventes (HIDALGO, et al., 2016; WU, et al., 2017), com fluido supercrítico (NISHA et al., 2012) e extração assistida por ultrassom (ZHOU et al., 2013) vêm recebendo atenção. Dentre os métodos de extração, a extração com fluido supercrítico e assistida por ultrassom apresentam algumas vantagens quando aplicadas.

O dióxido de carbono (CO_2) é o solvente mais utilizado na extração supercrítica (ESC), já que permite operações com pressões relativamente baixas e temperaturas próximas a ambiente. Além disso, o CO_2 apresenta algumas vantagens, pois é reconhecido como seguro (MANTELL et al., 2013), é inerte, não tóxico e não inflamável, e está facilmente disponível com elevada pureza e baixo custo (BRUNNER, 2005; POULIOT et al., 2014). Outro solvente que vem sendo utilizado na extração de óleos é o gás liquefeito de petróleo (GLP) (SOARES et al., 2015; ABAIDE et al., 2016), que é uma mistura de propano e n-butano. A extração usando GLP como solvente tem apresentado benefícios como, por exemplo, maior rendimento e seletividade, menor tempo de extração e menor consumo de solvente (SOARES et al., 2015; ABAIDE et al., 2016). Já a extração assistida por ultrassom têm sido amplamente desenvolvida na indústria nos últimos anos, provavelmente porque é uma tecnologia-chave para atingir o objetivo da química "verde" sustentável (ADAM et. al., 2012). Tem sido considerada uma técnica alternativa capaz de resolver problemas associados aos métodos convencionais, uma vez que o processo simplifica as condições de manuseio e processamento, proporciona maior pureza do produto final, elimina o pós-tratamento das águas residuais. Também reduz a quantidade de solvente, bem como a energia necessária em comparação com métodos convencionais, trabalhando a temperaturas mais baixas ou evitando a dispendiosa eliminação do solvente (CHEMAT et al., 2011).

Portanto, em vista do que foi exposto, esta dissertação pretende avaliar diferentes métodos de extração de lipídeos obtidos por processo fermentativo com o intuito de definir uma metodologia para a extração e obtenção de ácidos graxos poli-insaturados.

2. OBJETIVOS

2.1.OBJETIVO GERAL

O principal objetivo deste trabalho foi avaliar a extração com fluido supercrítico e a extração assistida por ultrassom para obtenção de ácidos graxos produzidos por fermentação submersa de *Mortierella isabellina*.

2.2.OBJETIVOS ESPECÍFICOS

- Produzir ácidos graxos poli-insaturados por fermentação submersa de *Mortierella isabellina*;
- Avaliar a extração do óleo microbiano da biomassa fúngica através de extração supercrítica utilizando CO_2 e GLP comprimido como solventes;
- Avaliar a extração do óleo microbiano da biomassa fúngica através de extração assistida por ultrassom utilizando etanol e mistura de metanol/clorofórmio/água como solventes.

3. REVISÃO DA LITERATURA

3.1. ÁCIDO GRAXOS POLI-INSATURADOS: BENEFÍCIOS E PRODUÇÃO

Ácidos graxos (AG) são ácidos carboxílicos com cadeias de hidrocarbonetos contendo de 4 a 24 carbonos (LEHNINGER et al., 2005). São classificados quanto à presença de insaturações: saturados (contendo apenas ligações simples), monoinsaturados (MUFA) (uma ligação dupla) ou poli-insaturados (PUFA) (duas ou mais ligações duplas) (RÚBIO-RODRIGUEZ et al., 2010).

Dependendo da posição da primeira dupla ligação, contando a partir do grupo metílico localizado ao final da molécula do AG, os AG podem ser classificados como poli-insaturados, AG pertencentes a família ômega-3 (n-3) ou ômega-6 (n-6) (GIUDETTI e CAGNAZZO, 2012). Estes AG contêm de 18 a 22 carbonos. Os AG n-3 apresentam a primeira dupla ligação entre o terceiro e o quarto átomo de carbono, enquanto os AG n-6 têm a primeira dupla ligação entre o sexto e o sétimo átomo de carbono (CARVALHO et al., 2003).

Esses PUFA (n-3 e n-6) são considerados AG essenciais (EFAs), pois são necessários na saúde humana, porém não são sintetizados pelo organismo. Dessa forma, devem ser obtidos a partir de fontes dietéticas. Nos seres humanos os PUFA não são sintetizados em quantidades suficientes dentro do corpo, portanto, eles devem ser adquiridos por fontes externas (DYAL e NARINE, 2005).

Os PUFA são precursores de eicosanóides (prostaglandinas, leucotrienos e tromboxanos), que são mediadores lipídicos com uma ampla gama de efeitos regulatórios, autócrinos e parácrinos. Devido às suas múltiplas ações fisiológicas, os AG ômega-3 desempenham um papel crucial no metabolismo humano, bem como a manutenção de um estado saudável. Portanto, a suplementação de ômega-3 pode ter efeitos clínicos relevantes que não se limitam ao sistema cardiovascular, mas também incluem saúde materna e reprodutiva, crescimento e desenvolvimento, distúrbios do sistema imunológico, câncer, função cognitiva e estado psicológico (PELLICCIA et al., 2013). Da mesma maneira, os AG ômega-6 são necessários para o desenvolvimento fetal e do cérebro infantil. Além disso, podem contribuir para diminuir alguns fatores relacionados a doença cardiovascular humana (DECKELBAUM e CALDER, 2010).

As plantas e alguns micro-organismos marinhos podem sintetizar os PUFA precursores da série n-3 e n-6. Estes ácidos graxos podem ser encontrados em peixes marinhos, frutos do

mar (RUIZ-LOPEZ et al., 2012), linho, linhaça, canola, soja, óleos de perila, de cártamo, de soja e óleos de milho (GONG et al., 2014). Além disso, muitos micro-organismos, como microalgas, fungos e bactérias, têm a capacidade de acumular lipídeos, sendo estes sintetizados durante o processo de crescimento como parte de seu processo metabólico e como reserva de carbono. Sendo que a composição dos lipídeos, a quantidade e a qualidade são variáveis que dependem da espécie do micro-organismo, da fase de crescimento, das condições ambientais e dos substratos do meio de cultivo (SILVA, 2011).

Os micro-organismos estão ganhando popularidade como fontes potenciais destes ácidos graxos. Dentre as vantagens da sua produção por via biotecnológica, destaca-se a utilização da estratégia de engenharia metabólica, incluindo elevadas taxas de crescimento, exigência de nutrientes simples, a composição de ácidos graxos e fácil manipulação genética (GONG et al., 2014). Outra razão é que a produção industrial a partir de micro-organismo tem vantagem de não necessitar grandes extensões de terra agriculturável e de enormes quantidades de água. Além disso, a produção do óleo microbiano pode ser feita durante todo o ano, pois não há dependência sazonal ou climática, e o óleo microbiano apresenta elevada percentagem de PUFA o que confere melhor valor de mercado quando comparado a outros óleos de menor qualidade (WELLBAUM, 2006; JINGYANG et al., 2012).

As principais classes de micro-organismos utilizados para a produção de lipídeos são as leveduras e os fungos filamentosos (RATLEDGE, 1996). Dessa forma, os fungos oleaginosos, principalmente do gênero *Mortierella* têm sido utilizados para a produção de lipídeos contendo PUFA. Os principais PUFA produzidos pelos fungos são o ácido γ-linolênico (GLA-C18:3 n-6), o ácido dihomo-γ-linolênico (DHGLA-C20:3 n-6), o ácido araquidônico (ARA-C20:4 n-6) e ácido docosa-hexaenóico (DHA-C22:3 n-3) (DEMIR et al., 2013). Dentro do gênero *Mortierella*, destaca-se o fungo *Mortierella isabellina* que vem sendo muito estudado devido a sua capacidade de acumular uma quantidade considerável de lipídeos, até 80% de biomassa celular (CHATZIFRAGKOU et al., 2010). *Mortierella isabellina* pode ser cultivada em vários substratos, incluindo açúcares monoméricos (CHATZIFRAGKOU et al., 2010), glicerol (FAKAS et al., 2008), xilose (GAO et al., 2013), bem como hidrolisados de biomassa lignocelulósica (casca de arroz e palha de milho) (ECONOMOU et al., 2011 e RUAN et al., 2012). Além disso, Zheng et al. (2012) demonstraram que *Mortierella isabellina* teve melhor desempenho na produção de lipídeos entre 5 fungos oleaginosos crescendo em palha de trigo pré-tratada com ácido sulfúrico diluído. Estas características, juntamente com uma boa tolerância a inibidores derivados de materiais lignocelulósicos (ZENG et al., 2012), sugerem

que a *Mortierella isabellina* poderia ser um bom fungo para a produção de lipídeos a partir de matéria-prima renovável de baixo custo.

3.2. EXTRAÇÃO DE LIPÍDEOS

3.2.1. Extração com fluido supercrítico

Um fluido está no estado supercrítico se a sua temperatura e pressão são maiores que os valores críticos (KNEZ et al., 2014). A temperatura crítica (Tc) é considerada como a temperatura máxima na qual o gás pode ser convertido em líquido pelo aumento da pressão, já a pressão crítica (Pc) é considerada a pressão máxima na qual o líquido pode ser convertido em gás pelo aumento da temperatura (RIZVI et al., 1986). Sendo assim, a extração supercrítica é definida como um processo de transferência de massa sob condições de pressão e temperatura acima do ponto crítico do solvente (MANTELL et al., 2013).

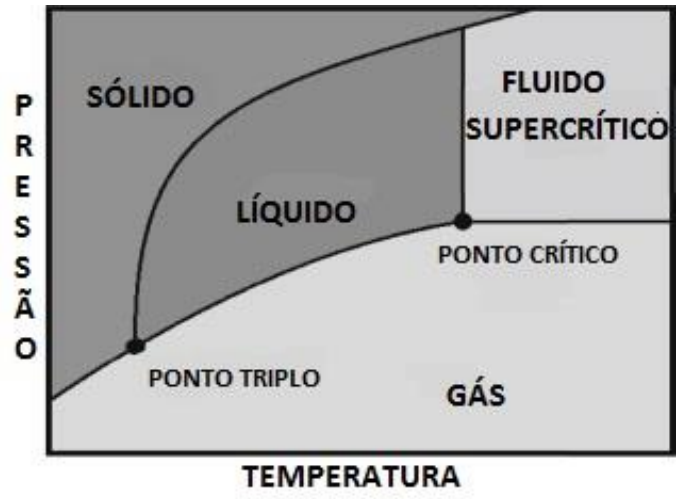


Figura 1- Diagrama de fases (Adaptado Knez et al., 2014).

A extração com fluido supercrítico vem se destacando, pois, é um método que oferece vantagens em relação a extração convencional, como por exemplo, aumento da seletividade, automaticidade, segurança ambiental, qualidade superior de extratos e uma diminuição na utilização de solventes orgânicos que resultam em extratos sem resíduo de solvente (XYNOS et al., 2012). Além disso, nos dias de hoje, existe um crescente interesse no desenvolvimento

de processos tecnológicos alternativos que reduzam os impactos ambientais, como por exemplo, redução do consumo de energia e produção de resíduos, melhor utilização de subprodutos e também melhor qualidade dos produtos finais (KNEZ et al., 2014). Portanto, processos que envolvem fluidos supercríticos requerem menos energia e são ecologicamente corretos comparados aos processos que envolvem solventes orgânicos, e isso se deve principalmente às vantagens de fluidos supercríticos serem associadas às suas propriedades físicas e químicas (KNEZ et al., 2014). No estado supercrítico, a densidade fica próxima à dos líquidos e está relacionada com o poder de solvatação do solvente. A viscosidade, que está próxima a dos gases, e a difusividade, que é intermediária entre a dos gases e dos líquidos, estão relacionados com a taxa de transferência de massa entre o soluto e o fluido (BRUNNER, 2005). Os valores característicos para o estado gasoso, líquido, e estado supercrítico são listados na Tabela 1.

Tabela 1 - Comparação entre as propriedades de gases, líquidos e fluídos supercríticos.

Propriedades físicas	Gás	Fluido supercrítico		Líquido
	(1 atm, 15 - 30°C)	*Tc, Pc*	*Tc, 4xPc*	15 - 30 °C
Coeficiente de difusão (cm²/s)	*0,1-0,4*	$0,7x10^{-3}$	$0,2x10^{-3}$	$(0,2-2)x10^{-5}$
Viscosidade (g/cm.s)	$(1-3)x10^{-4}$	$(1-3)x10^{-4}$	$(3-9)x10^{-4}$	$(0,2-3)x10^{-2}$
Densidade (g/L)	$(0,6-2)x10^{-3}$	*0,2 -0,5*	*0,4 -0,9*	*0,6 - 1,6*

Fonte: (TZIA et al., 2003).

Uma das principais características de um fluido supercrítico é a possibilidade de alterar a densidade do fluido ao ajustar a sua pressão e/ou sua temperatura, e assim, também alterar a sua solubilidade (HERRERO, CIFUENTES e IBANEZ, 2006), pois, a solubilidade de extratos no fluido supercrítico é função da densidade do solvente e da pressão de vapor do soluto (BRUNNER, 2005). Dessa forma, para pressões supercríticas mais baixas, próximas ao ponto crítico, a solubilidade dos compostos diminui. Por outro lado, em pressões mais elevadas a mudança da densidade com a temperatura é mais moderada, sendo a pressão de vapor do soluto o fator dominante. Sendo assim, o aumento da temperatura aumenta a pressão de vapor do soluto, resultando no aumento da solubilidade dos compostos (BRUNNER, 2005).

Na extração com fluido supercrítico (EFS) há um aumento na eficiência da extração, em comparação com os solventes líquidos utilizados no processo de extração convencional. Isso,

porque o fluido supercrítico apresenta baixa viscosidade, difundindo-se facilmente na matriz sólida, e baixa tensão superficial, permitindo a penetração rápida do solvente (POULIOT et al., 2014). Além disso, o processo de extração convencional necessita de grandes quantidades de solventes tóxicos que demandam uma etapa difícil de separação. Dessa forma, pode haver a degradação térmica dos compostos de interesse devido às elevadas temperaturas do solvente aplicadas durante longos tempos de extração (GIL-CHÁVEZ et al., 2013; KIM et al., 1999).

Diversos solventes têm sido estudados na EFS, como o dióxido de carbono (CO_2), a água (HSUEH et al., 2013), o etanol (AKALIN et al., 2013) e o propano (DEO et al., 1992). Porém, o solvente mais utilizado tem sido o dióxido de carbono, pois apresenta algumas vantagens: não é tóxico, não inflamável, facilmente disponível com elevada pureza e baixo custo, é inerte e reconhecido como seguro (Generally Recognized As Safe –GRAS) (BRUNNER, 2005; MANTELL et al., 2013; POULIOT et al., 2014). Além disso, o CO_2 permite operações supercríticas com pressões relativamente baixas e temperaturas próximas à ambiente (Tc = 31,1°C; Pc = 73,8 bar) (POULIOT et al., 2014; REVERCHON; DE MARCO, 2006). Dessa maneira, como o CO_2 é gasoso a temperatura ambiente, o processo de recuperação do extrato livre de solvente se torna muito simples e a extração de compostos termolábeis ou facilmente oxidáveis podem ser realizadas (HERRERO et al., 2010).

Na literatura alguns autores reportaram a utilização de CO_2 na extração de lipídeos. Entre estes, destaca-se os trabalhos de Zinnai et al (2016) que estudaram os efeitos das condições de operação sobre a cinética na extração com fluido supercrítico (EFS), em rendimentos de processos e na composição de ácidos graxos de extratos de lipídeos, também em comparação com a extração convencional por percolação com n-hexano. Neste trabalho, os autores obtiveram um modelo cinético adequado e concluíram que a EFS foi mais rápida que a extração convencional. Nisha et al (2012) analisaram a extração de lipídeos da biomassa liofilizada de *Mortierella alpina* utilizando a EFS e a extração utilizando solvente orgânico. O rendimento da EFS foi 38,73%, já o rendimento utilizando o solvente orgânico pelo método Sox Tec™ foi 51,47%. O rendimento utilizando o solvente orgânico foi melhor, pois extraiu lipídeos polares. Crampon et al (2017) avaliaram a influência de condições experimentais nos rendimentos de extração e cinética em escalas laboratoriais e piloto na extração de lipídeos neutros e antioxidantes a partir de microalgas enriquecidas com *Spirulina platensis* utilizando CO_2 supercrítico. Dentre as respostas foi observada perda de massa quando a pressão, temperatura e razão CO_2 /massa de microalgas aumentaram. As análises dos extratos mostraram que os extratos de óleo continham as clorofilas a e b, bem como o beta-caroteno.

3.2.2. Extração com líquido pressurizado

Embora o CO_2 seja o principal solvente utilizado na extração supercrítica, alguns estudos (CORSO et al., 2010; HAMDAN et al., 2008; PEDERSSETTI et al., 2011) utilizando propano comprimido como solvente de extração obtiveram rendimentos superiores aos obtidos com CO_2 supercrítico (RIBAS et al., 2014). Com isso, tem aumentado o interesse em estudos de extração em condições mais brandas de pressão e temperatura que alcancem rendimentos satisfatórios, levando em consideração a qualidade do óleo extraído, rendimento e a eficiência do processo (ABAIDE et al., 2016).

A extração com propano tem sido relatada como uma tecnologia promissora, uma vez que é possível obter rendimento mais elevado de extrato com tempo de extração mais curto, quando comparado com o CO_2 supercrítico (CORSO et al., 2010). No entanto, o custo deste solvente é cerca de vinte vezes mais elevado do que o CO_2. Sendo assim, um solvente alternativo mais barato seria o gás liquefeito de petróleo (GLP) comprimido que contém propano e n-butano como os principais constituintes. O GLP é muito abundante e barato, além disso, pode ser utilizado em pressões muito mais baixas em comparação com o CO_2 (DAL PRÁ et al., 2016). Dessa forma, alguns estudos tem utilizado GLP como alternativa aos processos tradicionais, devido a suas propriedades físico-químicas ideais para extração e por não apresentarem toxicidade (YANG et al., 2004).

Soares et al. (2016) estudaram a extração de farelo de arroz utilizando CO_2 supercrítico e GLP comprimido. Para as extrações utilizando CO_2 supercrítico foram estudadas as condições de pressão (150-250 bar) e temperatura (40-80 ºC). Já para as extrações utilizando GLP comprimido foram avaliadas as condições de pressão (5-25 bar) e temperatura (20-40 ºC). Neste trabalho os autores concluíram que o GLP comprimido é o solvente mais promissor para a extração de óleo de farelo de arroz, já que o tempo de extração foi consideravelmente reduzido. Abaide et al. (2016) quando avaliaram a extração de óleo da polpa de abacate utilizando CO_2 supercrítico e GLP comprimido nas mesmas condições também observaram redução significativa no tempo de extração utilizando gás liquefeito de petróleo como solvente, quando comparado com CO_2, e ainda um melhor rendimento na extração de 57,95 % para o primeiro e 39,76 % para o segundo nas melhores condições de extração avaliadas. Apesar das vantagens na utilização do gás liquefeito de petróleo, até o momento, nenhum estudo tem sido relatado na literatura utilizando este solvente na extração de PUFA de *Mortierella isabellina*.

3.3. EXTRAÇÃO ASSISTIDA POR ULTRASSOM

A extração assistida por ultrassom é uma técnica de baixo custo, simples e eficiente comparada com as técnicas de extração convencionais. O mecanismo da extração assistida por ultrassom é atribuído a eficácia mecânica e a cavitação que pode resultar no rompimento da parede celular, redução do tamanho de partícula e melhora na transferência de massa através da membrana celular (KHOEI et al., 2016).

Durante o processo de aplicação de ultrassom, ondas longitudinais são criadas quando uma onda sonora encontra um meio líquido, criando regiões alternadas de compressão e expansão induzidas sobre as moléculas (Figura 2). Nessas regiões de mudança de pressão, a cavitação ocorre e bolhas de gás são formadas. Essas bolhas têm uma maior área de superfície durante a expansão do ciclo, o que aumenta a difusão do gás, fazendo com que a bolha se expanda. Um ponto crítico é alcançado durante o ciclo de compressão em que a energia ultrassônica fornecida não é suficiente para manter a fase de vapor dentro da bolha. Como consequência, ocorre a condensação rápida e grandes quantidades de energia são liberadas (SORIA et al., 2010).

O ultrassom pode ser dividido em diferentes faixas de frequência. Recentemente, a maioria das aplicações de ultrassom na tecnologia de alimentos envolveu análise não-destrutiva que se refere particularmente à avaliação da qualidade. Tais aplicações usam alta frequência (100 kHz^{-1} a 1 MHz) de baixa potência (tipicamente <1 W cm^{-2}) de ultrassom. Ultrassom de baixa intensidade é mais comumente aplicado como técnica analítica para fornecer informações sobre as propriedades físico-químicas dos alimentos, como por exemplo, maturação, teor de açúcar, acidez, etc. Por outro lado, os níveis de potência utilizados em aplicações de baixa frequência (16-100 kHz) são tão grandes (tipicamente na faixa de 10-1000 W cm^{-2}) que eles são usados para alterar, fisicamente ou quimicamente, as propriedades dos alimentos (DEMIRDÖVEN e BAYSAL, 2009; MCCLEMENTS, 1995; SORIA et al., 2010).

A extração assistida por ultrassom quando comparada com outros métodos pode acelerar o processo a baixas temperaturas, causando menor dano as propriedades estruturais e moleculares dos compostos em materiais de plantas (YUAN et al., 2015). Sendo assim, o ultrassom tem sido utilizado para acelerar processos e reduzir os tempos de extração (REÁTEGUI et al., 2014).

A extração utilizando ultrassom vem sendo aplicada na extração de lipídeos de micro-organismos. No trabalho de Rezende dos Santos et al. (2015) foram comparados diferentes mé-

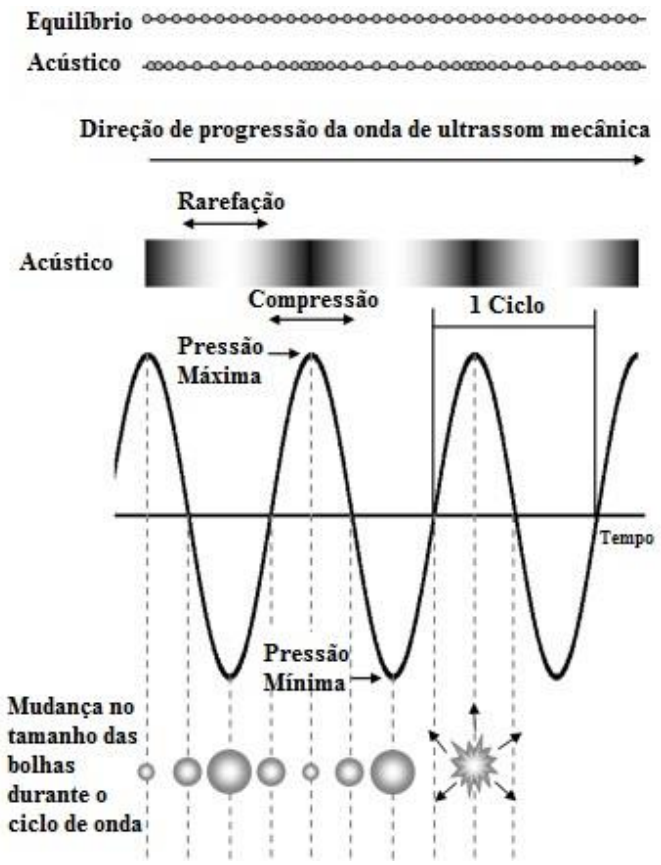


Figura 2 - Cavitação ultrassônica.

Fonte: Adaptado de Soria et al., (2010)

todos de extração de lipídeos totais da biomassa de *Chlorella vulgaris* com diferentes solventes. Etanol, hexano e mistura de clorofórmio:metanol em proporções 1:2 e 2:1 foram associados com outros mecanismos de ruptura de célula, como homogeneizador Potter e tratamento com ultrassom. A mistura de clorofórmio:metanol (2:1) assistida por ultrassom foi mais eficiente, extraindo uma média de 19 % do total de lipídeos.

Zhou et al. (2013), estudaram a extração de óleo unicelular de *Mortierella isabelina*. A melhor condição de extração foi quando utilizaram potência de ultrassom 300 W, tempo de ultrassom de 12,2 minutos, temperatura de ultrassom 53,42 ºC, ácido clorídrico 30 mmol em cada grama de micélio fúngico, tempo de extração 19,45 minutos e razão de solvente de extração 2:1. Sob essas condições a taxa de extração de óleo foi de 90,63 ± 1,35 % e o rendimento foi de 109,88 ± 0,02 mg g^{-1}. Keris-sen et al. (2014), avaliaram diferentes intensidades de potência de ultrassom (0,1-0,5 W mL^{-1}) a frequência de 30 kHz e por durações de 5-60 minutos em culturas de microalgas misturadas. O efeito do ultrassom sobre a ruptura

celular foi revelado pelo aumento das concentrações de proteínas e carboidratos liberados para a solução, e diminuição na concentração de sólidos totais suspensos na suspensão celular. A maior liberação de material intracelular foi obtida com intensidade de ultrassom de 0,4 kWh L^{-1}. Dey e Rathod (2013), estudaram a extração assistida por ultrassom de β-caroteno de *Spirulina platensis*. Foram explorados vários parâmetros tais como, tempo de extração, tipo de solvente, razão biomassa/solvente, temperatura, intensidade acústica elétrica, comprimento da ponta da sonda mergulhada no solvente, ciclo de trabalho e efeito do pré-tratamento para a extração de β-caroteno. Do ponto de vista econômico, as condições ótimas para a extração de β-caroteno da *Spirulina* foram 1,5 g de *Spirulina* (2 min previamente banhados em metanol) em 50 mL de n-heptano a 30 ° C, 167 W cm^{-2} de intensidade acústica elétrica e 61,5 % de ciclo de trabalho durante 8 min com o comprimento da ponta da sonda de 0,5 cm mergulhado no solvente de extração a partir da superfície. A extração máxima obtida sob os parâmetros ótimos acima mencionados foi 47,10%.

4. ARTIGO 1

Obtaining fatty acids from *Mortierella isabellina* using supercritical carbon dioxide and compressed liquefied petroleum gas

Publicado: The Journal of Supercritical Fluids

Abstract

The objective of this article was to provide the extraction yields, the kinetic parameters, and the fatty acids compositions in the oil obtained from freeze-dried cells of *Mortierella isabellina*. The oil was obtained by supercritical CO_2 (SFE-CO_2) and compressed liquefied petroleum gas (LPG) extractions in different conditions of temperature and pressure. According to the kinetic curves, the mass transfer rates, and the yields of extract, 80 ºC/250 bar (SFE-CO_2) and 40 ºC/20 bar (LPG) were the most favorable conditions. The highest yield was 4.45 wt.% (LPG). According to the fatty acids composition, SFE-CO_2 provided a slightly higher performance than compressed LPG. The highest concentration of fatty acids was 361 mg/g oil at 40 ºC/150 bar. Stearic, cis-10-pentadecanoic, cis-10-heptadecanoic, oleic, linoleic and linolenic acids were the major fatty acids extracted. These results highlight the possibility of using pressurized fluids to break the cells for increasing the fatty acids extraction from a lipids-rich fungal biomass.

Keywords: Oleaginous fungi, kinetic curves, linolenic acid, omega-3 fatty acid, extraction yields, spline model.

1. Introduction

Polyunsaturated fatty acids (PUFA) can belong to the omega-3 or omega-6 families, depending on the position of the first double bond from the methyl end [1]. These PUFA are considered essential fatty acids because they are required for optimal human health but are not synthesized by the body [2]. Several studies have reported that PUFA has important benefits for health, as presenting positive influence on the brain system functions [3] and on some types of cancer [4]. The PUFA also act on the cardiovascular system [5].

Overall, the fatty acids are found in natural sources, as in marine fish and seafood [6], flaxseed, linseed, canola, perilla, safflower, soy, cumbaru, corn and annatto seed oils [7–11], among others. Additionally, fatty acids can be produced by microorganisms such as marine bacteria, fungi, protists and microalgae [12]. Even though the plant sources are the main focus of studies for human consumption, microorganisms sources are also recently gaining popularity as potential and promising sources. Some microorganisms offer some advantages for the production of fatty acids, such as requiring simple nutrient input, allowing controllable culture conditions and providing simple fatty acids composition [13].

Oleaginous fungi from the genus *Mortierella* have been used for producing lipids rich in PUFA. The main PUFA produced by these fungi are -linolenic acid (GLA-C18:3 -6), dihomo-linolenic acid (DHGLA-C20:3 -6), arachidonic acid (ARA-C20:4 -6), and docosahexaenoic acid (DHA-C22:3 -3) [14]. Certain fungi accumulate intracellular lipids, especially the triacylglycerol (TG). Specifically, the filamentous fungus *Mortierella isabellina* is capable of accumulating a considerable amount of lipids [15].

Depending on the culture conditions, a unitary operation like extraction is needed to remove the PUFA from the fungi cells. Supercritical fluid extraction (SFE) has been employed for the extraction of PUFA from these cells [16,17]. SFE is a technology that offers advantages

over conventional extraction methods, such as increased selectivity, automaticity, environmental safety, superior quality of extracts and a considerably reduced consumption of organic solvents, thus resulting in extracts without solvent residue [18]. The most widely solvent used in SFE process is the CO_2, because it has some advantages such as being environmentally benign, non-toxic, non-flammable and non-polluting, and it is the most economical and compatible fluid which extracts oxygen sensitive compounds without any molecular alteration [19].

Despite that, some studies have recently reported the use of compressed liquefied petroleum gas (LPG) to extract mainly fatty acids because the extraction time could be shortened and the use of solvent could be reduced [20,21]. The selectivity is also another feature of this fluid composed of propane [22], with the additional advantage of being low-cost when compared with the pure propane or CO_2 [20]. However, there are no studies reporting the extraction of PUFA from oleaginous fungi cells using LPG. Therefore, this is an interesting gap that suggests discriminating the influence of different solvents on the extraction processes in pressurized media.

Based on this context, the purpose of this study was to evaluate process conditions that provide high extraction of fatty acids from freeze-dried cells of *Mortierella isabellina* produced by submerged fermentation. Among the process conditions, the objective was to assess the influence of temperature and pressure, and the solvent: supercritical CO_2 and compressed LPG. In order to provide the more suitable condition, the responses taken into account have been the extraction yields, the extraction kinetic parameters, and the fatty acids composition.

2. Material and methods

2.1. Microorganism

Mortierella isabellina was purchased from Tropical Culture Collection André Tosello (Campinas, Brazil). Before using, the culture was maintained in a potato dextrose agar (PDA) at 4 ºC and subcultured every month.

2.2. Solvents and reagentes

CO_2 (purity > 99.5%) was purchased from White Martins S.A. (Santa Maria, Brazil). LPG was purchased from Liquigás S.A. (Santa Maria, Brazil) and consisted of a mass mixture of propane (50.3 wt.%), n-butane (28.4 wt.%), isobutane (13.7 wt.%), ethane (4.8 wt.%), and other minor constituents (2.8 wt.% of methane, pentane, isopentane, etc.). N-hexane, methyl tridecanoate (C23:0 Me) and isooctane were supplied by Sigma-Aldrich (São Paulo, Brazil).

2.3. Fermentation in orbital shaker

Cell production for pre-inoculum was obtained by incubating the culture in a Petri dish containing PDA for 5 days at 28 ºC. Afterward, fungal mycelium of one Petri dish was transferred to a 125 mL Erlenmeyer flasks containing 25 mL of potato dextrose. The fungal mycelium and the potato dextrose were stirred for 48 h at 28 ºC and 120 rpm (rotations per minute) (Inova 44R, New Brunswick) for the pre-inoculum production. The fermentation was performed in 500 mL Erlenmeyer flasks containing 250 mL of medium. The fermentation medium was composed of (g/L): sucrose (50), peptone (1.5), yeast extract (3.75), $(NH_4)_2SO_4$ (2.0), $FeSO_4 \cdot 7H_2O$ (1.0), $MnSO_4 \cdot H_2O$ (1.0) and $MgSO_4$ (0.5). The fermentation process was fulfilled during 5 days at 24 ºC and 200 rpm (Inova 44R, New Brunswick), and the initial pH of 5.0 was fixed. The cells were filtered (Whatman qualitative filter paper, grade 1) and then washed with distilled water. Finally, the cells were freeze-dried (L 101, Liotop, São Carlos, Brazil) for 48 h.

2.4. Supercritical CO_2 and compressed LPG extractions

The laboratory scale plant (Fig. 1) used for the assays is composed by: (i) a 100 mL extraction vessel (stainless steel) with internal diameter of 2.5 cm and 19.5 cm of height, supporting up to 35 MPa; (ii) a syringe pump (ISCO 500 D, Lincoln, USA); (iii) a cooling bath (Quimis, ultrathersmostatic bath, São Paulo, Brazil) for controlling the temperature of CO_2 and LPG at the syringe pump; (iv) a heating bath (Quimis, ultrathersmostatic bath, São Paulo, Brazil) with thermocouples; (v) a heating electric jacket to control the temperature inside the extraction vessel; (vi) blocking valves and micrometering valves (HIP 15-11AF2 316SS, Erie, USA); and (vii) 1/8" tubing of stainless steel (HIP, Erie, USA).

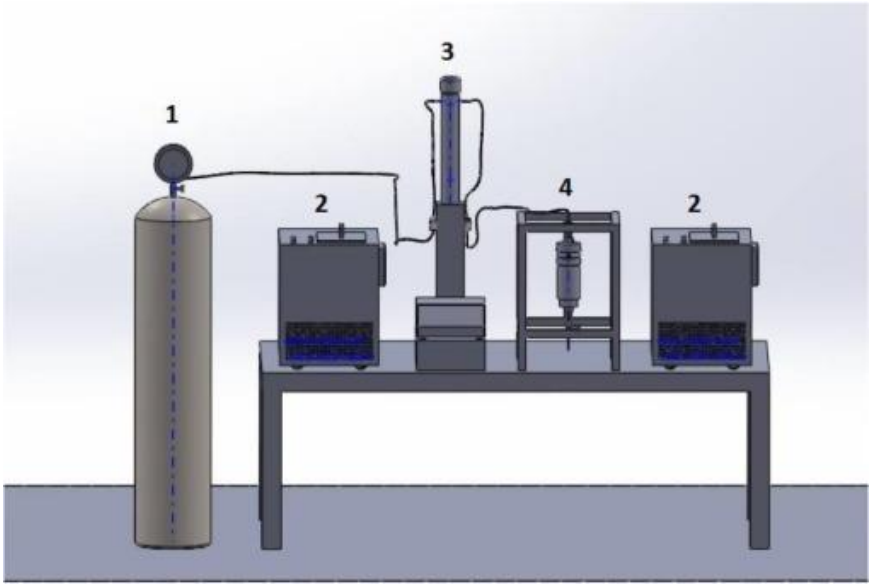


Fig.1: Experimental apparatus for CO_2 and LPG extractions. The main devices are: (1) solvent reservoir; (2) cooling (left) and heating (right) baths; (3) syringe high-pressure pump; (4) extraction vessel.

One micrometering valve was located immediately after the syringe pump to allow solvent loading at the entrance of the high-pressure extraction vessel. The other one was located just after the high-pressure extraction vessel in order to control the solvent flow. The outlet extremity of the extraction vessel was filled with filter paper (205 m) and a support disk of 300

mesh. The filter paper and the support disk were fixed on the bottom of the extraction vessel for avoiding the passage of particles that could obstruct the pipeline.

For the extraction procedure with CO_2, approximately 5 g of cells were loaded in the extraction vessel. In the sequence, CO_2 was pumped in the bed and the condition of pressure and temperature was established. After a short time for reaching the pressure and temperature equilibria inside the bed, the dynamic extraction was started. The extraction time was fixed to 60 min for the assays, with a solvent flow rate of 4 g/min. This time (60 min) was defined after evaluating the extraction curve obtained in a preliminary test. During the kinetic extractions, the oil was collected at equal intervals of 5 min, totalizing 12 samples for each assay. The experimental assays were performed using a 2^2 central composite design (CCD) with triplicate at the central point (60 °C and 200 bar). The variables temperature (40–80 °C) and pressure (150–250 bar) were evaluated.

For the extraction procedure with LPG, approximately 5 g of cells were also loaded in the extraction vessel. In the sequence, LPG was pumped in the bed and the condition of pressure and temperature was established. After reaching the pressure and temperature equilibria inside the bed, the dynamic extraction was started. Extraction curves obtained in a preliminary test were evaluated, and then the extraction time was fixed to 36 min for all the assays, with a solvent flow rate of 4 g/min. During the kinetic extractions, the oil was collected at equal intervals of 2 min, totalizing 18 samples for each assay. The experimental assays were performed using a 2^2 CCD with triplicate at the central point (30 °C and 15 bar). The variables temperature (20–40 °C) and pressure (10–20 bar) were evaluated.

2.5 Kinetic extraction curves

The experimental data of extract yields for CO_2 and LPG assays were fitted to a spline model with 2 straight lines [23]:

1) For $t \leq t_{CER}$:

$$Y(t) = b_0 + b_1 \cdot t \quad (1)$$

2) For $t > t_{CER}$:

$$Y(t) = b_0 - b_2 \cdot t_{CER} + (b_1 + b_2) \cdot t \quad (2)$$

Where: b_0 is the linear coefficient of line 1; b_1 and b_2 are the slopes of lines 1 and 2, respectively; t is the extraction time; t_{CER} is the end of the CER period; Y(t) is the yield of oil as a function of time.

Then, the SAS 9.2® package was used to estimate: (i) the end of the constant extraction rate (CER) period (t_{CER}); (ii) the mass transfer rate for the CER period (M_{CER}); (iii) the yield for the CER period (R_{CER}); and (iv) the mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period (Y_{CER}). These parameters were used to have a quantitative description of the kinetic extraction curves.

2.6 Determination of fatty acids in the oil

An amount of 10 μL of each sample of the oil was solubilized in 1 mL of *n*-hexane and 250 μL of a 4 mg/mL solution of methyl tridecanoate (C23:0 Me) in isooctane was added to each fraction. Solvents were evaporated at 40 °C under vacuum. Fatty acids methyl esters (FAME) derivatizations were performed according to the method described by Visentainer [24]. The FAME were analyzed in the GC system (Shimadzu, GCMS-QP2010 Ultra, Japan) by injecting 1 μL into a capillary column Ptx-Wax (Restek-USA) (30 m × 0.25 mm × 0.25 μm). The carrier gas (Helium, purity > 99%, White Martins, Santa Maria, Brazil) flowed at a constant pressure of 103.4 kPa. The following column temperature gradient was used: 50 °C; 3 °C/min up to 140 °C (10 min). The injector was maintained at

140 °C and the split ratio was 60:1. The detector was maintained at 140 °C. The compositions were expressed as milligram of each fatty acid per gram of oil (mg/g oil).

3. RESULTS AND DISCUSSION

3.1. Extraction yields

Some differences in the extraction yields are observed when the extractions of oil with supercritical CO_2 and compressed LPG from the freeze-dried cells of *Mortierella isabellina* are compared (Table 1). The yields using CO_2 ranged from 0 wt.% (assay 3) to 3.21 wt.% (assay 4), while the yields using LPG ranged from 3.43 wt.% (assay 2) to 4.45 wt.% (assay 4). These results are corroborated by Fakas et al. [15], which the oil extracted from *Mortierella isabellina* mycelia of different maturities varied between 3.5 wt.% and 4 wt.%.

Table 1: Comparison of the extraction yields obtained by SFE-CO_2 (S/F = 48) and compressed LPG (S/F = 29) from *Mortierella isabellina*.

	CO_2				**LPG**			
Assay	**T (°C)**	**P (bar)**	**Yield (g/100 g raw material)**	**Ext. ratio $\times 10^3$ (g ext./g sol.)**	**T (°C)**	**P (bar)**	**Yield (g/100 g raw material)**	**Ext. ratio $\times 10^3$ (g ext./g sol.)**
1	(-1) 40	(-1) 150	1.76	7.3	(-1) 20	(-1) 10	3.48	24.0
2	(-1) 40	(+1) 250	2.94	12.3	(-1) 20	(+1) 20	3.43	23.7
3	(+1) 80	(-1) 150	0.00	0.0	(+1) 40	(-1) 10	4.17	28.8
4	(+1) 80	(+1) 250	3.21	13.4	(+1) 40	(+1) 20	4.45	30.7
5	(0) 60	(0) 200	2.08	8.7	(0) 30	(0) 15	3.70	25.5
6	(0) 60	(0) 200	1.91	8.0	(0) 30	(0) 15	3.77	26.0
7	(0) 60	(0) 200	1.77	7.4	(0) 30	(0) 15	3.55	24.5

S/F: solvent mass to feed mass ratio; T: temperature; P: pressure; ext.: extract; sol.: solvent

At isobaric and at isothermal conditions, the higher the temperature and pressure the higher the extraction yields for both solvents. In some cases, the influence of the vapor pressure could overlap the influence of the solvent density on the yields. Curiously, for the SFE-CO_2 condition of 80 °C and 150 bar (assay 3), none extract was obtained. In this condition, the CO_2 density is 445 kg/m^3 [25], the smallest one amongst those evaluated. Then, the solvent density rather hampered the solubility of compounds in the solvent in such condition. According to Pereda et al. [26], the effect of fluid density is dominant at high pressures near the critical point, but far from the critical point, the effect is reduced. Even though low CO_2 densities commonly enable lower extraction yields, it is not common to have none yield in an assay. Considering the assay 3 was replicated two times, this finding is most likely associated with the retrograde condensation phenomenon [27] and [28], where there is an inversion point of pressure and temperature on the extractions yields. Therefore, the combination of temperature and pressure in such condition has not been effective in obtaining the bulk extract. It is important to point out that the deviation on the extraction yields around the average value in the central point was low (1.92 ± 0.15 wt.%), thus corroborating the findings.

When evaluating the yields obtained with compressed LPG (Table 1), all the assays produced higher content of oil than SFE-CO_2 did. The properties of the compressed LPG in such conditions (as solvating power, viscosity, diffusivity, etc.) were favorable for solubilizing the target components from the freeze-dried cells of *Mortierella isabellina*. This is evidenced in the extract ratio, which the compressed LPG provided oil ratios up to 3.3 higher than the supercritical CO_2. At the central point (30 °C and 15 bar), the average yield was equal to 3.67 ± 0.11 wt.%. Similar findings on the extraction of rice bran oil using compressed LPG are reported [20]. As the CO_2, the LPG is removed from the final extract by simple decompression of the sample at room pressure (1 bar).

The behavior of the extractions was also evaluated during the time. Kinetic curves were generated by the cumulative mass of oil from *Mortierella isabellina* as a function of extraction time and solvent mass to feed mass (S/F) ratio (Fig. 2 and Fig. 3). The behavior is typical of extraction curves reported from studies that obtained extracts using pressurized fluids [10], [29] and [30].

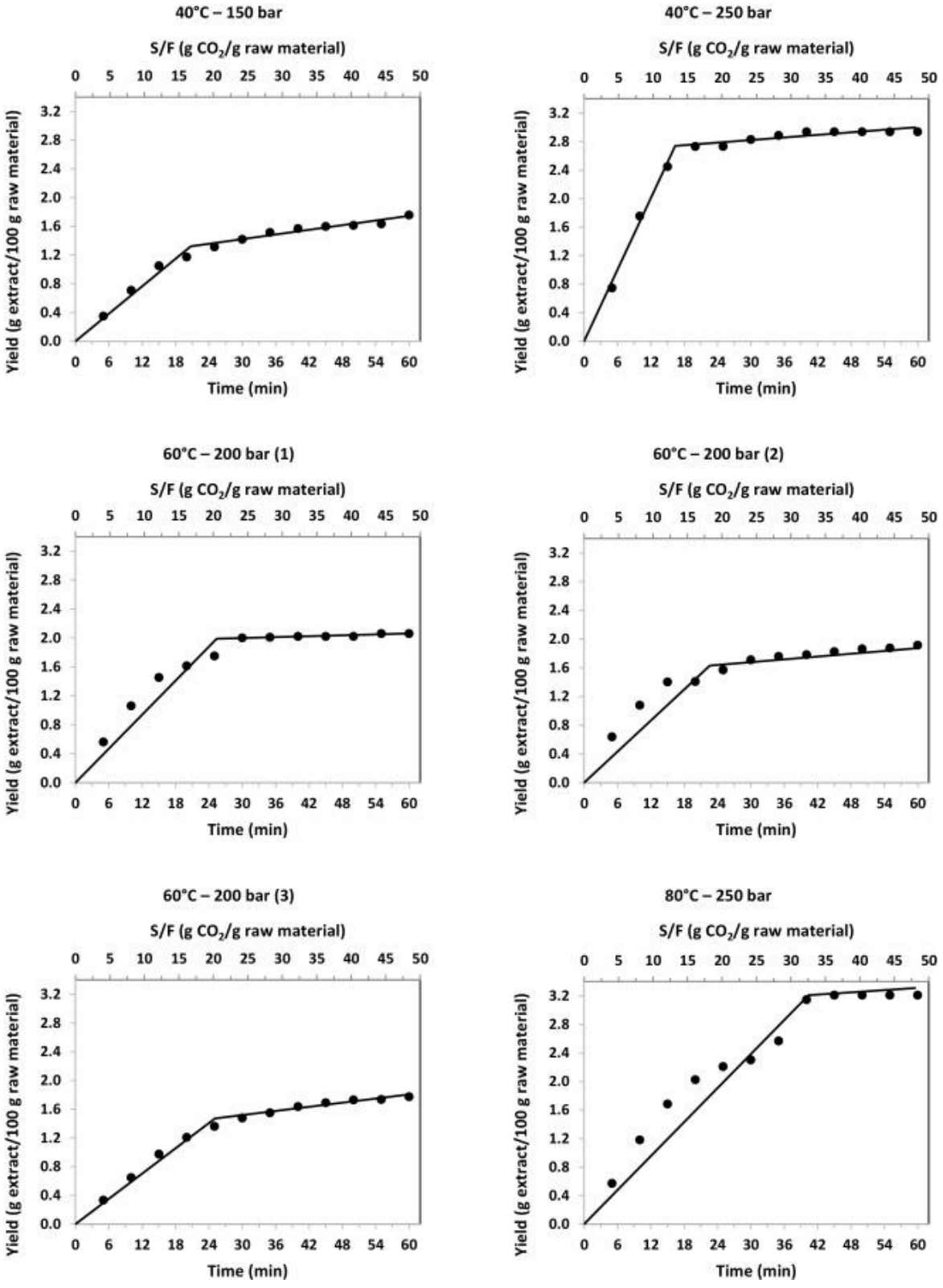


Fig.2: Kinetic yields of the oil obtained from *Mortierella isabellina* by SFE-CO_2: experimental data and fitted curves using the spline model; S/F: solvent mass to feed mass ratio.

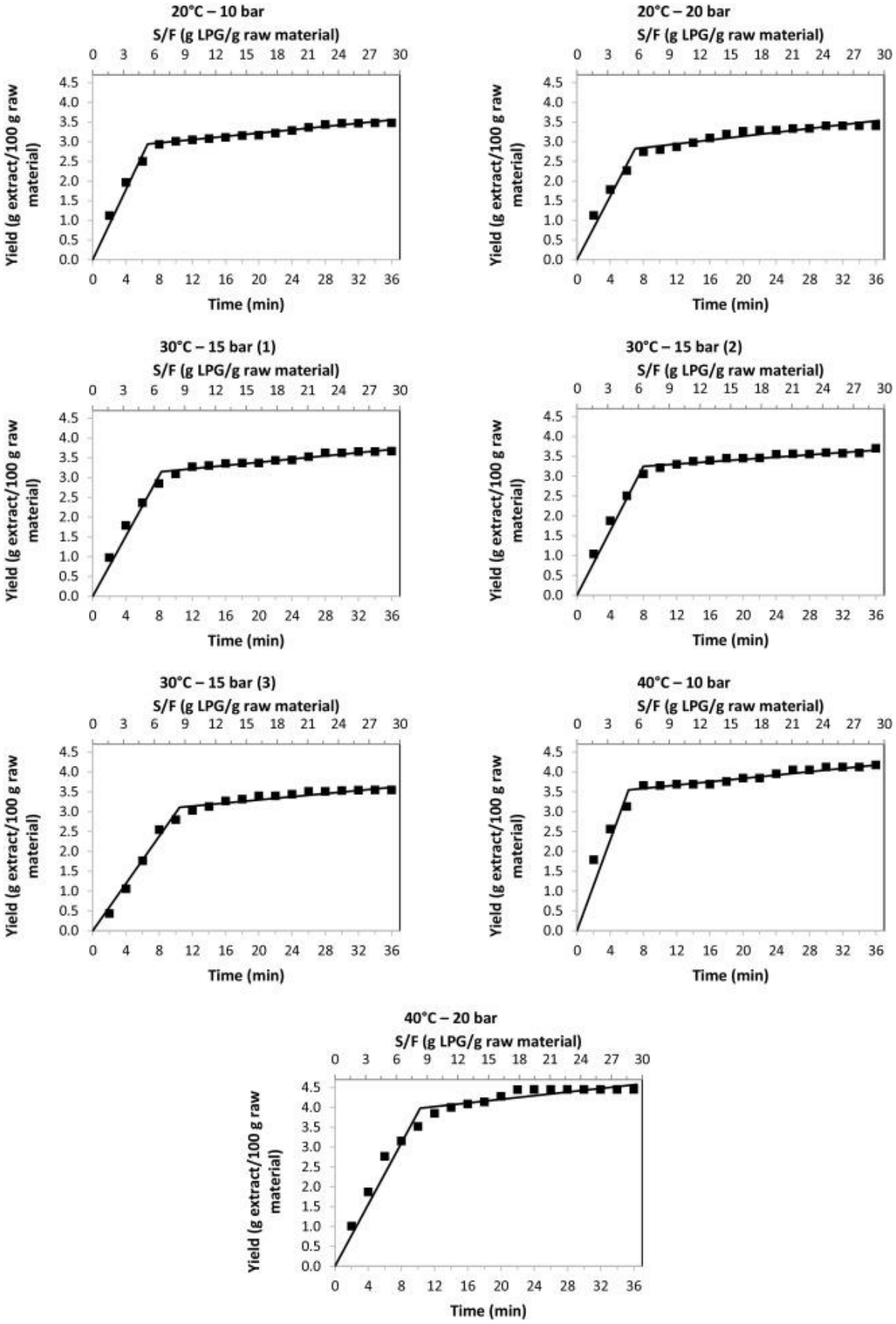


Fig.3: Kinetic yields of the oil obtained from *Mortierella isabellina* by compressed LPG: experimental data and fitted curves using the spline model; S/F: solvent mass to feed mass ratio.

For both solvents, the behavior of the extractions is typical of two distinguished periods: the first one means the higher extraction rate and the second one means the smaller extraction rate (or falling extraction rate). The transition between these periods represents the end of the CER period (t_{CER}). Most of the oil was obtained in the first period, indicating that the solvents could solubilize the bulk oil. Approximately 75–95 wt.% of compounds were recovered in the CER period, which succeeded in the range of 6–10 min for compressed LPG and 17–40 min for supercritical CO_2. The extraction time was considerably smaller and the extraction yields were higher when the compressed LPG is compared with supercritical CO_2. The compressed LPG could interact with the solute in an effective manner, thus solubilizing the compounds in a high rate. Our findings are also corroborated by the findings reported by Ribas et al. [22], where candeia oil was better extracted with propane (one of the main constituent of LPG). Besides of reducing the extraction time, an important factor for the economic feasibility of extraction processes [31] and [32], the energy required for the recompression of LPG is lower than that required for CO_2. For instance, at 65 °C and 25 bar, LPG shows the molar density of 9.27 mol/L, while CO_2 reaches the same molar density (at 65 °C) only at 124 bar [10].

Overall, the phenomena occurring along the extraction bed influenced the behavior of the extraction curves. Considering all the assays, the binomial effect (temperature and pressure) at 40 °C and 20 bar was the most feasible one to obtain a high yield of extract using $S/F \leq 18$ and operation time of approximately 20 min (Fig. 3). Under this condition, the mass transfer rate was higher, which the mechanism of mass transfer was mainly controlled by convection in the fluid film around the freeze-dried cells [23].

3.2 Extraction kinetic parameters

Extraction curves are important responses for further decisions involving the scale up of processes. In spite of that, other approaches could be applied for analyzing extraction yields. One of these approaches is describing the curves by a simple and usable spline model. Several studies dealing with the extraction of target compounds using pressurized fluids report the use of the spline model for fitting extraction kinetic parameters [9], [33] and [34]. Thus, the curves can be assessed through a quantitative description.

Then, the extraction kinetic parameters fitted by the spline model for the CER period are shown (Table 2). These parameters (t_{CER}, R_{CER}, M_{CER}, and Y_{CER}) were evaluated because they describe the moment at which the diffusional contribution starts to be important compared to the convective contribution. Fitting the kinetic parameters in this period is common because the mass transfer rate is constant and the productivity of oil is high.

Table 2: Extraction of the fatty acids-rich extract by SFE-CO_2 and compressed LPG: fitted data using the spline model.

Assay	t_{CER} (min)	R_{CER} (wt.%)	$M_{CER}\times10^3$ (g/min)	$M_{Sol,CER}$ (g)	S/F_{CER} (g solvent/g raw material)	$Y_{CER}\times10^3$ (g extract/g solvent)
CO_2						
40°C – 150 bar	20.9	1.32	3.16	84	16.7	0.79
40°C – 250 bar	16.5	2.74	8.33	66	13.2	2.08
60°C – 200 bar	25.3	1.47	2.90	101	20.2	0.73
60°C – 200 bar	22.8	1.63	3.58	91	18.2	0.90
60°C – 200 bar	25.6	1.99	3.90	102	20.4	0.98
80°C – 250 bar	40.7	3.21	3.95	163	32.5	0.99
LPG						
20°C – 10 bar	6.7	2.94	22.0	27	5.4	5.49
20°C – 20 bar	7.1	2.82	19.9	28	5.7	4.97
30°C – 15 bar	8.4	3.15	18.8	34	6.7	4.69
30°C – 15 bar	8.1	3.25	20.0	32	6.5	5.01
30°C – 15 bar	10.6	3.11	14.7	42	8.5	3.67
40°C – 10 bar	6.3	3.55	28.2	25	5.0	7.04
40°C – 20 bar	10.4	2.82	19.1	42	8.3	4.78

CER; Constant Extraction Rate; t_{CER}: end of the CER period; R_{CER}: yield of extract for the CER period; M_{CER}: mass transfer rate for the CER period; $M_{Sol,CER}$: mass of solvent used in the CER period; S/F_{CER}: solvent to feed mass ratio in the CER period; Y_{CER}: mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period.

The kinetic parameters were rather different when the assays are compared (Table 2). The t_{CER} ranged from 16.5 to 40.7 min and from 6.3 to 10.6 min when using supercritical CO_2 or compressed LPG, respectively. At the central point, the t_{CER} was equal to 24.6 ± 1.5 and 9.0 ± 1.3 min for supercritical CO_2 and compressed LPG, respectively. The remarkable differences of t_{CER} between the solvents stand for the higher capacity of LPG for solubilizing the solute from *Mortierella isabellina.* With an S/F lower than 8 g LPG/g freeze-dried cells, approximately 85 wt.% of the extract is recovered, representing a faster process. Therefore, the first step of extraction ($t < t_{CER}$) demonstrated that the external mass transfer rate dominated the process.

In addition, the kinetic curves can be evaluated in terms of M_{CER}. If the slope of the line in the first extraction period is increased, the M_{CER} is increased as well. Consequently, the higher the R_{CER} and the lower the t_{CER}, the higher is the M_{CER}. Such parameter ranged from $2.90 \times 10^{-3.}$ to 8.33×10^{-3} g/min and from 14.7×10^{-3} to 28.2×10^{-3} g/min when using supercritical CO_2 or compressed LPG, respectively. Overall, the extraction rates were approximately 6-fold higher when LPG was applied. These differences appear because the extraction time is shorter and less solvent is used when LPG is considered. Once it is not possible to compare the M_{CER} values with other studies for oil obtained by SFE-CO_2 or compressed LPG from *Mortierella isabellina* because there are none study dealing with such fit, values reported in the literature for some raw materials in closer laboratory scale and solvent flow rates are in the same range. For instance, values of M_{CER} were equal to $4.8 \times 10^{-3.}$ g/min for cumbaru oil [9] and 17.6×10^{-3} g/min for turmeric extract [27] when using supercritical CO_2.

Another kinetic parameter, the Y_{CER}, is assessed in extraction processes because it means the amount of oil solubilized in the solvent. If the Y_{CER} is high, it means a large amount of solute has been reached by the solvent. In the extraction of oil containing fatty acids

from *Mortierella isabellina*, such parameter ranged from $0.73 \times 10^{-3.}$ to 2.08×10^{-3} g/g CO_2 and from 3.67×10^{-3} to 7.04×10^{-3} g/g LPG (Table 2). In terms of bulk oil, the best performance was obtained when the highest yield was reached with a faster rate. Therefore, taking into account the t_{CER}, R_{CER}, M_{CER}, Y_{CER}, and S/F_{CER}findings, the condition that seems to be favorable is 40 °C and 250 bar for supercritical CO_2, and 40 °C and 10 bar for compressed LPG.

The quantitative description through the kinetic parameters helps to interpret the kinetic curves. The integrated evaluation of the kinetic yields (Fig. 2 and Fig. 3) and the kinetic parameters (Table 2) favors selecting a suitable condition that promotes the best extraction in an ideal time. In order to have additional information for facilitating this selection, the bulk oil was measured in terms of fatty acids, which the discussion is provided in the next section.

3.3 Fatty acids

Fourteen fatty acids were identified and quantified in the extracts obtained in different conditions by SFE-CO_2 and compressed LPG (Table 3). The major fatty acids identified in the oil from *Mortierella isabellina* were: estearic acid (C18:0), *cis*-10-pentadecenoic acid (C15:1), *cis*-10-heptadecenoic acid (C17:1), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), palmitic acid (C16:0) and α-linolenic acid (C18:3n3). These fatty acids were also identified in the lipid fractions of *Mortierella isabellina* mycelia [15]. According to Demir et al. [14], the microbial lipids produced by this fungus contained fatty acids with carbon chain lengths from 14 (myristic acid, C14:0) to 20 (arachidic acid, C20:0), which the palmitic acid (C16:0), the linoleic acid (C18:2n6c) and the oleic acid (C18:1n9c) were some of the main fatty acids produced. Taking into account the seeking for renewable fuels, the lipids extracted from *Mortierella isabellina* biomass can be also a potential microbial source for high-quality biodiesel production [35].

Table 3: Fatty acids (mg/g oil) identified in the extracts obtained by SFE-CO_2 and compressed LPG from *Mortierella isabellina*.

	CO_2				**LPG**				
	1	**2**	**4**	**5/6/7**	**1**	**2**	**3**	**4**	**5/6/7**
C13:0	3.28	1.32	1.95	1.85	1.76	1.32	0.36	1.28	1.22
C14:1	2.08	0.84	1.25	1.17	1.12	0.89	0.24	0.81	0.82
C15:1	104.00	45.21	63.70	65.84	61.22	50.10	12.75	41.63	47.30
C16:0	5.47	2.56	3.74	3.56	3.53	3.16	0.95	2.37	2.87
C17:1	27.66	12.99	18.12	18.45	16.93	14.22	3.68	10.96	13.29
C18:0	189.66	91.85	127.41	133.86	123.47	130.35	29.08	75.91	123.04
C18:1n9c	10.24	5.59	8.84	7.85	8.32	9.27	1.73	4.13	8.59
C18:2n6c	4.82	2.64	4.80	3.68	4.37	4.21	0.80	1.90	3.90
C18:3n3	4.16	2.19	3.01	3.03	2.71	2.81	0.69	1.61	2.60
C22:0	2.20	1.31	1.02	2.13	1.75	1.23	0.81	0.95	1.18
C23:0	10.93	6.54	5.99	9.24	5.53	5.61	4.61	4.31	5.20
C24:0	2.89	1.64	2.28	2.45	2.07	1.86	0.46	1.12	1.72
C22:6	1.59	0.00	1.30	1.36	1.16	1.34	0.28	0.65	1.22
C24:1	2.86	0.00	1.66	1.58	0.83	0.55	0.13	1.18	0.31
SFA	214.43	105.22	142.39	153.09	138.11	143.53	36.27	85.94	135.23
MUFA	119.18	51.64	75.45	76.44	71.49	60.81	14.85	47.75	57.02
PUFA	10.57	4.83	9.11	8.07	8.24	8.36	1.77	4.16	7.72
UFA/SFA	0.61	0.54	0.60	0.55	0.58	0.48	0.46	0.60	0.48
MUFA/SFA	0.56	0.49	0.53	0.50	0.52	0.42	0.41	0.55	0.42
PUFA/SFA	0.05	0.05	0.07	0.05	0.06	0.06	0.05	0.05	0.06
Total[a]	360.9	168.1	239.1	246.8	229.2	221.3	52.0	144.5	208.1
Total[b]	635.6	494.3	767.4	513.4	797.8	759.1	216.7	643.0	784.4

C13:0 - (Tridecanoic acid); C14:1 - (Myristoleic Acid); C15:1 - (cis-10-Pentadecanoic acid); C16:0 - (Palmitic acid); C17:1 - (cis-10-heptadecanoic acid); C18:0 - (Estearic acid); C18:1n9c - (Oleic acid); C18:2n6c - (Linoleic acid); C18:3n3 - (α-linolenic acid); C22:0 – (Behenic acid); C23:0 - (Tricosanoic acid); C24:0 - (Lignoceric acid); C22:6 – (Docosahexaenoic acid); C24:1 – (Nervonic acid); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids.

[a] Means mg/g oil.

[b] Means mg/100 g freeze-dried cells.

In this study, differences in the concentration of fatty acids in the extracts as a function of the solvent used and the condition of temperature and pressure applied can be observed (Table 3). Considering the total concentration of fatty acids, the use of supercritical CO_2 favored the extraction of these compounds in most of the conditions. The highest total concentration of fatty acids was obtained at 40 °C and 150 bar using SFE-CO_2 (approximately 361 mg/g oil), while the lowest total concentration of fatty acids was obtained at 40 °C and 10 bar using compressed LPG (approximately 52 mg/g oil). For the assay 4 (the assay with the highest yield and mass of oil solubilized in the solvent), the total concentration of fatty acids was 144.5 mg/g oil when using compressed LPG. Even though the concentration of fatty acids in the oil in the assay 4 (LPG) was approximately 2.5 times lower than the highest one obtained in the oil extracted by SFE-CO_2 (assay 1), the total fatty acids extracted from the freeze-dried cells of *Mortierella isabellina* was quite similar. The total fatty acids extracted from the biomass were 643 mg/100 g raw material and 636 mg/100 g raw material (Table 3) through the assays 4 (LPG) and 1 (CO_2), respectively. These findings indicate the SFE-CO_2 was most selective for extracting the higher amount of fatty acids in a lower amount of oil, while the compressed LPG enabled the extraction of other compounds together with the fatty acids identified, thus enhancing the total yields of oil (Table 1 and Fig. 2).

The concentrations of fatty acids presented in the oil (Table 3) obtained from the freeze-dried cells of *Mortierella isabellina* using both pressurized fluids are higher than those obtained using conventional methods, as Soxhlet and autoclaving [35] and [36], thus indicating the selectivity of using pressurized fluids. The supercritical CO_2 and compressed LPG could break the mycelia to a large extent (our study) than chloroform/methanol did [36]. In the same trend, Nisha et al. [17] compared the fatty acids composition of oil extracted from *Mortierella alpina* biomass using SFE and Soxtec extraction (organic solvent extraction), and concluded the SFE was favorable for extracting some fatty acids, especially the C18:1 and C18:2 ones. In

this study, LPG showed a slight advantage against CO_2 when the total concentration of fatty acids as a function of the raw material (mg/100 g freeze-dried cells) is evaluated, which this compressed fluid could break down the cell structure, increase the contact surface area between solid and liquid phase, and release intracellular components into the solvent. Otherwise, CO_2 showed an advantage against LPG when the fatty acids are evaluated in terms of oil produced (mg/g oil, Table 3).

Therefore, the fatty acids profile in the oil obtained from *Mortierella isabellina* freeze-dried cells demonstrated that the extraction conditions influenced the concentration of saturated (SFA) and unsaturated fatty acids (UFA). For SFE-CO_2, the assay 1 (40 °C and 150 bar) provided the highest concentration of UFA in the oil (129.75 mg/g oil), indicating that low values of temperature and pressure are enough and appropriate for UFA extraction. For compressed LPG, the assay 1 (20 °C and 10 bar) provided the highest concentration of UFA in the oil (79.73 mg/g oil). Although the UFA in the extracts obtained by compressed LPG were slightly lower than those obtained by SFE-CO_2, the UFA/SFA ratio was similar (0.46–0.61) because when the UFA increased the SFA increased as well. Most of the UFA measured in the extracts were composed by monounsaturated chains (approximately 85–90%, mass basis), while the polyunsaturated chains represented approximately 10–15% (mass basis). The best PUFA/SFA ratio was obtained in the assay 4 (CO_2).

Among the PUFA, α-linolenic acid (C18:3n3) has health benefits and it is being supplemented in many products. An optimal diet with a balance of PUFA may reduce brain dysfunctions, such as Parkinson's and Alzheimer's neurodegenerative disorders [37]. In this study, the α-linolenic acid accounted approximately 4 mg/g oil, which is in agreement with the findings reported by Demir et al. [14], Xian et al. [38] and Ruan et al. [39]. The α-linolenic acid in the oil extracted by centrifugation from *Mortierella isabellina* produced in whey treated with lactase ranged from 2.8 to 3.3 mg/g oil [14]. Likewise, the effect of initial sugar concentrations

on fatty acid composition of *Mortierella isabellina* stored lipid has been evaluated. Consequently, the α-linolenic acid ranged from 0.4 to 3.4 mg/g oil [39].

As presented [14], [36] and [39], the content of α-linolenic acid (and consequently all the PUFA) depends on the conditions of cultivation of *Mortierella isabellina*, and the conditions of rupturing the cells and extracting the compounds accumulated in the mycelia. The PUFA represented up to 10.6 mg/g oil (approximately of 85% of linoleic and α-linolenic acids, mass basis, Table 3), which is a remarkable value if compared to other findings with the same fungus. For example, the linolenic acid ranged from 6.6 to 10.2 mg/100 g oil obtained with *n*-hexane and diethyl ether at room pressure (approximately 1 bar) [15]. Such fatty acid was also present in a low content in the oil obtained through a homogenization method with chloroform and methanol, accounting 0.4–3.4 mg/100 g oil [39]. Furthermore, Yu et al. [36] reported that autoclaving, HCl digestion, bead-beating, sonication, and microwave extraction methods did not produce any content of linolenic acid in the oil obtained from *Mortierella isabellina*.

Therefore, the findings of oil composition indicate the SFE-CO_2 is the preferable extraction technique when compared with compressed LPG. Despite the LPG could extract the higher amount of total oil in lower time (lower solvent consumption), the CO_2could be more selective in obtaining fatty acids, especially the unsaturated fatty acids. One of the main characteristics of oils is their composition in terms of target compounds. Then, in a broader evaluation of all findings and reports, SFE-CO_2 is one technique with several advantages of selectivity. The characterization of the oil obtained from the filamentous fungus *Mortierella isabellina* indicates the mild conditions of CO_2 (40 °C and 150 bar) are suitable for extracting oil rich in target fatty acids.

4. Conclusion

The course of obtaining fatty acids and total oil from *Mortierella isabellina* was influenced by the solvent (in pressurized media) and by the process conditions. Overall, when the total oil is desired, the use of compressed LPG is slightly advantageous against the supercritical CO_2. Comprising the total oil, the highest extraction yields were obtained with the highest temperature and pressure amongst those evaluated. For SFE-CO_2 (80 °C and 250 bar) and compressed LPG (40 °C and 20 bar), the maximum yields were 3.21 wt.% and 4.45 wt.%, respectively. The fitting of kinetic parameters provided a quantitative description of the extraction curves, which also indicates the aforementioned conditions as the more appropriate for presenting the highest mass transfer rates and the largest yields of oil in the CER period. However, when high concentration of fatty acids in the oil is desired, the supercritical CO_2 is advantageous against compressed LPG because the CO_2 shows more selectivity properties. For fatty acids, approximately 361 mg/g oil and 229 mg/g oil were reached with CO_2 at 40 °C/150 bar and with LPG at 20 °C/10 bar, respectively. The fraction of polyunsaturated fatty acids was mostly composed of linoleic and linolenic acids. Up to now, the compressed LPG has been not used for recovering fatty acids from oleaginous cells of *Mortierella isabellina*. Thus, the results presented and discussed in this article are noteworthy outcomes and could encourage ongoing studies, once the use of compressed LPG to obtain oil from fungi is still a novelty for the scientific community, which should be further compared with supercritical CO_2.

References

[1] A.M. Giudetti, R. Cagnazzo, Beneficial effects of n-3 PUFA on chronic airway inflammatory diseases, Prostaglandins and Other Lipid Mediators, 99 (2012) 57-67.
[2] S.D. Dyal, S.S. Narine, Implications for the use of *Mortierella fungi* in the industrial production of essential fatty acid, Food Research International, 38 (2005) 445-467.
[3] J. Bradbury, Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain, Nutrients, 3 (2011) 529-544.
[4] P. Bougnoux, N. Hajjaji, K. Maheo, C. Couet, S. Chevalier, Fatty acids and breast cancer: Sensitization to treatments and prevention of metastatic re-growth, Progress in Lipid Research, 49 (2010) 76-86.
[5] D.B. Jump, C.M. Depner, S. Tripathy, Omega-3 fatty acid supplementation and cardiovascular disease, Journal of Lipid Research, 53 (2012) 2525-2545.
[6] I. Schneedorferová, A. Tomčalaa, I. Valterová, Effect of heat treatment on the n-3/n-6 ratio and content of polyunsaturated fatty acids in fish tissues, Food Chemistry, 176 (2015) 205-211.
[7] M. Koubaa, H. Mhemdi, F.J. Barba, S. Roohinejad, R. Greiner, E. Vorobiev, Oilseed treatment by ultrasounds and microwaves to improve oil yield and quality: An overview, Food Research International, 85 (2016) 59-66.
[8] E.K. Silva, G.L. Zabot, M.A.A. Meireles, Ultrasound-assisted encapsulation of annatto seed oil: Retention and release of a bioactive compound with functional activities, Food Research International, 78 (2015) 159-168.
[9] P. Santos, A.C. Aguiar, J. Viganó, J.S. Boeing, J.V. Visentainer, J. Martínez, Supercritical CO_2 extraction of cumbaru oil (*Dipteryx alata* Vogel) assisted by ultrasound: Global yield, kinetics and fatty acid composition, Journal of Supercritical Fluids, 107 (2016) 75-83.
[10] J.F. Soares, G.L. Zabot, M.V. Tres, F.C. Lunelli, V.M. Rodrigues, M.T. Friedrich, C.A. Pazinatto, D. Bilibio, M.A. Mazutti, N. Carniel, W.L. Priamo, Supercritical CO_2 extraction of black poplar (*Populus nigra* L.) extract: Experimental data and fitting of kinetic parameters, Journal of Supercritical Fluids, 117 (2016) 270-278.
[11] E.K. Silva, G.L. Zabot, M.A. Bargas, M.A.A. Meireles, Microencapsulation of lipophilic bioactive compounds using prebiotic carbohydrates: Effect of the degree of inulin polymerization, Carbohydrate Polymers, 152 (2016) 775-783.
[12] Y. Gong, X. Wan, M. Jiang, C. Hu, H. Hu, F. Huang, Metabolic engineering of microorganisms to produce omega-3 very long-chain polyunsaturated fatty acids, Progress in Lipid Research, 56 (2014) 19-35.
[13] D. Jacomini, R.C. Sinzker, C.A. Mangolin, P.A. Grande, S.R. Nocchi, C.V. Nakamura, A.J.B.d. Oliveira, R.A.C. Gonçalves, Lipid profile and antiproliferative activity of callus cultures of *Cereus peruvianus* Mill, Industrial Crops and Products, 69 (2015) 408-414.
[14] M. Demir, I. Turhan, A. Kucukcetin, Z. Alpkent, Oil production by *Mortierella isabellina* from whey treated with lactase, Bioresource Technology, 128 (2013) 365-369.
[15] S. Fakas, A. Makri, M. Mavromati, M. Tselepi, G. Aggelis, Fatty acid composition in lipid fractions lengthwise the mycelium of *Mortierella isabellina* and lipid production by solid state fermentation, Bioresource Technology, 100 (2009) 6118-6120.
[16] A. Zinnai, C. Sanmartin, I. Taglieri, G. Andrich, Supercritical fluid extraction from microalgae with high content of LC-PUFAs. A case of study: Sc-CO_2 oil extraction from *Schizochytrium* sp, Journal of Supercritical Fluids, 116 (2016) 126-131.
[17] A. Nisha, K.U. Sankar, G. Venkateswaran, Supercritical CO_2 extraction of *Mortierella alpina* single cell oil: Comparison with organic solvent extraction, Food Chemistry, 133 (2012) 220-226.
[18] G.L. Zabot, M.A.A. Meireles, On-line process for pressurized ethanol extraction of onion peels extract and particle formation using supercritical antisolvent, Journal of Supercritical Fluids, 110 (2016) 230-239.
[19] G. Brunner, Gas Extraction: An Introduction to Fundamentals ofSupercritical Fluids and the Application to Separation Processes, Springer, Darmstadt, New York, USA, 1994.

[20] J.F. Soares, V.D. Prá, M. Souza, F.C. Lunelli, E. Abaide, J.R.F. Silva, R.C. Kuhn, J. Martínez, M.A. Mazutti, Extraction of rice bran oil using supercritical CO_2 and compressed liquefied petroleum gas, Journal of Food Engineering, 170 (2016) 58-63.
[21] V.D. Prá, J.F. Soares, D.L. Monego, R.G. Vendruscolo, D.M.G. Freire, M. Alexandri, A. Koutinas, R. Wagner, M.A. Mazutti, M.B.d. Rosa, Extraction of bioactive compounds from palm (*Elaeis guineensis*) pressed fiber using different compressed fluids, Journal of Supercritical Fluids, 112 (2016) 51-56.
[22] M.C. Ribas, D. Mantovani, J.A. Awadallak, R.L. Canevesi, N.M. Tazinafo, L. Cardozo-Filho, F. Palú, E.A. Silva, Study of candeia oil extraction using pressurized fluids and purification by adsorption process, Journal of Supercritical Fluids, 92 (2014) 177-182.
[23] M.A.A. Meireles, Extraction of bioactive compounds from Latin American plants, in: J. Martinez (Ed.) Supercritical fluid extraction of nutraceuticals and bioactive compounds, CRC Press – Taylor & Francis Group, Boca Raton, 2008, pp. 243-274.
[24] J.V. Visentainer, Analytical aspects of the flame ionization detector response of fatty acid esters in biodiesels and foods, Química Nova, 35 (2012) 274-279.
[25] NIST - Chemistry WebBook. Accessed in: 19 sep. 2016 Available in: http://webbook.nist.gov/chemistry/.
[26] S. Pereda, S.B. Bottini, E.A. Brignole, Fundamentals of supercritical fluid technology, in: J.L. Martínez (Ed.) Supercritical Fluid Extraction of Nutraceuticals and Bioactive Compounds, CRC Press, Taylor and Francis Group, Boca Raton, USA, 2008.
[27] P.I.N. Carvalho, J.F. Osorio-Tobón, M.A. Rostagno, A.J. Petenate, M.A.A. Meireles, Techno-economic evaluation of the extraction of turmeric (*Curcuma longa* L.) oil and ar-turmerone using supercritical carbon dioxide, Journal of Supercritical Fluids, 105 (2015) 44-54.
[28] S.J. Park, T.Y. Kwak, G.A. Mansoori, Statistical mechanical description of supercritical fluid extraction and retrograde condensation, International Journal of Thermophysics, 8 (1987) 449-471.
[29] G.L. Zabot, M.N. Moraes, A.J. Petenate, M.A.A. Meireles, Influence of the bed geometry on the kinetics of the extraction of clove bud oil with supercritical CO_2, Journal of Supercritical Fluids, 93 (2014) 56-66.
[30] M.N. Moraes, G.L. Zabot, M.A.A. Meireles, Extraction of tocotrienols from annatto seeds by a pseudo continuously operated SFE process integrated with low-pressure solvent extraction for bixin production, Journal of Supercritical Fluids, 96 (2015) 262-271.
[31] G.L. Zabot, M.N. Moraes, P.I.N. Carvalho, M.A.A. Meireles, New proposal for extracting rosemary compounds: Process intensification and economic evaluation, Industrial Crops and Products, 77 (2015) 758-771.
[32] N. Mezzomo, J. Martínez, S.R.S. Ferreira, Supercritical fluid extraction of peach (*Prunus persica*) almond oil: Kinetics, mathematical modeling and scale-up, Journal of Supercritical Fluids, 51 (2009) 10-16.
[33] G.L. Zabot, M.N. Moraes, M.A.A. Meireles, Influence of the bed geometry on the kinetics of rosemary compounds extraction with supercritical CO_2, Journal of Supercritical Fluids, 94 (2014) 234-244.
[34] J. Hussain, Z. Ruan, I.A. Nascimento, Y. Liu, W. Liao, Lipid profiling and corresponding biodiesel quality of *Mortierella isabellina* using different drying and extraction methods, Bioresource Technology, 169 (2014) 768-772.
[35] K.A. Youdim, A. Martin, J.A. Joseph, Essential fatty acids and the brain: possible health implications, International Journal of Developmental Neuroscience, 18 (2000) 383-399.
[36] M. Xian, J. Yan, Y. Kang, J. Liu, Y. Bi, K. Zhen, Production of c-linolenic acid by *Mortierella isabellina* grown on hexadecanol, Letters in Applied Microbiology, 33 (2001) 367-370.
[37] Z. Ruan, M. Zanotti, X. Wang, C. Ducey, Y. Liu, Evaluation of lipid accumulation from lignocellulosic sugars by *Mortierella isabellina* for biodiesel production, Bioresource Technology, 110 (2012) 198-205.
[38] X. Yu, T. Dong, Y. Zheng, C. Miao, S. Chen, Investigations on cell disruption of oleaginous microorganisms: Hydrochloric acid digestion is an effective method for lipid extraction, European Journal of Lipid Science and Technology, 117 (2015) 730-737.

5. ARTIGO 2

Ultrasound-assisted extraction of fatty acids from *Mortierella isabellina*

Abstract

This work is focused in the optimization of process conditions that promote high extraction yields of fatty acids from freeze-dried cells of *Mortierella isabellina* produced by submerged fermentation. The influence of ultrasound intensity and pulse cycle were investigated for the extraction of fatty acids. Ultrasound intensity of 75.11 $W.cm^{-2}$ and pulse factor of 0.93 were the optimized conditions, yielding 14.46 wt% of fatty acids using ethanol and 19.49 wt% fatty acids using chloroform:methanol:water as solvents. Estearic, *cis*-10-pentadecenoic, *cis*-10-heptadecenoic, oleic, arachidic, linoleic, α-linolenic and palmitic acids were the major fatty acids extracted using both solvents. These results highlight the possibility of using ultrasound to break the cells for increasing the fatty acids extraction from a lipids-rich fungal biomass.

1. Introduction

Several microorganisms have been used for the production of lipids, mainly *Mortierella isabellina* fungus for the production of lipids containing polyunsaturated fatty acids (PUFA) (DEMIR et al., 2013). The PUFA can belong to the omega-3 or omega-6 family, depending on the position of the first double bond, counting from the methyl group located at the end of the fatty acid molecule (GIUDETTI and CAGNAZZO, 2012). These fatty acids are identified as potential food additives or pharmaceuticals because of their biological activities (JANG et al., 2005). The fatty acids have important benefits for health, positive influence on the brain system functions (BRADBURY, 2011), cardiovascular system (JUMP and DEPNER, 2012) and on some types of cancer (BOUGNOUX et al., 2010). The PUFA (n-3 and n-6) are considered essential fatty acids (EFA). Therefore, they are necessary in human health and they are not synthesized by the body. However, they must be obtained from dietary sources (DYAL and NARINE, 2005). The oleaginous fungus *Mortierella isabellina* has been extensively studied for single cell oil (SCO) production since it is able of accumulating a considerable amount of lipids (CHATZIFRAGKOU et al., 2010).

Lipids produced from *Mortierella isabelina* fungus are intracellular. Then, a unitary operation such as extraction is need to remove the PUFA from the fungi cells. Several solvents have been intensively studied for the lipids extraction, such as, hexane and diethylether (FAKAS et al., 2009); methanol and chloroform (HARDE et al., 2016); Bligh & Dyer method with a modified methanol:chloroform:water ratio of 2:1:0.8 (v/v/v); hexane and isopropanol (HALIM et al., 2012); dichloromethane and methanol (GUCKERT et al., 1988); hexane extraction (HUSSAIN et al., 2014); petroleum ether in a Soxhlet apparatus (XING et al., 2012); supercritical CO_2 and compressed liquefied petroleum gas (SALLET et al., 2017) and ultrasound-assisted extraction (UAE) (ZHOU et al., 2013). Some of these treatments are more economical, but most of these methods are either not effective or difficult to scale up (GECIOVA et al., 2002). More recently, ultrasound-assisted extraction as one of disruption methods has received attention in the literature for algal cell disruption.

Ultrasound is able to disrupt cells with less energy loss compared with high-shear force methods (CHEMAT et al., 2011; WANG et al., 2014), and also intensify the extraction process due to a cavitation phenomenon. Ultrasonic waves produce bubbles in the solvent, the bubbles burst near the cell walls, which make shock waves, causing the release of lipid in the solvent (WEI et al., 2008). Furthemore, applications of ultrasound generally involve processes that can increase rates, improve quality and/or safety, and reduce processing time (SICAIRE et al., 2016). Considering the ultrasound extraction of lipids from *Mortierella isabellina*, solely the work of Zhou et al., (2013) evaluated the ultrasound-assisted extraction using hydrochloric acid in extraction of single cell oil, no other studied using ultrasound-assisted extraction from cells of the *Mortierella isabellina* has been reported. Therefore, the novelty of this study was to apply the ultrasound extraction for improving the lipids yield and decreasing the time for extraction from cells.

Based in this context, the purpose of this study was to evaluate the ultrasound extraction of fatty acids from freeze-dried cells of *Mortierella isabellina* produced by submerged fermentation. The influence of ultrasound (power and pulse cicle) and the solvent: ethanol and mixture of chloroform:methanol:water were evaluated. In order to provide the more suitable condition, the extraction yields and fatty acids composition were evaluated.

2. Material and methods

2.1. Materials

The *Mortierella isabellina* cells were purchased from Tropical Culture Collection André Tosello (Campinas, Brazil). The culture was maintained in potato dextrose agar (PDA) at 4ºC and subcutured every month. Ethanol (99.8%) was purchased from Alphatec (Brazil), chloroform (P.A) and methanol (P.A) were purchased from Dinâmica Contemporary Dynamics Chemistry Ltda (Brazil) and hexane was supplied by Sigma-Aldrich (Brazil).

2.2. Fermentation

The fatty acids were produced in submerged fermentation according with conditions of Sallet et al. (2017).

2.3. Experimental apparatus and procedure for the extractions

The experimental apparatus was composed of a jacketed reactor (250 mL capacity) connected to a thermostatic water bath (temperature accuracy of ± 1.0 °C) for temperature control, a high-intensity ultrasound processor of 400 W and frequency of 24 kHz (Hielscher, Model UP 400S). The ultrasound was equipped with a titanium probe (Model H22, Tip 22) presenting a maximum ultrasound intensity of 85 $W.cm^{-2}$. For the extractions, the ultrasonic probe was placed at the center of the jacketed reactor containing 2.5 g of cells and 100 mL of ethanol.

In the extractions using chloroform:methanol:water, the ultrasonic probe was placed at the center of the jacketed reactor containing 2.5 g of cells and 26 mL of chloroform, 53 mL of methanol and 21 mL of water. Afterwards, the temperature was adjusted to 10 °C ± 2 °C by circulating water through the jacket. All extractions were carried out for 30 min (defined according experimental assays, data not showed) at specified ultrasound power and pulse cycle. After this, the samples were centrifuged at 4500 rpm for 5 min. The liquid phase was carefully collected and the solvents were evaporated at 40 ºC under vacuum. Besides, extractions without the use of ultrasound were done for each solvent. Where the same conditions (biomass, amount of solvent and time) were used.

The effects of ultrasound intensity (17 - 85 $W.cm^{-2}$) and pulse cycle (0.5-1.0) in extraction yield of cells were evaluated through a central composite rotational design (CCRD). Pulse cycle is related to the time that ultrasound is on. After analysing the results of CCRD, three additional assays were carried out to validate the results.

2.4. Determination of fatty acids in the extracts

An amount of 10 µL of each sample of the extract was solubilized in 1 mL of n-hexane and 250 µL of a 4 mg/mL solution of methyl tridecanoate (C23:0 Me) in isooctane was added to each fraction. Solvents were evaporated at 40°C under vacuum. Fatty acids methyl esters (FAME) derivatizations were performed according to the method described by Visentainer (2012) . The FAME were analyzed in the GC system (Shimadzu, GCMS-QP2010 Ultra, Japan) by injecting 1 µL into a capillary column Ptx-Wax (Restek-USA) (30 m × 0.25 mm × 0.25 µm). The carrier gas (Helium, purity > 99%, White Martins, Santa Maria, Brazil) flowed at a constant pressure of 103.4 kPa. The column temperature gradient was 50°C; 3°C/min up to 140°C (10 min). The injector was maintained at 140°C and the split ratio was 60:1. The detector was maintained at 140°C. The compositions were expressed as mg of fatty acid per g of extract.

3. Results and discussion

3.1 Ultrasound-assisted extractions

Table 1 shows the extraction yields obtained in the eleven runs of the CCRD, which ranged from 2.92 wt% (run 1) to 14.47 wt% (run 4) using ethanol and ranged from 14.08 wt% (run 5) to 19.49 wt% (run 4) using mixture of the solvents (chloroform:methanol:water). When compared the results from ultrasound extraction with ethanol and mixture of solvents, the results using the mixture were better, probably because the presence of non-polar/polar organic solvent (chloroform:methanol) is able to completely extract both neutral and polar lipids from biomass (HUSSAIN et al., 2014). Comparing the assay 4 and 12, the second without ultrasound-assisted the extraction improved approximately 900 % the lipid yield using ethanol and approximately 40 % using the mixture of solvents. The main reason for this result is the enhancement of the mass transfer in the system when ultrasound is employed. The cavitation was an important phenomenon which enables the extraction of strongly linked compounds and consequently increases the yield. Analyzing Table 1, the yield of lipid was strongly improved

for the ultrasound intensity. The best results were observed in the assays 4 and 6 for ethanol and mixture of solvents.

Table 1- Experimental design (CCRD) for ultrasoun-assisted extraction of biomass from *Mortierella isabellina*

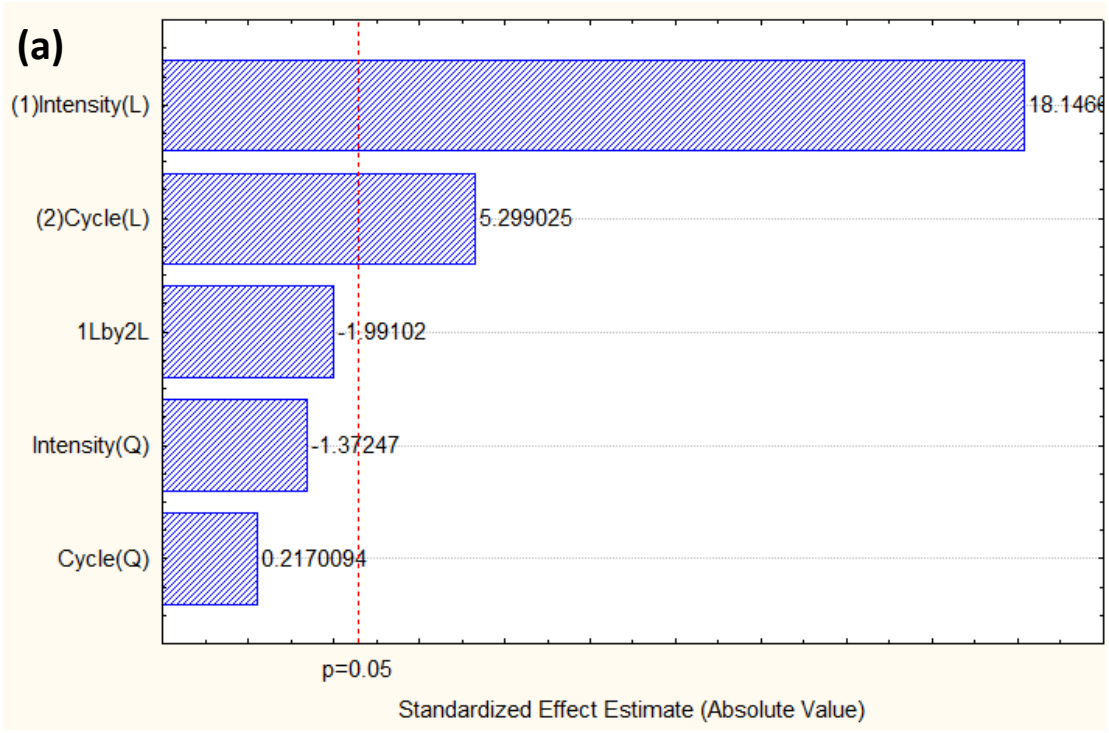
Assay	Ultrasound intensity ($W.cm^{-2}$)	Pulse cycle (-)	Yield[a] (wt%)	Yield[b] (wt%)
1	26.89 (-1)	0.57 (-1)	2.92	15.38
2	75.11 (1)	0.57 (-1)	13.04	19.08
3	26.89 (-1)	0.93 (1)	6.90	16.40
4	75.11 (1)	0.93 (1)	14.47	19.49
5	17 (-1.41)	0.75 (0)	3.70	14.08
6	85 (1.41)	0.75 (0)	14.43	18.90
7	51 (0)	0.50 (-1.41)	8.44	17.36
8	51 (0)	1.0 (1.41)	11.40	18.65
9	51 (0)	0.75 (0)	9.95	17.99
10	51 (0)	0.75 (0)	8.91	18.16
11	51 (0)	0.75 (0)	10.31	18.13
12*	0	0	1.45	13.92

[a] Extraction using ethanol

[b] Extraction using chloroform:methanol:water

[*] Extraction without ultrasound

Data from Table 1 were used to compute linear, quadratic and interaction between process variables on lipid yield. The results were expressed in the form of Pareto chart in Fig. 1. The linear terms for pulse cycle and ultrasound intensity were statistically significant ($p < 0.05$) using ethanol as solvent. In the mixture of solvents, the linear terms for pulse cycle and ultrasound intensity, as well as quadratic term for pulse cycle were statistically significant ($p < 0.05$). Ultrasound intensity presented a positive effect, indicating that its increase can lead to higher yields for both solvents. By comparing runs 1–2 and 3–4 (pulse cycle is maintained constant at levels −1 and +1, respectively), the highest yields were obtained at the highest ultrasound intensity. The quadratic term for the ultrasound intensity present negative effect on the lipid yield using mixture of solvents, the negative signs of the quadratic term indicate the presence of a maximum point for ultrasound intensity in the evaluated range.



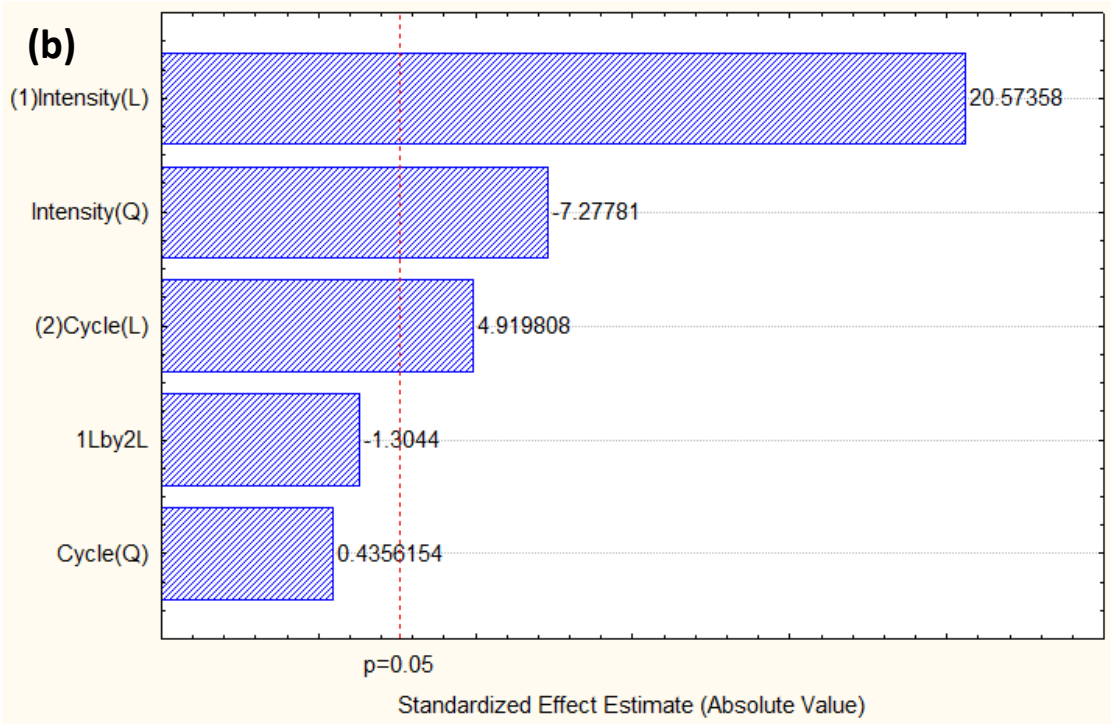


Fig. 1. Pareto chart expressing the effect of process variables on the extraction yield using ethanol (a) and mixture of solvents (b).

In order to better understand the influence of ultrasound intensity on the extraction yield, data from Table 1 were used to estimate the terms of a linear and quadratic model for extraction yield, which is presented in Eq. (1) (ethanol) and Eq. (2) (mixture of the solvents), considering the significant terms ($p < 0.05$).

$$Y = 9.50 + 4.11.I + 1.20.C \quad (1)$$

$$Y = 18.13 + 1.70.I - 0.73.I^2 + 0.41.C \quad (2)$$

where Y is the extraction yield (wt%), I is the coded ultrasound intensity, and C is the coded pulse cycle. The aforementioned models were statistically validated by the analysis of variance (ANOVA, Table 2). The calculated F-test to ethanol was about 28 times greater than the tabulated one and the determination coefficient (R^2) was 0.9695. For mixture of solvents, the calculated F-test was about 39 times greater than the tabulated one and the determination coefficient (R^2) was 0.9866.

Table 2. ANOVA for yield extraction using ethanol and mixture of solvents.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F Test	R^2
Ethanol					
Regression	146.55	2	73.28	126.34[a]	0.9695
Residual	4.62	8	0.58		
Total	151.17	10			
Mixture					
Regression	27.74	3	9.25	171.30[b]	0.9866
Residual	0.38	7	0.054		
Total	28.12	10			

[a] $F_{0.05;2;8} = 4.46$
[b] $F_{0.05;3;7} = 4.35$

Fig. 2 show the influence of ultrasound intensity and pulse cycle on the extraction yield. Maximum extraction yield was obtained for ultrasound intensity ranging from 75.11 to 85 $W.cm^{-2}$ and pulse cycle from 0.75 to 1.00 to ethanol and ranging from 60 to 85 $W.cm^{-2}$ and pulse cycle from 0.60 to 1.0 to mixture of the solvents.

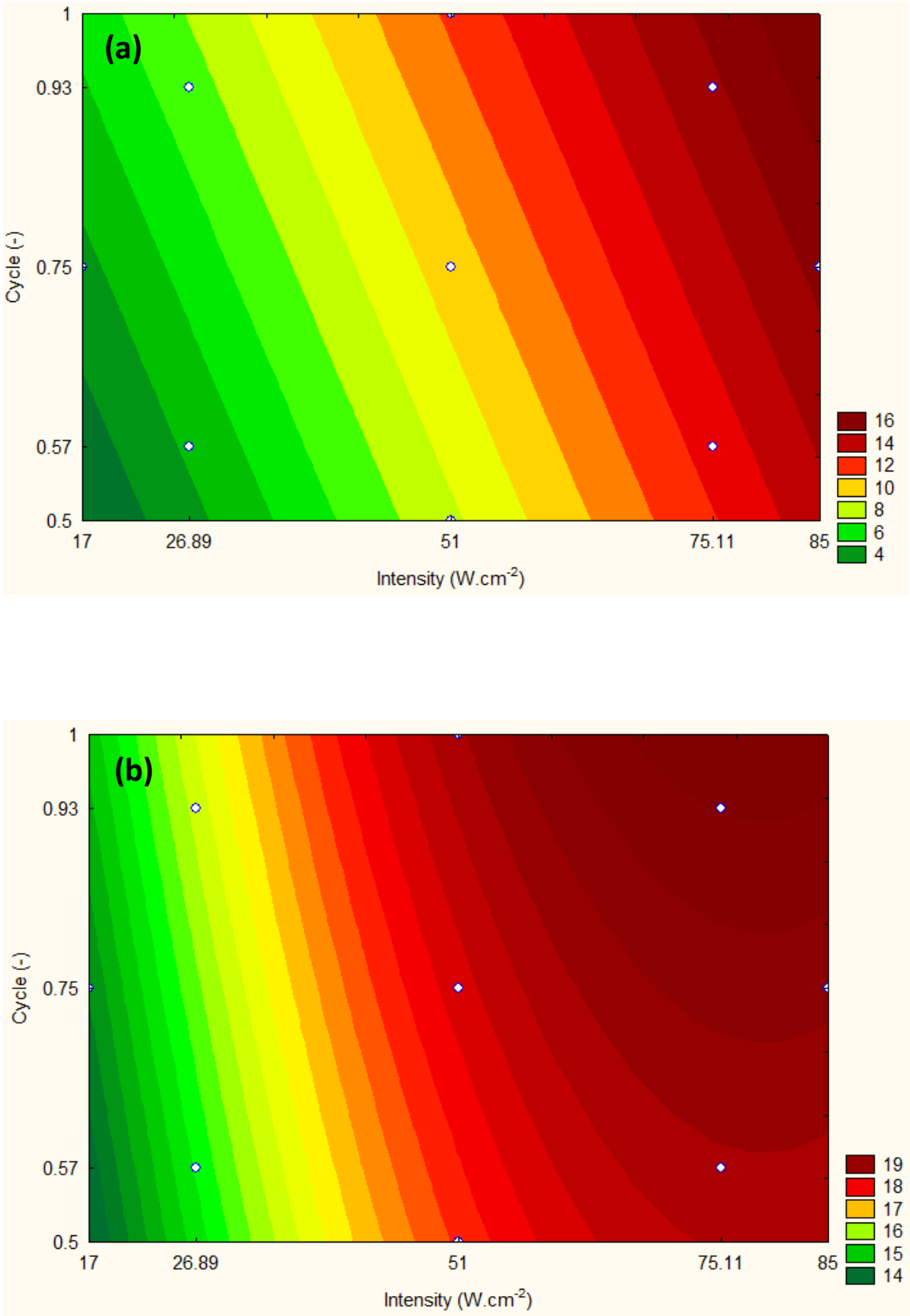


Fig. 2. Contour plots expressing the influence of process variables on the extraction yield using ethanol (a) and mixture of solvents (b).

From Fig. 2, the range for both variables that lead to maximum extraction yield were predicted. However, the process optimization can be achieved after validation of the predicted results. Therefore, three additional experiments were carried out at same conditions. This condition was estimated at the maximum intensity (85 $W.cm^{-2}$) and the maximum pulse cycle (1) of the equipment according to the contour plots of the CCRD. In these conditions, the yield of lipids using ethanol as solvent was 14.69±0.32 and for mixture of solvents was 20.13±0.60. These results are in agreement with data from CCRD, mainly assay 4. However, it is not necessary used higher conditions of intensity (85 $w.cm^{-2}$) and pulse cycle (1) for better results. The optimized conditions for ultrasound-assisted extraction of cells from *Mortierella isabellina* are range from 75-85 $W.cm^{-2}$ and pulse cycle of 0.9-1.0.

The lipids yield from *Mortierella isabellina* obtained in this work were better than the maximum extraction yield achieved with compressed liquefied petroleum gas (40 ºC/20 bar – 4.45 wt%) and supercritical CO_2 extraction (80 °C/250 bar – 3.21 wt%) (SALLET et al., 2017). In the study of Hussain et al., (2014) yields of 40.8 % from freeze-dried fungal biomass, when methanol:chloroform:water ratio of 2:1:0.8 was used. Zhou et al. (2013) obtained an extraction rate of 90.63% of lipids from *Mortierella isabellina* using ultrasound and hydrochloric acid at 53.42 ºC. When comparing the extraction yields, it is important to take into account the culture, the fermentation conditions and the solvent used for extraction.

3.2. Chemical characterization

Tables 3 and 4 shows the data from fatty acids profile obtained at runs 1–11 from CCRD and 12* (without ultrasound). Fourteen fatty acids were identified in the oil. The major fatty acids identified were the estearic acid (C18:0), *cis*-10-pentadecenoic acid (C15:1), *cis*-10-heptadecenoic acid (C17:1), oleic acid (C18:1n9c), tricosanoic acid (C23:0), arachidic acid (C20:0), linoleic acid (C18:2n6c), α-linolenic acid (C18:3n3) and palmitic acid (C16:0). These similar fatty acids were also identified in the lipid fractions of *Mortierella isabellina* mycelia by Sallet et al. (2017) and Fakas et al. (2009).

Table 3- Fatty acids profile of lipids obtained with ultrasound-assisted extraction using ethanol.

	ETHANOL											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12***
C13:0	4.55	3.28	3.48	3.48	4.02	3.24	4.01	3.57	4.04	3.83	3.92	4.78
C14:1	3.13	1.91	2.17	1.92	2.49	1.79	2.32	2.03	2.25	2.22	2.25	3.03
C15:1	123.51	90.17	100.82	98.79	107.62	91.12	109.84	100.30	110.89	108.18	108.52	126.10
C16:0	11.68	5.53	10.84	6.92	15.99	6.07	12.18	6.88	9.61	10.30	8.94	7.53
C17:1	29.02	21.66	25.88	24.12	26.30	22.35	26.45	24.01	26.94	26.34	26.05	31.34
C18:0	249.27	162.82	196.66	183.00	219.35	168.76	215.03	181.55	209.22	205.08	203.46	248.79
C18:1n9c	24.70	14.18	17.01	15.24	20.65	14.55	19.87	16.30	18.56	18.47	18.49	21.08
C18:2n6c	15.08	8.83	9.96	9.67	12.56	9.43	12.79	10.42	11.98	11.84	12.11	12.21
C18:3n3	4.35	3.35	4.19	3.75	4.23	3.51	4.10	3.68	4.19	4.12	3.96	4.99
C20:0	14.31	8.31	10.35	9.31	11.88	9.04	10.67	9.22	10.23	10.51	10.41	13.71
C22:0	4.39	1.65	3.26	2.04	8.88	1.71	4.92	2.37	3.03	3.20	2.74	1.74
C23:0	37.56	4.70	9.36	4.40	16.44	4.27	7.46	5.52	6.01	7.99	6.91	26.42
C24:0	3.56	3.00	3.77	3.51	3.72	3.21	3.70	3.54	3.82	3.74	3.60	3.81
C22:6	1.57	1.46	1.97	1.64	1.71	1.59	1.95	1.65	1.81	1.88	1.94	1.57
C24:1	0.00	4.70	3.06	5.44	0.09	0.66	2.18	4.53	5.47	3.53	5.98	0.00
SFA	287.76	184.59	228.36	208.26	263.84	192.03	250.51	207.13	239.95	236.66	233.07	280.36
MUFA	180.36	132.62	148.94	145.51	157.15	130.47	160.66	147.17	164.11	158.74	161.29	181.55
PUFA	21	13.64	16.12	15.06	18.5	14.53	18.84	15.75	17.98	17.84	18.01	18.77
TOTAL[a]	489.12	330.85	393.42	368.83	439.49	337.03	430.01	370.05	422.04	413.24	412.37	480.68

C13:0 – (Tridecanoic acid); C14:1 – (Myristoleic Acid); C15:1 – (cis-10-Pentadecanoic acid); C16:0 – (Palmitic acid); C17:1 – (cis-10-heptadecanoic acid); C18:0 – (Estearic acid); C18:1n9c – (Oleic acid); C18:2n6c – (Linoleic acid); C18:3n3 – (linolenic acid); C20:0 – (Arachidic acid); C22:0 – (Behenic acid); C22:6 – (Docosahexaenoic acid); C23:0 – (Tricosanoic acid); C24:0 – (Lignoceric acid); C24:1–(Nervonic acid); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

[a] Means mg/g oil.

12* Means a condition without using ultrasound.

Table 4 - Fatty acids profile of lipids obtained with ultrasound-assisted extraction using mixture of solvents (chloroform:methanol:water).

	CHLOROFORM:METHANOL:WATER											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12***
C13:0	2.53	1.01	1.43	1.07	1.23	1.04	1.44	1.28	1.66	1.58	1.74	2.35
C14:1	1.77	0.73	1.00	0.77	0.87	0.75	1.02	0.91	1.09	1.18	1.17	2.29
C15:1	71.89	30.65	39.55	33.47	36.26	32.26	43.31	38.74	50.12	49.80	50.89	72.83
C16:0	3.63	1.32	2.19	1.80	2.07	1.66	2.44	2.04	2.72	2.79	2.87	3.63
C17:1	16.53	7.88	10.28	8.23	9.69	7.81	10.20	10.21	12.62	12.26	11.88	16.24
C18:0	121.32	50.56	62.24	58.05	65.92	58.31	75.92	64.69	91.51	87.09	88.80	116.09
C18:1n9c	8.81	2.95	3.59	4.46	5.49	4.86	6.50	4.27	7.49	6.63	7.38	10.66
C18:2n6c	3.97	1.15	1.30	2.31	2.84	2.73	3.83	1.80	4.30	3.48	4.23	5.68
C18:3n3	2.19	1.16	1.33	1.10	1.31	1.15	1.39	1.60	1.80	1.72	1.70	2.26
C20:0	8.29	3,46	3.69	2.59	3.23	2.78	4.22	2.90	4.89	4.88	4.29	6.60
C22:0	0.82	0.48	0.43	0.40	0.56	0.42	0.57	0.78	0.73	0.64	0.68	1.01
C23:0	26.13	25.11	45.36	31.73	48.56	28.70	32.27	49.56	35.09	47.13	26.06	26.42
C24:0	1.72	0.85	1.12	1.01	1.19	0.95	1.15	1.25	1.56	1.53	2.50	1.69
C22:6	1.28	0.34	0.00	0.33	0.39	0.38	0.38	0.51	0.60	0.54	0.50	0.59
C24:1	0.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51
SFA	138.31	57.68	71.10	64.92	74.20	65.16	85.74	72.94	103.07	98.51	100.88	131.37
MUFA	99.36	42.21	54.42	46.93	52.31	45.68	61.03	54.13	71.32	69.87	71.32	102.53
PUFA	7.44	2.65	2.63	3.74	4.54	4.26	5.60	3.91	6.70	5.74	6.43	8.53
TOTAL[a]	245.11	102.54	128.15	115.59	131.05	115.10	152.37	130.98	181.09	174.12	178.63	242.43

C13:0 – (Tridecanoic acid); C14:1 – (Myristoleic Acid); C15:1 – (cis-10-Pentadecanoic acid); C16:0 – (Palmitic acid); C17:1 – (cis-10-heptadecanoic acid); C18:0 – (Estearic acid); C18:1n9c – (Oleic acid); C18:2n6c – (Linoleic acid); C18:3n3 – (linolenic acid); C20:0 – (Arachidic acid); C22:0 – (Behenic acid); C22:6 – (Docosahexaenoic acid); C23:0 – (Tricosanoic acid); C24:0 – (Lignoceric acid); C24:1–(Nervonic acid); SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

[a] Means mg/g oil.

12[*] Means a condition without using ultrasound.

Differences in the concentration of fatty acids as a function of the solvent used and the ultrasound power and pulse cycle applied can be observed (Tables 3 and 4). The extraction of fatty acids using ethanol was better than with the mixture of solvents. These findings indicate the ethanol was most selective for extracting higher amount of fatty acids, while the mixture (chloroform:methanol:water) enabled the extraction of other compounds together with the fatty acids identified, thus enhancing the total oil yields.

The concentrations of fatty acids using ethanol were higher than those obtained in the work reported by Sallet et al. (2017) using non-polar solvents, as CO_2 and liquefied petroleum gas (LPG). This result can be explained by the highest solubility of *Mortierella isabellina* cells in ethanol in comparison with LPG and CO_2. Some researches verified that ultrasonic treatment can improve the yield and rate of extraction and increase the contact surface area between solid and liquid phase (VILKHU et al., 2008). Consequentially, the ultrasonic treatment can increase the oil extraction from microbial cell (ZHOU et al., 2013).

Analyzing Table 3, the highest total concentration of fatty acids was obtained at 26.89 $W.cm^{-2}$ ultrasound intensity and 0.57 pulse cycle (approximately 490 mg/g oil), while the lowest total concentration of fatty acids was obtained at 75.11 W cm^{-2} ultrasound intensity and 0.57 pulse cycle using chloroform:methanol:water (approximately 103 mg/g oil). Zhou et al. (2013) reported a mean value of 109.88 mg/g of single cell oil using ultrasound-assisted extraction using hydrochloric acid. In the assay 3 from Table 4 (assay with highest yield and mass of oil solubilized in the solvent), the total concentration of fatty acids was 115.59 mg/g oil when using chloroform:methanol:water solvent in the extraction. The ultrasound extraction of PUFA with ethanol was higher (21 mg/g oil) than extraction using the mixture of solvents (8.53 mg/g oil).

4. Conclusions

The ultrasound-assisted extraction of PUFA from *Mortierella isabelina* was studied and the best conditions were ultrasound intensity of 75.11 W cm^{-2} and pulse factor of 0.93, yielding 14.47 wt% oil using ethanol and 19.49 wt% oil using mixture of solvents (chloroform:methanol:water). The ultrasound-assisted extraction with ethanol was advantageous for higher concentration of fatty acids in the oil. Estearic acid, *cis*-10-pentadecenoic acid, *cis*-10-heptadecenoic acid, oleic acid, arachidic acid, linoleic acid, α-linolenic acid and palmitic acid were the main fatty acids identified. The fraction of

polyunsaturated fatty acids was mostly composed of linoleic and linolenic acids. Ultrasound-assisted extraction is an efficient method for lipid extraction from fungal cells. This methodology decrease solvent consumption, reduce time extraction, improved yield. Therefore, the technology is promising for commercial application.

References

BOUGNOUX, P.; HAJJAJI, N.; MAHEO, K.; COUET, C.; CHEVALIER, S.Fatty acids and breast cancer: Sensitization to treatments and prevention of metastatic re-growth. **Progress in Lipid Research,** v. 49, p. 76-86, 2010.

BRADBURY, J.; Docosahexaenoic Acid (DHA): An Ancient Nutrient for the Modern Human Brain. Nutrients, v. 3, p. 529-544, 2011.

CHATZIFRAGKOU, A.; FAKAS, S.; GALIOTOU-PANAYOTOU, M.; KOMAITIS, M.; AGGELIS, G.; PAPANIKOLAOU, S. Commercial sugars as substrates for lipid accumulationin *Cunninghamella echinulata* and *Mortierella isabellina* fungi. **European Journal of Lipid Science and Technology,** v. 112, p. 1048–1057, 2010.

CHEMAT, F.; HUMA, Z.; KHAN, M.K. Applications of ultrasound in food technology: Processing, preservation and extraction. **Ultrasonics Sonochemistry**, v. 18, p. 813-835, 2011.

DEMIR, M.; TURHAN, I.; KUCUKCETIN, A.; ALPKENT, Z. Oil production by *Mortierella isabellina* from whey treated with lactase, **Bioresource Technology,** v. 128, p. 365–369, 2013.

DYAL, S. D.; NARINE, S. S. Implications for the use of *Mortierella* fungi in the industrial production of essential fatty acid. **Food Research International**, v. 38, p. 445-467, 2005.

FAKAS, S.; MAKRI, A.; MAVROMATI, M.; TSELEPI, M.; AGGELIS, G. Fatty acid composition in lipid fractions lengthwise the mycelium of *Mortierella isabellina* and lipid production by solid state fermentation. **Bioresource Technology.** v. 100, p. 6118-6120, 2009.

GECIOVA, J.; BURY, D.; JELEN, P. Methods for disruption of microbial cells for potential use in the dairy industry – a review. **International Dairy Journal,** v. 12, p.541-553, 2002.

GIUDETTI, A. M.; CAGNAZZO, R. Beneficial effects of n-3 PUFA on chronic airway inflammatory diseases. **Prostaglandins and Other Lipid Mediators,** v. 99, p. 57–67, 2012.

HARDE, S. M.; WANG, Z.; HORNE, M.; ZHU, J. Y.; PAN, X. Microbial lipid production from SPORL-pretreated Douglas fir by *Mortierella isabelina*. **Fuel.** v. 175, p. 64-74, 2016.

HUSSAIN, J.; RUAN, Z.; NASCIMENTO, I. A.; LIU, Y.; LIAO, W. Lipid profiling and corresponding biodiesel quality of *Mortierella isabellina* using different drying and extraction methods. **Bioresource Technology,** v. 169, p. 768-772, 2014.

JANG, H.; LIN, Y.; YANG, S. Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*. **Bioresource Technology,** v. 96, p. 1633-1644, 2005.

JUMP, D. B.; DEPNER, C. M.; TRIPATHY, S. Omega-3 fatty acid supplementation and cardiovascular disease. **Journal of Lipid Research**, v. 53, p. 2525-2545, 2012.

SALLET, D.; ABAIDE, E.; MARCUZ, C.; ARIOTTI, G.; DAL PRÁ, V.; UGALDE, G.; ZABOT, G. L.; MAZUTTI, M. A.; KUHN, R.C. Obtaining fatty acids from *Mortierella*

isabellina using supercritical carbon dioxide and compressed liquefied petroleum gas. **The Journal of Supercritical Fluids**, v. 122, p. 79-87, 2017.

SICAIRE, A.; VIAN, M. A.; FINE, F.; CARRÉ, P.; TOSTAIN, S.; CHEMAT, F. Ultrasound induced green solvente extraction of oil from oleaginous seeds. **Ultrasonics Sonochemistry,** v. 31, p. 319-329, 2016.

VILKHU, K.; MAWSON, R.; SIMONS, L.; BATES, D. Applications and opportunities for ultrasound assisted extraction in the food industry – A review. **Innovative Food Science & Emerging Technologies,** v. 9, p. 161-169, 2008.

VISENTAINER, J. V. Analytical aspects of the flame ionization detector response of fatty acid esters in biodiesels and foods, **Quim. Nova,** v. 35, p. 274–279, 2012.

XING, D.; WANG, H.; PAN, A.; WANG, J.; XUE, D. Assimilation of corn fiber hydrolysates and lipid accumulation by *Mortierella isabelina*. **Biomass and Bioenergy,** v. 39, p. 494-501, 2012.

WANG, M.; YUAN, W.; JIANG, X.; JING, Y.; WANG, Z. Disruption of microalgal cells using high-frequency focused ultrasound. **Bioresource Technology**, v. 153, p. 315-321, 2014.

WEI, F.; GAO, G.; WANGA, X.; DONG, X.; LI, P.; HUA, W.; WANG, X.; WU, X.; Chen, H. Quantitative determination of oil content in small quantity of oilseed rape by ultrasound-assisted extraction combined with gas chromatography, **Ultrason Sonochem,** v. 15, p. 938–942, 2008.

ZHOU, C.; ZHU, C.; REN, Y. Optimization of Ultrasound-Assisted Extraction of Single Cell Oil from *Mortierella isabelina*. **Taylor & Francis Group,** v. 48, p. 2188-2195, 2013.

5. DISCUSSÃO

Neste trabalho foram avaliadas diferentes metodologias para extração de ácidos graxos obtidos por via biotecnológica. A tecnologia de extração de lipídeos com fluido supercrítico tem recebido destaque na literatura nos últimos anos, dentre as principais vantagens deste método destaca-se a recuperação do solvente utilizado e os elevados rendimentos de extração na matriz. Os principais resultados deste trabalho demonstraram que a extração com fluido supercrítico utilizando dióxido de carbono e gás liquefeito de petróleo possui vantagens em relação aos métodos de extração tradicionais com solvente. Entretanto, utilizando fluido supercrítico foram obtidos menores rendimentos em lipídeos. Porém, comparando com os principais resultados na literatura, foram encontradas boas concentrações de ácidos graxos. Uma alternativa para melhorar o rendimento da extração seria a utilização de cossolventes. Desta maneira, poder-se-ia possivelmente aumentar a polaridade das células liofilizadas de *Mortierella isabellina* e com isso, aumentar o rendimento de óleo obtido.

Na extração assistida por ultrassom os resultados foram promissores sendo obtidos maiores rendimentos de lipídeos que na extração com fluido supercrítico, tanto utilizando etanol quanto com a mistura de solventes (clorofórmio, metanol e água). Na extração com ultrassom utilizando etanol foram obtidos menores rendimentos de óleo microbiano, porém a concentração de ácidos graxos obtidos foi superior da obtida quando utilizada a mistura de solventes. Portanto, analisando-se os métodos estudados, pode-se dizer que o método mais eficaz foi a extração assistida por ultrassom utilizando etanol como solvente. Essa técnica apresentou bons rendimentos, boa concentração de ácidos graxos, além de utilizar um solvente não tóxico.

Apesar da mistura de solvente ter possibilitado um maior rendimento de lipídeos, o clorofórmio e o metanol são solventes bastante tóxicos e após a extração devem ser evaporados para que se obtenha apenas os lipídeos. Sendo assim, este tipo de solvente deve ser separado destes lipídeos, uma técnica que poderia ser aplicada é a separação desses solventes utilizando um sistema de membranas. Essa técnica permitiria a recuperação dos solventes utilizados sem degradar os compostos de interesse, como também poderia permitir um processo de separação mais rápido do que o processo de evaporação. Em relação a separação do solvente a técnica de extração supercrítica apresenta vantagem sobre a técnica de ultrassom, pois, não necessita de outro processo para recuperar o produto final (lipídeos), sendo o solvente despressurizado e imediatamente se obtêm os lipídeos de *Mortierella isabellina*. Entretanto, em vista dos resultados que foram obtidos no presente trabalho o rendimento em óleo microbiano foi

superior utilizando a extração por ultrassom, o que definiria esta metodologia como mais interessante na extração de ácidos graxos de *Mortierella isabellina*.

6. CONCLUSÕES GERAIS

Dentre os solventes utilizados na extração com fluido supercrítico, pode-se concluir que a extração com gás liquefeito de petróleo pressurizado foi a que apresentou melhores resultados quando comparada com a extração com dióxido de carbono supercrítico, principalmente com relação às variáveis do processo, como o menor tempo de extração e o melhor rendimento de óleo obtido (4,45 % (m/m)). No entanto, quando se compara a concentração de ácidos graxos no óleo, o CO_2 supercrítico apresenta vantagem em relação ao GLP comprimido, 361 mg/g de óleo e 229 mg/g de óleo, respectivamente. A fração de PUFA foi composta principalmente por ácido linoleico e ácido linolênico.

Para a extração assistida por ultrassom, concluiu-se que a extração com a mistura (clorofórmio:metanol:água) resultou em melhores rendimentos quando comparada com a extração utilizando etanol como solvente, sendo obtidos 19,49 % (m/m) e 14,47 % (m/m) de lipídeos, respectivamente. A concentração de ácidos graxos para o etanol e a mistura de solventes foi de 489 e 245 mg/g de óleo, respectivamente. Além disso, o etanol foi mais interessante, pois, apresentou melhor perfil de ácidos graxos e maior concentração de ácidos graxos poli-insaturados.

7. SUGESTÕES PARA TRABALHOS FUTUROS

- Otimizar as condições de cultivo através da diferentes formas de condução da fermentação com o intuito de aumentar a produção de ácidos graxos, principalmente os ácidos graxos poli-insaturados;

-Avaliar a extração assistida por ultrassom utilizando solventes com diferentes polaridades;

- Avaliar um processo de separação por membranas acoplado a extração assistida por ultrassom.

8. REFERÊNCIAS

ABAIDE, E. R.; ZABOT, G. L.; TRES, M. V.; MARTINS, R. F.; FAGUNDEZ, J. L.; NUNES, L. F.; DRUZIAN, S.; SOARES, J. F.; DAL PRÁ, V.; SILVA, J. R. F.; KUHN, R. C.; MAZUTTI, M. A. Yield, composition, and antioxidant activity of avocado pulp oil extracted by pressurized fluids. **Food and Bioproducts Processing**, v. 102, p. 289-298, 2017.

ADAM, F.; ABERT-VIAN, M.; PELTIER, G.; CHEMAT, F. "Solvent-free" ultrasound assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process. **Bioresource Technology**,,v. 114, p. 457–465, 2012

AKALIN, M. K.; KARAGÖZ, S.; AKYÜZ, M. Supercritical ethanol extraction of bio-oils from German beech wood: Design of experiments. **Industrial Crops and Products**, v. 49, p. 720–729, 2013.

BARRELES, F. M.; REZENDE, C. A.; MARTÍNEZ, J. Supercritical CO_2 extraction of passion fruit (Passiflora edulis sp.) seed oil assisted by ultrasound. **The Journal of Supercritical Fluids**. v. 104, p. 183- 192, 2015.

BELAYNEH, H. D.; WEHLING, R. L.; CAHOON, E.; CIFTCI, O. Extraction of omega-3-rich oil from *Camelina sativa* seed using supercritical carbon dioxide. **The Journal of Supercritical Fluids**. v. 104, p. 153- 159, 2015.

BRUNNER, G. Supercritical fluids: technology and application to food processing. **Journal of Food Engineering**, v. 67, p. 21-33, 2005.

CARVALHO, P. de O.; CAMPOS, P. R. B.; NOFFS, M. D.; DE OLIVEIRA, J. G.; SHIMIZU, M. T.; DA SILVA, D.M. Aplicação de lipases microbianas na obtenção de concentrados de ácidos graxos poliinsaturados. **Química Nova**, v. 26, n. 1, p. 75–80, 2003.

CHATZIFRAGKOU, A.; FAKAS, S.; GALIOTOU-PANAYOTOU, M.; KOMAITIS, M.; AGGELIS, G.; PAPANIKOLAOU, S. Commercial sugars as substrates for lipid accumulationin *Cunninghamella echinulata* and *Mortierella isabellina* fungi. **European Journal of Lipid Science and Technology,** v. 112, p. 1048–1057, 2010.

CHEMAT, F.; HUMA, Z.; KHAN, M. K. Applications of ultrasound in food technology: Processing, preservation and extraction. **Ultrasonics Sonochemistry,** v. 18, p. 813-835, 2011.

CORSO, M. P.; FAGUNDES-KLEN, M. R.; SILVA, E. A.; CARDOZO FILHO, L.; SANTOS, J. N.; FREITAS, L. S.; DARIVA, C. Extraction of sesame seed (*Sesamun indicum* L.) oil using compressed propane and supercritical carbon dioxide, **Journal of Supercritical Fluids,** v. 52, p. 56–61, 2010.

CRAMPON, C.; NIKITINE, C.; ZAIER, M.; LÉPINE, O.; TANZI, C. D.; VIAN, M. A.; CHEMAT, F.; BADENS, E. Oil extraction from enriched *Spirulina platensis* microalgae using supercritical carbon dioxide. **The Journal of Supercritical Fluids,** v. 119, p. 289-296, 2017.

DAL PRÁ, V.; SOARES, J. F.; MONEGO, D. L.; VENDRUSCOLO, R. G; FREIRE, D. M. G.; ALEXANDRI, M.; KOUTINAS, A.; WAGNER, R.; MAZUTTI, M. A.; DA ROSA, M.

B. Extraction of bioactive compounds from palm (*Elaeis guineensis*) pressed fiber using different compressed fluids. **The Journal of Supercritical Fluids**, v. 112, p. 51-56, 2016.

DECKELBAUM, R. J.; CALDER, P. C. Dietary n-3 and n-6 fatty acids: are there 'bad' polyunsaturated fatty acids? **Curr. Opin. Clin. Nutr. Metab. Care,** v. 13, p. 123–124, 2010.

DEMIR, M.; TURHAN, I.; KUCUKCETIN, A.; ALPKENT, Z. Oil production by *Mortierella isabellina* from whey treated with lactase, **Bioresource Technology,** v. 128, p. 365–369, 2013.

DEMIRDÖVEN, A. & BAYSAL, T. The use of ultrasound and combined technologies in food preservation. **Food Reviews International,** v. 25, p. 1-11, 2009.

DEO, M. D.; HWANG, J.; HANSON, F. V. Supercritical fluid extraction of a crude oil, bitumen-derived liquid and bitumen by carbon dioxide and propane. **Fuel**, v. 71, p. 1519- 1526, 1992.

DEY, S.; RATHOD, V. K. Ultrasound assisted extraction of β-carotene from *Spirulina platensis*. **Ultrasonics Sonochemistry**, v. 20, p. 271-276, 2013.

DUQUE, A. M. R.; LONDONÕ-LONDONÕ, J.; ÁLVAREZ, D. G.; PAZ, Y. B.; SALAZAR, B. L. C. Comparación del aceite de aguacate variedade Hass cultivado em Colombia, obtenido por fluidos supercríticos y métodos convencionales: una perpectiva desde la calidad**. Lasallista de Investigación,** v. 9, p. 151-161, 2012.

DYAL, S. D.; NARINE, S. S. Implications for the use of *Mortierella* fungi in the industrial production of essential fatty acid. **Food Research International**, v. 38, p. 445-467, 2005.

ECONOMOU, C. N.; AGGELIS, G.; PAVLOU, S.; VAYENAS, D.V. Single cell oil production from rice hulls hydrolysate. **Bioresource Technology**, v. 102, p. 9737–9742, 2011.

FAKAS, S.; PAPANIKOLAOU, S.; BATSOS, A.; GALIOTOU-PANAYOTOU, M.; MALLOUCHOS, A.; AGGELIS, G. Evaluating renewable carbon sources as substrates for single cell oil production by *Cunninghamella echinulata* and *Mortierella isabelina*. **Biomass and Bioenergy**, v. 33, p. 573–580, 2008.

GAO, D.; ZENG, J.; ZHENG, Y.; YU, X.; CHEN, S. Microbial lipid production from xylose by *Mortierella isabelina*. **Bioresource Technology, v.** 133, p. 315–321, 2013.

GIL-CHÁVEZ, G. J.; VILLA, J. A.; AYALA-ZAVALA, J. F.; HEREDIA, J. B.; SEPULVEDA, D.; YAHIA, E. M.; GONZÁLEZ-AGUILAR, G. A. Technologies for Extraction and Production of Bioactive Compounds to be Used as Nutraceuticals and Food Ingredients: An Overview. **Comprehensive Reviews in Food Science and Food Safety**, v. 12, p. 5-23, 2013.

GIUDETTI, A. M.; CAGNAZZO, R. Beneficial effects of n-3 PUFA on chronic airway inflammatory diseases. **Prostaglandins and Other Lipid Mediators,** v. 99, p. 57–67, 2012.

GONG, Y.; WAN, X.; JIANG, M.; HU, C.; HU, H.; HUANG, F. Metabolic engineering of microorganisms to produce omega-3 very long-chain polyunsaturated fatty acids, **Progress in Lipid Research,** v. 56, 19–35, 2014.

HAMDAN, S.; DAOOD, H.G.; TOTH-MARKUS, M.; ILLÉS, V. Extraction of cardamom oil by supercritical carbon dioxide and sub-critical propane. **Journal of Supercritical Fluids,** v. 44 p. 25–30, 2008.

HERRERO, M.; CIFUENTES, A.; IBAÑEZ, E. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-byproducts, algae and microalgae. A review. **Food Chemistry,** v. 98, p. 136–148, 2006.

HERRERO, M.; MENDIOLA, J. A.; CIFUENTES, A.; IBÁÑES, E. Supercritical fluid extraction: Recent advances and applications. **Journal of Chromatography A**, v. 1217, p. 2495–2511, 2010.

HIDALGO, P.; CIUDAD, G.; NAVIA, R. Evaluation of different solvent mixtures in esterifiable lipids extraction from microalgae *Botryococcus braunii* for biodiesel production. **Bioresource Technology,** v. 201, p. 360-364, 2016.

HSUEH, K. A.; CHANG, F. C.; WANG, H. P.; WANG, H. C.; HUANG, Y. J.; HUANG, H. L.; TUAN, Y. J. Enhanced extraction of PAHs hindered in fly ashes with supercritical water. **Journal of Supercritical Fluids**, v. 73, p.116–119, 2013

JINGYANG, X.; WEI, D.; XUEBING, Z.; GUOLING, Z.; DEHUA, L. Microbial oil production from various carbon sources and its use for biodiesel preparation. **Biofuels, Bioproducts and Biorefining**, v. 7, n. 1, p. 65–77, 2013.

KERIS-SEN, U. D.; SEN, U.; SOYDEMIR, G.; GUROL, M. D. An investigation of ultrasound effect on microalgal cell integrity and lipid extraction efficiency. **Bioresource Technology,** v. 152, p. 407-413, 2014.

KHOEI, M.; CHEKIN, F. The ultrasound-assisted aqueous extraction of rice bran oil. **Food Chemistry**. v. 194, p. 503-507, 2016.

KIM, H. J.; LEE, S. B.; PARK, K. A.; HONG, I. K. Characterization of extraction and separation of rice bran oil rich in EFA using SFE process. **Separation and Purification Technology**, v. 15, p. 1-8, 1999.

KNEZ, Z.; MARKOČIČ, E.; LEITGEB, M.; PRIMOŽIČ, M.; HRNČIČ, M. K.; ŠKERGET, M. Industrial applications of supercritical fluids: A review. **Energy,** v. 77, p. 235-243, 2014.

LEHNINGER, A. L.; NELSON, D. L.; COX, M. M. Lehninger Principles of Biochemistry. W.H. Freeman, v. 1, p. 1119, 2005.

MANTELL, C. et al. Supercritical fluid extraction. In: RAMASWAMY, S.; HUANG, H.-J.; RAMARAO., B. V. **Separation and Purification Technologies in Biorefineries**. First ed. John Wiley & Sons, Ltd., p. 79–100, 2013.

MARAN, J. P.; PRIYA, B. Supercritical fluid extraction of oil from muskmelon (*Cucumis melo*) seeds. **Journal of the Taiwan Institute of Chemical Enfineers**, v. 47, p. 71-78, 2015.

MCCLEMENTS, D. J. Advances in the application of ultrasound in food analysis and processing. Trends. **Food Science and Technology,** v. 6, p. 293-299, 1995.

NISHA, A.; SANKAR, K. U.; VENKATESWARAN, G. Supercritical CO_2 extraction of *Mortierella alpina* single cell oil: Comparison with organic solvent extraction. **Food Chemitry**, v. 133, p. 220-226, 2012.

PEDERSSETI, M. M.; PALÚ, F.;FERNANDO, E.A.; SILVA, J.H.; ROHLING, L.; CARDOZO-FILHO, C. D. Extraction of canola seed (Brassica napus) oil using compressed propane and supercritical carbon dioxide. **Journal of Food Engineering,** v. 102, p. 189–196, 2011

PELLICCIA, F.; MARAZZI, G.; GRECO, C.; et al. Current evidence and future perspectives on n-3 PUFAs. **International Journal of Cardiology**, v. 170, n. 2 Suppl 1, p. S3–7, 2013.

POULIOT, Y.; CONWAY, V.; LECLERC, P. Separation and Concentration Technologies in Food Processing. In: CLARK, S.; JUNG, S.; LAMSAL, B. **Food Processing: Principles and Applications.** Second ed. John Wiley & Sons, Ltd, p. 33–60, 2014.

RATLEDGE, C. Microorganisms for lipids. In: MEESTERS, P. A.; HUIJBERTS, G. N. High-cell density cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as a carbon source. **Applied Microbiology Biotechnology,** v.45, n.5, p.575-579, 1996.

REÁTEGUI, J. L. P.; MACHADO, A. P. da F.; BARBERO, G. F.; REZENDE, C. A.; MARTÍNEZ, J. Extraction of antioxidant compounds from blackberry (*Rubus* sp.) bagasse using supercritical CO2 assisted by ultrasound. **The Journal of Supercritical Fluids**. v. 94, p. 223-233, 2014.

REVERCHON, E.; DE MARCO, I. Supercritical fluid extraction and fractionation of natural matter. **Journal of Supercritical Fluids**, v. 38, p. 146–166, 2006.

REZENDE DOS SANTOS, R.; MOREIRA, D. M.; KUNIGAMI, C. N.; ARANDA, D. A. G.; TEIXEIRA, C. M. L. L. Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass. **Ultrasonics Sonochemistry,** v. 22, p. 95–99, 2015.

RIBAS, M. C.; MANTOVANI, D.; AWADALLAK, J. A.; CANEVESI, R. L.; TAZINAFO, N. M.; FILHO, L. C.; PALÚ, F.; DA SILVA, E.A. Study of candeia oil extraction using pressurized fluids and purification by adsorption process. **The Journal of Supercritical Fluids,** v. 92, p. 177-182, 2014.

RIZVI, S. S. H.; BENADO, A. L.; ZOLLWEG, J. A.; DANIELS, J. A. Supercritical fluid extraction: fundamental principles and modeling methods. **Food Technology**, v. 40, n. 6, p. 55-65, 1986.

RUAN, Z.; ZANOTTI, M.; WANG, X.; DUCEY, C.; LIU, Y. Evaluation of lipid accumulation from lignocellulosic sugars by *Mortierella isabellina* for biodiesel production**. Bioresource Technology,** v. 110, p. 198–205, 2012.

RÚBIO-RODRIGUEZ, N.; BELTRÁN, S.; JAIME, I.; DIEGO, S. M. de; SANZ, M. T.; CARBALLIDO, J. R. Production of omega-3 polyunsaturated fatty acid concentrates: A review. **Innovative Food Science & Emerging Technologies,** v. 11, p. 1-12, 2010.

RUIZ-LÓPEZ, N.; SAYANOVA, O.; NAPIER, J. A.; HASLAM, R. P. Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants. **Journal of Experimental Botany,** v. 63, p. 2397-2410, 2012.

SANTOS, P.; AGUIAR, A. C.; BARBERO, G.F.; REZENDE, C. A.; MARTÍNEZ, J. Supercritical carbon dioxide extraction of capsaicinoids from malagueta pepper (*Capsicum frutescens* L.) assisted by ultrasound. **Ultrasonics Sonochemistry,** v. 22, p. 78-88, 2015.

SILVA, G. K. B. **Produção de ácidos graxos poli-insaturados (PUFAs) por *cunninghamella elegans* ucp 542 em substratos alternativos.** Dissertação (Mestrado em Ciências Biológicas). Universidade Federal de Pernambuco, Recife, 2011.

SILVA, J. R. F.; CANTELLI, K. C.; TRES, M. V.; DALLA ROSA, C.; MEIRELLES, M. A. A.; SOARES, M. B. A.; OLIVEIRA, D.; OLIVEIRA, J. V.; TREICHEL, H.; MAZUTTI, M. A. Treatment with compressed liquefied petroleum gas and ultrasound to improve cellulase activity. **Biocatalysis and Agricultural Biotechnology**, v. 2, p. 102–107, 2013.

SOARES, J. F.; DAL PRÁ, V.; DE SOUZA, M.; LUNELLI, F.C.; ABAIDE, E. R.; DA SILVA, J.R.F.; KUHN, R.C.; MARTÍNEZ, J.; MAZUTTI, M.A. Extraction of rice bran oil using supercritical CO2 and compressed liquefied petroleum gas. **Journal of Food Engineering,** v. 170, p. 58-63, 2016.

SORIA, A. C. e VILLAMIEL, M. Effect of ultrasound on the technological properties and bioactivity of food: a review. **Trends in Food Science eamp; Technology,** v. 21**,** p. 323-331, 2010.

TUR, J. A.; BIBILONI, M. M.; SUREDA, A.; PONS, A. Dietary sources of omega 3 fatty acids: public health risks and benefits. **The British Journal of Nutrition**, v. 107 Suppl, p. S23–52, 2012.

TZIA, C.; LIADAKIS, G. **Extraction optimization in food engineering.** New York: Marcel Dekker, 442p., 2003.

WELLBAUM, C. **Produção de ácidos graxos por linhagens de *mucor* spp. Isoladas de solo de área de Cerrado município de Corumbataí, Estado de São Paulo.** (Tese de doutorado), UNESP, 70p., 2006.

WU, J.; ALAM, M. A.; PAN, Y.; HUANG, D.; WANG, Z.; WANG, T. Enhanced extraction of lipids from microalgae with eco-friendly mixture of methanol and ethyl acetate for biodiesel production. **Journal of the Taiwan Institute of Chemical Engineers,** v. 71, p. 323-329, 2017.

XYNOS, N.; PAPAEFSTATHIOU, G.; PSYCHIS, M.; ARGYROPOULOU, A.; ALIGIANNIS, N.; SKALTSOUNIS, A.L. Development of a green extraction procedure with super/subcritical fluids to produce extracts enriched in oleuropein from olive leaves. **The Journal of Supercritical Fluids,** v. 67, p. 89-93, 2012.

YANG, C.; TEO, K. C.; XU, Y. R. Butane extraction of model organic pollutants from water. **Journal of Hazardous Materials,** v. 108, p. 77–83, 2004.

YEHUDA, S.; RABINOVITZ, S.; CARASSO, R.L.; MOSTOFSKY, D. I. The role of polyunsaturated fatty acids in restoring the aging neuronal membrane. **Neurobiology of Aging,** v. 23, p. 843-853, 2002.

YOUDIM, K. A.; MARTIN, A.; JOSEPH, J. A. Essential fatty acids and the brain: possible health implications. **International Journal Developmental Neuroscience,** v. 18, p. 383-399, 2000.

YUAN, J.; HUANG, J.; WU, G.; TONG, J.; XIE, G.; DUAN, J.; QIN, M**.** Multiple responses optimization of ultrasonic-assisted extraction by response surface methodology (RSM) for rapid analysis of bioactive compounds in the flower head of *Chrysanthemum morifolium* Ramat. **Industrial Crops and Products,** v. 74, p. 192-199, 2015.

ZENG, J.; ZHENG, Y.; YU, X.; YU, L.; GAO, D.; CHEN, S. Lignocellulosic biomass as a renewable carbohydrate source for microbial lipid production. **Bioresource Technology,** v. 128, p. 385–391, 2012.

ZHENG, Y.; YU, X.; ZENG, J.; CHEN, S. Feasibility of filamentous fungi for biofuel production using hydrolysate from dilute sulfuric acid pretreatment of wheat straw. **Biotechnology for Biofuels,** v. 5, p. 1-10, 2012.

ZHOU, C.; ZHU, C.; REN, Y. Optimization of Ultrasound-Assisted Extraction of Single Cell Oil from *Mortierella isabelina*. **Taylor & Francis Group,** v. 48, p. 2188-2195, 2013.

ZINNAI, A.; SANMARTIN, C.; TAGLIERI, I.; ANDRICH, G. Supercritical fluid extraction from microalgae with high content of LC-PUFAs. A case of study: Sc-CO2 oil extraction from *Schizochytrium sp*. **The Journal of Supercritical Fluids,** v. 116, p. 126-131, 2016.