UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA QUÍMICA

Denise Tonato

PRODUÇÃO DE ÓLEO E MATERIAL ADSORVENTE A PARTIR DA BIOMASSA DO FUNGO *Nigrospora* sp.

Santa Maria, RS

2019

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do grau de **Doutora em Engenharia Química**.

Orientador: Dr. Marcio Antonio Mazutti

Santa Maria, RS

2019

Tonato, Denise
PRODUÇÃO DE ÓLEO E MATERIAL ADSORVENTE A PARTIR DA BIOMASSA DO FUNGO Nigrospora sp. / Denise Tonato.- 2019.
131 p.; 30 cm

Orientador: Marcio Antônio Mazutti
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Tecnologia, Programa de Pós-Graduação em Engenharia Química, RS, 2019

1. Fungo Nigrospora sp. 2. Fermentação submersa 3. Extração supercrítica 4. Óleo fúngico 5. Adsorção de corante I. Mazutti, Marcio Antônio II. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 24 de julho de 2019.

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Santa Maria, RS, 2019.

AGRADECIMENTO

Gostaria de agradecer primeiramente a Deus, que me iluminou, protejeu e me guiou nas escolhas dos melhores caminhos;

A minha família, meus pais, Divaldino e Teresinha, e meus irmãos, Daniela, Dalva e Rafael, pelo apoio e incentivo e por proporcionar todas as condições para eu poder concluir o doutorado;

Ao meu orientador, Prof. Dr. Marcio Antônio Mazutti, pela oportunidade de realizar este projeto, pela orientação e confiança depositada;

Aos colegas do Programa de Pós-graduação em Engenharia Química pelo companheirismo e auxílios prestados durante o período de realização do doutorado;

Ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal de Santa Maria, incluindo professores e funcionários, pelo auxílio e colaboração durante a elaboração desta pesquisa;

RESUMO

PRODUÇÃO DE ÓLEO E MATERIAL ADSORVENTE A PARTIR DA BIOMASSA DO FUNGO *Nigrospora* sp.

AUTORA: DENISE TONATO
ORIENTADOR: Dr. MARCIO ANTONIO MAZUTTI

Este trabalho teve o objetivo de produzir óleo microbiano a partir da biomassa do fungo *Nigrospora* sp., obtida do processo de fermentação submersa e extraída a partir dos métodos de *Soxhlet* e extração supercrítica, e avaliar a sua posterior utilização na adsorção de corante. Para isso, este trabalho foi dividido em três partes. Para realizar a primeira parte, foi necessário estudar uma estratégia para maximizar a concentração de biomassa e o acúmulo de lipídeos do fungo *Nigrospora* sp., investigando diferentes variáveis de composição e processo dos meios em frascos agitados, e a influência da taxa de agitação e aeração em biorreator, no modo batelada e batelada alimentada. As extrações de lipídeos nesta primeira etapa do trabalho foram realizadas somente através do método *Soxhlet*. A concentração máxima de biomassa e rendimento de lipídeos obtidos foi de 18,80 g L^{-1} e 3,94% em frascos agitados e de 40,17 g L^{-1} e 21,32% no biorreator em batelada alimentada, respectivamente. Na segunda parte do trabalho, foi avaliado o rendimento e composição do óleo obtido do processo de extração de lipídeos com tecnologia supercrítica e pré-tratamento da biomassa do fungo *Nigrospora* sp., obtida da primeira parte do trabalho através de fermentação em frascos agitados na sua melhor condição. As extrações foram avaliadas em três etapas: Utlizando somente CO_2 supercrítico (etapa 1), ultrassom e CO_2 supercrítico (etapa 2) e ultrassom mais CO_2 pressurizado com co-solvente etanol (etapa 3). Os parâmetros avaliados foram temperatura (40-80 °C), pressão (15-25 MPa), tempo de pré-tratamento de ultrassom (15-60 min) e a concentração de etanol (30-70 g/100 g de biomassa). O maior rendimento de óleo foi de 3,94% e de ácidos graxos totais de 45,5 g/100 g de óleo, obtidos a 80 °C e 25 MPa com 50% de etanol após pré-tratamento da biomassa com ultrassom por 60 min. Na terceira e última parte do trabalho, foi avaliada a utilização da biomassa residual desengordurada (RDB) do fungo *Nigrospora* sp., obtida a partir do processo de extração supercrítica de lipídeos, como um biossorvente para a remoção do corante Vermelho Procion (PR H – E7B) de soluções aquosas. Foi avaliada a influência de diferentes variáveis de processo na biossorção a partir dos pontos de vista da cinética e equilíbrio. O RDB mostrou ter potencial como biossorvente, com capacidade de biossorção de 188,79 mg g^{-1}.

Palavras-chave: fungo; óleo microbiano; fermentação submersa; extração supercrítica; biossorção.

ABSTRACT

PRODUCTION OF OIL AND ADSORBENT MATERIAL FROM BIOMASS OF FUNGUS *Nigrospora* sp.

AUTHOR: DENISE TONATO
ADVISOR: Dr. MARCIO ANTONIO MAZUTTI

This works was focused on the production of microbial oil from the biomass of the fungus *Nigrospora* sp. and the reuse of defated residual biomass as a biosorbent to recovery dye from aqueous solution. The study was divided into three parts. In the first one, it was necessary to study a strategy to maximize the biomass concentration and lipid accumulation of the fungus *Nigrospora* sp., by investigating different composition and process variables of the media in shaken flasks, and the influence of the stirring rate and aeration in bioreactor, in batch and fed batch modes. The extraction of lipids in this first stage wase carried out by the *Soxhlet* method. The maximum concentration of biomass and lipid yield obtained was 18.80 g L^{-1} and 3.94% in shaken flasks and 40.17 g L^{-1} and 21.32% in the bioreactor, respectively. In the second part of the study, it was investigated three strategies to extract the oil from microbial cells: i) using only supercritical CO_2 ; ultrasound treatment followed by supercritical CO_2 extraction; co-solvent combined with ultrasound treatment followed by supercritical CO_2 extraction. The parameters evaluated were temperature (40-80 °C), pressure (15-25 MPa), pre-treatment time of ultrasound (15-60 min) and addition of ethanol (30-70 g / 100 g of biomass). The highest oil yield was 3.94% with a total fatty acids of 45.5 g/100 g oil, obtained at 80 °C and 25 MPa with 50% by weight ethanol after ultrasound pretreatment of the biomass for 60 min. In the third and final part of the study, the use of the defatted residual biomass (RDB) of the fungus *Nigrospora* sp., obtained from the process of supercritical extraction of lipids was used as a biosorbent for the removal of the red dye Procion (PR H-E7B) from aqueous solutions. For this, it was necessary to evaluate the influence of different process variables on biosorption from the kinetic and equilibrium points of view. The RDB showed potential as a biosorbent, with a biosorption capacity of 188.79 mg g^{-1}.

Keywords: fungus; microbial oil; submerged fermentation; supercritical extraction; biosorpti

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LISTA DE SÍMBOLOS E ABREVIATURAS

SFE-CO_2	Extração com CO_2 supercrítico
SFE	Extração com fluidos supercríticos
CO_2	Dióxido de carbono
SC-CO2	Dióxido de carbono supercrítico
PUFA	Ácido graxo poli-insaturado
MUFA	Ácido graxo monoinsaturado
SFA	Ácido graxo saturado
DHA	Ácido docosahexaenóico
GLA	Ácido gama-linolênico
EPA	Ácido eicosapentaenóico
ARA	Ácido araquidônico
TAGs	Triglicerídeos
EFAs	Ácidos graxos essenciais
AGL	Ácidos graxos livres
C/N	Carbono/nitrogênio
GC-FID	Cromatografia Gasosa com Detector por Ionização de Chama
SSF	Fermentação em estado sólido
SmF	Fermentação submersa
STRs	Biorreatores de tanque agitado
CSTRs	Reatores de tanque continuamente agitado
m	Massa do adsorvente (g)
C_0	Concentração inicial de corante (mg L^{-1})
C_e	Concentração de equilíbrio na solução (mg L^{-1})
q_1	Capacidade de adsorção (mg g^{-1}) de Pseudo-primeira Ordem
k_1	Constante cinética (min^{-1}) de Pseudo-primeira Ordem
q_2	Capacidade de adsorção (mg g^{-1}) de Pseudo-segunda Ordem
k_2	Constante cinética (g mg^{-1} min^{-1}) de Pseudo-segunda Ordem
k_{av}	Constante cinética (min^{-1}) de Avrami
q_{av}	Capacidade de adsorção (mg g^{-1}) de Avrami
n_{av}	Exponente de Avrami

qn	Capacidade de adsorção (mg g^{-1}) de Ordem Geral
kn	Constante cinética min^{-1}(g mg^{-1}) n^{-1}) de Ordem Geral
n	Exponente de Ordem Geral
a	Velocidade inicial (mg g^{-1} min^{-1})
b	Constante de dessorção do modelo de Elovich (g mg^{-1})
k_F	Constante ((mg g^{-1}) (mg $L^{-1})^{-1/nf}$) de Freundlich
$1/n_F$	Fator de heterogeneidade de Freundlich
k_L	Constante (L mg^{-1}) de Langmuir
q_m	Capacidade máxima de adsorção (mg g^{-1}) de Langmuir
t	Tempo (min)
T	Temperatura (K)
V	Volume de solução (L)
K_e	Constante de equilíbrio (L mg^{-1})
DRX	Difração de raios-X (XRD)
FTIR	Espectroscopia no Infravermelho por Transformada de Fourier
MEV	Microscopia eletrônica por varredura
PR H-E7B	Corante vermelho procion
RR 141	Corante vermelho reativo
RDB	Biomassa residual desengordurada
NaOH	Hidróxido de sódio
HCl	Ácido clorídrico
PFO	Pseudo-primeira ordem
PSO	Pseudo-segunda ordem

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CAPÍTULO 1 – INTRODUÇÃO

Alguns microrganismos são capazes de acumular óleo no interior de suas células como compostos de armazenamento intracelular (ZENG et al., 2013). Por esta característica, estas espécies microbianas são denominadas como oleaginosas (RATLEDGE; LIPPMEIER, 2017). Os principais microrganismos que apresentam esta particularidade "oleaginosa", são as microalgas, bactérias, fungos e leveduras (VICENTE et al., 2009), os quais são capazes de acumular quantidades significativas de óleo, mais de 20% em relação à biomassa (MENG et al., 2009). No entanto, alguns fatores como a fisiologia do microrganismo, condições ambientais, composição do substrato, quantidade de nutrientes, além da temperatura e pH, podem influenciar consideravelmente o conteúdo lipídico e a composição de ácidos graxos destas espécies oleaginosas (BEOPOULOS et al., 2009; SUBRAMANIAM et al., 2010).

Óleos microbianos acumulados por microrganismos oleaginosos como bactérias, algas e fungos estão sendo vistos como fonte de combustível sustentável/renovável (SUBHASH; MOHAN, 2014). Estas espécies microbianas podem acumular quantidades significativas de triacilgliceróis (ECONOMOU et al., 2011), uma fonte atraente e adequada de óleo para a produção de biodiesel (DONG et al., 2016; MA et al., 2018; TANG et al., 2018, MARELLA et al., 2018). Além disso, estes óleos microbianos são uma importante fonte de ácidos graxos poli-insaturados (PUFAs), (RATLEDGE; LIPPMEIER, 2017).

Diversas espécies de fungos como *Mortierella alpina*, *Mortierella isabelina*, *Aspergillus terreus*, entre outros, apresentam a capacidade de acumular lipídeos (THEVENIEAU; NICAUD, 2013). O óleo produzido por estas espécies de fungos oleaginosos, além de apresentarem diversas vantagens biotecnológicas para produção de biocombustível (CARVALHO et al., 2018), são muito explorados para a produção de lipídeos especiais, como os ácidos graxos poli-insaturados (PUFAs): ácido docosahexaenóico (DHA), ácido gama-linolênico (GLA), ácido eicosapentaenóico (EPA) e ácido araquidônico (ARA) (THEVENIEAU; NICAUD, 2013).

Estes ácidos graxos poli-insaturados (PUFAs) especiais são necessários e muito importantes para a dieta humana, uma vez que eles não são produzidos pelo organismo, se fazendo necessário serem incorporados a partir de fontes alimentares (TONATO et al., 2018). Ácidos graxos poli-insaturados (PUFAs) especiais apresentam um grande potencial na aplicação na área alimentar, terapêutica e nutracêutica (RATLEDGE et al., 2013; BÉLIGON et al., 2016; BELLOU et al., 2016). Estes apresentam diversos benefícios para a saúde

humana (VADIVELAN; VENKATESWARAN, 2014), devido à suas propriedades estruturais e funcionais (CERTÍK; ADAMECHOVÁ; LAOTENG, 2012), podendo ser utilizados em tratamento de doenças cardiovasculares, inflamatórias, circulatórias, câncer e depressão (BAYIZIT, 2014).

Microrganismos oleaginosos são capazes de utilizar uma grande variedade de substratos de carbono para produção de lipídeos, como: biomassa lignocelulósica, resíduos agroindustriais, águas residuais, glicose, glicerol, lactose, amidos, óleos, água de maceração de milho, xilose, ácido acético e etanol, entre outros (SUBHASH; MOHAN, 2014; AMIRSADEGHI et al., 2015; LIU et al., 2017; CARVALHO et al., 2018; CAROTA et al., 2018). Em específico, os fungos oleaginosos são microrganismos conhecidos na literatura por apresentarem um rápido crescimento com ciclos de vida curtos e a capacidade de utilizar diversas fontes de carbono na síntese de lipídeos. No entanto, para a obtenção de um maior acúmulo de óleo se faz necessário a otimização das condições de cultivo e fatores nutricionais (SUBHASH; MOHAN, 2014).

O modo de cultivo (batelada, batelada alimentada, contínuo), para a acumulação de lipídeos em microrganismos oleaginosos é um parâmetro de extrema importância e imprescindível para otimizar a produção de lipídeos e, portanto, têm um impacto econômico direto no processo. O cultivo em batelada refere-se a culturas de células em um volume fixo de meio de cultura sob condições específicas (tipo de nutriente, temperatura, pressão, aeração), que permite determinar as condições ideais para o acúmulo de lipídeos (CHRISTOPHE et al., 2012). Já no processo de batelada alimentada os nutrientes são fornecidos em etapas, enquanto as células e produtos permaneceram até o final no biorreator, o que diminui a inibição do crescimento do microrganismo pela alta concentração da fonte de carbono (QU et al., 2013; FEI et al., 2016). No entanto, a eficiência do cultivo em batelada alimentada está diretamente relacionada à estratégia de alimentação (CHEN et al., 2018).

O cultivo de microrganismos em processos fermentativos pode gerar quantidade significativa de biomassa, a qual pode ser convertida em diversos produtos (DONG et al., 2016). Em relação aos microrganismos oleaginosos, o óleo é armazenado intracelularmente, desta forma para a obtenção dos lipídeos se faz necessário que esta biomassa produzida passe por um processo de extração (OCHSENREITHER et al., 2016).

Para a extração destes lipídeos microbianos, a ruptura celular é um fator muito importante, pois pode influenciar diretamente a eficiência do processo de extração (OCHSENREITHER et al., 2016). O rompimento celular aumenta a liberação de lipídeos

intracelulares armazenados na biomassa microbiana aumentando assim recuperação de lipídeos com menores custos operacionais. Diversos métodos para a ruptura celular microbiana vêm sendo utilizados, como: micro-ondas, ultrassom, moagem de esferas, secagem e extração com fluido supercrítico (BYREDDY et al., 2015).

Os lipídeos produzidos por microrganismos geralmente são extraídos por métodos de extração que utilizam solventes orgânicos (clorofórmio, metanol, hexano), uma vez que estes solventes são baratos e altamente eficazes na extração de lipídeos. No entanto, os processos de extração lipídica com solventes orgânicos necessitam de alto consumo de energia para a recuperação do óleo e separação do solvente (DO YOOK et al., 2019).

Muitos estudos vêm sendo realizados nesta área de extração de lipídeos microbianos. Além do aumento do rendimento de óleo, há a intenção de substituir, em um futuro próximo, os solventes orgânicos utilizados nestes processos em escala piloto e industrial, por solventes não inflamáveis, menos tóxicos a fim de obter processos sustentáveis (HEGEL et al., 2011). A extração com fluidos supercríticos (SFE) vem recebendo uma maior atenção como uma importante alternativa aos métodos convencionais que utilizam solventes orgânicos para extração de lipídeos microbianos (TONATO et al., 2019 a). E este aumento de interesse em utilizar este método esta vinculado principalmente ao fato que esta tecnologia supercrítica é um processo sustentável verde em que o poder solvente e a seletividade podem ser ajustados conforme as condições de operação. Além disso, o dióxido de carbono (CO_2) supercrítico, uns dos fluidos usados neste método de extração, é um solvente inerte, de baixo custo, facilmente disponível, inodoro, insípido e ecologicamente correto (HEGEL et al., 2011; CABEZA, et al., 2017).

Processos de extração, como de óleo, e transformações bioquímicas a partir de processos de fermentação industrial geram uma grande quantidade de resíduos (biomassa) (SVECOVA et al., 2006). Atualmente o mundo está enfrentando umas das piores crises ambientais de sua história. Nas últimas décadas, a recuperação de resíduos gerados em diversos processos industriais se destaca como um dos temas mais desafiadores dentre preservação ambiental. A reutilização destes resíduos como materiais adsorventes de baixo custo para a remoção de vários poluentes de efluentes contaminados faz destes substratos (resíduos) um recurso atraente, principalmente na redução dos custos de tratamento dos resíduos gerados em processamentos industriais e, além disso, contribui com a diminuição da poluição ambiental (BELLO et al., 2015).

Atualmente, diversas técnicas vêm sendo empregadas com sucesso, na despoluição de efluentes contaminados, o que inclui processos como: fotocatálise, coagulação, eletrocinética, troca iônica, adsorção, filtração por membrana, degradação anaeróbica e aeróbica (KAUSAR et al., 2018). Entre estes métodos a adsorção tem atraído atenção considerável devido à sua viabilidade técnica, flexibilidade e simplicidade de operação. No entanto os adsorventes que são amplamente utilizados como carvão ativado, materiais de troca iônica, zeólitas, argilas bentoníticas ainda são considerados caros se aplicados em grande escala em tratamento de efluentes. Com o intuito de diminuir os custos, diversos bioadsorventes foram desenvolvidos a partir de diferentes recursos biológicos para remoção de poluentes (LI; UM; YANG, 2019).

Neste contexto, várias pesquisas já foram realizadas a respeito do reaproveitando de resíduos de biomassa, subprodutos de atividades industriais e agrícolas (bagaços, cascas, etc), resíduos naturais entre outros (PARK; YUN; PARK, 2010; KHARAT, 2015), alternativas de baixo custo e com grande potencial como adsorventes (KRISHNAN; SREEJALEKSHMI; BAIJU, 2011; BELLO et al., 2015; NOOR et al., 2017), para remover corantes, metais e outros contaminantes de águas residuais (YU et al., 2012; KEBAILI et al., 2018) com alta eficiência de adsorção. A utilização de biomassa inativa microbiana como de algas, fungos para remoção de corantes e metais, poluentes de efluentes residuais da indústria, também vem sendo bastante pesquisada (FONTOURA et al., 2017; DHAL; PANDEY, 2018; LI et al., 2018; TONATO et al., 2019 b).

O fungo do gênero *Nigrospora* sp., isolado do Bioma Pampa, se trata de um fungo filamentoso, largamente disseminado no ambiente como solo, plantas e sementes, apresentando-se como um contaminante comum (KANIA, 2014). Com relação à produção de lipídeos, há um relato de fungo deste gênero, *Nigrospora* sp., cultivado em estado sólido utilizando palha e farelo de trigo como substrato, apresentando rendimento de óleo de 21,3%, (PENG; CHEN, 2007). Outro trabalho realizado nos mesmos moldes a este já mencionado, foi desenvolvido pelo nosso grupo de pesquisa (TONATO et al., 2018), que avaliou a capacidade de produção de lipídeos de 150 cepas fúngicas, isoladas de plantas daninhas do Bioma Pampa. Destas cepas, a do gênero *Nigrospora* sp., foi a que apresentou melhor resultado na produção de lipídeos em fermentação submersa, e os ácidos graxos em maior concentração, identificados na composição dos lipídeos deste fungo foram: ácido palmítico (C16:0), ácido oléico (C18:1n9c), ácido linoléico (C18:2n6c) e ácido esteárico (C18:0).

Este microrganismo, *Nigrospora* sp., por ser um fungo produtor de lipídeos, apresenta um grande potencial como fornecedor de matéria-prima para a produção de biocombustível (TONATO et al., 2018), lipídeos especiais, ácidos graxos poli-insaturados (PUFAs), e além disso, o seu resíduo de biomassa de processo de extração de lipídeos, tem potencial para ser utilizado com grande eficiência como bioadsorvente na remoção de corante de efluente.

Nesse sentido, a produção de óleo e bioadsorvente do fungo do gênero *Nigrospora* sp., isolado a partir do Bioma Pampa, se torna uma alternativa para o desenvolvimento econômico e sustentável do país a partir de processos biotecnológicos. Em vista do que foi exposto, o presente trabalho teve por objetivo principal produzir óleo fúngico e reutilizar o resíduo (biomassa) gerado no processo de extração de lipídeos do fungo *Nigrospora* sp. isolado no bioma Pampa, como um bioadsorvente de corante. Para isto, foi necessário realizar a otimização da capacidade de produção deste microrganismo oleaginoso, através de fermentação submersa em frascos agitados e biorreator do tipo STR, em batelada e batelada alimentada, e com extração supercrítica, utilizando pré-tratamento com ultrassom e co-solvente (etanol). Além disso, se fez necessário avaliar a capacidade de adsorção, em batelada, do resíduo de biomassa como adsorvente de corante.

1.1. Justificativa

Nos últimos anos várias pesquisas vêm sendo desenvolvidas com o interesse de obter novas fontes renováveis para a produção de óleo e de adsorventes, de forma eficiente e barata. Desta forma, aumenta a necessidade de investigar a possibilidade de obter lipídeos e adsorventes de maneira ecológica e economicamente viável a partir desses novos recursos renováveis. Sendo assim, a utilização de novas espécies microbianas oleaginosas, como o fungo do gênero *Nigrospora* sp., isolado do Bioma Pampa, se torna uma nova alternativa para a produção de óleo e adsorvente.

Uma nova possibilidade para busca de novas matérias-primas, sem competir com a produção de alimentos, é a utilização de processos biotecnológicos, tais como a produção de óleo microbiano, uma nova fonte de lipídeos para produção de biocombustível como também uma nova fonte de ácidos graxos poli-insaturados (PUFAs) específicos para consumo humano (RATLEDGE, 2004; DONG et al., 2016; RATLEDGE; LIPPMEIER, 2017; NOURI et al., 2019). Óleos sintetizados por microrganismos apresentam composição semelhante aos óleos e

gorduras obtidos a partir de vegetais e animais, o que contribui para a produção de biocombustível (SUBHASH et al., 2014; DONG et al., 2016; CHO; PARK, 2018). Além disso, o óleo microbiano tem muitas vantagens, tais como matéria-prima em grandes quantidades e de baixo custo, menos influência do ambiente, estação e do clima, e da facilidade de aumentar a sua produção (POLI, 2014).

Óleos produzidos por microrganismos além de poderem ser utilizados na produção de biocombustíveis (DONG et al., 2016) podem ser empregados como substitutos de lipídeos raros (óleos contendo grande quantidade de ácidos graxos poli-insaturados (PUFAs) essenciais), encontrados nas plantas ou animais, muito aplicados na indústria de alimentos e farmacêutica (MARTÍNEZ et al., 2015; BÉLIGON et al., 2016; BELLOU et al., 2016;). Fungos oleaginosos vêm sendo muito estudados para a produção de PUFAS especiais, de alto valor, porque os lipídeos armazenados por estas espécies são caracterizados por um número maior de insaturações do que os lipídeos acumulados por outros microrganismos oleaginosos (MARTÍNEZ et al., 2015; ATHENAKI et al., 2018).

Os ácidos graxos poli-insaturados (PUFAs) essenciais, como um todo, têm um impacto sobre várias atividades bioquímicas celulares e estão implicados em condições fisiológicas e patológicas, incluindo doenças carcinogênicas e doenças cardiovasculares. Uma vez que estes ácidos não são produzidos pelo organismo humano, são atualmente amplamente utilizados como suplementos dietéticos para saúde e fórmulas infantis (SAKURADANI et al., 2013; DEELAI et al., 2015; BELLOU et al., 2016; KIKUKAWA et al., 2018;).

Atualmente, os PUFAs são obtidos principalmente através do óleo de peixes marinhos. No entanto além de algumas características desagradáveis deste produto (óleo de peixe), como gosto e odor, alguns peixes produtores deste óleo, especialmente salmão, sardinha, atum e pescada, estão frequentemente contaminados com metais pesados (cádmio, chumbo e mercúrio) e poluentes orgânicos (dioxinas, compostos semelhantes à dioxina e furanos) que são tóxicos para o homem. Desta forma, considerando o risco a saúde humana, novas alternativas de fontes de PUFAs, têm sido muito pesquisada utilizando principalmente microrganismos (microalgas, bactérias, leveduras, fungos) e plantas transgênicas (DEELAI et al., 2015).

Desta forma, o fungo do gênero *Nigrospora* sp., pode ser explorado como uma nova fonte renovável para o desenvolvimento de biocombustível, produção de PUFAs especiais e de adsorvente, baseado em alguns princípios. Primeiramente, ele contém um perfil de ácidos graxos semelhantes ao dos óleos vegetais o que facilita a produção de biocombustível, tendo a

vantagem de não ser utilizado como fonte comestível, desta forma ele não competiria diretamente com o setor de alimentos. Em segundo lugar, este óleo microbiano apresenta em sua composição alguns PUFAs especiais, que poderiam ser aplicados na indústria de alimentos e farmacêutica. Em terceiro lugar, o reaproveitamento do resíduo de biomassa do processo de extração do óleo como um novo adsorvente para corante, torna o processo sustentável. Por fim, este fungo foi isolado do Bioma Pampa, o qual constitui importante biodiversidade para o estado, tratando-se de um patrimônio natural, genético e cultural de importância nacional e global. Assim, a exploração deste óleo para a produção de óleo e adsorvente seria benéfico para a economia local como também serve como incentivo a novas pesquisas para desenvolvimentos de novos produtos biotecnológicos.

1.2. Objetivos

O objetivo geral deste trabalho consistiu em otimizar o processo fermentativo (fermentação submersa) para a produção de óleo a partir do fungo do gênero *Nigrospora* sp., avaliar diferentes processos de extração e reaproveitar a biomassa residual para a remoção de corante de águas residuárias por adsorção. Para atender ao objetivo geral, fez-se necessário o cumprimento dos seguintes objetivos específicos:

i. Avaliar o comportamento da cinética de fermentação do fungo, em escala de bancada, através do estudo da influência da temperatura na produção de biomassa e lipídeos.

ii. Otimizar o processo de fermentação, avaliando a influência da composição do meio de fermentação em batelada e batelada alimentada em frascos agitados e biorreator do tipo STR, em relação ao rendimento e composição dos lipídeos.

iii. Otimizar o processo de extração de lipídeos com CO_2 supercrítico, utilizando pré-tratamento com ultrassom e a combinação de ultrassom e co-solvente (etanol).

iv. Avaliar a aplicação do resíduo de biomassa desengordurada (RDB) do fungo do *Nigrospora* sp., obtido do processo de extração de óleo, como um adsorvente alternativo na adsorção de corante.

v. Avaliar a eficiência desse novo biossorvente (RDB), na adsorção do corante vermelho procion H-E7B, em adsorção em batelada, estudando a influência de diferentes variáveis de processo como, pH e dosagem de adsorvente.

CAPÍTULO 2 - REVISÃO BIBLIOGRÁFICA

2.1. HISTÓRIA DO ÓLEO MICROBIANO

A ocorrência de óleos microbianos, como o de leveduras e fungos, já é relatada desde o final do século XIX. As primeiras pesquisas a respeito de óleo microbiano nas décadas do século XX mostraram que os ácidos graxos encontrados nestes óleos eram proporcionais aos encontrados em plantas e animais. No período da ocorrência da Segunda Guerra Mundial, nos anos de 1939 a 1945, pesquisadores alemães, percursores destas pesquisas, consideravam que os óleos produzidos por microrganismos poderiam ser consumidos por seres humanos. No entanto, isto não ocorreu. Em vez disso, o óleo e biomassa produzida em uma escala industrial modesta, serviram de alimentos para os animais (cavalos) do exército (RATLEDGE; LIPPMEIER, 2017).

O apelo dos lipídeos microbianos era devido à necessidade de uma alternativa aos óleos produzidos por vegetais e ácidos graxos poli-insaturados específicos para dieta humana. No entanto, o alto custo de produção inviabilizou a produção industrial deste óleo. Mas com melhoria e avanço de técnicas de fermentação, os lipídeos de microrganismos, atualmente podem ser produzidos em grande escala (SHIELDS-MENARD et al., 2018). Nos últimos tempos, com a necessidade de buscar novas fontes alternativas renováveis para produção de óleos, para a aplicação em biocombustível, e por razões médicas e nutricionais, que utilizam os PUFAs na dieta humana, o interesse pelos lipídeos microbianos vem aumentado consideravelmente (AKPINAR-BAYIZIT, 2014).

2.2. MICRORGANISMOS PRODUTORES DE LIPÍDEOS

A produção de óleo utilizando microrganismos oleaginosos como microalgas, levedura, fungos e bactérias vem sendo bastante pesquisada. Os lipídeos que se acumulam na célula microbiana em sua maioria são nas formas de triglicerídeos (TAGs), ácidos graxos livres (AGL), hidrocarbonetos, lipídeos polares, esteróis e pigmentos (DONG et al., 2016).

Dentre os microrganismos produtores de lipídeos, as leveduras oleaginosas, apresentam um grande destaque devido a sua eficiência no acúmulo de óleo, representadas principalmente pelo gênero *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhizopus*, e *Thichosporon*

Yarowia, que podem acumular em média 40% de sua biomassa como lipídeos e em condições óptimas esta acumulação pode atingir 70% da sua biomassa (BEOPOULOS et al., 2009).

Os lipídeos sintetizados por fungos oleaginosos, por apresentar um número elevado de insaturações, quando comparado ao óleo produzido por outros microrganismos como leveduras, têm sido muito estudados para a produção de ácidos graxos poli-insaturados (PUFAs), (PAPANIKOLAOU; AGGELIS, 2011). Muitas espécies de fungos, como *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, *Mortierella isabellina*, também são conhecidos na literatura como oleaginosas, produtores de lipídeos e a maioria dessas espécies de fungos produzem ácidos graxos da família ômega-3 como o ácido graxo eicosapentaenóico (EPA) e o ácido docosahexaenóico (DHA) e da família ômega 6 como o ácido araquidônico (ARA), (LI; DU; LIU, 2008; THEVENIEAU; NICAUD, 2013).

Outro microrganismo oleaginoso que se destaca na produção de lipídeos são as microalgas do gênero *Chlorella* sp., *Nannochloropsis* sp. e *Scenedesmus* sp., que são consideradas uma fonte promissora para a produção de biocombustível devido apresentar uma alta produtividade de lipídeos e crescer rapidamente (DONG et al., 2016). As bactérias oleaginosas, como *Arthrobacter* sp., *Rhodococcus opaco* e *Acinetobacter calcoaceticus*, podem acumular um elevado teor de óleo em relação a sua biomassa seca e além disso apresenta uma alta taxa de crescimento, produzindo quantidades elevadas de biomassa durante um curto período de tempo (DONG et al., 2016).

Os microrganismos oleaginosos são capazes de acumular mais de 20% de sua biomassa como lipídeos, podendo variar de 20% a mais de 70% da biomassa da célula (SHIELDS-MENARD et al., 2018). Na Tabela 1, mostrada a seguir, pode-se verificar uma lista de espécies oleaginosas de vários grupos microbianos capazes de acumular grandes quantidades de lipídeos intracelulares, com grande potencial para a produção de biodiesel.

Tabela 1- Teor lipídico de diversos microrganismos oleaginosos.

Espécies microbianas	Teor de Óleo (% g/100g)
Alga	
Chlorella vulgaris	56,6
Chlorella emersonii	63
Chlorella minutissima	57
Chlorella sorokiniana	22
Levedura	
Lipomyces starkeyi	52,6
Cryptococcus curvatus	34,6
Yarrowia lipolytica'	58,5
Rhodotorula glutinis	53
Rhodosporidium toruloides	64,5
Cryptococcus albidus	65
Chlorella potothecoides	46,13
Rhodosporidium toruloides	37,6
Cryptococcus curvatus	37,8
Lipomyces starkeyi	38
Cryptococcus curvatus	56,4
Fungo	
Mortierella isabellina	50,5
Mortierella ramanniana	42
Mortierella vinacea	66
Bactéria	
Rhodococcus opacus PD630	42,1
Rhodococcus opacus PD630	87
Rhodococcus rhodochrous	50
Bacillus alcalophilus	24
Rhodococcus jostii	55
Rhodococcus opacus	27
Rhodococcus opacus	29

Fonte: (SHIELDS-MENARD et al., 2018).

Nos microrganismos oleaginosos, o conteúdo e a composição dos lipídeos variam de acordo com o metabolismo do organismo, mas pode ser modificado por condições de cultura como temperatura, estágio de crescimento, nutrientes e pH, (DONG et al., 2016). Estas espécies de microrganismos produzem lipídeos sob condições de *estresse*, com excesso de carbono e uma quantidade limitante de fontes de nitrogênio. O conteúdo lipídico produzido por estes microrganismos oleaginosos, cultivado sob condições limitantes, as quais contribuem para o alto acúmulo de lipídeos, permite que este produto microbiano (lipídeo) se torne uma fonte promissora para a produção de biodiesel (CORTES; CARVALHO, 2015).

Os lipídeos sintetizados por estes microrganismos oleaginosos apresentam composição de ácidos graxos semelhantes aos de óleos vegetais, contendo principalmente ácidos graxos C16 e C18 esterificados na forma de triacilgliceróis (CHRISTOPHE et al., 2012). A

semelhança na composição deste óleo inclui ácidos graxos, tais como ácidos: palmítico (C16: 0), esteárico (C18: 0), oleico (C18: 1), linoleico (C18: 2) e α-linolênico (C18: 3). Por esta semelhança, os lipídeos microbianos podem substituir óleos vegetais na produção de biodiesel, manteiga de cacau, suplementos alimentares, (MARTÍNEZ et al., 2015; TONATO et al., 2018). Esta semelhança da composição do óleo microbiano em relação ao vegetal, pode ser observada na Tabela 2, que compara a composições químicas dos óleos obtido a partir de sementes oleaginosas e microrganismos (levedura, fungos oleaginosos e microalgas), (THEVENIEAU; NICAUD, 2013).

Tabela 2 - Conteúdo lipídico (%) e perfil de ácidos graxos de sementes e microrganismos oleaginosos.

	Composição do ácido graxo (% g/100g)							
	Lipídeo (%)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Oleaginosas								
Amendoim	50	N/D	11	0	2	48	32	N/D
Colza	45	N/D	4	N/D	2	62	22	10
Girassol	45	N/D	7	N/D	5	19	68	1
Soja	20	N/D	11	N/D	4	24	54	7
Frutos de árvore								
Coco	50	18	9	N/D	3	6	2	N/D
Oliva	6 kg/l	N/D	13	1	3	71	10	1
Palma	50	1	44	N/D	4	38	10	1
Bagaço de palma	N/D	16	8	N/D	3	15	2	N/D
Microrganismos: ***Levedura***								
Cryptococcus albidus	60	N/D	12	1	3	73	12	N/D
Lipomyces starkeyi	63	N/D	34	6	5	51	3	N/D
Rhodosporidium toruloides	66	N/D	18	3	3	66	N/D	N/D
Rhodotorula glutinis	72	N/D	37	1	3	47	8	N/D
Yarrowia lipolytica	36	N/D	11	6	1	28	51	N/D
Rhizopus arrhizus	57	N/D	18	N/D	6	22	10	12
Fungos								
Mortierella isabellina	50	N/D	29	N/D	3	55	3	3 (n-6)
Mucor circinelloides	25	N/D	22	N/D	5	38	10	15 (n-6)
Pythium ultimum	48	N/D	15	N/D	2	20	16	1
Aspergillus terreus	N/D	2	23	N/D	traços	14	40	N/D
Pellicularia praticola	N/D	N/D	8	N/D	2	11	72	N/D
Claviceps purpurea	N/D	N/D	23	N/D	2	19	8	N/D
Bactérias								
Rhodococcus opacus	19–26	N/D	N/D	N/D	3–19	6–74	N/D	N/D
Microalgas								
Chlorella sp.	28–32	N/D	7–19	10,9	1–4	8–9	1–14	16–19
Chlorella zofingiensis	28–32	N/D	23	2	2	36	18	8
Crypthecodinium cohnii	23	13	23	N/D	3	8	N/D	N/D
Chatoceros muelleri	31–68	18–40	5–40	N/D	0–25	0–4	0–5	0–5
Schizochytrium linacinum	50–77	3–4	54–60	N/D	1–4	N/D	N/D	N/D

Miristico-(C14:0); Palmítico-(C16:0); Palmitoleico-(C16:1); Esteárico- (C18:0); Oleico- (C18:1); Linoleico-(C18:2); Linolênico-(C18:3).
Nota: N/D = Não disponível
Fonte: (Adaptado de: THEVENIEAU; NICAUD , 2013).

2.3. ÓLEO FÚNGICO E SUA APLICAÇÃO

Nos últimos anos, o interesse pela produção de óleo microbiano vem aumentado devido à necessidade de utilizar recursos renováveis alternativos como fontes de carbono para produção de biocombustivel e por razões médicas e nutricionais, que utlizam os PUFAs para aplicações de fins práticos, como na dieta humana (AKPINAR-BAYIZIT, 2014). O óleo produzido por fungos oleaginosos apresenta mais insaturações do que o óleo produzido por leveduras. Este é um dos principais motivos pelo qual os fungos oleaginosos são principalmente utilizados, a fim de produzir lipídeos ricos em PUFAs (ácidos graxos poli-insaturados) de interesse médico e dietético (PAPANIKOLAOU; AGGELIS, 2011).

Várias espécies de fungos filamentosos são bastante divulgadas na literatura como produtoras de lipídeos, entre elas se destacam: *Aspergillus oryzae*, *Claviceps purpurea*, *Humicola lanuginose*, *Mortierella isabellina*, *Mortierella vinacea* e *Mucor circinelloides* (MATSAKAS; GIANNAKOU; VÖRÖS, 2017). Lipídeos sintetisados por fungos apresentam um grande potencial para a produção de biocombustível (MENARD et al., 2018; CARVALHO et al., 2018), e além disso, o óleo fúngico é uma alternativa para a produção de PUFAs essenciais, como o ácido araquidônico (ARA), ácido γ- linolênico (GLA), ácido eicosapentaenóico (EPA), ácido docosahexaenóico (DHA), (THEVENIEAU; NICAUD, 2013).

A grande maioria das espécies fúngicas oleaginosas contém em sua composição lipídica, em ordem de abundância, os ácidos graxos, oleico (C18:1), palmítico (C16:0), linoléico (C18:2), esteárico (C18:0), linolênico (C18:3) e palmitoleico (C16:1). Além de apresentar grande quantidade de PUFAs, como linoléico (C18:2) e linolênico (C18:3), estas espécies oleaginosas contém em sua composição lipídica os ácidos graxos poli-insaturados de cadeia longa (ARA, GLA, EPA, DHA), (AKPINAR-BAYIZIT, 2014).

Os PUFAs apresentam um arranjo estrutural vital e funções funcionais em organismos superiores, incluindo humanos. Os neurônios e as células sensoriais, (membranas celulares), são ricos em ácidos graxos poli-insaturados (PUFAs) contendo fosfolipídeos, que influenciam a fluidez da membrana, o qual se trata de um importante fator, pois afeta a atividade das proteínas da membrana e suas interações com outras moléculas (BELLOU et al., 2016). Os PUFAs são importantes para a saúde, e apresentam grande potencial para serem utilizados como aditivos alimentares ou produtos farmacêuticos devido às suas atividades biológicas (TONATO et al., 2018).

Os ácidos graxos poli-insaturados são aqueles que apresentam mais de uma dupla ligação, normalmente separada por um único grupo metileno. De acordo com a localização da

dupla ligação, os PUFAs são classificados em quatro grupos: ômega-3 (ω3), ômega-6 (ω6), ômega-7 (ω7) e ômega-9 (ω9), (AKPINAR-BAYIZIT, 2014). Os PUFAs que contêm três até seis ligações duplas como os encontrados em óleo de peixe ou no tecido cerebral, também são conhecidos como ácidos graxos essenciais (EFAs), e eles são compostos por duas famílias de ácidos graxos, o ômega-3 e ômega-6 (GOLDBERG; ROKEM, 2017). Os EFAs vêm sendo muito utilizados como suplementos dietéticos para saúde e fórmulas infantis, pois estes ácidos graxos esenciais são necessários para um saúde humana, uma vez que não são sintetizados pelo organismo, havendo necessecidade de serem obtidos a partir de fontes alimentares (TONATO et al., 2018).

Atualmente a principal fonte lipídica rica em PUFAs essenciais é o óleo de peixe. No entanto, os estoques globais deste óleo produzidos por peixes são gradualmente limitados, e, além disso, este produto (óleo de peixe) apresenta algumas características negativas como odor e mau gosto. Desta forma os óleos produzidos por microrganismos oleaginosos têm grande potencial de ser uma fonte alternativa de PUFAs para consumo humano (TONATO et al.,2018). Os fungos filamentosos oleaginosos, principalmemte do gênero *Mortierella*, são muito conhecidos como uma fonte promissora de PUFAs essenciais e recentemente com várias inovações em engenharia metabólica usando engenharia genética esta produção de ácidos graxos poli-insaturados pode ser aumentada consideravelmente (KIKUKAWA et al., 2018).

2.4. FUNGO DO GÊNERO *NIGROSPORA* SP.

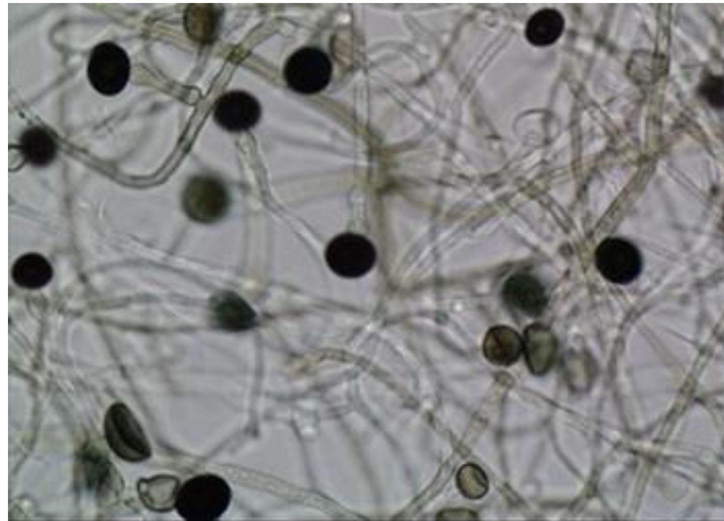
Nigrospora sp. é um fungo dematiáceo filamentoso, largamente disseminado no ambiente como solo, plantas e sementes, apresentando-se como um contaminante comum. Cresce rapidamente na forma de colônias com textura lanosa, e num período de três a quatro dias recobre totalmente uma placa de meio BDA (batata, dextrose, ágar), numa temperatura de 25 °C. As colônias apresentam-se inicialmente com micélio branco que ao passar do tempo torna-se cinza com áreas negras. Na cultura, a cor da colônia escurece em proporção com o aumento da quantidade de esporulação durante a incubação. Algumas espécies deste fungo podem levar mais de três semanas para esporular (KANIA, 2014).

Os fungos do gênero *Nigrospora* sp., são frequente patógenos de plantas, afetando grãos e frutas. São muito comuns em sementes de um grande número de hospedeiros, sendo frequentemente encontrado em sementes de soja (GOULART, 2004). Analisado

microscopicamente em laboratório, o *Nigrospora* sp. apresenta hifas septadas e hialinas, conidióforos também hialinos, ou levemente pigmentados. As células conidiogênicas nos conidióforos são infladas e com formato de ampola. Essas células apresentam um único conídio de 14-20 micra de diâmetro em seu ápice. Os conídios são pretos, unicelulares, levemente achatados (KANIA, 2014).

Como o próprio nome indica o gênero *Nigrospora* sp., ou "esporos preto" em latim, é muito fácil de ser identificado através do microscópio, por ser unicelular, preto, brilhante, possuir esporos assexuais (conídios) achatados e muitas vezes com uma linha equatorial incolor (Figura 1). Os fungos deste gênero são encontrados principalmente em países tropicais e subtropicais e em regiões temperadas (DELGADO; SANTO-PIETRO, 2006).

Figura 1 - Fungo do gênero *Nigrospora* sp. analisado microscopicamente em laboratório.



Fonte: (DELGADO; SANTO-PIETRO, 2006).

Os fungos do gênero *Nigrospora* sp. são ricos em fonte de metabólitos secundários bioativos com diversas estruturas químicas, com atividade inibidora no crescimento de plantas *nigrosporolida* e *fomamalactona*, *nigrosporinas* antibacterianas fitotóxicas, *lactonas* fitotóxicas, *antraquinonas* antibacterianas, *griseofulvina* antifúngica, *heptacetides* antivirais, *cioclioquinona* citotóxicos entre outros (METWALY et al., 2014; ZHANG et al., 2016). Já em relação à produção de óleo, poucos estudos são relatados utilizando este gênero de fungo. A exploração do potencial deste microrganismo para produção de lipídeos foi pesquisada por PENG e CHEGN (2007), que utilizando fungos isolados, entre eles uma das espécies *Nigrospora* sp., cultivados em fermentação em estado sólido (SSF), obteveram resultados satisfatórios de produção de lipídeos. Em trabalho mais recente nosso grupo de pesquisa

(TONATO et al., 2018) avaliaram a capacidade de produção lipídica de cepas fúngicas isoladas do Bioma Pampa, em fermentação submersa (SmF), e obtiveram resultados promissores com acúmulo lipídico rico em PUFAs com o fungo do gênero *Nigrospora* sp.

2.5. PROCESSOS DE FERMENTAÇÃO

Para a produção de lipídeos microbianos, dois métodos de fermentação são utilizados, fermentação em estado sólido e fermentação submersa (BAYIZIT, 2014). O processo de fermentação submersa (SmF), envolve o crescimento de microrganismos em meio líquido com nutrientes dissolvidos ou suspensos. Já o processo de fermentação de substrato sólido (SSF), utiliza substratos sólidos ou nutrientes com ou sem a adição de certa quantidade de água. Embora o processo submerso crie um ambiente de crescimento mais homogêneo, a fermentação em estado sólido está mais próxima do habitat natural para o microrganismo crescer (DURAND et al., 1997).

A SSF, definida como um processo que ocorre na ausência ou quase ausência de líquido livre, empregando um substrato inerte ou natural como suporte sólido, é utilizada para a bioconversão de resíduos de plantas em alimentos, forragens, enzimas e metabolitos secundários (por exemplo, medicamentos, suplementos alimentares). Por outro lado, as culturas líquidas submersas, funcionando como sistemas homogêneos sob o controle do processo completo (pH, agitação, concentração de componentes do meio, oxigenação, densidade média), permite a produção de biomassa fúngica com alto valor nutritivo ou biossíntese de metabólitos com composição previsível. O processamento a jusante após o cultivo submerso é mais fácil em comparação com a SSF. No entanto, a cultura submersa induz alto custo energético requerido para a agitação, fornecimento de oxigênio, estabilização da temperatura do meio (TURŁO, 2014). Sendo assim, estes dois processos apresentam em função de suas características, vantagens e desvantagens, as quais são mostradas na Tabela 3.

Tabela 3 - Comparação das características da SSF e SmF.

Fermentação em estado sólido (SSF)	Fermentação em estado submerso (SmF)
Vantagens	
✓ Consumo de energia pequeno	✓ O alto teor de água e a natureza diluída do meio facilitam o controle da temperatura, reduzindo a degradação dos produtos;
✓ Substratos baratos (materiais lignocelulósicos naturais, resíduos da indústria alimentar)	✓ A purificação de moléculas ativas é facilitada pela ausência ou baixa concentração de substrato;
✓ Meios concentrados resultando em menores dimensões de biorreator	✓ Processos de misturas são facilitados devido ao caráter homogêneo do sistema;
Desvantagens	
✓ Problemas com o isolamento e a purificação dos produtos	✓ Quando operado com elevadas concentrações de substrato, podem ocorrer problemas reológicos no sistema;
✓ Controle difícil ou impossível dos parâmetros do processo (pH, temperatura, arejamento).	✓ Maior demanda energética associada à esterilização do meio e à remoção de produto do meio fermentado;

Fonte: (Adaptado de: TURŁO, 2014).

Microrganismos oleaginosos como fungos e leveduras apresentam taxa de crescimento rápida e seu crescimento é favorecido com a utlização de fontes de carbono simples, como glicose derivada de milho, cana-de-açúcar ou biomassa (DONG et al., 2016). O conteúdo e composição dos ácidos graxos produzidos por microrganismos oleaginosos podem variar muito dependendo do tipo de processo e do substrato utilizado na síntese óleo microbiano (THEVENIEAU; NICAUD, 2013). Diversos estudos relatam o crescimento de fungos filamentosos em diversas fontes baratas de carbono, incluindo resíduos agroindustriais em cultura líquida, fermentação submersa (SmF), e cultura sólida, fermentação com substrato sólido (SSF), para produção de lipídeos (DEY; BANERJEE; MAITI, 2011). Desta forma, na Tabela 4 pode-se verificar a influência do modo de cultivo (estado sólido e líquido) como também a influência de diversas fontes de carbono no acúmulo de lipídeos em diferentes espécies de fungos produtores de lipídeos.

Tabela 4 - Produção de lipídeos por diferentes espécies de fungos oleaginosos utilizando diversas fontes de carbono.

Fungo	Fonte de carbono	Biomassa	Rendimento lipídico	Modo de cultura
Mortierella isabellina	Glicose	27,0 (g/L)	14,0 (g/L)	Líquido
	Xilose	9,5 (g/L)	6,1 (g/L)	Líquido
	Glicerol cru	6,2 (g/L)	3,3 (g/L)	Líquido
	Amido	10,4(g/L)	3,7 (g/L)	Líquido
	Pectina	8,4(g/L)	2,0 (g/L)	Líquido
Mucor sp. RRL001	Goma de tapioca	28,0(g/L)	5,0 (g/L)	Líquido
Cunninghamella echinulata	Glicose	15,0 (g/L)	6,9 (g/L)	Líquido
	Xilose	12,6 (g/L)	6,7 (g/L)	Líquido
	Glicerol cru	7,8 (g/L)	2,0 (g/L)	Líquido
	Amido	13,5(g/L)	3,8 (g/L)	Líquido
	Pectina	4,1(g/L)	0,4 (g/L)	Líquido
Colletotrichum sp. (DM06)	Glicose	18,4 (g/L)	7,8 (g/L)	Líquido
	Sacarose	16,7 (g/L)	7,5(g/L)	Líquido
	Xilose	7,4 (g/L)	2,2 (g/L)	Líquido
Alternaria sp. (DM09)	Glicose	15,2 (g/L)	8,6 (g/L)	Líquido
	Sacarose	11,5 (g/L)	4,6 (g/L)	Líquido
	Xilose	10,4 (g/L)	4,3 (g/L)	Líquido
Aspergillus oryzae A-4	Palha de trigo e farelo	N/D	36,6 (mg/gds)	Sólido
Microsphaeropsis sp.	Palha de trigo e farelo	10,9 (mg/gds)	42 (mg/gds)	Sólido
Colletotrichum sp. (DM06)	Palha de arroz e farelo de trigo	10,8 (mg/gds)	68,2 (mg/gds)	Sólido
Alternaria sp. (DM09)	Palha de arroz e farelo de trigo	8,97 (mg/gds)	60,32 (mg/gds)	Sólido

Nota: N/D = Não disponível; (mg /gds) = mg de glucosamina produzida por g de substrato seco.
Fonte: (Adaptado de: DEY; BANERJEE; MAITI, 2011).

A produção de lipídeos microbianos pode ser conduzida tanto por SmF quanto por SSF, e esses dois procesos (fermentação submersa e sólida) apresentam tanto pontos positos quanto negativos para este fim (produção de lipídeos). No entanto na SmF pode-se ter um controle maior dos parâmetros de fermentação (temperatuea, pH, etc) quando comparado a SSF, devido a esta caracteristica a SmF vem sendo muito estudada para síntese de óleo microbiano (HANSEN et al., 2015; OCHSENREITHER et al., 2016).

2.6. FERMENTAÇÃO SUBMERSA

No processo de fermentação submersa, o substrato usado para fermentação é sempre dissolvido ou em suspensão em meio líquido contendo os nutrientes (açúcar, vitaminas, minerais e outros) necessários para o crescimento do microrganismo. A aeração é uma

operação importante no cultivo do microrganismo, pois proporciona o oxigênio necessário para crescimento, e o calor que é gerado durante o cultivo, pode ser removido utilizando um dispositivo de arrefecimento. A biomassa microbiana pode ser colhida por vários métodos, célula única como fermento e as bactérias são recuperadas por centrifugação, enquanto a biomassa de fungos filamentosos é recuperada por filtração (SUMAN et al., 2015).

A capacidade de controlar a morfologia de um fungo em cultura submersa é importante para manter o rendimento do produto elevado. Em fermentação em estado submerso (SmF), os parâmetros do processo podem ser controlados em maior grau em relação a fermentação em estado sólido (SSF), especialmente a temperatura, que é um dos fatores mais importantes, pois afeta a taxa de crescimento, tensão de oxigênio dissolvido, taxa de evaporação do meio, formação de grânulos. Os biorreactores mais utilizados para SmF de micoorganismos, são tanques de agitação, que podem ser utilizados continuamente para reduzir a intensidade de trabalho e onde a transferência de oxigénio é controlável (HANSEN et al., 2015).

Para cultivo microbiano, as condições fornecidas pelo biorreator devem atender às necessidades específicas dos microrganismos a fim de obter elevada produtividade dos bioprodutos desejados (SALMON et al., 2016). Tradicionalmente os biorreatores de tanque agitado em batelada (STRs) e reatores de tanque continuamente agitado (CSTRs) são utlizados há muito tempo e ainda são amplamente adotados na indústria química e de bioprocessos, devido à sua simplicidade de operação. Além desses dois bioreatorres, outros tipos como os de tambor rotativo, névoa, membrana, leito empacotado e semidimensionado, coluna de bolhas e ar entre outros, foram desenvolvidos para atender determinadas aplicações e processos específicos (SINGH; KAUSHIK; BISWAS, 2014). A utilização de tecnologias atuais de reatores, permitem que estes novos tipos de biorreatores sejam constantemente desenvolvidos para otimizar e melhorar produtividade. No entanto os reatores de tanque agitados mecanicamente (STR) continuam a ser a base da indústria, devido à sua versatilidade e flexibilidade (SPIER et al., 2011).

O objetivo principal do biorreator é proporcionar um ambiente adequado e controlado para crescimento celular e síntese de produtos, mantendo durante o processo de fermentação as condições monosépticas e taxa uniforme de cisalhamento. Sendo assim, alguns fatores importantes devem ser considerados na construção do biorreator, como: *design*, investimento de capital, custo operacional, esterilidade, aeração e mistura do sistema, controle de

temperatura e pH, baixo consumo de energia e tamanho e material adequados (SPIER et al., 2011; SINGH; KAUSHIK; BISWAS, 2014).

O cultivo submerso de células microbianas em biorreatores garante um ambiente controlado para a produção eficiente de produtos finais de alta qualidade e para produtividade e rendimento ótimos. Biorreatores industriais operados em processo de batelada (descontínuo), batelada alimentada e em processo contínuo, são utilizados para cultivar diferentes tipos de microrganismos que produzem uma vasta gama de produtos (PAULOVÁ; PATÁKOVÁ; BRÁNYIK, 2013). Cada um desses processos tem características particulares e se adapta melhor ao microrganismo utilizado e as condições de processo (SCHMIDELL; FACCIOTTI, 2001). A Tabela 5 mostra as diferentes formas de operação de um processo de fermentação submersa e suas principais características.

Tabela 5 – Modo de operação de um processo de fermentação submersa e suas principais características.

Forma de condução do processo	Principais características
Batelada (descontínua)	Melhor controle da assepsia; Inoculação direta no meio esterilizado; Adição somente de oxigênio e nutrientes; Volume reacional constante; Baixos rendimentos e possibilidade de inibição
Batelada alimentada	Adição de nutrientes durante o cultivo do microrganismo; Adição do mosto contínua ou intermitentemente
Contínuo	Alimentação contínua do meio de cultura e uma vazão constante; Volume reacional mantido constante através da retirada contínua de caldo fermentado; Operação em estado estacionário; Operação por longos períodos de tempo

Fonte: (SCHMIDELL; FACCIOTTI, 2001).

O processo de cultivo submerso para desenvolvimento industrial de microrganismos oleaginosos geralmente requer três unidades de operação: fermentação, separação de células e extração e refinação. O planejamento da composição de meios de crescimento é um fator importante para fermentações industriais, do ponto de vista econômico que é estritamente afetado pelo custo de materiais utilizados. Os substratos mais rentáveis são os resíduos e subprodutos da indústria de alimentos e da agroindústria. Estes substratos após os seus pré-tratamentos apropriados são utilizados por fungos sob condições favoráveis em sistemas de

fermentação em batelada (descontínuo), batelada alimentada ou processos contínuos (CERTÍK; ADAMECHOVÁ; LAOTENG, 2012).

A fisiologia de fermentação de microrganismos oleaginosos é baseada na aplicação de meios ricos em fonte de carbono e quantidades restritas de outros nutrientes, especialmente nitrogênio. Este procedimento tipicamente resulta em um processo bifásico, onde a primeira fase é caracterizada por crescimento fúngico rápido até que os nutrientes de crescimento, diferente da fonte de carbono, sejam esgotados. O lipídeo é acumulado durante a segunda fase por meio de conversão do substrato de carbono em ácidos graxos e subsequente incorporação em triacilgliceróis (CERTÍK; ADAMECHOVÁ; LAOTENG, 2012).

Os resíduos agroindustriais normalmente apresentam quantidade considerável de carboidratos, lipídeos e, portanto, podem ser usados como fonte de carbono para o crescimento microbiano (BANAT et al., 2014). Assim, a utilização de uma fonte de carbono orgânico barato e abundante pode contribuir substancialmente para a redução de custos de produção. Consequentemente, várias fermentações de substratos de baixo custo como glicerol, efluente de resíduo de óleo de palma, águas residuais municipais, melaço, águas residuais de amido, e os hidrolisados de celulose, já foram testados com sucesso na capacidade de apoiar o crescimento de produção de lipídeos em microrganismos oleaginosos (SCHNEIDER et al., 2013).

2.7. PROCESSO DE EXTRAÇÃO DE LIPÍDEOS

A extração de lipídeos de biomassa microbiana exige o rompimento celular, que pode ser realizado utilizando processos químicos, físicos ou bioquímicos. A definição dos solventes a serem utilizados no processo, é um fator importante que deve ser levado em consideração. A escolha dos solventes para a extração de lipídeos deve ser realizada baseando-se em vários fatores adicionais como, volatilidade, capacidade para formar um sistema de duas fases com água (para remover não lipídeos), custo, toxicidade, poder de extração para as diferentes classes de lipídeos, entre outros (TONATO, 2015).

Vários métodos de extração de lipídeos foram desenvolvidos e estudados nos últimos tempos, utilizando na sua maioria solvente orgânico (JEON et al., 2013; ANTHONY; STUART, 2015). A utilização de equipamentos de extração automatizada, como o aparelho *Soxhlet* ou *Goldfisch*, também são muito utilizados para este processo. Apesar destes métodos de extração apresentarem inúmeras vantagens no processo extrativo ainda mostram certas características negativas em relação aos requisitos de extração ideal de lipídeos. O método

Bligh e *Dyer* utilizando misturas contendo clorofórmio e metanol (não polar/polar) é capaz de extrair completamente os lipídeos neutros e polares da biomassa, entretanto, a principal desvantagem desta técnica é a elevada toxicidade dos solventes e a geração grandes quantidades de resíduos de solventes perigosos. O interesse pela extração de lipídeos e a falta de eficiência de métodos já conhecidos, levaram ao desenvolvimento de novas técnicas de extração, entre elas, destacam-se o uso de micro-ondas e fluido supercrítico (SAHENA et al, 2009; HUSSAIN et al., 2014).

A redução nas etapas operacionais, operação segura devido ao uso de solventes não orgânicos e uso de temperatura moderada na faixa crítica favoráveis para alimentos termo sensíveis, são algumas vantagens da utilização da extração com fluido supercrítico (SFE) em relação aos demais processos convencionais de extração. Além disso, a utilização da SFE de lipídeos proporciona uma boa qualidade do produto resultante (SAHENA et al., 2009). Na Tabela 6 podem-se verificar as comparações entre as extrações utilizando os métodos de SFE e extração por solvente.

Tabela 6 - Comparação entre a extração com fluido supercrítico com CO_2 com extração por solvente.

Nº	Extração com solvente	Extração supercrítica
I-	A presença de solventes é inevitável. O nível residual (geralmente ppm) do solvente depende do tipo de solvente utilizado.	É totalmente livre de solventes e, portanto, muito puro.
II-	O teor de metais pesados também é inevitável e depende do solvente, do método de reciclagem do solvente, da fonte da matéria-prima e do material usado para construir as partes de contato da máquina.	Totalmente livre de metais pesados, uma vez que não são extraíveis, mesmo se estiverem presentes na matéria-prima. Não há metais pesados presentes no CO_2 ou no equipamento.
III-	O teor de sal inorgânico não pode ser evitado, usando o mesmo conceito acima.	
IV-	As substâncias polares dissolvem-se juntamente com as substâncias lipofílicas da matéria-prima do material devido à fraca seletividade do solvente. Durante as operações de remoção de solvente, estas substâncias polares formam polímeros, que levam à descoloração do extrato e empobrecem as características de fluxo. Tudo isso faz com que o extrato pareça diferente dos componentes básicos na matéria-prima.	Totalmente livre de sais inorgânicos usando a mesma explicação acima não existe essa possibilidade, uma vez que o CO_2 é altamente seletivo e não existe a possibilidade de substâncias polares formarem polímeros. Além disso, a temperatura de operação é de apenas 40-80 °C.
V-	A remoção de solvente requer operações unitárias extras, resultando em maior custo e menor recuperação de material útil.	Nenhuma operação extra da unidade é necessária e o rendimento do material útil é muito alto.

Fonte: (Adaptado de: SAHENA et al., 2009).

O processo de extração com fluido supercrítico vem sendo bastante relatado na literatura principalmente na área de bioprocesso, devido a suas diversas aplicações que incluem: extração de produtos de fermentação, compostos bioativos, bio-óleo, entre outros (DA SILVA; ROCHA-SANTOS; DUARTE, 2016; GANDHI; ARORA; KUMAR, 2017).

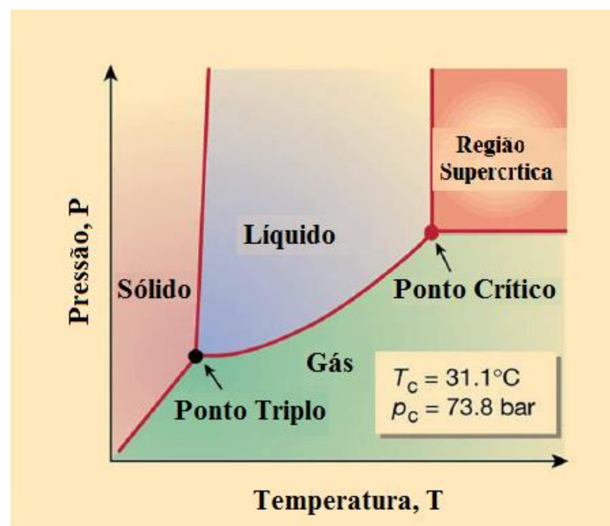
A extração com fluidos supercríticos (SFE) vem recebendo atenção como uma importante alternativa aos métodos convencionais. Entre os fluidos usados, o dióxido de carbono (CO_2) supercrítico é um importante solvente comercial e industrial na extração de compostos químicos, pois apresenta características como, baixa toxicidade, ser um solvente

"verde", seguro de manipular. Além disso, a temperatura relativamente baixa utilizada no processo de extração e a estabilidade do CO_2 permitem que a maioria dos compostos sejam extraídos sem que ocorra a sua desnaturação. Desta forma a extração supercrítica utilizando CO_2 como solvente tem sido usada para extração de compostos em áreas como ciência alimentar, produtos farmacêuticos, resíduos químicos, biocombustíveis e polímeros (NISHA; SANKAR; VENKATESWARAN, 2012; CABEZA et al., 2017).

2.7.1. Extração de lipídeos com CO_2 supercrítico

Nas extrações que utilizam tecnologia supercrítica, o dióxido de carbono (CO_2) é talvez o fluido mais procurado, devido apresentar algumas características como: ser uma substância inerte, não tóxica, não inflamável, não explosivo e por fim apresentar alta pureza a um valor razoável (GANDHI; ARORA; KUMAR, 2017). O dióxido de carbono age como um fluído supercrítico acima de sua temperatura crítica (304,25 K ou 31,1 ℃) e pressão crítica (7,38 Mpa ou 73,8 bar), (Figura 2), expandindo para encher o seu recipiente como um gás, mas com densidade similar à de um líquido. No momento em que os fluidos e gases são aquecidos acima da temperatura crítica e comprimidos acima de sua pressão crítica eles entram em uma fase supercrítica e, nesta etapa, algumas propriedades, como o poder solvente, pode ser bastante alterado (CABEZA et al., 2017).

Figura 2 - Diagrama Supercrítico de CO_2 p-T.



Fonte: (Adaptado de: CABEZA et al., 2017).

As extrações com SC-CO_2 são uma opção relevante na obtenção de óleos comestíveis. Atualmente várias pesquisas vêm sendo realizadas a respeito da utilização do solvente CO_2 supercrítico na extração de óleos ricos em PUFAs, extraídos de microrganismos oleaginosos como: alga marron, *Sargassum Hemiphyllum* e fungos, *Cunninghamella echinulata* e *Pythium irregulare*, para uso potencial em alimentos funcionai e nutracêuticos (SAHENA et al., 2009).

As extrações que utilizam somente CO_2 supercrítico normalmente promovem uma boa recuperação de lipídeos apolares. Por outro lado, os lipídeos polares, por serem menos solúveis em CO_2 supercrítico podem dificultar o processo. Para melhorar a extração destes lipídeos polares, a polaridade do fluido supercrítico CO_2 pode ser variada adicionando ao processo de extração co-solventes como metanol, etanol e água. Além da adição de co-solvente, para obter o máximo de recuperação de lipídeos no processo de extração com SC-CO_2, deve-se levar em consideração algumas características importantes da amostra como: tamanho da partícula, que interfere na recuperação lipídica uma vez que influencia na área de superfície e teor de umidade, que afeta a eficiência de extração uma vez que o alto teor de umidade age como uma barreira para a difusão de SC-CO_2 na amostra, fazendo-se necessário para melhorar a eficiência destas extrações a liofilização da amostra antes de ser extraída (SAHENA et al., 2009).

2.8. ADSORÇÃO

Adsorção é uma operação unitária que envolve transferência de massa de uma fase fluída (liquida ou gás) para a superfície e interior de uma fase sólida. Quando as moléculas contidas em um fluído (adsorbato) interagem com um sólido (adsorvente), uma força de atração entre o sólido e as moléculas do fluído provocam sua fixação na superfície do sólido (GOMIDE, 1980; MCCABE; SMITH; HARRIOTT, 1993).

Adsorção pode ser realizada por duas abordagens, sorção química e sorção física (KATHERESAN; KANSEDO; LAU, 2018). A adsorção química ou quimissorção é definida pela formação de fortes associações químicas entre moléculas ou íons de adsorbato, que é geralmente devido à troca de elétrons, onde geralmente o processo é irreversível. Já a adsorção física ou fisiossorção envolve as ligações intrapartículas fracas de *van der Waals* entre adsorbato e adsorvente, sendo assim a fisiossorção na maioria dos casos é reversível (YAGUB et al., 2014).

Entre os diversos métodos utilizados na remoção de corantes a adsorção surgiu como uma das técnicas preferidas de remoção destes contaminantes (corantes) devido à sua alta eficiência para remover quase qualquer tipo de corante. A adsorção é uma técnica muito utilizada para purificação de águas residuais indústriais e água potável (KATHERESAN; KANSEDO; LAU, 2018). A adsorção ainda é o método mais favorável para a remoção dos contaminantes (poluentes) das águas devido ao seu baixo custo de operação, facilidade operar e design simples (MO et al., 2018). A única desvantagem deste método era o alto custo dos adsorventes utilizados no processo, mas com as novas descobertas de adsorventes baratos, eficientes, esta técnica se tornou a mais utilizada na remoção de poluentes em todo o mundo (KATHERESAN; KANSEDO; LAU, 2018).

Diversas pesquisas vêm sendo realizadas a respeito do reaproveitando de resíduos de biomassas como biossorventes. Alternativas de baixo custo com grande potencial de adsorção (KHARAT, 2015; NOOR et al., 2017), para remover corantes, metais e outros contaminantes de águas residuais (YU et al., 2012; KEBAILI et al., 2018) com alta eficiência de adsorção.

Os biossorventes se enquadram principalmente nas seguintes categorias: microbiana, (fungos, algas, bactérias), subprodutos de atividades industriais (resíduos de fermentação, resíduos de alimentos/bebidas, lamas ativadas, lamas anaeróbias, etc.), subprodutos agrícolas (resíduos de frutas/vegetais, palhas de arroz, farelo de trigo, casca de soja, etc.), resíduos naturais (resíduos de plantas, serragem, cascas de árvores, ervas daninhas) e outros biomateriais (PARK; YUN; PARK, 2010). Na Tabela 7, podem-se verificar diversos tipos de resíduos (microbianos, naturais, subprodutos de atividades industriais e agrícolas) que vem sendo utilizados como biossorventes na literatura.

Tabela 7 - Diferentes tipos de biossorventes aplicados na remoção de poluentes de soluções aquosas.

Categoria	Espécie	Tipo de Biomassa	Poluente	Referência
Microrganismos				
Fungo	*Fusarium equiseti* - KR706303 e *Penicillium citrinum* - KR706304	Biomassa fúngica tratada quimicamente	Íons de chumbo e cobre	AKINKUNMI et al. (2016)
Fungo	*Flammulina velutipes*, *Auricularia polytricha*, *Pleurotus eryngii* e *Pleurotus ostreatus*	Resíduo de biomassa fúngica	Cobre, zinco e mercúrio	LI et al. (2018)
Fungo	*Pleurotus ostreatus* - BWPH, *Gleophyllum odoratum* - DCa e *Polyporus picipes* - RWP17	Biomassa imobilizada	Corantes - Diazo azul Evans e Verde brilhante	PRZYSTAŚ; ZABŁOCKA-GODLEWSKA; GRABIŃSKA-SOTA, (2018)
Fungo	*Phoma* sp.	Resíduo de biomassa fúngica de processo de produção de bioherbicida	Corante Vermelho Ácido 18 (AR 18)	DRUMM et al. (2019)
Fungo	*Diaporthe schini*	Resíduo de biomassa gerado na produção de metabólitos secundários.	Corante Cristal violeta (CV)	GRASSI et al. (2019)
Fungo	*Nigrospora* sp.	Resíduo de biomassa obtido na extração de óleo fúngico	Corante vermelho Procion H – E7B (PR H – E7B)	TONATO et al. (2019b)
Alga	*Scenedesmus* sp.	Residuo de biomassa obida da produção de biodiesel	Corante Ácido Azul 161 (AB-161)	DA FONTOURA et al. (2017)

Tabela 7 - *Continuação*

Categoria	Espécie	Tipo de Biomassa	Poluente	Referência
Alga	*Spirulina platensis*	Residuo de biomassa obida após o processo de produção de biodiesel	Íons de cromo	NITHYA et al. (2019)
Subprodutos de atividades industriais				
Casca de laranja	-	Resíduos de casca de laranja obtido na fábrica de suco.	Corantes – Azul de metileno (MB) e Índigo carmim (IC)	KEBAILI et al. (2018)
Bagaço de cana	-	Fuligem de bagaço de cana	Corante Azul de metileno	GIUSTO et al. (2017)
Subprodutos agrícolas				
Casca de arroz	-	Resíduo de casca de arroz	Corante vermelho Procion H – E7B (PR H – E7B	FOLETTO et al. (2013)
Farelo de trigo	-	Resíduo de farelo de trigo	Corante Vermelho Ácido 18 (AR 18)	ZHANG et al. (2018)
Casca de soja	-	Casca de soja crua	Corante reativo vermelho BF-4B	MODENES et al. (2019)
Resíduos naturais				
Casca de cedro	*Cedrella fissilis*	Resíduo de processamento de madeira	Corante vermelho 97	GEORGIN et al. (2019)
Serragem da árvore Terminalia arjuna	-	Resíduo de serragem	Cristal violeta (CV)	SHAKOOR; NASAR, (2018)

Fonte: (Autora).

CAPÍTULO 3 – RESULTADOS

OS RESULTADOS E DISCUSSÃO DESTA TESE ESTÃO APRESENTADOS NA FORMA DE TRÊS ARTIGOS:

I - ARTIGO 1: SUBMERGED CULTIVATION OF *NIGROSPORA* SP. IN BATCH AND FED-BATCH MODES FOR PRODUCTION OF MICROBIAL OIL. Authors: Denise Tonato, Thiarles Brun, Luciana Luft, Marcela Bromberger Soquetta, Fernanda Caroline Drumm, Patrícia Grassi, Jordana Georgin, Raquel C. Kuhn, Marcio A. Mazutti. Submetido à *Biochemical Engineering Journal* de qualis A1, na área de engenharias II.

II - ARTIGO 2: ENHANCEMENT OF FATTY ACIDS IN THE OIL EXTRACTED FROM THE FUNGUS *NIGROSPORA* SP. BY SUPERCRITICAL CO_2 WITH ETHANOL AS A COSOLVENT. Authors: Denise Tonato, Luciana Luft, Tássia C. Confortin, Giovani L. Zabot, Marcio A. Mazutti. Artigo publicado na revista *Journal of Supercritical Fluids* (2019), https://doi.org/10.1016/j.supflu.2019.02.001 de qualis A1, na área de engenharias II.

III - ARTIGO 3: RESIDUAL BIOMASS OF *NIGROSPORA* SP. FROM PROCESS OF THE MICROBIAL OIL EXTRACTION FOR THE BIOSORPTION OF PROCION RED H–E7B DYE. Authors: Denise Tonato, Fernanda C. Drumm, Patrícia Grassi, Jordana Georgin, Ademir E. Gerhardta, Guilherme L. Dotto, Marcio A. Mazutti. Artigo publicado na revista *Journal of Water Process Engineering* (2019), https://doi.org/10.1016/j.jwpe.2019.100818 de qualis B1, na área de engenharias II.

3.1. ARTIGO I: SUBMERGED CULTIVATION OF *NIGROSPORA SP.* IN BATCH AND FED-BATCH MODES FOR PRODUCTION OF MICROBIAL OIL

Submerged cultivation of *Nigrospora* sp. in batch and fed-batch modes for production of microbial oil

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ABSTRACT

In this study was presented a strategy to maximize the biomass concentration and the lipid accumulation by the fungus *Nigrospora* sp. in submerged fermentation. Different media composition and process variables, in batch and fed-batch modes, were investigated in shaken flasks. In the bioreactor the influence of stirring rate and aeration in the batch and fed-batch mode was investigated. Maximum biomass concentration and lipid accumulation were 40.17 $g.L^{-1}$ and 21.32 wt% in bioreactor that were 2.1 and 5.4 times higher than the same condition in shaken flasks, respectively. The results presented information relevant to the production of fungal lipids, since there are few works exploring the fed-batch strategy to increase the yield of fungi lipids, as well as few works regarding the exploitation of the fungus of the genus *Nigrospora* sp. to produce lipids.

Keywords: Lipid accumulation; oleaginous microorganisms; fatty acids; bioreactor operation

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3.1.1. INTRODUCTION

Oleaginous microorganisms are microbial species that can accumulate significant amounts of triacylglycerols [1]. Bacteria, algae and fungi are used to produce microbial oil. Several species of filamentous fungi such as *Aspergillus oryzae*, *Claviceps purpurea*, *Humicola lanuginose*, *Mortierella isabellina*, *Mortierella vinacea* and *Mucor circinelloides* have been widely reported in the literature as microorganisms capable of accumulating lipids [2]. In particular, the oils produced by fungi, besides presenting a great potential for the production of biofuels [3,4,5], are also an alternative for the production of essential polyunsaturated fatty acids (PUFAs) [6], such as arachidonic (ARA) [7,8,9], γ- linolenic (GLA) [10], eicosapentaenoic (EPA) [11] and docosahexaenoic acids (DHA) [12].

The fungi of the genus *Nigrospora* sp. are widely studied in the literature to produce several bioactive secondary metabolites [13,14,15]. However, the production of lipids is not well addressed. Peng and Cheng [16] isolated 5 fungi with potential in the production of microbial oil, among them one of the species *Nigrospora* sp., which produced about 20 wt% of microbial oil by solid state fermentation. Tonato et al. [17] evaluated the lipid production capacity of 150 fungal strains isolated from the Pampa Biome, the genus *Nigrospora* sp., showed promising results with maximum lipid accumulation of 11.28 wt% of which 51% were PUFA. However, the lipid accumulation should be higher to feasible an industrial process for production of microbial oil. Alternatives to increase the lipid accumulation is to modify the fermentation media and process conditions, as well as investigate the possibility to operate the process in the fed-batch mode, [18,19,20,21].

The choice of the carbon source is an important factor, since it can significantly influence the yield and lipid composition of the microbial oil, due to differences in the metabolism of microorganisms [22,23]. In addition, the cost of raw material is an important factor to be considered in the definition of total production costs [4]. Substrates as glucose, lactose, starch, oils, corn steep liquor, xylose, glycerol, acetic acid, ethanol are some of the carbon sources used to produce microbial oil, [4,24,25,26,27,28].

The nature and mode of cultivation of the microorganism, can considerably influence the accumulation of lipids of an oleaginous species [29]. The fermentation strategy (batch, fed-batch, repeated fed-batch) is also a very important factor to be analyzed in the microbial fermentation processes, since it can significantly influence the final yield of the bioproduct [30]. Several studies are reported in the literature, using batch fermentation to produce lipids

by different species of fungi, [31,32,5,33,34]. The fed-batch cultivation has shown to be very efficient in the biotechnological process to produce several bioproducts. Several studies are reported in the literature using this technique to increase lipid production in different microorganisms as a strategy to increase the yield of microbial lipids [35,36,37].

Based on these aspects, the aim of the present study was to maximize the biomass concentration and the lipid accumulation by the fungus *Nigrospora* sp. in submerged fermentation. Initially, different media composition and process variables, in batch and fed-batch modes, were investigated in orbital shaker. In bioreactor, the influence of stirring rate and aeration was investigated in batch and fed-batch mode. Detailed composition of microbial oil was presented for all fermentations.

3.1.2. MATERIAL AND METHODS

3.1.2.1 Microorganism

The fungus *Nigrospora* sp. was isolated and identified in previous study of Tonato et al. [17]. The fungus was cultivated in Petri dishes with potato dextrose agar (PDA) medium for 7 days at 28°C under dark conditions.

3.1.2.2. Fermentations

3.1.2.2.1 Shaken flasks – Batch

A disc (6 mm) of the mycelium of *Nigrospora* sp. was inoculated in Erlenmeyer flasks containing 150 mL of sterilized liquid medium (autoclaved at 121°C for 20 minutes) at pH 5.8. The medium was composed of glucose (10 $g.L^{-1}$), yeast extract (7.5 $g.L^{-1}$), peptone (10 $g.L^{-1}$), ammonium sulfate (2 $g.L^{-1}$), magnesium sulfate (0.5 $g.L^{-1}$), ferrous sulfate (1 $g.L^{-1}$) and manganese sulfate (1 $g.L^{-1}$; $MnSO_4$. H_2O). The submerged fermentation was performed in an orbital shaker (Innova 44R, New Brunswick, EUA) at 150 rpm for 7 days in triplicate.

The influence of temperature (25, 28 e 33 °C), variation of pH, glucose uptake on microbial growth, and lipids accumulation were evaluated. In the most appropriated temperature (28°C), the media was modified. The first two experiments were carried out at glucose concentration of 10 (B_{G10}) and 100 $g.L^{-1}$ (B_{G100}), maintaining other nutrients fixed.

The third one was carried out using 100 $g.L^{-1}$ of glucose, nitrogen and micronutrients were substituted by 10 % ($v.v^{-1}$) of corn steep liquor (B_{G100_CSL10}).

3.1.2.2.2 Shaken flasks – Fed-batch

Fed-batch fermentation was carried out by supplementing aseptically the media at second day of fermentation (point where all initial glucose was uptake) with a mass of glucose (FB_{G20}, FB_{G60}, FB_{G100}) or sucrose (FB_{S20}, FB_{S60}, FB_{S100}) corresponding to 20, 60 and 100 $g.L^{-1}$, respectively. The initial media (150 mL) was composed of glucose (10 $g.L^{-1}$), yeast extract (7.5 $g.L^{-1}$), peptone (10 $g.L^{-1}$), ammonium sulfate (2 $g.L^{-1}$), magnesium sulfate (0.5 $g.L^{-1}$), ferrous sulfate (1 $g.L^{-1}$) and manganese sulfate (1 $g.L^{-1}$; $MnSO_4$. H_2O). Other fed-batch fermentation was carried out by adding a mass of glucose corresponding to 100 $g.L^{-1}$ in the second day of fermentation in the initial media composed of 100 $g.L^{-1}$ of glucose and 10 % ($v.v^{-1}$) of corn steep liquor (FB_{G100_CSL10}).

3.1.2.2.3 Bioreactor – Batch

The process was scaled-up to a STR bioreactor (Tecnal, model Biotec-C, Brazil) with a useful volume of 5L. All fermentations were carried out with a working volume of 2L during 7 days at specified aeration and stirring rate, according experimental design. The media was composed of glucose (100 $g.L^{-1}$), yeast extract (7.5 $g.L^{-1}$), peptone (10 $g.L^{-1}$), ammonium sulfate (2 $g.L^{-1}$), magnesium sulfate (0.5 $g.L^{-1}$), ferrous sulfate (1 $g.L^{-1}$) and manganese sulfate (1 $g.L^{-1}$; $MnSO_4.H_2O$) at pH 5.8. Inoculum (150 mL) presented the same composition of fermentation media. The inoculum was obtained using a disc (6 mm) of the mycelium of *Nigrospora* sp. was inoculated in Erlenmeyer flasks and incubated at 28°C at 150 rpm during 5 days in an orbital shaker (Innova 44R, New Brunswick, USA). A central composite design was conceived to evaluate the influence of aeration (0.5 to 2.0 vvm – volume of air per volume of media per minute) and stirring rate (100 – 200 rpm) in the biomass concentration and lipid accumulation.

3.1.2.2.4 Bioreactor – Fed-batch

Fed-batch fermentation was carried out with initial volume of 1 L with ascetic addition

of 200 mL of media each 24 hours from second day until sixth day of fermentation, resulting in a final volume of 2 L. The total cultivation time was kept constant at 7 days. The aeration and stirring rate were maintained at 2 vvm and 100 rpm, respectively.

3.1.2.2.5 Analyses

After the fermentations, the samples were filtered (Whatman filter paper, grade 1) using a vacuum pump. Sugar concentration was determined by DNS method [38] in the liquid media. The retained biomass was washed with distilled water and filtered again. The samples of this washed retained biomass were frozen in ultra-freezer (FV-500, New Brunswick, Canada) for 48 h. Thereafter, the samples of fungal biomass were freeze-dried (L 101, Liotop, Brazil) for 24 h. The dried solid material was stored in sealed impermeable flasks until the extractions and also used for determination of biomass concentration.

Kinetics of the fermentations were carried out to determine the influence of temperature on microbial growth and lipid accumulation. The kinetics was determined in duplicate. For determination of kinetic curves, it was adopted the destructive sampling procedure, where each point (interval of 24 hours) of curve corresponds to one Erlenmeyer. For other fermentations, only initial and final values of dependent variables were determined. Yields parameters such as: cells from substrate ($Y_{X/S}$), products from cells ($Y_{P/X}$) and products from substrate ($Y_{P/S}$) were calculated for some conditions.

3.1.2.3. Microbial oil extraction

The microbial oil was extracted from fungal biomass in a *Soxhlet* apparatus (MA491/6, Marconi, Brazil) for 2 hours using n-Hexane (purity of 99%, Alphatec, Brazil) as solvent. Experimental details may be found in the study of Tonato et al. [39].

3.1.2.4. Characterization of fatty acids

For characterization of fatty acids profile, a fraction of microbial oil was first esterified, derivatized and, in the sequence, analyzed in a gas chromatograph (Shimadzu, model MDGC/GCMS-2010), equipped with an autoinjector (AOC-20i), an autosampler (AOC-20s) and a flame ionization detector (FID-2010 Plus). Quantification of fatty acids

(mg/g of oil) was made by comparing the retention time of the sample with external standards (FAME Mix-37, P/N 47885-U, Sigma-Aldrich, USA). Experimental details may be found in the study of Tonato et al. [39].

3.1.2.5. Statistical analysis

Data were submitted to analysis of variance (ANOVA) followed by Tukey´s test with a significance level of 95%, using the software Statistica® 8.0.

3.1.3. RESULTS AND DISCUSSION

3.1.3.1. Microbial growth and lipid accumulation in shaken flasks

Figure 1 presents the sugar, biomass and pH profiles obtained in the different temperatures (25, 28 and 33 °C) during 7 days of fermentation. The pH of medium, independently of temperature, showed two distinct behavior. In the first two days of fermentation the pH decreased slightly (5.8 to 5.3). In the same period, ~80% of initial sugar was consumed by the fungus. From second to seventh day of fermentation the pH raised until close to 8.0. These characteristics show a relationship between pH variation in the fermentation process and the consumption of glucose by the fungus. According to Patakova et al. [40], these changes in the pH during the period of microbial growth are dependent on the carbon and nitrogen sources of the fermentation medium. The reduction of pH in the first two day is due to the production of organic acids during the metabolism of carbon source [41,25]. Probably, after the depletion of sugar the fungus metabolize the acids produced in the first stage, increasing the pH of the medium [42].

The biomass showed similar profiles for the three temperatures evaluated. At 25 and 28°C, the steady state was reached in the fourth day of fermentation, whereas for 33°C biomass was slightly increasing until seventh day. Temperature affects the initial rate of sugar consumption, where the highest initial rate was obtained for the temperature of 28°C. The growth of *Nigrospora* sp. was related to the consumption of sugar in the media, since biomass was accumulated until all sugar be metabolized. Afterwards, the growth was stabilized, showing that the final concentration of biomass is dependent of sugar concentration in the

media [43].

Table 1 presents the biomass concentration, lipid accumulation and biochemical parameters for three temperatures evaluated after 7 days of fermentation. Biomass concentration was not influenced by temperature ($p<0.05$), highest value was obtained at 33°C (11.88 $g.L^{-1}$). Temperatures around 30 ºC are frequently most appropriated for fungi cultivation aiming to obtain a high biomass concentration, because around this temperature the enzymes show increased metabolic activity [43]. These data are also in agreement with [44], which cultivated the *Mortierella alpina* for the production of lipids in a temperature range of 20 to 28 ºC and obtained the highest concentration of biomass (17.7 $g.L^{-1}$) at 28°C.

At higher temperatures (28 and 33 ºC), the lipid yield was also higher (1.24 and 1.02 wt%), respectively, in comparison with 0.84% obtained at 25ºC. Many studies in literature report maximum production of lipids for temperature close to 30°C for different fungi [45,46,44]. The highest values of biochemical parameters such as μ_{max}, $Y_{X/S}$, $Y_{P/S}$ and $Y_{P/X}$ were observed at the temperature of 28°C.

From analysis of data presented in Figure 1 and Table 2 is possible to obtain two main responses: 28°C is the most appropriated temperature and biomass concentration is dependent of sugar concentration. It is evident that the values of the biomass concentration and lipid accumulation obtained in this first set of experiments is not enough to feasible the bioprocess for production of microbial oil. So, other strategies should be proposed.

Table 2 presents data referring to cultivation of *Nigrospora* sp. in shaken flasks using different media composition in batch and fed-batch fermentations. All fermentations were carried out at 28°C. Increasing initial glucose concentration from 10 to 100 $g.L^{-1}$ in batch fermentations increased the final biomass concentration as well as the lipid accumulation. The batch fermentation using glucose and CSL as nutrient source lead to a biomass concentration of 22.2 $g.L^{-1}$, but the lipid accumulation was lower in comparison with $B_{G100.}$ The low value of lipid accumulation in the fermentation B_{G100_CSL10} may be attributed to the reduction of C/N ratio, since CSL is a nitrogen-rich source [47]. The fed-batch fermentations were not effective to increase the biomass concentration and lipid accumulation. FB_{G100_CSL10} was effective to increase biomass concentration but fail in the lipid accumulation. Both carbon sources evaluated in the fed-batch fermentations (glucose and sucrose) presented similar biomass concentration (~13 $g.L^{-1}$) at concentration higher than 60 $g.L^{-1}$, however, glucose, showed to be more effective on lipid accumulation (~15% more lipid than using sucrose as carbon source). These results are corroborated by [48], that using the fungus *Cunninghamella*

echinulata with several carbon sources (glucose, xylose and lignocellulosic residues) in shaken flasks, found that the ideal condition to obtain the accumulation of lipid and high biomass concentration was using glucose as carbon source at concentration of 100 gL^{-1}.

3.1.3.2. Microbial growth and lipid accumulation in STR bioreactor

In the third set of experiments, aiming to increase the biomass concentration in the media as well as the lipid accumulation, fermentations were carried out in a STR bioreactor in batch and fed-batch modes (Table 3). The use of bioreactor was effective to increase biomass concentration and lipid accumulation. In batch mode, maximum biomass concentration was 32.50 $g.L^{-1}$ with 16.7 wt% of lipid accumulation (Assay 3). Comparing with fermentation in shaken flasks (B_{G100}), biomass concentration increased 72.9% and lipid accumulation 324%. One reason for this result is the aeration that was considerably improved in the STR bioreactor in relation to shaken flasks [49]. The importance of aeration in the biomass production and lipid accumulation may be done by comparing assays 1 and 3 (stirring rate of 100 rpm) and 2 and 4 (stirring rate of 200 rpm). In both situations, the increase of the aeration from 0.5 to 2 vvm improved the biomass production and lipid accumulation. This increase was more accentuated in runs with the lowest stirring rate (100 rpm).

The interaction of stirring rate and aeration on biomass and lipid accumulation is well explained in the literature. The increase of stirring rate increases the shear forces, which may influence the morphology of the microorganism, decrease viscosity and increase the rate of oxygen transfer, as well as damaging the mycelium, causing a reduction of productivity. In contrast, low stirring rates promote a reduction of dissolved oxygen concentration in the system, which may be detrimental to the microorganism in the biosynthesis of secondary metabolites [50,51,49,52].

Saad et al. [32] , using filamentous fungus *Cunninghamella bainieri* 2A1 in a 5 L bioreactor for lipid production, reported that the control of stirring rate and aeration was important to increase lipid production in the bioreactor. Chatzifragkou et al. [33] cultivated *Mortierella isabellina* in a 3-L batch bioreactor with glucose as substrate, verifying that with a lower stirring rate and high concentration of dissolved oxygen (DO), obtained higher values of biomass and lipid content when compared to the values obtained in the assays in shake flasks.

A fed-batch fermentation was carried out in the best condition (Assay 8) at 100 rpm and 2 vvm to biomass production and lipid accumulation in batch fermentations in STR bioreactor. According to the results, in fed-batch STR fermentations, biomass and lipid increased 23.6 and 27.6%, respectively. At this point, it was reached a process condition with satisfactory biomass and lipid accumulation and comparable to other successful studies focusing on production of microbial oil with conventional oleaginous microorganism.

The use of fed-batch fermentation to increase the yield of biomass and lipids of oil-producing microorganisms is more efficient when compared to batch mode. In batch cultivation, the nutrients are added only once while in the fed-batch the nutrients are added during the fermentation, preventing the inhibition by carbon source [53]. Several studies have been carried out using this fermentation strategy (fed batch) to increase the accumulation of lipids in various oil-producing microorganisms. Tchakouteu et al. [35] produced 37.2 $g.L^{-1}$ of biomass and 64.5 wt% of lipid in fed-batch in bioreactor with *Rhodosporidium toruloides*. These values were higher than those obtained in batch fermentation. In other studies, using oleaginous microorganisms grown in fed-batch mode, such as the *Chlorella sorokiniana* microalgae [37], the yeasts *Trichosporon oleaginosus* [20,36], *Rhodotorula glutinis* [54], and *Rhodosporidium toruloides* Y4 [55], the efficacy of this mode of cultivation to increase the biomass and lipid concentration was confirmed.

3.1.3.3. Fatty acids profile

The fatty acids composition of the lipids obtained from the biomass of the fungus *Nigrospora* sp. is presented in Table 4. The fatty acids with the greatest concentration, independent of fermentation mode, identified in the lipids extracted from this fungus were: oleic acid (C18:1n9c), linoleic acid (C18:2n6c), palmitic acid (C16:0). In general way, it can be observed that the concentration of fatty acids was different in function of cultivation in shaken flasks or bioreactor. The mode of conduction of fermentation also affected the fatty acid composition. In shaken flasks, higher concentration of monounsaturated fatty acids (MUFA) was obtained, in sequence, saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), ranging from 130.785 to 370.927 $mg.g^{-1}$ oil, 98.329 to 207.833 $mg.g^{-1}$ oil, and from 49.442 to 169.052 $mg.g^{-1}$ oil, respectively.

In bioreactor, the fatty acid profile was like the shaken flasks (MUFA>SFA>PUFA), but the concentrations of fatty acids were lower. On the other hand, only in fermentations

carried out in bioreactor was verified the synthesis of essential PUFA such as arachidonic fatty acid (ARA C20:4n6), docosahexaenoic fatty acid (DHA, C22:6n3) and acid eicosapentaenoic (EPA, C20:5n3). The improvement in the mixing and oxygen transfer rate inside the bioreactor had a positive influence on the synthesis of long chain PUFAs [56], mainly ARA, DHA and EPA, which are highly desirable due to their positive effect of human health [57]. Similar result was obtained by [58] that obtained arachidonic acid only in the fed-batch cultivation in bioreactor.

3.1.4. CONCLUSIONS

In this study was presented a strategy to maximize the biomass concentration and the lipid accumulation by the fungus *Nigrospora* sp. in submerged fermentation. Maximum biomass concentration and lipid accumulation were 40.17 $g.L^{-1}$ and 21.32 wt%, respectively. These values were obtained with bioreactor operating at 100 rpm and 2 vvm in the fed-batch mode, with glucose concentration of 100 $g.L^{-1}$ and intermittent feeding strategy from second to sixth day of fermentation. Final biomass concentration and lipid accumulation were 2.1 and 5.4 times greater than the same condition in shaken flasks, respectively. The results presented provide information relevant to the production of fungal lipids, since there are few works exploring the fed-batch strategy to increase the yield of fungi lipids, as well as few works regarding the exploitation of the fungus of the genus *Nigrospora* sp. to produce lipids.

ACKNOWLEDGEMENTS

The authors thank the National Council for Scientific and Technological Development (CNPq) for financial this study.

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Figure captions

Figure 1 - Influence of temperature on kinetic profiles of sugar, biomass and pH in batch shaken flasks

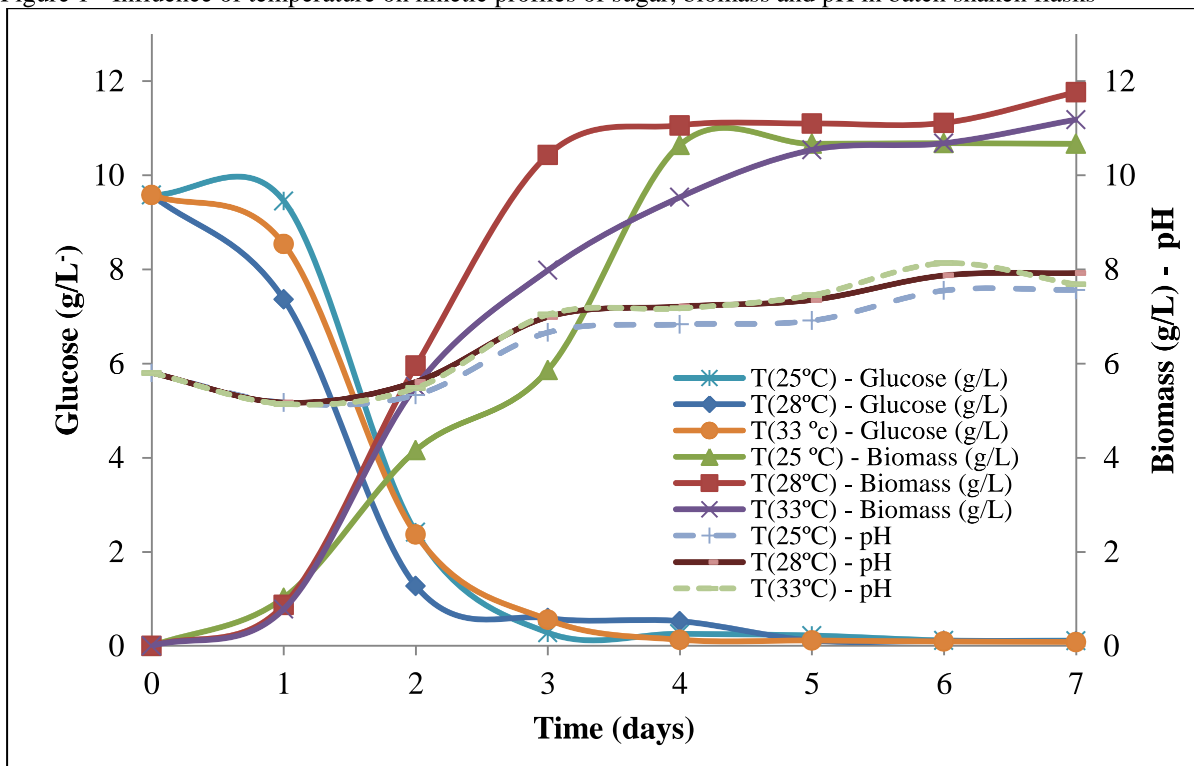


Table 1 - Influence of temperature on biomass concentration, lipid accumulation and biochemical parameters.

Temperature (ºC)	Biomass ($g.L^{-1}$)	μ_{max} (h^{-1})	$Y_{X/S}$ ($g.g^{-1}$)	$Y_{P/X}$ ($g.g^{-1}$)	$Y_{P/S}$ ($g.g^{-1}$)	Lipid accumulation (wt%)
25	10.67^{a}	1.40	1.13^{b}	0.0084^{c}	0.0896^{c}	0.84^{c}
28	11.76^{a}	1.81	1.24^{a}	0.0124^{a}	0.0145^{a}	1.24^{a}
33	11.88^{a}	1.76	1.18^{b}	0.0102^{b}	0.0114^{b}	1.02^{b}

[a-c] Different letters in the same column indicates significant statistical difference ($p<0.05$) by Tukey´s Test

Table 2 - Lipid accumulation and biomass concentration obtained in the cultivation of *Nigrospora* sp. in shaken flasks using different media composition in batch and fed-batch.

Fermentation	Biomass ($g.L^{-1}$)	Microbial oil (wt%)	$Y_{X/S}$ ($g.g^{-1}$)	$Y_{P/X}$ ($g.g^{-1}$)	$Y_{P/S}$ ($g.g^{-1}$)
B_{G10}	11.76^{cd}	1.24^{f}	1.24^{a}	0.012^{e}	0.015^{a}
B_{G100}	18.80^{b}	3.94^{a}	0.20^{b}	0.039^{a}	0.008^{b}
B_{G100_CSL10}	22.19^{b}	1.92^{d}	0.21^{b}	0.019^{bcde}	0.004^{c}
FB_{G20}	6.20^{d}	1.40^{ef}	0.06^{e}	0.015^{de}	0.001^{d}
FB_{G60}	13.07^{c}	3.33^{b}	0.12^{cd}	0.033^{ab}	0.004^{c}
FB_{G100}	13.13^{c}	3.06^{b}	0.13^{c}	0.030^{abc}	0.004^{c}
FB_{S20}	9.40^{cd}	1.30^{f}	0.09^{de}	0.013^{e}	0.001^{d}
FB_{S60}	12.00^{c}	2.73^{c}	0.11^{cd}	0.028^{abcd}	0.003^{c}
FB_{S100}	13.07^{c}	2.60^{c}	0.13^{c}	0.026^{abcde}	0.003^{c}
FB_{G100_CSL10}	29.93^{a}	1.70^{de}	0.15^{c}	0.017^{cde}	0.003^{c}

[a-f] Different letters in the same column indicates significant statistical difference ($p<0.05$) by Tukey´s Test.
B: batch
FB: fed-batch

Table 3 - Influence of stirring rate and aeration on biomass concentration and lipid accumulation in STR bioreactor in batch and fed-batch.

Run	Fermentation mode	Stirring rate (rpm)	Aeration (vvm)	Biomass ($g.L^{-1}$)	Lipid (wt%)
1	Batch	100 (-1)	0.5 (-1)	10.15^{f}	9.02^{de}
2	Batch	200 (+1)	0.5 (-1)	17.55^{e}	15.09^{bc}
3	Batch	100 (-1)	2.0 (+1)	32.50^{b}	16.71^{b}
4	Batch	200 (+1)	2.0 (+1)	27.79^{bc}	15.93^{bc}
5	Batch	150 (0)	1.25 (0)	23.26^{cd}	10.57^{d}
6	Batch	150 (0)	1.25 (0)	22.34^{de}	12.05^{cd}
7	Batch	150 (0)	1.25 (0)	24.15^{cd}	9.67^{de}
8	Fed-batch	100 (-1)	2.0 (+1)	40.17^{a}	21.32^{a}

$^{a-f}$ Different letters in the same column indicates significant statistical difference ($p<0.05$) by Tukey´s Test

Table 4 - Fatty acids (mg.g^{-1} oil) quantified in the oil extracted from the fungus

Fatty acid	Fermentation in shaken flasks									Fermentation in STR Bioreactor					
	FB_{G100}	FB_{G60}	FB_{G20}	FB_{S100}	FB_{S60}	FB_{S20}	B_{G100}	B_{G100_CSL10}	FB_{G100_CSL10}	1	2	3	4	5/6/7	8
C4:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.003	0.009	0.042	0.003	0.010	0.004
C6:0	0.168	0.211	0.142	0.288	0.296	0.248	0.938	0.019	0.030	1.034	2.394	7.770	1.255	1.486	0.129
C8:0	0.109	0.069	0.112	0.261	0.116	0.092	0.278	0.024	0.046	1.120	1.061	1.272	0.245	0.825	0.049
C10:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.111	0.106	0.108	0.049	0.125	0.026
C11:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.022	0.018	0.032	0.013	0.019	0.000
C12:0	0.131	0.095	0.547	0.241	0.111	0.147	0.158	0.084	0.196	0.110	0.115	0.167	0.055	0.110	0.065
C13:0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.078	0.090	0.096	0.109	0.106	0.187
C14:0	1.946	2.211	1.650	4.629	2.028	1.019	4.543	2.187	3.545	1.711	1.580	1.678	0.710	2.103	1.180
C14:1	0.122	0.198	0.332	0.278	0.073	1.566	0.246	0.097	0.138	0.065	0.045	0.040	0.121	0.054	0.019
C15:0	0.978	1.090	1.056	1.375	1.483	1.065	1.535	1.078	1.051	0.572	0.578	0.328	0.171	0.542	0.214
C16:0	69.976	70.762	49.444	151.579	99.004	53.546	141.965	87.279	75.940	57.469	56.567	57.303	26.842	65.666	35.357
C16:1	8.042	10.597	0.150	16.056	8.549	5.060	17.314	10.147	9.302	3.378	2.220	0.801	0.615	1.035	1.966
C17:0	0.517	0.584	0.704	0.681	1.036	0.826	0.693	0.696	0.398	0.705	0.748	0.767	0.403	1.420	0.268
C17:1	0.954	1.105	1.201	1.282	1.758	1.290	0.998	1.601	0.710	1.237	0.910	0.435	0.228	0.593	0.394
C18:0	9.843	11.166	10.838	15.457	18.476	12.031	38.903	14.676	16.062	19.269	17.851	24.933	15.992	49.657	12.957
C18:1n9c	171.510	204.981	168.961	331.660	328.986	164.545	211.036	277.781	109.603	149.410	112.527	112.345	40.238	108.810	39.466
C18:1n9t	9.750	14.126	7.632	18.767	14.441	6.420	17.881	20.790	9.154	0.113	0.062	0.064	0.085	0.064	0.023
C18:2n6c	52.002	47.777	68.312	84.240	86.194	64.390	54.807	162.874	87.338	22.625	12.378	9.324	14.452	29.444	41.820
C18:2n6t	0.163	0.165	0.395	0.248	0.270	0.228	0.220	0.191	0.344	0.233	0.277	0.288	0.251	0.635	0.087
C18:3n3	0.099	0.121	2.769	2.247	2.007	2.307	1.362	5.665	2.183	0.571	0.222	0.109	0.655	0.449	2.449

Table 4 - Continued

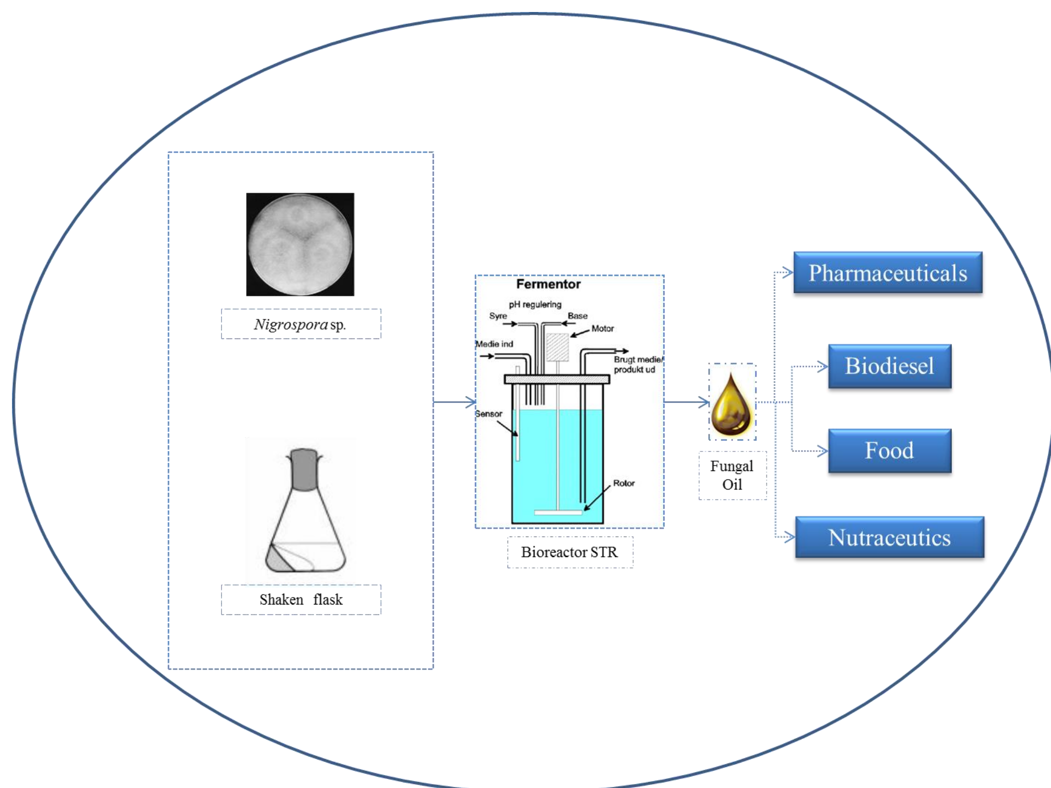
Fatty acid	Fermentation in shaken flasks									Fermentation in STR Bioreactor					
	FB_{G100}	FB_{G60}	FB_{G20}	FB_{S100}	FB_{S60}	FB_{S20}	B_{G100}	B_{G100_CSL10}	FB_{G100_CSL10}	1	2	3	4	5/6/7	8
C20:0	0.189	0.098	0.168	0.153	2.618	2.008	3.172	1.908	1.469	1.479	1.281	1.573	0.682	1.690	0.658
C20:1n9c	1.280	1.735	1.446	2.392	2.332	1.253	1.539	3.153	1.216	3.230	3.162	3.508	1.613	2.572	0.498
C20:2	0.293	0.317	0.416	0.491	0.420	0.435	0.358	0.966	0.398	0.178	0.251	0.065	0.056	0.163	0.220
C20:3 n3	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.089	0.063	0.042	0.035	0.036	0.041
C20:4 n6	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.031	0.024	0.048	0.041	0.019	0.049
C20:5n3	0.736	1.380	1.016	0.862	1.690	1.905	1.325	0.322	0.217	0.090	0.070	0.144	0.057	0.769	0.057
C22:0	0.408	1.084	0.989	0.506	1.414	1.871	0.895	0.203	0.215	0.934	1.496	1.844	0.749	1.825	0.653
C22:1n9	0.666	2.073	1.176	1.148	3.345	2.104	2.484	0.689	0.662	0.893	0.603	0.645	0.866	1.732	0.415
C22:2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.597	0.267	0.246	0.107	0.331	0.081
C23:0	12.525	12.493	35.108	20.643	19.433	39.915	10.400	12.192	30.250	4.559	4.435	3.618	4.931	3.873	8.314
C24:0	1.538	1.879	2.029	2.060	2.437	2.578	4.353	3.044	2.214	2.316	1.300	1.204	0.768	1.392	1.235
C22:6 n3	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.147	0.942	0.806	0.427	0.626	0.261
C24:1	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.942	1.640	1.430	0.750	1.023	0.153
∑SFA	98.329	101.742	102.786	197.873	148.453	115.347	207.833	123.392	131.417	92.594	91.730	105.878	57.027	135.956	67.323
∑MUFA	191.952	233.058	180.139	370.927	356.559	180.568	251.499	314.259	130.785	160.270	121.169	119.267	44.517	115.881	42.933
∑PUFA	53.000	49.442	72.492	87.598	90.161	68.830	57.714	169.052	90.081	25.560	14.494	11.073	16.080	32.474	45.064

C4:0 - Butyric acid; C6:0 - Caproic acid; C8:0 - Caprylic acid; C10:0 - Capric acid; C11:0 - Hectic Acid; C12:0 - Lauric acid; C13:0 - Isomyrisic acid; C14:0 - Myristic acid; C14:1 - Myristoleic acid; C15:0 - Pentadecanoic acid; C16:0 - Palmitic acid; C16:1 - Palmitoleic acid; C17:0 - Margaric acid; C17:1 - Heptadecenoic acid; C18:0 - Stearic acid; C18:1n9c - Oleic acid; C18:1n9t - Elaidic acid; C18:2n6c - Linoleic acid; C18:2n6t - Linolelaidic acid; C18:3n3 - Linolenic acid; C20:0 - Arachidic acid; C20:1n9c - Cis-11-Eicosenoic acid; C20:2 - Cis-11,14-Eicosadienoic acid; C20:3 n3 - Eicosatrienoic acid; C20:4 n6 - Arachidonic acid (ARA); C20:5n3 - Eicosapentaenoic acid (EPA); C22:0 - Behenic acid; C22:1n9 - Erucic acid; C22:2 - 13,16-docosadienoic acid; C23:0 - Tricosanoic acid; C24:0 - Lignoceric acid; C22:6 n3 - Docosahexaenoic acid (DHA); C24:1 - Nervonic acid.; ΣSFA - Sum of saturated fatty acids; ΣMUFA - Sum of monounsaturated fatty acids ; ΣPUFA – Sum of polyunsaturated fatty acid.
Note: nd = Not detected

HIGHLIGHTS

- The increase of the carbon source positively influenced the accumulation of lipids by the fungus *Nigrospora* sp.
- The use of the batch STR bioreactor had a significant influence on the accumulation of essential polyunsaturated fatty acids.
- Optimization of the fed-batch STR bioreactor has improved lipid yield

GRAPHICAL ABSTRACT



3.2. **ARTIGO II**: ENHANCEMENT OF FATTY ACIDS IN THE OIL EXTRACTED FROM THE FUNGUS *NIGROSPORA* SP. BY SUPERCRITICAL CO_2 WITH ETHANOL AS A COSOLVENT

Enhancement of fatty acids in the oil extracted from the fungus *Nigrospora* sp. by supercritical CO_2 with ethanol as a cosolvent

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ABSTRACT

Fatty acids rich-oil was extracted from freeze-dried fungal biomass of *Nigrospora* sp. using ultrasound with ethanol as a cosolvent + supercritical CO_2. The parameters of temperature (40-80°C), pressure (15-25 MPa), ultrasound pre-treatment time (15-60 min), and the addition of ethanol (30-70 g/100 g biomass) were evaluated on the oil yield and composition. The best oil yield (3.94 wt.%) and total fatty acids (45.5 g/100 g oil) were obtained at 80°C and 25 MPa with 50 wt.% ethanol after pre-treating the biomass for 60 min using 132 W of ultrasound power at 40 kHz. The main fatty acids were oleic, palmitic, linoleic, elaidic and palmitoleic acids. The combination of CO_2 under supercritical conditions and ultrasound with ethanol as a pre-treatment of the biomass from *Nigrospora* sp. concentrated up to 4.0 times fatty acids in the oil when compared to the *Soxhlet* technique, especially the unsaturated ones.

Keywords: supercritical fluid extraction; SFE; oleic acid; linoleic acid; ultrasound; fungal biomass.

3.2.1. INTRODUCTION

Microbial oils accumulated by oleaginous microorganisms such as bacteria, algae, and fungi are being used as a sustainable/renewable source of fuels and/or a valuable source of phytochemicals to food-related areas [1, 2]. Some oleaginous microorganisms can accumulate lipids quickly and with the composition of fatty acids like those of vegetable oils [3]. The similarity in the composition includes C16 and C18 fatty acids, such as palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and α-linolenic (C18:3) acids. Microbial oils can replace vegetable oils in the production of biodiesel [4] and in the production of cocoa butter and food supplements [5, 6]. Therefore, they do not compete with the use of food raw materials to produce biofuels and phytochemicals.

Recently, interest in lipids produced by fungi has increased because they are known for their rapid growth with short life cycles, and their cultivation is independent of climatic variations. Furthermore, lipid-producing fungi can be grown using a wide range of carbon sources as renewable substrates such as glycerol, sewage, whey, and molasses [7]. Oleaginous fungi have several unique characteristics, such as a fatty acid composition containing a high content of saponifiable lipids and free fatty acids, which are suitable for the production of biodiesel. Another important characteristic of these oleaginous fungi is that they can accumulate a large amount of fatty acids with a high content of polyunsaturated fatty acids (PUFA), such as γ-linolenic and arachidonic acids [8]. PUFA present several uses in food, biomedical and nutraceutical areas due to their structural and functional properties [9, 10].

The fungi belonging to the genus *Nigrospora* present a great potential for the production of a wide range of bioactive secondary metabolites [11]. Recently, our Research Group carried out a screening study for lipid production containing 150 fungal strains and the most promising ones belong to the genus *Nigrospora* sp. [12].

Several methods for oil extraction have been developed and studied, mostly using an organic solvent. Although these extraction methods present advantages, they still show certain drawbacks in relation to the ideal lipid extraction requirements. The interest in the extraction of lipids and the lack of efficiency of conventional methods led to the increasing use of green extraction techniques such as ultrasound and supercritical/pressurized fluids [13-15].

Among the fluids used in supercritical/pressurized extraction, CO_2 is an important solvent. It has characteristics such as low toxicity and it is environmentally friendly, cheap, and safe. It has been used to extract compounds in areas such as food science, pharmaceuticals, chemical residues, biofuels, and polymers [16, 17]. Specifically about fungal

oil extraction, CO_2 has been used for recovering compounds from *Botryosphaeria dothidea* [18], *Mortierella isabellina* [19], *Pythium irregulare* [20], and *Saprolegnia parasitica* [21, 22], among others.

However, one of the main difficulties is the slow rates of extraction during the kinetics, especially at the beginning of extractions. Therefore, the use of ultrasound technology can be an interesting alternative as a pre-treatment step [23]. The mechanical effect of ultrasound allows breaking the structure of cellular materials through cavitation effects and it can facilitate the release of the solutes during the extraction [24]. The increase in the extraction efficiency with supercritical CO_2 can be done with the addition of cosolvents (or solvent modifiers) to CO_2, such as ethanol. The presence of more polar mixtures can improve the solubility of different compounds due to the increase of chemical forces, such as hydrogen bonds or physical interactions of the solutes with the cosolvent [25].

Nevertheless, only a few studies report the separated use of supercritical extraction with cosolvent and ultrasound to improve the oil yields obtained from microorganisms. One of the studies was developed with oleaginous yeasts, where an oil yield of 4.96 wt.% was obtained using cell wall disruption by supercritical fluid assisted by ultrasound [26]. Another study reported a positive and significant influence of ethanol on a supercritical mixture, where 9% ethanol (w/w) increased the solubility of compounds from *Saccharomyces cerevisiae* [27]. Up to now, none study could be found in the scientific database that reports the integrated use of ultrasound with ethanol and a solvent under supercritical conditions for recovering compounds from fungal biomass.

Based on this context, this work aims at obtaining fungal oil from *Nigrospora* sp. cultivated in submerged fermentation using an integrated process for extraction. The integrated process consisted of studying ultrasound with ethanol as a cosolvent + supercritical CO_2 under different operational conditions. This integrated process was compared with ultrasound (without cosolvent) + supercritical CO_2 extraction and with only supercritical CO_2 (without a pre-treatment by ultrasound) extraction. The oil yield, kinetic curves, and compositions in terms of fatty acids have been compared for the steps studied. *Soxhlet* has been used as a conventional and referenced method.

3.2.2. MATERIAL AND METHODS

3.2.2.1. Microorganism and fermentation

The fungus *Nigrospora* sp. was identified and used in this work after a screening performed and reported in a previous study [12]. For the submerged fermentation, a disc (6 mm) of the mycelium of *Nigrospora* sp., cultivated in Petri dishes with potato dextrose agar (PDA) medium for 7 days at 28°C under dark conditions, was inoculated in Erlenmeyer flasks containing a sterilized liquid medium. This aqueous medium (pH of 5.8) was composed of glucose (100 g/L; $C_6H_{12}O_6$), yeast extract (7.5 g/L), peptone (10 g/L), ammonium sulfate (2 g/L; $(NH_4)_2SO_4$), magnesium sulfate (0.5 g/L; $MgSO_4$. 7 H_2O), ferrous sulfate (1 g/L; $FeSO_4$. 7 H_2O), and manganese sulfate (1 g/L; $MnSO_4$. H_2O). The submerged fermentation was performed in an orbital shaker (Inova 44R, New Brunswick, Canada) at 150 rpm and 28°C for 7 days.

3.2.2.2. Preparation of samples of fungal biomass

After the submerged fermentation, the samples were filtered (Whatman filter paper, grade 1) using a vacuum pump. The retained biomass was washed with distilled water and filtered again. The samples of this washed retained biomass were frozen in ultra-freezer (FV-500, New Brunswick, Canada) for 48 h. Thereafter, the samples of fungal biomass were freeze-dried (L 101, Liotop, Brazil) for 24 h. The dried solid material was stored in sealed impermeable flasks until the extractions.

3.2.2.3. Conventional extraction (*Soxhlet*)

The dried fungal biomass was ground before the extractions using a mortar and pestle until it had a homogenous appearance with a fine granulometry. Afterward, 1 g of sample was submitted to the extraction of oil for 2 h in a *Soxhlet* apparatus (MA491/6, Marconi, Brazil) using 150 mL of n-Hexane (purity of 99%, Alphatec, Brazil). The n-Hexane was evaporated and the mass of extracted oil was quantified in an analytical balance. The assays were performed in triplicate. The conventional extraction was performed to make possible a comparison of yields and composition with the other techniques (supercritical CO_2, ultrasound + supercritical CO_2, and ultrasound + supercritical CO_2 with cosolvent).

3.2.2.4. Extraction with supercritical CO_2

The extraction with supercritical CO_2 was performed in a laboratory apparatus composed of a solvent reservoir, two thermostatic baths (Q214M2, Quimis, Brazil), a high-pressure syringe pump (260 D, ISCO, USA) and a jacketed stainless steel 316L extraction vessel (100 mL). The freeze-dried fungal biomass (3 g) was loaded into the extraction vessel and mixed with glass spheres to fill completely the vessel volume. CO_2 (purity > 99.5%, White Martins, Brazil) was loaded in the vessel and conditions of pressure were defined. The system was maintained pressurized under a static time for 1 h. Thereafter, the extract was recovered for 85 min at intervals of 5 min (in the first 15 min) and of 10 min (from 15 min to 85 min). In the dynamic extraction, CO_2 flow rate was defined as 4 g/min based on other reports [16, 19]. The pump pressurizes the CO_2 at a constant volumetric flow rate, where the outlet CO_2 is at 5°C (controlled by a thermostatic bath) and at the desired pressure (according to the experimental design). Therefore, the volumetric flow rate is converted to mass flow rate based on the solvent density [28].

A factorial design (2^2) with triplicate of the central point was applied to evaluate the influence of pressure (15 – 25 MPa) and temperature (40 – 80°C) on the yield and oil composition. Considering the oil was collected at intervals of 5 min or 10 min, each kinetic curve had 10 points. Near to the end of the total extraction time (85 min), low amount of oil was recovered (diffusion-controlled period), indicating that the time was sufficient for obtaining the oil from the fungal biomass in those conditions. The yield of oil in the kinetic curves was determined according to Eq. (1).

$$Yield\ (wt.\%) = \frac{oil\ (g)}{fungal\ biomass\ (g)} \cdot 100 \qquad (1)$$

3.2.2.5. Pre-treatment with ultrasound and extraction with supercritical CO_2

After the extraction using pure supercritical CO_2 without a pre-treatment, the largest yield of oil was obtained at 80°C and 25 MPa. Therefore, three new assays were performed in triplicate to evaluate the influence of ultrasound as a pre-treatment. Previously to the supercritical fluid extraction (SFE), 3 g of the dried and ground fungal biomass was loaded in a beaker and maintained in an ultrasonic water bath (USC-1800A, Unique, Brazil) at 132 W of power and 40 kHz of frequency during all time of pre-treatment. The beaker was sealed and all water covered the beaker. Three times of pre-treatment were studied: 15 min, 30 min, and 60 min. Thereafter, each biomass pre-treated was removed from the beaker and loaded in

the high-pressure extraction vessel (stainless steel) while the procedures of extraction followed those presented into Section 2.4 (the only one exception is that the pressure and temperature were fixed at 25 MPa and 80°C). The yield and composition of oil in the kinetic curves were also determined.

3.2.2.6. Addition of ethanol, pre-treatment with ultrasound, and extraction with supercritical CO_2

After the extraction using pure supercritical CO_2 at 80°C and 25 MPa with ultrasound as a pre-treatment, the highest yield of lipids was obtained using a pre-treatment time of 60 min. Therefore, three new assays were performed in triplicate for evaluating the influence of adding ethanol to the biomass (30%, 50%, and 70% (w/w); the percent refers to the dried and ground fungal biomass) before the pre-treatment using ultrasound and previously to SFE. Afterward, the pre-treated biomass was loaded in the extraction vessel and the procedures of extraction followed those presented into Section 2.4 (the only one exception is that the pressure and temperature were fixed at 25 MPa and 80°C). The ethanol in the extracted samples was evaporated and the yield of oil in the kinetic curves was determined as well.

3.2.2.7. Analysis of fatty acids

Each sample of oil (10 μL) was solubilized in n-Hexane (Alphatec, Brazil) (1 mL) and centrifuged at 3,220 × g (centrifugal force) to separate the insoluble substances. In the sequence, the solution (supernatant) was divided into two fractions of 0.5 mL to have the duplicate of the analysis. A total of 250 μL of a solution of methyl tridecanoate (Sigma-Aldrich, USA) in isooctane (4 mg/mL) was added to each fraction. The solvents were evaporated at 40°C under vacuum. The fatty acids methyl esters (FAME) derivatization was performed as procedures reported by Visentainer [29]. The FAME were identified and quantified by injecting 1 μL of the derivatized solution in a gas chromatograph equipped with a flame ionization detector (GC-FID, 3400 CX-CA, Varian, USA). The FAME was separated in a capillary column (CP-Wax 52 CB, Agilent, The Netherlands) of 50 m × 0.32 mm × 0.20 m using hydrogen at 0.1 MPa. The detector was maintained at 240°C and the injector was maintained at 250°C with a split/splitless ratio of 20:1. The temperature profile of the column was defined as 50°C (1 min), 50-200°C (20°C/min), 200-230°C (10°C/min), and 230°C (8 min). The FAME was identified by comparing the retention times with external standards

(FAME Mix-37, P/N 47885-U, Sigma-Aldrich, USA). The compositions were expressed as g of fatty acid per 100 g of oil, as reported elsewhere [30].

3.2.2.8. Statistical analysis

The statistical analysis of the experimental data was carried out using the Statistica 8.0® software (Statsoft Inc., USA). The Tukey's test was applied and a significance level of 95% (*p-value* < 0.05) was set for all analyses.

3.2.3. RESULTS AND DISCUSSION

3.2.3.1. Influence of treatments on total oil yields

3.2.3.1.1. Supercritical CO_2

The oil yields obtained from the fungal biomass using *Soxhlet*, supercritical CO_2, ultrasound + supercritical CO_2, and ultrasound + supercritical CO_2 with cosolvent (ethanol) are presented in Table 1.

Table 1 - Yield of lipids obtained from the fungus *Nigrospora* sp. by supercritical CO_2, ultrasound + supercritical CO_2, ultrasound with cosolvent (ethanol) + supercritical CO_2, and n-Hexane (*Soxhlet*).

Assay	Pressure (MPa)	Temperature (ºC)	Ultrasound time (min)	Cosolvent (%, g ethanol/100 g biomass)	Yield (wt.%)
Supercritical CO_2					
1	15	40	-	-	2.80 ^{cE}
2	25	40	-	-	2.84 ^{cE}
3	15	80	-	-	1.10 ^{bF}
4	25	80	-	-	3.27 ^{aC}
5	20	60	-	-	2.71 ^{dE}
6	20	60	-	-	2.72 ^{dE}
7	20	60	-	-	2.71 ^{dE}
Ultrasound + Supercritical CO_2					
8	25	80	15	-	3.06 ^{bD}
9	25	80	30	-	3.01 ^{bD}
10	25	80	60	-	3.39 ^{aC}
Ultrasound with cosolvent (ethanol) + Supercritical CO_2					
11	25	80	60	30.0	3.54 ^{bB}
12	25	80	60	50.0	3.94 ^{aA}
13	25	80	60	70.0	3.12 ^{cD}
n-Hexane (*Soxhlet*)					
14	-	-	-	-	3.84 ^{A}

Coefficient of variation (CV): 0.21% (assays with supercritical CO_2).
a-d Different letters in the same column indicate a significant difference at a confidence level of 95% (p-value < 0.05 – Tukey's test) for the assays of each extraction technique.
A-F Different capital letters in the same column indicate a significant difference at a confidence level of 95% (p-value < 0.05 – Tukey's test) for the assays among the extraction techniques.

Regarding the assays with pure supercritical CO_2 without a pre-treatment, the highest yield (3.27 wt.%) was obtained in the assay 4: 80°C and 25 MPa. Otherwise, the lowest yield (1.10 wt.%) was obtained in the assay 3: 80°C and 15 MPa. In such case, the condition of higher pressure favored the extraction of more quantity of oil. Amongst the 7 assays, an interaction of parameters is seen when taking into account the statistical analysis. The

pressure had an influence when the highest level of temperature was tested (80°C), that is, at 40°C the oil yield was similar between assays developed at 15 MPa or 25 MPa.

The yields presented in this study are higher than those reported elsewhere [18, 31], which the authors produced oil from fungal biomass by supercritical CO_2. For example, the fungus *Botryosphaeria dothidea* produced 1.14 wt.% oil [18] and the fungus *Ganoderma lucidum* produced a maximum 2.98 wt.% extract [31]. However, the oil yield obtained from *Nigrospora* sp. (this work) is lower than other lipid-producing biomass such as microalgae *Chlorella protothecoides* [32] and the microalgae *Scenedesmus obliquus*, *Chlorella protothecoides* and *Nannochloropsis salina* [33].

The oil yield obtained from *Nigrospora* sp. (this work) is similar or even that larger than biomasses as black poplar [34] and tea seed (*Camellia oleifera*) [35]. Considering different fungal biomass processed by supercritical technology for recovering oil, there are similarities in terms of defining favorable conditions to the extractions. For example, the oil recovered from a filamentous fungus (*Mortierella isabellina*) using supercritical CO_2 presented its highest yield (3.21 wt.%) when the condition of 80°C and 25 MPa was applied [19]. On the other hand, the authors reported no recovery at 80°C and 15 MPa.

The pressure favors the extraction of oil as the density of the supercritical solvent increases. The vapor pressure of solutes increases with temperature. At a given pressure, the temperature influences both the solvent density and the vapor pressure of the solute, and this influence may occur in an opposite manner with respect to the solubility of the solute. For this reason, the crossing behavior can be observed. This behavior is corroborated by the findings reported by Scapin et al. [36], where the highest yield of chia oil (21.93 wt.%) using supercritical CO_2 was obtained at 25 MPa (the highest level tested). Furthermore, Scapin et al. [36] indicated that the increase of temperature from 20°C to 60°C (at a constant pressure of 10 MPa) led to a reduction of oil yield. This phenomenon was attributed to the abrupt decrease of solvent density with increasing temperature when pressures closer to the critical pressure were used. Higher pressures (e. g., 25 MPa) also favored the increase of oil yields obtained from dry biomass of the fungus *Botryosphaeria dothidea* using supercritical CO_2 [18]. However, the total yield of oil did not exceed 0.86 wt.% when pure CO_2 was used. Therefore, this work provides findings with respect to fungal biomass (*Nigrospora* sp.) with more oil than some others (e. g., *Botryosphaeria dothidea*).

3.2.3.1.2. Pre-treatment with ultrasound and extraction with supercritical CO_2

The extraction using pure supercritical CO_2 without a pre-treatment produced the largest oil yield (3.27 wt.%) at 80°C and 25 MPa. Therefore, another step was studied using this condition of temperature and pressure which included a pre-treatment with ultrasound previously to the extraction with supercritical CO_2 at 80°C and 25 MPa. The results are presented in Table 1.

The purpose of pre-treating the freeze-dried fungal biomass during three different times (15, 30 and 60 min) did not provide a significant increase in the oil yields. Ultrasound forms high-frequency waves that cause cavitation as a consequence of the expansion/contraction cycles, and it can cause rupture of cellular structures of different biomass. Indeed, according to the Tukey's test (Table 1), the pre-treatment with 15 min and 30 min was not sufficient to break the cell structure of the fungal biomass of *Nigrospora* sp. The pre-treatment for 60 min favored an oil yield of 3.39 wt.%, but it has not been statistically different from the assay 4 (3.27 wt.%, without pre-treatment).

One recent work has used ultrasound as a pre-treatment technique for obtaining lipids from the yeast *Trichosporon* sp. [37]. Higher lipid content was reached when using ultrasonic assisted extraction (43.00 ± 0.33%, w/w) if compared to the conventional *Soxhlet* extraction method (30.00 ± 0.28%, w/w). Furthermore, this alternative of ultrasound pre-treatment has been used to favor oil extraction from other biomass. For example, the oil yield obtained from adlay (*Coix lachrymal*-jobi L. var. *Adlay*) seed was increased by 14% when ultrasound (power of 100 W and frequency of 20 kHz) was used with supercritical CO_2 at 20 MPa and 40°C [38]. Passion fruit oil was also obtained by supercritical CO_2 and ultrasound, where the yields were 29% higher than those reported for the extractions with pure supercritical CO_2 [39]. Taking into account the pre-treatment of the fungal biomass of *Nigrospora* sp. with ultrasound performed in this work, the behavior presented in Table 1 (no significant influence) has occurred due to the characteristics of the biomass. We infer that the conditions of power and frequency used in the study could not rupture the complex structure of the fungal biomass to a large extent.

3.2.3.1.3. Addition of ethanol, pre-treatment with ultrasound, and extraction with supercritical CO_2

Even though no significant difference was seen between the oil yields for extractions with supercritical CO_2 and ultrasound (60 min) + supercritical CO_2, the pre-treatment with ultrasound (60 min) was used for comparing the addition of different proportions of ethanol as

cosolvent on the yields (Table 1). Three proportions of ethanol added to the biomass were studied: 30%, 50% and 70% (w/w, g ethanol/100 g biomass).

The purpose of adding ethanol to the biomass before the pre-treatment was a positive option to increase the oil yields, according to the Tukey's test. Two conditions favored the highest yields: 3.54 wt.% (30% ethanol, w/w) and 3.94 wt.% (50% ethanol, w/w). The polar characteristic of ethanol favors recovering compounds such as non-saponifiable lipids. In such case, the increase on the concentration of ethanol from 50 wt.% to 70 wt.% could have hindered the extraction of non-polar compounds. In the study reported by Yang and Wei [40], triterpenic acids from *Hedyotis corymbosa* were better extracted when ethanol was added to the CO_2 solution. Oleanolic and ursolic acids were extracted in larger amount when performing a hyphenated procedure for ultrasound–assisted supercritical extraction combining 15.4% ethanol (v/v) with CO_2, pressure of 32 MPa, temperature of 60°C, power of 185 W and frequency of 40 kHz. Likewise, considering fungal biomass, Valente et al. [18] reported that the mixture CO_2 + 10 wt.% ethanol provided an extraction yield of 1.14 wt.% from *Botryosphaeria dothidea*, which is approximately 1.3 times higher than the yield obtained by using pure supercritical CO_2 (0.86 wt.%). In our work, the best proportion of ethanol (50%, w/w) was suitable for improving the oil yield by a factor of approximately 1.2. The addition of a small quantity of ethanol modified the properties of the solvent, such as the solvation, viscosity, and diffusivity, which were favorable in solubilizing more quantity of solutes.

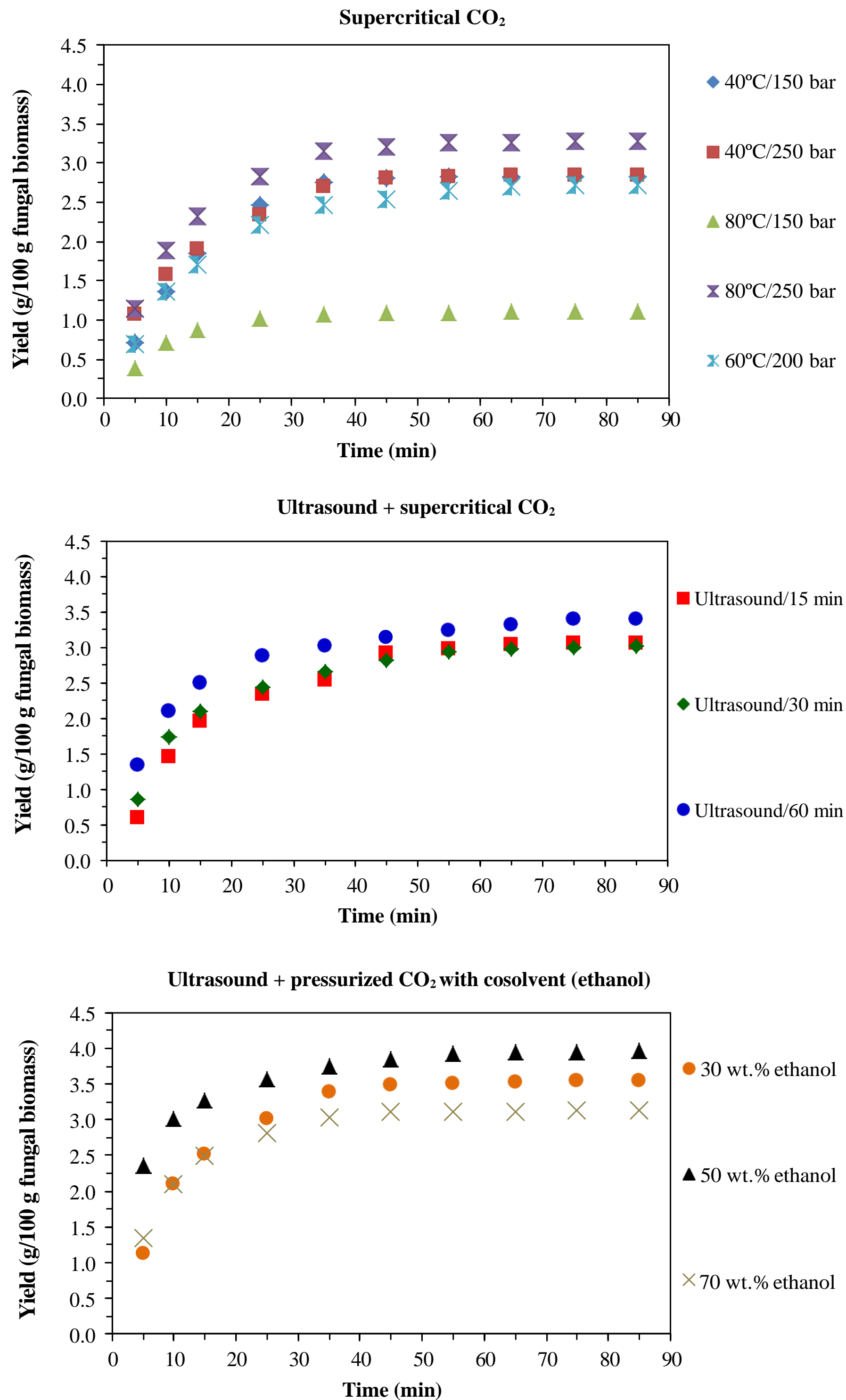
3.2.3.1.4. Soxhlet

The conventional extraction presented an oil yield of 3.84 wt.%. This value was higher when compared to the oil yields in the extractions using supercritical CO_2 without a pre-treatment (3.27 wt.%) and ultrasound + supercritical CO_2 (3.39 wt.%), with statistical differences ($p\text{-}value < 0.05$) observed in Table 1. However, when comparing the yields of samples of oil obtained by *Soxhlet* and ultrasound + supercritical CO_2 with ethanol (50%; w/w), no statistical differences ($p\text{-}value < 0.05$) were observed. The relatively higher oil yield obtained by *Soxhlet* can be attributed to undesirable compounds that could be extracted with n-hexane, as phosphatides and waxes [41, 42]. This becomes clear when a lower quantity of fatty acids obtained by *Soxhlet* is presented and discussed in Section 3.3.

3.2.3.2. Extraction kinetics

The three steps of obtaining oil from the freeze-dried fungal biomass of *Nigrospora* sp. (supercritical CO_2, ultrasound + supercritical CO_2, ultrasound with ethanol + supercritical CO_2) were also evaluated in terms of kinetic curves (Figure 1). The dynamic extraction time was 85 min.

Figure 1 - Kinetic curves of oil obtained from freeze-dried fungal biomass of *Nigrospora* sp. using supercritical CO_2, ultrasound + supercritical CO_2, and ultrasound + supercritical CO_2 with ethanol.



Overall, when using supercritical CO_2 without a pre-treatment, the extractions curves reached the diffusion-controlled (DC) period at approximately 45 min. Specifically in the condition of 80°C and 25 MPa, the DC was reached at approximately 35 min. Besides, in this condition (80°C/25 MPa), the yield at 5 min of extraction was larger than the yield at 85 min in the condition of 80°C and 15 MPa. As aforementioned, the increase on pressure increased the solubility of compounds in the supercritical solvent. It is corroborated by the mass transfer rate in the period of constant extraction rate (CER), which was approximately 2.7 times higher when the highest investigated pressure was applied. In the first 15 min of extraction, for each 100 g of biomass loaded in the extraction vessel as a reference mass, the mass transfer rates could reach 0.154 g/min and 0.057 g/min for the conditions of 80°C/25 MPa and 80°C/15 MPa, respectively. In the same way, the mass ratio of solute in the fluid phase at the extraction vessel outlet for the CER period was higher in the condition of higher pressure (for a constant temperature of 80°C).

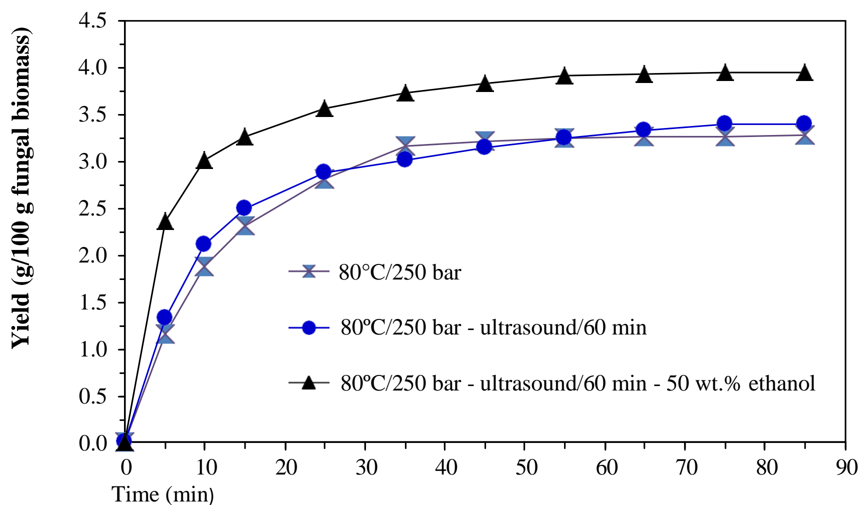
The pre-treatment with ultrasound caused a few changes in the behavior of the kinetic curves. Overall, based on the quantitative description of the curves, the DC period started some minutes later, that is, at approximately 55 min. There is a more visible intermediary stage defined as falling extraction rate (FER) period. It lasted approximately 30 min for the conditions of ultrasound for 15 min and 30 min, and approximately 20 min for the condition of ultrasound for 60 min. Considering the mass transfer rate for the CER period, it was 0.166 g/min (considering 100 g of biomass loaded in the extraction vessel as a reference mass). Even though the final amount of oil was similar for the assays either using or not a pre-treatment with ultrasound for 60 min, the decision of testing the addition of cosolvent for a sample submitted to ultrasound was based on this mass transfer rate. As evidenced, it was slightly larger (0.166 g/min) than the assay performed without ultrasound pre-treatment (0.154 g/min).

Consequently, when the step of ultrasound + supercritical CO_2 extraction with cosolvent was performed, the differences on the kinetic curves were quite evident (Figure 1). Firstly, the three different proportions of ethanol led to different total oil yields. Secondly, the addition of 50 wt.% ethanol favored obtaining both the highest oil yield (3.94 wt.%) and mass transfer rate (0.217 g/min).

For a comparison purpose, the kinetics for the three best assays (one of each step) is presented in Figure 2. It is clear that the addition of ethanol as a cosolvent (50 wt.%) was a positive alternative to improve the oil yield from fungal biomass of *Nigrospora* sp. This

behavior has occurred due to the access of non-polar and polar compounds using supercritical CO_2 with ethanol. The solutes of easy access are achieved faster. Thereafter, the solutes entrapped in the pores of the biomass are achieved later, becoming the extraction rate slower. In the scientific literature, similar kinetic curves (based on an analysis of a qualitative description) for extractions from fungal biomass by using green technologies have been reported [18, 19, 43].

Figure 2 - Comparison of the best kinetic curves of oil obtained from freeze-dried fungal biomass of *Nigrospora* sp. using supercritical CO_2, ultrasound + supercritical CO_2, and ultrasound + supercritical CO_2 with ethanol.



3.2.3.3. Fatty acids

In order to have additional information about the steps of oil extraction from the freeze-dried biomass of *Nigrospora* sp., the bulk oil was measured in terms of fatty acids (Table 2). A total of 24 fatty acids were identified and quantified.

Table 2 - Fatty acids (mg/g oil) quantified in the oil extracted from the fungus *Nigrospora* sp. by supercritical CO_2, ultrasound + supercritical CO_2, ultrasound with cosolvent (ethanol) + supercritical CO_2, and n-Hexane (*Soxhlet*).

Fatty acid		**Supercritical CO_2**					**Ultrasound + Supercritical CO_2**		**Ultrasound with cosolvent+Supercritical CO_2**				***Soxhlet***
		1	**2**	**3**	**4**	**5/6/7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
C6:0	Caproic acid	0.09	0.10	0.24	0.20	0.05	0.22	0.22	0.07	0.07	0.45	0.12	0.01
C8:0	Caprylic acid	0.06	0.06	0.13	0.11	0.03	0.12	0.14	0.04	0.04	0.20	0.12	0.08
C12:0	Lauric acid	0.09	0.06	0.09	0.07	0.04	0.09	0.08	0.04	0.04	0.13	0.13	0.09
C14:0	Myristic acid	1.54	1.57	0.86	1.39	0.48	2.42	1.37	0.81	0.89	3.73	2.46	0.25
C14:1	Myristoleic acid	0.12	0.12	0.05	0.11	0.03	0.17	0.10	0.06	0.06	0.24	0.17	0.12
C15:0	Pentadecanoic acid	0.45	0.48	0.29	0.42	0.15	0.70	0.42	0.24	0.25	1.08	0.72	0.13
C16:0	Palmitic acid	42.10	43.83	23.97	36.20	12.81	68.31	36.43	21.12	25.38	111.47	69.27	24.05
C16:1	Palmitoleic acid	7.81	7.65	5.38	7.38	2.70	10.73	6.77	3.99	4.03	16.54	11.01	8.70
C17:0	Margaric acid	0.15	0.15	0.08	0.12	0.04	0.24	0.12	0.06	0.08	0.37	0.23	0.50
C17:1	Heptadecenoic acid	0.11	0.14	0.32	0.39	0.15	0.56	0.38	0.21	0.21	0.37	0.61	0.75
C18:0	Stearic acid	5.40	5.56	2.96	3.94	1.54	8.93	4.02	2.41	3.44	15.67	9.48	2.85
C18:1n9c	Oleic acid	95.10	96.77	62.46	86.91	32.47	143.13	81.14	48.04	55.13	224.65	148.27	43.15
C18:1n9t	Elaidic acid	9.41	9.35	6.70	8.63	3.22	13.58	8.03	4.69	5.00	21.73	13.47	9.41
C18:2n6c	Linoleic acid	29.12	28.92	25.26	28.30	11.85	33.15	19.94	16.60	18.09	38.40	42.91	9.39

Table 2 - *Continued*

Fatty acid		Supercritical CO_2					Ultrasound + Supercritical CO_2		Ultrasound with cosolvent+Supercritical CO_2				*Soxhlet*
		1	**2**	**3**	**4**	**5/6/7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
C18:2n6t	Linolelaidic acid	0.09	0.08	0.08	0.11	0.03	0.12	0.10	0.05	0.05	0.19	0.19	0.17
C18:3n3	Linolenic acid	1.43	1.42	0.73	0.84	0.49	0.99	0.58	0.50	0.74	1.07	1.51	1.72
C20:0	Arachidic acid	0.53	0.53	0.31	0.15	0.16	0.15	0.21	0.09	0.10	0.69	0.86	1.34
C20:1n9c	Cis-11-Eicosenoic acid	1.09	1.09	1.04	1.02	0.47	1.50	0.83	0.54	0.56	2.00	1.42	1.27
C20:2	Cis-11,14-Eicosadienoic acid	0.24	0.24	0.18	0.22	0.10	0.28	0.18	0.12	0.20	0.36	0.28	0.29
C20:5n3	Eicosapentaenoic acid	0.35	0.34	0.45	0.48	0.40	0.65	0.83	0.21	0.30	1.50	0.84	0.63
C22:0	Behenic acid	0.57	0.56	0.54	0.46	0.30	0.43	0.70	0.22	0.25	0.90	1.18	0.64
C22:1n9	Erucic acid	0.85	0.84	0.72	0.72	0.35	0.94	0.70	0.35	0.38	1.82	1.77	1.17
C23:0	Tricosanoic acid	12.17	12.09	28.89	9.87	12.62	14.85	14.85	9.63	14.94	9.70	31.33	8.23
C24:0	Lignoceric acid	0.65	0.65	0.36	0.46	0.21	1.22	0.47	0.28	0.37	2.44	1.44	0.34
UFA/SFA*		2.28	2.24	1.76	2.53	1.84	2.11	2.03	2.15	1.85	2.10	1.90	1.99
TFA		209.52	212.60	162.09	188.50	80.69	303.48	178.61	110.37	130.60	455.70	339.79	115.28

UFA: unsaturated fatty acids; SFA: saturated fatty acids; TFA: total fatty acids.

Experimental conditions: 1 – 15 MPa/40°C; 2 - 25 MPa/40°C; 3 - 15 MPa/80°C; 4- 25 MPa/80°C; 5,6,7 - 20 MPa/60°C; 8 - 25 MPa/80°C/ 15 min of ultrasound pre-treatment; 9 - 25 MPa/80°C/ 30 min of ultrasound pre-treatment; 10 - 25 MPa/80°C/ 60 min of ultrasound pre-treatment; 11 - 25 MPa/80°C/ 60 min of ultrasound pre-treatment with 30.0 wt% ethanol; 12 - 25 MPa/80°C/ 60 min of ultrasound pre-treatment with 50.0 wt% ethanol; 13 - 25 MPa/80°C/ 60 min of ultrasound pre-treatment with 70.0 wt% ethanol.

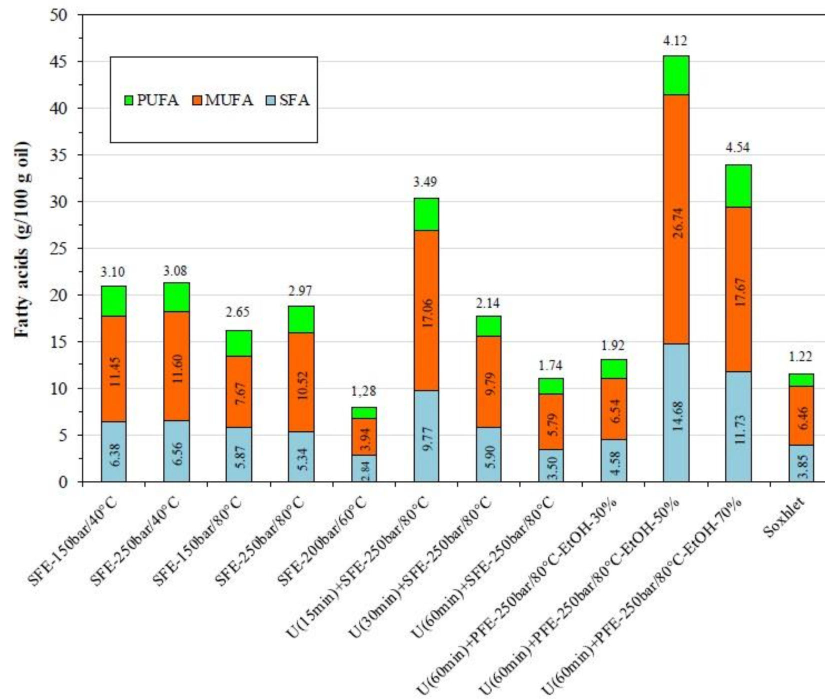
* The UFA/SFA ratio is dimensionless

The main fatty acids found in the fungal oil were oleic acid (C18:1n9c), palmitic acid (C16:0), linoleic acid (C18:2n6c), elaidic acid (C18:1n9t), and palmitoleic acid (C16:1). Considering all the steps, the composition of oleic acid in the bulk oil ranged from 32.47 mg/g oil to 224.65 mg/g oil. A considerable variation was also seen for the other fatty acids, such as linoleic (11.85-42.91 mg/g oil) and palmitoleic (2.70-16.54 mg/g oil) acids.

When comparing the composition for the samples of oil obtained by supercritical CO_2, ultrasound + supercritical CO_2, ultrasound + supercritical CO_2 with cosolvent (ethanol), and n-Hexane (*Soxhlet*), some trends are observed, which corroborate the findings reported and discussed into Sections 3.1 and 3.2. The procedure of sonicating the fungal biomass for 60 min and extracting the oil at 25 MPa/80°C with 50% (w/w) ethanol also yielded oil more concentrated in fatty acids. As aforementioned, this step yielded 3.94 wt.% total oil and the highest mass transfer rate (0.217 g/min) in the CER period. For example, when comparing the assays 12 and 4, the sonication and addition of ethanol (assay 12) provided oil approximately 2.6 and 2.2 times more concentrated in oleic and palmitoleic acids, respectively. When comparing the assays 12 and 14 (*Soxhlet*), the integration of ultrasound and supercritical fluid extraction with cosolvent yielded oil approximately 5.2 and 4.1 times more concentrated in oleic and linoleic acids, respectively. These findings agree with the information commonly reported in the scientific literature, where conventional extractions (such as *Soxhlet*) can extract some untargeted substances, thus reducing or diluting the concentration of the targeted ones [44, 45].

The alternative of pre-treating the fungal biomass with ultrasound (60 min) and extracting oil with a mixture of CO_2 and ethanol (50%, w/w) under supercritical condition (25 MPa/80°C) was also positive in terms of total unsaturated fatty acids (UFA) (Figure 3). The sum of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) ranged from 5.22 g/100 g oil to 30.86 g/100 g oil.

Figure 3 - Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) in the samples of oil obtained from freeze-dried fungal biomass of *Nigrospora* sp. using supercritical CO_2 (SFE), ultrasound (U) + supercritical CO_2, and ultrasound (U) with ethanol (EtOH) + supercritical CO_2 (SFE).



There is a large difference on the composition of SFA, MUFA, and PUFA between assays 11 and 12 (Table 2), where the only modification in the conditions is the content of ethanol (30% versus 50%, w/w). This behavior is attributed to the interaction of the mixture of CO_2/ethanol and the bonds of fatty acids. Covalent interactions (hydrogen bonds) and dipole-dipole could affect the solutes in different degree. The addition of 50% ethanol (w/w) was suitable to have 3.5 times more total fatty acids (TFA) than the addition of 30% ethanol (w/w). Likewise, the UFA/SFA ratio increased from 1.8 to 2.1 when this proportion of ethanol was increased (Table 2). However, 70% ethanol (w/w) was excessive in terms of obtaining both total oil (Table 1) and concentrated fatty acids (Figure 3). The 70% ethanol (w/w) increased the polarity of the solution, which could have hindered the access of CO_2 to the non-polar solutes during the SFE step. Therefore, in a mid-term, the most indicated concentration of ethanol found to be 50% (w/w) ethanol.

The definition of the assay 12 (codified as “U(60min)-EtOH50%+SFE-25MPa/80°C” in Figure 3) as the best one amongst those studied is also based on the actions of the compounds found in the oil. According to Martínez et al. [46], special fatty acids, such as γ-linolenic acid and eicosapentaenoic acid, have detached importance in the pharmaceutical field because they present positive effects on the development of the infant's brain and on

cardiovascular diseases. In fact, in the food and bioproducts processing area, several opportunities can be exploited to recover a wide range of compounds [47].

3.2.4. CONCLUSION

Three steps of extracting oil from the freeze-dried biomass of *Nigrospora* sp. were performed: (1) supercritical CO_2, (2) ultrasound + supercritical CO_2, and (3) ultrasound with cosolvent (ethanol) + supercritical CO_2. After comparing oil yields and composition among these steps and with the conventional one (*Soxhlet*), some conclusions have been attained:

i) At a constant temperature of 80°C, a higher pressure favored the extraction of more quantity of oil; at 40°C, the pressure did not influence the oil yield;

ii) A pre-treatment of the biomass with ultrasound (132 W and 40 kHz) for 60 min was more appropriate than 15 min or 30 min;

iii) The addition of 50% ethanol (g ethanol/100 g biomass) as a cosolvent in the extraction solvent was a positive alternative to increase oil yields, mass transfer rates in the CER period and composition of fatty acids;

iv) In the best condition, the oil was composed of up to 45.5% fatty acids, mostly containing oleic, palmitic, linoleic, elaidic and palmitoleic acids.

The use of green solvents (CO_2 and ethanol) and promising technologies (supercritical and ultrasound) have concentrated up to 4.0 times fatty acids in the oil when compared to the conventional one (*Soxhlet*). Therefore, we show that the SFE is a promising high-pressure technology to obtain oil with a high content of polyunsaturated fatty acids (as oleic and linoleic acids) from fungal biomass. The oil can be used in pharmaceutical and food-related areas due to their structural and functional properties.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors thank the Coordination for the Improvement of Higher Education Personnel (CAPES) for the scholarships and the National Council of Technological and Scientific Development (CNPq) for the financial support.

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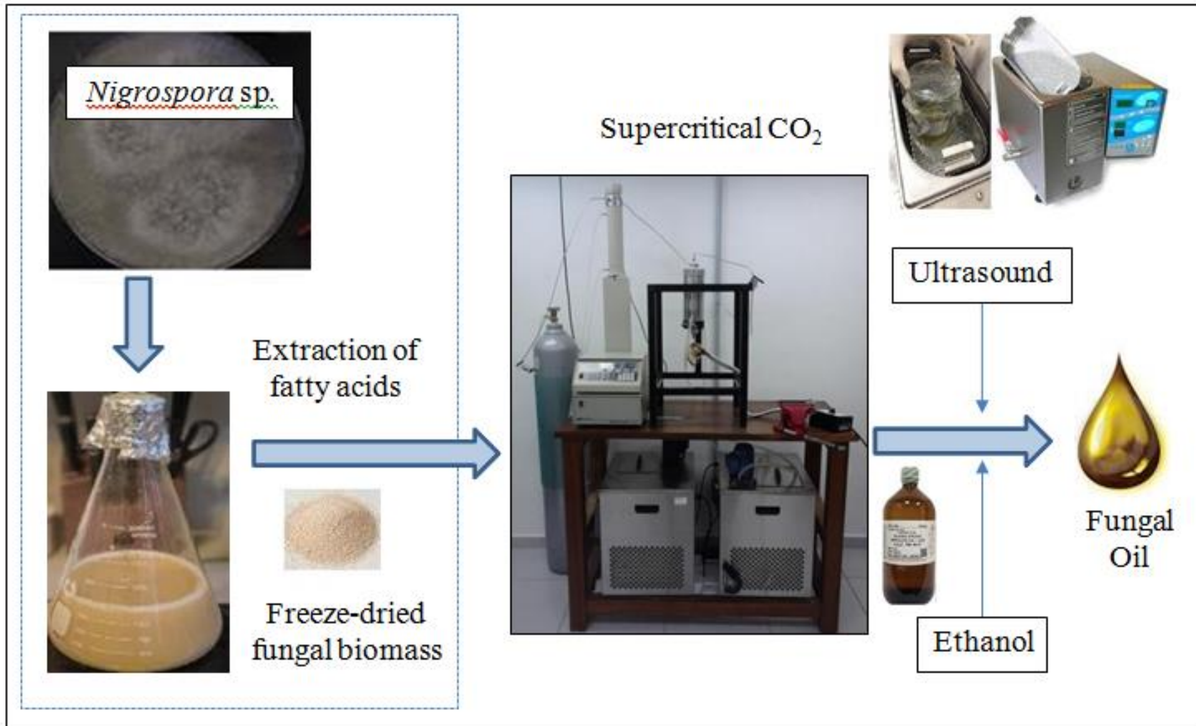
HIGHLIGHTS

- Fungal oil was obtained from freeze-dried biomass of *Nigrospora* sp.
- Supercritical CO_2 with ethanol as a cosolvent yielded 3.94 wt.% oil
- The addition of 50 wt.% ethanol increased 38% polyunsaturated fatty acids
- Oleic, palmitic, linoleic, elaidic and palmitoleic acids were quantified

GRAPHICAL ABSTRACT



3.3. **ARTIGO III:** RESIDUAL BIOMASS OF *NIGROSPORA* SP. FROM PROCESS OF THE MICROBIAL OIL EXTRACTION FOR THE BIOSORPTION OF PROCION RED H–E7B DYE

Residual biomass of *Nigrospora* sp. from process of the microbial oil extraction for the biosorption of procion red H–E7B dye

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ABSTRACT

The residual defatted biomass (RDB) of the fungus *Nigrospora* sp. obtained from the oil extraction process using supercritical technology was used as a low cost biosorbent for the removal of Procion red H–E7B dye (PR H–E7B) from aqueous solutions. The biosorption was favored using a biosorbent dosage of 0.78 g L^{-1} at pH 2. Biosorption kinetics was evaluated for different dye concentrations (50–200 mg L^{-1}) and the General order model presented the best fit of experimental curves. Experimental biosorption equilibrium was determined for different temperatures (298–328 K). The Freundlich isotherm model showed the best fit of the equilibrium data. The maximum biosorption capacity was 188.79 mg g^{-1} at 298 K. These results show that the RDB of *Nigrospora* sp. has potential as biosorbent. In this way, the *Nigrospora* sp. biomass can be used to obtain oil and as biosorbent at the same time, contributing in the fields of process integration and cleaner production.
Keywords: biosorption; cleaner production; residual biomass; oil extraction; process integration.

3.3.1. INTRODUCTION

Several industrial activities use a variety of dyes in different areas [1]. Textile industries are responsible to generate 54% of effluents contaminated with dyes around the world [2]. The residual waters of these activities contain a large quantity of dyes, which are harmful to the aquatic environment [3]. Many dyes found in these waters are toxic with mutagenic and carcinogenic potential, adversely affecting human health and aquatic biota [4]. An example is Procion Red H–E7B, a dye used extensively in the textile and tanning industries. This dye contains N=N in the structure, which can generate aromatic amines, being extremely harmful [5].

The treatment of dye containing wastewaters is normally performed by several separation techniques, including photolysis, photocatalytics, coagulation, electrokinetics, ion exchange, adsorption and biosorption [6]. Among these, adsorption and biosorption are considered the more suitable methods [6], due its simple design, ease operation and high efficiency [7]. The use of adsorption or biosorption should present low cost. There are a considerable number of studies using low cost materials for this purpose, such as, residual biomass, by–products of industrial and agricultural activities, bagasse, shells, among others [2,6,8–15].

The capacity of inactive microbial biomasses used as alternative materials for the removal of dyes and metals from industrial wastewaters has also been widely reported [16–20]. The use of microbial biomass as biosorbent is advantageous because it is less contaminated by harmful wastes and can be regenerated and reused [19]. In addition, the composition of cell–wall biomass, mainly from fungi (chitin, glucan, mannan, proteins, amino and hydroxyl groups), make this residue attractive for biosorption [21].

Species of fungus of the genus *Nigrospora* sp. are widely studied in the literature for the production of a wide range of bioactive secondary metabolites [22,23]. Moreover, this genus of fungus is also studied as a potential microorganism in the production of microbial oil by solid–state fermentation [24] and submerged fermentation [25]. The extraction of microbial oil from fungal biomass generates about 800 kg of solid residue per ton of biomass [25]. The reuse of the residual biomass from the oil extraction process as biosorbent would be an alternative to add value to the production of this biotechnological product (microbial oil) and reduce total expenses with treatment of the substrates generated in the process.

This study evaluated the application of the RDB of the fungus *Nigrospora* sp., obtained from the process of oil extraction, as an alternative biosorbent for the removal of PR H–E7B dye from aqueous media. The biosorbent and the extracted oil were characterized. To evaluate the efficiency of this new biosorbent (RDB), the influence of different process variables on the biosorption was studied. The biosorption was analyzed in detail from the kinetic and equilibrium viewpoints.

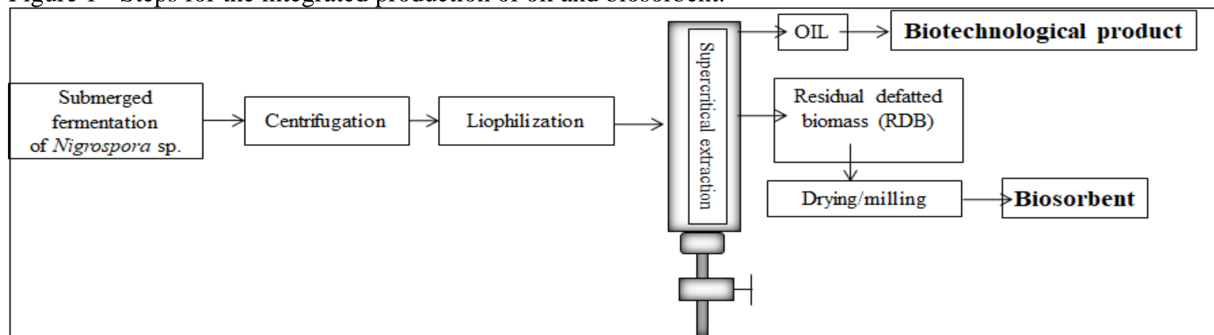
3.3.2. MATERIALS AND METHODS

3.3.2.1. Biosorbent, oil and dye

The fungus *Nigrospora* sp. was cultivated by submerged fermentation and the biomass was obtained by separation from liquid culture using centrifugation followed by lyophilization [25]. This biomass was used for oil extraction using supercritical CO_2 (80°C and 250 Bar). As a result of the supercritical extraction, two products were generated: the residual defatted biomass (RDB) and the oil. The oil was characterized. RDB was oven dried at 60 °C for 12 h, sieved until 60 mesh, characterized and used as biosorbent. Figure 1 shows the scheme of process integration.

It was used the red dye Procion H–E7B (PR H–E7B) ($C_{52}H_{26}O_{26}S_8Cl_2N_{14}Na_8$, CAS number 61931–52–0). This dye was as chosen because it is common in textile and tanning effluents [26].

Figure 1 - Steps for the integrated production of oil and biosorbent.



3.3.2.2. Biosorbent and oil characterization

RDB was characterized by Fourier transform infrared spectroscopy (FTIR) (Prestige, 21210045, Japan) by the direct transmittance method using the KBr tablet technique. The spectra were obtained in the range of 400 to 4500 cm^{-1}, with scanning of 45 scans and resolution of 2.0 cm^{-1}. The biosorbent structure was determined by X–ray diffraction (XRD) (Rigaku, Miniflex 300, Japan), operating with Cu–Kα (λ = 1.5418 Å), 30 kV, 10 mA, step of 0.03° and acquisition time of 0.5 s. The morphology was visualized by scanning electron microscopy (SEM), using an apparatus (Vega 3, Tescan, Czech Republic). The zero–loading point (pH_{ZPC}) was determined according to Georgin et al. [5]. The oil was characterized by gas chromatography (Shimadzu, GCMS–QP2010 Ultra, Japan) according to Sallet et al. [27].

3.3.2.3. Biosorption experiments

For the biosorption assays, a stock solution of the PR H–E7B dye was prepared (1000 mg L^{-1}) with deionized water and then diluted to obtain the desired concentrations. All experiments were performed in a thermostated shaker (Marconi, MA 093, Brazil), with a solution volume of 50 mL and agitation rate of 150 rpm. After the tests, solid–liquid separation was performed by centrifugation (Centribio, 80–2B, Brazil) at 4000 rpm for 10 min. The supernatant was used for determination of absorbance using a spectrophotometer (Biospectro SP–22, Brazil) at wavelength of 538 nm.

Initially, the pH effect (2.0–10.0) was evaluated at 298 K, using 1.5 g L^{-1} of biosorbent, 100 mg L^{-1} of dye concentration and stirring time of 2 h. The pH was adjusted with HCl and NaOH. The influence of biosorbent dosage was evaluated at 298K, dye concentration was 100 mg L^{-1} and contact time of 2 h, using the best pH above determined. The effects of pH and biosorbent dosage were measured in terms of dye recovery efficiency (R) and biosorption capacity (q_t), by means of a one–factor–at–a–time method. Dye recovery efficiency (R, %) and biosorption capacity (q_t, mg g^{-1}), were calculated conform the following equations:

$$R = 100\left(\frac{C_0 - C_t}{C_0}\right) \quad (1)$$

$$q_t = \left(\frac{C_0 - C_t}{m}\right)V \quad (2)$$

where C_0 is the initial dye concentration in liquid phase (mg L^{-1}), C_t is the dye concentration in the liquid phase at time t (mg L^{-1}), m is the mass of biosorbent (g) and V is the solution volume (L).

3.3.2.4. Kinetic and equilibrium studies

The kinetic study was carried out at pH 2.0, biosorbent dosage of 0.78 g L^{-1}, temperature of 298 K for different initial dye concentrations (50, 100, 200 mg L^{-1}) and contact time from 0 to 270 min. To fit the experimental data, four kinetic models were used, conform presented in Table S1 (supplementary material).

The equilibrium was determined for different initial dye concentrations (0, 25, 50, 100, 200, 300, 400, 500 mg L^{-1}) and temperatures (298, 308, 318 and 328 K) after 5 h of contact time. This time was considered after three equal measurements of dye concentration in liquid phase. The biosorbent dosage was 0.78 g L^{-1} and pH of solution 2.0. The biosorption capacity at equilibrium (q_e, mg g^{-1}) was calculated according to Equation (2), but using the equilibrium values of dye concentration (C_e, mg L^{-1}). The isotherm models of Freundlich, Langmuir and Sips were tested to fit the experimental data. These models and the respective parameters are presented in Table S2 (supplementary material).

3.3.3. RESULTS AND DISCUSSION

3.3.3.1. Characteristics of microbial oil and biosorbent

The microbial oil yield obtained by supercritical CO_2 at 80°C and 250 bar from the biomass of *Nigrospora* sp. was 3.27 wt%. The main fatty acids found in the oil are presented in Table 1.

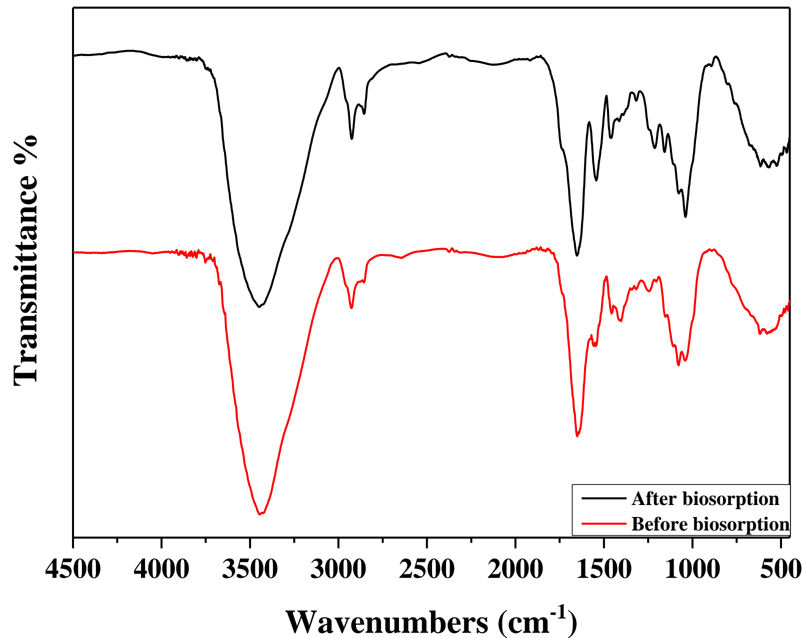
Table 1 - Fatty acids profile (mg g^{-1} oil) of the microbial oil extracted from the fungus *Nigrospora* sp. by supercritical CO_2 at 80 ° C and 250 bar.

Fatty Acid		(mg g^{-1} oil)
C6:0	Caproic acid	0.202
C8:0	Caprylic acid	0.108
C12:0	Lauric acid	0.065
C14:0	Myristic acid	1.387
C14:1	Myristoleic acid	0.111
C15:0	Pentadecanoic acid	0.416
C16:0	Palmitic acid	36.200
C16:1	Palmitoleic acid	7.383
C17:0	Margaric acid	0.115
C17:1	Heptadecenoic acid	0.392
C18:0	Stearic acid	3.942
C18:1n9c	Oleic acid	86.906
C18:1n9t	Elaidic acid	8.633
C18:2n6c	Linoleic acid	28.300
C18:2n6t	Linolelaidic acid	0.114
C18:3n3	Linolenic acid	0.844
C20:0	Arachidic acid	0.147
C20:1n9c	Cis−11−Eicosenoic acid	1.020
C20:2	Cis−11,14−Eicosadienoic acid	0.221
C20:5n3	Eicosapentaenoic acid	0.482
C22:0	Behenic acid	0.464
C22:1n9	Erucic acid	0.724
C23:0	Tricosanoic acid	9.866
C24:0	Lignoceric acid	0.463

The main fatty acids of the microbial oil were oleic acid, linoleic acid and palmitic acid. Others fatty acids in lower concentrations also were reported such as, palmitoleic, stearic, elaidic and tricosanoic acids. The microbial oil can be used for specific food and pharmaceutical applications. As a result of the oil extraction, a residual defatted biomass of the fungus *Nigrospora* sp. was generated (Figure 1). In this work, the residual defatted fungal biomass was dried, milled and used as biosorbent. This practice is an alternative to add value to the production of microbial oil and reduce total expenses with treatment of the residues generated in the process.

In order to verify the structure of the biosorbent (RDB), the XRD study was performed, as shown in Figure S1 (supplementary material). The XRD patterns show that the material did not show any crystalline phase, only a pronounced wide band at 2θ in the range of 10° to 50°, indicating an amorphous structure [28]. The amorphous region presents a disarranged structure, with empty spaces, which provides a better arrangement of large adsorbate molecules (dyes, for example). This characteristic can favor dye biosorption [26].

Figure 2 - FTIR spectra of the residual defatted biomass (RDB) before (red line) and after (black line) the PR H−E7B biosorption.



Infrared spectra of the RDB before (red line) and after (black line) the biosorption experiments with PR H−E7B are shown in Figure 2. It is possible to observe the nature of the different functional groups present on the surface of RDB. The major bands found in the RDB before biosorption were close to 3446, 2926, 2878, 1650, 1556, 1457, 1412, 1318, 1246, 1152, 1078, 1041 cm^{-1}. In the FTIR of RDB before biosorption (red line), the broad band with a vibrational elongation around 3446 cm^{-1} indicates the presence of the hydroxyl (OH) and amino (NH) groups on the surface of the material [18]. The bands at 2926 and 2877 cm^{-1} correspond to the stretching vibrations of the CH groups [26]. The vibrations near to 1650 cm^{-1} and 1556 cm^{-1} can be attributed to C=O and N−H of amide group bonds [29]. The bands at 1457 cm^{-1} and 1318 cm^{-1} correspond to angular deformation vibrations of CH_2 [28] and, the bands of 1246−1041 cm^{-1} reflect the C−O vibrations [30]. From this analysis, it can be verified that RDB contains hydroxyl, carboxyl, amide and amine groups, which are the main functional groups present in the polysaccharides and fungal biomass proteins [31]. The presence of these functional groups can be an indicative of good biosorption capacity of RDB [32].

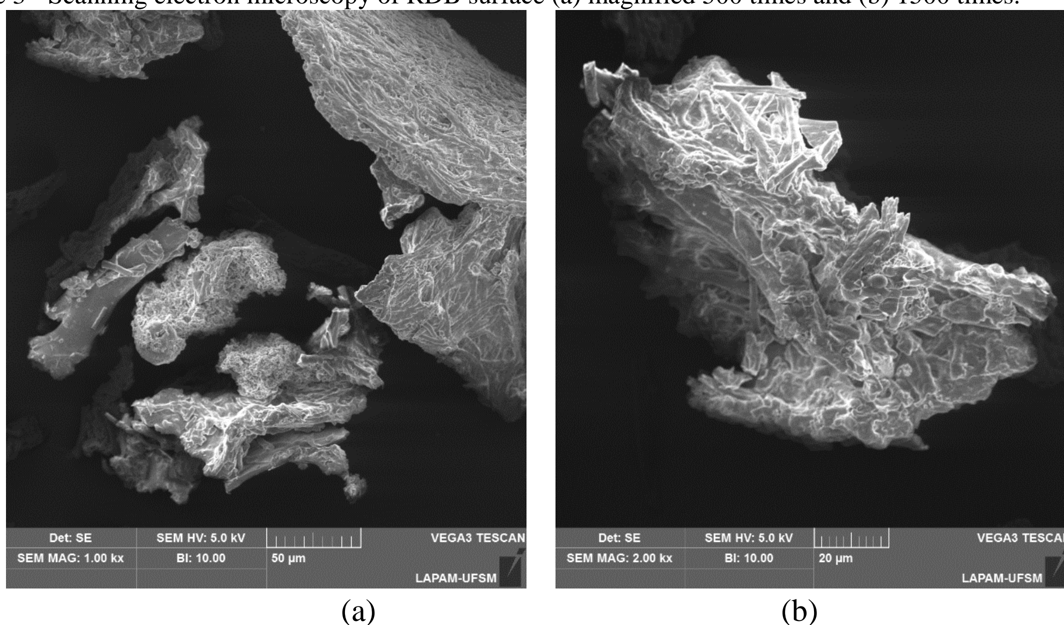
In relation to the infrared spectrum of the RDB after the biosorption, it is possible to verify some changes in the bands, especially in the range of (1700−1500 cm^{-1}) referring to the groups C=O and NH, bonds of the amide group [29] and in the range of (1220−1000 cm^{-1})

corresponding to the CO vibrations [30], suggesting that an interaction occurred between these groups present on the surface of the material and the dye PRH−E7B. This corroborates the hypothesis that the presence of these groups is responsible for the biosorption capacity of the material.

Figure 3 (a and b) show the surface morphology details of the biosorbent before the biosorption, obtained by scanning electron microscopy (SEM) at 500× and 1500×. Through these images, it can be observed that RDB presents an irregular surface structure, with different particle sizes, characterizing a rough texture. These characteristics of the material benefit the impregnation of the adsorbate more quickly and easily during the biosorption process [33]. These properties allow a greater interaction of the dye in the structure of the biosorbent [26].

These surface characteristics of the RDB, besides being a property of the nature of the material, can also be attributed to the fact that this residue was obtained from the process of lipid extraction, through supercritical technology. This technology promotes the rupture of the microbial cell wall, increasing the release of intracellular lipids [34] and consequently causes an increase in the porosity of this material. These characteristics found in this fungal biomass, after the lipid extraction process, were also observed in the work done by Fontoura et al. [18], which used residual microbial biomass (microalgae) after the lipid extraction process with organic solvents to remove the blue acid dye 161 (AB−161) from aqueous solution.

Figure 3 - Scanning electron microscopy of RDB surface (a) magnified 500 times and (b) 1500 times.

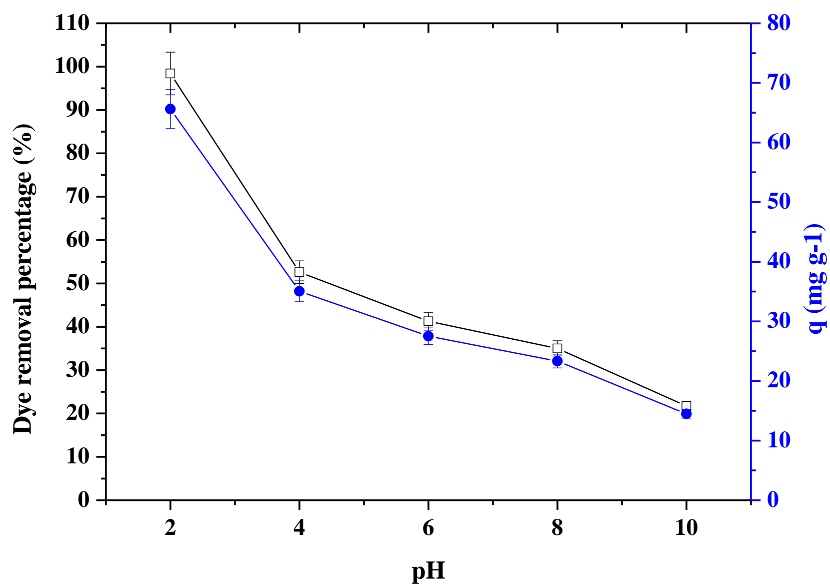


3.3.3.2. Effects of pH and biosorbent dosage

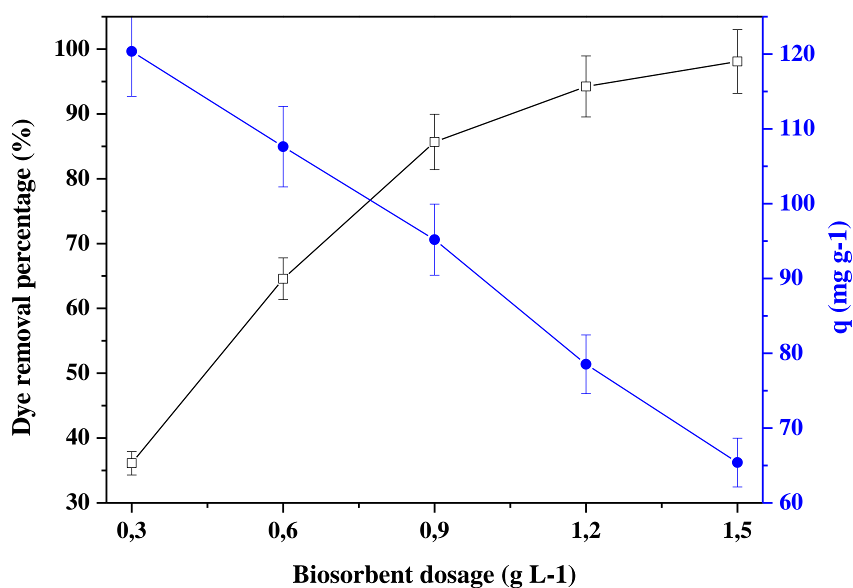
In this work, the effect of initial pH on the biosorption of PR H−E7B dye was investigated using 1.5 g L^{-1} of biosorbent, with initial dye concentration of 100 mg L^{-1}, varying the pH from 2−10. The results are presented in Figure 4 (a). The best results for dye removal percentage (98.4%) and biosorption capacity (65.61 mg g^{-1}) were obtained at pH 2. For pH higher than 2, it was verified a sharply decrease in the removal percentage and biosorption capacity. For example, at pH 10, the removal percentage was only 21.7% and the biosorption capacity was 15 mg g^{-1}. This improvement in the dye removal at pH 2 can attributed to the occurrence of the electrostatic interactions between the adsorbate (dye) and biosorbent, which are normally affected by the ionic charges of the functional groups [35]. In acid solutions, the biosorbent is charged with a positive charge (the point of zero charge of RDB was 7.0). This allows the interactions between the functional groups of the biosorbent with the sulfonated (SO_3^-) groups contained in the dye [36]. When the pH is increased, biosorption of the dye in the RDB decreases, since the functional groups of the dye molecules are repealed electrostatically by the negatively charged surface of the biosorbent. The results found here were similar to some studies in the literature. Akar et al. [3] used the geosorbent (natural mineral) to remove orange reactive dye 13 and obtained a greater percentage of dye removal (98.48%) at pH 2. Vanaamudan et al. [37] employed hydrotalcite as adsorbent to remove the dyes reactive blue 21 and reactive red 141 from aqueous solution and the maximum adsorption capacity for the two dyes (266.7 mg g^{-1} and 320.5 mg g^{-1}, respectively) was obtained at pH 2.

The effect of the biosorbent dosage (0.3, 0.6, 0.9, 1.2 and 1.5 g L^{-1}) on the dye removal percentage and biosorption capacity is presented in Figure 4 (b). The removal percentage increased from 37 to 99% when the biosorbent dosage increased from 0.3 to 1.5 g L^{-1}. This behavior is attributed to the increase of total biosorption sites. On the contrary, the biosorption capacity decreased from 120 to 65 mg g^{-1}. This decrease in biosorption capacity of the dye with increasing biosorbent dosage can be attributed to a greater number of sites that remain unloaded during biosorption, as well as the agglomeration of biosorbent particles. To ensure good values for both, biosorption capacity and dye removal percentage, the intersection of the curves in Figure 4 (b) was selected as the more suitable biosorbent dosage. This value was 0.78 g L^{-1}, which provided removal percentage around 80% and biosorption capacity around 100 mg g^{-1}.

Figure 4 - (a) Effect of pH on PR H−E7B biosorption by defatted fungal biomass; (b) Influence of biosorbent dosage on PR H−E7B biosorption by defatted fungal biomass (V = 50 mL, C_0 = 100 mg L^{-1}, 150 rpm, pH = 2, t = 2 h, T = 298 K).



(a)



(b)

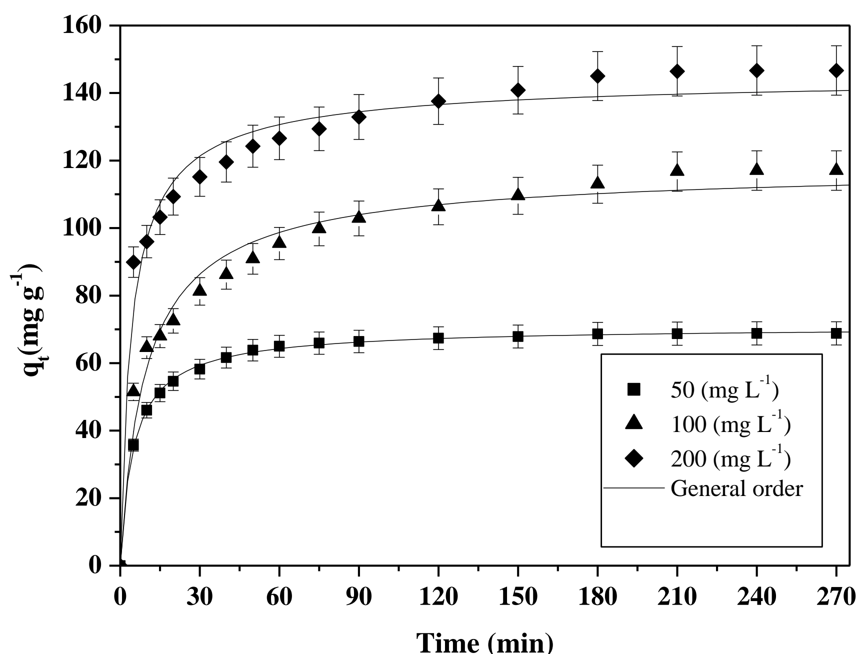
3.3.3.3. Biosorption kinetic profile

The kinetic study is fundamental to understand the biosorption process, since it provides data on the mechanism of the process and biosorption rate, which are of great

importance in the treatment of aqueous solutions containing dyes [38]. The biosorption kinetic curves were determined at different initial dye concentrations (50, 100 and 200 mg L^{-1}) and are shown in Figure 5. The dye biosorption occurred in three stages. In the first stage (first 30 min), it is verified a high biosorption rate of the dye in the RDB. This behavior occurs due to the large number of active sites available on the surface of the biosorbent. In the second step, ranging from 30 to 120 min, the biosorption rate decreased, since the active sites are gradually filled. Other point that reduces the biosorption rate is the occurrence of the repulsive forces of the dye molecules that have already been biosorbed in the process [39]. In the last step (120 to 270 min) there is a little variation in the biosorption capacity, since the process is near to the equilibrium and the sites are practically saturated.

Different kinetic models (see Table S1) were used to fit the experimental data. The estimated parameters and the fit quality are presented in Table S3. The highest values for the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2_{adj}) associated with the lowest values of average relative error (*ARE*) were obtained using the General order model, which was the best to fit the experimental data, when compared with the PPO, PSO and Avrami models. The General order model also provided the best fit of the experimental data on the adsorption of the reactive violet dye 5 using cocoa shell activated carbon as biosorbent [40], adsorption of red procion H−E7B using pretreated avocado peels [5], adsorption of reactive red 120 dye using *Spirulina platensis* and commercial activated charcoal as adsorbents [41] and adsorption of direct blue 53 dye using carbon nanotubes and activated carbon powder as adsorbents [42].

Figure 5 - Biosorption kinetic curves of PR H−E7B dye into RDB (T = 298 K, 150 rpm, biosorbent dosage = 0.78 g L^{-1}, pH=2).



The q_n values increased with the initial dye concentration, reaching maximum values of 145.74 mg g^{-1}, for dye concentration of 200 mg L^{-1}. There is a good agreement among experimental (q_e) and calculated (q_n) values for all dye concentrations, confirming the good fitting of the model with experimental data. An important factor to consider is that the kinetic equation of the General order model presents different orders (n). At the moment that the concentration of the dye is modified, it is necessary to estimate the initial biosorption rate h_0, determined by ($h_0 = k_n q_n^n$). The values for h_0 increased with the increase of the initial dye concentration, indicating that there is coherence in the experimental data. This indicates that in the initial biosorption stages, the process was fast at higher dye concentrations.

3.3.3.4. Biosorption equilibrium isotherms

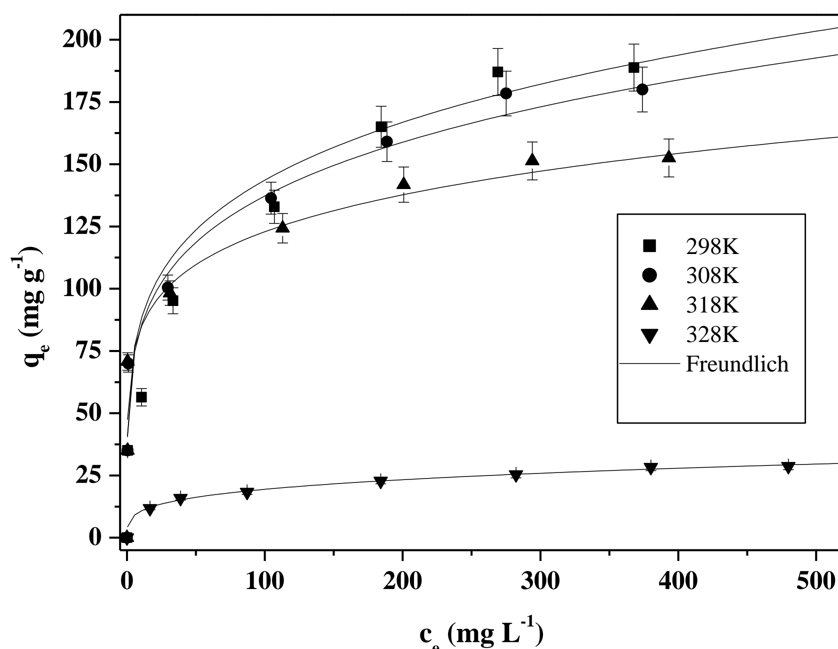
The equilibrium isotherms, which describe the relationship between the adsorbate concentrations (dye) remaining in the solution and biosorption capacity at equilibrium are shown in Figure 6 for different temperatures (298, 308, 318 and 328 K). The curves presented an inclined step in lower C_e values, indicating that there are several biosorption sites to be occupied by the dye. Also, this inclined portion indicates affinity between the dye and the

RDB. At higher concentrations in liquid phase, the curves tend to a plateau. This shows that practically all the biosorption sites were occupied by the dye.

The biosorption capacity of the dye at equilibrium decreased with the temperature increase. This trend was strongly pronounced at 328 K. Comparing the isotherms at 298 and 328 K, it was found that the biosorption capacity was around 7 times lower at 328 K. This behavior can be related with the thermosensitivity of the functional groups of the biosorbent, which can be damaged at higher temperatures [43]. A similar result was found in the adsorption of methylene blue dye using acid washed black cumin seeds at temperatures from 300 to 318 K [29].

The parameters of biosorption equilibrium models (Freundlich, Langmuir and Sips) estimated from experimental data are presented in Table S4. The Freundlich model (Figure 6 and Table S4) presented the best fit with the experimental data, with the highest coefficients of determination and the lowest average relative errors. The Freundlich model was also the most adequate to interpret the adsorption equilibrium in others studies available in literature [18,26,28]. The Freundlich constant k_F was similar in the temperatures from 298 to 308 K, but was strongly lower at 328 K. The evolution of this parameter confirms the explanation presented above, that higher temperatures are not adequate in this biosorption system.

Figure 6 - Biosorption equilibrium isotherms of dye into RDB (biosorbent dosage of 0.78 g L^{-1}, pH = 2).



To verify if the RDB of fungus *Nigrospora sp*. is suitable as a biosorbent, a comparison with other materials for uptake the dye PR H−E7B was presented in Table S5. It is possible to verify that there is a great difference in capacity among the materials, which varies from 0.76 mg g^{-1} to 212.6 mg g^{-1}. The capacities presented in Table S5 were obtained in different experimental conditions, but are the higher values obtained in each work. The RDB evaluated in this work, presented the second best biosorption capacity, which was 188.79 mg g^{-1}. This satisfactory result makes this material (RDB), a promising material in the removal of the dye PR H−E7B from aqueous solutions. In addition to this, another advantage of the use of this material is the low cost, since it is a residue of a biotechnological process (extraction of fungal oil).

3.3.4. CONCLUSION

It was demonstrated that the residual defatted biomass (RDB) of the fungus *Nigrospora* sp. is an alternative material that can be used as biosorbent to treat colored dyes. RDB demonstrated high efficiency (removal percentage around 98%) and high biosorption capacity (188.79 mg g^{-1}) to uptake PR H−E7B dye from aqueous solution. Furthermore, the RDB has low cost, since is generated from the extraction of a biotechnological product (microbial oil). The application of RDB as biosorbent is an alternative to add value for the production of microbial oil from *Nigrospora* sp., reducing the total expenses with treatment of the substrates (residues) generated in the oil extraction process.

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SUPPLEMENTARY MATERIAL

Table S1 - Kinetic models used to fit the biosorption data.

Models	Name	Parameters
$q_t = q_1(1-\exp(-k_1 t))$ (3)	Pseudo–first order [1S]	k_1 (min^{-1}) rate constant of pseudo–first order. q_1 ($mg\ g^{-1}$) theoretical value of adsorption capacity.
$q_t = \frac{t}{(1/k_2 q_2^2) + (t/q_2)}$ (4)	Pseudo–second order [2S]	k_2 ($g\ mg^{-1}\ min^{-1}$) rate constant of pseudo–second order q_2 ($mg\ g^{-1}$) theoretical value of adsorption capacity
$q_t = q_{AV}(1-\exp(-k_{AV} t)^{nAV})$ (5)	Avrami [3S]	k_{AV} (min^{-1}) rate constant of Avrami model. q_{AV} ($mg\ g^{-1}$) theoretical value of adsorption capacity. n_{AV} exponent of the Avrami model
$q_t = q_n - \frac{q_n}{\left[k_n(q_n)^{n-1} t(n-1)+1\right]^{1/(n-1)}}$ (6)	General order [4S]	k_n ($min^{-1}(g\ mg^{-1})^{n-1}$) rate constant of general order model. q_n ($mg\ g^{-1}$) theoretical value of adsorption capacity. n reaction order

Table S2 - Isotherm models used to fit the biosorption data.

Models	Name	Parameters
$q_e = \frac{q_m k_L C_e}{1 + k_L C_e}$ (7)	Langmuir [5S]	q_m (mg g^{-1}) maximum adsorption capacity. k_L (L mg^{-1}) Langmuir constant.
$q_e = k_F C_e^{1/n}$ (8)	Freundlich [6S]	k_F (mg g^{-1})(mg L^{-1})$^{-1/n}$ Freundlich constant. $1/n$ heterogeneity factor.
$q_e = \frac{q_m (k_S C_e)^m}{1 + (k_S C_e)^m}$ (9)	Sips [7S]	q_m (mg g^{-1}) maximum adsorption capacity. ks (L mg^{-1}) Sips constant. m heterogeneity factor.

The model parameters of the kinetic (Table S1) and isotherm (Table S2) were estimated by nonlinear regression, through the minimization of the least squares function, with the aid of the software *Statistic* 8.0 (Statsoft, USA). The quality of fitting was evaluated by means of determination coefficient (R^2), adjusted determination coefficient (R^2_{adj}) and average relative error (ARE, %) [8S].

Table S3 - Biosorption kinetic parameters for the system RDB and PR H−E7B dye

Models	Initial dye concentration (mg L^{-1})		
	50	100	200
q_e [exp] (mg g^{-1})	68.80	117.05	146.68
PFO			
q_1 (mg g^{-1})	66.03	106.26	133.37
k_1(min^{-1})	0.1131	0.0671	0.1341
R^2	0.9830	0.9445	0.9411
R^2_{adj}	0.9816	0.9398	0.9362
ARE (%)	5.21	11.04	9.33
PSO			
q_2 (mg g^{-1})	70.19	116.17	142.19
k_2 (g mg^{-1} min^{-1})	0.0027	0.0009	0.0015
R^2	0.9993	0.9837	0.9812
R^2_{adj}	0.9992	0.9823	0.9797
ARE (%)	0.96	6.14	5.24
General order			
q_n (mg g^{-1})	71.19	119.23	145.74
k_n (min^{-1}(g $mg^{-1})^{n-1}$))	0.0011	0.0003	0.0003
n	2.1669	2.2452	2.3409
h_0 (mg g^{-1} min^{-1})	11.37	12.67	36.10
R^2	0.9996	0.9871	0.9864
R^2_{adj}	0.9995	0.9860	0.9853
ARE (%)	0.78	5.45	4.40
Avrami			
q_{av} (mg g^{-1})	66.03	106.26	133.37
k_{av} (min^{-1})	0.2567	0.4413	0.0034
n_{av}	0.4405	0.1521	39.8743
R^2	0.9830	0.9445	0.9411
R^2_{adj}	0.9816	0.9398	0.9362
ARE (%)	5.21	11.04	9.33

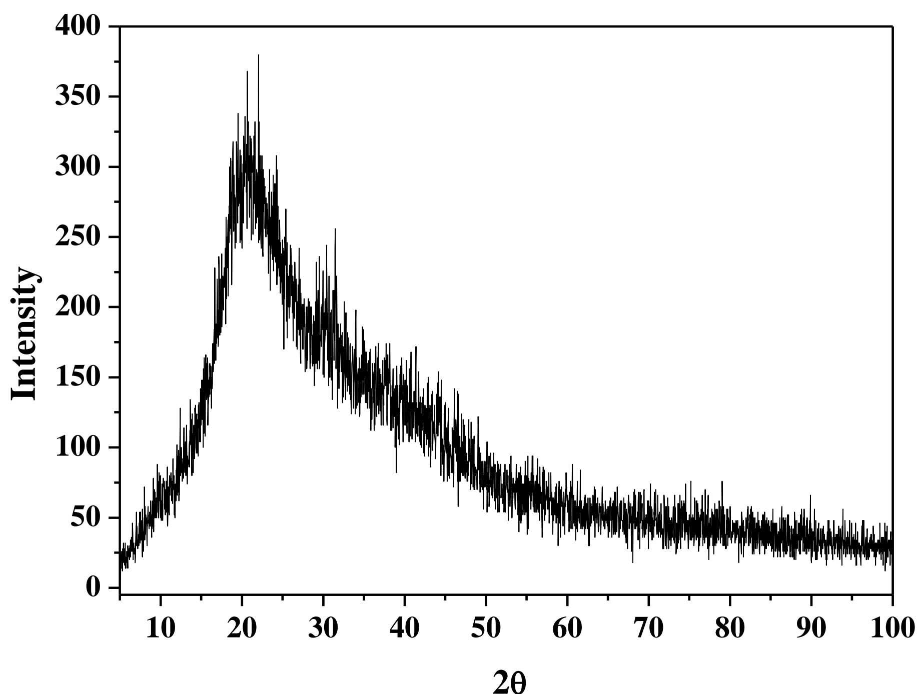
Table S4 - Biosorption isotherm parameters for the system RDB and PR H−E7B dye

Models	Temperature (K)			
	298	308	318	328
Freundlich				
k_F (mg g^{-1}) (mg L^{-1})$^{-1/nF}$	53.01	51.98	57.64	5.85
$1/n_F$	0.2164	0.2108	0.1644	0.2608
R^2	0.9826	0.9822	0.9672	0.9964
R^2_{adj}	0.9797	0.9792	0.9617	0.9958
ARE (%)	9.38	9.20	11.32	1.93
Langmuir				
q_m (mg g^{-1})	159.96	153.03	133.92	29.11
k_L (L mg^{-1})	0.6410	0.6547	1.3492	0.0293
R^2	0.8811	0.8822	0.8798	0.9580
R^2_{adj}	0.8613	0.8626	0.8598	0.9510
ARE (%)	14.99	13.94	18.80	7.16
Sips				
q_{mS} (mg g^{-1})	494.65	454.53	902.99	65.04
$k_S \times 10^3$(L mg^{-1})	0.40	0.50	0.04	1.20
m_S	0.2796	0.2756	0.3616	0.3821
R^2	0.9784	0.9776	0.8216	0.9956
R^2_{adj}	0.9698	0.9687	0.7502	0.9939
ARE (%)	9.52	9.42	20.28	1.93

Table S5 - Comparison of biosorption/adsorption capacities of different materials for the removal of PR H−E7B dye from aqueous solutions.

Adsorbent	**q_m (mg g^{-1})**	**Reference**
RDB	188.79	This work
Banana peel	0.76	[9S]
Zn_2SnO_4 oxide	48.80	[10S]
Papaya seeds	73.26	[11S]
Acidetreated palm shell	13.95	[12S]
Biochar	130.00	[22]
Orange peel	5.12	[9S]
Chitosan	22.48	[12S]
Untreated charcoal	8.52	[13S]
Ecological adsorbent	78.74	[14S]
Rice hull	20.40	[11S]
Sonicated TiO_2	5.46	[9S]
Avocado shells (AS–H_2SO_4)	167.00	[5]
Avocado shells AS–HNO_3)	212.60	[5]
Sawdust	2.12	[9S]
Activated carbon	41.50	[15S]
Woodeshaving bottom ash/H_2O	24.30	[15S]
Treated flute−reed	7.58	[16S]

Figure S1 - XRD pattern of the residual defatted biomass (RDB) of *Nigrospora* sp.



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HIGHLIGHTS

- One step production of microbial oil and biosorbent from *Nigrospora* sp.
- The microbial oil is rich in oleic, linoleic and palmitic acid.
- The generated biosorbent presented maximum capacity of 188.79 mg g–1.

CAPÍTULO 4 - DISCUSSÃO DOS RESULTADOS

Este presente trabalho proporcionou a elaboração de três artigos, com resultados relevantes, que serão discutidos de modo geral a seguir.

O primeiro artigo teve como objetivo aumentar a produção de biomassa e o teor de óleo a partir da implementação de diferentes estratégias, que incluía a definição de temperatura ótima de fermentação, otimização do meio de fermentação em frascos agitados, além de cultivo em biorreator STR operando nos modos batelada e batelada-alimentada. Para isso, primeiramente foi avaliado o cultivo submerso em batelada do fungo *Nigrospora* sp., em meio de fermentação com concentração inicial de glicose de 10 g L^{-1} (BG_{10}), realizado em diferentes condições de temperatura 25, 28 e 33 ºC sob agitação em incubadora a 150 rpm, pH 5,8 por 7 dias. O maior rendimento lipídico de 1,24% g g^{-1} foi alcançado na temperatura de 28 ºC.

Após a avaliação da cinética de fermentação foi avaliada a composição dos meios de fermentação, utlizando diferentes quantidades de fontes de carbono, glicose e sacarose, como também diferente fonte de nitrogênio (água de maceração de milho), em dois modos de cultivo, batelada e batelada alimentada, em frascos agitados. Os meios de fermentação avaliados em batelada foram, BG_{100} e BG_{100}_CSL_{10}, e em batelada alimentada foram, FBG_{100}_CSL_{10}, FBG_{20}, FBG_{60}, FBG_{100}, FBS_{20}, FBS_{60}, FBS_{100}. O melhor resultado em relação a rendimento lipídico foi utilizando o meio de fermentação BG_{100} (concentração inicial de glicose de 100 g L^{-1}), valor correspondente a 3,94% g g^{-1}. Quando comparando os resultados de rendimento de óleo, obtido na cinética de fermentação, 1,24% g g^{-1}, com o obtido na otimização dos meios de fermentação, 3,94% g g^{-1}, foi observado que a otimização do meio de fermentação apresentou influência positiva no rendimento lipídico.

Em sequência, foi avaliado o processo de fermentação em biorreator STR em batelada utilizando o meio otimizado BG_{100}, através de um delineamento definido pela matriz experimental do tipo fatorial (2^2), considerando as variáveis aeração (0,5-2,0 vvm) e agitação (100-200 rpm). Na condição experimental de (2 vvm e 100 rpm) foi obtido o maior rendimento lipídico (16,71% g g^{-1}) e de biomassa 32,50 (g L^{-1}). Como estratégia para aumentar a produção de lipídeos, com esta condição otimizada foi realizada uma nova fermentação no biorreator STR de forma de batelada alimentada, obtendo um rendimento lipídico de (21,32 % g g^{-1}) e de biomassa de 40,17 (g L^{-1}). Fazendo a comparação deste último resultado, utilizando batelada alimentada em biorreator, com o obtido em frasco agitado nas

mesmas condições, foi possível verificar que a concentração de biomassa e o acúmulo de lipídeos foram de 2,1 e 5,4 vezes maior, respectivamente.

As extrações dos lipideos foram realizadas pelo método de *Soxhlet* e a determinação do perfil de ácidos graxos do óleo fúngico foi através de Cromatografia Gasosa com Detector por Ionização de Chama (GC-FID). Os resultados obtidos desta análise da composição dos ácidos graxos mostraram que os ácidos graxos monoinsaturados (MUFA) foram encontrados em maior concentração, e em sequência, os ácidos graxos saturados (SFA) e os ácidos graxos polinsaturados (PUFA). Os ácidos graxos em maior concentração (mg g^{-1} de óleo) identificados nos lipídeos extraídos deste fungo foram: ácido oleico (C18:1n9c), ácido linoleico (C18:2n6c), ácido palmítico (C16:0), tanto em biorreator do tipo frascos agitados quanto do tipo STR. No entanto, somente as fermentações realizadas no biorreator STR, ocorreu a produção de ácidos graxos polinsaturados (PUFAs), como o ácido araquidônico (C20:4n6 - ARA) e o ácido docosahexaenóico (C22:6n3 - DHA).

Estes resultados mostraram a viabilidade da utilização do uso do biorreator do tipo STR em batelada alimentada, tanto, no aumento da produção de lipídeos quanto no aumento da concentração de ácidos graxos poli-insaturados essenciais do óleo sintetizado pelo fungo do gênero *Nigrospora*.sp.

No segundo artigo as fermentações foram realizadas em frascos agitados em batelada com o meio otimizado BG_{100}, obtido no trabalho anterior (Artigo 1). Neste trabalho, foram avaliados os parâmetros cinéticos de extração do óleo do fungo do gênero *Nigrospora* sp., com fluido CO_2 supercrítico e com o auxílio dos pré-tratamentos de extração supercrítica, ultrassom e adição de co-solvente etanol, em relação a rendimento e a composição dos lipídeos extraídos.

Nas extrações realizadas com somente CO_2 supercrítico, foram avaliadas diferentes condições de temperatura (40, 60, 80 ºC) e pressão (15, 20, 25 MPa). Na condição de maior temperatura e pressão (80ºC/25MPa), posteriormente utilizada nas etapas de extração com pré-tratamento, foi obtido o maior rendimento lipídico (3,27% g g^{-1}). O efeito da utilização de pré-tratamento com ultrassom no processo de extração com CO_2, foi avaliado, variando o tempo (15, 30, 60 mim) de exposição da amostra a ultrassom. O maior rendimento lipídico foi de (3,39% g g^{-1}), com o tempo de exposição ao ultrassom de 60 min. Para avaliar o efeito do pré-tratamento com etanol, foi adicionada à amostra concentrações de (30, 50, 70%) de etanol. Com as melhores condições definidas anteriormente, (80ºC/25MPa, 60min ultrassom), novas extrações com CO_2 supercrítico foram realizadas. O maior rendimento foi de (3,94% g

g^{-1}) com (50%) de etanol.

As análises de ácidos graxos, foram realizadas da mesma forma que o no artigo 1. Os ácidos graxos, monoinsaturados (MUFA) foram obtidos em maior concentração, e em sequência, os ácidos graxos saturados (SFA) e os ácidos graxos polinsaturados (PUFA). Os ácido palmítico (C16:0), ácido palmitoléico (C16:1), ácido esteárico (C18:0), ácido oleico (C18:1n9c), ácido eláidico (C18:1n9t), ácido linoleico (C18:2n6c), ácido tricosanóico (C23:0), foram os ácidos graxos extraídos em maior concentração.

De modo geral, estes resultados obtidos, mostraram a viabilidade da utilização dos pré-tramentos (ultrassom e co-solvente etanol) na extração com fluido CO_2 pressurizado, no aumento do rendimento e concentração de lipídeos fúngicos.

No terceiro artigo, foi avaliada a utilização do resíduo de biomassa desengordurada (RDB), do fungo do gênero *Nigrospora* sp., obtido do processo de extração de óleo através de tecnologia supercrítica (artigo 2), como um adsorvente para a remoção do corante vermelho procion H-E7B (PR H-E7B), também conhecido como vermelho reativo 141, (RR 141), de soluções aquosas. A biomassa foi caracterizada por técnicas analíticas de difração de raios-X (DRX), espectroscopia no infravermelho por transformada de Fourier (FTIR), microscopia eletrônica de varredura (MEV). O resultado de caracterização de acordo com o DRX mostrou que a amostra (RDB), apresentou uma estrutura amorfa. Já, a partir da caracterização com FTIR foi possível observar a natureza dos diferentes grupos funcionais presentes na superfície do adsorvente (RDB). E por fim, através das imagens obtidas por microscopia eletrônica de varredura (MEV), foi verificado que o material (RDB) apresentou uma estrutura superficial irregular, com diferentes tamanhos de partículas, caracterizando uma textura rugosa.

Os experimentos em batelada foram realizados para avaliar a variação dos parâmetros como: pH da solução (2-10), concentração de corante (50–200 mg L^{-1}), dose de adsorvente (0,3-1,5 g L^{-1}), tempo de contato (0–270 min), temperatura (298–328 K), para a determinação das melhores condições para remoção do corante PR H-E7B. A adsorção do corante PR H-E7B no RDB foi favorecida utilizando uma dosagem de 0,78 g L^{-1} de adsorvente em solução ácida, pH 2. Os modelos cinéticos de Pseudo-primeira Ordem, Pseudo-segunda Ordem, Ordem Geral e Avrami foram utilizados para explicar a cinética de adsorção. O modelo cinético de Ordem Geral apresentou o melhor ajuste, desta forma foi o mais adequado para representar a adsorção do corante PR H-E7B. Os modelos de isoterma de equilíbrio de Freundlich, Langmuir, Sips, foram usados para determinar capacidade de adsorção. O modelo de isoterma de Freundlich mostrou o melhor ajuste dos dados de equilíbrio, com isso foi o

modelo mais adequado para representar os dados isotérmicos. A capacidade máxima de adsorção foi de 188,79 mg g^{-1} a temperatura de 298 K. Em resumo, estes resultados mostram a viabilidade da utilização do RDB do fungo do gênero *Nigrospora* sp., como uma opção de adsorvente de baixo custo e eficiente para a remoção do corante PR H-E7B de soluções aquosas.

CAPÍTULO 5 - CONCLUSÃO

A partir da análise dos resultados obtidos neste trabalho, divido em três artigos, algumas conclusões foram alcançadas:

Artigo I:

i) Na cinética de fermentação, o maior rendimento lipídico foi de 1,24% g g^{-1} alcançado na temperatura de 28 ºC e a maior concentração de biomassa foi de 11,88 g L^{-1} obtida na temperatura de 33 ºC;

ii) O maior rendimento lipídico utilizando frascos agitados foi de 3,94% g g^{-1} em batelada, utilizando o meio de fermentação BG_{100} e em biorreator STR em batelada o maior rendimento lipídico obtido foi de 16,71% g g^{-1} na condição experimental de (2 vvm e 100 rpm);

iii) Em batelada alimentada no biorreator STR o rendimento lipídico foi de 21,32 % g g^{-1} o que correspondeu a um aumento de 5,4% g g^{-1} de lipídeos, quando comparado ao obtido em biorreator STR em batelada, nas mesmas codições;

iv) Os ácidos graxos em maior concentração (mg g^{-1} de óleo) identificados nos lipídeos, tanto em biorreator do tipo frascos agitados quanto do tipo STR, foram: os ácidos oléico, palmítico, linoleico, elaidico e palmitoleico;

Artigo II:

i) O maior rendimento lipídico obtido nas extrações com CO_2 supercrítico, foi alcançado na condição de maior temperatura e pressão (80ºC/25MPa), correspondente a 3,27% g g^{-1};

ii) Com um pré-tratamento da biomassa com ultrassom (132 W e 40 kHz) por 60 minutos foi obtido um maior rendimento lipídico de 3,39% g g^{-1}, quando comparado ao tempo de 15 min e 30 min;

iii) O melhor efeito do pré-tratamento com etanol, foi com uma concentração de 50%;

iv) Com a combinação da melhor condição de extração de pressão e temperatura (80ºC/25MPa), e com as melhores condições de pré-tratamento, ultrassom (60 mim) e concentração de etanol (50 %), foi obtido o maior rendimento lipídico correspondente a 3,94% g g^{-1};

v) Nas extrações com CO_2 supercrítico e com pré-tratamento, os ácidos graxos extraídos em maior concentração foram: ácido palmítico (C16:0), ácido palmitoléico (C16:1), ácido esteárico (C18:0), ácido oleico (C18:1n9c), ácido eláidico (C18:1n9t), ácido linoleico (C18:2n6c), ácido tricosanóico (C23:0);

Artigo III:

i) A adsorção do corante PR H-E7B no resíduo de biomassa desengordurada (RDB) foi favorecida utilizando uma dosagem de 0,78 g L^{-1} de adsorvente em solução ácida, pH 2;

ii) A capacidade máxima de adsorção do resíduo de biomassa desengordurada (RDB) do fungo do gênero *Nigrospora* sp., foi de 188,79 mg g^{-1} a temperatura de 298 K;

Desta forma, este estudo além de contribuir com informações revelantes para novas pesquisas relacionadas a produção e extração de lipídeos microbianos também serve como incentivo á proteção ambiental, uma vez que, o resíduo gerado no processo de obtenção do óleo microbiano, foi utilizado com sucesso como uma nova alternativa de material adsorvente no tratamento de efluentes líquidos contaminados com corante.

5.1. Sugestões para trabalhos futuros

- Aplicar um planejamento experimental no processo de fermentação em batelada alimentanda em biorreator STR, levando em consideração o número de alimentação e o intervalo de tempo em que estas ocorrem, visando o aumento de rendimento lipídico;

- Investigar a influência do ultrassom combinado a fermentação alimentanda em biorreator STR no rendimento de lipídeos e na concentração de ácidos graxos poli-insaturados;

- Investigar a influência do pré-tratamento com ultrassom nas extrações de óleo com CO_2 supercrítico utilizando um maior tempo de exposição da amostra ao ultrassom;

- Investigar a influência da utilização de uma coluna de adsorção (leito fixo) em relação aos resultados obtidos neste trabalho, que utilizou o processo de adsorção em batelada.

CAPÍTULO 6 - REFERÊNCIAS BIBLIOGRÁFICAS

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