UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

OTIMIZAÇÃO DA PRODUÇÃO E CARACTERIZAÇÃO DO ÓLEO MICROBIANO PRODUZIDO PELA LEVEDURA *Yarrowia lipolytica* QU21

JANDORA SEVERO POLI

ENGENHEIRA DE ALIMENTOS - UNISINOS

MESTRE EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

Porto Alegre

2014

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS GRADUAÇÃO EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

JANDORA SEVERO POLI

ENGENHEIRA DE ALIMENTOS - UNISINOS

MESTRE EM MICROBIOLOGIA AGRÍCOLA E DO AMBIENTE

OTIMIZAÇÃO DA PRODUÇÃO E CARACTERIZAÇÃO DO ÓLEO MICROBIANO PRODUZIDO PELA LEVEDURA *Yarrowia lipolytica* QU21

Tese apresentada como um dos requisitos para obtenção do grau de Doutor em Microbiologia Agrícola e do Ambiente - Área de concentração: Microbiologia Industrial

Orientador: Patricia Valente

Co-Orientador: Neila Silvia Pereira dos Santos Richards

Porto Alegre, Rio Grande do Sul, Brasil

Fevereiro, 2014

Família
Danske-Brasilianske families
Patricia Valente
CAPES
Irini Angelidaki
Neila Richards
Lene Kirstejn Jensen
Bioenergy Group-DTU
Dimitar Karakashev
Ezequias Siqueira
Paula Mattanna
Inês Silva
LabFungos-UFMG
DTU
Ana Tomás
Fiocruz
Hans-Christian Lützhøft
Yifeng Zhang
LEC
Hector Garcia
Ioannis Fotidis
Luiz Rosa
Mirra Angelina
UFRGS
Marilene Vainstein
Jonas
Vânya Pasa
Carlos Rosa
Colegas-UFRGS
Morten Andreasen

Otimização da produção e caracterização do óleo microbiano produzido pela levedura *Yarrowia lipolytica* QU21

Autor: Jandora Severo Poli

Orientador: Patricia Valente

Co-Orientador: Neila Silvia Pereira dos Santos Richards

[1]RESUMO

O tradicional biodiesel de 1ª geração (produzido a partir de óleo de origem vegetal, como soja ou canola) possui muitas desvantagens e limitações como sazonalidade, uso de grandes áreas de cultivo, competição com alimentos, dentre outras. Uma alternativa são os óleos produzidos por microrganismos. Com o objetivo de otimizar a produção do óleo microbiano, o presente trabalho avaliou a produção de biomassa, lipídios e composição de ácidos graxos da levedura *Yarrowia lipolytica* QU21 quando cultivada em diferentes fontes de carbono (glicose e glicerol), nitrogênio (sulfato de amônio, triptona, ureia, nitrato de amônio e extrato de levedura), assim como diferentes condições de cultivo (agitação, aeração e razão carbono/nitrogênio). Dois resíduos industriais, glicerina bruta e resíduo de indústria cervejeira (FYE) também foram testados como substitutos da fonte de carbono e nitrogênio, respectivamente. Este trabalho também apresenta uma técnica de triagem de leveduras oleaginosas, de forma a quantificar os lipídios utilizando solventes menos agressivos, tanto para o manipulador quanto para o meio ambiente. A composição de ácidos graxos do óleo produzido pela *Y. lipolytica* QU21 quando cultivada em glicerina bruta e sulfato de amônio apresentou potencial utilização como matéria prima para o biodiesel. O uso combinado dos dois resíduos industriais pela *Y. lipolytica* QU21 resultou na produção de óleo com elevado teor de ácidos graxos poliinsaturados. Além de tornar o óleo microbiano da levedura *Y. lipolytica* QU21 uma matéria prima competitiva para a produção de biodiesel, a utilização da glicerina bruta poderia atenuar problemas ambientais, como a disposição inadequada no meio ambiente.

[1] Tese de Doutorado em Microbiologia Agrícola e do Ambiente - Microbiologia Industrial: Microbiologia de Matérias Primas e Bioprocessos, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (95p.) Fevereiro, 2014.

Production optimization and characterization of microbial oil produced by the yeast *Yarrowia lipolytica* QU21

Author: Jandora Severo Poli

Advisor: Patricia Valente

Co-Supervisor: Neila Silvia Pereira dos Santos Richards

[2]ABSTRACT

The traditional 1st generation biodiesel (produced from plant oils, such as soybeans and canola) has many drawbacks and limitations as season and climate-dependent cultivation, agricultural land competition for food, among others. Possible alternative oil sources is microbial oil produced by oleaginous microorganisms. With the purpose of optimizing the production of microbial oil, this study evaluated the production of biomass, lipid and fatty acid composition of the yeast *Yarrowia lipolytica* QU21 when grown on different carbon source (glucose and glycerol), nitrogen source (ammonium sulfate, tryptone, urea, ammonium nitrate and yeast extract) as well as different culture conditions (agitation, aeration and carbon/nitrogen ratio). Two industrial waste were also evaluated, crude glycerol and brewery waste (FYE) as surrogate carbon and nitrogen sources, respectively. This work also presents a technique for sorting oleaginous yeast in order to quantify the lipids using less aggressive solvent for both the handler and to the environment. The fatty acid composition of the oil produced by the *Y. lipolytica* QU21 growing on crude glycerol and ammonium sulfate showed potential use as a feedstock for biodiesel. The combined wastes resulted on microbial oil produced by *Y. lipolytica* QU21 with high polyunsaturated fatty acid content. Besides making the microbial oil a competitive feedstock for biodiesel production, the use of crude glycerol could mitigate environmental issues such as improper waste disposal.

[2] Doctoral thesis in Environmental and Agricultural Microbiology, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (95p.) February, 2014.

SUMÁRIO

LISTA DE FIGURAS

LISTA DE SÍMBOLOS

AMP: adenosina monofosfato desaminase

B10: 10% de biodiesel misturado ao diesel

B5: 5% de biodiesel misturado ao diesel

BP: British Petroleum

C/N: razão carbono/nitrogênio

$CHCl_3$: chloroform (clorofórmio)

$C_{i\text{-}PrOH}$: isopropanol concentration (concentração de isopropanol)

CoA: coenzima A

CrGly: crude glycerol (glicerina bruta)

CS: carbon source (fonte de carbono)

DHA: docosa-hexaenóico

DMK: acetone (acetona)

DMSO: dimethylsulfoxide (dimetilsulfóxido)

EPA: eicosapentaenoic acid (ácido eicosapentaenóico)

ES: esterol éster

EtOH: ethanol (etanol)

FAME: Fatty acid methyl ester (metil éster de ácidos graxos)

FYE: fresh yeast extract (extrato de levedura fresco)

Gly: glicerol

GRAS: generally regarded as safe (geralmente considerado seguro)

GTL: glyceryltrilinoleate (trilinoleína)

GTO: glyceryltrioleate (trioleína)

GTP: glyceryltripalmitate (tripalmitina)

i-PrOH: isopropanol

LCFAs: Long chain fatty acids (ácidos graxos de cadeia longa)

LNS: Liquid nitrogen with sonication (nitrogênio líquido com sonicação)

MeOH: methanol (metanol)

MUFAs: monounsaturated fatty acids (ácidos graxos monoinsaturados)

NADPH: nicotinamida adenina dinucleotídeo fosfato

NS: nitrogen source (fonte de nitrogênio)

PMT: photomultiplier tube (tubo fotomultiplicador)

ppmv: partes por milhão

PUFAs: polyunsaturated fatty acids (ácidos graxos poliinsaturados)

SCO: single cell oil (óleo microbiano)

TAG: triacilglicerol

TBBD: thousand barrels of biodiesel per day (mil barris de biodiesel por dia)

USGB: Ultrasonic bath and glass beads (banho ultrasônico com pérolas de vidro)

V_{ef}/V_{cm}: ratio between the volume of the Erlenmeyer flask and the volume of culture medium (razão entre o volume do frasco de Erlenmeyer e o volume do meio de cultura).

VGB: Vortex with glass beads (agitador tipo vórtex com pérolas de vidro)

YE: yeast extract (extrato de levedura)

1. INTRODUÇÃO GERAL

Em 2012, 87% do consumo mundial de energia foi proveniente de combustíveis fósseis: petróleo, gás natural e carvão (33, 24, 30%, respectivamente) (BP, 2013). A queima de combustíveis fósseis, com conseqüente liberação dos gases do efeito estufa, como o dióxido de carbono (CO_2), é uma das principais causas das mudanças climáticas e distúrbios associados, como catástrofes ambientais, acidificação dos oceanos, queimadas, entre outros, além de aumentar os níveis de poluição do ar (IPCC, 2007).

Com o objetivo de modificar este panorama, pesquisadores vêm direcionando cada vez mais seus trabalhos para encontrar alternativas limpas, sustentáveis, eficientes e viáveis de fontes de energia. Em 2012, os restantes 13% do consumo mundial de energia foram provenientes de energia nuclear (4,4%), hidroeletricidade (6,7%) e outras energias renováveis (1,9%), como os biocombustíveis bioetanol e biodiesel (BP, 2013). Dentre estes últimos, o biodiesel parece uma alternativa interessante por várias razões: é altamente biodegradável; possui uma toxicidade muito baixa; pode substituir o diesel em várias aplicações, como caldeiras e motores de combustão interna sem grandes modificações, sendo relatada apenas uma pequena diminuição no

desempenho; a emissão de sulfatos, compostos aromáticos e outras substâncias químicas destrutivas para o ambiente é praticamente zero (Atabani *et al*., 2012).

No que se refere à produção, a baixa diversificação e o alto custo da principal matéria-prima (óleos vegetais) para a produção do biodiesel têm sido apontados como as maiores dificuldades para dinamizar o setor. A produção é altamente dependente da soja, que responde por 80% do volume produzido de biodiesel e, apesar de possuir alta homogeneidade e disponibilidade, possui baixa produtividade de óleo (19% da massa total) (Biodiesel no Brasil,2012).

As leveduras são fontes promissoras de óleo microbiano (Papanikolaou e Aggelis, 2011) e algumas linhagens chegam a acumular mais de 70% do seu peso seco em lipídios (Angerbauer *et al.,* 2008). De mais de 1600 espécies de leveduras já conhecidas, o número de espécies consideradas oleaginosas é em torno de 40 (Sitepu *et al*., 2013). Dentre elas, pode-se destacar *Rhodotorula glutinis, Rhodosporidium toruloides, Lipomyces starkeyi, Cryptococcus curvatus e Yarrowia lipolytica* (Ageitos*et al*., 2011).

Um dos principais gargalos para se descobrir novas espécies de micro-organismos oleaginosos é a metodologia utilizada. A quantidade de lipídios intracelulares extraída está diretamente relacionada com o método empregado na sua extração. No método tradicional, além de ser extremamente trabalhoso, é necessário a utilização de grandes volumes de solventes orgânicos (clorofórmio e metanol) prejudiciais tanto à saúde do manipulador quanto ao meio ambiente. Este método é necessário para saber a quantidade

mais próxima de lipídios que a linhagem de levedura estudada consegue acumular, assim como para verificar o perfil de ácidos graxos presentes no óleo para o estudo de sua aplicação. No entanto, quando se trata da realização de uma triagem a partir de um número muito grande de linhagens a serem estudadas, faz-se necessário uma metodologia mais rápida, eficiente e segura.

Outro tópico importante e essencial para fazer do óleo microbiano uma matéria prima alternativa na produção de biodiesel é torná-lo competitivo e viável no mercado atual. Para a produção de óleo microbiano ser considerada competitiva, o primeiro passo é a redução do custo de produção e isso envolve pesquisas com micro-organismos oleaginosos capazes de acumularem lipídios utilizando substratos de baixo valor comercial. Para tanto, outras fontes de carbono, que não a glicose, devem ser pesquisadas.

Com base nestes fatos, a padronização de uma metodologia de extração de lipídios, o desenvolvimento de uma metodologia eficiente, rápida e segura para a triagem de micro-organismos com potencial oleaginoso, assim como a otimização da produção de óleo microbiano de forma sustentável serviram como motivação para a investigação que resultou nesta tese de Doutorado.

O objetivo deste trabalho foi produzir óleo microbiano a partir de uma linhagem de levedura oleaginosa utilizando resíduos industriais como substrato. Uma triagem de leveduras potencialmente oleaginosas para uso do óleo na síntese de biodiesel foi realizada. Metodologias para seleção de leveduras potencialmente oleaginosas e para extração do óleo microbiano

foram desenvolvidas e padronizadas. Neste estudo, a otimização da produção do óleo microbiano em escala laboratorial, assim como a utilização de resíduos industriais para a sua produção foram avaliados.

Esta tese foi elaborada com base nos artigos produzidos durante a realização do presente trabalho e será organizada por meio da apresentação dos Artigos I, II e III.

2. REVISÃO BIBLIOGRÁFICA GERAL

2.1 Fontes de energia

A revolução industrial teve início na metade do Século XVIII e forneceu à população recursos para muito além do poder humano e animal. Trens e navios a vapor e, a seguir, motores de combustão interna transformaram a maneira como as pessoas se locomovem e produzem bens ao redor do mundo. Eletrificação e tecnologias relacionadas deram continuidade à revolução industrial nos Séc. XIX e XX. Atualmente, um crescente número de pessoas mantêm suas casas aquecidas no inverno, refrigeradas no verão e iluminadas à noite; vão aos mercados locais em seus carros com o poder de mais de uma centena de cavalos e voam entre continentes em aeronaves de fuselagem larga com o poder de cem mil cavalos. Este poder é derivado, em grande parte, da nossa capacidade de explorar fontes fósseis de energia. No entanto, com toda esta transição, as emissões de carbono, que resultam do equivalente a mais de um bilhão de cavalos trabalhando continuamente, criaram riscos significativos de mudanças climáticas (Chu & Majumdar, 2012).

A demanda crescente de energia caminha lado a lado com o crescimento e desenvolvimento mundial. A oferta global de energia primária mais do que dobrou entre 1971 e 2011, sendo que os combustíveis fósseis

ainda contam com mais de 80% da oferta mundial de energia (IEA, 2013). Com isso, a emissão de CO_2 na atmosfera vem aumentando drasticamente. Somente no período entre 1970 e 2004, este aumento ficou em torno de 80%, de 21 para 38 gigatoneladas (Gt), e representou 77% do total de gases do efeito estufa (IPCC, 2007). De acordo com a Agência Internacional de Energia (IEA, 2013), em 2012 a concentração de CO_2 na atmosfera foi de 394 ppmv (partes por milhão em volume), sendo que nos últimos 10 anos, a média de aumento foi de 2 ppmv/ano.

A Figura 1 apresenta o panorama atual do consumo mundial de energia, comparando os combustíveis fósseis (petróleo, carvão, gás) e energia não emissora de carbono (hidrogênio, nuclear e outros combustíveis renováveis, como bioetanol e biodiesel).

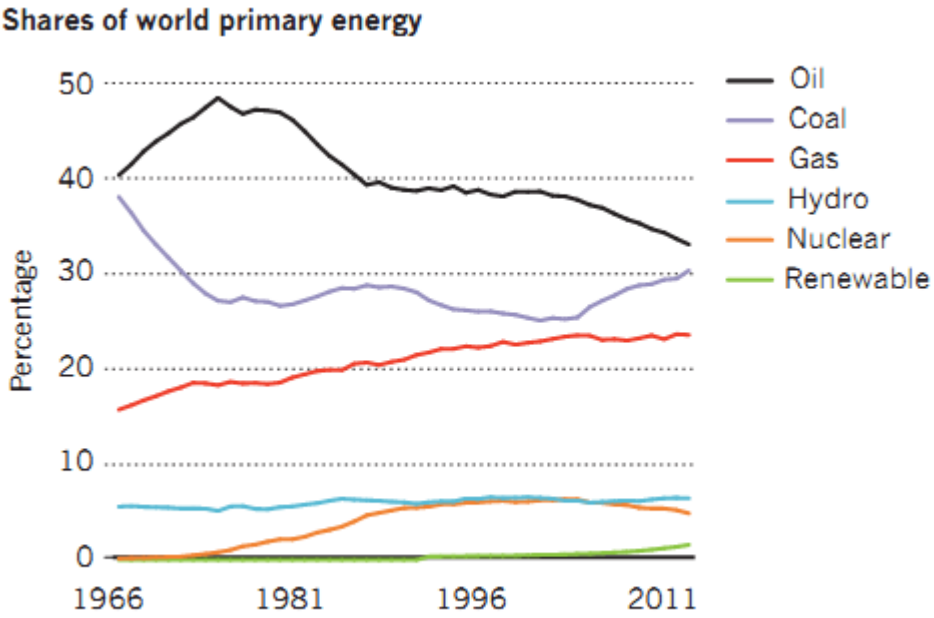

Figura 1: Revisão estatística da energia mundial: Energia fóssil compreende mais de 80% do consumo de energia mundial. A soma da fração de fontes de energia não emissoras de carbono permaneceu praticamente constante entre os anos de 1966 e 2011. Fonte: Chu & Majumdar, 2012.

2.2 Biodiesel

De acordo com a Resolução ANP nº 14, (2012), biodiesel é o combustível composto de ésteres alquila de ácidos carboxílicos de cadeia longa, produzido a partir da transesterificação e/ou esterificação de matérias graxas, de gorduras de origem vegetal ou animal.

O Brasil destaca-se como país líder na produção de biodiesel devido à sua capacidade de produção de uma gama de grãos dos quais o óleo vegetal é extraído. O Programa Nacional de Uso e Produção de Biodiesel (PNPB) é

uma iniciativa inovadora, criada em 2005, para fomentar a produção desse biocombustível no Brasil com o objetivo de se obter uma energia mais limpa e renovável, além de aumentar a renda e o emprego no campo (O Biodiesel, 2010). Devido aos inúmeros incentivos governamentais e a crescente importância do consumo do biodiesel, a produção deste combustível no Brasil aumentou significativamente. Em 2010, o Brasil alcançou um lugar de destaque na produção de biodiesel, tornando-se o segundo maior produtor mundial com 2,4 mil m^3, atrás apenas da Alemanha, que obteve uma produção de 2,7 mil m^3. Já em 2011, o Brasil caiu uma posição, ficando atrás dos Estados Unidos e Alemanha (OECD/FAO, 2011; Index Mundi).

O biodiesel é uma alternativa usada para aditivação do diesel (Gerpen, 2005). No Brasil, a mistura comercializada atualmente nos postos de combustíveis é a B5, ou seja, 5% de biodiesel misturado ao diesel. Em 2011, 2,55 milhões de m^3 de biodiesel foram produzidos (Biodiesel no Brasil, 2012). A capacidade instalada atualmente é de aproximadamente 5,1 milhões de m^3, superior à necessidade da demanda gerada pelo B5, já sendo suficiente para atender a adoção do B10 sem necessidade de construção de novas usinas. Porém, com a projeção de aumento da composição de biodiesel na mistura para 20%, previsto para 2020, a produção de biodiesel precisará atingir 14,3 milhões de m^3, ou seja, existe uma necessidade de ampliação para a produção de 9,2 milhões de m^3 de biodiesel (O Biodiesel, 2010).

2.3 Glicerol: principal subproduto da cadeia produtiva do biodiesel

A síntese de biodiesel ocorre através da transesterificação dos triglicerídeos com alcoóis, geralmente metanol ou etanol, sendo estimulada principalmente por catalisadores químicos ou físicos (Ma & Hanna, 1999). Os catalisadores alcalinos têm sido mais utilizados, em particular, devido ao seu baixo custo (Gonçalves *et al*., 2009). Após o fim da reação, a mistura é decantada (ou centrifugada) para separação das fases de onde são obtidos o biodiesel (fase superior) e o glicerol (fase inferior). Em todas as formas de catálise, seja ácida, básica ou heterogênea, ocorre formação da glicerina, representando um subproduto do processo (Ma & Hanna, 1999; Fukuda *et al*., 2001). A Figura 2 ilustra a reação de produção do biodiesel.

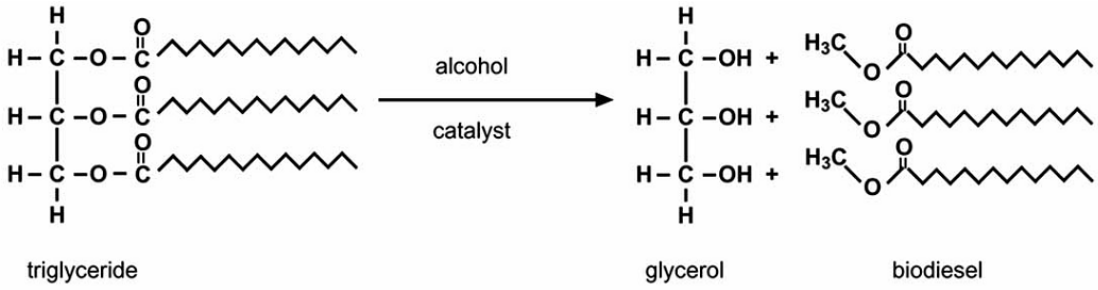

Figura 2: Reação de transesterificação do triglicerídeo com álcool: Triglicerídeos de óleos e/ou gorduras reagem com um álcool em uma reação conhecida como transesterificação ou alcoólise na presença de um catalisador. Os produtos desta reação são ésteres de ácidos graxos (biodiesel) e glicerina. Fonte: Manzanera *et al*., 2008.

O glicerol é um dos principais subprodutos da cadeia produtiva do biodiesel. Possui cor clara, alta concentração de sais, é higroscópico, com pouco odor e solúvel em água (Donkin & Doane, 2007). Este triol conjugado às impurezas inerentes da produção do biodiesel é denominado de glicerina (também chamado de glicerol bruto), possuindo baixo valor de mercado, inclusive devido a grande quantidade produzida e ofertada. No seu estado puro, ele é utilizado por diferentes indústrias, tais como farmacêuticas, cosméticas e de alimentos (Solaiman *et al*., 2006; Lammers *et al*., 2008; Pagliaro *et al*., 2007; Johnson & Taconi, 2007). No entanto, a purificação do glicerol obtido da produção do biodiesel a níveis aceitáveis para o seu uso em diversas indústrias envolve custos em procedimentos físico-químicos para aumentar a pureza da glicerina. Com a crescente produção de biodiesel nos últimos anos, a quantidade de glicerina gerada por esse processo tem sido considerável. Estima-se que para cada tonelada de biodiesel produzido são gerados 100 kg de glicerina, acarretando efeitos negativos na cadeia produtiva do biodiesel (Bowker *et al*., 2008). Com a projeção do aumento da composição de biodiesel na mistura com diesel para 20% (B20) em 2020, a produção de biodiesel deverá atingir 14,3 milhões de m^3, consequentemente, serão gerados no Brasil aproximadamente 1,43 milhões de m^3 de glicerina. Desse modo, surgem as questões sobre o aproveitamento de tal subproduto, pois um dos fatores mais preocupantes nesse processo seria o seu descarte inadequado no ambiente, ou seja, este é um potencial poluidor dos ecossistemas.

2.4 Mudanças climáticas e o seu impacto na área disponível para plantio

Um fator preocupante é a utilização de extensas áreas de terra destinadas à plantação de oleaginosas para a produção de biodiesel, gerando uma competição com atividades do setor de alimentos, resultando em diminuição da área plantada para alimentação e consequente aumento do custo dos alimentos. Atualmente, existe uma pressão internacional para a redução do uso de vegetais na produção de biocombustíveis.

O aquecimento global, causado principalmente pela emissão de gases do efeito estufa, é atualmente o maior problema ambiental mundial. De acordo com as projeções do Painel Intergovernamental de Mudanças Climáticas (IPCC, 2007), a temperatura média global poderá aumentar 2°C nos próximos 20 anos. A análise destas mudanças climáticas sobre várias áreas de cultivo brasileiras indica uma forte redução de áreas de plantio para 2030, incluindo o cultivo de algodão (Assad *et al*., 2013), soja, feijão, milho e arroz de terras altas (Assad *et al*., 2007), café Arabica (Zullo *et al*., 2011) assim como a redução na produção de frutas na região sul do país (Wrege *et al*., 2007). À luz destes fatos, a busca por alternativas limpas de fontes de energia vem sendo alvo de pesquisas mundialmente.

2.5 Óleo microbiano

O óleo microbiano (ou *single cell oil, SCO*) pode ser definido como óleo obtido de micro-organismos, similar em tipo e composição aos óleos e gorduras obtidos de plantas e animais (Ratledge, 2005).

2.5.1 Síntese de ácidos graxos e fatores que afetam a produção

Os lipídios intracelulares podem ser acumulados através de duas vias diferentes: a) síntese *de novo*, envolvendo a produção, em condições definidas, de ácidos graxos precursores, como acetil-coenzima A (acetil-CoA) e malonil-CoA, a partir de carboidratos não oleaginosos e sua integração na via biossintética de armazenamento de lipídios (via Kennedy) e b) via de acumulação *ex novo*, envolvendo a captação de ácidos graxos, óleos e triacilgliceróis (TAG) do meio de cultura e seu acúmulo em forma não-modificada ou modificada no interior da célula. Esta última via requer a hidrólise do substrato hidrofóbico, transporte dos ácidos graxos liberados para dentro da célula, sua remontagem nas frações de TAG e esterol éster (ES) e seu acúmulo dentro dos corpos lipídicos (Beopoulos *et al*. 2009)

Na síntese *de novo*, quando a glicose é a fonte de carbono utilizada pelo micro-organismo oleaginoso, ela é convertida à piruvato no citoplasma após várias etapas da via glicolítica. Ao entrar na mitocôndria, o piruvato é convertido à acetil-CoA, que condensa com oxaloacetato (um intermediário do ciclo de Krebs) formando citrato. Quando o nível de citrato mitocondrial é suficientemente alto, ele entra no citoplasma, onde é clivado, formando acetil-CoA e oxaloacetato (Liang e Jiang, 2013). Esta etapa é denominada lançadeira citrato-piruvato (*citrate-pyruvate shuttle*) e as etapas da reação podem ser obsevadas na Figura 3.

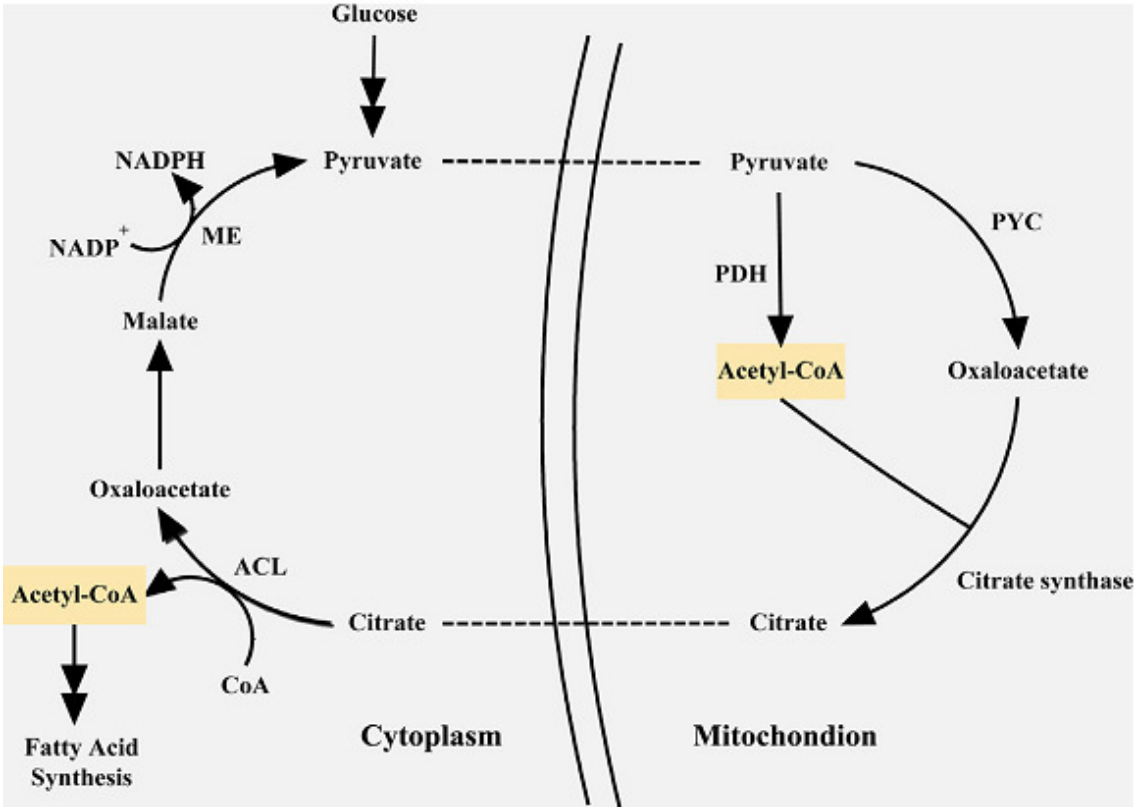

Figura 3: Lançadeira citrato-piruvato em micro-organismos eucariotos. Esta lançadeira transporta o grupo acetil da mitocôndria para o citoplasma. O grupo acetil sai da mitocôndria como citrato; no citoplasma ele é convertido em acetil-CoA para a síntese de ácidos graxos. PDH= piruvato desidrogenase; PYC= piruvato carboxilase; ACL= ATP:citrato liase; ME= enzima málica. Fonte: Liang e Jiang, 2013.

O principal fator que determina o acúmulo de lipídios em micro-organismos é a razão carbono/nitrogênio (C/N), sendo que o excesso de carbono e condições limitantes de nitrogênio favorecem o processo. O micro-organismo esgota rapidamente a fonte de nitrogênio, mas continua a assimilar a fonte de carbono, levando ao acúmulo de triglicerídeos. Para que ocorra a

síntese de lipídios, é necessário um grande suprimento intracelular de acetil-CoA e de nicotinamida adenina dinucleotídeo fosfato (NADPH).

As espécies de micro-organismos oleaginosos possuem uma enzima, denominada ATP citrato liase, que não é encontrada nas não-oleaginosas. Esta enzima catalisa a reação entre o citrato e coenzima A (CoA), formando acetil-CoA e oxaloacetato. A limitação de nitrogênio no meio de cultura induz uma cascata de acontecimentos que resulta no acúmulo de lipídios. Inicialmente, há um aumento na atividade de adenosina monofosfato desaminase (AMP-desaminase), diminuindo o conteúdo de AMP na célula. Essa diminuição na quantidade de AMP resulta na inibição da enzima isocitrato desidrogenase, que é dependente de AMP nos micro-organismos oleaginosos. Portanto, o isocitrato não é metabolizado e é equilibrado com citrato via ação da aconitase, havendo acúmulo de citrato na mitocôndria. O citrato é exportado da mitocôndria por meio de um sistema de efluxo, entrando no citoplasma e sendo utilizado pela ATP citrato liase na formação de oxaloacetato e acetil-CoA, que é utilizada na síntese de ácidos graxos. O oxaloacetato é convertido em malato e é utilizado no sistema de efluxo para saída do citrato da mitocôndria. O outro componente essencial é o NADPH, que é produzido nos micro-organismos oleaginosos via enzima málica. Esta enzima catalisa a reação entre o malato e NADP+, formando piruvato e NADPH (Wynn *et al*., 1999). Enzimas alternativas geradoras de NADPH também podem estar presentes (Ratledge, 2004).

2.5.2 Aplicações

O óleo microbiano, quando comparado com óleo vegetal, possui vantagens como: curto ciclo de vida, matérias-primas em grande quantidade e de baixo custo, menos influência do ambiente, estação e clima, além de facilidade de expandir sua produção. Sua aplicação vai depender do perfil de ácidos graxos característico do óleo que, por sua vez, é dependente do micro-organismo, assim como das condições de cultivo empregadas. Leveduras, diferentemente de muitos fungos filamentosos, tendem a produzir uma quantidade limitada de ácidos graxos poliinsaturados (PUFAs), sendo que algumas linhagens podem ter teores relativamente elevados de ácido esteárico (C18:0) (Ratledge, 2005). *Yarrowia lipolytica* acumula lipídios a níveis mais baixos quando comparada com outras espécies oleaginosas, mas é a única levedura conhecida capaz de acumular uma elevada proporção de ácido linoléico (mais de 50% do total de ácidos graxos) (Beopoulos *et al.*, 2009). Algumas espécies de fungos filamentosos, por exemplo, são explorados pela sua capacidade de produzir lipídios especiais, como os ácidos docosaexanóico, Y-linolênico, eicosapentaenóico e araquidônico (Liang & Jiang, 2013) com finalidade nutricional por meio de suplementos alimentares para grávidas, prematuros e recém-nascidos (Ratledge, 2005). A reengenharia do metabolismo microbiano com a finalidade de favorecer a produção de óleo para fabricação de combustíveis parece ser o caminho a seguir para os biocombustíveis de 3ª geração: óleos podem ser produzidos a partir de

materiais mais apropriados como celulose, glicerol ou, até mesmo, resíduo de óleo (Beopoulos *et al*., 2009).

2.5.3 *Yarrowia lipolytica*

A levedura *Y. lipolytica* é frequentemente encontrada em ambientes ricos em substratos hidrofóbicos, como alcanos ou lipídios, e desenvolveu sofisticados mecanismos para o uso eficiente de substratos hidrofóbicos como única fonte de carbono (Barth & Gaillardin, 1997; Beopoulos *et al*., 2009; Groenewald *et al*., 2013). *Y. lipolytica* é encontrada principalmente em alimentos com elevada proporção de gordura e/ou proteína, particularmente em produtos lácteos e carnes (ambos fermentados) (Groenewald *et al*., 2013).

A maioria das linhagens não é capaz de crescer em temperaturas superiores a 32°C e a espécie é estritamente aeróbica. *Y. lipolytica* vem sendo utilizada como uma levedura hospedeira de produtos com uma grande variedade de aplicações biotecnológicas. É considerada uma levedura não patogênica e foi classificada como GRAS (*Generally Regarded as Safe*) pela FDA (*Food and Drug Administration*) para a produção de ácido cítrico (Barth & Gaillardin, 1997; Fickers *et al*., 2005; Nicaud, 2012), eritritol, ácido eicosapentaenóico e β-caroteno (Groenewald *et al*., 2013).

O uso industrial de *Y. lipolytica* teve início com a empresa British Petroleum (BP) no final da década de 50. O aumento acentuado nos preços da matéria-prima, juntamente com o fato de autoridades recusarem a liberação das licenças necessárias para a produção, levaram a BP a encerrar suas

atividades com *Y. lipolytica* no final da década de 70 (Groenewald *et al*., 2013). Desde 2009, uma empresa polonesa chamada Polish Skotan Company SA, juntamente com um grupo de pesquisa da Wroclaw University (na cidade de Breslávia), introduziu um novo processo para a produção em larga escala de biomassa de *Y. lipolytica*, assim como lipídios para alimentação humana e animal. A produção anual de biomassa de levedura, no momento, alcança 1200 toneladas (Rywinska *et al*., 2013).

3. ARTIGOS

I Poli JS, Dallé P, Senter L, Mendes S, Ramirez M, Vainstein MH, Valente P. Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. Revista Brasileira de Biociências, Porto Alegre, v. 11, n. 2, p. 203-208, abr./jun. 2013.

II Poli JS, Lützhøft HCH, Karakashev DB, Valente P, Angelidaki I. An environmentally-friendly fluorescent method for quantification of lipid contents in yeast. Bioresource Technology, v. 151, p. 388-391, 2014. DOI: 10.1016/j.biortech.2013.09.128.

III Poli JS, Silva MAN, Siqueira EP, Pasa VMD, Rosa CA, Valente P. Microbial lipid produced by *Yarrowia lipolytica* QU21 using industrial waste: a potential feedstock for biodiesel production. Submetido.

I - Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils.

Poli JS, Dallé P, Senter L, Mendes S, Ramirez M, Vainstein MH, Valente P.

Revista Brasileira de Biociências, Porto Alegre, v. 11, n. 2, p. 203-208, abr./jun. 2013.

Revista Brasileira de Biociências
Brazilian Journal of Biosciences

ISSN 1980-4849 (on-line) / 1679-2343 (print)

ARTICLE

Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils

Jandora Severo Poli[1], Priscila Dallé[1] , Luciana Senter[1] , Sandra Mendes[1], Mauricio Ramirez[1], Marilene Henning Vainstein[2] and Patricia Valente[2]*

Received: February 20 2013 Received after revision: April 7 2013 Accepted: May 2 2013
Available online at http://www.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/2524

ABSTRACT: (Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils). Fatty acid methyl esters produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. An alternative for vegetable oil is microbial oil. There is, nowadays, an increasing interest in microbial lipidic compounds, called *single cell oils* (SCO), due to their several potential biotechnological applications, such as biodiesel production, food ingredients and antimicrobial activity, among others. Oleaginous yeasts are able to accumulate lipids up to 20% of their cellular dry weight, and some species can accumulate up to 70% of lipids. We have screened 86 yeast strains isolated from artisanal cheese using Nile red stain for the detection of intracellular lipid droplets by fluorescence microscopy, and 27% of them were promising for the production of microbial oil. *Yarrowia lipolytica* QU21 was selected for comparison of five different methods of cell wall disruption for lipid extraction at a laboratory scale (dry biomass maceration, lysis with vortex and glass beads, ultrasonic bath and glass beads, maceration using liquid nitrogen, and liquid nitrogen followed by sonication). The method which showed the highest oil yield value (26.5%) was liquid nitrogen with sonication. Oil yield was highly influenced by the method used for cell wall lysis. Fatty acid methyl esters (FAME) composition of strain QU21 after GC analysis was myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:1), stearic acid (C18:0) and oleic acid (C18:1).
Key words: cell disruption, lipid extraction, microbial oil, biodiesel.

RESUMO: (Metil ésteres de ácidos graxos produzidos pela levedura oleaginosa *Yarrowia lipolytica* QU21: uma alternativa para óleos vegetais). Uma alternativa para óleos vegetais é o óleo microbiano. Atualmente, existe um aumento no interesse em compostos lipídicos de origem microbiana, chamados *single cell oils* (SCO), devido ao seu grande potencial de aplicação biotecnológica, como a produção de biodiesel, suplementos alimentares, atividade microbiana, entre outros. Leveduras oleaginosas são capazes de acumular mais de 20% do seu peso seco em lipídios e algumas espécies conseguem acumular mais de 70% de lipídios. Foram analisadas 86 linhagens de leveduras isoladas de queijo artesanal, utilizando o corante vermelho de Nilo para a detecção de gotas lipídicas intracelulares em microscópio de fluorescência. Dessas, 27% se mostraram promissoras para a produção de óleo microbiano. *Yarrowia lipolytica* QU21 foi selecionada para comparar cinco métodos de rompimento da parede celular para a extração de lipídios em escala laboratorial (maceração da biomassa seca, lise com pérolas de vidro em agitador vórtex, lise com pérolas de vidro em banho ultrasônico, maceração utilizando nitrogênio líquido e maceração utilizando nitrogênio líquido seguido por sonicador). O método que apresentou maior rendimento lipídico (26,5%) foi maceração utilizando nitrogênio líquido seguido por sonicador. O rendimento lipídico é amplamente influenciado pelo método utilizado na etapa de lise da parede celular. A composição de metil ésteres de ácidos graxos (FAME) da linhagem QU21 utilizando cromatografia gasosa (GC) foi ácido mirístico (C14:0), ácido miristoleico (C14:1), ácido palmítico (C16:0), ácido palmitoleico (C16:1), ácido heptadecanóico (C17:1), ácido esteárico (C18:0) e ácido oleico (C18:1).
Palavras-chave: rompimento celular, extração de lipídios, óleo microbiano, biodiesel.

Abbreviations: DB, dry biomass maceration; VGB, vortex and glass beads; USGB, ultrasonic bath and glass beads; LN, liquid Nitrogen; LNS, liquid nitrogen with sonication.

INTRODUCTION

Oleaginous microorganisms, such as yeasts, fungi, and microalgae, can accumulate high amounts of neutral storage lipids under appropriate cultivation conditions. There is, nowadays, an increasing interest in microbial lipidic compounds, called single cell oils (SCO), due to their several potential biotechnological applications, such as biodiesel production, food ingredients, antimicrobial activity, and so on (Papanikolaou *et al.* 2004, Li *et al.* 2008, Beopoulos *et al.* 2009, Amaretti *et al.* 2010). Yeasts are a promising source of microbial oil, since they can accumulate up to 70% of their dry weight in lipids (Angerbauer *et al.* 2008), possess a GRAS (generally regarded as safe) status, and are easy to cultivate.

The development of reliable methods for screening oleaginous yeasts is necessary for time optimization and cost reduction in microbial oil production, as well as methods for accurate quantification and determination of the composition of the accumulated lipids. Greenspan *et al.* (1985) examined a variety of dye agents for the observation of lipid droplets, and found that Nile

1. Department of Microbiology, Immunology and Parasitology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.
2. Biotechnology Center (CBiot) , Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil.
* Author for correspondence. E-mail: patricia.valente@ufrgs.br

red is an excellent stain for the detection of intracellular lipid droplets by fluorescence microscopy and flow citometry. Many well-known extraction methods have been applied to food lipids, such as Soxhlet, Bligh & Dyer (1959) and Folch *et al.* (1957) processes, but extraction of lipids accumulated inside yeast cells needs, as a preliminary step, the disruption of the cell wall. It is known that yeast cell wall is hard to break, and the solvents used for lipid extraction are not sufficient to disrupt the cell wall by their own.

The purpose of the present investigation was to optimize a reliable protocol for screening of oleaginous yeasts and lipid extraction in laboratory scale, and evaluate Brazilian artisanal cheese as a source of oleaginous yeasts. Yeast screening was done using Nile red dye to observe the lipid droplets inside the cell. One oleaginous strain was chosen for the comparison of different methods for cell wall disruption. Lipid extraction was then performed according to Folch *et al.* (1957), and the lipid yield was analyzed in order to evaluate the efficacy of the different methods. Finally, the lipid profile was analyzed by gas chromatograph.

MATERIAL AND METHODS

Yeast screening with Nile red

Microorganism and culture conditions

Eighty six yeast strains, previously isolated from Brazilian artisanal cheeses (Landell *et al.* 2006), maintained in GYP agar (0.5% yeast extract, 1% peptone, 2% glucose, 2% agar) at 4 °C, were inoculated in conical flasks containing 25mL of a culture medium prepared with 10% glucose, 0.1% ammonium sulfate, 0.1% monopotassium phosphate, and 0.05% magnesium chloride hexahydrate (C:N ratio 100:1), and incubated at 150 rpm and room temperature (22-25 °C) during 96 hours.

Yeast molecular identification

Strain QU21 was grown aerobically in GYP broth (2% glucose, 1% peptone, 0.5% yeast extract) at 28 ºC. Total genomic DNA was extracted and purified from 5mL cultures as described by Osorio-Cadavid *et al.* (2009). Sequencing of the D1/D2 domain of the large subunit (26S) ribosomal DNA was performed according to Kurtzman & Robnett (1998) using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'). The amplification conditions were: initial denaturation at 94 ºC for 5 min, 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, and final extension at 72ºC for 10 min. The PCR product was purified by the polyethylene glycol precipitation method (Lis 1980), and sequenced at the Biotechnology Center of Rio Grande do Sul Federal University (Cbiot/UFRGS), Brazil. The sequence was assembled and compared with sequences reported in GenBank using the basic local alignment search tool (BLAST) algorithm.

Nile red solution

Nile red solution was prepared by dissolving 0.1 mg of Nile red in 1 mL of acetone (Greenspan *et al.* 1985, Kimura *et al.* 2004). Due to the heterocyclic nature of Nile red, it was handled as a carcinogen, though there is no evidence proving it. As Nile red is sensitive to light (Fowler & Greespan 1985), it was always handled with reduced light conditions, and stored at 4 °C.

Procedure

The samples were prepared as described below. Aliquots of 1 mL of each inoculum were centrifuged at 2000 rpm during five minutes. The supernatant was removed and the cells were resuspended with 1 mL of PBS buffer 10 mM (pH 7.4). The centrifugation and resuspension steps were repeated twice, and a smear was prepared spreading aliquots of 10 µL of the final cell suspension in a glass slide, which was air dried to fix the cells. 10 µL of Nile red solution were added to the smear and kept at room temperature for five minutes. The excess of Nile red was removed with PBS buffer 10 mM (pH 7.4), the glass slide was covered with a coverslip, and analyzed in a fluorescence microscope using lenses with wavelengths ranging between 450-500 nm. Lipids were observed as yellow gold droplets, whose size was visually estimated in relation to cell area.

Lipid extraction

Five different methods to disrupt yeast cell wall were tested. Lipid extraction was done according to Folch *et al.* (1957) using a mixture of chloroform and methanol in a proportion of 2:1. The procedure and steps are described below.

Microorganism and culture conditions

The yeast strain QU21 was chosen to analyze the total lipid content. It was maintained in GYP agar, inoculated in conical flasks containing 150mL of the culture medium described above, and incubated at 150rpm and room temperature (22 – 25 °C) for 72 hours.

Chemical Solutions

Pure solvent to upper and lower phase: This solution was prepared by mixing chloroform, methanol and distilled water in 8:4:3 proportions, respectively. A biphasic system is obtained and the two phases shall be collected and stored at amber glass bottles separately. The upper phase shall be denominated *pure solvent to upper phase* and the lower phase shall be denominated *pure solvent to lower phase*. These solutions shall be stored at room temperature.

Methods for yeast cell wall disruption

Dry biomass (DB): After 72 hours, the inoculum was filtered in a Kitassato flask with a membrane of 0.45 µm

pore diameter. The membrane containing the biomass was incubated at 40 ºC during two hours for drying. Then, dry biomass was transferred into a mortar and macerated with 20 mL chloroform and methanol solution with a pistil during 30 minutes.

Vortex and glass beads (VGB): After 72 hours, the inoculum was transferred into falcon tubes (15mL) and centrifuged at 5000 x g in a refrigerated centrifuge for 20 minutes. The supernatant was discarded, and 1 mL of chloroform and methanol solution, and a volume of 0.2 g of glass beads (diameter 200 μm) were added to the biomass in the falcon tubes. The mixture was vigorously vortexed during 15 minutes. Then, biomass and solvent were transferred into a mortar (at this step, it was necessary to wash the falcon tubes several times with the chloroform and methanol solution to remove total biomass without transferring the glass beads, which pelleted at the bottom of the tube), and macerated with 20mL chloroform and methanol solution with a pistil during 30 minutes.

Ultrasonic bath and glass beads (USGB): After 72 hours, the inoculum was transferred into falcon tubes (15 mL), and centrifuged at 5000 x g in a refrigerated centrifuge for 20 minutes. The supernatant was discarded, and 1 mL of chloroform and methanol solution and a volume of 0.3 g of glass beads (diameter 200 μm) were added to the biomass in the falcon tubes. The tubes were vortexed and transferred to Ultrasonic bath (Model USC700) with ice for two hours. Then, biomass and solvents were transferred into a mortar (at this step it was necessary to wash the falcon tubes several times with the chloroform and methanol solution to remove total biomass without transferring the glass beads, which pelleted at the bottom of the tube). The maceration step was not performed due to the amount of solvent needed to remove the glass beads, but the biomass remained in contact with the chloroform and methanol solution during the same 30 minutes used in the other treatments.

Liquid Nitrogen (LN): After 72 hours, the inoculum was filtered in a Millipore filtration system with a membrane of 0.45 μm pore diameter. The biomass retained in the membrane was transferred into a mortar. Liquid nitrogen was added to biomass, which was macerated with a pistil to break the cells. Then, 20 mL of chloroform and methanol solution was added, and the biomass with the solvents was macerated during 30 minutes.

Liquid Nitrogen with Sonication (LNS): After 72 hours, the inoculum was filtered in a Millipore filtration system with a membrane of 0.45 μm pore diameter. The biomass retained in the membrane was transferred into a mortar. Liquid nitrogen was added to biomass, which was macerated with a pistil to break the cells. After this step, the biomass was transferred into a falcon tube, distilled water was added to the biomass and cells were sonicated ten times for 30 seconds (Vibra Cell Sonicator, Model VC601, Sonics and Materials Inc.). Falcon tubes remained on ice during sonication. Sonicated cells remained in contact with 20 mL chloroform and methanol solution during 30 minutes.

Procedure

After the step of yeast cell wall disruption, the technique of lipid extraction was performed according to Folch *et al.* (1957). The mixture of biomass and chloroform/methanol solution was filtered through a filter paper MN-615 (Macherey-Nagel), and the dry weight of the biomass was determined. The filtered (crude) extract was collected in a graduate cylinder, its volume was determined and it was added 0.2 times its volume of distilled water, vigorously mixed in a flat bottom flask and transferred back into the graduate cylinder. The graduate cylinder with the sample was kept at rest until complete separation of the biphasic system. According to Folch *et al.* (1957), this step can be performed centrifuging the sample, but we were not able to successfully separate the phases by centrifugation (data not shown). The upper phase was removed with a pipette without disturbing the interface. The graduate cylinder walls were carefully washed several times with pure solvent to upper phase avoiding interface disturbance, and the excess of solvent was removed with a pipette. The upper phase was discarded, and 10 mL of methanol was added to the lower phase, which contains the extracted lipids, mixed in order to homogenize the phases, and transferred into a rotary evaporator. The graduate cylinder walls were washed several times with pure solvent to lower phase, and the solvents were eliminated with a flash evaporator at these conditions: 40 °C and 120 rpm. After evaporation of solvents, lipids were removed from the evaporating flask with a pipette and transferred into a falcon tube (50 mL). The residual sample was removed from the rotary evaporator with the help of chloroform and methanol solution until complete removal. The falcon tube was incubated at 37-40 ºC for complete evaporation of the solvent, and weighted (final weight).

In the LN and LNS procedures for cell wall disruption, the crude extract was collected in a 50 mL falcon tube. The procedure for lipid extraction was as described above, except that the tubes were incubated at 37-40 ºC, without using rotary evaporator.

Quantification of total lipids was estimated according to the difference between the final weight and the weight of the falcon tubes and filter paper used, allowing the estimation of the proportion of accumulated lipids in relation to dry biomass (oil yield).

Statistical analysis

All values shown for the yeast cell wall disruption methods are the average of two separate experiments. The mean values are reported and compared by analysis of variance (One-way ANOVA) ($P \leq < 0.05$). Difference among the observed averages of the treatments were tested using the Tukey test ($P < \leq 0.05$).

Lipid profile

In order to obtain the lipid profile (qualitative analysis), the lipids extracted from strain QU21 were methylated according to Hartman & Lago (1973) to obtain fatty acid methyl esters (FAME). FAME were analyzed in a gas chromatograph equipped with FID (flame ionization detection) and the capillary column DB-Wax (60 m x 0.25 mm x 0.2 µm). Temperatures from the detector and injector were 250 ºC. The following temperature program was used for the separation of FAME: 100 ºC for 1 minute, with a gradual increase of 2 ºC per minute until the final temperature of 240 ºC, where it was held for 10 minutes. The carrier gas was H_2 with constant flow (20 mL/min). Volume of injection was 1 microliter with split rate of 1:100. The identification of FAME was evaluated using Supelco 37 Component Fame Mix (10 mg/mL – SIGMA) as standard.

RESULTS

Yeast screening with Nile red

All yeasts analyzed (n = 86) presented lipid droplets in different proportions, whose size was visually estimated in relation to cell area, based on the yellow gold fluorescence emitted by neutral lipids after treatment with Nile red dye (Fowler & Greespan 1985). Of these, 32 and 31 yeast strains accumulated lipids up to 30% and between 30 to 50% of the cell area, respectively. However, 23 yeasts strains filled more than 50% of cell area with lipid droplets, and were selected as potentially oleaginous yeasts. Yeast strains representative of each of the three lipid accumulation groups after screening with Nile red dye can be visualized in Figure 1.

Molecular identification of strain QU21

Based on the results obtained in the screening step using Nile red dye, strain QU21 was chosen for lipid extraction, as it belonged to the group in which lipid droplets filled more than 50% of cell area. The 26S rDNA D1/D2 region of this strain showed a 99% sequence identity with *Yarrowia lipolytica*, therefore strain QU21 was identified as belonging to this species.

Table 1. Oil yield obtained using five different methods to disrupt the *Yarrowia lipolytica* QU21 cell wall.

Methods for yeast cell wall disruption	Dry biomass (g)	Total lipid (g)	Oil yield (%)
LNS*	0.34	0.09	26.5 A**
DB	0.28	0.04	14.3 B
LN	0.17	0.02	12.8 B
USGB	0.41	0.03	7.3 C
VGB	0.27	0.01	3.7 C

Abbreviations: LNS, liquid nitrogen with sonication; DB, Dry biomass maceration; LN, liquid nitrogen; USGB, ultrasonic bath and glass beads.VGB, vortex and glass beads.

**Values followed by the same letter do not differ statistically by the ANOVA Test ($P \leq 0.05$).

Lipid extraction comparing different methods for yeast cell wall disruption and lipid profile of strain QU21

We tested five different methods for disruption of yeast cell wall before lipid extraction using the oleaginous strain QU21, selected after screening with Nile Red. USGB and VGB showed the lowest values of oil yield, with 7.3 and 3.7% of the dry biomass composed by oil, while DB and LN showed similar values with 14.3 and 12.8% of oil yield (Table 1). The method showing the highest oil yield value was LNS with 26.5% of oil yield, with significant difference compared with any other method tested here. The lipid profile of strain QU21 can be seen in Table 2.

DISCUSSION

The treatment with Nile Red proved to be efficient for a preliminary large scale screening of oleaginous yeasts. The whole screening process lasted 96 hours for yeast cultivation, and 20 to 30 minutes per sample for Nile Red smear preparation and fluorescence detection. This process would greatly benefit from a reduction in the

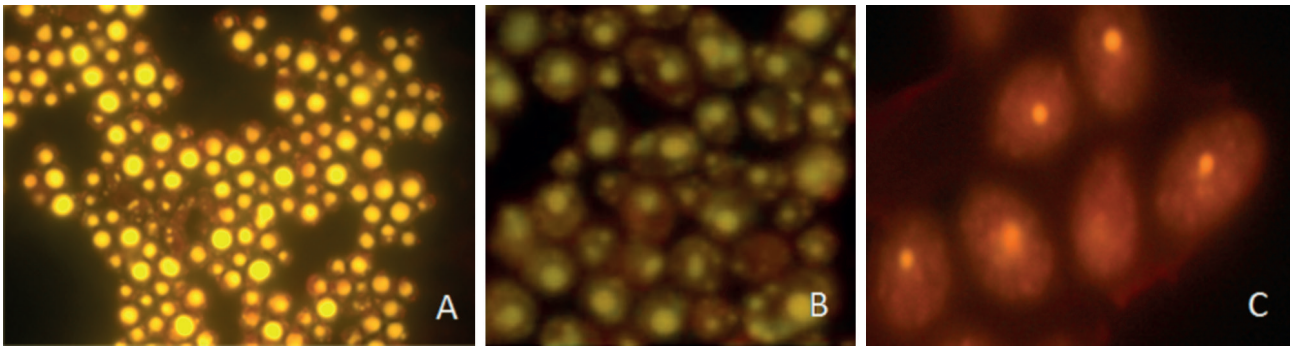

Figure 1. Representative photos of yeast strains classified according to the area covered by lipid droplets (yellow-gold) inside the cell visualized with the oil immersion objective lenses (100x). A. Lipid droplets filling more than 50% of cell area. B. Lipid droplets filling between 30 to 50% of cell area. C. Lipid droplets filling less than 30% of cell area.

Table 2. Long chain fatty acid profile of *Yarrowia lipolytica* QU21 and its comparison with two types of vegetable oils usually applied in biodiesel production.

LCFAs	QU 21 yeast strain	Soybean oil*	Rapeseed oil*
Myristic acid (C14:0)	✓	✓	
Myristoleic acid (C14:1)	✓	✓	
Palmitic acid (C16:0)	✓	✓	✓
Palmitoleic acid (C16:1)	✓	✓	✓
Hexadecanoic acid (16:2)		✓	
Heptadecanoic acid (C17:1)	✓		
Stearic acid (C18:0)	✓	✓	✓
Oleic acid (C18:1)	✓	✓	✓
Linoleic acid (C18:2)		✓	✓
Linolenic acid (C18:3)		✓	✓
Arachidic acid (20:0)		✓	
Eicosenoic acid (C20:1)			✓
Behenic acid (22:0)		✓	
Erucic acid (22:1)		✓	

✓ presence of fatty acid.

* Data from Downey & Craig 1964, Goodrum & Geller 2005, Saka & Kusdiana 2001, Koutb & Morsy 2011.

cultivation time, especially if a high throughput methodology is established. The strain QU21 was chosen for lipid extraction, as it belonged to the group in which lipid droplets filled more than 50% of cell area, as well as it was identified as belonging to *Y. lipolytica* species. *Y. lipolytica* is largely known as an oleaginous yeast (Beopoulos *et al.* 2009, Papanikolaou *et al.* 2011a, Papanikolaou *et al.* 2011b) proving that the screening step with Nile red was efficient.

Lipid extraction from oleaginous yeasts is an extremely important issue. Unfortunately, it is generally accepted that there is no extraction method capable of resulting in 100% oil yield (Jacob 1992, Ageitos *et al.* 2011). In our preliminary experiments, we observed that there was little correspondence between the fluorescence image after staining with Nile Red and the lipid yield obtained after extraction according to Folch *et al.* (1957). We tentatively explained this lack of correspondence by an inefficient cell wall breakage prior to lipid extraction with solvents. According to Jacob (1992), yeasts have several disadvantages for lipid extraction, including the presence of a thick cell wall that renders the yeast cells resistant to many solvents, as well as the possible presence of lipases in their cell extract. The inefficiency in this step could act as an important barrier during the process of intracellular lipid extraction.

Of the five different methods for disruption of yeast cell wall before lipid extraction using the oleaginous strain QU21, LNS yielded the highest oil content (26.5%). Oleaginous yeasts are able to accumulate more than 20% of their cellular dry weight in lipids (Ageitos *et al.* 2011, Koutb *et al.* 2011). Therefore, strain QU21 can be considered an oleaginous yeast. Examples of oleaginous yeasts besides *Y. lipolytica* include the species *Rhodotorula glutinis*, *Rhodosporidium toruloides Lipomyces starkeyi*, and *Cryptococcus curvatus* (Ageitos *et al.* 2011). Some yeast species, like *Lipomyces starkeyi*, can accumulate oil up to 70% of their cellular dry weight (Angerbauer *et al.* 2008).

Lipids produced by yeasts are classified as neutral (triglycerides) and polar (glycolipids and phospholipids), and are mainly composed by saturated and monounsaturated triglycerides. Microbial oil can be an alternative to vegetable oil in some industrial processes, such as biodiesel production, thus a comparative lipid profile between QU21 oil and two types of vegetable oils usually applied in biodiesel production can be seen in Table 2 (Downey *et al.* 1964, Goodrum *et al.* 2005, Clemente & Cahoon 2009, Saka *et al.* 2001). In the conditions analyzed in this work, strain QU21 does not accumulate two types of polyunsaturated fatty acids (Linoleic and Linolenic acid). According to Papanikolaou & Aggelis (2011a), the oil obtained from yeasts is less unsaturated than oil from other oleaginous fungi. Biodiesel constituted by unsaturated methyl esters, especially poly-unsaturated methyl esters, such as methyl linoleate (C18:2) and methyl linolenate (C18:3) is most susceptible to oxidation (Xin *et al.* 2009). Therefore, the oil obtained with yeast strain QU21 is advantageous for biodiesel production, since it is devoid of polyunsaturated fatty acids.

In summary, cheese proved to be a promising source of oleaginous yeasts, and the Nile red dye was an adequate tool for rapid screening of these yeasts. The oil yield obtained after lipid extraction was highly dependent on the method used for disrupting yeast cell wall. In the conditions we used, the most efficient method for laboratory scale use was Liquid Nitrogen with Sonication. The lipid profile from *Yarrowia lipolytica* QU21 shows that this strain should be thoroughly investigated due to its potential industrial application, such as oil source for biodiesel production.

ACKNOWLEDGMENTS

The authors thank Dr JEC Kanan (Universidade Federal do Rio Grande do Sul) for helping with the fluorescence microscope, Dr Simone Flores and Aline Leuven for providing the GC analysis. This work was financially supported by CNPq (process 558515/2010-0) and FAPERGS (process 11/2047-0).

REFERENCES

AGEITOS, J.M., VALLEJO, J.A., VEIGA-CRESPO, P. & VILLA, T.G. 2011. Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol*., *90*: 1219-27.

AMARETTI, A., RAIMONDI, S., SALA, M., RONCAGLIA, L., LUCIA, M. DE, LEONARDI, A. & ROSSI, M. 2010. Single cell oils of the cold-adapted oleaginous yeast *Rhodotorula glacialis* DBVPG 4785. *Microb. cell fact*., *9*: 1-6.

ANGERBAUER, C., SIEBENHOFER, M., MITTELBACH, M. & GUEBITZ, G.M. 2008. Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. *Bioresource Technol*., *99*: 3051-3056.

BEOPOULOS, A., CESCUT, J., HADDOUCHE, R., URIBELARREA, J.-L., MOLINA-JOUVE, C. & NICAUD, J.-M. 2009. *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res., 48*: 375-87.

BLIGH, E.G. & DYER, W.J. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol*., *37*: 911-917.

CLEMENTE, T.E. & CAHOON, E.B. 2009. Soybean oil: genetic approaches for modification of functionality and total content. *Plant Physiol., 151*: 1030-40.

DOWNEY, R. & CRAIG, B. 1964. Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus L.*). *J. Am. Oil Chem. Soc*., *41*: 475-478.

FOLCH, J., LEES, M. & STANLEY, G.H.S. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*., *226*: 497-509.

FOWLER, S.D. & GREENSPAN, P. 1985. Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: comparison with oil red O. *J. Histochem. Cytochem*., *33*: 833-836.

GOODRUM, J.W. & GELLER, D.P. 2005. Influence of fatty acid methyl esters from hydroxylated vegetable oils on diesel fuel lubricity. *Bioresour. Technol*., *96*: 851-5.

GREENSPAN, P., MAYER, E.P. & FOWLER, S.D. 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol., 100*: 965-73.

HARTMAN, L. & LAGO, R.C. 1973. Rapid preparation of fatty acid methyl esters from lipids. *Lab. Pract*., *22*: 475-476.

JACOB, Z. 1992. Yeast lipids: Extraction, quality analysis and acceptability. *Crit. Rev. Biotechnol*., *12*: 463-491.

KIMURA, K., YAMAOKA, M. & KAMISAKA, Y. 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *J. Microbiol. Methods*, *56*: 331-8.

KOUTB, M. & MORSY, F.M. 2011. A potent lipid producing isolate of *Epicoccum purpurascens* AUMC5615 and its promising use for biodiesel production. *Biomass Bioenergy*, *35*: 3182-3187.

KURTZMAN, C.P. & ROBNETT, C.J. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek*, *73*: 331-371.

LANDELL, M.F., HARTFELDER, C. & VALENTE, P. 2006. Identification and enzymatic profile of yeasts isolated from artisanal cheese in Southern Brazil. *Acta Sci. Vet*., *34*: 49-55.

LI, Q., DU, W. & LIU, D. 2008. Perspectives of microbial oils for biodiesel production. *Appl Microbiol. Biotechnol*., *80*: 749-56.

LIS, J.T. 1980. Fractionation of DNA fragments by polyethylene glycol induced precipitation. *Methods Enzymol*., *65*: 347-353.

OSORIO-CADAVID, E., RAMÍREZ, M., LÓPEZ, W.A. & MAMBUSCAY, L.A. 2009. Estandarización de un protocolo sencillo para la extracción de ADN genómico de levaduras. *Ver. Colomb. Biotecnol*., *XI*: 125-131.

PAPANIKOLAOU, S. & AGGELIS, G. 2011a. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *Eur. J. Lipid Sci. Technol., 113*: 1031-1051.

PAPANIKOLAOU, S. & AGGELIS, G. 2011b. Lipids of oleaginous yeasts. Part II: Technology and potential applications. *Eur. J. Lipid Sci. Technol., 113*: 1052-1073.

PAPANIKOLAOU, S., KOMAITIS, M. & AGGELIS, G. 2004. Single cell oil (SCO) production by *Mortierella isabellina* grown on high-sugar content media. *Bioresource Technol*., *95*: 287-91.

SAKA, S. & KUSDIANA, D. 2001. Biodiesel fuel from rapeseed oil as prepared in supercritical methanol. *Fuel*, *80*: 225-231.

XIN, J., IMAHARA, H. & SAKA, S. 2009. Kinetics on the oxidation of biodiesel stabilized with antioxidant. *Fuel*, *88*: 282-286.

II - An environmentally-friendly fluorescent method for quantification of lipid contents in yeast

Jandora Severo Poli, Hans-Christian Holten Lützhøft, Dimitar Borisov Karakashev, Patricia Valente, Irini Angelidaki

Bioresource Technology, v. 151, p. 388-391, 2014.

DOI: 10.1016/j.biortech.2013.09.128

An environmentally-friendly fluorescent method for quantification of lipid contents in yeast

Jandora Severo Poli[a,b], Hans-Christian Holten Lützhøft[a,*], Dimitar Borisov Karakashev[a], PatriciaValente[b], Irini Angelidaki[a]

[a]Department of Environmental Engineering, Technical University of Denmark, Miljøvej 113, 2800 Kongens Lyngby, Denmark.

[b]Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500 sala 154, Cep: 90.050-170, Porto Alegre, RS, Brazil.

E-mail of the authors: jandora.poli@gmail.com; hhlu@env.dtu.dk; dbka@env.dtu.dk; patricia.valente@ufrgs.br; iria@env.dtu.dk

[*]Corresponding author: Tel.: +45-45251425.

Bioresource Technology, v. 151, p. 388-391, 2014.

DOI: 10.1016/j.biortech.2013.09.128

Highlights

- Nile red staining offers short analysis time for neutral lipid determination in yeast.
- Nile red staining is energy efficient and uses minute volumes of organic solvents.
- Nile red staining is suitable for fast screening and high-throughput analysis.

Abstract

This study aimed to develop an efficient, fast and environmentally-friendly method to quantify neutral lipid contents in yeast. After optimizing the fluorescence instrument parameters and influence of organic solvent concentrations, a new method to quantify neutral lipids in yeast based on fluorescence was demonstrated. Isopropanol and Nile red in concentrations of 5% (final volume %) and 500 µg/L, respectively, were added to washed cells suspended in potassium chloride phosphate buffered saline (PBSKCl). Fluorescence was measured after 10 min in the dark. Glyceryltrioleate (GTO) was used as model lipid and the calibration curve showed linearity (R^2=0.994) between 0.50-25 mg/L. Compared with traditional gravimetric analysis, the developed method is much faster and uses less organic solvents. Lipid contents determined by fluorescence and gravimetry were the same for some strains, but for other strains the lipid contents determined by fluorescence were less. This new method will therefore be suitable for fast screening purposes.

Keywords: Yeast, lipid, quantification, Nile red, biodiesel

1. Introduction

The use of biofuels as a renewable, environmentally-friendly alternative to fossil fuels has been subject of research for many years. The most common biofuel in Europe is biodiesel. The global biodiesel market is estimated to reach 37 billion gallons by 2016 with an average annual growth of above 40 % (Li et al., 2008). Nevertheless, traditional 1st generation (1G) biodiesel production (transesterification of triacylglycerols from plant oils) has various drawbacks and limitations (Kalscheuer et al., 2006) such as: season and climate-dependent cultivation of the plant oil feedstock (rapeseed, soybean); the agricultural land competition for food, resulting in reduction of

cultivated area for feed and consequently increasing food prices; and international pressure to reduce the use of terrestrial plants in biofuels production. It is therefore of ultimate importance to evaluate other lipid sources in addition to plants. Possible alternative oil sources are oleaginous microorganisms, such as microalgae, yeasts and fungi. Fermentation employing oleaginous microorganisms (Amaretti et al., 2012; Papanikolaou, 2011) (2G biodiesel production) is a very promising alternative solution to overcome the critical bottlenecks of 1G biodiesel production.

In comparison to other oleaginous fungi, the oleaginous yeasts are much more robust and easy to handle in the laboratory and large scale applications (Papanikolaou, 2011). Traditional methods for measurement of lipid content of microbial biomasses are based on solvent extractions followed by gravimetric determination of either total, polar or neutral lipids (e.g. Poli et al., 2013; Sitepu, et al., 2012). There are two major drawbacks of the conventional method: 1) the results are quite dependent on the cell wall lysis step (Poli, et al., 2013; Sitepu et al., 2012) hence highly time and labour consuming, infeasible for screening; 2) the method uses non-environmental friendly and strong organic solvents such as chloroform ($CHCl_3$) that should be handled with care. Therefore, there is a need for a rapid, robust and highly efficient method for quantifying lipid contents in microbial biomasses. Particular attention has recently been paid to a fluorescence-based method to evaluate lipid levels in different cells using the fluorescent dye Nile red. Kimura et al. (2004) published a method to determine lipid contents of yeast cells applying Nile red to yeast cells re-suspended in potassium phosphate buffer (PBS). Quite recently Sitepu et al. (2012) evaluated the method by Kimura et al. (2004) and suggested addition of 5% dimethylsulfoxide (DMSO) for improving the penetration of Nile red into the yeast cells. However, neither of the methods could adequately quantify the lipids, which is

essential for allowing screening and evaluation of oleaginous yeasts for their ability to produce lipids. The aim of this study was therefore to develop a highly efficient and fast method to quantify neutral lipid contents in yeasts using less aggressive organic solvents and to investigate the factors affecting the performance.

2. Materials and methods

2.1. Microorganism and culture conditions

The yeast strain *Yarrowia lipolytica* QU 21 (Poli et al., 2013) and three yeast isolates (YI1, YI2 and YI3) obtained from a biodiesel plant (Emmelev A/S, Denmark), were used for evaluation of the method and were pre-grown on YEPD or YEPG agar (1% yeast extract, 2% peptone, 2% glucose or glycerol, 2% agar). The liquid medium for cell culture growth was composed of: 10% glucose or glycerol, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, and 0.05% $MgCl_2 \cdot 6H_2O$. Glucose was used for *Y. lipolytica* and glycerol was used for YI1, YI2 and YI3. All microorganisms were pre-cultured for 24 hours, followed by inoculation in 100 mL of the corresponding liquid media using 250-mL Erlenmeyer flasks. The submerged cultures were incubated in a rotating shaker at 150 rpm and 25°C for 5 days. Liquid cultures were centrifuged (Heraus Multifuge X3R Centrifuge, Thermo Scientific, AXEB, Denmark) at 4700 x g for 10 min to remove the supernatant, and used for dry biomass determination, analyses of yeast lipids using the fluorescence method and gravimetric determination. The cell pellets were washed twice with 0.15 M potassium chloride (KCl) in 0.01 M phosphate buffered (pH 7.0) saline (PBSKCl) and, if necessary, suspended in PBSKCl.

2.2. Dry biomass determination

Cell pellets (described in section 2.1) were kept at 70°C to constant weight.

2.3. Fluorescence spectroscopy

2.3.1 General procedure

Samples were diluted to 10 mL with PBSKCl containing 5% (volume %) isopropanol (i-PrOH), unless otherwise stated. Fluorescence in arbitrary units (A.U.) was measured using a Cary Eclipse fluorescence spectrophotometer controlled by the Cary Eclipse software version 1.1.1.3 (Varian, Analytical Instruments, Værløse, Denmark) before and after addition of 50µL Nile red, in order to subtract the intrinsic fluorescence of the sample. Samples added Nile red remained 10 minutes in the dark before analysis. To measure the neutral lipids, an excitation and emission wavelengths of 488 and 585 nm, respectively, were used (Kimura et al., 2004). A 10 mg/L solution of the standard lipid resulted in a fluorescence intensity in A.U. of about 23. 100 mg/L Nile red was prepared in acetone (DMK).

2.3.2 Determining the optimal model lipid as standard

Three different model lipids, glyceryltripalmitate, $C16:0_3$ (GTP), glyceryltrioleate, $C18:1^{\Delta 9}{}_3$ (GTO) and glyceryltrilinoleate, $C18:2^{\Delta 9,12}{}_3$ (GTL), where the acyl-groups represent different degrees of saturation, were selected to determine the possibility to differentiate among the degree of saturation in the fluorescence assay, and thereby determine the lipid saturation profile of the yeast strains. Stock solutions of GTP, GTO and GTL were prepared in i-PrOH. Working solutions were prepared by diluting the stock solutions 10 times in PBSKCl, reaching the final concentration of 5, 15 and 1.5 mg/L, respectively.

2.3.3 Influence of organic solvents and yeast cell quantities

A range of organic solvents (ethanol (EtOH), isopropanol (i-PrOH), acetone (DMK), DMSO and combinations thereof) were assessed for their ability to enhance/suppress the Nile red penetration into the cell and compared with water as well

as their influence on the fluorescence signal of the Nile red-lipid complex. According to the general procedure, the fluorescence of a mixture of growth medium:organic solvent in the ratio 1:4 was measured before and after addition of Nile red.

An experiment using increasing quantities of yeast cells and different organic solvents was set up. The aim was to study the influence of yeast cell quantity on the lipid determination and if the type of organic solvent affected the Nile red penetration through the cell membrane. Linear regression was used to evaluate the potential influence of quantities of yeast cells and organic solvent on the lipid determination. 10-200 µL volumes (corresponding to 2.77-55.3 mg/L according to the cell dry weight) were diluted to 10 mL with PBSKCl with or without 10% (volume %) i-PrOH or DMSO. The fluorescence of the samples was measured as described in Section 2.3.1.

2.3.4 Optimizing i-PrOH concentration, mixing intensity and fluorescence signal

An experimental design using Plackett & Burman with 12 assays (PB12) and 3 centre points run was performed to evaluate i-PrOH concentration, mixing intensity and fluorescence signal. The i-PrOH concentration was tested at 5, 10 and 20% (volume %) in the analysis solution. Vortexing for one min, gentle manual mixing three times were compared with no mixing at all to find the optimal mixing intensity. The fluorescence signal is influenced by when the signal is recorded, how long the instrument scans the emitted light, at which voltage the photomultiplier tube (PMT voltage) is operated and the slit width. The signal was recorded immediately, after 30 s and after 180 s. The instrument scan times were studied at 0.1; 3 and 10 s. PMT voltages of 400, 600 and 800 V and slit widths of 2.5; 5 and 10 nm were tested.

2.4. Lipids extraction

An equivalent of 0.14g dry biomass was re-suspended in 10 mL PBSKCl, kept on ice while sonicated for 10 minutes using Branson Sonifier Cell Disruptor S-250A (Branson Ultrasonics Corporation, VWR Bie & Berntsen A/S, Herlev, Denmark), and centrifuged at 4700 x g for 10 minutes at 20°C. The lipids were extracted from the disrupted cells using a $CHCl_3$:MeOH solution (2:1) according to Folch et al. (1957), the organic phase was isolated and evaporated to constant weight.

2.5. Calibration curves and Statistical analysis

Linear regression of GTO (0.50-25 mg/L) was used to quantify yeast cell lipid contents using the Nile Red fluorescence method. The experimental design analysis (PB12) was carried out using Statistic 5.0 software (Statsoft, USA). The lipid content mean values (n=3) are reported and compared by analysis of variance (One-way ANOVA) (P=0.05) using excel. Difference among the observed averages of the treatments were tested using the Tukey test (P=0.05).

3. Results and discussion

3.1. Selecting the model lipid for determining yeast lipid contents

The acyl moieties of yeast lipids are mainly represented by the oleic ($C18:1^{\Delta 9}$), palmitic (C16:0) and linoleic ($C18:2^{\Delta 9,12}$) long chain fatty acids (LCFAs) with a minor content of other LCFAs (Juszczyk et al., 2013; Beopoulos et al., 2009). However, experiments performed to distinguish among degree of saturation, did not show any differences in the fluorescence signal (neither spectrum, nor intensity) obtained by the Nile red-lipid complex (data not shown). GTO was therefore selected as the lipid standard for the rest of the experiments.

The excitation and emission wavelengths for total lipid (neutral and polar) measurements are dependent on the individual organism tested and the composition and content of intracellular lipids, ranging from 470 to 549 nm and 540 to 628 nm, respectively (Chen et al., 2009). Therefore the excitation wavelength that previously has been used for neutral lipid analysis has been in the range 470 to 530 nm (Chen et al., 2009). According to Kimura et al. (2004) the emitted fluorescence measured using 488 nm as the excitation wavelength showed higher intensity than using 522 nm as the excitation wavelength. Similarly, for all the tested microorganisms (oleaginous fungi and yeasts), the peak wavelengths of emission varied between 565 and 585nm (Kimura et al., 2004). Based on these data and according to a determination of the excitation and emission wavelengths for *Y. lipolytica* QU21 stained with Nile red (data not shown), the excitation and emission wavelengths were set to 488 and 585 nm, respectively, as mentioned in section 2.3, were used throughout the work.

3.2. Yeast cell quantities diluted in different solvents

For other microorganisms in other assays, addition of organic solvents (medium:DMK:DMSO 2:4:4 after 20 min and medium:EtOH 3:7 overnight) enables repeatable measurements of e.g. chlorophyll A in algae (Mayer et al., 1997; Lützhøft et al., 1999). However, experiments on yeast cells showed that adding solvents (EtOH, i-PrOH, DMK and DMSO) or mixtures thereof to the medium in a concentration of 80% resulted in quenching of the fluorescence signal, and were therefore proved not to be useful, see supporting information Figure S1.

Nevertheless, in order to be able to quantify the lipid contents of the yeast cells, using GTO as the model lipid, an organic solvent is needed to keep GTO in solution. From the initial experiments (see supporting information Figure S1), the fluorescence signal using pure DMSO was lower than the fluorescence signal when

using e.g. i-PrOH. The organic solvent concentration was therefore reduced to a minimum, still allowing GTO to be dissolved. An organic solvent concentration of 10% (volume %) resulted in the graphs as shown in Fig. **1**, where an increase of the yeast cell concentration resulted in an increase of the fluorescence intensity, thereby showing that the fluorescence signal was not quenched by the added quantity of organic solvent. Different organic solvents showed significant differences in the slope and linearity, see Fig. **1** (P=0.009399). However, comparing DMSO with i-PrOH did not show any difference (P=1 for the slope and P=0.1891 for the intercept), and though using DMSO as the organic solvent resulted in a higher slope/sensitivity, i-PrOH gave a better R^2 for the regression. Due to higher R^2 (better linearity and less variation) and because i-PrOH is a less aggressive solvent with lower toxicity compared with the more harmful DMSO, i-PrOH was chosen as the organic solvent for the rest of the experiments.

3.3. Selection of significant variables by Plackett & Burman

t-tests were used to identify the effect of each factor on the fluorescence signal emitted by the Nile red-GTO complex. Supporting information Table S1 shows that PMT voltage and slit width were the most significant factors ($P<0.05$) with positive effect. However, increasing the values of the settings of these two variables resulted in fluorescence signals that were out of the measurement range, i.e. the equipment was saturated. Mixing (if the mixing intensity showed influence on the signal), signal function (whether instant reading or reading after a certain period of time influenced the signal) and scan time (to see if the signal changed with longer scan time) exerted non-significant negative effects on the fluorescence signal. The organic solvent concentration ($C_{i\text{-}PrOH}$) exerted a non-significant positive effect on the fluorescence signal emitted by the Nile red-GTO complex. For future experiments, these four non-significant variables were set at their low levels (5% i-PrOH content; immediate read

and 0.1 s scan time), except the mixing intensity, which was settled at gentle manual mixing three times, while the two significant values (PMT voltage and slit witdh) were fixed at central levels (PMT voltage: 600 V and slit width: 5 nm).

3.4. Linear relationship of the model lipid

A calibration curve using GTO as model lipid was obtained, see supporting information Figure S2. The linearity was good with an R^2 of 0.994 and a linear range of 0.50-25 mg/L. This is in agreement with Bertozzini et al. (2011), who also obtained similar data (R^2=0.9934) using Nile red on algal lipids.

3.5. Fluorescence method *versus* gravimetric measurement of total lipids

In order to assess the fluorescence method for evaluation of yeast lipid contents, the results were compared with the traditional gravimetric determination of total lipids in biomasses. The lipid content obtained by fluorescence intensity from cells of *Y. lipolytica* QU21 (calculated based on the calibration curve using GTO shown in supporting information Figure S2) was compared to the total lipid content obtained by gravimetric analysis. The results showed no significant difference (P=0.6181) between the methods with 492 and 460 mg/L, respectively. However, for YI1, YI2 and YI3 the results showed 293, 459 and 298 mg/L for the fluorescence method, and 719, 756 and 453 mg/L for the gravimetric method, respectively. This larger deviation for the YIs indicates, that the method could not adequately quantify the lipid contents. Using linear regression, an R^2 of 0.6 was obtained (supporting information Figures S3 and S4). Compared with Sitepu et al. (2012), who obtained an R^2 of 0.5 using DMSO to enhance the fluorescence signal, this method showed better results with a less harmful solvent.

Recently, other research groups have proposed new methods for measuring lipid contents in e.g. yeast, fungi and microalgae by testing different solvents as i-PrOH

(Bertozzini et al., 2011), DMK and EtOH (Chen et al., 2009) and DMSO (Sitepu et al., 2012; Chen et al., 2011; Chen et al., 2009). As also suggested by these authors, one factor that could explain the difference among the proposed methods is the cell wall thickness of the microorganism, which could prevent the Nile red to reach the intracellular compartments and form the Nile red-lipid complex. This suggests that one universal method will not suit all different microorganisms for quantitative lipid determination, but rather individual methods may be used for individual organisms.

In summary, although the fluorescence method proposed here showed slight variations among the analyzed strains, it has advantages over the traditional gravimetric method, such as: 1) *Short time for sample preparation and analysis*: Using the gravimetric analysis method, up to 6 samples can be processed by a skilled technician in 1 day including overnight processing time (21 h). However, at least 60 samples can be processed when using the fluorescence procedure within a working day of 8 h, see Table 1. 2) *Environmentally-friendly amounts and types of solvents*: Using the fluorescence procedure only minute volumes of DMK (50 µL) and i-PrOH (500 µL) (used for dissolving Nile red and improving cell penetration) are used per sample, whereas several millilitre of MeOH (8 mL) and $CHCl_3$ (16 mL) are used applying the traditional gravimetric method. Moreover we can mention the energy savings for centrifugation and evaporation of solvents. In other words, the fluorescence procedure applies less aggressive solvents and is less energy demanding. 3) *Possibility of automation and thereby applying high-throughput equipment*: For high throughput screening of oleaginous yeast strains, the fluorescence procedure is much more suited than the traditional gravimetric analysis. By adjusting the procedure to high throughput screening instruments, e.g. using a 96-well microplate reader, an even higher number of samples could be produced during a day.

4. Conclusions

The procedure established in this article is very rapid, easy and environmentally-friendly compared to the traditional methods. The new method is useful for rapid screening of oleaginous yeast cells and for certain strains it is a tool for quantitative determination of neutral lipids.

Acknowledgements

This work was supported by Inter Reg IVA programme Biorefinery Øresund financed by EU and by FAPERGS (process nº 11/2047-0). Comments and grammatical proof reading by Jonathan Van Wagenen is appreciated.

References

1. Amaretti, A., Raimondi, S., Leonardi, A., Rossi, M.,2012. *Candida freyschussii*: an oleaginous yeast producing lipids from glycerol. Chem Eng Trans 27, 139-144.
2. Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J., Molina-Jouve, C., Nicaud, J., 2009. *Yarrowia lipolytica* as a model for bio-oil production. Prog Lipid Res 48, 375-387.
3. Bertozzini, E., Galluzzi, L., Penna, A., Magnani, M.,2011. Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. J Microbiol Methods 87, 17-23.
4. Chen, W., Zhang, C., Song, L., Sommerfeld, M., Hu, Q., 2009. A high throughput method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods77, 41-47.
5. Chen, W., Sommerfeld, M., Hu.,Q., 2011.Microwave-assisted Nile red method for *in vivo* quantification of neutral lipids in microalgae. Bioresour Technol 102, 135-141.

6. Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226, 497-509.
7. Juszczyk, P., Tomaszewska, L., Kita, A., Rymowicz, W., 2013. Biomass production by novel strains of *Yarrowia lipolytica* using raw glycerol, derived from biodiesel production. Bioresour Technol 137, 124-131.
8. Kalscheuer, R., Stolting T., Steinbuchel, A., 2006. Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152, 2529-2536.
9. Kimura, K., Yamaoka, M., Kamisaka, Y., 2004. Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. J Microbiol Methods 56, 331-8.
10. Li, Q., Du, W., Liu, D., 2008. Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol 80, 749-756.
11. Lützhøft, H.C.H., Halling-Sørensen, B., Jørgensen, S.E., 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. Arch Environ Contam Toxicol 36, 1-6.
12. Mayer, P., Cuhel, R., Nyholm, N.,1997. A simple in vitro fluorescence method for biomass measurements in algal growth inhibition tests. Water Res 31, 2525-2531.
13. Papanikolaou, S., 2011. Oleaginous Yeasts: Biochemical events related with lipid synthesis and potential biotechnological applications. Ferment Technol 1, 1-3.
14. Poli, J.S., Dallé, P., Senter, L., Mendes, S., Ramirez, M., Vainstein, M., Valente, P., 2013. Fatty acid methyl ester produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. Rev Bras Biocienc 11, 203-208.
15. Sitepu, I.R., Ignatia, L., Franz, A.K., Wong, D.M., Faulina, S.A., Tsui, M., Kanti, A., Boundy-Mills, K., 2012. An improved high-throughput Nile red fluorescence

assay for estimating intracellular lipids in a variety of yeast species. J Microbiol Methods 91, 321-328.

Table 1

Comparison between the traditional method used to measure lipids (lipid extraction with cell wall disruption step) and the new fluorescence method presented in this paper.

Method	Chemicals	Steps	Manipulation time	Total time
Traditional method: Lipid extraction	$CHCl_3$ MeOH PBSKCl	Cell disruption Centrifugation Extraction Filtration Solvent evaporation (compressed air) Solvent traces evaporation (incubator) Weighing	10 minutes 10 minutes 30 minutes 2 hours 3 hours 15 hours 2 minutes	21 hours
New method: fluorescence measurement	i-PrOH Nile red DMK PBSKCl	Sample preparation: mixing the yeast cell suspension with i-PrOH and fill up with PBSKCl. Time required for the Nile red-lipid complex to be established Reading	20 minutes 10 minutes 1minute	31 minutes

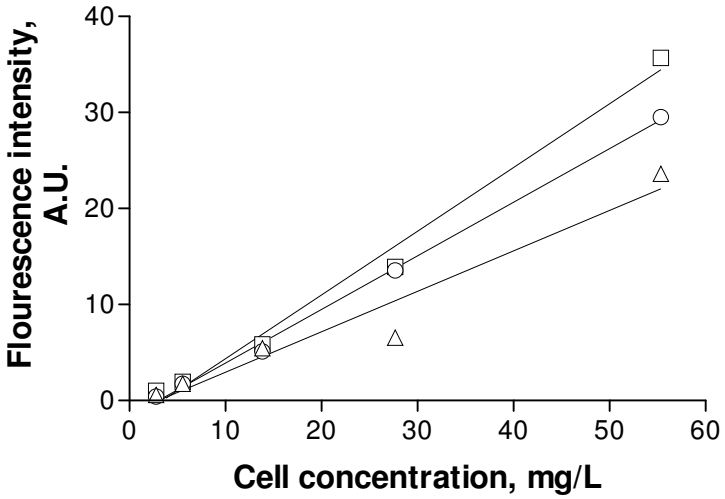

Fig. 1: Fluorescence intensity obtained from the Nile red-lipid complex at increasing yeast cell concentrations (*Y. lipolytica* QU21) (calculated according to dry weight) diluted in PBSKCl (△, R^2=0.9454), 10% i-PrOH in PBSKCl (○, R^2=0.9976) and 10% DMSO in PBSKCl (□, R^2=0.9881). Excitation and emission wavelengths were 488 and 585nm, respectively.

Appendix A - Suplementary data 1

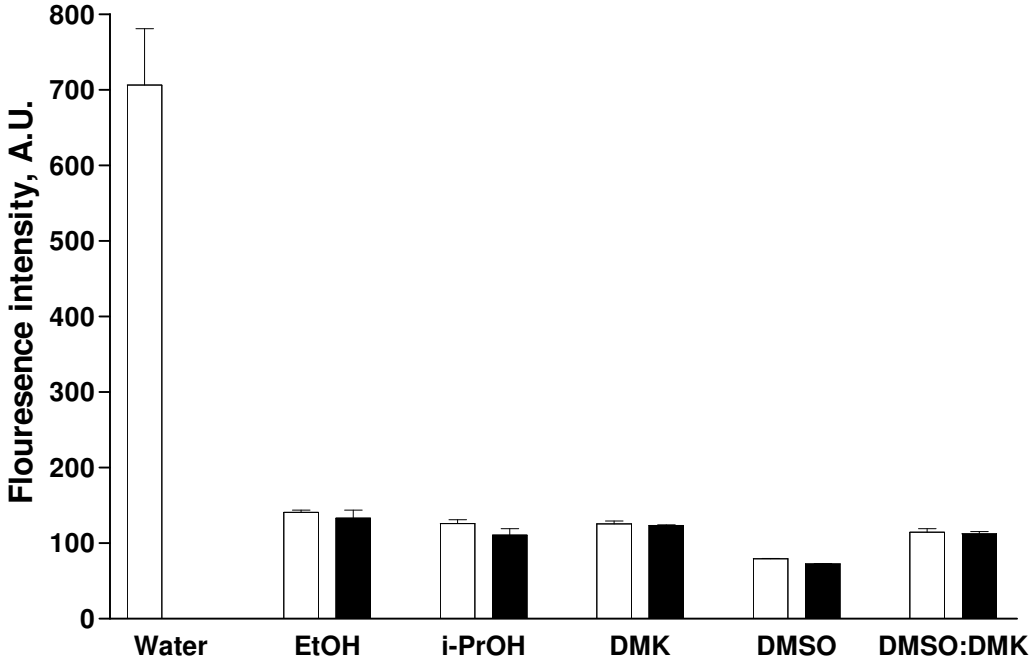

Figure S1: Influence of organic solvent on the fluorescence signal of the Nile red-lipid complex from *Y. lipolytica* QU21 after 10 min (□) and 24 h (■), n=2, error bars indicate standard deviation which for water is 74.81 (relative standard deviation is 11%) and for the organic solvents ranges from 0.5798 to 10.47 (relative standard deviation ranges from 0.43-7.9%). Medium:organic solvent 1:4.

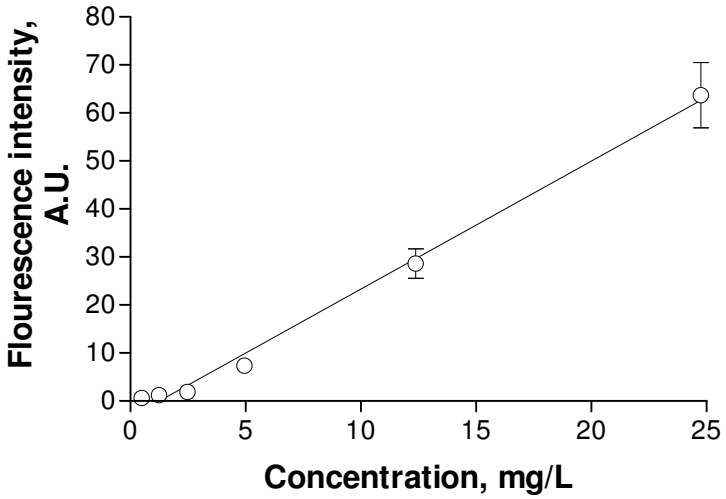

Figure S2: Fluorescence intensity from GTO in 5% i-PrOH in PBSKCl (n=3, R^2=0.994, error bars indicate standard deviation, which ranges from 0.08705 to 6.785 corresponding to 2.2-14% as relative standard deviation). Excitation and emission wavelengths were 488 and 585nm, respectively.

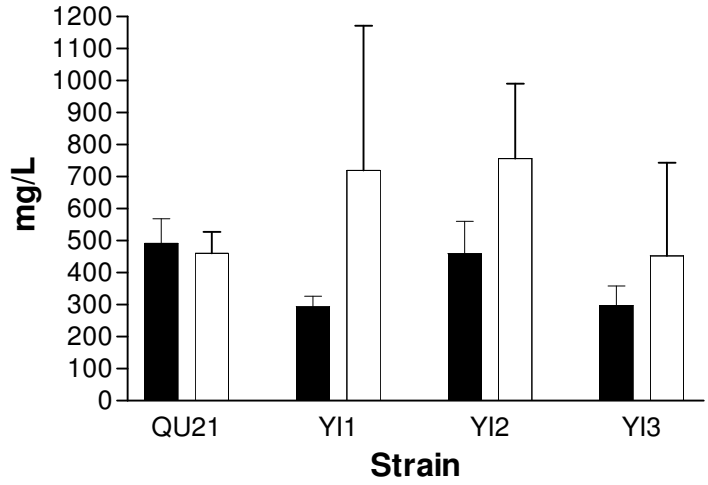

Figure S3: A comparison between the fluorescence (■) and the gravimetric (□) methods for the four studied yeast strains (*Y. lipolytica* QU21, YI1, YI2 and YI3) (n=3, error bars indicate standard deviation). The standard deviation for the fluorescence method ranges from 33.55 to 76.88, corresponding to relative standard deviations of 11-22%, whereas for the gravimetric method the standard deviation ranges from 67.58 to 452.4, corresponding to relative standard deviations of 15-64%.

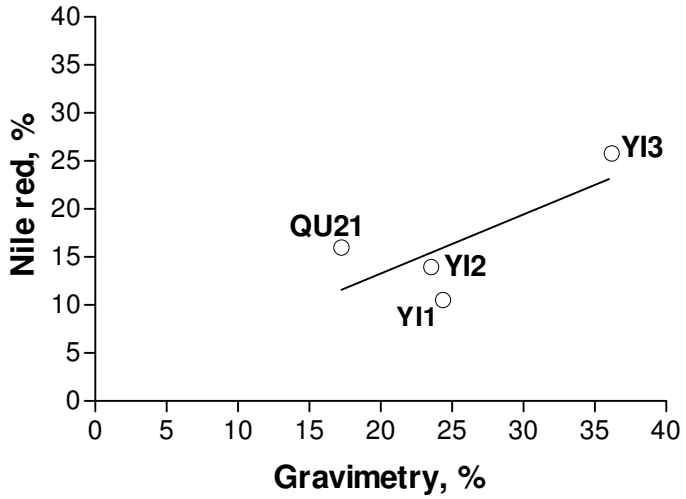

Figure S4: Relationship between the lipid content determined by fluorescence and gravimetric analysis. The lipid content determined by each method has been converted to percentage based on their dry weight biomass (R^2=0.6).

Table S1: Identification of significant variables for measuring the fluorescence signal of the Nile red-GTO complex using the Plackett-Burman design.

Variables	t-ratio	P-value
Intercept	4.83	0.0169
$C_{i\text{-}PrOH}$	1.50	0.2299
Mixing	-1.58	0.2112
Signal function	-1.53	0.2227
Scan time	-1.60	0.2070
PMT voltage	4.98	0.0155[a]
Slit	4.83	0.0169[a]

[a]Statistically significant at 95% of confidence level.

III - Microbial lipid produced by *Yarrowia lipolytica* QU21 using industrial waste: a potential feedstock for biodiesel production

Jandora Severo Poli, Mirra Angelina Neres da Silva, Ezequias P. Siqueira, Vânya M.D. Pasa, Carlos Augusto Rosa, Patricia Valente

Submetido para a Bioresource Technology, 2014.

Microbial lipid produced by *Yarrowia lipolytica* QU21 using industrial waste: a potential feedstock for biodiesel production

Jandora Severo Poli[1,2*], Mirra Angelina Neres da Silva[3], Ezequias P. Siqueira[4], Vânya M.D. Pasa[3], Carlos Augusto Rosa[2], Patricia Valente[1]

[1]Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500 sala 154, CEP:90050-170, Porto Alegre, RS, Brazil.

[2]Departamento de Microbiologia, ICB, C.P. 486, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil.

[3]Fuel Laboratory, Chemistry Department, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, MG, Brazil.

[4]Centro de Pesquisas René Rachou, Av. Augusto de Lima 1715, CEP 30190-002, Belo Horizonte, MG, Brazil.

[*]Corresponding author: Jandora Severo Poli

Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500 sala 154, CEP:90050-170, Porto Alegre, RS, Brazil; telephone: +55-5133084196. E-mail: 00162027@ufrgs.br, jandora.poli@gmail.com

Abstract

This study aimed to evaluate the effect of medium composition and culture conditions on lipid content, fatty acid profile and biomass production by the yeast *Yarrowia lipolytica* QU21. Lipid production by the yeast growing on glycerol/$(NH_4)_2SO_4$ (10%/0.1%) reached 1.48 g/L (30.1% according to total cell dry weight). When glycerol was replaced by crude glycerol (industrial waste), the lipid yield was 1.27 g/L, with no significant difference. Some particular fatty acids were found when crude glycerol was combined with fresh yeast extract (FYE, brewery waste), as linolenic acid (C18:3n3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3n3) and eicosapentaenoic acid (C20:5n3). In addition, the FYE promoted an increase of more than 300% on polyunsaturated fatty acid content (PUFA), which is an undesirable feature for biodiesel production. The fatty acid composition of the oil produced by *Y. lipolytica* QU21 growing on crude glycerol/$(NH_4)_2SO_4$ presented a potential use as biodiesel feedstock, with low PUFA content.

Keywords: Oleaginous yeast; *Yarrowia lipolytica*; biodiesel; industrial wastes; microbial oil.

1 Introduction

Concerns about the climate change have attracted attention of many researchers, and boosted recent investigations on biofuels as a renewable, environmentally friendly alternative to fossil fuels. In 2011, the United States jumped from the 4th to 1st place with 63 thousand barrels of biodiesel per day (TBBD) while Germany and Brazil produced 52 and 46 TBBD, respectively. From 2010 to 2011, Argentina increased its total production from 36 to 47 TBBD and has remained on 3rd place in world's largest producer rank (http://www.indexmundi.com/energy.aspx?product=biodiesel&graph=production). The most common biofuel in Europe is the biodiesel. The blend of biodiesel to diesel changes according to each country. Currently, the blend commercialized in Brazil is called B5, i.e., 5% of biodiesel blended to diesel, and the country has the capacity to produce B10 without need of new biorefineries. Nevertheless, the projected increase of the blend to 20% (B20), planned for 2020, requires a total production of 14.3 million litters, which means an extra 9.2 million litters to the current production capacity (FGV, 2010).

The primary feedstock for biodiesel production is vegetable oil as rapeseed and canola oil in Europe and soybean oil in Brazil and North America. With the increasing incentives for biofuels production in the last years, there is a crescent demand for land areas destined to biofuel production, which results in competition with existing food plantations and a consequent increase of food prices.

The crude glycerol is the main byproduct of the biodiesel production chain. The volume of crude glycerol has increased considerably with the recent growth of biodiesel production. For each ton of biodiesel produced it is estimated 100Kg of crude glycerol

is generated (Bowker et al., 2008). Thereby, questions regarding the utilization of such byproduct arise, due to the concerns with their inadequate disposal into the environment.

Fermentation employing oleaginous microorganisms (Papanikolaou, 2011) (2nd generation (2G) biodiesel production) is a very promising alternative to overcome the critical bottlenecks of 1st generation (1G) biodiesel production. Yeasts are a promising source of microbial oil (Papanikolaou and Aggelis, 2011), since some strains can accumulate up to 70% of their dry weight in lipids (Angerbauer et al., 2008). Moreover, the production of microbial oil is of particular interest due to the capacity of various microorganisms to synthesize lipids of medical and dietetical interest, like the polyunsaturated ϒ-linolenic fatty acid, among others (Papanikolaou and Aggelis, 2011). Examples of oleaginous yeasts (and their carbon sources for lipid production) include the species: *Yarrowia lipolytica* (animal fats and industrial lipids/glycerol), *Rhodotorula glutinis* (glucose), *Rhodosporidium toruloides* (glucose and xylose), *Lipomyces starkeyi* (xylose, ethanol and L-arabinose) and *Cryptococcus curvatus* (culture media containing oils) (Ageitos et al., 2011). *Yarrowia lipolytica* has the advantage to be considered as GRAS (generally regarded as safe) (Groenewald et al., 2013), and some strains are capable of accumulating lipids using crude glycerol (Juszczyk et al., 2013; Cheirsilp and Louhasakul, 2013; Rywinska et al., 2013), as well as sugarcane bagasse hydrolysate (Tsigie et al., 2011) and rice bran hydrolysate (Tsigie et al., 2012) as carbon sources. For these reasons, it is considered a robust and promising microorganism to work with.

Among the factors that are known to promote lipid accumulation in the oleaginous microorganisms, the high carbon/nitrogen (C/N) ratio is considered the most important. In most of the performed studies, the nitrogen limitation is the easiest condition to

control the carbon/nitrogen (C/N) ratio in order to induce the lipid accumulation in microorganisms (Beopoulos et al., 2009; Papanikolaou and Aggelis, 2011). Moreover, the modification of culture conditions affects the lipid composition (Chen et al., 2013; Sitepu et al., 2013), which may determine the oil application, e.g. biodiesel production or nutritional use. The aim of the present work is to evaluate the effect of medium composition and culture conditions on lipid content, fatty acid profile and biomass production by the yeast *Yarrowia lipolytica* QU21.

2 Materials and Methods

2.1 Yeast strain and culture conditions

The yeast strain *Yarrowia lipolytica* QU21 (Poli et al., 2013) was maintained at 4°C. This strain was pre-grown on YEPD agar (%, yeast extract 1, peptone 2, glucose 2, agar 2) at 28°C for 24 hours. The cells (an equivalent of 0.03g dry biomass) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of liquid media comprised of (%): carbon source (CS) 10, nitrogen source (NS) 0.1 and incubated in a rotating shaker at 150 rpm, 28°C for 4 days, unless otherwise stated. All liquid media were enriched with KH_2PO_4 0.1% and $MgCl_2 \cdot 6H_2O$ 0.05%. Liquid cultures were centrifuged at 3000 x g for 10 min to remove the supernatant, the cell pellets were washed twice with 0.15 M potassium chloride (KCl) in 0.01 M phosphate buffered saline (PBSKCl, pH 7.0), and used for dry biomass determination (section 2.6) and lipid extraction (section 2.7).

2.2 Effect of carbon and nitrogen sources on yeast growth and lipid production

Pure glucose and glycerol (grade reagents) were chosen to evaluate the effect of carbon source (CS) on growth, lipid accumulation and lipid profile of *Y. lipolytica* QU21. The fermentation was carried out using each sugar as the single CS, and $(NH_4)_2SO_4$ (0.1%) as the nitrogen source (NS). The culture conditions were as described in section 2.1.

The influence of organic NS (yeast extract, tryptone and urea) and inorganic NS [$(NH_4)_2SO_4$ and NH_4NO_3] were also evaluated using the best CS from the previous experiment at a concentration of 10%. The fermentation was performed with each component as a sole NS in a concentration of 0.1%. The culture conditions were as described in section 2.1.

2.3 Evaluation of culture conditions: Aeration, agitation speed and their effect on yeast growth and lipid production

With the culture media obtained in section 2.2, experiments were conducted to evaluate the culture conditions on the fermentation process. The effect of aeration on biomass, lipid production and fatty acid composition was evaluated by modifying the volume of liquid media (100 and 75mL), while keeping the size of the Erlenmeyer flask (250mL) the same (the ratio between the volume of the Erlenmeyer flask and the volume of culture medium were 2.5 and 3.33, respectively) (Silva et al., 2010). The aeration was not measured. The influence of agitation speed (150 and 200 rpm) was also investigated.

2.4 Evaluation of C/N ratio on yeast growth and lipid production

With the best results obtained in sections 2.2 and 2.3, the effect of C/N ratio was evaluated on the yeast growth, lipid production and fatty acid profile. The five treatments were designed by varying the glycerol or $(NH_4)_2SO_4$ contents according to the specifications listed on Table 1. For the calculation of C/N ratio, carbon content in glycerol of 39.1% and nitrogen content in $(NH_4)_2SO_4$ of 21.2% were assumed. The culture conditions were as described in section 2.1., except for the liquid media volume that was 75mL.

2.5 Industrial wastes as substrates for growth

Two residual industrial wastes were investigated as substrates for lipid production. The crude glycerol (CrGly) (glycerol content 82.73% w/w) obtained from a biodiesel industry as the sole carbon source was used in a concentration of 10%. Impurities of crude glycerol were sulphated ash (6.3%), NaCl (5.15%), chloride (3.13%), sodium (2.02%), methanol (0.008%), pH 6.0. The nitrogen sources used were 0.1% $(NH_4)_2SO_4$ or fresh yeast extract (FYE) (obtained from a brewery) at 50% (FYE_{50}) or 100% (FYE_{100}). The FYE was prepared as described by Silva and Almeida (2006). All liquid media were enriched with 0.1% KH_2PO_4 and 0.05% $MgCl_2 \cdot 6H_2O$. The culture conditions were as described in section 2.1., except for the liquid media volume that was 75mL.

2.6 Determination of dry biomass and glycerol

Cell pellets obtained from 30 or 35mL of submerged cultures (described in section 2.1) were kept at 65°C to constant weight. Glycerol was determined in filtered aliquots

of the culture medium analysed by a Shimadzu (Japan) HPLC in a SUPELCOGEL C-610H 30 cm x 7.8 mm column (Sigma-Aldrich). The eluant used was H_2SO_4 5mM. The glycerol was detected by an RI detector (RID 10-A).

2.7 Lipid extraction and fatty acids characterization

The lipids from cell pellets obtained from 30 or 35mL of submerged cultures (described in section 2.1) were extracted according to Poli et al. (2014). Briefly, the cell pellets were re-suspended in 10mL of PBSKCl or distilled water, and kept on ice while sonified for 10 or 15 cycles using Branson Sonifier 450 (Branson Ultrasonics Corporation, VWR Scientific, USA). The lipids from the disrupted cells were extracted using $CHCl_3$:MeOH solution (2:1) (Folch et al., 1957). The resulting liquid phase was filtered, and the solvents were evaporated using Speed Vac Concentrator Savant SPD121P (Thermo Scientific, USA). Cellular lipids were converted to their fatty acid methyl-esters (FAME) according to Hartman and Lago (1973). FAMEs from CS and NS assays were analyzed in a gas chromatograph equipped with mass spectroscopy (GCMS-QP5050A, Shimadzu, Japan) and the capillary column SP 2340 (60m x 0.25mm x 0.2µm). Temperatures from the detector and injector were 300 and 250°C, respectively. The following temperature program was used for the separation of FAME: 140°C for 5 minutes, with gradual increase of 4°C per minute until the final temperature of 240°C, where it was held for 5 minutes. The carrier gas was helium with constant flow (33mL.min^{-1}). Volume of injection was 1 µL with split rate of 1:50. FAMEs from aeration, agitation speed, period of incubation and industrial waste assays were analyzed in a gas chromatograph equipped with flame ionization detector (GC-FID) with auto injector AOC-20i (GC-2010, Shimadzu, Japan). FAMEs separation were

performed on a Supelco capillary column SP 2340 (60m x 0.25mm x 0.2µm, USA Sigma-Aldrich). The column temperature was maintained at 140°C for 5 min, upgraded from 140°C to 240°C at a rate of 4°C min^{-1} and kept at 240°C for 9 min. Helium was used as the carrier gas with flow at (0.78mL.min^{-1}). Split ratio was 1:50. The injector and detector temperatures were set at 260°C and 300°C, respectively. Volume of injection was 1 µL. The identification of FAME was evaluated using Supelco 37 Component Fame Mix (10mg/mL - Sigma) as standard and the quantification by normalization of the area.

2.8 Statistical analysis

The lipid content, dry biomass and fatty acid composition were determined in triplicates. The mean values are reported and compared by analysis of variance (One-way ANOVA) (P=0.05). Difference among the observed averages of the treatments were tested using the Tukey test (P=0.05).

3 Results and discussion

3.1 Evaluation of carbon and nitrogen sources on growth and lipid accumulation by the yeast strain *Y. lipolytica* QU21

The biomass production and lipid accumulation by *Y. lipolytica* QU21 in the medium with glucose as sole carbon source were 3.28 ±0.27 g/L and 0.75 ± 0.1 g/L respectively. When glycerol was used as the only carbon source, *Y. lipolytica* QU21 showed a significant decrease on biomass production, with 2.22 ± 0.2 g/L (decrease of 67.7%), but the lipid content remained almost the same, with 0.77 ± 0.01g/L. Differences on glycerol tolerance during growth and lipid production by other strains of

Y. lipolytica have already been reported. *Y. lipolytica* LGAM S(7)1, when grew on pure glycerol in flask cultures produced between 6.0 to 7.5 g/L of biomass, however, this strain produced low amounts of storage lipid (0.05-0.10 g/g, i.e., 0.3-0.75g/L) (Papanikolaou and Aggelis, 2002). *Y. lipolytica* ACA-DC 50109 growing in an initial glycerol concentration of 104.9 g/L, in a fermentation time of 50 and 240 h, presented 6.71 and 5.92 g/L of biomass, respectively, and lipid production of 20.4 and 13.4% (i.e., 1.37 and 0.79 g/L of lipids, respectively) (Makri et al., 2010). Note that both studies reported a higher biomass production and lower lipid yield than that obtained in the present study (i.e., 34.5 ± 2.5 %, according to dry weight).

Glycerol was chosen as the sole carbon source for the rest of the experiments, as it best fits this study. Five nitrogen sources (NS), comprised of three organic NS (yeast extract, tryptone and urea) and two inorganic NS [$(NH_4)_2SO_4$ and NH_4NO_3] were evaluated for their capacity to promote lipid accumulation. The results presented on Figure 1 shows that tryptone was the best NS for biomass production, with 2.94 ± 0.27 g/L, followed by yeast extract with 2.43 ± 0.09 g/L. Although tryptone presented significant difference on biomass production to all other NS, $(NH_4)_2SO_4$ was the NS that most induced lipid accumulation on *Y. lipolytica* QU21 with 0.77 ± 0.01g/L, showing significant difference to all NS. These results are in agreement with those found by Hansson and Dostáliek (1986) for *Cryptococcus albidus var. albidus* CBS 4517, where inorganic nitrogen as $(NH_4)_2SO_4$ and NH_4Cl better induced lipid accumulation than organic nitrogen. These results are different from the ones obtained with the yeast *Rhodosporidium toruloides*, for which L-glutamate induced a lipid production of 52% (according to dry weight) which was significantly superior to the 18% of lipids produced in $(NH_4)_2SO_4$ (Evans and Ratledge, 1984a). The lipid production rate and

regulation of lipid biosynthesis in *Rh. toruloides* CBS 14 were affected by the intracellular NH_4^+ concentration (Evans and Ratledge, 1984b). According to these authors, fundamental differences in the nitrogen metabolism between different species of oleaginous yeasts may account for the ability of various organic nitrogen compounds to stimulate lipid production.

3.2 Aeration, agitation speed and their effect on the growth and lipid productionby the yeast strain *Y. lipolytica* QU21

Yarrowia lipolytica is a strictly aerobic yeast (Beopoulos et al., 2009) and the oxygen availability in the culture medium might affect the lipid production (Naganuma et al., 1985; Papanikolaou et al., 2002). In order to evaluate the effect of oxygen availability in flask cultures on the growth and lipid production, two experiments were performed: a) aeration: the ratio between the volume of the Erlenmeyer flask and the volume of culture medium (V_{ef}/V_{cm}), i.e., 2.5 and 3.33, were evaluated, and b) the speed agitation was increased from 150 to 200 rpm. All the results are shown in Table 2.

Increasing the ratio from 2.5 to 3.33 and keeping the agitation speed at 150 rpm, the biomass and lipid production jumped from 2.28 to 4.01g/L and 0.77 to 0.94g/L, respectively. With the speed agitation increase to 200 rpm, even though the biomass production reached 5.1 g/L, the lipid accumulation remained the same (i.e., 0.94 g/L). In our previous work, it was concluded that the methodology employed for cell disruption had a strong influence on the lipid extraction efficiency (Poli et al., 2013). As the ratio of 3.33 resulted in a higher biomass (a 2-fold increase), maybe it could affect the efficiency of the cell disruption step. Accordingly, the assays were repeated and the period of sonication was increased from 10 to 15 cycles. As expected, in the 150 rpm

assay (V_{ef}/V_{cm} ratio of 3.33), the lipid content increased from 0.94 to 1.48 g/L when the sonication period increased from 10 to 15 cycles, respectively. Also, in 200 rpm assay (V_{ef}/V_{cm} ratio of 3.33), the lipid content increased from 0.94 to 1.29 g/L. Although lipid production is not usually associated to cell growth, the cultures must be aerated (Ageitos et al., 2011). Therefore, increasing the aeration by increasing the V_{ef}/V_{cm} ratio proved to induce the lipid accumulation on *Y. lipolytica* QU21, while there was no significant difference between the tested agitation speeds (150 and 200 rpm). Therefore, 150 rpm was chosen for the following experiments.

3.3 Effect of various C/N ratios on growth and lipid accumulation by the yeast strain *Y. lipolytica* QU21

The lipid production usually takes place when excess carbon in the medium is associated with a nutrient depletion, normally the nitrogen source (Beopoulos et al., 2009; Ageitos et al., 2011; Papanikolaou and Aggelis, 2011). At this point the transition from catalytic growth to lipid accumulation occurs and, in *Y. lipolytica*, large amounts of TCA-cycle intermediates are produced and the excess carbon is converted into triacylglycerols (Beopoulos et al., 2009).

As shown in Figure 2, the biomass achieved its highest value when *Y. lipolytica* QU21 grew in C/N 276 and C/N 184, with 5.1 ± 0.56 g/L and 4.9 ± 0.68 g/L, respectively, with no significant difference among them. For lipid production, the profile was different, with a gradual increase up to 1.48 ± 0.27 g/L on C/N 184, followed by a slow decrease as the C/N ratio increased. The lipid production when *Y. lipolytica* QU21 grew in C/N 184 showed significant difference to all other treatments. These results are in agreement with Chen et al. (2013), who obtained a similar behavior

for *Trichosporon cutaneum* growing in various C/N ratios, and with Papanikolaou et al. (2002), who reported that the lipid production by *Y. lipolytica* growing in nitrogen-rich media was lower than that observed in low-nitrogen media.

3.4 *Y. lipolytica* growth on industrial wastes

For the microbial oil to be competitive in the biofuels area, the production costs must be reduced. The use of carbon sources other than glucose has been subject of research in the last years (Papanikolaou et al., 2007; Angerbauer et al., 2008; Papanikolaou et al., 2008; Chen et al., 2013). The present work evaluated two industrial wastes. The crude glycerol (CrGly) from a biodiesel industry and the fresh yeast extract (FYE) from brewery waste were investigated as the carbon and the nitrogen source, respectively. All the results are shown in Table 3. Crude glycerol and $(NH_4)_2SO_4$ were the best combination for the growth of *Y. lipolytica* QU21, reaching the highest value for biomass production in this work (6.7 ± 0.67 g/L). However, the higher biomass was not accompanied by an increase of storage lipids [1.27 ± 0.25 g/L; $Y_{L/S}$ (lipid yield on glycerol consumed, g/g) = 0.06 ± 0.01 g/g)], whose value was similar to that obtained with pure glycerol (1.48 ± 0.27g/L; $Y_{L/S}$ = 0.07 ± 0.02 g/g). Cheirsilp and Louhasakul (2013) found similar results for *Y. lipolytica* strains growing on palm oil mill effluent (1.6-1.7g/L of lipids) and for *Y. lipolytica* TISTR 5151 (approximately 2g/L on crude glycerol). *Y. lipolytica* S6 growing in a bioreactor using crude glycerol as the carbon source produced 12.3g/L of biomass and 1.37g/L (reported as 11.1%) of lipids (Juszczyk et al., 2013). Although the lipid production by *Y. lipolytica* S6 was similar to the yield obtained in the present work, note that the biomass was much higher (almost

2-fold higher). This could be undesirable due to the high content of cellular waste produced as a byproduct of oil extraction.

The enhanced biomass production in crude glycerol could be related to the impurities present on the glycerol waste, as some amounts of peptides and proteins are additional sources of nitrogen for yeasts (Juszczyk et al., 2013). Accordingly, a possible explanation for the fact that crude glycerol promoted biomass production without increment on storage lipids is the C/N ratio, which may not be as high as with pure glycerol.

When the $(NH_4)_2SO_4$ was replaced by both FYE_{100} and FYE_{50}, the lipid production had a slight decrease when compared to the inorganic nitrogen source, with 0.85 ± 0.14 g/L and 0.84 ± 0.14g/L, respectively, while the growth of *Y. lipolytica* on both combinations was similar between them, as well as to the growth obtained on pure reagents. As this work is the first study using FYE for lipid production, further adjustments on FYE and crude glycerol concentrations might be necessary.

3.5 Fatty acid composition

Slight variations in the fatty acid composition were observed when the medium composition was modified (Table 4). The main fatty acids produced by the yeast growing in the culture medium composed of glycerol and $(NH_4)_2SO_4$ with V_{ef}/V_{cm} ratio of 2.5 were C18:1n9c (45.48%), C16:0 (19.18%), C18:0 (18.76%), C18:2n6c (7.93%) and C16:1 (6.62%). When the V_{ef}/V_{cm} ratio increased to 3.33 (assay named as C/N 184), the changes were mainly on the fatty acids C16:1 and C18:0, which increased 73% and decreased 56.9%, respectively. In all experiments, the oleic acid (C18:1n9c) was the dominant fatty acid, varying from 39.0 to 62.2%. These findings are in

accordance with those observed by other authors (Makri et al., 2010, Sitepu et al. 2013, Cheirsilp and Louhasakul, 2013). The synthesis of linoleic acid (C18:2n6) decreased with a longer incubation time. Unlike the results from these experiments, Sitepu et al. (2013) observed an increase of such fatty acid with nitrogen starvation and longer incubation time, denoting that each yeast strain could display a particular behavior concerning fatty acid composition. The concentration of polyunsaturated fatty acids (PUFAs) increased more than 80% when nitrogen concentration increased in the culture medium (C/N 18 and C/N 36), and traces of eicosapentaenoic acid (EPA) (C20:5n3) were observed on the C/N 18 and crude glycerol/$(NH_4)_2SO_4$ assays. For the above mentioned assays, no linolenic acid (C18:3n3) was found (Table 4) and, despite the variations observed in the assays, there was always a low content of PUFAs in the lipid composition of the oil produced by *Y. lipolytica* QU21. Biodiesel fuels derived from different sources have significantly variation on fatty acid profiles and properties and thus affect the performance of biodiesel (Knothe, 2008). The fatty acid composition of soybean oil is highly unsaturated, making the soybean-derived biodiesel very prone to oxidation (Canakci, et al., 1999). Furthermore, the buildup of viscous materials in soybean-derived biodiesel may clog oil filters (Canakci, et al., 1999). Therefore, the oil produced by *Y. lipolytica* QU21 can be suitable for biodiesel production.

Nevertheless, some particular fatty acids were found when crude glycerol was combined with FYE on both concentrations (50 and 100%). In addition to the presence of C18:3n3 (6.22 and 6.97, respectively) and an average increase of 185% of linoleic acid (C18:2n6c) when compared to CrGly/$(NH_4)_2SO_4$ (Table 4), the PUFAs eicosadienoic acid (C20:2) and eicosatrienoic acid (C20:3n3) were found in the CrGly/FYE_{100} assay (4.19 ± 1.54% and 2.04 ± 0.92%, respectively) and cr gly/FYE_{50}

assay (4.15 ± 3.63% and 3.28 ± 2.88%, respectively). The last assay also presented traces of C20:5n3. The higher amounts of total PUFAs (i.e., 32.48 and 32.17%) for both assays (CrGly/FYE_{100} and CrGly/FYE_{50}, respectively) could be attributed to some properties of FYE and/or its combination with crude glycerol, since when replaced by the $(NH_4)_2SO_4$, the total PUFAs was 7.34% (i.e, an increase of 342.3% with FYE). The C18:2n6c and C20:2 belong to the omega-6 fatty acid group, and the C18:3n3, C20:3n3 and C20:5n3 to the omega-3 fatty acid group. Both omega-3 and omega 6 are essential fatty acids and must be ingested for good health.

A recent study with four strains of *Y. lipolytica* using wastes as substrates reported that only the strain TISTR 5151 could grow well on crude glycerol and accumulate high lipid content (Cheirsilp and Louhasakul, 2013). In addition, the authors reported, for all evaluated waste substrates and their combinations, that the C18:2n6c and C18:3n3 contents were 9.4-13.6% and 0 to 0.6%, respectively. According to Cheirsilp and Louhasakul (2013), the impurities of crude glycerol could repress the cell growth, therefore, the results presented here reinforces the robust feature of *Y. lipolytica* QU21 and the wide application of its oil.

4 Conclusion

The fatty acid composition of the oil produced by *Y. lipolytica* QU21 growing on crude glycerol/$(NH_4)_2SO_4$ presented a potential use as biodiesel feedstock. The combined wastes also seemed to be a potential source for microbial oil with high PUFAs content. Reports on the literature, together with the present findings, are consolidating *Y. lipolytica* as a robust yeast with a wide capacity to adapt, grow and accumulate lipids on a large variety of wastes. Besides making the microbial oil a

competitive feedstock for biodiesel production, the use of crude glycerol could mitigate environmental issues such as improper waste disposal.

Aknowledgments

This work was supported by FAPERGS (process nº 11/2047-0), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). The authors thank Dr. Luiz de Macêdo Faria and Luiz Henrique Rosa for providing essential equipments to perform this work. Comments and grammatical proof reading by Jonas Bragagnolo are appreciated.

References

1. Ageitos, J.M, Vallejo, J.A., Veiga-Crespo, P., Villa, T.G., 2011. Oily yeasts as oleaginous cell factories. Appl. Microbiol. Biotechnol. 90, 1219-1227.
2. Angerbauer, C., Siebenhofer, M., Mittelbach, M., Guebitz, G.M., 2008. Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. Bioresour. Technol. 99, 3051-3056.
3. Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.L., Jouve, C.M., Nicaud, J.M., 2009. *Yarrowia lipolytica* as a model for bio-oil production. Prog. Lipid Res. 48, 375-387.
4. Bowker, M.; Davies, P.R.; Al-Mazroai, L.S., 2008. Photocatalytic reforming of glycerol over gold and palladium as an alternative fuel source. Catal. Lett. 128, 253-255.

5. Canakci, M., Monyem, A., Gerpen, J.V., 1999. Accelerated oxidation processes in biodiesel. Trans ASAE 42, 1565-1572.
6. Cheirsilp, B. and Louhasakul, Y., 2013. Industrial wastes as a promising renewable source for production of microbial lipid and direct transesterification of the lipid into biodiesel. Bioresour. Technol. 142, 329-337.
7. Chen, X.F., Huang, C., Yang, X.Y., Xiong, L., Chen, X.D., Ma, L.L., 2013. Evaluating the effect of medium composition and fermentation condition on the microbial oil production by *Trichosporon cutaneum* on corncob acid hydrolysate. Bioresour. Technol. 143, 18-24.
8. Evans, C.T., Ratledge, C., 1984a. Effect of nitrogen source on lipid accumulation in oleaginous yeasts. J. Gen. Microbiol. 130, 1693-1704.
9. Evans, C.T., Ratledge, C., 1984b. Influence of nitrogen metabolism on lipid accumulation by *Rhodosporidium toruloides* CBS 14. J. Gen. Microbiol. 130, 1705-1710.
10. FGV, Fundação Getúlio Vargas, 2010. O Biodiesel e sua contribuição ao desenvolvimento brasileiro. União Brasileira do Biodiesel-UBRABIO.
11. Folch, J., Lees, M., Stanley, G.H.S., 1957.A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.
12. Groenewald, M., Boekhoult, T., Neuvéglise, C., Gaillardin, C., Dijck, P.W.M. van, Wyss, M., 2013. *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. Crit. Rev. Microbiol., Early on line, 1-20.
13. Hansson, L. and Dostalek, M., 1986. Effect of culture conditions on fatty acid composition in lipids produced by the yeast *Cryptococcus albidus var. albidus.* J. Am. Oil Chem. Soc. 63, 1179-1184.

14. Hartman, L. and Lago, R.C., 1973. Rapid preparation of fatty acid methyl esters from lipids. Lab. Pract., 22, 475-476.

15. Index Mundi, available on http://www.indexmundi.com/energy.aspx?product=biodiesel&graph=production (acessed on 4thoctober, 2013).

16. Juszczyk, P., Tomaszewska, L., Kita, A., Rymowicz, W., 2013. Biomass production by novel strains of *Yarrowia lipolytica* using raw glycerol, derived from biodiesel production. Bioresour. Technol. 137, 124-131.

17. Knothe, G., 2008. "Designer" biodiesel: Optimizing fatty ester composition to improve fuel properties. Energy Fuels 22, 1358-1364.

18. Makri, A., Fakas, S., Aggelis, G., 2010. Metabolic activities of biotechnological interest in *Yarrowia lipolytica* grown on glycerol in repeated batch cultures. Bioresour. Technol. 101, 2351-2358.

19. Naganuma, T., Uzuka, Y., Tanaka, K., 1985. Physiological factors affecting total cell number and lipid ocntent of the yeast, lipomyces starkeyi. J. Gen. Appl. Microbiol. 31, 29-37.

20. Papanikolaou, S. , Panayotou, M.G., Fakas, S., Komaitis, M., Aggelis, G., 2007. Lipid production by oleaginous *Mucorales* cultivated on renewable carbon sources. Eur. J. Lipid. Sci. Technol. 109, 1060-1070.

21. Papanikolaou, S., 2011. Oleaginous Yeasts: Biochemical events related with lipid synthesis and potential biotechnological applications. Ferment. Technol. 1, 1-3.

22. Papanikolaou, S., Aggelis, G., 2002. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour. Technol. 82, 43-49.

23. Papanikolaou, S., Aggelis, G., 2011. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur. J. Lipid Sci. Technol. 113, 1031-1051.

24. Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I., 2002. Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Appl. Microbiol. Biotechnol. 58, 308-312.

25. Papanikolaou, S., Fakas, A., Fick, M., Chevalot, Is., Panayotou, M.G., Komaitis, M., Marc, I., Aggelis, G., 2008. Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid metgyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. Biomass Bioenergy 32, 60-71.

26. Poli, J.S., Dallé, P., Senter, L., Mendes, S., Ramirez, M., Vainstein, M., Valente, P., 2013. Fatty acid methyl ester produced by oleaginous yeast *Yarrowia lipolytica* QU21: an alternative for vegetable oils. Rev. Bras. Biocienc. 11, 203-208.

27. Poli, J.S., Lützhøft, H.C.H., Karakashev, D.B., Valente, P. Angelidaki, I., 2014. An environmentally-friendly fluorescent method for quantification of lipid contents in yeast. Bioresour. Technol. 151, 388-391.

28. Rywinska, A., Juszczyk, P., Wojtatowicz, M., Robak, M., Lazar, Z., Tomaszewska, L, Rymowicz, W., 2013. Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. Biomass Bioenergy 48, 148-166.

29. Silva, G.A. and Almeida, E.A., 2006. Production of yellow-green fluorescent pigment by *Pseudomonas fluorescens*. Braz. Arch. Biol. Technol. 49, 411-419.

30. Silva, J.P.A., Mussato, S.I., Roberto, I.C., 2010. The influence of initial xylose concentration, agitation and aeration on ethanol production by *Pichia stipitis* from rice straw hemicellulosic hydrolysate. Appl. Biochem. Biotechnol. 162, 1306-1315.
31. Sitepu, I.R., Sestric, R., Ignatia, L., Levin, D., German, J.B., Gillies, L.A., Almada, L.A.G., Mills, K.L.B., 2013. Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. Bioresour. Technol. 144, 360-369.
32. Tsigie, Y.A., Wang, C.Y., Kasim, N.S., Diem, Q.D., Huynh, L.H., Ho, Q.P., Truong, C.T., Ju, Y.H., 2012. Oil production from *Yarrowia lipolytic*a Po1g using rice bran hydrolysate. J. Biomed. Biotechnol. 2012, Article ID 378384, 1-10.
33. Tsigie, Y.A., Wang, C.Y., Truong, C.T., Ju, Y.H., 2011. Lipid production from *Yarrowia lipolytica* Po1g grown in sugarcane bagasse hydrolysate. Bioresour. Technol. 102, 9216-9222.

Table 1: Content of glycerol and $(NH_4)_2SO_4$ on liquid media and the final C/N ratio for each assay.

Treatment	Glycerol (%)	$(NH_4)_2SO_4$ (%)	C/N ratio
C/N 18	10	1	18.45
C/N 36	10	0.5	36.89
C/N 184	10	0.1	184.5
C/N 276	15	0.1	276.7
C/N 368	20	0.1	368.9

Table 2: Lipid content (g/L; % according to total cell dry weight), and biomass (g/L) produced by *Y. lipolytica* QU21 growing on two different liquid media volume (75 or 100 mL) in Erlenmeyer flasks (250mL) with cotton plugs and two different agitation speeds (150 or 200 rpm). Values followed by the same letter do not differ statistically (P=0.05). V_{ef}/V_{cm} : The ratio between the volume of the Erlenmeyer flask and the volume of culture medium, used in the experiments; * samples submitted to 10 cycles of sonication; ** samples submitted to 15 cycles of sonication.

V_{ef}/V_{cm}/Agitation speed (rpm)	Lipid (g/L)	Biomass (g/L)	Lipid (%)
3.33/150 **	1.48± 0.27 [a]	4.92 ± 0.68 [a]	30.1
3.33/200**	1.29± 0.04 [ab]	5.14± 0.31 [a]	25.1
3.33/200*	0.94 ± 0.20 [bc]	5.10 ± 1.52 [a]	18.4
3.33/150*	0.94 ± 0.30 [bc]	4.01 ± 0.33 [ab]	23.4
2.5/150*	0.77 ± 0.01 [c]	2.28 ± 0.23 [b]	33.8

Table 3: Lipid accumulation (g/L; % according to total cell dry weight), biomass production and oil characteristics by the *Y. lipolytica* QU21 growing in a combination of pure carbon/nitrogen sources (grade reagents): Gly/$(NH_4)_2SO_4$ and industrial wastes. Abbreviations: Gly, glycerol; CrGly, crude glycerol; FYE_{100}: 100% of fresh yeast extract; FYE_{50}: 50% of fresh yeast extract. All the culture medium were supplemented with 0.1% KH_2PO_4 and 0.05% $MgCl_2 \cdot 6H_2O$. The culture conditions were 150 rpm, 28°C for 4 days. The V_{ef}/V_{cm} ratio in all treatments was 3.33 and the cell pellets were submitted to 15 cycles of sonication. Values followed by the same letter do not differ statistically (P=0.05).

Substrate	Lipid (g/L)	Biomass (g/L)	Lipid (%)	Saturated (%)	PUFAs(%)
Gly/$(NH_4)_2SO_4$	1.48 [a]	4.92 [b]	30.1	39.50	7.93
CrGly/$(NH_4)_2SO_4$	1.27 [ab]	6.7 [a]	18.9	34.15	7.34
CrGly/ FYE_{100}	0.85 [b]	3.85 [b]	22.1	12.73	32.48
CrGly/FYE_{50}	0.84 [b]	4.41 [b]	19.0	17.54	32.17

Table 4: Fatty acid profile (%) of lipid content produced by *Y. lipolytica* QU21 in various carbon/nitrogen sources combinations and different culture conditions. Glu: glucose; Gly: glycerol; YE: yeast extract; C/N 18, 36, 184, 276, 368 as Table 1; CrGly/$(NH_4)_2SO_4$, CrGly /FYE_{100}, CrGly / FYE_{50} as Table 3; tr: traces (≤ 1%); ni: not identified.*The difference between both assays is the V_{ef}/V_{cm} ratio: 2.5 and 3.33 for Gly/$(NH_4)_2SO_4$ and C/N 184, respectively.

Carbon/nitrogen sources	Fatty acids (%)				
	C16:0	C16:1	C17:1	C18:0	C18:1n9c
Glu/$(NH_4)_2SO_4$	17.12 ± 0.47	8.2 ± 0.79	3.02 ± 0.30	17.40 ± 0.59	49.03 ± 0.92
Gly/$(NH_4)_2SO_4$*	19.18 ± 1.05	6.62 ± 0.55	tr	18.76 ± 0.73	45.48 ± 0.49
Gly/NH_4NO_3	26.08 ± 1.46	8.46 ± 0.18	ni	22.11 ± 1.83	37.35 ± 2.88
Gly/YE	24.22 ± 3.12	9.05 ± 0.96	tr	21.62 ± 2.31	39.00 ± 2.52
Gly/Tryptone	18.29 ± 0.79	9.65 ± 0.34	tr	16.63 ± 0.35	45.63 ± 1.59
Gly/Urea	19.72 ± 0.98	14.99 ± 1.20	ni	14.51 ± 1.15	41.6 ± 0.10
Culture conditions					
C/N 18	16.16 ± 0.64	6.6 ± 0.85	tr	14.76 ± 1.94	50.59 ± 0.93
C/N 36	12.8 ± 2.20	11.30 ± 1.40	3.10 ± 2.80	6.50 ± 3.20	53.80 ± 6.80
C/N 184*	14.59 ± 2.32	11.45 ± 0.73	1.32 ± 0.70	8.08 ± 2.22	53.38 ± 5.42
C/N 276	13.64 ± 0.65	11.20 ± 1.42	tr	7.61 ± 0.71	60.89 ± 1.45
C/N 368[a]	11.57 ± 0.71	12.66 ± 0.78	ni	6.58 ± 0.35	62.22 ± 2.02
200 rpm/15 cycles	12.75 ± 1.56	10.81 ± 1.44	1.37 ± 0.45	5.71 ± 0.46	60.16 ± 4.32
48 hours	18.38 ± 1.27	10.75 ± 1.03	1.85 ± 0.62	14.74 ± 1.11	44.29 ± 1.28
72 hours	14.95 ± 0.47	12.5 ± 1.36	tr	7.33 ± 1.03	55.96 ± 0.53
CrGly/$(NH_4)_2SO_4$	15.52 ± 0.74	5.49 ± 0.64	tr	17.49 ± 1.91	52.52 ± 2.24
CrGly/FYE_{100}	8.46 ± 0.17	5.97 ± 0.77	2.29 ± 0.27	4.27 ± 1.35	46.52 ± 5.72
CrGly/ FYE_{50}	9.41 ± 4.12	6.04 ± 1.12	1.99 ± 0.37	8.13 ± 4.55	42.26 ± 5.25

(continued on the next page)

Table 4 (continued)

Carbon/nitrogen sources	Fatty acids (%)				
	C18:2n6c	C18:3n3	Saturated	MUFAs**	PUFAs***
Glu/$(NH_4)_2SO_4$	4.39 ± 0.46	ni	34.94	60,67	4.39
Gly/$(NH_4)_2SO_4$*	7.93 ± 0.44	ni	39.50	52,47	7.93
Gly/NH_4NO_3	5.99 ± 0.17	ni	48.19	45,81	5.99
Gly/YE	5.44 ± 0.86	ni	45.84	48,28	5.44
Gly/Tryptone	6.90 ± 0.32	ni	36.84	56,27	6.90
Gly/Urea	8.76 ± 0.58	ni	34.23	56,85	8.76
Culture conditions					
C/N 18	10.48 ± 0.72	ni	30.92	58.01	11.07
C/N 36	12.60 ± 4.40	ni	19.27	68.2	12.6
C/N 184*	6.18± 0.34	ni	22.67	71.15	6.18
C/N 276	5.67 ± 0.39	ni	21.50	72.83	5.67
C/N 368	6.68 ± 1.54	ni	18.20	75.10	6.70
200 rpm/15 cycles	9.20 ± 1.08	ni	18.46	72.34	9.20
48 hours	9.99 ± 0.31	ni	33.12	56.89	9.99
72 hours	8.31 ± 0.87	ni	22.28	69.41	8.31
CrGly/$(NH_4)_2SO_4$	6.49 ± 1.17	ni	34.15	58.51	7.34
CrGly/FYE_{100}	19.29 ± 3.57	6.97 ± 1.17	12.73	54.79	32.48
CrGly/ FYE_{50}	17.81 ± 2.78	6.22 ± 5.46	17.54	50.29	32.17

** Monounsaturated fatty acids; *** Polyunsaturated fatty acids.

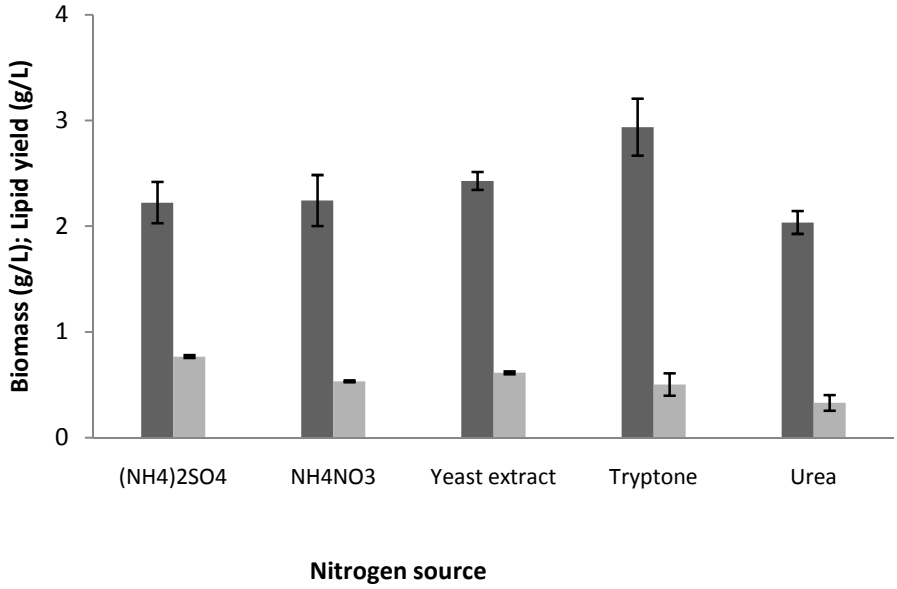

Figure 1: Inorganic [$(NH_4)_2SO_4$ and NH_4NO_3] and organic (yeast extract, tryptone and urea) nitrogen sources and their effect on biomass (■) and lipid accumulation (■) by *Y. lipolytica* QU21 (error bars indicate standard deviation, which for biomass ranges from 0.09 to 0.27 and lipid production ranges from 0.01 to 0.11). The culture medium was comprised of 10% glycerol, 0.1% KH_2PO_4, 0.05% $MgCl_2 \cdot 6H_2O$ and each nitrogen source (0.1%) separately. The culture conditions were 150 rpm, 28°C for 4 days.

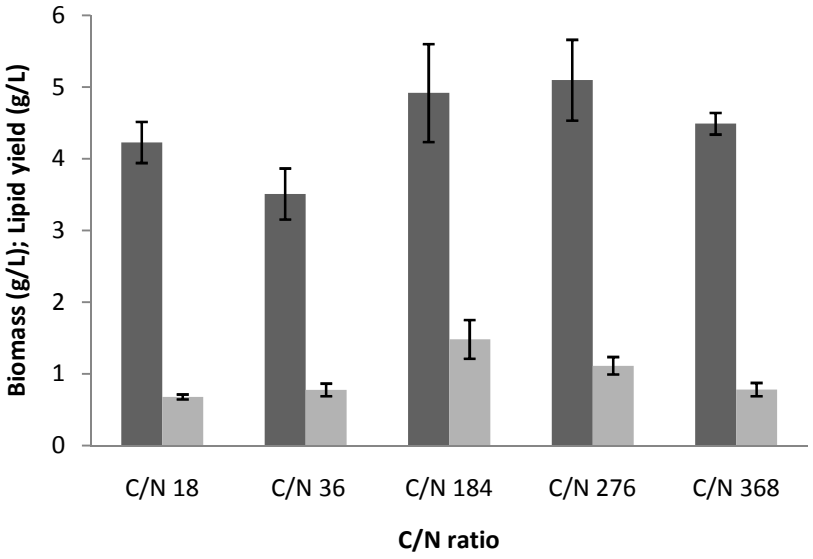

Figure 2: Effect of C/N ratio on the biomass production (■) and lipid accumulation (■) by *Y. lipolytica* QU21 (error bars indicate standard deviation, which for biomass ranges from 0.15 to 0.68 and lipid production ranges from 0.03 to 0.27). All the culture medium were supplemented with 0.1% KH_2PO_4 and 0.05% $MgCl_2 \cdot 6H_2O$. The culture conditions were 150 rpm, 28°C for 4 days. The V_{ef}/V_{cm} ratio for all treatments was 3.33 and the cell pellets were submitted to 15 cycles of sonication.

4. RESULTADOS E DISCUSSÃO GERAL

O método tradicional para análise de lipídios, além de ser laborioso, exige a utilização de solventes orgânicos fortes, como clorofórmio e metanol. Quando o objetivo é avaliar um número elevado de micro-organismos quanto ao seu potencial oleaginoso, um método mais rápido faz-se necessário. Oitenta e seis linhagens de leveduras, previamente isoladas de queijo artesanal do Rio Grande do Sul, foram avaliadas quanto ao seu potencial de acumular lipídios utilizando o corante vermelho de Nilo e visualização em microscópio de fluorescência. Do total, 23 linhagens apresentaram gotas lipídicas preenchendo, pelo menos, 50% da área celular. Esta metodologia tem caráter qualitativo e o tamanho das gotas lipídicas é estimado visualmente com relação à área celular. Portanto, para quantificar o conteúdo de lipídios totais intracelulares, bem como o estudo do perfil de ácidos graxos do óleo, a metodologia tradicional é necessária.

Por apresentar o melhor resultado na análise de triagem com o corante vermelho de Nilo, a linhagem de levedura QU21 foi selecionada para padronizar a técnica de extração de lipídios. Primeiramente, a extração foi realizada de acordo com Folch *et al.* (1957), extraindo os lipídios através da mistura de solventes (clorofórmio:metanol, 2:1) em contato com a biomassa,

macerando-a durante 30 minutos. O total de lipídios extraídos (experimento realizado em triplicata) representou 4% (de acordo com o peso seco) da biomassa total. Ao comparar este resultado com a técnica de triagem, ficou evidente que a técnica utilizada não foi capaz de extrair boa parte dos lipídios intracelulares (Fig.1A - Artigo I). Sabe-se que nenhum método de extração de lipídios possui uma eficiência de 100% (Jacob 1992, Ageitos *et al*., 2011). Por outro lado, a linhagem de levedura QU21 foi identificada como sendo da espécie *Yarrowia lipolytica*, conhecida por sua capacidade de acumular lipídios. Esta espécie que vem sendo estudada ao longo dos anos por sua capacidade de produzir uma gama de produtos de importância biotecnológica, como ácido cítrico (Papanikolaou *et al*., 2008), bio-óleo com diferentes aplicações (dietética, médica e biodiesel) (Papanikolaou & Aggelis, 2011) e, até mesmo, biomassa para produção de bioetanol (Tsigie *et al*., 2012). Além do mais, *Y. lipolytica* é considerada uma espécie de levedura modelo para produção de bio-óleo (Beopoulos *et al*., 2009). Desta forma, sustentou-se a hipótese de que os lipídios não estavam sendo extraídos de forma correta. Portanto, cinco técnicas de rompimento da parede celular da levedura *Y. lipolytica* foram testadas.

Cinco técnicas utilizadas para romper a parede celular da levedura *Yarrowia lipolytica* QU21 foram realizadas, seguidas por uma etapa de extração de lipídios, como descrito por Folch *et al.* (1957). As técnicas foram avaliadas através da quantidade total de lipídios extraídos da levedura *Y. lipolytica* QU21. O uso concomitante de nitrogênio líquido para maceração da biomassa seguido de sonicação foi a melhor técnica dentre as testadas no presente trabalho,

sendo capaz de promover o rompimento das células de forma que 26,5% de lipídios intracelulares (em relação ao peso seco) puderam ser extraídos (equivalente a 0,6g/L).O uso do nitrogênio líquido como única técnica de rompimento, não foi tão eficiente, pois promoveu a extração de apenas 12,8% de lipídios totais. O uso do sonicador, sem a prévia utilização do nitrogênio líquido, não foi avaliado nesta etapa. Estes resultados confirmam a hipótese levantada anteriormente, de que os lipídios estavam presentes, mas não estavam sendo completamente extraídos, e o método de Folch *et al.* (1957), quando empregado para extração de lipídios de células de levedura da espécie *Y. lipolytica*, necessita de uma etapa prévia para a ruptura da parede celular.

O método de triagem empregado nesta etapa (Artigo I) foi eficaz, e se confirmou quando a levedura selecionada por apresentar maiores gotas lipídicas foi identificada como sendo da espécie *Y. lipolytica*, assim como, quando o resultado da extração de lipídios apresentou 26,5% de óleo, caracterizando-a como uma linhagem oleaginosa (Ageitos *et al*. 2011; Koutb *et al*. 2011). No entanto, a técnica é de caráter qualitativo, sendo a característica oleaginosa estimada visualmente. Além do mais, embora seja mais rápida quando comparada à metodologia tradicional (extração de lipídios), o procedimento exige o preparo de lâminas, sendo que as amostras deverão ser preparadas uma por vez pelo fato de a luz e o tempo interferirem com o corante vermelho de Nilo, e este acabar perdendo a sua intensidade. Assim, torna-se necessário o desenvolvimento de um método de triagem de leveduras com característica oleaginosa, de caráter quantitativo, de forma a utilizar o mínimo

possível de solventes orgânicos e, ainda, com menor potencial agressivo, tanto para o manipulador quanto para o meio ambiente.

Com o objetivo de quantificar os lipídios, este trabalho utilizou como padrão a trioleína. Desta forma, uma curva padrão pôde ser obtida e, através dela, pôde-se calcular os lipídios intracelulares presentes em leveduras. O método de fluorescência, proposto no presente trabalho, possui duas grandes diferenças quando comparado com o método de triagem descrito por Sitepu *et al*. (2012). Embora estes autores também tenham feito a utilização do corante vermelho de Nilo, seu método não quantifica o teor lipídico. O solvente orgânico utilizado para facilitar a penetração do vermelho de Nilo nas células de leveduras foi o isopropanol, que é um solvente com característica menos agressiva, tanto para o manipulador quanto para o meio ambiente, quando comparado ao dimetilsulfóxido (DMSO), utilizado na metodologia proposta por Sitepu *et al*. (2012).

O método de fluorescência quantitativo para a utilização em triagem de leveduras permite o preparo de várias amostras ao mesmo tempo e, portanto, a metodologia torna-se mais rápida e menos trabalhosa quando comparada à técnica de triagem apresentada inicialmente. O método proposto não substitui totalmente o método tradicional pois, quando o objetivo é estudar o perfil de ácidos graxos, a extração de lipídios é uma etapa obrigatória. Entretanto, ele poderá substituí-lo quando o objetivo for avaliar e/ou realizar um estudo inicial do conteúdo lipídico de uma grande coleção de linhagens de leveduras ainda não estudadas. A leitura do método proposto foi realizada em espectrofotômetro de fluorescência com o preparo de várias amostras ao

mesmo tempo, mas com a leitura realizada individualmente. Este método já foi testado, com sucesso, com as amostras em placa de 96 poços e a leitura realizada em leitor de ELISA (dados não apresentados).

Como demonstrado no Artigo I, a técnica de rompimento da parede celular que melhor promoveu a extração de lipídios foi a maceração da biomassa com nitrogênio líquido seguido da sonicação. Nesta mesma etapa, o sonicador como único método de rompimento da parede celular não foi estudado. Entretanto, sua eficiência foi demonstrada nos Artigos II e III.

Os ensaios de otimização da produção de lipídios, bem como a caracterização do perfil de ácidos graxos do óleo produzido foram, também, realizados com a levedura *Y. lipolytica* QU21. Nos ensaios realizados em frascos de Erlenmeyer, esta linhagem foi capaz de produzir uma boa quantidade de lipídios (1,48 g/L) em quatro dias de crescimento em glicerol e sulfato de amônio como fontes de carbono e nitrogênio, respectivamente. Vale ressaltar que, quando o glicerol foi substituído pela glicerina bruta, não houve diferença significativa com relação à produção de lipídios (1,27 g/L). Além disso, é importante destacar que a quantidade de biomassa produzida em glicerol foi menor que em glicerina bruta (4,9 g/L e 6,7 g/L, respectivamente). Os resultados apresentados no Artigo III mostram que a quantidade de biomassa pode interferir na extração de lipídios, sendo necessário mais ciclos de sonicação para rompimento da parede celular. Portanto, existe a possibilidade de que a levedura *Y. lipolytica* QU21, quando cultivada em glicerina bruta e sulfato de amônio, possa acumular uma quantidade ainda maior de lipídios que não estão sendo completamente extraídos.

A composição dos ácidos graxos é outro fator que merece destaque. Os resultados obtidos no Artigo III mostram que, quando as condições de cultivo, bem como o meio de cultura, foram modificados, a composição dos ácidos graxos também sofreu alterações. Portanto, o óleo obtido a partir de uma determinada espécie de levedura pode ter mais de uma aplicação à medida que as condições e/ou o meio de cultivo forem modificados. Um exemplo são os resultados apresentados no Artigo III, onde o resíduo de cervejaria (*fresh yeast extract - FYE*), utilizado como única fonte de nitrogênio, combinado com a glicerina bruta, promoveram um aumento de mais de 300% na concentração de ácidos graxos poliinsaturados quando comparados à glicerina bruta com sulfato de amônio.

Globalmente, existem mais de 300 culturas oleaginosas identificadas como potenciais fontes para a produção de biodiesel. A viabilidade das matérias-primas comumente utilizada na produção de biodiesel (de origem vegetal) depende de vários fatores como, clima, localização geográfica, condições do solo e práticas agrícolas (Shahid e Jamal, 2011; Atabani *et al*., 2012). Quanto a este aspecto, a utilização do óleo microbiano possui uma grande vantagem sobre o óleo vegetal pois não depende de nenhum dos fatores citados acima. A matéria-prima, sozinha, responde por 75% do custo da produção do biodiesel e, para ser levada em conta, ela deverá cumprir duas principais exigências: baixo custo de produção e capacidade de produção em larga escala (Atabani *et al*., 2012). A utilização de um substrato de baixo valor comercial está diretamente relacionada com o baixo custo de produção na obtenção do óleo microbiano. A levedura *Y. lipolytica* QU21, capaz de acumular

1,27 g/L de lipídios utilizando glicerina bruta como principal substrato em apenas 4 dias, uniu dois aspectos de grande importância. Além de utilizar um substrato de baixo valor comercial diminuindo os custos de produção do óleo, a utilização da glicerina bruta, proveniente da cadeia produtiva de biodiesel, faz do óleo da levedura *Y. lipolytica* QU21 um produto ecologicamente correto, ou seja, a levedura utiliza como insumo um resíduo industrial, minimizando, ou até mesmo, evitando o seu descarte no ambiente, prevenindo assim a contaminação do solo (caso a glicerina não seja adequadamente tratada), e preservando o meio ambiente para não comprometer os recursos naturais das gerações futuras. Vale lembrar que a projeção do aumento da composição de biodiesel na mistura para B20 em 2020, acarretará na produção de, aproximadamente 1,43 milhões de m^3 de glicerina como resíduo industrial, e um dos fatores mais preocupantes nesse processo seria o seu descarte inadequado no ambiente. Além do mais, a utilização da glicerina como substrato para a produção de óleo pela *Y. lipolytica* QU21 evita a etapa de purificação deste subproduto pela indústria de biodiesel. A purificação do glicerol, obtido da produção do biodiesel, a níveis aceitáveis para o seu uso em diversas indústrias, envolve custos em procedimentos físico-químicos para aumentar a pureza da glicerina (Bowker *et al.*, 2008).

De acordo com Atabani *et al.*, (2012), para que qualquer matéria prima seja considerada uma fonte para produção de biodiesel, a porcentagem de óleo e o rendimento/hectare são parâmetros essenciais. A soja, sendo a principal matéria-prima utilizada na produção de biodiesel no Brasil, apresenta um conteúdo lipídico que varia entre 15 e 20%, e o rendimento de óleo fica em

torno de 446 L/hectare/ano. A colza, principal matéria-prima utilizada para produção de biodiesel na Europa, apresenta um conteúdo lipídico que varia entre 38 e 46% e o rendimento de óleo de 1190 L/hectare/ano. Já as fontes alternativas que vêm sendo estudadas, como a semente de pinhão manso, apresentam conteúdo lipídico entre 35 e 40% e 1892 L/hectare/ano de rendimento de óleo.

Entrando no ramo dos micro-organismos utilizados como fonte alternativa para a produção de biodiesel, pode-se falar em microalgas. A microalga de baixo conteúdo lipídico (30%) pode apresentar um rendimento de óleo de 58.700 L/hectare/ano. Já uma microalga de alto conteúdo lipídico (70%) pode alcançar um rendimento de 136.900 L/hectare/ano (Atabani *et al*., 2012). Com relação à levedura *Y. lipolytica* QU21, considerando a quantidade de óleo obtida de 1,13 mL de óleo (aproximadamente 20% de conteúdo lipídico) (1,27 g/L, convertido em mL, utilizando como parâmetro a densidade do óleo de soja, 0,891 g/cm^3) quando cultivada em glicerina bruta e $(NH_4)_2SO_4$ por 4 dias, e uma planta industrial com capacidade de 1000 L ocupando uma área de 55m^2, resultará em um rendimento de óleo de 18.747,72 L/hectare/ano. Vale lembrar que a biomassa produzida neste experimento pode ter interferido na quantidade de óleo extraído, diminuindo assim, o rendimento total. Portanto, é importante mencionar os resultados obtidos com glicerol e $(NH_4)_2SO_4$ por 4 dias. A média de produção de óleo pela *Y. lipolytica* QU21 foi 1,48 g/L (média) (31% de conteúdo lipídico), sendo que em um dos experimentos (resultante da menor biomassa obtida, 4,39 g/L) a quantidade de óleo extraída foi 1,77g/L (40,32%). Neste caso, os rendimentos de óleo seriam 21.878,10 L/hectare/ano

e 26.165,025 L/hectare/ano, respectivamente. Estes rendimentos foram calculados sem levar em consideração a área utilizada na produção do substrato utilizado pela levedura, neste caso a glicerina bruta proveniente de uma empresa de biodiesel. Outro aspecto que deve ser levado em conta é que estes resultados foram obtidos a partir de experimentos realizados em frascos de Erlenmeyer, podendo sofrer alterações quando realizados em escala industrial.

O rendimento estimado do óleo da levedura *Y. lipolytica* QU21 foi inferior ao de microalga. Por outro lado, é bastante superior ao rendimento de qualquer óleo de origem vegetal, de acordo com os rendimentos apresentados por Atabani *et al*., (2012). Além do mais, outros fatores devem ser levados em conta. De acordo com Chisti (2007), o óleo de microalga difere da maioria dos óleos vegetais por ser rico em PUFAs com quatro ou mais ligações duplas. Ácidos graxos, assim como FAME com quatro ou mais duplas ligações são suscetíveis à oxidação durante o armazenamento e isso reduz a sua aceitabilidade para o uso em biodiesel. Alguns óleos vegetais enfrentam este tipo de problema, como o óleo de canola por conter grandes quantidades de ácido linoléico (C18:2n6, duas ligações duplas) e linolênico (C18:3n3, três ligações duplas). Embora estes ácidos graxos possuam maior estabilidade oxidativa quando comparados aos ácidos docosa-hexaenóico (DHA, C22:6n3, 6 ligações duplas) e eicosapentaenóico (EPA, C20:5n3, cinco ligações duplas), a norma européia de biodiesel (*European Standard EN* 14214) limita o conteúdo do ácido linolênico metil éster em biodiesel para uso em veículos em 12% (mol) (Chisti, 2007). Ainda de acordo com o autor, tendo em vista a

composição de muitos óleos de microalga, a maioria não é suscetível de cumprir as normas européias de biodiesel, sendo imprescindível a realização de uma etapa de redução do conteúdo de insaturações por hidrogenação catalítica parcial. De acordo com Clemente e Cahoon (2009), o óleo de soja é composto por 68% de PUFAs, sendo 55% ácido linoléico e 13% ácido linolênico. O perfil de ácidos graxos do óleo da levedura *Y. lipolytica* QU21, quando cultivada em glicerina bruta e $(NH_4)_2SO_4$, apresentou um total de 7,34% de PUFAs em sua composição sendo que, nestas condições, não houve produção de ácido linolênico. Quanto a este aspecto, o óleo de levedura possui grande vantagem tanto com relação ao óleo de microalga, quanto com relação ao óleo de soja.

Ao longo do desenvolvimento desta pesquisa, a extração de lipídios foi o ponto crítico durante todas as etapas. Além de se constatar que a extração com solventes necessita de um método de rompimento da parede celular (Artigo I), no Artigo III pôde-se observar que a quantidade de biomassa pode interferir no processo de sonicação. Estas limitações puderam ser detectadas em experimentos realizados em escala de bancada (frascos de Erlenmeyer de 250 mL). Por exemplo, a levedura *Yarrowia lipolytica* ACA-DC 50109, quando cultivada em biorreator de 1,5L utilizando gordura industrial (resíduo) como substrato, produziu 15 g/L de biomassa em 120 horas de cultivo, mas a síntese de lipídios foi reportada como insignificante (Papanikolaou *et al*., 2002). Atualmente, extração de lipídios de microalgas vem sendo fortemente debatida por ser o processo mais oneroso da produção, o que pode determinar a sustentabilidade do biodiesel a partir de microalga

(OILGAE, 2013). Portanto, a produção de biodiesel a partir de óleo de levedura também deverá enfrentar este desafio, sendo imprescindível a realização de pesquisas para viabilizar o processo de extração em escala industrial.

Com o cenário previsto das mudanças climáticas, a busca por fontes alternativas de combustíveis renováveis de modo a não competir com a produção de alimentos é um assunto que só tende a crescer. E o desenvolvimento de novas tecnologias de biocombustíveis renováveis capazes de suprir a demanda da população mundial, assim como, colocá-las em prática, é uma corrida contra o tempo para minimizar os efeitos do aquecimento global.

5. CONCLUSÕES

Esta tese teve como foco a produção de óleo a partir de resíduos industriais, por meio da seleção de uma linhagem de levedura oleaginosa e análise de seu comportamento sob diferentes condições de cultivo. Além disso, a padronização do método de extração de lipídios, identificando as principais limitações da técnica, assim como o desenvolvimento de uma metodologia de triagem de leveduras para facilitar a identificação de novas espécies oleaginosas foram necessários. As principais contribuições resultantes destes estudos estão resumidas abaixo.

- Foram selecionadas 23 leveduras com potencial oleaginoso a partir de queijo artesanal.
- A metodologia de extração de lipídios necessita de uma etapa prévia, visando o rompimento da parede celular da levedura, sendo que a técnica de rompimento da parede celular que possibilitou a maior extração de lipídios foi o uso de sonicador.
- Uma metodologia de triagem de leveduras oleaginosas, de caráter quantitativo, rápida, fácil de ser empregada, ecologicamente amigável foi demonstrada.

- A levedura *Yarrowia lipolytica* QU21 foi capaz de crescer e acumular lipídios tanto em glicose quanto em glicerol, como únicas fontes de carbono.
- A levedura *Y. lipolytica* QU21 foi capaz de crescer e acumular lipídios utilizando dois tipos de resíduos industriais que se complementaram: a glicerina industrial, subproduto da cadeia produtiva do biodiesel (como fonte de carbono) e o extrato de levedura fresco, resíduo de uma cervejaria (como fonte de nitrogênio).
- O meio de cultura, assim como as condições de cultivo, influenciaram no perfil de ácidos graxos presentes no óleo produzido pela levedura *Y. lipolytica* QU21.

6. PROPOSTAS DE TRABALHOS FUTUROS

Com base nos resultados obtidos ao longo da presente tese, algumas propostas para trabalhos futuros podem ser sugeridas, tais como:

- Utilização de outros resíduos industriais como matéria prima para a produção de óleo microbiano a partir de leveduras oleaginosas;
- Otimização da produção de óleo microbiano em biorreator para maior controle das variáveis do processo, como aeração, agitação, temperatura e pH;
- Desenvolvimento de um método de extração do óleo microbiano em escala industrial.

7. REFERÊNCIAS BIBLIOGRÁFICAS

AGEITOS, J.M et al. Oily yeasts as oleaginous cell factories. **Applied Microbiology and Biotechnology**, v.90, p.1219-1227, 2011.

ANGERBAUER, C. et al. Conversion of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. **Bioresource Technology,** v.99, p.3051-3056, 2008.

ASSAD, E.D. et al. Impacts of climate change on the agricultural zoning of climate risk for cotton cultivation in Brazil. **Pesquisa Agropecuária Brasileira**, v. 48, p.1-8, 2013.

ASSAD, E.D.; PINTO, H.S.; ZULLO, J.Jr. Impacts of global warming in the Brazilian agroclimatic risk zoning. In: da Silva Dias P.L.S., Ribeiro W.C, Nunes, L.H., A contribution to understanding the regional impacts of global change in South America. **Instituto de Estudos Avançados da Universidade de São Paulo**, São Paulo, p.175-182, 2007. Disponível em: http://www.iea.usp.br/pesquisa/grupos/meio-ambiente-e-sociedade/publicacoes/globalchangeinsouthamerica.pdf/at_download/file (acesso: 18/12/2013).

ATABANI, A.E. et al. A comprehensive review on biodiesel as an alternative energy resource and its characteristics. **Renewable and Sustainable Energy Reviews**, v.16, p.2070-2093, 2012.

BARTH, G.; GAILLARDIN, C. Physiology and genetics of the dimorphic fungus *Yarrowia lipolytica*. **FEMS Microbiology Reviews**, v.19, p.219-237, 1997.

BEOPOULOS, A. et al. *Yarrowia lipolytica* as a model for bio-oil production. **Progress in Lipid Research***,* v.48, p.375-87, 2009.

Biodiesel no Brasil: desafios das políticas públicas para a dinamização da produção. Comunicado do IPEA nº 137, 2012.

BOWKER, M.; DAVIES, P.R.; AL-MAZROAI, L.S. Photocatalytic reforming of glycerol over gold and palladium as an alternative fuel source. **Journal Catalysis Letters**, v.128, p.253-255, 2008.

BP Statistical Review of World Energy, 2013. Disponível em: http://www.bp.com/content/dam/bp/pdf/statistical-review/statistical_review_of_world_energy_2013.pdf (acesso: 18/12/2013).

CHISTI, Y. Biodiesel from microalgae. **Biotechnology Advances**, v.25, p.294-306, 2007.

CHU, S.; MAJUMDAR, A. Opportunities and challenges for a sustainable energy future. **Nature**, v.488, p.294-303, 2012.

CLEMENTE, T.E.; CAHOON, E.B. Soybean oil: genetic approaches for modification of functionality and total content. **Plant Physiology**, v.151, p.1030-40, 2009.

DONKIN, S.S.; DOANE, P. Glycerol as a feed ingredient in dairy rations. **Proceedings of the 2007 Tri-State Dairy Nutrition Conference**, Fort Wayne, Indiana, USA, p.24-25, April, 2007.

FICKERS, P. et al. Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. **FEMS Yeast Research**, v.5, p.527-543, 2005.

FOLCH, J.; LEES, M.; STANLEY, G.H.S. A simple method for the isolation and purification of total lipids from animal tissues. **The Journal of Biological Chemistry**, v.226, p.497-509, 1957.

FUKUDA, H.; KONDO, A.; NODA, H. Biodiesel fuel production by transesterification of oils. **Journal of Bioscience and Bioengineering**, v.92, p.405-416, 2001.

GERPEN, J.V. Biodiesel processing and production. **Fuel Processing Technology**, v. 86, p.1097-1107, 2005.

GONÇALVES, B.R.L.; PEREZ, L.; ÂNGELO, A.C.D. Glicerol: uma inovadora fonte de energia proveniente da produção de biodiesel. **2nd International Workshop | Advances in Cleaner Production**, São Paulo, p.1-6, 2009.

GROENEWALD, M. et al. *Yarrowia lipolytica*: Safety assessment of an oleaginous yeast with a great industrial potential. **Critical Reviews in Microbiology**, Early on line, p.1-20, 2013.

IEA (INTERNATIONAL ENERGY AGENCY). CO_2 emissions from fuel combustion-Highlights, 2013. Disponível em: http://www.iea.org/publications/freepublications/publication/name,43840,en.html (Acesso: 22/11/2013).

INDEX MUNDI. Disponível em:

http://www.indexmundi.com/energy.aspx?product=biodiesel&graph=production (Acesso: 22/11/2013).

IPCC - CLIMATE CHANGE 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Core writing team, Pachauri, R.K and Reisinger, A., **IPCC**, Geneva, Switzerland. p.104, 2007.

JACOB, Z. Yeast lipids: Extraction, quality analysis and acceptability. **Critical Reviews in Biotechnology**, v.12, p.463-491, 1992.

JOHNSON, D.T.; TACONI, K.A. The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. **Environmental Progress**, v.26, p.338-348, 2007.

KOUTB, M.; MORSY, F.M. A potent lipid producing isolate of *Epicoccum purpurascens* AUMC5615 and its promising use for biodiesel production. **Biomass Bioenergy**, v.35, p.3182-3187, 2011.

LAMMERS, P.J. et al. Nitrogen-corrected apparent metabolizable energy value of crude glycerol for laying hens. **Poultry Science**, v.87, p.104-107, 2008.

LIANG, M.H.; JIANG, J.G. Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. **Progress in Lipid Research**, v.52, p.395-408, 2013.

MA, F.; HANNA, M.A. Biodiesel production: a review. **Bioresource Technology**, v.70, p.1-15, 1999.

MANZANERA, M.; MUÑOS, M.L.M.; LÓPEZ, J.G. Biodiesel: An alternative Fuel. **Recent Patents on Biotechnology**, v.2, p.25-34, 2008.

NICAUD, J.M. *Yarrowia lipolytica*. **Yeast**, v.29, p.409-418, 2012.

O BIODIESEL e sua contribuição ao desenvolvimento brasileiro. **Fundação Getúlio Vargas-União Brasileira do Biodiesel** (UBRABIO), 2010.

OECD-FAO Agricultural Outlook 2011-2020, **OECD Publishing and FAO**. Disponível em: http://www.oecd-ilibrary.org/agriculture-and-food/oecd-fao-agricultural-outlook-2011_agr_outlook-2011-en (Acesso: 22/11/2013).

OILGAE, 2013. Disponível em: http://www.oilgae.com/algae/oil/extract/extract.html (Acesso: 22/11/2013)

PAGLIARO, M. et al. From glycerol to value-added products. **Angewandte Chemie International**, v.46, p.4443-4440, 2007.

PAPANIKOLAOU, S.; AGGELIS, G. Lipids of oleaginous yeast. Part I: Biochemistry of single cell oil production. **European Journal of Lipid Science and Technology**, v.113, p.1031-1051, 2011.

PAPANIKOLAOU, S.; AGGELIS, G. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. **European Journal of Lipid Science and Technology**, v.113, p.1031-1051, 2011.

PAPANIKOLAOU, S. et al. Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. **Applied Microbiology and Biotechnology**, v.58, p.308-312, 2002.

PAPANIKOLAOU, S. et al. Biotechnological valorization of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. **Biomass and Bioenergy**, v.32, p.60-71, 2008.

RATLEDGE, C. Fatty acid biosynthesis in microorganisms being used for single cell oil production. **Biochimie**, v.86, p.807-815, 2004.

RATLEDGE, C. Single cell oils for the 21st century. In: Cohen, Ratledge, editors. Single cell oils. **Champaign, Illinois: Aocs Press**, p.1-20, 2005.

RESOLUÇÃO ANP Nº 14, de 11.5.2012, DOU 18.05.2012. Disponível em: http://nxt.anp.gov.br/nxt/gateway.dll/leg/resolucoes_anp/2012/maio/ranp%2014%20-%202012.xml (Acesso em 22/11/2013).

RYWINSKA, A. et al. Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. **Biomass and Bioenergy**, v.48, p.148-166, 2013.

SHAHID, E.M.; JAMAL, Y. Production of biodiesel: A technical review. **Renewable and Sustainable Energy Reviews**, v.15, p.4732-4745, 2011.

SITEPU, I.R. et al. An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. **Journal of Microbiological Methods**, v.91, p.321-328, 2012.

SITEPU, I.R. et al. Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. **Bioresource Technology,** v.144, p.360-369, 2013.

SOLAIMAN, D.K.Y. et al. Conversion of agricultural feedstock and coproducts into poly(hydroxyalkanoates). **Applied Microbiology and Biotechnology**, v.71, p.783-9, 2006.

TSIGIE, Y.A. et al. Bioethanol production from *Yarrowia lipolytica* Po1g biomass. **Bioresource Technology**, v.145, p.210-216, 2012.

WREGE, M.S. et al. Simulação do impacto do aquecimento global nas horas de frio no Rio Grande do Sul. **Revista Brasileira de Agrometeorologia,** v.14, p.347-352, 2007.

WYNN, J.P.; BIN, A.H.A.; RATLEDGE, C. The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. **Microbiology**, v.145, p. 1911-1917,1999.

ZULLO, J.Jr. et al. Potential for growing Arabica coffee in the extreme south of Brazil in a warmer world. **Climatic Change**, v.109, p.535-548, 2011.