ANDREÍSA TEIXEIRA DE CASTRO

PRODUCTION OF LIPIDS AND CAROTENOIDS BY YEASTS USING AGROINDUSTRIAL BY-PRODUCTS

LAVRAS – MG
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Tese apresentada a Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para obtenção do título de Doutora.

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PRODUÇÃO DE LIPIDEOS E CAROTENOIDES POR LEVEDURAS UTILIZANDO SUBPRODUTOS AGROINDUSTRIAIS

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APROVADA em 22 de Junho de 2023.
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2023

Aos meus pais, Luís Carlos e Adelimar, pelo amor, dedicação, incentivo e por não medirem esforços para o alcance de cada conquista em minha vida.

Dedico

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RESUMO

Lipídeos e carotenoides são compostos de alto valor e demanda em diversos setores industriais. Devido à crescente preocupação com o desenvolvimento de processos sustentáveis, há interesse em fontes alternativas de produção destes compostos para substituir a extração vegetal e síntese química. Leveduras são microrganismos capazes de sintetizar lipídeos e carotenoides e possuem diversas vantagens de cultivo. No entanto, um número restrito de espécies de leveduras são descritas para a síntese desses metabólitos e ainda não há utilização em escala industrial. Este estudo teve como objetivo identificar novas cepas de leveduras capazes de produzir lipídeos e carotenoides utilizando subprodutos agroindustriais como substrato. Na primeira etapa, foram selecionadas 26 cepas de leveduras e realizados testes para avaliar a produção desses compostos. Nove cepas foram selecionadas como oleaginosas, apresentando perfis de ácidos graxos ricos em ácido palmítico, palmitoleico, esteárico, oleico e linoleico. A levedura *Torulaspora maleeae* CCMA 0039 mostrou a maior acumulação lipídica (77,7%) em meio de glicose, enquanto a levedura *Exophiala spinifera* CCMA 2073, não descrita anteriormente como oleaginosa, apresentou um teor lipídico de 35% em glicerol puro. Quatro cepas foram identificadas como carotenogênicas. O meio de glicerol bruto foi eficiente para a produção de lipídeos e carotenoides, resultando em maior diversidade no perfil de ácidos graxos e maiores concentrações de carotenoides totais para todas as cepas testadas. A *Cystofilobasidium ferigula* CCMA 1623 foi identificada como promissora para estudos posteriores, apresentando um acúmulo lipídico de 41,88% e 2,76 µg/mL de carotenoides totais sob cultivo em glicerol bruto. A segunda etapa do estudo consistiu na otimização de parâmetros de cultivo da cepa *C. ferigula* CCMA 1623 utilizando glicerol bruto como fonte de carbono, visando a produção concomitante de lipídeos e carotenoides. A concentração de extrato de levedura mostrou-se como fator determinante para a síntese desses compostos. A condição ótima para o acúmulo de lipídeos e carotenoides foi de 1,5 g/L de extrato de levedura, a 26°C e rotação de 140 rpm. Sob essas condições, foram obtidos 52,59% de teor lipídico e 3,03 µg/mL de carotenoides. O perfil de ácidos graxos sintetizados consistia principalmente de ácido palmítico, esteárico e ácidos ômega (oleico e linolênico). O extrato de carotenoides totais mostrou um alto poder antioxidante nos ensaios de ABTS, FRAP e DPPH. Este estudo possibilitou a identificação de cepas de leveduras oleaginosas e carotenogênicas ainda não exploradas, além de abrir novas perspectivas para o estabelecimento de processos fermentativos utilizando a levedura *C. ferigula* para a produção simultânea de lipídeos e carotenoides, utilizando glicerol bruto como substrato.

Palavras-chave: Leveduras. Glicerol bruto. Ácidos graxos. Carotenoides.

ABSTRACT

Lipids and carotenoids are valuable compounds in high demand across various industrial sectors. Due to increasing concerns about sustainable processes, there is interest in alternative sources for the production of these compounds to replace plant extraction and chemical synthesis. Yeasts are microorganisms capable of synthesizing lipids and carotenoids and offer several cultivation advantages. However, only a limited number of yeast species are described for the synthesis of these metabolites, and industrial-scale utilization is still lacking. This study aimed to identify new yeast strains capable of producing lipids and carotenoids using agro-industrial byproducts as substrates. In the first stage, 26 yeast strains were selected, and tests were conducted to evaluate the production of these compounds. Nine strains were selected as oleaginous, exhibiting fatty acid profiles rich in palmitic, palmitoleic, stearic, oleic, and linoleic acids. The yeast *Torulaspora maleeae* CCMA 0039 showed the highest lipid accumulation (77.7%) in glucose medium, while the previously undescribed oleaginous yeast *Exophiala spinifera* CCMA 2073 exhibited a lipid content of 35% in pure glycerol. Four strains were identified as carotenogenic. Crude glycerol medium was efficient for lipid and carotenoid production, resulting in greater diversity in fatty acid profiles and higher concentrations of total carotenoids for all tested strains. *Cystofilobasidium ferigula* CCMA 1623 was identified as promising for further studies, presenting a lipid accumulation of 41.88% and 2.76 µg/mL of total carotenoids under cultivation in crude glycerol. The second stage of the study consisted of optimizing the cultivation parameters of the *C. ferigula* CCMA 1623 strain using crude glycerol as the carbon source, aiming for concomitant production of lipids and carotenoids. The concentration of yeast extract proved to be a determining factor for the synthesis of these compounds. The optimal condition for lipid and carotenoid accumulation was found to be 1.5 g/L of yeast extract at 26°C and rotation of 140 rpm. Under these conditions, lipid content of 52.59% and 3.03 µg/mL of carotenoids were obtained. The synthesized fatty acid profile consisted mainly of palmitic, stearic, and omega fatty acids (oleic and linoleic). The total carotenoid extract exhibited high antioxidant power in the ABTS, FRAP and DPPH assays. This study enabled the identification of unexplored oleaginous and carotenogenic yeast strains, opening new perspectives for the establishment of fermentation processes using the *C. ferigula* yeast for simultaneous production of lipids and carotenoids, utilizing crude glycerol as the substrate.

Keywords: Yeasts. Crude glycerol. Fatty acids. Carotenoids.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Lipídeos microbianos são uma fonte alternativa aos óleos vegetais, com aplicações nas indústrias de alimentos, cosméticos, farmacêutica e de biocombustíveis. A medida que crescem as preocupações com o meio ambiente, os óleos microbianos surgem como potencial contribuintes para o desenvolvimento de processos sustentáveis (KARAMEROU; WEBB, 2019). A produção de óleos microbianos é vantajosa por necessitar de pequenas áreas para cultivo, ter facilidade de expansão e taxas de produção superiores aos óleos vegetais ao se considerar a área utilizada (BHARATHIRAJA *et al*., 2017; CAPORUSSO, CAPECE, DE BARI**,** 2021).

Compostos carotenoides também podem ser sintetizados por microrganismos e possuem vasta aplicabilidade nos diferentes setores industriais (RODRIGUEZ-CONCEPCION *et al*., 2018). Eles constituem um grupo diverso de pigmentos utilizados como corantes alimentares, suplementos, e aditivos cosméticos e farmacêuticos (PEREIRA *et al*., 2019). Essa diversidade de aplicações se deve à comprovação de efeitos benéficos à saúde como atividades antioxidante, anti-inflamatória, antitumoral, e de prevenção no desenvolvimento de doenças crônicas degenerativas (MAPELLI- BRAHM *et al*., 2020).

As leveduras têm sido alvo de pesquisas relacionadas à produção destes metabólitos devido a facilidade de cultivo, capacidade de assimilar substratos diversificados de baixo custo, produção de biomassa em biorreatores, eficiência para acumulação lipídica e robustez nos processos otimizados (KARAMEROU; WEBB, 2019). Além disso, biomassa de algumas espécies pode acumular, concomitantemente, elevado teor lipídico e pigmentos carotenoides, considerados produtos de valor com representatividade no mercado industrial (LIU *et al*., 2020).

A coprodução destes compostos por leveduras é relevante, uma vez que as principais formas de obtenção são a extração vegetal para óleos e síntese química para carotenoides. No entanto, tais processos são limitados pela sazonalidade, condições geográficas, e geração de resíduos prejudiciais à saúde, respectivamente (MATA-GOMEZ *et al*., 2014). Neste contexto, a utilização de leveduras conferem vantagens devido ao apelo de marketing de consumo de produtos naturais e seguros para a alimentação, concorrendo com a produção vegetal (CUTZU *et al*., 2013).

A espécie *Yarrowia lipolytica* é reconhecida como espécie modelo de interesse para a indústria, visando sua lipogênese. Possui capacidades fisiológicas únicas, inclusive de

metabolizar fontes de carbono diversificadas de baixo custo, acumulando mais de 30% de lipídeos de seu peso celular seco (BAO *et al*., 2021). A espécie é considerada como microrganismo geralmente seguro para alimentação (GRAS), sendo empregada para produção de suplementos dietéticos e nutracêuticos, além da síntese de lipídeos para biocombustíveis (WANG *et al*., 2020; CAPORUSSO, CAPECE, DE BARI**,** 2021).

Outra espécie de grande importância é a *Rhodosporidium toruloides*, uma levedura vermelha emergente para aplicação industrial. Pode acumular até 70% de lipídeos de sua biomassa seca, podendo ser capaz de coproduzir carotenoides, e assim como as outras espécies amplamente estudadas, também podem utilizar diversos substratos para tal finalidade (LIU *et al*., 2019; KAMAL *et al*., 2020; LEE *et al*., 2021).

A escolha de substratos para os processos fermentativos é importante para amenizar o processo dispendioso, que é um impasse nessa cadeia produtiva. Diversas matérias primas de baixo custo têm sido avaliadas como fontes de carbono tanto para a produção de lipídeos quanto para carotenoides microbianos. Normalmente são resíduos industriais, como hidrolisados lignocelulósicos, águas residuárias da fabricação de bebidas, resíduos de alimentos, glicerol bruto, licor de milho, melaço, entre outros compostos, com característica biodegradável, que não podem ser usados nos sistemas relevantes (BENTO *et al*., 2020; CAI *et al*., 2016; DIAS *et al*., 2020; MA *et al*., 2019; PAPADAKI *et al*., 2019; PEREIRA *et al*., 2019; SAISRIYOOT *et al*., 2019).

Atualmente não há produção comercial de lipídeos e carotenoides sintetizados por leveduras, e as espécies identificadas para este fim são restritas (LIU *et al*., 2020). Além do mais, as pesquisas nesta área utilizando resíduos industriais têm como foco a viabilidade econômica da produção de biomassa e biodiesel. Portanto, o objetivo do trabalho foi selecionar potenciais cepas de leveduras para a síntese de lipídeos e carotenoides utilizando glicerol bruto ou melaço de cana-de-açúcar como fontes de carbono. Por fim, buscou-se identificar novas cepas oleaginosas e carotenogênicas, otimizar parâmetros de cultivo para produção concomitante dos metabólitos, e ampliar o conhecimento acerca destes compostos como produtos de valores para aplicações em processos fermentativos industriais.

2 REFERENCIAL TEÓRICO

2.1 Síntese de lipídeos por microrganismos

Os lipídeos constituem um grupo diverso de compostos químicos orgânicos formados basicamente por moléculas de triacilglicerol (TAG) e ácidos graxos. Em menores quantidades, participam também da constituição dos óleos, mono e diacilgliceróis, esteróis, fosfolipídeos, álcoois graxos e vitaminas lipossolúveis, entre outras substâncias (CHAVES; BARRERA-ARELLANO; RIBEIRO, 2018).

Algumas espécies de algas, fungos filamentosos, leveduras e bactérias são capazes de sintetizar lipídeos a partir da conversão metabólica de substâncias como açúcares, dióxido de carbono e ácidos orgânicos (SITEPU *et al.*, 2014; DÍAZ-FERNÁNDEZ *et al*., 2019). Estes microrganismos são denominados espécies oleaginosas, e geralmente a estimulação metabólica para síntese lipídica ocorre quando estão sob condições de estresse como escassez de nutrientes, por exemplo (PARSONS; CHUCK; MCMANUS, 2017).

Os óleos microbianos também denominados óleos *single cell* (SCOs), têm sido considerados bons substitutos dos óleos vegetais devido às semelhanças de composição de ácidos graxos, aliado a redução dos danos ambientais decorrente da busca por processos sustentáveis (CHEIRSILP; LOUHASAKUL, 2013; PARSONS; CHUCK; MCMANUS, 2017). Explorando os sistemas microbianos é possível produzir óleo o ano todo obtendo uma taxa de produção de até cem vezes superior à óleos vegetais em litros por hectare por ano (BHARATHIRAJA *et al*., 2017). Além disso, podem ser utilizadas terras impróprias para a agricultura e aproveitar resíduos industriais como substratos para o crescimento microbiano. São consideradas também outras vantagens como taxa de crescimento rápida e facilidade de expansão (MAHAJAN; SENGUPTA; SEN, 2019).

O rendimento e o tipo de lipídeo sintetizado pelos microrganismos dependem principalmente da espécie, condições de cultura, e do substrato utilizado (BHARATHIRAJA *et al*., 2017). Condições de cultivo controladas e otimizadas podem propiciar uma taxa de acumulação de lipídeos de até 85% (p/p) da biomassa microbiana (AROUS; JAOUANI; MECHICHI, 2019). Os parâmetros que devem ser considerados são fontes de carbono e nitrogênio, pH, temperatura, teor de aeração, salinidade e tempo de incubação (BHARATHIRAJA *et al*., 2017; VERMA *et al*., 2020).

O acúmulo de lipídeos em microrganismos oleaginosos ocorre quando há excesso de fontes de carbono como açúcares ou compostos semelhantes metabolizados, associado à

limitação de nitrogênio, principalmente, ou outros elementos como sulfato, biotina, ferro e enxofre que são cofatores celulares (AROUS; JAOUANI; MECHICHI, 2019). O excesso de fonte de carbono é assimilado pelas células e convertido em triacilgliceróis. No entanto, a condição mais eficaz para induzir a lipogênese microbiana é a limitação de nitrogênio, que ao se esgotar, cessa a divisão celular e o lipídio formado é armazenado intracelularmente (RATLEDGE, 2004; ROSSI *et al*., 2011; BEOPOULOS, 2012).

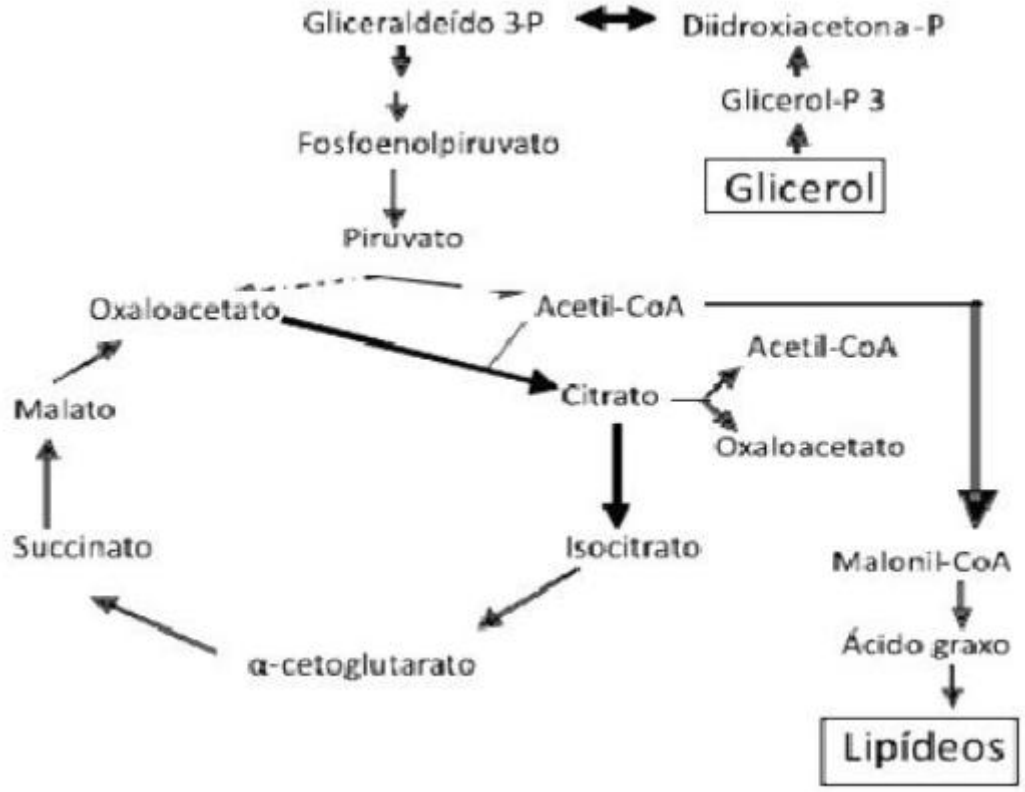
São descritas duas vias de acumulação lipídica: (1) síntese *de novo* que envolve a produção de acetil-CoA e malonil-CoA, precursores de ácidos graxos, e sua integração à via de armazenamento de lipídeos (FIGURA 1), e (2) via *ex novo*, que consiste na absorção de ácidos graxos, óleos e triacilgliceróis do meio de cultura e sua acumulação intracelular de forma inalterada (BEOPOULOS *et al.*, 2009; BEOPOULOS, 2012, MOTA; MÚGICA; SÁ-CORREIA, 2023). A biossíntese de lipídeos por meio da via *de novo* é atribuída à atividade das enzimas acetil-CoA carboxilase (ACC), acil graxo sintase (FAS) e diacilglicerol aciltransferase (DGAT). Em decorrência à escassez de nitrogênio, ocorre um aumento da atividade de ATP-citrato liase (ACL), levando a um fornecimento contínuo de acetil- CoA (precursor da síntese de ácidos graxos) que induz o acúmulo de lipídeos. Outra hipótese estudada para algumas espécies de leveduras, também relacionada à limitação de nitrogênio é que por possuírem uma atividade metabólica reduzida de isocitrato liase (ICL) e alto nível de aconitase, estas produzem citrato em excesso ativando a via de armazenamento de ácidos graxos (CHATTOPADHYAY, MITRA, MAITI, 2021).

Os fatores metabólicos que desencadeiam a lipogênese microbiana após o esgotamento de nitrogênio são: (1) acúmulo de ATP (Adenosina Trifosfato) e o esgotamento de AMP (Adenosina Monofosfato); (2) inativação mitocondrial de NAD+: isocitrato desidrogenase; (3) o transporte de citrato a partir da mitocôndria para o citosol; (4) expressão citosólica de ATP: citrato liase; (5) inibição do retorno de ATP: atividade liase citrato e citrato de efluxo por ésteres de ácidos graxos de acil-CoA de cadeia longa (SITEPU et al., 2014). Dos fatores citados acima, a produção de acetil-CoA é definitiva para a síntese de lipídeos, pois são constituintes dos ácidos graxos, e estes, por condensação de um esqueleto glicerol-3- fosfato formam os lipídeos (LIU et al., 2013). Quando há limitação de nitrogênio, a AMP desaminase é ativada para suprir a célula de NH_4^+, e diminui o conteúdo celular e mitocondrial de AMP. Este processo provoca bloqueio na metabolização de isocitrato, pois a enzima isocitrato desidrogenase é dependente de AMP. Ocorre um acúmulo de citrato na mitocôndria, que posteriormente é transportado para o citosol via malato/citrato e convertido em acetil - CoA e oxaloacetato. O último é convertido a malato e utilizado no sistema de efluxo de citrato (RATLEDGE, 2004). Finalmente, o acetil-

CoA e malonil-CoA produzidos são adicionados na cadeia de ácidos graxos complexos (FAS), e estes são direcionados para a via de armazenamento de lipídeos (BEOPOULOS, 2012).

Os lipídeos microbianos, uma vez acumulados na célula microbiana podem ser obtidos por meio de solventes ou técnicas de extração mecânicas ou enzimáticas. Após a extração existe uma vasta área de aplicação para os óleos microbianos na produção de biodiesel, indústrias farmacêuticas, cosméticas, aditivos alimentares e produção de biopolímeros (BHARATHIRAJA *et al.*, 2017).

Figura 1 - Via simplificada de biossíntese de ácidos graxos em leveduras.



Fonte: Wang et al., 2001.

2.2 Microrganismos oleaginosos

Muitos microrganismos como microalgas, leveduras, bactérias e fungos filamentosos possuem a capacidade de acumular óleos sob condições de cultivo especiais, porém poucas espécies produzem de forma significativa. Somente as espécies capazes de acumular mais de 20% de lipídeos de sua biomassa são classificadas como oleaginosas (BEOPOULOS *et al.*, 2009).

Microalgas, fungos filamentosos e leveduras sintetizam triacilgliceróis como principais componentes lipídicos. As bactérias têm a capacidade de sintetizar lipídeos complexos específicos, porém o rendimento é muito baixo. O conteúdo lipídico encontra-se aderido à membrana bacteriana dificultando o processo de extração, havendo portanto, pouco investimento pelos pesquisadores (AROUS; JAOUANI; MECHICHI, 2019). A classe de lipídeos mais abundantes em bactérias são os poli-hidroxialcanoicos, que tem função de reserva de carbono intracelular e fonte de energia (LIANG; JIANG, 2013). A biossíntese e acumulação de triacilgliceróis foram identificadas em bactérias heterotróficas anaeróbicas e cianobactérias do grupo *Actinomycetes*, gêneros *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia* ou *Gordionia* (SHIELDS-MENARD *et al*., 2018).

Algumas espécies de fungos filamentosos foram identificadas como produtoras e acumuladoras de lipídeos principalmente para ácidos graxos poliinsaturados de cadeia longa (PUFAs), com alto valor de biomassa. As espécies mais descritas são *Aspergillus terreus, Claviceps purpurea, Tolyposporium, Mortierella alpina, Mortierella isabelina* e *Mucor circinelloides* (ECONOMOU *et al*., 2011; ZHENG *et al*., 2012; AROUS; JAOUANI; MECHICHI, 2019; YAO *et al*., 2019).

As microalgas são os microrganismos mais utilizados para produção lipídica, pois apresentam muitas vantagens em relação aos outros. As culturas fotoautotróficas podem crescer em águas residuais com baixas concentrações de nutrientes, pois utilizam dióxido de carbono como fonte de carbono e luz solar como fonte energética. Microalgas heterotróficas requerem substratos orgânicos como fonte de carbono, e as mixotróficas combinam a fotossíntese e assimilação heterotrófica de compostos orgânicos (AROUS; JAOUANI; MECHICHI, 2019).

Outro fator relevante relacionado a este grupo é o crescimento extremamente rápido, podendo dobrar a biomassa a cada 24 horas. Seu conteúdo lipídico médio é 70%, podendo chegar a 90% do peso seco em condições específicas. As microalgas *Chlorophyta* e *Bacillariophyceae* são as que mais se destacam por apresentarem maior teor de acumulação de óleo e facilidade de cultivo. A *Chlorella*, particularmente, é a mais atrativa devido a exploração industrial em larga escala para a produção de biodiesel (AROUS; JAOUANI; MECHICHI, 2019; LIANG; JIANG, 2013). As desvantagens do cultivo de microalgas são a necessidade de uma área grande e um período de fermentação maior do que os outros microrganismos, além dos fatores físico-químicos como intensidade de luz e temperatura, inerentes ao cultivo, interferirem rigorosamente limitando a produção lipídica (BAO *et al*., 2021).

As leveduras apresentam vantagens relacionadas à síntese de lipídeos devido a facilidade de cultivo, capacidade de consumir matérias primas diversificadas de baixo custo,

expansão da produção de biomassa em biorreatores, altas taxas de acumulação lipídica e robustez nos processos otimizados (KARAMEROU; WEBB, 2019). Os gêneros de leveduras oleaginosas mais conhecidos são *Lipomyces*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhizopus*, *Trichosporon* e *Yarrowia*. Essas leveduras acumulam, em média, uma taxa de lipídeos de 40% de sua biomassa. Entretanto, sob condições adequadas de escassez de determinados nutrientes, como a fonte de nitrogênio, esse nível pode ser elevado a até 70% (BEOPOULOS et al., 2009). As leveduras *Yarrowia sp*., *Cryptococcus sp*., *Lipomyces sp*., *Rhodotorula sp*. e *Rhodosporidium sp*. possuem capacidade de consumo de diversos substratos orgânicos para a produção estável de lipídeos em um curto tempo (CHO e PARK, 2018).

A organela especializada em armazenamento de lipídeos na maioria das espécies de leveduras é o corpúsculo lipídico ou partícula lipídica. Este compartimento consiste em um núcleo lipídico revestido por uma camada fosfolipídica com proteínas embebidas com diversas funções. Algumas espécies acumulam lipídeos na parede celular (RADULOVIC; KNITTELFELDER, 2013).

Os lipídeos sintetizados por leveduras em maiores quantidades são diacil e triacilgliceróis (DAG e TAG), ácidos graxos livres, carotenoides, esteróis, poliprenois, fosfolipideos, esfingolipideos e glicolipideos, podendo variar de acordo com a espécie. Os lipídeos neutros acumulam, em sua maioria, na forma de triacilglicerois (80-90%), e em menor quantidade como ácidos graxos livres, considerando a glicose como substrato (KHOT *et al*., 2020).

Souza, Schwan e Dias (2014), relataram que *Yarrowia lipolytica,* em concentrações de 30g/L de glicerol bruto, levou à obtenção de 63,4% de lipídeos, com predomínio de ácidos esteárico e palmítico no perfil de ácidos graxos. As espécies *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Lipomyces starkeyi*, *Trichosporon oleaginosus*, *Candida tropicalis* entre outras, são alvos de pesquisas em engenharia genética por possuírem vias metabólicas únicas que diferem de leveduras não oleaginosas (CHATTOPADHYAY, MITRA, MAITI, 2021). Buscando assim, estratégias para aprimorar a utilização e relevância destes microrganismos no contexto industrial. De acordo com Chatzifragkou (2011); Madani, Enshaeieh e Abdoli (2017); Masri et al. (2018), as leveduras sintetizam principalmente o ácido palmítico (C16: 0), esteárico (C18: 0), oleico (C18: 1), linoleico (C18: 2), linolênico (C18: 3) e pequenas quantidades de ácidos graxos de cadeia longa quando submetidas a fontes de glicerol em quantidades que variam de 30 a 90 g/L.

Apesar das diversas vantagens na síntese de lipídeos por leveduras, um grande impasse desse processo é a forma de recuperação, pois são utilizados solventes químicos caros e que

necessitam de formas específicas para descarte após o uso. São descritas diversas metodologias para a extração dos lipídeos de leveduras, no entanto a relação custo-benefício ainda não é favorável para a aplicação em escala industrial (KHOT *et al*., 2020).

2.3 Fatores que influenciam no acúmulo de lipídeos por leveduras

A seleção de cepas potenciais que constituam uma robusta fábrica de metabólitos é o fator primordial para o estudo de produção e acumulação lipídica por microrganismos. Juntamente a essa seleção o que se almeja é obter o meio de cultivo melhor e mais barato para que se mantenha um processo de produção eficaz. Para desenvolver um sistema confiável deve-se monitorar o acúmulo de ácidos graxos avaliando os fatores que interferem no processo fermentativo como fonte de carbono, fonte de nitrogênio, relação carbono/nitrogênio (C/N), temperatura, pH e aeração (AMI *et al*., 2014; GRIFFITHS; HARRISON, 2009).

A acumulação de lipídeos em leveduras acontece após a etapa de crescimento, quando há um excesso de carbono associado a uma limitação de nutrientes, sendo a limitação de nitrogênio a mais utilizada (MENG *et al*., 2009). Essa estratégia envolve duas fases em função do metabolismo da levedura. Na primeira fase, de crescimento, o nitrogênio é rapidamente consumido durante o aumento do número de células. Quando o nitrogênio se torna limitado, começa a segunda fase, de acúmulo de lipídeos. A escassez de nitrogênio retarda o crescimento e o carbono continua ser assimilado resultando na acumulação lipídica (BEOPOULOS *et al*, 2009).

O rendimento global de conversão da fonte de carbono em lipídeos depende da duração da fase de crescimento e da transição para a fase de acumulação, que são determinadas pela razão C/N (carbono/ nitrogênio) do meio. Estudos têm demonstrado que quanto mais compostos nitrogenados presentes no meio, menos lipídeos contidos nas células. Diante disso, a medida que se aumenta a razão C/N, aumenta-se, também, o acúmulo de lipídeos em leveduras (LIU, YANG; SHI, 2000).

O uso de diferentes fontes de nitrogênio também influencia a produção de lipídeos. As fontes de nitrogênio mais comumente utilizadas são extrato de levedura, peptona e sulfato de amônio, visto que o extrato de levedura se destaca por contribuir para uma quantidade elevada de biomassa e conteúdo lipídico (KUMAR *et al*., 2010).

A maioria das leveduras oleaginosas são aeróbias obrigatórias, carecendo de oxigênio para o metabolismo energético e síntese de componentes celulares. Portanto a aeração exerce influência na produtividade lipídica obtida, em virtude da necessidade de bons níveis de

oxigênio dissolvido para resultar em maior crescimento celular (VASCONCELOS *et al*., 2019). A taxa de aeração também interfere na composição dos ácidos graxos. Em condições de aeração há aumento de ácidos graxos insaturados, e a limitação de oxigênio provoca um aumento na proporção de ácidos graxos saturados (VALERO, MILLAN; ORTEGA, 2001). Portanto, a concentração de oxigênio dissolvido no meio de cultura apresenta uma correlação positiva com o acúmulo de óleos pelos microrganismos (LIANG *et al*., 2006).

O perfil lipídico é modificado também pela temperatura e pH inicial dos cultivos. A temperatura ideal para o crescimento de cada linhagem é variável, porém geralmente em torno de 25ºC. Temperaturas baixas reduzem o metabolismo microbiano, pode diminuir o grau de saturação dos ácidos graxos e resultar em menor taxa de acúmulo de lipídeos, devido à baixa atividade celular (BEOPOULOS *et al*., 2009). O pH inicial do cultivo altera a atividade enzimática e o metabolismo microbiano. Para leveduras oleaginosas, o estudo do pH inicial faz-se necessário visando modificações no produto final. Estudos apontam que geralmente, o pH de cultivos de leveduras oleaginosas deve ser controlado entre 5,0 e 6,0, o que leva a uma maior produção lipídica, sendo os pH ácidos desfavoráveis para o crescimento e produção (KARATAY; DÖNMEZ, 2010).

Segundo Beopoulos *et al*. (2008), o acúmulo de lipídeos na célula começa aumentar na fase de crescimento (fase log), pois esses lipídeos são utilizados na síntese da membrana da célula para suportar seu crescimento e, quando a célula alcança seu tamanho ideal, passa a acumular lipídeos em gotículas no interior da célula. O conteúdo lipídico atinge seu máximo na fase estacionária. Assim que a quantidade de nutrientes no meio começa a diminuir, os lipídeos acumulados são rapidamente degradados em ácidos graxos livres. A retirada das células no início da fase estacionária impede a degradação dos lipídeos. Para alcançar produção elevada de lipídeos, a otimização dos parâmetros de cultivo acima são importantes.

2.4 Ácidos graxos

Ácidos graxos (AGs) são ácidos carboxílicos derivados de hidrocarbonetos que possuem o estado de oxidação baixo. Suas cadeias variam de quatro à trinta e seis carbonos (C4 a C36), as quais podem ser classificadas como saturados, que não contêm ligações duplas; monoinsaturados, que contêm uma única ligação dupla; e poli-insaturados, que contêm múltiplas ligações duplas. Alguns poucos contêm anéis de três carbonos, grupos hidroxila ou ramificações de grupos metila (NELSON; COX, 2016).

Os ácidos graxos poli-insaturados (PUFAs), ou essenciais, são importantes para o funcionamento do organismo dos seres humanos, porém, não podem ser sintetizados endogenamente por eles. Por isso, sua aplicação farmacêutica e alimentícia tem atraído considerável interesse comercial e atenção dos pesquisadores (GIULIANO *et al*., 2018).

Os PUFAs podem ser definidos como ácidos graxos de 18 ou mais carbonos, contendo duas ou mais ligações duplas (BEAUDOIN *et al*., 2000) e são classificados como ω 3 e ω 6, com base na localização da última ligação dupla em relação ao grupo metil terminal da molécula (SCHMITZ; ECKER, 2008). As famílias ω-3 e ω-6 abrangem ácidos graxos que apresentam insaturações separadas apenas por um carbono metilênico, com a primeira insaturação no sexto e terceiro carbono, respectivamente, enumerado a partir do grupo metil terminal (MARTIN *et al*., 2006). Entre os principais membros das famílias ω-3 e ω-6, se destacam o ácido linoleico e o ácido linolênico, respectivamente.

Segundo Ratledge (2013) os principais ácidos graxos sintetizados por microrganismos como nutracêuticos, são os PUFAs ácido alfa- linolênico (ALA), gama linolênico (GLA, 18:3 n-6), docohexanoico (DHA, 22:6 n-3), araquidônico (ARA, 20:4 n-6) e eicosapentaenoico (EPA, 20:5 n-3). No começo dos 90 os ácidos graxos poliinsaturados (PUFAs) chamavam atenção devido seu conteúdo nutricional importante para o desenvolvimento de bebês recém-nascidos. Foi constatado que os ácidos araquidônico (ARA;20: 4, n-6) e ácido docosahexaenóico (DHA; 22: 6, n-3) estão presentes no leite materno e formam a maioria dos ácidos graxos presentes no tecido cerebral contribuindo para o desenvolvimento neural, função ocular e memória em adultos também (BOSWELL *et. al*., 1996; SINCLAIR; JAYASOORIYA, 2010). PUFAs de cadeia muito longa produzidos por microrganismos são muito visados atualmente porque não ocorrem em óleos vegetais e constituem importantes nutrientes dietéticos para bebês neonatais e para suas mães (RATLEDGE, 2004).

Os PUFAs incluem também os ácidos graxos conjugados que são assim denominados por possuírem ligações duplas conjugadas em diferentes posições e geometrias (GONG *et al*., 2019). Os principais representantes dos ácidos graxos conjugados são o ácido linolênico e o linoleico, que desempenham efeitos fisiológicos benéficos em animais como como controle do peso corporal, atividade anticarcinogênica, regulação do metabolismo lipídico, atividades antioxidantes e anti-inflamatórias (KIM *et al*., 2013; MALINSKA *et al*., 2015).

Os triacilglicerois ou gorduras neutras, são constituídos de três ácidos graxos simples. Aqueles que contêm o mesmo tipo de ácido graxo em todas as três posições são chamados de triacilgliceróis simples, e sua nomenclatura é derivada do ácido graxo que contêm. Os triacilgliceróis simples de 16:0, 18:0 e 18:1, por exemplo, são tripalmitina, triestearina e

trioleína, respectivamente. Para dar nome a esses compostos sem gerar ambiguidade, o nome e a posição de cada ácido graxo devem ser especificados (NELSON; COX, 2011).

Em estudo realizado por Sitepu e colaboradores (2013) sobre o perfil de ácido graxos produzidos por leveduras, os mais expressivos foram o oleico, palmítico, esteárico e linoleico.

2.5 Aplicações de lipídeos microbianos

Os óleos microbianos são explorados para a produção de biodiesel, aplicação nas indústrias farmacêuticas, de cosméticos e de alimentos, como aditivos alimentares, além da utilização para a produção de biopolímeros, biossurfactantes, solventes, tintas, lubrificantes e ceras (AYADI *et al*., 2016; VINARTA *et al*., 2016; BHARATHIRAJA *et al.*, 2017). Características como alto teor de ácidos graxos livres e conteúdo nutritivo, aproveitamento de resíduos industriais e matérias primas de baixo custo como substrato, vantagens em relação à facilidade de cultivo microbiana permitem essa aplicação diversificada de lipídeos microbianos com prospecção de importância remuneratória industrialmente num futuro próximo (VASCONCELOS *et al*., 2019).

O segmento biotecnológico que atrai mais pesquisas e investimentos para a utilização de lipídeos microbianos é a produção de biocombustíveis, devido ao caráter sustentável (SHIELDS-MENARD *et al*., 2018). A alta demanda nos últimos anos por biodiesel de primeira geração, produzidos a partir de óleos vegetais provocou um aumento no valor de produtos alimentícios, que levou à necessidade de buscar fontes alternativas de óleos. Os lipídeos microbianos foram descobertos como promissores fontes de óleos para a produção de biodiesel de segunda geração (PAPANIKOLAOU; AGGELIS, 2011).

Os microrganismos podem consumir biomassa subutilizada e produzir combustível alternativo a partir da transesterificação de triglicerídeos em ésteres metílicos e etílicos de ácidos graxos (FAMEs e FAEEs), respectivamente. Esse biocombustível pode substituir o combustível fóssil utilizados em motores a diesel (BHARATHIRAJA *et al.*, 2017; SHIELDS-MENARD *et al*., 2018; MA *et al*., 2018; SADASIVAN *et al*., 2020). Os óleos microbianos podem ser altamente nutritivos e substituir óleos vegetais e gorduras, como manteiga de cacau, óleo de palma e alguns ácidos graxos específicos, atraindo o interesse de indústrias de alimentos. Podem ser utilizados também como aditivos em alimentos com finalidade de preservação de organismos deteriorantes, patogênicos ou para eliminar componentes indesejáveis (BHARATHIRAJA *et al*., 2017).

A indústria farmacêutica e de cosméticos também podem utilizar os óleos microbianos como aditivos e suplementos. Eles são usados para a preparação de linimentos, demulcentes, emolientes, laxantes, produção de cosméticos para cuidados com a pele e formulações para crescimento capilar, e suplementos para terapia nutricional em formulações farmacêuticas (BHARATHIRAJA *et al*., 2017). Alguns alimentos são enriquecidos com ácidos graxos poliinsaturados (PUFAs) e trazem benefícios à saúde como redução da pressão arterial, reduz risco de câncer de cólon, prevenção de doenças cardiovasculares e epidérmicas, e atenuação manifestações inflamatórias no organismo (ZHAO *et.al*., 2014).

2.6 Síntese de carotenoides por microrganismos

Os carotenoides são pigmentos naturais vermelho, amarelo e laranja capazes absorver a luz entre 400 e 500 nm; possuem caráter lipofílico e podem ser biossintetizados em diversidade por plantas, algas, bactérias, leveduras e fungos filamentosos (RODRIGUEZ-CONCEPCION *et al*., 2018). Esses compostos consistem em oito unidades de isoprenos tetraterpenoides altamente insaturados. A molécula contém uma espinha dorsal de grandes cadeias de polieno C35- C40, que consiste na característica fundamental para o desempenho da função antioxidante dos carotenoides, reagindo no sequestro de radicais livres e inibidores de moléculas oxigênio singlete (SHARMA; GHOSHAL, 2020). Os carotenoides são classificados em dois grupos de acordo com a presença ou ausência de oxigênio em sua estrutura: os carotenos (β-caroteno, α- caroteno, neurosporeno, licopeno) não possuem oxigênio, são chamados de hidrocarbonetos puro, e o grupo xantofila (astaxantina, zeaxantina, luteína, criptoxantina) que apresenta oxigênio em sua molécula (REHMAN *et al*., 2020).

Atualmente já foram identificados cerca de mil compostos carotenoides que se diferenciam por modificações estruturais do tipo ciclização, adição, eliminação, rearranjo substituição e oxidação (SIKORSKI, 2007; YABUZAKI, 2017). Carotenoides naturais utilizados na suplementação nutricional humana e animal eram extraídos quase em sua totalidade de plantas. Nos últimos 25 anos outras fontes naturais como as algas, fungos, bactérias e leveduras também são exploradas comercialmente para a produção de carotenoides do tipo β-caroteno e astaxantina, principalmente. A partir desses mais estudos vem sendo realizados acerca de outros carotenoides microbianos como licopeno, luteína, zeaxantina e cantaxantina (ARUNKUMAR; GORUSUPUDI; BERNSTEIN, 2020).

A produção de carotenoides microbianos é mais vantajosa que a produção a partir de plantas pois requer menor área, é independente de sazonalidade, mudanças climáticas e composição do solo (VALDUGA *et al*., 2009). Parâmetros como composição do meio de cultura, temperatura, pH, aeração e luminosidade interferem na produção destes compostos resultando em diferentes classes e quantidades relativas (MASSOUD; KHOSRAVI-DARANI, 2017). O estresse de condições ambientais referentes ao meio de cultivo acarreta, como resposta, o acúmulo de vários tipos de carotenoides pelos microrganismos. Isso acontece porque ocorre alteração no nível de atividade enzimática envolvidas no processo (VALDUGA *et al*., 2009).

A biossíntese dos compostos carotenoides (FIGURA 2) apresenta algumas reações principais que são padrões e posteriormente, a partir de modificações se diferenciam entre as classes carotenos e xantofilas. O ácido mevalônico é o primeiro precursor da síntese de terpenoides, que após sofrer uma série de reações forma geranil difosfato (10 C), farnesil difosfato (15 C) e geranil-geranil difosfato (20 C). O geranil- geranil difosfato sofre dimerização e forma o fitoeno, o primeiro composto de quarenta carbonos ainda sem coloração. O fitoeno forma fitoflueno, caroteno, neurosporeno e, finalmente licopeno a partir de reações de desaturação. O neurosporeno ou licopeno podem ser ciclizados gerando β-caroteno e α-caroteno. Nessas moléculas ciclizadas pode ocorrer substituição com introdução de hidroxilas e é gerado as xantofilas (VALDUGA *et al*., 2009).

Figura 2 - Via de biossíntese de produção de carotenoides.

Fonte: Frengova; Emilina; Beshkova (2003)

2.7 Microrganismos produtores de carotenoides

As microalgas exibem um complexo metabolismo carotenoide envolvido em suas funções fotossintéticas. Dependendo do meio e condições as quais estão expostas podem acumular diferentes tipos de pigmentos carotenoides (RODRIGUEZ-CONCEPCION *et al*., 2018). Em algumas algas, a biossíntese de carotenoides está relacionada à resposta ao estresse e acoplada à produção de lipídeos, ocorrendo a acumulação em vesículas lipídicas situadas nos cloroplastos ou citoplasma. As espécies mais conhecidas como acumuladoras de carotenoides são *Dunaliella salina* para β-caroteno (14% do peso seco), *Haematococcus pluvialis* para astaxantina (6% do peso seco) e luteína por *Muriellopsis sp*. (5,5%) e *Scenedesmus almeriensis* (5,3%) (BOROWITZKA, 1999; RODRIGUEZ-CONCEPCION *et al.*, 2018).

Algumas bactérias sintetizam carotenoides com 30, 45 e 50 átomos de carbonos, consideradas uma exceção, pois a maioria dos carotenoides sintetizados por microrganismos são tetraterpenos (C40) (VALDUGA *et al*., 2009). Foi descrita a biossíntese de carotenoides em algumas espécies de arquéias como *Halobacterium salinarum*, *Halovrubrum chaoviator*, *Halococcus morrhuae*, *Natromonas pharaonis* e *Haloarcula japonica*, porém é bem menos frequente. O principal produto carotenoide sintetizado por esse grupo é a bacterioruberina C50 (MANDELLI; MIRANDA, 2012).

Para as bactérias, condições de anaerobiose favorecem a formação de compostos carotenoides menos oxigenados, em contrapartida a oxigenação promove a produção de cetocarotenoides. Em bactérias fotossintéticas a carotenogênese é afetada pela exposição à luz e oxigênio, desta forma, os grupos *Myxococcus*, *Streptomyces*, *Mycobacterium*, *Agromyces* e *Sulfolobus* produzem carotenoides somente quando iluminados (TAKANO *et. al*., 2005; TAKAICHI, 2009). As bactérias púrpuras *Rhodospirillum rubrum*, *Rhodobacter capsulatus* e *Rhodobacter sphaeroides* produzem espiriloxantina e esferoideno. As bactérias púrpuras e verdes sulfurosas, *Chlorobium tepidum* e *Clorobium phaeobacteroides*, produzem isorenierateno e clorobacteno. As espécies *Heliobacillus mobilis*, *Heliophilum fasciatum* e *Heliobacterium chlorum* produzem diapolicopeno (RODRIGUEZ-CONCEPCION *et al*., 2018).

A síntese de carotenoides por bactérias não fotossintetizantes, bem como nos outros microrganismos eucarióticos, está interligada às condições de cultivo podendo haver diferenças nas vias metabólicas ativadas. Alguns exemplos desses microrganismos e os respectivos carotenoides sintetizados são: *Erwinia* (β-criptoxantina, zeaxantina e xantofilas); *Myxococcus fulvus* (4-cetotoruleno, mixobactina e mixobactona); *Staphylococcus aureus*, *Streptococcus*

faecium, Heliobacillus fasciatum e *Heliobacterium* (carotenoides C30) (RODRIGUEZ-CONCEPCION *et al*., 2018).

A biossíntese de carotenoides em fungos é metabolicamente mais simples que dos organismos fotossintéticos por haver menor quantidade de genes e enzimas envolvidas no metabolismo. As espécies *Phycomyces blakesleeanus, Mucor circinelloides* e *Blakeslea trispora*, pertencentes ao grupo mucormicotina, se destacam pela produção de β-caroteno, assim como *Cercospora nicotianae* e *Ustilago maydis* dos grupos ascomicotina e basidiomicotina, respectivamente (DAUB, PAYNE, 1989; RODRIGUEZ-CONCEPCION *et al*., 2018). A produção de xantofilas como neurosporaxantina se destaca nas espécies *Neurospora crassa* e *Fusarium fujikuroi* (AVALOS *et al*., 2017). Outras espécies fúngicas muito pesquisadas para produção de carotenoides são as leveduras.

As leveduras possuem vantagens para produção de compostos carotenoides comparada ao outros microrganismos devido às facilidades e menor custo para seu cultivo, além de serem espécies GRAS, reconhecidas como seguras para a alimentação (PEREIRA *et al*., 2019). Uma dificuldade de ampliar a produção de carotenoides por microrganismos a nível industrial são os custos. Sendo assim, as leveduras se destacam pela eficiência em metabolizar diferentes substratos e atraem mais pesquisas na busca por novas fontes de resíduos que possam ser utilizados para esse fim. Além dos diversos parâmetros já descritos que interferem na produção de carotenoides, foi demonstrado também que algumas leveduras biossintetizam carotenoides como mecanismo de defesa contra os danos causados pela luz (VALDUGA *et al*., 2009). A quantidade e intensidade de luz tolerada pelo gênero *Rhodotorula* varia conforme a espécie.

Os gêneros *Sporobolomyces* e *Rhodotorula* são bons produtores de compostos carotenoides (CHENG; YANG, 2016). Espécies bastante estudadas como *Xenophyllomyces dendrorhous, Rhodotorula glutinis, Rhodotorula mucilaginosa, Rhodotorula graminis, Rhodosporidium sp., Sporobolomyces roseus* e *Sporidiobolus salmonicolor*, sintetizam principalmente os carotenoides torularrodina, β-caroteno, toruleno e astanxantina (MASSOUD; KHOSRAVI-DARANI, 2017; VALDUGA et al., 2009). As espécies *Rhodotorula mucilaginosa, Sporidiobolus pararoseus* e *Pichia fermentans*, cultivadas em diferentes fontes de carbono (melaço de cana, glicerol bruto e licor de maceração de milho) apresentaram uma produção de β-caroteno acima de 63% (PEREIRA *et al*., 2019). Em estudo realizado por Moreira e colaboradores (2018) foi reportado a produção de carotenoides totais pela levedura *Rhodotorula mucilaginosa* utilizando resíduos do processamento de café, sendo identificadas também atividades antioxidante, antimicrobiana e antifúngica.

2.8 Aplicações dos carotenoides microbianos

Os carotenoides são sintetizados principalmente por organismos fotossintéticos com função de fotoproteção e pigmentação. Eles são responsáveis pelas cores amarela e vermelha de vegetais como milho, cenoura, abóbora, tomate, melancia, entre outros (RODRIGUEZ-CONCEPCION *et al*., 2018).

A maioria dos animais, bem como os humanos, não sintetizam carotenoides, portanto devem obtê-lo a partir da alimentação, pois sabe-se que eles desempenham funções fundamentais em nosso organismo. Os compostos carotenoides possuem ampla utilização nas indústrias alimentícia, farmacêutica e de ração animal. Eles são compostos versáteis, com propriedades de pró-vitamina A, antioxidantes, são pigmentos naturais e estão envolvidos em processos metabólicos para a promoção da saúde proporcionando atividades antitumoral, anticardíaca, anti-envelhecimento e anti-inflamatória (MAPELLI-BRAHM *et al*., 2020; REHMAN *et al*., 2020).

Na nutrição humana os carotenoides precursores da vitamina A, são convertidos em retinol, importantes para a visão e regulação de genes. Eles acumulam na retina e atuam como filtros contra o estresse oxidativo pela luz (GRUNE *et al*., 2010; ARUNKUMAR; GORUSUPUDI; BERNSTEIN, 2020). Carotenoides podem ser consumidos como suplementos alimentares para complementar ou aumentar a ingestão de carotenoides dietéticos, ou para obtenção dos que não podem ser adquiridos a partir da dieta alimentar, como a astaxantina (MAPELLI-BRAHM *et al*., 2020).

As diversas aplicações médicas e nutricionais atribuídas aos carotenoides deve-se principalmente à sua atividade antioxidante, pois eles podem reduzir danos causados por radicais livres gerados na fase inicial de doenças. Alta ingestão de carotenoides também é importante para manutenção do sistema imunológico, ajudando na prevenção do desenvolvimento de doenças crônica degenerativas relacionadas à idade e outras como diabetes tipo 2, obesidade, certos tipos de câncer, doenças cardiovasculares, arteriosclerose, catarata, esclerose múltipla (FERNÁNDEZ-GARCÍA *et al*., 2012; BONET *et al*., 2015; MILANI *et al*., 2017; ARUNKUMAR; GORUSUPUDI; BERNSTEIN, 2020).

Processos fermentativos para a produção de carotenoides têm sido avaliados com o intuito de agregar novas características, aprimorar a qualidade e garantir maior preservação destes compostos (MAPELLI-BRAHM *et al*., 2020). A aplicação prática dos carotenoides tem relevância indiscutível para os setores de alimentos funcionais, como nutracêuticos, no contexto

de nutri cosméticos e indústria farmacêutica; e importância no ponto de vista comercial para o emprego na aquicultura e pecuária (MELÉNDEZ-MARTÍNEZ *et al*., 2018; RODRIGUEZ-CONCEPCION *et al*., 2018).

2.9 Fontes de carbono para síntese de lipídeos e carotenoides

A síntese de lipídeos e carotenoides por microrganismos é uma alternativa promissora devido às diversas vantagens em relação ao seu cultivo, por não competir com as áreas utilizadas para o plantio de vegetais fontes de alimentos e pelo caráter sustentável atribuído a renovabilidade e biodegradabilidade dos subprodutos gerados (MENG *et al*., 2009). Entretanto, existe um obstáculo nesta cadeia produtiva referente ao custo de cultivo que pode ser superado utilizando resíduos industriais de baixo custo como substratos para o crescimento microbiano.

A escolha de substratos para processos de fermentação microbiana é arbitrária, geralmente dependente da disponibilidade de recursos da região, objetivando diminuir custos e garantir eficiência, alta produtividade e desperdício mínimo. Muitos pesquisadores têm se dedicado a investigar diversas matérias primas de baixo custo como fontes de carbono para a produção de lipídeos e carotenoides microbianos. Essas matérias primas normalmente são resíduos industriais, compostos que não podem ser usados nos sistemas relevantes, com característica biodegradável (FREITAS *et al*., 2014).

Alguns resíduos necessitam de pré- tratamentos para tornarem passíveis de metabolização pelos microrganismos, como o emprego da hidrólise de biomassa lignocelulósica, lenhocelulósica, resíduos de alimentos ou águas residuais (KARAMEROU; WEBB, 2019). Os polímeros, como celulose, lignina, hemicelulose, látex , resultantes do processamento de madeira, algodão, papel e combustível, látex e outros processos industriais, apesar de serem mais disponíveis, ainda necessitam de etapas de pré – tratamento, aumentando o custo de produção (SPALVINS *et al*., 2018).

A biomassa lignocelulósica é amplamente utilizada por ser a matéria orgânica mais abundante e renovável mundialmente (CLAASSEN, 1999). Ela é composta por celulose, hemicelulose e lignina, dos quais 70% do material seco correspondem a celulose e hemicelulose, com alto teor de açúcares que tornam esse resíduo adequado para a utilização como substrato microbiano (PALMQVIST; HAHN-HAGERDAL, 2000; PANDEY; KIM, 2011). Os resíduos lignocelulósicos tem a vantagem de não competir com a economia alimentar, correspondendo a madeira, grama, resíduos da silvicultura e resíduos agrícolas (PALMQVIST; HAHN-HAGERDAL, 2000). Como exemplo de biomassa lignocelulósica são

utilizados bagaço e casca de cana-de-açúcar, palhas de trigo, arroz e milho (KARAMEROU; WEBB, 2019).

A hidrólise de resíduos alimentares gera produtos orgânicos de baixo peso molecular e oligoelementos isentos de substâncias tóxicas adequados para a fermentação microbiana (CHI *et al*., 2011; PLEISSNER *et al*., 2013; MA *et al*., 2018). Uma dificuldade para a utilização desse tipo de resíduo é a acumulação de ácido lático após três a cinco dias de armazenamento devido a proliferação de bactérias do ácido lático (MA *et al*., 2018). A presença do ácido lático é inadequada para o acúmulo de lipídeos, portanto uma alternativa é promover a fermentação em duas etapas, uma direcionada à proliferação celular (alto teor de ácido lático) e outra para a acumulação lipídica (baixa concentração de ácido lático) (FU *et al*., 2018; LEONG *et al*., 2018).

O glicerol bruto também tornou-se uma alternativa muito viável para a síntese de lipídeos microbianos por ser gerado a partir de um processo co-dependente da produção de biodiesel (KARAMEROU; WEBB, 2019). Cerca de 10% da produção de biodiesel é glicerol bruto, gerado a partir de reações de transesterificação. Tais reações podem ser obtidas por meio de catalisadores químicos e enzimas levando à transformação direta de óleos vegetais e gorduras animais em ácidos graxos metil ésteres e glicerol (AYOUB; ABDULLAH, 2012).

A transesterificação de triglicerídeos advindos de óleos de palma, soja e girassol ganhou visibilidade na fabricação de biodiesel de alta qualidade (ZHOU *et al*., 2008; MOOTABADI *et al*., 2010). Como a composição de ácidos graxos de óleos vegetais e microbianos são muito semelhantes pode-se pressupor que lipídeos microbianos terão a mesma característica. Há ao menos quatro setores industriais que geram glicerol bruto residual: indústria de sabão, de ácidos graxos, de biodiesel e de ésteres graxos, tornando indispensável alternativas para o reaproveitamento deste subproduto (KARAMEROU; WEBB, 2019). Atualmente ele é bastante empregado para substituir fontes de carbono caras para meio de fermentação de microrganismos, gerando produtos como ácido cítrico, ácidos orgânicos, bioetanol e bioplásticos (ANITHA *et al*., 2016; MOTA *et al*., 2017; VIVEK *et al*., 2017; IYYAPPAN *et al*., 2018; RZECHONEK *et al*., 2019).

Meios com alto teor de açúcares simples como glicose, lactose, sacarose, soro de leite, e melaço de cana são muito utilizados como fontes de carbono para meios de cultura microbianos (FREITAS *et al*., 2014). A glicose é uma das fontes de carbono mais utilizadas para fermentação industrial, porém pode representar até 60% do custo total da produção tornando-se inviável quando há alternativas de utilização de resíduos (GONG *et al*., 2019). Mosto de uvas, melaço de beterraba, extrato de farinha de soja, extrato de farinha de milho,

soro de queijo, e o melaço de cana-de-açúcar são exemplos de resíduos agroindustriais já avaliado para o cultivo de leveduras (MAROVA *et al*., 2012; BANZATTO *et al*., 2013).

A indústria biotecnológica de países de clima tropicais como Brasil e Índia investem no plantio de cana-de-açúcar devido a sua abundância e baixo custo de produção nessas regiões. No Brasil, o melaço de cana é utilizado principalmente como substrato para a produção de etanol, mas mundialmente também é matéria-prima para a geração de biogás (DETMAN *et al*., 2017), proteínas (SUMAN *et al*., 2015), enzimas (MARIM *et al*., 2016) e biomassa microbiana (JIRU *et al*., 2018).

O suco ou caldo de cana possui uma composição química relativamente simples, sendo composto principalmente de sacarose, portanto ele é processado como matéria-prima para a produção de açúcar a partir de plantas. Esse processo gera o melaço de cana como subproduto, com composição mineral semelhante ao caldo de cana, com alta concentração de sacarose não cristalizada, glicose e frutose (BENTO *et al*., 2020). Outros componentes desse subproduto são aminoácidos, ácidos inorgânicos, compostos inorgânicos, vitaminas e oligoelementos (GONG *et al*., 2019). O baixo teor de nitrogênio do melaço o torna bom substrato para leveduras oleaginosas com capacidade para metabolizar sacarose (SPALVINS *et al*., 2018).

Segundo Machado e Burket (2015), o glicerol, melaço de cana-de-açúcar e licor de maceração de milho podem se tornar alternativas potenciais para a produção de carotenoides microbianos também, pois são encontrados em abundância e contêm os nutrientes necessários ao metabolismo dos microrganismos. Em estudo realizado com leveduras utilizando glicerol com licor de maceração de milho, e melaço com licor de maceração de milho foi obtido mais de 63% de β-caroteno nos experimentos (PEREIRA *et al*., 2019).

3 CONSIDERAÇÕES FINAIS

Leveduras são microrganismos vantajosos e promissores para a utilização em processos biotecnológicos industriais. Algumas espécies são capazes de sintetizar lipídeos e carotenoides, que constituem bioprodutos com alto valor de mercado e ampla utilização em diversos setores industriais.

Existem limitações para a implementação de leveduras em escalas industriais devido ao alto custo para cultivo, extração e purificação dos compostos. No entanto, uma forma de amenizar o processo dispendioso é utilizar subprodutos e resíduos industriais como fonte de carbono para o cultivo, contribuindo para o desenvolvimento de processos sustentáveis.

Leveduras oleaginosas de coloração vermelha, rósea ou alaranjada acumulam concomitantemente lipídeos e carotenoides. Com o intuito de promover a industrialização destes compostos, são necessários esforços em diferentes aspectos da cadeia de produção, incluindo seleção e desenvolvimento de uma cepa com potencial para ser utilizada em processos de larga escala, e estabelecimento de um bioprocesso adequado capaz de maximizar a formação do produto pela levedura.

Estudos futuros são necessários para ampliar o conhecimento de espécies de leveduras oleaginosas e carotenogênicas, e estabelecer parâmetros de cultivo em condições otimizadas. A produção simultânea dos dois compostos de alto valor, em alta concentração e condições de fermentação semelhantes ajuda reduzir tempo, instalações, equipamentos e outras despesas que seriam necessárias para sua produção individual. Dessa forma, uma cepa robusta para acumulação concomitante de lipídeos e carotenoides permite a implementação do bioprocesso em larga escala com maior viabilidade econômica.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1- USE OF AGRO-INDUSTRIAL BY-PRODUCTS FOR THE PRODUCTION OF LIPIDS AND CAROTENOIDS BY YEAST

Artigo elaborado de acordo com a NBR 6022 (ABNT, 2018)

ABSTRACT

The synthesis of lipids and carotenoids by microorganisms using renewable resources has stimulated research focused on developing economic and sustainable biotechnological alternatives. However, previous studies have demonstrated a limited number of yeast species identified for this purpose. This study aimed to select yeast strains from the Collection of Agricultural Microbiology Cultures (CCMA) for the production of lipids and carotenoids using crude glycerol and sugarcane molasses as carbon sources. A pre-selection of twenty-six yeasts was performed to synthesize metabolites and submerge cultivation of oleaginous and carotenogenic strains in different culture media. Subsequently, lipid extraction was carried out, and fatty acids were determined by gas chromatography, along with carotenoid extraction and quantification of total carotenoids by spectrophotometry. Nine strains were considered oleaginous, with fatty acid profiles mainly composed of palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2). The yeast *Exophiala spinifera*, CCMA 2073, not previously described in the literature as oleaginous, exhibited a lipid content of 35% in pure glycerol. The yeast *Torulaspora maleeae* CCMA 0039 achieved the glucose medium's highest lipid accumulation (77.7%). *Rhodotorula dairenensis* (CCMA 945), *Rhodotorula mucilaginosa* (CCMA 0156), *Rhodosporidium toruloides* (CCMA 2032), and *Cystofilobasidium ferigula* (CCMA 1623) were classified as carotenogenic. The crude glycerol medium was the most efficient for lipogenesis and carotenogenesis, as it provided greater diversity in the fatty acid profile and higher concentrations of total carotenoids for all strains. The strain *C. ferigula* is promising for the co-production of lipids and carotenoids using crude glycerol as a substrate, as it exhibited a lipid accumulation of 41.88% and 2.76 µg/mL of total carotenoids in this substrate.

Keywords: Carotenoids. Crude glycerol. Fatty acids. Sugarcane molasses. Yeasts.

1 INTRODUCTION

Microbial lipids and carotenoids are high-value compounds that can be employed in various sectors, such as the pharmaceutical, food, and animal feed industries. Oils can be primarily used to produce biofuels, biopolymers, and biosurfactants. The versatility of these compounds is due to the chemical composition of the metabolites and the beneficial nutritional and biological activities they provide to the organism (MUSSAGY *et al*., 2019; SZCZEPAŃSKA *et al*., 2021; MAPELLI-BRAHM *et al*., 2020).

Industrial production of lipids predominantly occurs through plant extraction, while chemical synthesis is employed for carotenoid production. However, due to concerns about environmental damage and the increasing demand for healthy eating habits, there is a need for alternative sources of obtaining these compounds (MATA-GOMEZ *et al*., 2014). In this regard, the use of microorganisms has attracted the attention of researchers as a sustainable and natural source that does not compete with food production and holds prospects for scale-up and commercial valorization (BHARATHIRAJA *et al*., 2017; LIU *et al*., 2020).

Among microorganisms, yeasts are more advantageous and attractive for biotechnological processes as they exhibit ease of cultivation and lower costs when exploring the efficiency in metabolizing carbon sources derived from industrial waste and by-products. Additionally, they possess other positive characteristics, such as rapid and robust biomass production and feasibility for process expansion (ABELN and CHUCK, 2021; KARAMEROU; WEBB, 2019; PEREIRA *et al*., 2019).

Some yeast species are recognized for their efficiency in lipid and carotenoid accumulation. However, considering the number of yeast species and the metabolic complexity of these microorganisms, the number of species described as oleaginous and carotenogenic is limited (VASCONCELOS *et al*., 2019; VARGAS SINISTERRA *et al*., 2020). The well-known genera of oleaginous yeasts include *Lipomyces*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon*, *Yarrowia*, and *Rhodosporidium* (LAKSHMIDEVI *et al*., 2020; VASCONCELOS *et al*., 2019). Regarding carotenoid production, species such as *Xenophyllomyces dendrorhous, Rhodotorula glutinis, Rhodotorula mucilaginosa*, *Rhodotorula graminis*, *Rhodosporidium* sp., *Sporobolomyces roseus*, and *Sporidiobolus salmonicolor* are widely studied (ASHOKKUMAR *et al*., 2023; MASSOUD; KHOSRAVI-DARANI, 2017).

The studies reported on these species for the production of both metabolites prioritize the use of industrial waste or by-products as cultivation substrates, aiming to characterize sustainable and cost-effective processes (AYADI *et al*., 2018; VASCONCELOS *et al*., 2019).

Reusing these compounds as carbon sources for fermentative processes depends on their availability and abundance in each country or region and the need to direct these wastes for utilization, considering environmentally friendly measures.

Sugarcane molasses and crude glycerol are abundant by-products in Brazil, with potential for application in microbial cultivation due to their chemical composition, high carbohydrate content, and presence of inorganic compounds. Sugarcane cultivation is primarily aimed at sugar and ethanol production, with sugarcane molasses being used by the biotechnology industry as a substrate for ethanol production and globally as a raw material for biogas generation (DETMAN *et al*., 2017), proteins (SUMAN *et al*., 2015), enzymes (MARIM *et al*., 2016), and microbial biomass (JIRU *et al*., 2018). Crude glycerol is generated by soap, fatty acids, biodiesel, and fatty esters industries, making it essential to find alternatives to reuse this by-product (KARAMEROU; WEBB, 2019; KUMAR *et al*., 2019). It is employed to replace expensive carbon sources in microbial fermentation media, resulting in the production of products such as citric acid, organic acids, bioethanol, and bioplastics (ANITHA *et al*., 2016; IYYAPPAN *et al*., 2018; MOTA *et al*., 2017; VIVEK *et al*., 2017; RZECHONEK *et al*., 2019).

In light of the above, the objective of this study was to select novel yeast strains for the production of lipids and carotenoids, using molasses and crude glycerol as alternative carbon sources. The fatty acid profile of the yeasts in different media was also characterized, and the quantification of total carotenoids was performed relative to the β-carotene standard.

2 MATERIALS AND METHODS

2.1 Microorganisms

Twenty-six yeast strains from the Agricultural Microbiology Culture Collection (CCMA, Federal University of Lavras, Lavras, MG, Brazil) were selected (Table 1) based on previous studies and genera described in the literature as potential producers of lipids and/or carotenoids (SAENGE *et al*., 2011; HUANG *et al*., 2013; FREITAS *et al*., 2014; SOUZA; SCHWAN; DIAS, 2014; SOUZA *et al*., 2017; KARAMEROU; WEBB, 2019; OTERO *et al*., 2019; PEREIRA *et al*., 2019; CHREPTOWICZ *et al*., 2019; DIAS *et al*., 2020). All microorganisms were reactivated in test tubes containing 2 mL of YEPG culture medium (g/L: yeast extract, 10; peptone, 20; glucose, 20) for 24 hours at 28 ºC.

Table 1 – Yeasts belonging to the Agricultural Microbiology Culture Collection (CCMA) used in tests for the production of lipids and carotenoids.

Code CCMA	Species	Origin
CCMA 0023	*Candida neerlandica*	Coffee
CCMA 0040	*Candida sojae*	Coffee
CCMA 0020	*Candida tropicalis*	Coffee
CCMA 2076	*Cryptococcus aspenensis*	Nelore cattle feces
CCMA 0036	*Cryptococcus humicola*	Coffee
CCMA 0025	*Cryptococcus laurentii*	Coffee
CCMA 2070	*Cryptococcus liquefaciens*	Nelore cattle feces
CCMA 1623	*Cystofilobasidium ferigula*	Coffee
CCMA 0468	*Debaryomyces hansenii*	Coffee
CCMA 0141	*Debaryomyces pseudopolymorphus*	Coffee
CCMA 2073	*Exophiala spinifera*	Nelore cattle feces
CCMA 2071	*Kalmonozyma brasiliensis*	Nelore cattle feces
CCMA 0019	*Pichia guilliermondii*	Fermented indigenous drink - caxiri
CCMA 0051	*Pichia kudriavzevii*	Cane silage
CCMA 0048	*Pichia manshurica*	Cane silage
CCMA 945	*Rhodotorula dairenensis*	Ruminal fluid
CCMA 2075	*Rhodotorula dairenensis*	Nelore cattle feces
CCMA 0151	*Rhodotorula mucilaginosa*	Coffee
CCMA 0156	*Rhodotorula mucilaginosa*	Coffee
CCMA 2032	*Rhodosporidium toruloides*	Sugar cane bagasse hydrolyzate
CCMA 0684	*Torulaspora delbrueckii*	Coffee
CCMA 0021	*Torulaspora globosa*	Coffee
CCMA 0039	*Torulaspora maleeae*	Coffee
CCMA 0029	*Trichosporon loubieri*	Coffee
CCMA 0358	*Wickerhamomyces anomalus*	Coffee
CCMA 0357	*Yarrowia lipolytica*	Amazon soil

Source: author (2023).

2.2 Characterization of substrates used for the production of lipids and carotenoids

The substrates used as alternative carbon sources were crude glycerol, provided by the Biodiesel Laboratory of the Federal University of Lavras, and sugarcane molasses, provided by the sugar and alcohol plant. They were presented for the composition of carbohydrates, organic acids, and the presence of furfural and 5- methylfurfural using high-performance liquid chromatography (HPLC) according to the methodology described by Ferreira *et al*., 2022. The samples were centrifuged (10,000 rpm) for 5 min at 4 °C. The supernatant was recovered and filtered with sterile syringe filters (0.22 µm pore size; Kasvi, Brazil). Perchloric acid (1 µL) was added to the Acid Exception to equalize the pH of the sample with that of the mobile phase (pH 2.1), followed by centrifugation and filtration as described above. Analyzes were performed using an HPLC (model LC-10Ai; Shimadzu Corp., Tokyo, Japan) equipped with a dual detection system consisting of a UV-vis detector (SPD-10Ai; Shimadzu) and a U.V. detector refractive index (RID-10Ai; Shimadzu). A Shimadzu ion exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was used for the preparation of carbohydrates (30 °C), and organic acid (50 °C). The mobile phase was ultrapure water (carbohydrates and alcohols) and acidified ultrapure water (pH 2.1) to absorb at a 0.6 mL/min flow rate. For the identification of furfural and 5-methylfurfural, the samples were acidified, and the same column was used, eluted with H_2SO_4 (0.005 M), at 60 °C, with a flow of 0.6 mL/min, for 50 min and light detector UV at 280 nm (LIU *et. al*., 2021). Compounds were identified based on the retention time of the standards, and the external container determined their concentrations. All samples were examined in triplicates.

2.3 Submerged fermentation for the production of lipids and carotenoids

2.3.1 Culture medium and cultivation conditions

The methodology was adapted from Souza *et al*. (2017), changing carbon sources and concentrations. Each yeast strain selected as oleaginous and/or carotenogenic was separately inoculated in a fermentation medium adapted from Papanikolaou *et al*. (2003), containing the following concentrations in g/L: KH_2PO_4, 7.0; NaH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H2O$, 0.06; $(NH_4)_2SO_4$, 0.5; yeast extract, 0.5; and glucose, 5.0; pH 6.0. The yeast strains were cultivated using glucose, pure glycerol (controls), and

alternative substrates (crude glycerol and sugarcane molasses) as carbon sources. All carbon sources (glucose, pure glycerol, sugarcane molasses, and crude glycerol) were evaluated at a concentration of 40 g/L for submerged cultivation.

The yeast isolates (10^7 cells/mL) were inoculated into 250 mL flasks containing 150 mL of fermentation medium and incubated for 96 hours at 28 °C with agitation at 150 rpm on an orbital shaker (New Brunswick Scientific, Excella E25 model; Edison, New Jersey, USA). Samples were taken at 0, 24, 48, and 96 h of fermentation to monitor yeast growth on different substrates by counting the number of cells in a Neubauer chamber. The experiments were performed in triplicates.

2.4 Step 1 - EVALUATION OF LIPID SYNTHESIS

2.4.1 Screening of oleaginous yeasts in submerged culture using pure glycerol medium

Submerged cultivation of the twenty-six yeasts was performed using pure glycerol as the substrate, following the conditions described in section 2.3 for subsequent lipid extraction. After cultivation in pure glycerol, the pre-selected oleaginous yeasts were subjected to other cultures using the other carbon sources (glucose, sugarcane molasses, and crude glycerol) under the conditions described in section 2.3. Samples were taken every 24 hours for viable cell counting, following a protocol adapted from Souza, Schwan and Dias (2014). The adaptations included the use of different carbon sources and their concentrations. At the end of fermentation, lipid extraction, and quantification were performed.

2.4.2 Extraction and determination of lipid content

At the end of fermentation, lipid extraction, and quantification were performed following the protocol of Bligh and Dyer (1959) with modifications. Samples (100 mL) were centrifuged at 5,000 rpm for 10 min at 4 °C, washed three times with distilled water, and the weight of the dried pellet was determined in an oven at 50 °C for 12 hours. To disrupt the yeast cell wall, 5 mL of 2M HCl was added to the dried biomass and placed in a water bath at 70 °C for 1 hour. After cooling to room temperature, centrifugation was performed at 5,000 rpm for 10 min, and the acid was discarded (SOUZA *et al*., 2017). The sediment was resuspended in 3.75 mL of chloroform/methanol (solution 2:1, v/v) and vortexed for 15 min, followed by the addition of 1.25 mL of chloroform and 1.25 mL of 1M NaCl, with vortexing at each step. The

mixture was centrifuged at 5,000 rpm for 15 min to separate the aqueous and organic phases. A three-phase system was formed, consisting of a lower liquid phase (chloroform and lipid content), an interfacial solid phase (biomass), and an upper liquid phase (methanol and water).

The lower organic phase was transferred to a pre-weighed and dried flask and taken to a fume hood for complete solvent evaporation. The weight of the flask was re-recorded. Therefore, the lipid content of the dried cell biomass was calculated using the formulas below (SINGH *et al*., 2020):

$$(1)\ \text{Lipid contend (g/L)} = \frac{\text{Weight of lipid content in the flask (g)} - \text{weight of empty flask (g)}}{\text{Volume of the cultivation sample (L)}}$$

$$(2)\ \text{Lipid content (\%)} = \frac{\text{Lipid content (g/L)}}{\text{Dry cell weight (g/L)}} \times 100$$

2.4.3 Lipid Transesterification

The transesterification of the lipids extracted from the cells to obtain fatty acid methyl esters (FAMEs) was performed according to the methodology of Singh et al. (2018). To each tube, 2 mL of n-hexane and 1 mL of 2 M methanolic KOH were added. The tubes were capped, vigorously shaken for 30 seconds, and then incubated at 70°C for 20 minutes. After cooling to room temperature, 1.2 mL of 1 M HCl was added with gentle agitation, followed by 1 mL of n-hexane. Subsequently, the mixture was allowed to settle for phase separation. The fatty acid methyl esters (FAMEs) were determined by gas chromatography-mass spectrometry (GC-MS).

2.4.4 Analysis of Fatty Acids by Gas Chromatography-Mass Spectrometry (GC-MS)

The chromatographic analyses were performed according to the methodology described by Singh *et al*. (2020) with adaptation of the chromatographic column. A GC/MS system (model GCMS-QP2010SE; Shimadzu, Tokyo, Japan) equipped with a capillary column (Rtx-5MS) of 30 m in length and 0.25 µm in thickness, coupled to a quadrupole detector, was used. An electron ionization system with an ionization energy of 70 eV was used, and the carrier gas (99.99% helium) was maintained at a constant flow rate of 1.1 mL/min. The mass transfer line and injector temperature were set at 220°C and 250°C, respectively. The oven temperature was programmed as follows: the initial temperature was 50°C for 2 min, followed by a ramp of 4°C/min to 220°C for 10 min, and then held at 250°C for 2 min after the run. Approximately 1

µl of the sample was injected in split mode (split ratio 10:1). The signals were recorded in full-scan mode (20-600 m/z). All components were identified by comparing their mass spectra with the NIST database in the GC/MS Solution software library version 2.6. After identifying the fatty acids synthesized in the different culture media, the relative quantification of these acids, as a percentage of the total peak area, was performed.

2.4.5 Qualitative analysis for vizualization of lipid accumulation

The yeast strains identified as oleaginous were subjected to the Sudan III staining technique to visualize the lipid content. For this purpose, oleaginous yeasts were inoculated on Petri dishes containing an inducing culture medium for lipid accumulation, as proposed by Evans and Ratledge (1983). The medium composition was as follows (g.L-1): 40.0 glycerol P.A.; 7.0 KH_2PO_4; 2.0 Na_2HPO_4; 1.5 $MgSO_4.7H_2O$; 0.1 $CaCl_2$; 0.008 $FeCl_3.6H_2O$; 0.0001 $ZnSO_4.7H_2O$; 0.8 yeast extract; 20.0 agar; pH adjusted to 5.5. The Petri dishes (in triplicates) were incubated at 28°C for 96 hours. Yeast colonies from the Petri dishes made make a culture smear on glass slides. The smear was heat-fixed, stained using a 0.3% Sudan III solution (Sigma Aldrich, Brazil), and left at room temperature for 15 minutes. Excess dye was washed off with 70% ethanol, and the slide was counterstained with safranin (0.5%) for 30 seconds, then rinsed with distilled water. The slide was allowed to air dry, and observations were made using an optical microscope. The lipid droplets were stained yellow, characterizing the oleaginous yeasts (SAGIA *et al*., 2020). The methodology was adapted using Sudan III dye (JIRU *et al*., 2016). The slides were observed using an Olympus Cx31 light microscope connected to an image capture system (Olympus, Tokyo, Japan), where the images were also obtained.

2.5 Step 2 - Evaluation of carotenoid synthesis

2.5.1 Pre-selection of carotenogenic yeasts

The 26 yeast strains were grown in YM medium (yeast extract, 3 g/L; malt extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L) at 28°C for 120 hours. Colonies showing red, orange, or yellow coloration were considered capable of carotenoid synthesis (MALDONADE; SCAMPARINI; RODRIGUEZ-AMAYA, 2007).

2.5.2 Selection of carotenogenic yeasts in submerged culture

The pre-selected yeasts from the previous step were subjected to submerged cultures as described in section 2.3, using glucose, pure glycerol, molasses, and crude glycerol as substrates. After 96 hours of cultivation at 28°C with agitation at 150 rpm in an orbital shaker, carotenoids were extracted and quantified using the standard β-carotene curve.

2.5.3 Carotenoid Extraction

The recovery of total carotenoids was performed according to the methodology described by Valduga *et al*. (2009) with modification, using a solvent solution of acetone: methanol (7:3, v/v). The biomass recovered from the fermentation medium was centrifuged (5.000 rpm, 4 °C, 10 min) and dried in an oven at 50°C for 24 h. Subsequently, the dried biomass was ground in liquid nitrogen, 2 mL of dimethyl sulfoxide (DMSO) was added, and it was heated in a water bath at 50°C for 30 min. Then, 2 mL of the mixed solvent was added, followed by centrifugation (5,000 rpm, 4°C, 10 min). The supernatant was separated, and successive extractions with the solvent were performed until the cells remained colorless.

At the end of the extraction process, the samples were analyzed using a UV-VIS spectrophotometer (Model: SPECORD 200/210 PLUS – Analytik Jena) at 450 nm to quantify total carotenoids based on the construction of the β-carotene standard curve described below.

2.5.4 Quantification of Total Carotenoids

To construct the calibration curve for β-carotene, Sigma Beta-carotene was used. A solution of β-carotene in acetone and methanol in a 1:1 (v/v) ratio was prepared. Triplicates of dilutions were prepared at concentrations of 3.0, 2.0, 1.25, 0.312, 0.156 µg/mL, followed by reading on a spectrophotometer (Model: SPECORD 200/210 PLUS – Analytik Jena) at 450 nm. From the absorbance values obtained from the reading, a concentration vs. absorbance graph was constructed to obtain the linear correlation, which generated the equation of the line and the R-squared value. Using the equation of the line, it was possible to quantify the total carotenoids produced during fermentation under cultivation in glucose, pure glycerol, molasses, and crude glycerol media. The methodology was adapted from Kot *et al*. (2020), with modifications to the concentration values of β-carotene and the acetone and methanol solution used for the dilutions.

2.6 Statistical Analysis

Quantitative data related to lipid production and β-carotene concentration of the yeasts under cultivation in different media (pure glycerol, glucose, molasses, and crude glycerol) were compared using the Scott-Knott test using the statistical software R (R i386 3.3.3). Differences in values were considered significant at $p < 0.05$.

3 RESULTS

3.1 Characterization of sugarcane molasses and crude glycerol substrates

In the sugar cane molasses sample, lactic and acetic acids were identified at 5.93 g/L and 37.65 g/L, respectively. The identified carbohydrates were sucrose (240.36 g/L), glucose (30.698g/L), fructose (40.75g/L), and glycerol (0.60 g/L), ethanol were also detected at a concentration of 6.41 g/L. The crude glycerol by-product was characterized by acetic acid at a concentration of 0.197 g/L and 386.83 g/L of glycerol. The presence of the toxic compounds furfural and 5-methylfurfural was not detected in the substrate samples.

3.2 Screening of oleaginous yeasts cultivated in pure glycerol

Table 2 shows the lipid production as a percentage for the 26 yeast strains.

Table 2 - Screening of lipid production of yeast under pure glycerol cultivation (to be continued).

Yeast code	Yeast	Lipid content (%)
CCMA 0358	*Wickerhamomyces anomalus*	2.73±0.505 i
CCMA 0151	*Rhodotorula mucilaginosa*	5.07 ±1.358 h
CCMA 0020	*Candida tropicalis*	6.63 ±0.760 g
CCMA 2075	*Rhodotorula dairenensis*	7.28 ±0.240 g
CCMA 0051	*Pichia kudriavzevii*	7,38 ±1.105 g
CCMA 0468	*Debaryomyces hansenii*	7.42 ±0.080 g
CCMA 2076	*Cryptococcus aspenensis*	9.23 ±0.415 f

Table 2 - Screening of lipid production of yeast under pure glycerol cultivation (conclusion).

Yeast code	Yeast	Lipid content (%)
CCMA 2070	*Cryptococcus liquefaciens*	9.60 ±3.594 f
CCMA 0141	*Debaryomyces pseudopolymorphus*	9,76 ±1.110 f
CCMA 0040	*Candida sojae*	10.76 ±0.570 f
CCMA 0019	*Pichia guilliermondii*	12.15 ±2.485 e
CCMA 0023	*Candida neerlandica*	12.57 ±2.035 e
CCMA 2071	*Kalmonozyma brasiliensis*	13.41 ±0.770 e
CCMA 0048	*Pichia manshurica*	13.89 ±0.720 e
CCMA 0684	*Torulaspora delbrueckii*	13.99 ±1.785 e
CCMA 0036	*Cryptococcus humicola*	14.88 ±4.125 e
CCMA 0029	*Trichosporon loubierii*	16.33 ±3.345 e
CCMA 0025	*Cryptococcus laurentii*	21.02 ±0.350 d
CCMA 0945	*Rhodotorula dairenensis*	21.42 ±1.140 d
CCMA 0156	*Rhodotorula mucilaginosa*	22.77 ±0.570 d
CCMA 0021	*Torulaspora globosa*	30.86 ±0.225 c
CCMA 1623	*Cystofilobasidium ferigula*	33.33 ±3.295 c
CCMA 2073	*Exophiala spinifera*	35.81±2.160 c
CCMA 0357	*Yarrowia lipolytica*	45.00 ±0.525 b
CCMA 2032	*Rhodosporidium toruloides*	45.85 ±1.205 b
CCMA 0039	*Torulaspora maleeae*	68.73 ±0.020 a

The same letters in the lines do not differ according to the Scott-Knott test at $p<0.05$.

Source: author (2023).

Among the 26 wild strains evaluated, 17 had lipid yields below 20%. At the same time, nine can be considered oleaginous (*Cryptococcus laurentii* CCMA 0025, *Rhodotorula dairenensis* CCMA 0945, *Rhodotorula mucilaginosa* CCMA 0156, *Torulaspora globosa* CCMA 0021, *Cystofilobasidium ferigula* CCMA1623, *Exophiala spinifera* CCMA 2073, *Yarrowia lipolytica* CCMA 0357, *Rhodosporidium toruloides* CCMA 2032, *Torulaspora*

maleeae CCMA 0039), with lipid accumulation ranging from 21% to 68% under pure glycerol cultivation.

Some evaluated species are considered models for lipid production, such as *Y. lipolytica* (CCMA 0357), which showed a lipid production of 45% in this study, and the strain *R. toruloides* (CCMA 2032) with lipogenesis of 42.5% under pure glycerol cultivation.

Two species of the *Torulaspora* genus exhibited significant lipid production, with *Torulaspora maleeae* having the highest lipid accumulation content (68.7%) and *Torulaspora globosa* (30.8%). *Exophiala spinifera* and *Cystofilobasidium ferigula* also deserve attention, as the former has not been previously described as oleaginous and showed a lipid content of 35.8%. There are few studies on *C. ferigula*, which yielded over 33% lipids from its dry biomass.

3.2 Lipid production of oleaginous yeasts in glucose, pure glycerol, sugarcane molasses, and crude glycerol culture media

The percentage rate of lipid accumulation in the four different media: glucose and pure glycerol (controls), sugarcane molasses, and crude glycerol (alternative substrates), for the nine strains pre-selected in the previous stage, is presented in Table 3.

Table 3- Lipid production (%) of yeasts in different culture media (to be continued).

Yeast	Culture medium				Standart error
	Glucose	Pure Glycerol	Molasses	Crude Glycerol	
T. maleeae (CCMA 0039)	77.70Aa	68.73Ab	31.38Ad	64.84Bc	1.78
Y. lipolytica (CCMA 0357)	17.58Fc	31.04Db	13.12Ed	51.32Ca	
R. toruloides (CCMA 2032)	35.30Bc	45.85Bb	6.60Fd	77.98Aa	
T. globosa (CCMA 0021)	2.11Id	30.86Db	13.68Ec	41.39Da	
C. laurentii (CCMA 0025)	12.46Gd	21.02Ec	22.96Cb	31.63Ea	
R. mucilaginosa (CCMA 0156)	4.36Hc	22.77Ea	22.63Ca	16.15Hb	

Table 3- Lipid production (%) of yeasts in different culture media (conclusion).

Yeast	Culture medium				Standart error
	Glucose	Pure Glycerol	Molasses	Crude Glycerol	
R. dairenensis (CCMA 0945)	23.20C	21.42E	22.42C	21.21G	1.78
C. ferigula (CCMA 1623)	19.55Ec	33.33Da	25.54Bb	41.88Ea	
E. spinifera (CCMA 2073)	21.35Dc	35.81Ca	19.28Dd	23.97Fb	

Lowercase letters in rows compare culture media and uppercase letters in columns compare yeasts. Different letters indicate a significant difference ($p<0.05$) by the Scott-Knott test. (Statistical program R (R i386 3.3.3).

Source: author (2023).

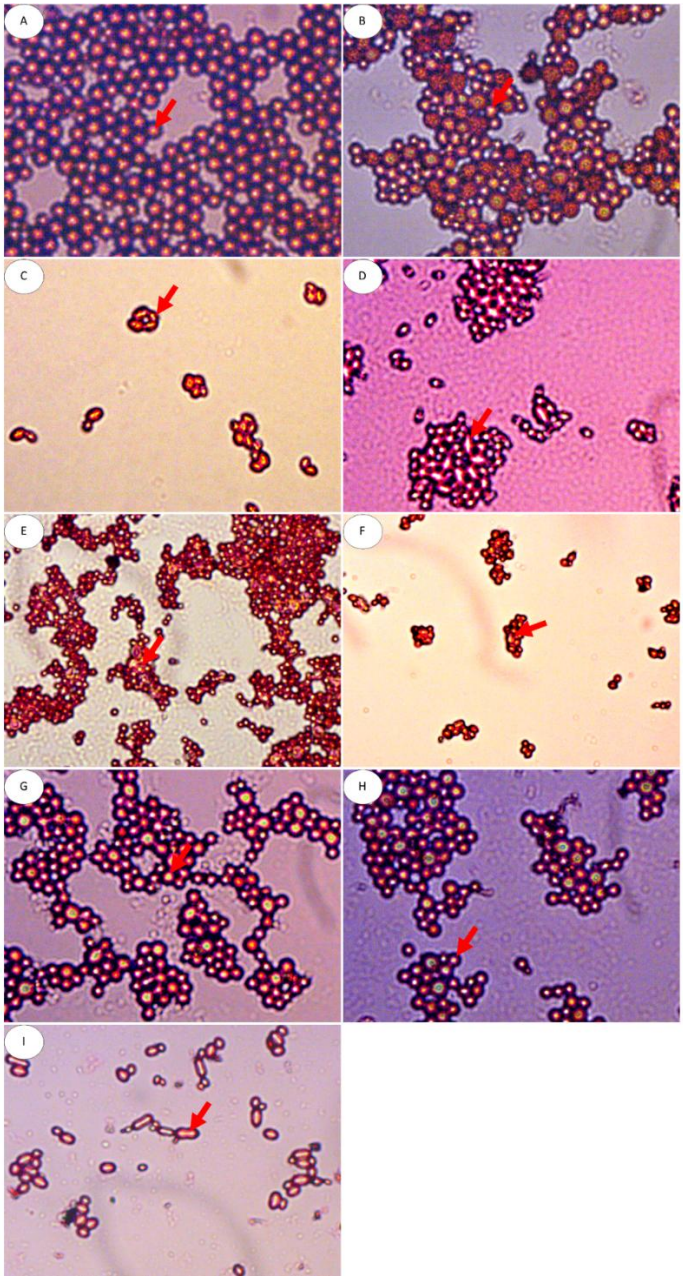
Lipid accumulation differs among strains and evaluated culture media (Table 3). The highest average lipid production for each strain concerning the culture media was as follows: *T. malleae* in glucose (77.7%); *R. toruloides* in crude glycerol (77.98%); *Y. lipolytica* in crude glycerol (51.32%); *C. ferigula* in crude glycerol (41.88%); *E. spinifera* in pure glycerol (35.81%); *T. globosa* in crude glycerol (41.39%); *C. laurentii* in crude glycerol (31.63%). *R. dairenensis* did not show a statistical difference in the mean production values between the media, and *R. mucilaginosa* under culture in pure glycerol (22.77%) and molasses (22.63%) also showed no statistical difference. More significant lipid accumulation was observed for the *T. malleae* strain in glucose (77.7%), pure glycerol (68.95%), and molasses (31.38%) media. Meanwhile, the highest accumulation of crude glycerol was observed in the *Rhodosporidium toruloides* strain (77.98%).

Evaluating the general averages of lipid production of the strains among the four substrates, the highest values, in descending order, were obtained for crude glycerol, pure glycerol, glucose, and sugarcane molasses. Cultivation in crude glycerol medium provided higher levels of lipid accumulation for five strains, *Y. lipolytica*, *R. toruloides*, *T. globosa*, *C. laurentii*, and *C. ferigula*. The strains *T. malleae* and *R. dairenensis* exhibited lipid accumulation percentages higher than 20% for all four substrates used for cultivation.

3.3 Qualitative characterization of lipid accumulation

The nine strains previously selected as oleaginous exhibited intracellular yellow staining in the qualitative Sudan III assay, confirming their ability to accumulate lipids (Figure 1).

Figure 1– Yeast cells stained with Sudan III.



The red arrows indicate the intracellular lipid content with yellow coloring. A: *T. globosa* CCMA 0021; B: *C. laurentii* CCMA 0025; C: *T. maleeae* CCMA 0039; D: *E. spinifera* CCMA 2073; E: *R. mucilaginosa* CCMA 0156; F:*Y. lipolytica* CCMA 0357; G: *R. dairenensis* CCMA 945; H: *C. ferigula* CCMA 1623; I: *R. toruloides* CCMA 2032.

Source: author (2023).

3.4 Identification and relative quantification of synthesized fatty acids in different culture media

Saturated, monounsaturated, and one polyunsaturated fatty acid (C18:2, linoleic acid) constituted the lipid profile of the strains. In all species and evaluated media, myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) were identified. All strains synthesized palmitoleic acid (C16:1) and linoleic acid (C18:2), although they were not identified in all media for some of them. With lower prevalence among the species, the fatty acid profile also included dodecanoic acid (C12:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), eicosanoic acid (C20:0), and docosanoic acid (C22:0).

In cultures using glucose as the carbon source, eleven different fatty acids synthesized by the strains were identified. The fatty acids with the highest concentrations were palmitic acid (C16:0) (50.5-29%), stearic acid (C18:0) (34-13.5%), and oleic acid (C18:1) (45.5-20.0%). The yeasts that exhibited the highest total peak areas were *T. globosa* (102,008), *E. spinifera* (19,854), and *T. maleeae* (17,507) (Table 4).

Table 4 - Profile of synthesized fatty acids (% peak area) by yeasts grown in glucose medium after 96 hours of cultivation, identified by GC-MS.

	Medium: glucose								
Fatty acids	*R. mucilaginosa*	*Y. lipolytica*	*R. toruloides*	*E. spinifera*	*C. ferigula*	*R. dairenensis*	*T. maleeae*	*C. laurentii*	*T. globosa*
	Peak area (%)								
C14:0	2.0	2.0	3.0	0.5	2.5	1.5	3.5	1.0	1.0
C15:0	0.0	1.0	0.0	0.5	0.0	0.0	0.5	0.0	0.0
C16:0	47.5	35.0	45.0	50.5	46.0	30.0	29.0	30.0	42.4
C16:1	7.0	5.0	1.0	0.8	0.6	5.5	27.5	0.0	13.0
C17:0	0.0	1.0	0.0	0.2	0.0	0.0	1.0	0.5	0.3
C17:1	0.0	1.0	0.0	0.0	0.0	0.0	0.5	0.0	0.3
C18:0	17.5	22.5	17.0	14.0	16.0	13.5	17.0	34.0	12.0
C18:1	26.0	24.5	33.0	22.0	29.0	45.5	20.0	28.0	31.0
C18:2	0.0	6.5	1.0	11.0	6.0	4.0	0.3	4.0	0.0
C20:0	0.0	1.5	0.0	0.2	0.0	0.0	0.5	1.5	0.0
C22:0	0.0	0.0	0.0	0.2	0.0	0.0	0.2	1.0	0.0
Total peak area	1,207	2,577	1,269	19,854	1,200	1,572	17,507	6,054	102,008

Source: author (2023).

Under cultivation in pure glycerol, it was possible to identify fifteen types of fatty acids. Similarly to the glucose medium, palmitic, oleic, and stearic acids were produced in higher proportions by the yeasts, with peak area percentages ranging from (46.5-35%), (36-17.5%), and (24.5-11%), respectively. Oleic and linoleic acids exhibited higher levels when compared to the glucose medium. The fatty acid heneicosanoic acid (C21:0) was synthesized only by the *C. ferigula* strain with a relative peak area of 21%. The yeast *E. spinifera* showed the highest total peak area (58982) of fatty acids for this substrate (Table 5).

Table 5 - Profile of fatty acids synthesized by yeasts cultivated in the medium of pure glycerol after 96 hours of cultivation, identified by GC-MS.

Medium: Pure glycerol									
Fatty acids	*R. mucilaginosa*	*Y. lipolytica*	*R. toruloides*	*E. spinifera*	*C. ferigula*	*R. dairenensis*	*T. maleeae*	*C. laurentii*	*T. globosa*
	Peak area (%)								
C9:1	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
C10:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
C12:0	0.2	0.0	0.0	0.0	0.0	0.0	1.0	0.3	0.5
C14:0	1.4	2.0	2.5	0.5	3.0	2.5	4.0	1.0	2.0
C15:0	0.0	1.0	0.0	0.5	0.0	0.0	0.5	0.3	0.0
C16:0	35.0	35.0	45.0	46.5	41.5	43.5	40.0	40.0	41.0
C16:1	0.6	5.0	1.5	3.5	0.0	0.0	1.5	0.3	17.0
C17:0	0.3	1.0	0.5	0.5	0.0	0.0	1.5	0.4	0.0
C17:1	0.2	1.0	0.0	0.5	0.0	0.0	2.0	0.0	0.0
C18:0	17.8	22.5	14.0	20.0	0.0	13.5	18.0	24.5	11.0
C18:1	33.0	24.5	32.0	22.5	29.0	36.0	17.5	23.0	26.5
C18:2	10.5	6.5	4.5	4.5	5.5	4.5	10.0	8.2	1.0
C20:0	0.3	1.5	0.0	0.5	0.0	0.0	1.0	1.0	0.0
C21:0	0.0	0.0	0.0	0.0	21.0	0.0	0.0	0.0	0.0
C22:0	0.7	0.0	0.0	0.5	0.0	0.0	2.0	1.0	0.0
Total peak area	7,544	2,577	3,794	58,982	384	1,104	6,943	14,839	4,397

Source: author (2023).

In molasses medium cultivation, thirteen fatty acids were identified, with the highest relative percentages being exhibited by palmitic acid (58.5 - 23%), oleic acid (47.5-8%), and stearic acid (21-12.5%). Higher total peak area values were determined for the *T. globosa* (50624) and *R. mucilaginosa* (48926) strains (Table 6).

Table 6 - Profile of fatty acids synthesized by yeasts cultivated in the medium of molasses after 96 hours of cultivation, identified by GC-MS.

Medium: Molasses									
Fatty acids	*R. mucilaginosa*	*Y. lipolytica*	*R. toruloides*	*E. spinifera*	*C. ferigula*	*R. dairenensis*	*T. maleeae*	*C. laurentii*	*T. globosa*
	Peak area (%)								
C10:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.5
C12:0	0.0	0.0	0.0	0.3	0.0	0.0	0.5	0.0	0.5
C14:0	3.0	0.5	3.0	0.7	1.5	2.0	1.5	2.5	2.0
C15:0	0.5	0.5	0.0	1.0	0.0	0.0	0.5	0.0	0.0
C16:0	58.5	23.0	42.0	39.0	26.5	40.5	35.0	56.5	41.0
C16:1	2.0	13.0	0.5	2.0	3.0	1.0	9.5	2.0	14.5
C17:0	0.5	0.5	0.5	1.0	0.2	0.0	2.0	0.0	0.0
C17:1	0.5	1.0	0.0	1.0	0.5	0.0	1.0	0.0	0.5
C18:0	21.0	13.5	14.5	13.5	17.8	19.0	20.0	0.0	12.5
C18:1	8.0	42.5	35.0	29.0	47.5	32.0	21.5	33.5	24.0
C18:2	3.0	4.5	4.5	12.0	1.5	5.0	8.5	5.5	2.5
C20:1	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C22:0	2.0	0.5	0.0	0.5	1.5	0.0	0.0	0.0	0.0
Total peak area	48,926	15,538	2,311	11,484	13,545	2,423	5,520	456	50,624

Source: author (2023).

In cultures using crude glycerol, of the thirteen identified fatty acids, higher percentages of accumulation were observed for palmitic, oleic, linoleic and stearic.The yeasts showed higher linoleic acid (C18:2) accumulation levels when utilizing crude glycerol as a carbon source, except for the *C. laurentii* and *T. globosa* strains. Higher total peak area values were also observed, except for the *R. mucilaginosa* and *T. globosa* strains (Table 7).

Table 7 - Profile of fatty acids synthesized by yeasts cultivated in the medium of crude glycerol.

Medium: Crude glycerol									
Fatty acids	*R. mucilaginosa*	*Y. lipolytica*	*R. toruloides*	*E. spinifera*	*C. ferigula*	*R. dairenensis*	*T. maleeae*	*C. laurentii*	*T. globosa*
	Peak area (%)								
C10:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
C12:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0
C14:0	1.0	2.0	2.5	9.0	3.5	1.5	2.0	0.0	13.0
C15:0	0.0	0.5	0.0	1.5	0.0	0.0	0.0	30.0	0.5
C16:0	34.0	44.5	35.5	15.5	35.5	31.5	35.0	1.0	17.5
C16:1	1.0	2.5	1.5	6.0	0.5	1.0	1.5	0.0	17.0
C17:0	0.0	1.0	0.0	0.5	0.0	0.0	0.0	0.0	0.5
C17:1	0.0	0.5	0.0	0.5	0.0	0.0	0.0	8.5	0.5
C18:0	9.0	19.0	3.0	35.5	3.5	11.0	9.0	17.5	39.0
C18:1	18.5	18.0	23.5	13.0	37.0	18.5	13.0	1.0	9.0
C18:1	1.0	0.0	0.0	0.0	0.5	1.0	1.0	40.5	0.0
C18:2	35.0	11.5	34.0	16.5	19.5	35.0	38.0	0.5	0.0
C20:2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.0
C22:0	0.5	0.5	0.0	2.0	0.0	0.5	0.5	0.0	0.0
Total peak area	13,483	47,914	19,285	99,452	15,504	10,351	27,936	25,300	75,673

Source: author (2023).

The fatty acid profile of the carbon sources was also evaluated and showed lower diversity compared to the fatty acids synthesized by the yeasts. However, the acids with higher relative peak areas are the same as those found in the yeasts, namely: palmitic, oleic, linoleic, and stearic acids. The crude glycerol byproduct had a total peak area of 19,549, higher than molasses (18,113) and pure glycerol (655) (Table 8).

Table 8 - Profile of fatty acids identified in the substrates used for the culture media.

Fatty acids	Substrates		
	Pure glycerol	Molasses	Crude glycerol
	Peak area (%)		
C10:0	0.0	0.0	0.5
C14:0	2.0	1.5	8.5
C16:0	38.0	38.0	16.5
C16:1	0.0	0.5	0.5
C18:0	9.0	6.0	3.5
C18:1	16.0	16.5	53.0
C18:2	35.0	37.5	17.5
Total peak area	655	1,813	19,549

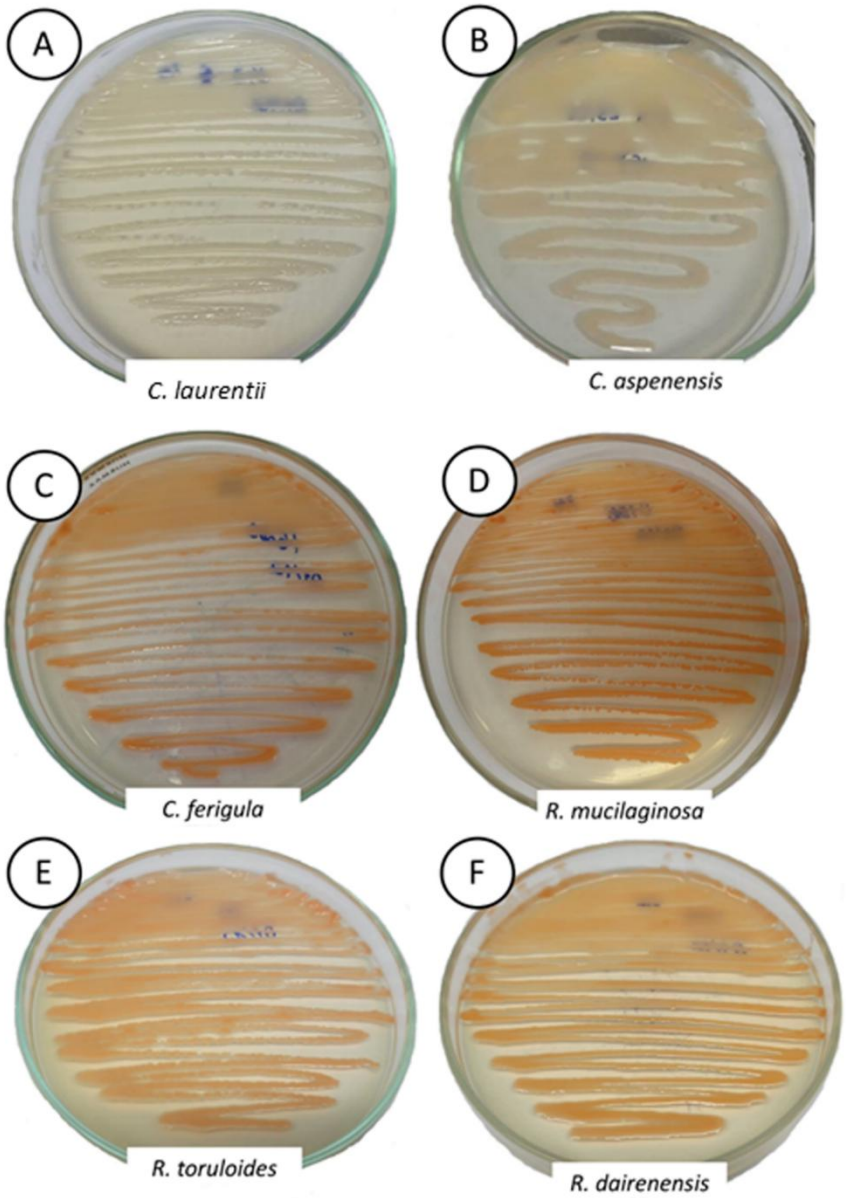
Source: author (2023).

Cultivating yeasts in pure glycerol medium provided greater diversity in the fatty acid profile, but higher relative percentages were identified for cultivation in crude glycerol. The level of linoleic acid production (C18:2) by the yeasts was higher under cultures in pure and crude glycerol media, indicating efficiency in converting substrates during accumulation, leading to changes in the lipid profile.

3.5 Pre-selection of carotenogenic yeasts on solid medium

Strains *C. laurentii* CCMA 0025 and *C. aspenensis* CCMA 2076 exhibited a light color when grown on YM medium, while strains *C. ferigula* CCMA 1623, *R. muciloginosa* CCMA 0156, *R. toruloides* CCMA 2032, and *R. dairenensis* CCMA 945 displayed shades ranging from pink to orange (Figure 2).

Figure 2 - Potentially carotenogenic yeasts cultured on YM medium.



Source: author (2023).

3.6 Quantification of total carotenoids in different growth media

The results of total carotenoid concentration were estimated based on the construction of the β-carotene standard curve. The light-colored species *C. laurentii* and *C. aspenensis* showed the lowest concentrations of carotenoids for all carbon sources used in submerged cultivation. The yeast *R. toruloides* exhibited the highest average production in all media, except for the molasses medium. When evaluating production under pure glycerol cultivation, there was no statistical difference among the yeasts *R. dairenensis* (1.42 µg/mL), *C. ferigula*

(1.20 µg/mL), and *R. toruloides* (1.34 µg/mL). The crude glycerol medium yielded the highest average concentrations of total carotenoids for all evaluated strains (Table 9), with the highest production by *R. toruloide* (3.30 µg/mL) and *C. ferigula* (2.76 µg/mL). Estimating the carotenoid concentration for the *C. laurentii* CCMA 0025 strain under glucose and pure glycerol cultivation was impossible, as the absorbance readings were below the lowest concentration used for the standard curve construction.

Table 9 - Production of total carotenoids (µg/mL) of yeasts cultivated in glucose, pure glycerol, molasses and crude glycerol media.

Yeasts (Y)	Culture medium (M)				P value		
	Glucose	Pure glycerol	Molasses	Crude glycerol	Y	M	Y x M
C. laurentii CCMA 0025	0.00Ce	0.00Cc	0.49Bc	0.88Ad	<0.001	<0.001	<0.001
R. mucilaginosa CCMA 0156	0.49Dd	0.77Cb	1.15Bb	2.39Ac			
R. dairenensis CCMA 945	1.25Bc	1.42Ba	0.52Cc	2.31Ac			
C. aspenensis CCMA 2076	0.35Ad	0.07Bc	0.04Bd	0.52Ae			
C. ferigula CCMA 1623	1.60Bb	1.20Ca	1.38Ca	2.76Ab			
R. toruloides CCMA 1623	1.89Ba	1.34Ca	1.14Cb	3.30Aa			

Uppercase letters in rows compare culture media (M) for each yeast strain and lowercase letters in columns compare yeasts (Y) in each culture medium. In case of significant difference ($p<0.05$) by the Scott-Knott test.

Source: author (2023).

4 DISCUSSION

Several factors interfere with the potential for lipid syntheses and accumulation, such as medium composition, cultivation conditions, and species-specific metabolic capacity. Yeasts are considered oleaginous when they accumulate lipids above 20% (w/w) of their dry biomass, with accumulation levels varying and reaching up to 80% (CAPORUSSO, 2021; CHEBBI *et al*., 2019; MADANI, ENSHAEIEH, ABDOLI, 2017). Among the 1,600 described yeast species, only about 70 species have been identified as oleaginous (SZCZEPAŃSKA, HAPETA,

and LAZAR, 2021). This number is expected to increase due to the search for non-conventional oleaginous yeasts with bioeconomically advantageous characteristics (SREEHARSHA, MOHAN, 2020).

The genera *Rhodotorula*, *Rhodosporidium*, and *Cryptococcus* are widely described in the literature as oleaginous, along with species such as *Rhodosporidium toruloides* and *Yarrowia lipolytica*, corroborating the strains identified in this study (CAPORUSSO, CAPECE, DE BARI, 2021; KARAMEROU; WEBB, 2019; BAO *et al*., 2021). These species are becoming popular and commonly explored for metabolic engineering modifications for industrial-scale applications (CHATTOPADHYAY, MITRA, MAITI, 2021; SREEHARSHA; MOHAN, 2020). Despite a large number of studies on these genera and species for lipid production, the percentage of accumulated content cannot be used as a basis for comparison, as these numbers vary even among species depending on the evaluated strain and established cultivation conditions (CAPORUSSO, CAPECE, DE BARI, 2021; KARAMEROU; WEBB, 2019; SREEHARSHA; MOHAN, 2020; VASCONCELOS *et al*., 2019).

This study identified oleaginous species from the genera *Torulaspora*, *Cystofilobasidium*, and *Exophiala*, which have not been extensively explored or described in the literature regarding this characteristic. These strains exhibited lipid accumulation above 30% in cultures using pure glycerol as a carbon source: *T. malleae* (68.7%), *T. globosa* (30.8%), *C. ferigula* (33.3%), and *E. spinifera* (35.8%). A study by Leesing and Karrapan (2011) reported lipid production by *Torulaspora malleae*, isolated from soil, classifying it as oleaginous. The species *Torulaspora globosa* has been identified in two studies with potential lipid accumulation and application for biodiesel production; however, the authors note that studies on this species for lipid production are still insufficient (KAMZOLOVA; MORGUNOV, 2021; LEESING; BAOJUNGHARN, 2011).

Its black coloration characterizes the genus *Exophiala* due to the presence of melanin in the yeast cell wall. Species of this genus are primarily studied as opportunistic pathogens, causing cutaneous and visceral infections in humans and animals (BORMAN *et al*., 2020; LAVRIN *et al*., 2020; LIU *et al*., 2019; VILA *et al*., 2019). Another unique characteristic of this genus is its ability to grow in environments contaminated with toxic hydrocarbons such as benzene and toluene, contributing to features like extreme tolerance and metabolism of toxic compounds (ZHANG *et al*., 2019). *Exophiala* species have been described for bioremediation, application in industrial air biofilters, arsenic accumulation (SEYEDMOUSAVI *et al*., 2011), and biosurfactant production (CHIEWPATTANAKUL *et al*., 2010; ERGIN *et al*., 2018). However, the evaluation of lipid accumulation capacity has not been described, which could

broaden the application of this strain for biotechnological purposes. In the present study, under cultivation in pure glycerol, the strain *E. spinifera* (CCMA 2073) exhibited a lipid accumulation of 35.8%, thus considered an oleaginous strain.

The species *Cystofilobasidium ferigula* was described in 2001 by Sampaio, Gadanho, and Bauer as a yeast strain with a pink color. There are few reports on this species and limited research on lipid production by *Cystofilobasidium* strains (ŘEZANKA; KOLOUCHOVÁ and SIGLER, 2016; SAMEK *et al*., 2015;), emphasizing the need for further studies. In this study, *Cystofilobasidium ferigula* (CCMA 1623) exhibited a lipid accumulation of over 30% (w/w) in pure glycerol medium and 41% under cultivation in crude glycerol.

Glucose and pure glycerol are typically used as controls for yeast cultivation as they are rich carbon sources that promote microbial growth when aiming to produce secondary metabolites. However, their large-scale cost becomes prohibitively high (KARAMEROU and WEBB, 2019). Evaluating the lipid production obtained in cultures using different carbon sources, only the strain *T. malleae* (CCMA 0039) showed higher accumulation in the glucose medium. In comparison, *R. mucilaginosa* (CCMA 0156) and *E. spinifera* (CCMA 2073) exhibited higher accumulation in pure glycerol medium.

Glucose is one of the primary sugars used by yeasts for growth, followed by fructose, mannose, galactose, and in some cases, glycerol, hydrocarbons, and oils (CAPORUSSO, CAPECE, DE BARI, 2021). Leesing and Karraphan (2011) reported using glucose as a carbon source for *Torulaspora malleae* cultivation at various concentrations and nitrogen limitations. The lowest concentration used (40g/L) resulted in a lipid yield of 1.98g/L, which is close to the yield obtained in this study (1.16 g/L) [unshown data]. *T. malleae* was the only strain with a higher accumulation of glucose. The low lipid accumulation by some strains using glucose may be related to osmotic stress. In high-sugar media, yeasts can trigger an osmotic stress response, inducing molecular, physiological, and morphological changes to synthesize and accumulate molecules acting as osmoprotectants, thus reducing lipogenesis rates (BABAZADEH *et al*., 2017).

Sugarcane molasses is an abundant residual byproduct in Brazil, derived from the sugar industry, and has also been widely used as a carbon source for microbial fermentation processes. Its main composition is non-crystallized sucrose (30 to 35%). Its monomeric units are glucose and fructose (15 to 20%), in addition to other constituents such as amino acids, phenolic compounds, and inorganic salts (BENTO *et al*., 2020; FREITAS *et al*., 2014; LUO *et al*., 2018). The strains evaluated in this work showed lower lipid accumulation under molasses cultivation, which may be related to the difficulty in sucrose assimilation.

It has been reported by Singh *et al*. (2020) that using concentrations greater than 30g/L of sugarcane molasses to supplement media for lipid accumulation resulted in decreased biomass and lipid production, suggesting osmotic stress by the yeast. Additionally, some wild strains do not assimilate sucrose as the sole carbon source since they lack the invertase enzyme, requiring a prior hydrolysis process, which limits investment in platforms using these strains (BAO *et al*., 2021). Cultures of specific strains in molasses showed higher biomass, suggesting that the yeast invested in cellular growth, consequently leading to low lipid accumulation, as accumulation occurs during the stationary phase of the microbial growth curve.

Crude glycerol is a byproduct of the oleochemical industry produced in quantities that exceed market demand. Its reuse becomes costly due to the need for expensive purification processes and high energy consumption, which must be more attractive to the industry (YANG *et al*., 2012). For these reasons, crude glycerol is inexpensive, abundant, and sought after for use as a substrate for microbial cultivation, with the potential for recycling to produce more lipids (VASCONCELOS *et al*., 2019). Its chemical composition may vary depending on the industry from which it is derived. It may contain about 25% carbon and elements such as Na, Ca, K, Mg, Na, P, and S, as well as proteins, fats, and carbohydrates, which can be used as nutrient sources by microorganisms (KUMAR *et al*., 2019). Impurities can also be found in crude glycerol, such as soap, methanol, free fatty acids (FFAs), sodium salts, carbon, and chemical elements (from the catalyst), which can limit cell growth and intracellular lipid production (GAO *et al*., 2016; SREEHARSHA and MOHAN, 2020).

Ex novo accumulation pathways can stimulate high lipid content in yeast cultured in crude glycerol. These pathways occur in yeasts that secrete lipases when cultured in hydrophobic media, absorbing compounds such as esters, triacylglycerols, sterols, and fatty acids from the medium. The yeast can metabolize free fatty acids to obtain energy or store it in lipid droplets (CAPORUSSO, CAPECE, DE BARI, 2021).

Few strains of oleaginous yeasts reported in the literature have demonstrated efficient bioconversion of crude glycerol into lipids, mainly due to impurities. The majority of these strains belong to the genus *Rhodosporidium*. High lipid accumulation yield (35.4%) was achieved in previous studies with *Rhodosporidium toruloides* using crude glycerol as the carbon source at a concentration of 50 g/L (GUERFALI *et al*., 2020; YANG *et al*., 2014). In this study, utilizing 40 g/L of crude glycerol, the percentage of lipid production was 77.98%, more than double the value reported in the study above. The high rate of lipid accumulation by yeasts using crude glycerol as a substrate, as identified in this work, is attractive for the application of these strains in biotechnological processes, indicating their efficiency and tolerance to

impurities, highlighting the importance of studies with wild or non-conventional strains (SREEHARSHA; MOHAN, 2020).

From a metabolic perspective, comparing the utilization of the two alternative substrates, sugarcane molasses, and crude glycerol, it is more advantageous for the cell to metabolize crude glycerol. Assuming the cells possess the invertase enzyme, the consumption of molasses requires prior hydrolysis of sucrose into glucose and fructose, enabling the utilization of these sugars through glycolysis to generate pyruvate, which must then enter the Krebs cycle to form acetyl-CoA, a precursor for lipid biosynthesis. In contrast, when crude glycerol is used as the substrate, it is directed towards the end of glycolysis (glycerol-3-phosphate), converted into pyruvate, and metabolized in the Krebs cycle, forming acetyl-CoA. Therefore, it is a less costly process for the cell (PATEL *et al*., 2017; CAPORUSSO, CAPECE, DE BARI, 2021). The different lipid accumulation rates obtained in cultures using different carbon sources highlight the importance of evaluating lipid production under diverse media and conditions to guide optimization experiments.

The diversity of fatty acids synthesized by microorganisms mainly depends on the species, their genetic conditions related to metabolism, and cultivation conditions. This diversity is limited in yeasts, but there are advantages, such as the ability to grow on diverse substrates and thus modulate the fatty acid profile, which is of great industrial interest. Oleaginous yeasts predominantly synthesize the fatty acids palmitic acid (C16:0), palmitoleic acid (16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), as also observed in this study. Myristic acid (C14:0) is found in lesser abundance (CAPORUSSO, CAPECE, DE BARI, 2021).

The fatty acid profile found in yeasts is similar to the composition of cocoa butter: C16:0 23%–30%; C18:1 30%–37%; C18:0 32%–37%; C18:2 2%–4%. Because cocoa butter is a high-value product with increasing demand and limited supply, oleaginous yeast platforms are promising alternatives for this purpose (PATEL *et al*., 2019).

A greater variety of fatty acids was observed in pure glycerol medium, and larger peak areas were observed in crude glycerol medium. Through *ex novo* synthesis, which occurs when yeasts are in a hydrophobic medium such as glycerol, there is specific selectivity regarding the absorption rate of free fatty acids, leading to changes in the profile of fatty acids synthesized by the microorganism over the fermentation time. This pathway can provide a more diverse fatty acid profile (CAPORUSSO, CAPECE, DE BARI, 2021).

Linoleic acid (C18:2) is a highly relevant polyunsaturated fatty acid (PUFA) and was found in the lipid profile of all evaluated oleaginous strains. However, it was not identified in

all culture media for all yeasts. PUFAs are essential fatty acids that provide health benefits such as maintaining brain functions and alleviating symptoms of chronic diseases like hypertension, Alzheimer's disease, and chronic intestinal disorders (BHARATHIRAJA *et al*., 2017). C18:2 is a conjugated linoleic acid from the ω-6 family, characterized by a pair of conjugated double bonds separated by a methylene group. Industrial production is achieved through synthesis, generating undesirable byproducts (SZCZEŚNIAK; HAPETA; LAZAR, 2021). In the glucose medium, linoleic acid was identified in lower percentages. It was not synthesized by the *R. mucilaginosa* and *T. maleeae* strains, whereas under pure glycerol and molasses cultures, it was synthesized by all strains.

In crude glycerol cultures, only the yeast *T. globosa* did not present linoleic acid, and *C. laurentii* exhibited it in low amounts. However, the relative percentages for the other yeasts were higher in this medium. Analyzing the presence of this fatty acid in crude glycerol byproduct, it was reported in lower percentages than in pure glycerol and molasses. This result indicates the metabolic efficiency of the strains in absorbing the fatty acid from the medium and metabolizing it, diversifying the profile composition.

Behenic acid (C22:0), primarily identified in the lipid composition of the strains *C. laurentii*, *E. spinifera*, *R. mucilaginosa*, *C. ferigula*, and *T. maleeae*, is considered a very-long-chain fatty acid, reported with low prevalence in previous studies (SITEPU *et al*., 2013). It is synthesized through the elongation of saturated fatty acids C16 or C18. Through desaturase reactions, it can form the polyunsaturated fatty acids docosahexaenoic acid (C22:6) or docosapentaenoic acid (C22:5) from the omega-3 and omega-6 families, respectively (BELLOU *et al*., 2016; REZANKA *et al*., 2019; WYNN; RATLEDGE, 2007). These PUFAs have high economic value due to their various health benefits. The possibility of large-scale production of PUFAs by yeasts sparks industrial interest to meet the demand for these fatty acids for supplements, nutraceuticals, and incorporation into cosmetic products (PATEL *et al*., 2019). The other reported long-chain fatty acid was heneicosanoic acid (C21:0) for *C. ferigula* under pure glycerol cultivation with a relative area of 21%.

Regarding evaluating carotenogenic strains, the species *C. laurentii* and *C. aspenensis* showed a light coloration. However, Gácser *et al*. described in 2001 strains of *Cryptococcus hungaricus* isolated from different environments (flower, seawater, and cereals) capable of growing under psychrophilic conditions and synthesizing carotenoid pigments. These strains were described as red yeasts and are believed to belong to a subclade of *Cystofilobasidium capitatum*, a genus with a pink coloration (Gácser *et al*., 2001).

Red yeasts are *Cystofilobasidium ferigula*, *Rhodotorula mucilaginosa*, *Rhodosporidium toruloides*, and *Rhodotorula dairenensis*. They exhibited an orange coloration on YM medium plates, indicating carotenoid production. They are extensively described and explored for their ability to synthesize carotenoids and other metabolites (CHREPTOWICZ *et al*., 2019; LAKSHMIDEVI *et al*., 2020; VARGAS SINISTERRA; RAMÍREZ CASTRILLÓN, 2020).

β-carotene is one of the most abundant carotenoids in carotenogenic extracts, with a significant presence in the carotenoid market and known antioxidant activity (CIPOLATTI *et al*., 2019; SANCHEZ *et al*., 2013). The present study used it as a parameter to quantify the production of carotenoids by yeasts cultivated in different media. Like most carotenoid compounds, it can be used in the food industry, animal feed supplements, and pharmaceutical industries due to its various biological activities that promote health (REHMAN *et al*., 2020).

Quantifying total carotenoids as β-carotene equivalents by a spectrophotometer is a standard methodology. Ribeiro *et al*. (2019) reported a high production of carotenoids (0.98 mg/L) by the species *Rhodotorula glutinis* cultivated in cassava wastewater. Cipolatti *et al*. (2019) also highlighted a high production of β-carotene by the wild strains *Rhodotorula mucilaginosa* (1.06 µg/mL) and *Sporidiobolus paraseus* (0.6 µg/mL) under YM medium cultivation.

The strain *R. toruloides* (CCMA 2032) exhibited higher carotenoid concentrations, except for the molasses medium. This species is recognized for its potential for high accumulation of lipids and carotenoids, even in a molasses medium (LAKSHMIDEVI *et al*., 2020). Cultivation in crude glycerol medium resulted in higher carotenoid production for all strains. Since carotenoids, like lipids, are lipophilic, their metabolic pathways for synthesis and storage coincide. The synthesis of fatty acids and carotenoids depends on acetyl-CoA as a carbon donor and NADPH for the reductive steps of biosynthesis (ARHAR; NATTER, 2019; SAENGE *et al*., 2011). Therefore, when cultivated in the same substrate, yeasts with efficient lipid accumulation under crude glycerol cultivation through *ex novo* pathways can concurrently trigger higher carotenoid accumulation. The *C. ferigula* strain (CCMA1623) is promising for further studies of the concomitant production of lipids and carotenoids using crude glycerol as substrate, as it showed 41.88% of lipid accumulation and 2.76 µg/mL of total carotenoids, values close to those of strains described as reference. In addition, the species *C. ferigula* is poorly studied, which can increase the visibility of its application in other biotechnological processes.

5 CONCLUSION

Five strains of unconventional wild yeasts, four isolated from coffee and one from Nelore bovine feces, were identified as oleaginous with lipid accumulation ranging from 21% to 68.7%. The species *E. spinifera* (CCMA 2073) stands out with 35% accumulation due to its novelty as an oleaginous yeast, along with *T. maleeae* (CCMA 0039), *C. ferigula* (CCMA 1623), *T. globosa* (CCMA 0021), and *C. laurentii* (CCMA 0025), which showed accumulations of 68.7%, 33%, 30%, and 21%, respectively, with rare reports in the literature. The fatty acid profile synthesized by the strains has a higher abundance of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), which are highly valued by the food, pharmaceutical, and cosmetics industries due to their similarity to vegetable oil and cocoa butter. Crude glycerol can be used as a carbon source for the cultivation of these strains since it provided the highest levels of lipid synthesis and total carotenoid concentration among the species, greater diversity in the fatty acid profile, as well as higher peak area percentages for the major fatty acids, characterizing efficient strains tolerant to impurities. The *C. ferigula* strain (CCMA 1623) is capable of synthesizing lipids and carotenoids, exhibiting 41% lipogenesis and 2.76 µg/mL of total carotenoids in crude glycerol medium. Therefore, it is a relevant strain for co-production exploration, adding value to industrial fermentation processes.

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ARTIGO 2- OPTIMIZATION OF CONCOMITANT PRODUCTION OF LIPIDS AND CAROTENOIDS BY NON CONVENTIONAL YEAST IN CRUDE GLYCEROL

Artigo elaborado de acordo com a NBR 6022 (ABNT, 2018)

ABSTRACT

This study aimed to select cultivation conditions capable of providing maximum concomitant production of lipids and carotenoids by the strain *Cystofilobasidium ferigula* CCMA 1623, cultivated in a crude glycerol medium. The optimization was evaluated through Central Composite Design (CCD), using response surface methodology to assess the effects of the parameters of yeast extract concentration, temperature, and oxygen incorporation through homogenization on the production of metabolites. The fatty acid profile synthesized by *C. ferigula* was identified by Gas Chromatography-Mass Spectrometry (GC/MS), and the antioxidant potential of the total carotenoid extract was also determined. The results revealed that the yeast extract concentration is the determining factor for lipogenesis and carotenogenesis, as it is related to the carbon-to-nitrogen ratio of the medium, which triggers the accumulation of metabolites. The optimal condition for the accumulation of lipids and carotenoids by *C. ferigula* was 1.5 g/L of yeast extract at 26°C with homogenization at 140 rpm. Under these conditions, a lipid content of 52.59% and 3.03 µg/mL of carotenoids were obtained. The fatty acid profile obtained was similar to vegetable oils and cocoa butter, mainly composed of palmitic acid, stearic acid, and two omega fatty acids (oleic and linoleic). The total carotenoid extract exhibited high antioxidant power in ABTS (365.37±1.9 µM Trolox/L), FRAP (385.02±19.6 µM ferrous sulfate/L), and DPPH (49.5% ± 1.9) assays. This study opens up new prospects for establishing a larger-scale process for producing lipids and carotenoids by the strain *C. ferigula* using a viable medium, ensuring a sustainable process.

Keywords: Bioprocess. Carotenoids. *Cystofilobasidium ferigula*. Subproduct. Fatty acids.

1 INTRODUCTION

Specific oleaginous yeasts can exhibit orange, pink, or red coloration, and in addition to accumulating lipids, they store carotenoid pigments in the cell wall and intracellular organelles called lipid droplets (LAKSHMIDEVI *et al*., 2021). Both compounds are lipophilic and share common points in their metabolic pathways, such as the requirement of acetyl-CoA as a carbon donor and NADPH for the reductive steps. Thus concurrent accumulation can occur in the cell through triacylglycerols and isoprenoids (ARHAR; NATTER, 2019; FAKANKUN; LEVIN, 2023).

Microbial lipids (single-cell oil) are high-value products due to their chemical composition similar to those obtained from plant extraction, and they can replace or serve as an alternative source for obtaining the same compounds. Lipids can be used in biofuels, detergents, and as fine chemicals in the food, pharmaceutical, and cosmetic industries (GONG *et al*., 2019; PATEL *et al*., 2020). Carotenoids also have a high and expanding market value, estimated to reach 2 billion dollars by 2026 (LIU *et al*., 2021). They can be employed as colorants in the food, feed, cosmetics, and pharmaceutical industries. They also perform various beneficial biological activities in the body, such as anti-tumor effects, regulation of metabolic activities, antioxidant action, and serving as precursors for vitamin A, among others. Similarly to lipids, they can be used as nutraceuticals (CIPOLATTI *et al*., 2019; VARGAS-SINISTERRA; RAMÍREZ-CASTRILLÓN, 2021).

Currently, the production of lipids and carotenoids is carried out through plant extraction and chemical synthesis, respectively, raising concerns about competition for land used for food production due to low yields, high purification costs, and long cultivation periods (ARHAR; NATTER, 2019). Yeasts are alternative microbial sources for synthesizing these compounds, capable of accumulating up to 70% of their dry weight as lipids. They are relevant microorganisms for biotechnological processes due to the advantages of easy and rapid cultivation, construction of robust platforms, and the ability to utilize byproducts and industrial waste as substrates, contributing to the development of sustainable processes (XUE *et al*., 2023). Moreover, obtaining natural compounds offers more significant potential for commercialization attributed to the health benefits to consumers compared to chemical production routes (CIPOLATTI *et al*., 2019).

Crude glycerol is the main byproduct generated from biodiesel production (approximately 10% w/w). Other industrial sectors, such as soap, fatty acids, and fatty esters industries, also generate this byproduct, albeit in smaller quantities (KUMAR *et al*., 2019). As

biodiesel production expands, several studies focus on using crude glycerol in biotransformation processes. The reuse of this byproduct in microbial fermentation processes is significant due to its composition, with 50 to 60% glycerol, constituting a cheap carbon source for microbial cultivation (CIPOLATTI *et al*., 2019; MANOWATTANA *et al*., 2018; SAENGE *et al*., 2011; UPRETY; DALLI; RAKSHIT, 2017).

The yeast *Cystofilobasidium ferigula* was described in 2001 by Sampaio *et al*. as an unconventional pink yeast. Some studies report the production of neutral lipids and accumulation of carotenoids by other species of the genus *Cystofilobasidium* using alternative substrates (CHREPTOWICZ *et al*., 2019; KOT *et al*., 2018; PETRIK *et al*., 2013; SZOTKOWSKI *et al*., 2019). However, research on the species *Cystofilobasidium ferigula* is scarce. In a previous study (Article 1), the strain *Cystofilobasidium ferigula* CCMA 1623 showed a lipid accumulation content above 20% of its dry biomass in cultures using pure glycerol (33.33% w/w), sugarcane molasses (25.54% w/w), and crude glycerol (41.88% w/w), thus being considered an oleaginous strain (unpublished data). Under cultivation in crude glycerol, in addition to higher lipogenesis, a higher concentration of total carotenoids (2.76 µg/mL) was also obtained, arousing interest for further studies focused on this species, which is still relatively unexplored.

The biosynthesis of fatty acids and carotenoids in yeasts is related to cultivation conditions such as carbon and nitrogen sources, carbon/nitrogen ratio, pH, temperature, aeration rate or dissolved oxygen, and the presence of cellular inhibitors (FAKANKUN; LEVIN, 2023; RAPOPORT *et al*., 2021). In order to enhance biotechnological processes to achieve maximized production of lipids and carotenoids by yeasts, optimization strategies for cultivation parameters are employed. This study optimized the fermentation parameters of nitrogen rate, temperature, and oxygen incorporation by homogenization to simultaneously produce lipids and carotenoids by *C. ferigula* CCMA 1623, using crude glycerol as the carbon source. Through optimization, combinations of conditions are selected to obtain higher metabolite yields at a lower cost, aiming for scale-up and industrialization of microbial lipid and carotenoid production (LIU *et al*., 2021; MAHAJAN; SENGUPTA; SEN, 2019).

2 MATERIALS AND METHODS

2.1 Microorganism

The strain *Cystofilobasidium ferigula* (CCMA 1623) from the Collection of Agricultural Microbiology Cultures (CCMA, Federal University of Lavras, Lavras, MG, Brazil) isolated from coffee, was selected based on a previous screening study of strains for the production of lipids and carotenoids using agro-industrial by-products as carbon sources (sugarcane molasses and crude glycerol). The microorganism was reactivated in test tubes containing 2 mL of YEPG culture medium (g/L: yeast extract, 10; peptone, 20; and glucose, 20) for 24 hours at a temperature of 28°C.

2.2 Characterization of the substrate for the production of lipids and carotenoids

Crude glycerol, provided by the Biodiesel Laboratory of the Federal University of Lavras, was used as an alternative carbon source for the cultivation of the strain *Cystofilobasidium ferigula* CCMA 1623 for the co-production of lipids and carotenoids. The substrate was characterized by High-Performance Liquid Chromatography (HPLC) for the composition of carbohydrates, organic acids, and the presence of furfural and 5-methylfurfural through high-performance liquid chromatography (HPLC). The samples were centrifuged (10,000 rpm) for 5 minutes at 4 °C. The supernatant was recovered and filtered using sterile syringe filters (pore size 0.22 µm; Kasvi, Brazil). Perchloric acid (1 µL) was added to acid samples only to equate the sample pH to the mobile phase (pH 2.1), followed by centrifugation and filtration as described above. The analyses were performed using an HPLC system (model LC-10Ai; Shimadzu Corp., Tokyo, Japan) equipped with a dual detection system consisting of a UV-Vis detector (SPD-10Ai; Shimadzu) and a UV refractive index detector (RID-10Ai; Shimadzu). A Shimadzu ion-exclusion column (Shim-pack SCR-101H, 7.9 mm × 30 cm) was used for the determination of alcohols, carbohydrates (30 °C), and organic acids (50 °C) (FERREIRA *et al*., 2022). The mobile phase consisted of ultrapure water (carbohydrates and alcohols) and acidified ultrapure water (pH 2.1) for acids at a 0.6 mL/min flow rate. For the identification of furfural and 5-methylfurfural, the samples were acidified, and the same column was used, eluted with H2SO4 (0.005 M), at 60 °C, with a flow rate of 0.6 mL/min, for 50 minutes, and UV light detection at 280 nm (LIU *et al*., 2021). The compounds were identified based on the retention time of standards, and their concentrations were determined by the external calibration method.

2.3 Culture medium and submerged cultivation conditions used in optimization tests

The yeast was inoculated in the fermentation medium adapted from Papanikolaou *et al*. (2003), containing g/L: KH_2PO_4, 7.0; NaH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 2H_2O$, 0.15; $ZnSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot H_2O$, 0.06; $(NH_4)_2SO_4$, 0.5; yeast extract, (0.5, 1.0, or 1.5); glucose, 5.0; crude glycerol, 40; pH 6.0. 10^7 cells/mL were inoculated in 250 mL flasks containing 150 mL of fermentation medium and incubated for 96 hours, varying the temperature (26 °C, 28 °C, or 30 °C) and agitation (140 rpm, 160 rpm, or 180 rpm) in an orbital shaker (New Brunswick Scientific, Excella E25 model; Edison, New Jersey, USA). Samples were taken at 0, 24, 48, and 96 hours of fermentation to monitor yeast growth in the assays by counting the number of cells in a Neubauer chamber. Methodology adapted from Souza *et al*. (2017).

2.4 Experimental design

The accumulation of lipids and carotenoids by the strain *Cystofilobasidium ferigula* (CCMA1623) using crude glycerol as a carbon source was optimized using the Central Composite Design (CCD). The parameters of yeast extract rate, temperature, and oxygen transfer by shaking were evaluated to obtain higher lipid and carotenoid yields from the yeast. Table 1 lists the process variables and their respective decoded values according to their lower, central, and upper levels.

Table 1 - Parameters used in the central composite design.

Parameters	**Code**	**Levels**		
		-1	**0**	**+1**
Yeast extract rate (g/L)	X1	0.5	1.0	1.5
Temperature (°C)	X2	26	28	30
Homogenization (rpm)	X3	140	160	180

Source: author (2023).

According to the 2^3 factorial planning matrix, seventeen trials were carried out of the Central Composite Design (CCD), as shown in Table 2.

Table 2 - Matrix of the central composite design 2^3 for evaluation of lipid and carotenogenic synthesis using different rates of yeast extract, temperatures and homogenization as independent variables.

Assay	Coded variables			Real variables		
	X1	X2	X3	Yeast extract rate (g/L)	Temperature (ºC)	Homogenization (rpm)
1	-1	-1	-1	0.5	26	140
2	+1	-1	-1	1.5	26	140
3	-1	+1	-1	0.5	30	140
4	+1	+1	-1	1.5	30	140
5	-1	-1	+1	0.5	26	180
6	+1	-1	+1	1.5	26	180
7	-1	+1	+1	0.5	30	180
8	+1	+1	+1	1.5	30	180
9	-1	0	0	0.5	28	160
10	+1	0	0	1.5	28	160
11	0	-1	0	1.0	26	160
12	0	+1	0	1.0	30	160
13	0	0	-1	1.0	28	140
14	0	0	+1	1.0	28	180
15	0	0	0	1.0	28	160
16	0	0	0	1.0	28	160
17	0	0	0	1.0	28	160

Source: author (2023).

The results obtained from the applied experimental model were evaluated using Statistica Release software version 7.1, Stat Soft. Inc., USA. After obtaining the optimization results, a validation assay was performed under the predicted optimal cultivation conditions to confirm lipid and carotenoid production.

2.5 Extraction and determination of lipid content

Lipid extraction was performed following the protocol of Bligh and Dyer (1959) with modifications. Cells (100 mL) were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C and washed twice with distilled water. The weight of the dried pellet was recorded after drying in an oven at 50 °C for 12 hours. 5 mL of 2 M HCl was added to the dried biomass to disrupt the yeast cell wall and placed in a water bath at 70 °C for 1 hour. After cooling to room temperature, centrifugation was carried out at 5,000 rpm for 10 min, and the acid was discarded (SOUZA *et al*., 2017). The sediment was resuspended in 3.75 mL of chloroform/methanol (solution 2:1, v/v) and vortexed for 15 min, followed by the addition of 1.25 mL of chloroform and 1.25 mL of 1 M NaCl, with vortexing at each step. The mixture was centrifuged at 5,000 rpm for 15 min to separate the aqueous and organic phases. It formed a three-phase system composed of a lower liquid phase (chloroform and lipid content), an interfacial solid phase (biomass), and an upper liquid phase (methanol and water).

The lower organic phase was transferred to a pre-weighed and dried flask and taken to the fume hood for complete solvent evaporation. The weight of the flask was re-recorded. Therefore, the lipid content of the dried cell biomass was calculated using the formulas below (SINGH *et al*., 2020):

$$(1)\ \text{Lipid content (g/L)} = \frac{\text{Weight of lipid content in the vial (g)} - \text{empty vial weight (g)}}{\text{Culture sample volume (L)}}$$

$$(2)\ \text{Lipid content (\%)} = \frac{\text{Lipid content (g/L)}}{\text{Cell dry weight (g/L)}} \times 100$$

2.6 Extraction of carotenoids

The recovery of total carotenoids was performed according to the methodology described by Valduga et al. (2009) with modifications, using acetone: methanol solvent solution (7:3, v/v). The biomass recovered from the fermentation medium was centrifuged (5,000 rpm, 4 °C, 10 min) and dried in an oven at 50 °C for 24 hours. Subsequently, the dried biomass was ground in liquid nitrogen, 2 mL of dimethyl sulfoxide (DMSO) was added, and it was heated in a water bath at 50 °C for 30 min. After this, 2 mL of the mixed solvent was added, followed by centrifugation (5,000 rpm, 4 °C, 10 min). The supernatant was separated, and successive extractions were performed with the solvent until the cells remained colorless.

At the end of the extraction process, the samples were analyzed using a UV-VIS spectrophotometer (Model: SP 2000 UV Spectrum) at 450 nm to quantify the produced and extracted carotenoids. For this purpose, a calibration curve was constructed, as described below.

2.6.1 Beta-carotene standard curve

To construct the beta-carotene calibration curve, Beta-carotene (SIGMA) was used. A beta-carotene solution in acetone and methanol in a 1:1 ratio (v/v) was prepared. Triplicate dilutions were prepared at concentrations of 3.0, 2.0, 1.25, 0.312, 0.156 µg/mL, followed by reading on a UV-VIS spectrophotometer (Model: SP 2000 UV Spectrum) at 450 nm. From the absorbance values obtained from the readings, a concentration vs. absorbance graph was constructed, obtaining the linear correlation that generated the equation of the line and the R^2 value. Using the equation of the line, the total carotenoids corresponding to the beta-carotene standard produced during fermentation were quantified. Methodology adapted from Kot *et al*. (2020), with modifications to the concentration values of β-carotene and the acetone and methanol solution used for dilutions.

2.7 Analysis of fatty acids by gas chromatography coupled with mass spectrometry

The transesterification of the lipids extracted from the cells to obtain the methyl esters of fatty acids was performed according to the methodology of Singh et al. (2018). 2 mL of n-hexane and 1 mL of 2 M methanolic KOH was added to the tubes, which were then capped and vigorously shaken for 30 seconds and then incubated at 70°C for 20 minutes. After cooling to room temperature, 1.2 mL of 1 M HCl with gentle agitation and 1 mL of n-hexane were added. Subsequently, the mixture was allowed to settle for phase separation. The upper phase containing the fatty acid methyl esters (FAMEs) was transferred to a glass vial for subsequent identification using gas chromatography (GC).

The chromatographic analysis of the samples was performed according to the methodology of Singh *et al*. (2020) with column adaptation. The GC/MS system (model GCMS-QP2010SE; Shimadzu, Tokyo, Japan) equipped with a capillary column (Rtx-5MS) of 30 m in length and 0.25 µm in thickness coupled to a quadrupole detector was used. An electron ionization system with an ionization energy of 70 eV was used, and the carrier gas (helium 99.99%) was supplied at a constant flow rate of 1.1 ml/min. The mass transfer line and injector

temperature were set at 220°C and 250°C, respectively. The oven temperature was programmed as follows: the initial temperature was 50°C for 2 min, then increased at a rate of 4°C/min to 220°C for 10 min, followed by 250°C for 2 minutes after the run. Approximately 1 µl of the sample was injected in a 10:1 split mode. The signals were recorded in full scan mode (20–600 m/z). All components were identified by comparing their mass spectra with the NIST database in the GC/MS Solution software library version 2.6.

2.8 Confocal microscopy for visualization of lipid storage

At the end of the 96-hour cultivation period, 10 mL samples were taken from the validation assay. The cells were centrifuged and washed with 10 mM potassium phosphate buffer (pH 7.0; PBS). A solution was prepared with an aliquot of 100 µL of cells and 2 mL of phosphate buffer, and then 10 µL of 0.1 mg/mL Nile Red dye (Sigma) in acetone was added to visualize lipid droplets. After 10 minutes, the stained yeast cells were visualized using a Zeiss Axio-Observer Z1 LSM 780 confocal microscope with an excitation filter of 540-580 nm and a 40x objective lens (KIMURA; YAMAOKA; KAMISAKA, 2004).

2.9 Antioxidant analysis of total carotenoid extract

The carotenogenic extracts obtained from the yeast biomass in the validation assay were tested for antioxidant activity under different oxidative conditions. The radical scavenging activity of the total carotenoid extract sample was evaluated using the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical according to Rufino *et al*. (2010) with modifications. Briefly, 30 µL of the extract samples or reference substance (Trolox) were added to 3 mL of the ABTS radical solution and allowed to react in the dark for 6 minutes, followed by absorbance measurement at 734 nm. Quantification was based on a Trolox standard curve, and the results were expressed as micromoles of Trolox equivalents (TE) per liter of the sample (µmol TE/L).

The ferric-reducing antioxidant power (FRAP) was determined based on the protocol developed by Rufino *et al*. (2010). The FRAP reagent was prepared from a 0.3 M acetate buffer (pH 3.6) solution, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ), and 20 mM ferric chloride. In the dark, 90 µL of each extract dilution was mixed with 2.7 mL of the FRAP reagent and 270 µL of distilled water. The mixture was homogenized and incubated in a water bath at 37°C for 30 minutes, followed by absorbance measurement at 593 nm using a spectrophotometer. A 2 mM

ferrous sulfate solution was used to construct the calibration curve at concentrations of 500, 1000, 1500, and 2000 µM/mL, and the results were expressed as µM of FeSO4 equivalents per liter of extract.

The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method described by Rufino *et al*. (2010), with a modified reaction time. A solution of 60 µM DPPH in methanol was prepared and protected from light, and the carotenogenic extract was added. After 60 minutes, the absorbance was determined at 515 nm. The results were expressed as a percentage of DPPH inhibition compared to control samples without carotenoids.

3 RESULTS

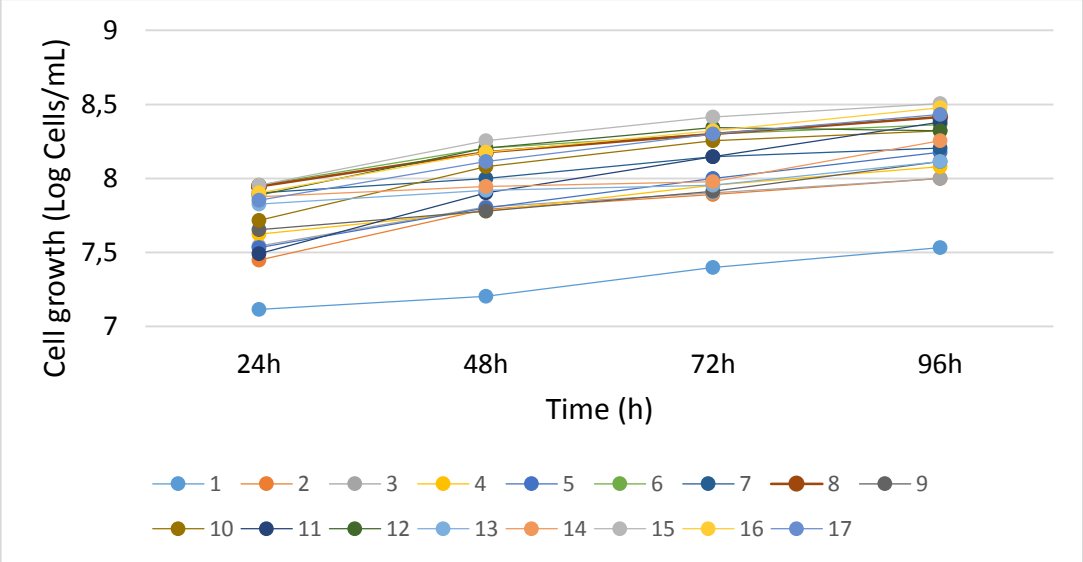
3.1 Substrate characterization

The crude glycerol byproduct was characterized by acetic acid at a concentration of 0.197 g/L and 386.83 g/L of glycerol. The sample did not show peaks related to potentially toxic compounds, furfural, and 5-methylfurfural.

3.2 Cell growth of *C. ferigula* CCMA 1623 in optimization assays for lipid and carotenoid production

The growth of *C. ferigula* CCMA 1623 in crude glycerol medium during 96 hours of fermentation under different cultivation conditions (referring to the 17 assays) is shown in Figure 1. The highest cell counts at the end of the 96 hours were obtained in assays 15, 16, 17 with concentrations of $3.2x10^8$, $3.0x10^8$, $2.7x10^8$ cells/mL, respectively. Assay 1 had the lowest cell count/mL ($3.4x10^7$).

Figure 1 - Cell growth of *C. ferigula* CCMA1623 under cultivation in crude glycerol and different parameters for the optimization of lipid and carotenoid production (assays 1-17).



Source: author (2023).

3.3 Optimization for concurrent production of lipids and carotenoids

The effects of yeast extract concentration, temperature, and oxygen incorporation by homogenization on the production of lipids and carotenoids by *Cystofilobasidium ferigula* CCMA 1623 were evaluated using a Central Composite Design (CCD). The lipid production content varied from 26.25% to 68.72%. Lipid contents above 60% were observed in assays 9 (0.5 g/L yeast extract, 28 °C, 160 rpm), 1 (0.5 g/L yeast extract, 26 °C, 140 rpm), 7 (0.5 g/L yeast extract, 30 °C, 180 rpm), and 3 (0.5 g/L yeast extract, 30 °C, 140 rpm), with 68.72%, 66.29%, 64.57%, and 60.67%, respectively (Table 3).

The carotenoid concentration ranged from 1.02 to 3.30 µg/mL (Table 3). Higher carotenogenic concentrations, above 3.0 µg/mL, were found in assays 2 (1.5 g/L yeast extract, 26 °C, 140 rpm), 12 (1.0 g/L yeast extract, 30 °C, 160 rpm), 13 (1.0 g/L yeast extract, 28 °C, 140 rpm), 14 (1.0 g/L yeast extract, 28 °C, 180 rpm), and at the central point, assays 15, 16, and 17 (1.0 g/L yeast extract, 28 °C, 160 rpm).

Table 3 - Experimental design matrix for the production of lipids and carotenoids by *Cystofilobasidium ferigula* (CCMA 1623) in submerged fermentation using crude glycerol as a substrate, and values of the independent variables used in the CCD (yeast extract, temperature, homogenization).

Assay	Coded variables			Real variables			Lipid content (%)	Concentration of total carotenoids (µg/mL)
	X1	X2	X3	Yeast extract concentration (g/L)	Temperature (°C)	Homogenization (rpm)		
1	-1	-1	-1	**0.5**	**26**	**140**	**66.29**	1.62
2	1	-1	-1	**1.5**	**26**	**140**	58.43	**3.14**
3	-1	1	-1	**0.5**	**30**	**140**	**60.67**	1.62
4	1	1	-1	1.5	30	140	50.75	2.33
5	-1	-1	1	0.5	26	180	51.84	1.02
6	1	-1	1	1.5	26	180	49.86	1.98
7	**-1**	**1**	**1**	**0.5**	**30**	**180**	**64.57**	1.89
8	1	1	1	1.5	30	180	42.29	2.31
9	-1	0	0	**0.5**	**28**	**160**	**68.72**	1.44
10	1	0	0	1.5	28	160	31.86	2.17
11	0	-1	0	1	26	160	29.63	1.84
12	0	1	0	**1**	**30**	**160**	28.91	**3.26**
13	0	0	-1	**1**	**28**	**140**	27.75	**3.27**
14	0	0	1	**1**	**28**	**180**	35.86	**3.25**
15	0	0	0	**1**	**28**	**160**	28.27	**3.30**
16	0	0	0	**1**	**28**	**160**	27.22	**3.25**
17	0	0	0	**1**	**28**	**160**	26.25	**3.27**

Source: author (2023).

The data were subjected to analysis of variance (ANOVA, Table 4.1 and 4.2), and the model fit was evaluated using the coefficient of determination (R^2). For lipid content and carotenoid concentration, the model indicated R^2=0.89. The model adequately fits the quadratic experimental data, explaining 89% of the variability in the response. The F-test was used to verify the statistical significance of the model, and the p-value indicates significant regression at a 5% probability level ($p \leq 0.05$).

Table 4.1 - Analysis of variance (ANOVA) for lipid production by *Cystofilobasidium ferigula* (CCMA 1623) in submerged fermentation.

Factors	**GL**	**SQ**	**QM**	**F-value**	**p-value**
Regression	9	3504.02	389.34	6.61	0.0105*
Residue	7	412.43	58.92		
Total	16	3916.45			

***** Significant at the 5% probability level; R^2: 0,8947.
Source: author (2023).

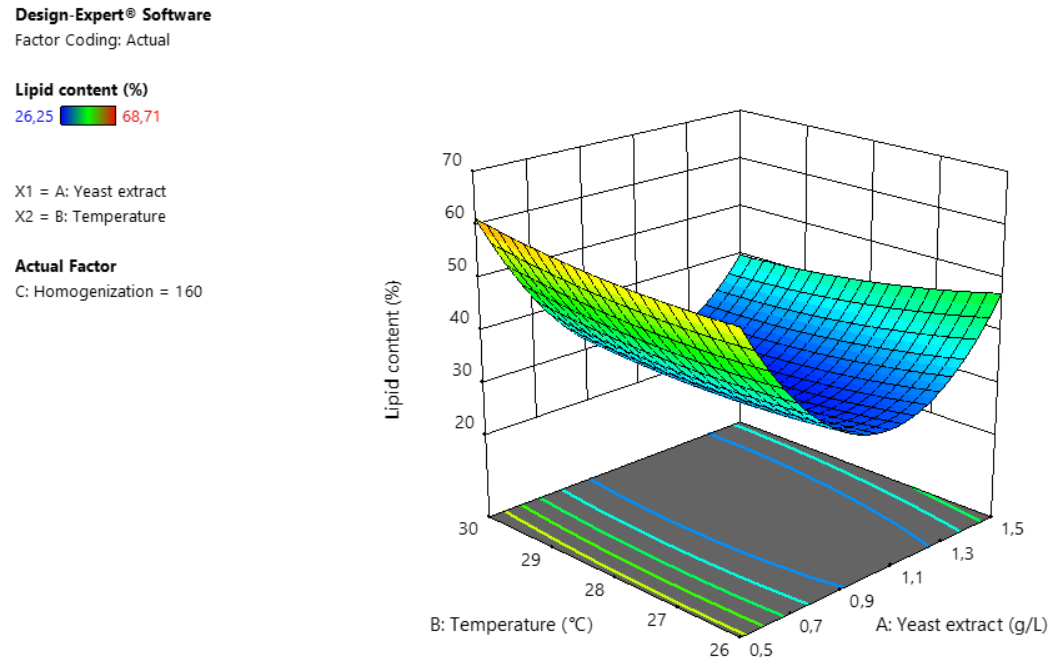
Table 4.2 - Analysis of variance (ANOVA) for carotenoid production by *Cystofilobasidium ferigula* (CCMA 1623) in submerged fermentation.

Fatores	**GL**	**SQ**	**QM**	**F-value**	**p-value**
Regression	9	8.83	0.9810	6.51	0.0109*
Residue	7	1.05	0.1507		
Total	16	9.88			

* Significant at the 5% probability level; R^2: 0,8933.
Source: author (2023).

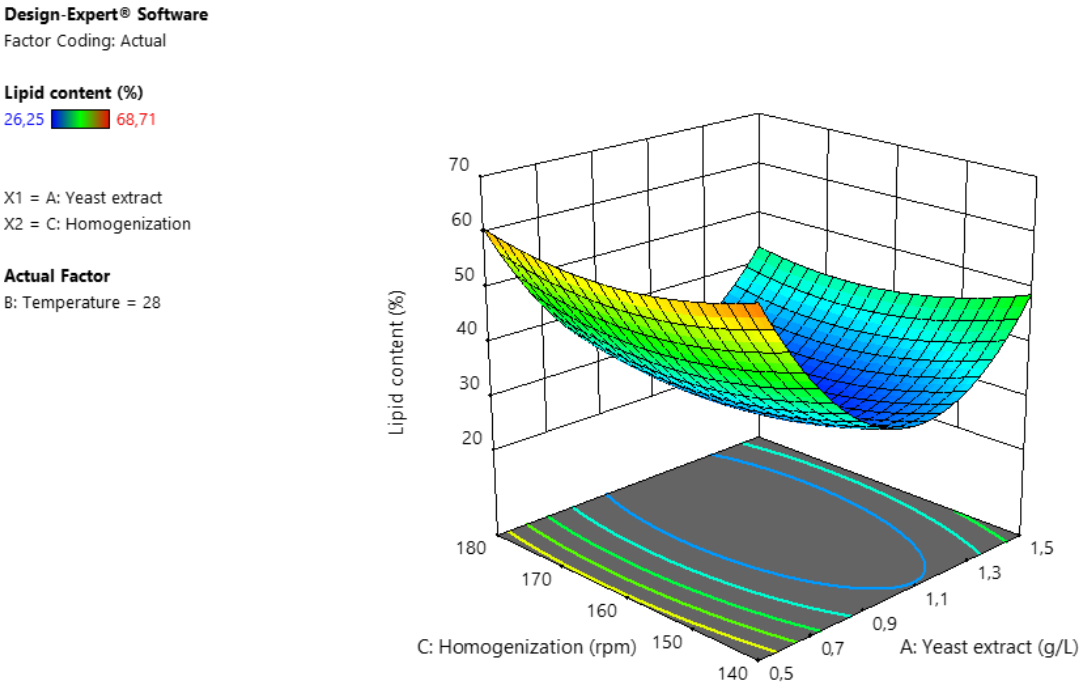
The response surface plots highlight the regions of optimal production for the studied compounds (lipids and carotenoids) based on the evaluated parameters. When evaluating the parameters of temperature and yeast extract concentration, it was observed that the temperature range analyzed did not influence the lipid content. However, the yeast extract concentration significantly influenced lipid production, with higher production when the cultivation was conducted with 0.5 g/L of yeast extract, regardless of the temperature used (Figure 2). When evaluating the parameters of homogenization and yeast extract, it was also possible to observe that the homogenization factor did not influence lipid production. In contrast, the yeast extract concentration (0.5 g/L) was more efficient, resulting in a higher lipid content (Figure 3). Using yeast extract at 1 g/L resulted in lower lipid accumulation. Temperature and homogenization did not significantly influence lipid production, as indicated by the flat and blue-colored graph, indicating a low lipid content (Figure 4).

Figure 2 – Three- dimensional response surface plot of the interactive effects of temperature and yeast extract concentration on lipid accumulation by *C. ferigula* CCMA1623.



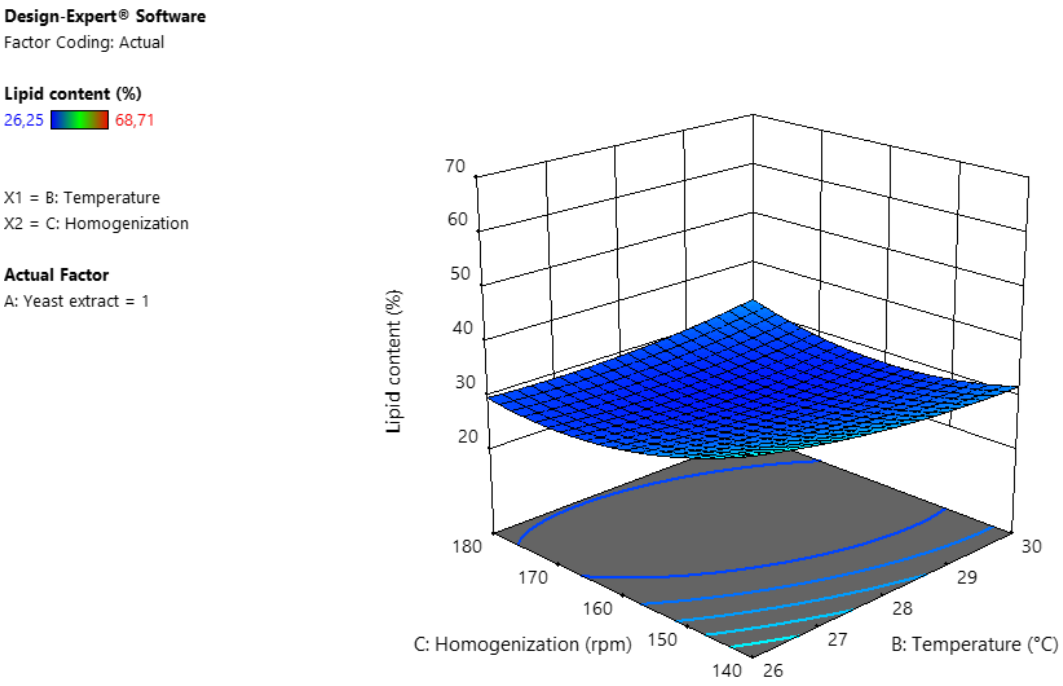
Source: author (2023).

Figure 3 - Three- dimensional response surface plot of the interactive effects of yeast extract concentration and homogenization on lipid accumulation by *C. ferigula* CCMA1623.



Source: author (2023).

Figure 4 - Three- dimensional response surface plot of the interactive effects of temperature and homogenization on lipid accumulation by *C. ferigula* CCMA1623.

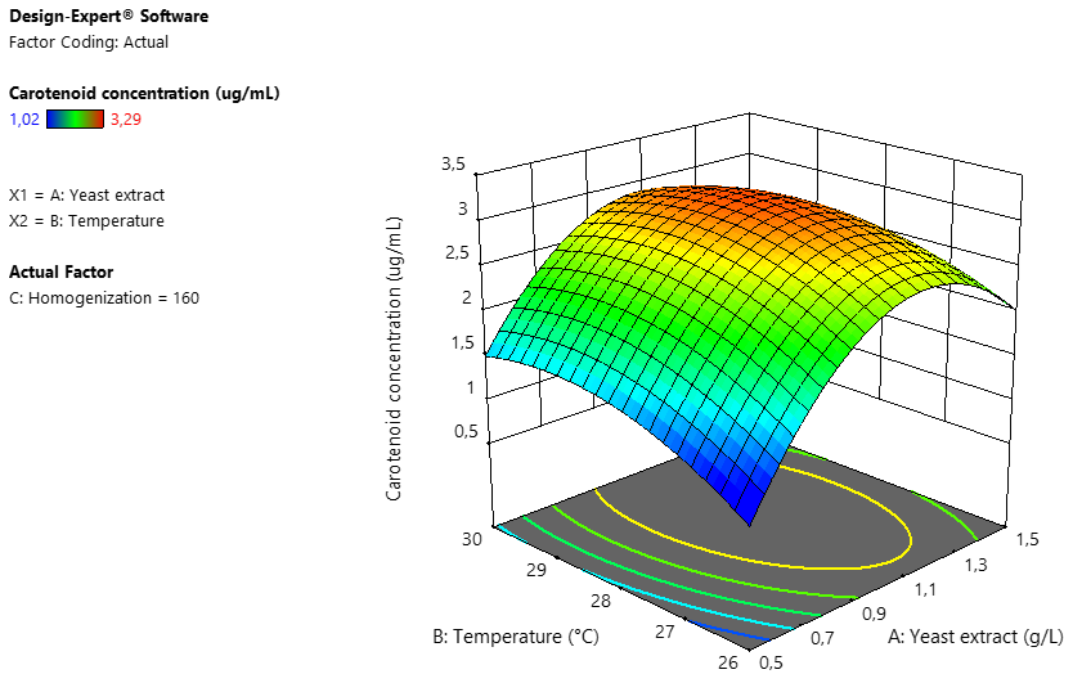


Source: author (2023).

Analyzing the three cultivation parameters (temperature, yeast extract concentration, and homogenization) used in the optimization assays, it is noted that at a lower yeast extract concentration, there was an increase in lipid production, regardless of the evaluated temperature and homogenization speed.

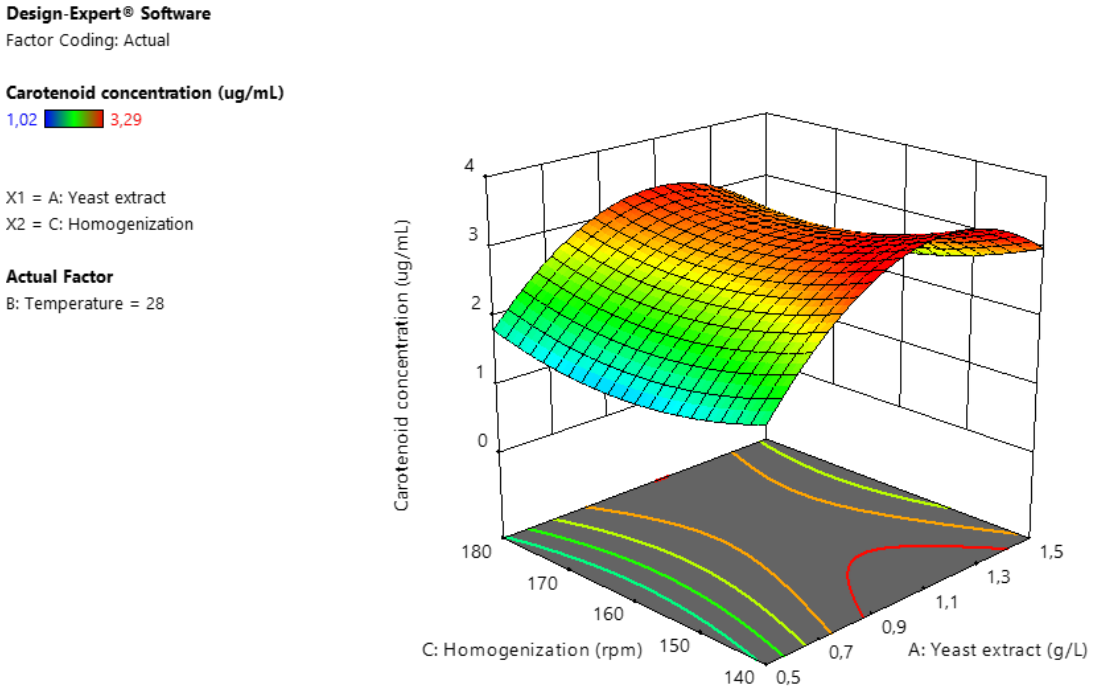
Regarding carotenoids, the temperature was not a factor that influenced production. However, higher carotenoid concentrations were obtained using yeast extract at 1.0 g/L (Figure 5). Comparing homogenization and yeast extract concentration, it was observed that the first parameter did not influence carotenoid production. Again, a higher accumulation was observed near the central point of the yeast extract concentration, decreasing as it approached the upper and lower points (Figure 6). In contrast to the results found for lipid production, higher production of carotenoids occurred in cultures using 1.0 g/L of yeast extract. This can be observed when fixing this value compared to the different temperature and homogenization parameters levels, where the graph exhibited a flat behavior, revealing the range of higher carotenoid production, ranging from 3.25 to 3.30 µg/mL (Figure 7).

Figure 5 - Total carotenoid concentration synthesized by *C. ferigula* CCMA1623 as a function of temperature and yeast extract concentration used in fermentation (response surface).



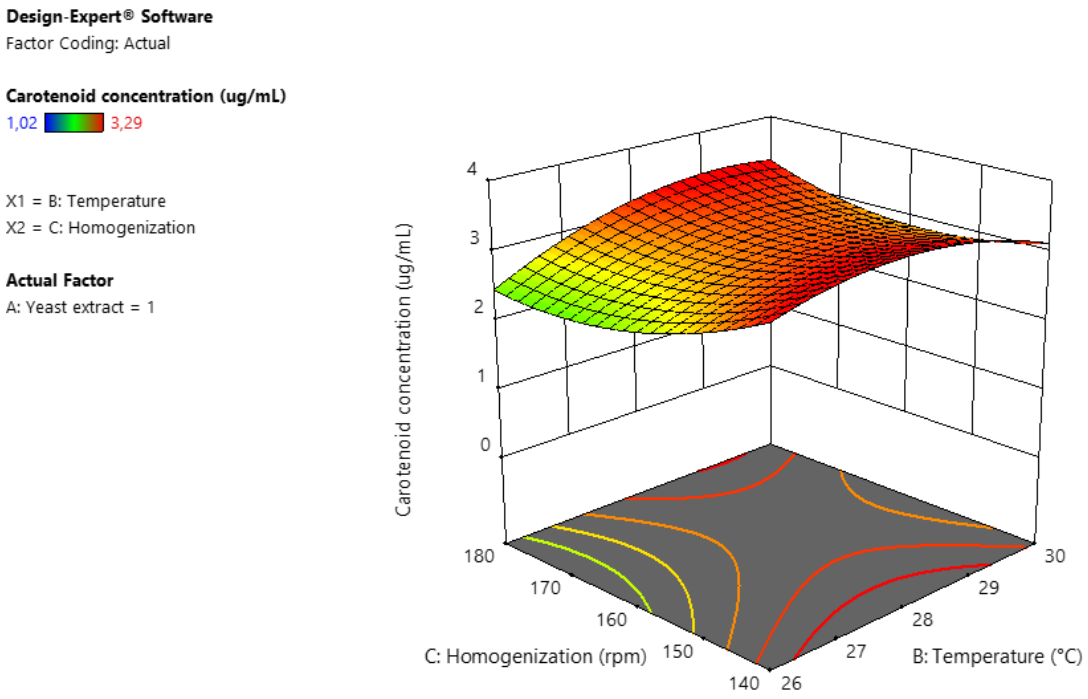
Source: author (2023).

Figure 6 - Concentration of total carotenoids synthesized by *C. ferigula* CCMA1623 as a function of homogenization and concentration of yeast extract used in fermentation (response surface).



Source: author (2023).

Figure 7 - Concentration of total carotenoids synthesized by *C. ferigula* CCMA1623 as a function of homogenization and temperature used in fermentation (response surface).



Source: author (2023).

Using 1.0 g/L of yeast extract resulted in higher carotenoid production, regardless of the variation in the other cultivation parameters (temperature and homogenization speed).

The parameter that influenced the production of lipids and carotenoids was the concentration of yeast extract used. The temperature and homogenization parameters did not show significant effects. However, when evaluating the production of metabolites separately, a lower nitrogen source concentration (0.5 g/L) favored lipid production and accumulation. In comparison, the intermediate concentration corresponding to the central point of yeast extract (1.0 g/L) led to higher carotenoid concentrations. The optimal point for lipid and carotenoid production, obtained through statistical analysis of the Central Composite Design, was 1.5 g/L of yeast extract at 26°C with homogenization at 140 rpm, resulting in a lipid content of 56.49% and a carotenoid concentration of 2.88 µg/mL. This point coincides with experiment 2, which yielded a lipid production of 58.43% and a carotenoid content of 3.14 µg/mL. The validation assay resulted in a lipid accumulation of 52.59% and a total carotenoid concentration of 3.03 µg/mL.

3.4 Fatty acid profile synthesized by *C. ferigula*

The strain *C. ferigula* CCMA 1623, cultivated in crude glycerol medium under the conditions of 26°C, 1.5 g/L of yeast extract, and 140 rpm, presented a profile composed of saturated fatty acids, three unsaturated fatty acids (hexadecenoic, heptadecenoic, and octadecenoic), and one polyunsaturated fatty acid (9,12 octadecadienoic). The fatty acids palmitic (C16:0), stearic (C18:0), linoleic (C18:2), and oleic (C18:1) showed higher relative peak area percentages of 54.72%, 13.66%, 11.53%, and 10.61%, respectively. Two long-chain fatty acids were also identified in low percentages: eicosanoic acid (C20:0) with 0.39% and docosanoic acid (C22:0) with 0.23%. Fatty acids with 10, 12, 14, and 15 carbon atoms were also present in the fatty acid profile, exhibiting low relative peak area percentages, except for tetradecanoic acid (5.15%) (Table 5).

Table 5 - Fatty Acid Profile Synthesized by the *Cystofilobasidium ferigula* strain (CCMA 1623) cultivated in crude glycerol medium.

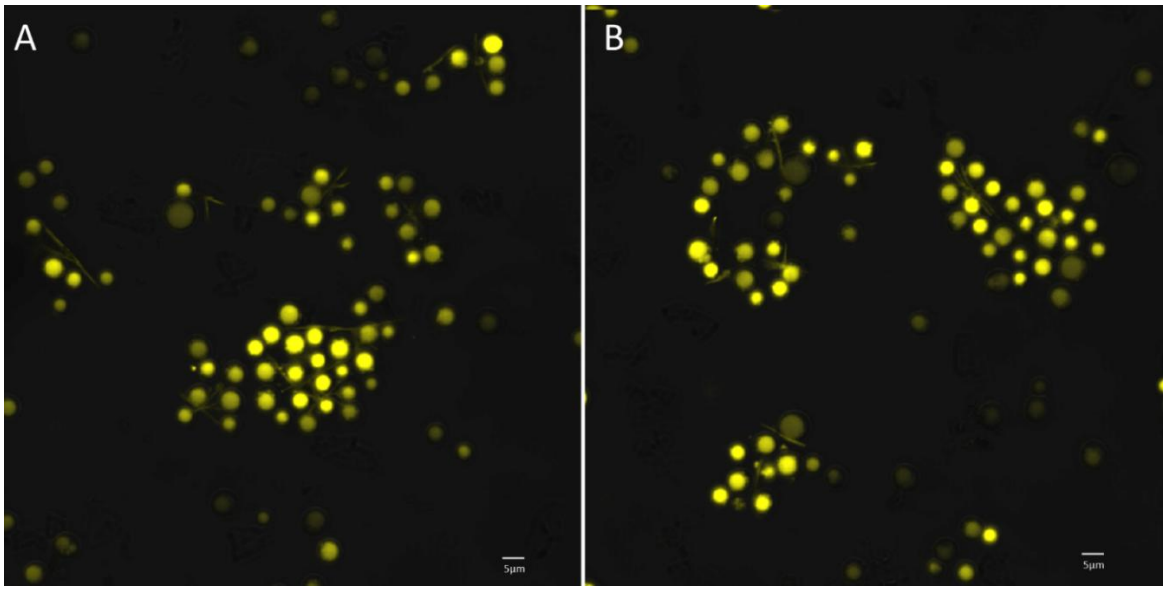
Fatty acid	Retention time (min)	Peak area	% Relative area
C10:0 Decanoic	22.952	47.278	0.07
C12:0 Dodecanoic	29.618	154.277	0.22
C14:0 Tetradecanoic	35.622	3524.07	5.15
C15:0 Pentadecanoic	38.38	328.855	0.48
C16:1 Hexadecenoic/palmitoleic	40.53	1778.147	2.64
C16:0 Hexadecanoic/ palmitic	41.185	37451.38	54.72
C17:1 Heptadecenoic	42.951	99.808	0.14
C17:0 Heptadecanoic	43.555	113.364	0.16
C18:2 9,12-Octadecadienoic/ linoleic	45.563	7893.827	11.53
C18:1 9-Octadecenoic/ oleic	45.657	7263.855	10.61
C18:0 Octadecanoic/ stearic	46.18	9351.347	13.66
C20:0 Eicosanoic	53.074	270.194	0.39
C22:0 Docosanoic	61.155	159.767	0.23
Total peak area		68,436.17	100%

Source: author (2023).

3.5 Qualitative analysis of lipid storage and total carotenoid extract antioxidant activity

Figure 8, images A and B, confirm the lipid accumulation in the yeast *Cystofilobasidium ferigula* (CCMA 1623), where it is possible to visualize the lipid droplets stained in golden yellow through exposure to the lipophilic dye Nile Red.

Figure 8 - Confocal fluorescence microscopy photographs of lipid accumulation by *C. ferigula* CCMA1623.



Source: author (2023).

The validation assay's total carotenoid extract (3.03 µg/mL) interacted with the ABTS radical, exhibiting an antioxidant activity equivalent to 365.37±1.9 µM Trolox/L. The ferric-reducing power of the extract, as determined by the FRAP assay, was 385.02±19.6 µM ferrous sulfate/L. In the DPPH assay, the extract inhibited 49.5%±1.9 of DPPH free radicals.

4 DISCUSSION

The synthesis of lipids and carotenoids by yeast is influenced by the carbon source used for cell growth and the cultivation conditions. The combination of factors such as carbon source, nitrogen source, carbon-to-nitrogen ratio (C/N), dissolved oxygen concentration, temperature, pH, and inoculum rate during the fermentation process is intentionally manipulated to direct yeast metabolism towards the production of fatty acids and carotenoid pigments (MASSOUD; KHOSRAVI-DARANI, 2017; MOTA; MÚGICA; SÁ-CORREIA, 2022).

Crude glycerol is an efficient carbon source for biomass production by oleaginous yeasts and specific metabolites such as lipids and carotenoids (PETRIK *et al*., 2013). In this study, crude glycerol obtained from the biodiesel laboratory provided cell growth and accumulation of lipids and carotenoids in the optimization assays using the *Cystofilobasidium ferigula* CCMA 1623 strain. The substrate had a composition of 386.83 g/L glycerol, and 0.197 g/L acetic acid was detected, but it did not contain toxic compounds such as furfural and 5-methylfurfural.

According to Bharathiraja *et al*. (2017), a concentration of 3 g/L acetic acid and 2.5 g/L furfural is required to inhibit microbial growth completely.

C. ferigula CCMA 1623 showed more significant growth in the assays related to the central values of the optimization parameters (28ºC, 1.0 g/L yeast extract, and 160 rpm homogenization), as well as in the assays with higher concentrations of yeast extract (1.5 g/L) and homogenization (180 rpm). The assay with the lowest cell count ($3.4x10^7$) was assayed 1, with all parameters evaluated at their lowest values (0.5 g/L yeast extract, 26°C, and 140 rpm). Therefore, the microbial growth of the strain under crude glycerol cultivation is related to the higher concentrations of nitrogen source used (1.5 and 1.0 g/L), combined with homogenization at the +1 or central point (180 or 160 rpm) to incorporate oxygen into the medium. When a higher nitrogen concentration is available, the cell invests in nucleic acid production and maintains cell division for longer, resulting in higher biomass production (ARHAR and NATTER, 2019).

Oleaginous yeasts generally require oxygen for rapid growth (MOTA; MÚGICA; SÁ-CORREIA, 2022). Different homogenization speeds (140, 160, and 180 rpm) were used to ensure uniform oxygen incorporation into the medium. This factor triggered more significant growth of *C. ferigula* in assays with higher rotation values. However, some studies on lipid production by yeasts have attributed a decrease in lipid content to high aeration levels (CALVEY *et al*., 2016). The optimal level of aeration can vary between yeast species, favoring either biomass production or metabolite biosynthesis depending on the evaluated strain (MOTA; MÚGICA; SÁ-CORREIA, 2022). The homogenization conditions evaluated in cultivating *C. ferigula* CCMA 1623 using the response surface model did not significantly affect lipid and carotenoid production.

Temperature is an essential factor for microbial growth as it controls enzymatic concentrations and consequently influences the biosynthesis and accumulation of metabolites. Regarding carotenoid production, different temperature ranges modulate the production of specific carotenoids (VALDUGA *et al*., 2009). According to Cheng and Yang (2016), temperatures between 20°C and 30°C favor yeast growth, but temperatures below 20°C can limit growth, and temperatures above 30°C are unfavorable for carotenoid production as they denature enzymes related to carotenogenesis. In a study conducted by Da Silva *et al*. (2020) on the production of lipids and carotenoids by *Rhodotorula mucilaginosa*, it was observed that cultivation with temperature variations between 22°C and 34°C influenced the production of specific carotenoids. However, it did not affect the biomass produced. It was also reported that an increase in temperature (34°C) unfavorably affected carotenoid production.

In the optimization assays of *C. ferigula*, biomass production, lipid content, and total carotenoid concentration were not significantly affected by the temperatures used in the cultivations (26°C, 28°C, and 30°C) as there was little difference among the evaluated temperatures. A study using disparate temperature values of 20°C and 28°C for the simultaneous production of lipids and carotenoids by *Rhodotorula gracilis* found that the temperature of 20°C resulted in higher lipid and carotenogenic accumulation within 96 hours. However, there were modifications in the profile of fatty acids and carotenoids depending on the temperature and fermentation time (KOT *et al*., 2020).

Among the parameters evaluated for the simultaneous production of lipids and carotenoids optimization by *C. ferigula* CCMA 1623 under crude glycerol cultivation, only the concentration of yeast extract significantly interfered. This result can be justified by the fact that the concentration of yeast extract is related to the carbon-to-nitrogen ratio in the culture medium, a crucial factor for triggering the accumulation of metabolites by inducing a stress condition on the cell (ASHOKKUMAR *et al*., 2023; MOTA; MÚGICA; SÁ-CORREIA, 2022). According to Petrik *et al*. (2013), in studies using response surface methodology for biomass, lipid content, and carotenoid concentration production by yeasts, the C/N ratio also contributed with a significant effect.

An essential condition for lipid accumulation in yeasts is an excess carbon source in the medium combined with nitrogen limitation (AROUS; JAOUANI; MECHICHI, 2019). *C. ferigula* showed higher lipogenesis levels, ranging from 60.67% to 68.72%, in all assays with a lower yeast extract concentration (0.5 g/L), regardless of the temperature and homogenization speed used. The excess carbon source is assimilated by the cells and converted into triacylglycerols. However, lipogenesis is determined by nitrogen scarcity, which, upon depletion, ceases cell division, and the formed lipid is stored intracellularly (RATLEDGE, 2004; BEOPOULOS, 2012). As a result of nitrogen scarcity, there is an increase in ATP-citrate lyase (ACL) activity, leading to a continuous supply of acetyl-CoA (a precursor for fatty acid synthesis) that induces lipid accumulation (CHATTOPADHYAY; MITRA; MAITI, 2021).

Limiting nitrogen conditions are achieved when its concentration in the medium is close to zero, compromising cell growth. Under this condition, lipid accumulation occurs if there is an excess of carbon. Thus, a high carbon-to-nitrogen ratio (C/N) favors lipogenesis, with an ideal C/N ratio of 100 reported (DIAS *et al*., 2020). The results obtained in this study for increased lipid production corroborate the above, as considering the concentration of 0.5 g/L of yeast extract and 40 g/L of crude glycerol, the C/N ratio will be 80. However, increased lipogenesis was not correlated with cell growth.

Higher carotenoid production by *C. ferigula* was obtained in the optimization assays with a yeast extract concentration of 1.0 g/L and temperatures of 28°C and 30°C. It can be inferred that the strain invested nitrogen in cell growth, resulting in higher carotenoid concentrations. In a study conducted by Aksu and Eren (2007) on growth characteristics and carotenoid production by *Rhodotorula glutinis* in batch cultivation, increased growth and total carotenoid concentrations were observed when ammonium sulfate concentrations were increased up to 2 g/L.

Using the Central Composite Design optimization technique and the response surface model, the optimal condition for the simultaneous production of fatty acids and carotenoids by *C. ferigula* CCMA 1623, using crude glycerol as a carbon source, was determined to be 1.5 g/L of yeast extract, 26°C, and homogenization at 140 rpm. Under these cultivation conditions, a lipid content of 52.59% and a carotenoid concentration of 3.03 µg/mL were obtained.

In red oleaginous yeasts, the biosynthesis pathways of fatty acids and carotenoids share a crucial common point, the cytosolic precursor Acetyl-CoA. The generation of Acetyl-CoA depends on carbohydrate metabolism and, therefore, the composition of the different carbon sources used for cultivation. The composition of the carbon source and its impact on the glycolytic pathway can positively regulate the generation of pyruvate and, consequently, Acetyl-CoA availability (FAKANKUN; LEVIN, 2023). The optimal point found in this study for the simultaneous production of both metabolites by *C. ferigula* highlights that 1.5 g/L of yeast extract, using the crude glycerol substrate containing 386.83 g/L of glycerol, along with the other parameters of temperature and oxygen incorporation, were sufficient to ensure high production of lipids and carotenoids. Previous studies emphasize the importance of choosing an appropriate C/N ratio according to the carbon source, yeast strain used, and other cultivation parameters (MOTA; MÚGICA; SÁ-CORREIA, 2022).

The composition of the fatty acid profile synthesized by yeasts is similar to that of vegetable oils, with saturated and monounsaturated fatty acids, predominantly those composed of 16 and 18 carbon atoms (DA SILVA et al., 2020). In this study, the lipid profile of the *C. ferigula* CCMA 1623 strain was mainly composed of palmitic acid (54.72%), stearic acid (13.66%), linoleic acid (11.53%), and oleic acid (10.61%). Linoleic acid (C18:2) and oleic acid (C18:1) are relevant, as they are omega fatty acids that confer medicinal properties and industrial value as nutraceuticals. The fatty acid profile found for *C. ferigula* is similar to that of cocoa butter, which is of great industrial interest due to increasing demand and scarcity of supply (PATEL *et al*., 2020).

The fatty acid profile is also determined according to the cultivation conditions used, primarily the carbon source that composes the substrate. Cultivating the *C. ferigula* CCMA 1623 strain in a crude glycerol medium can trigger fatty acid accumulation through the *ex novo* pathway. This alternative pathway occurs when yeasts are cultivated in hydrophobic media and can ensure additional fatty acid accumulation. Yeasts secrete lipases that help assimilate free fatty acids and triacylglycerols in the medium, incorporating and modifying the fatty acid profile (FAKANKUN; LEVIN, 2023).

The total carotenoid extracts from the *C. ferigula* strain exhibited high antioxidant activity for the three evaluation methods used: ABTS (365.37 µM Trolox/L), DPPH (49.5% inhibition), and FRAP (385.02 µM ferrous sulfate/L), when compared to antioxidant results obtained by Rufino *et al*. (2010) from fruit extracts. The antioxidant activity of the carotenogenic extracts from *C. ferigula* can be considered high, similar to those obtained by Silva *et al*. (2023) from carotenoids produced by the yeast *Paffia rhodozyma*, with ABTS activity of 72.51 µM Trolox g-1 and FRAP of 21.57 µM Trolox g-1.

The *C. ferigula* CCMA 1623 strain can be used on a larger scale in future studies to produce lipids and carotenoids under the optimized conditions described in this work using an airlift bioreactor. Cultivation in airlift bioreactors has the advantages of simple operation, low energy consumption, and greater precision in maintaining stable cultivation parameters. Therefore, it can grow red oleaginous yeasts relatively inexpensively, facilitating the commercial production of microbial oils and carotenoids (MANOWATTANA *et al*., 2018).

5 CONCLUSION

This study demonstrated that the rose-colored strain *Cystofilobasidium ferigula* CCMA 1623, isolated from coffee, efficiently produces lipids and carotenoids using crude glycerol as a substrate. The synthesis of lipids and carotenoids is mainly influenced by the concentration of the nitrogen source, which consequently alters the carbon-to-nitrogen ratio in the culture medium. Under optimized conditions, *C. ferigula* exhibited a lipid content of 52.59% and a total carotenoid concentration of 3.03 µg/mL. Critical fatty acids such as omega-6 and omega-9 and total carotenoids with high antioxidant power were identified as components of the biocompound profile synthesized by the strain.*C. ferigula* CCMA 1623 shows promise for further studies on larger scales to obtain lipids and carotenoids, aiming for robustness in implementing biotechnological processes in the food, chemical, pharmaceutical, and cosmetic industries.

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