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JOSÉ EVANGELISTA SANTOS RIBEIRO

UTILIZAÇÃO DE RESÍDUOS AGROINDUSTRIAIS PARA A OBTENÇÃO DE MOLÉCULAS BIOATIVAS A PARTIR DE MICRORGANISMOS

João Pessoa - PB
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Tese apresentada ao Programa de Pós-graduação em Ciência e Tecnologia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba, em cumprimento aos requisitos para obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Flávio Luiz Honorato da Silva

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Tese **APROVADA** em **02/03/2018**.

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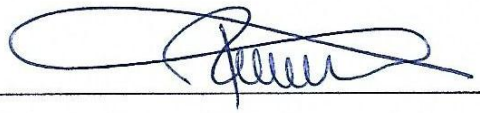

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À minha avó (in memoriam): Almerinda,

Por todo o seu amor na minha formação como pessoa.

DEDICO.

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RESUMO

Nos últimos anos, microalgas e leveduras vermelhas emergiram como fontes importantes de muitas moléculas bioativas, como carotenoides e ácidos graxos. No entanto, o uso de culturas de microrganismos para produzir produtos comerciais tem sido historicamente limitado, devido ao elevado custo destes processos biotecnológicos. O objetivo deste estudo foi avaliar o potencial do soro de queijo ricota (*scotta*) e da manipueira para serem utilizados como substratos alternativos de baixo custo para o cultivo da microalga *Chlorella protothecoides* e da levedura *Rhodotorula glutinis*. Além disso, estratégias de cultivo foram aplicadas para melhorar a produção dos metabólitos de interesse. Com os resultados observa-se que tanto o soro de queijo ricota (*scotta*) quanto a manipueira têm um grande potencial para serem usados como meios de cultura para o crescimento destes microrganismos. A microalga *C. protothecoides*, cultivada na *scotta*, cresceu em modo mixotrófico, utilizando a fonte de carbono orgânica fornecida. Além disso, a estratégia de estresse salino e luminoso aplicada melhorou a carotenogênese, permitindo o acúmulo celular dos carotenoides desejáveis, astaxantina e luteína/zeaxantina. Para a manipueira, foi observado que este subproduto pode ser usado como fonte única de nutrientes para o crescimento celular e acúmulo de metabólitos na levedura *R. glutinis*. Uma elevada produção de biomassa (10,28 g/L), carotenoides (0,98 mg/L) e lipídeos (1,34 g/L) foi obtida quando a levedura *R. glutinis* foi cultivada na manipueira. Além disso, os perfis lipídicos da biomassa cultivada nos meios contendo este subproduto demonstraram um maior percentual (acima de 50 %) de ácidos graxos insaturados. Os subprodutos agroindustriais estudados foram eficientemente utilizados como fontes de carbono pelos microrganismos testados e os resultados obtidos para as produções de biomassa e de moléculas bioativas de interesse nos meios contendo os substratos alternativos foram ainda melhores em comparação com o cultivo em meio sintético contendo teor similar de carbono orgânico, possivelmente em decorrência do adequado balanço de nutrientes nestes subprodutos.

Palavras-chave: Microalga · Levedura vermelha · Ingredientes alimentares · Processos biotecnológicos

ABSTRACT

In recent years, microalgae and red yeasts have emerged as attractive sources for many bioactive molecules such as carotenoids and fatty acids. However, the use of microorganism cultures to produce commercial products has historically been limited, due to the high costs of these biotechnological processes. The aim of this study was to evaluate the potential of ricotta cheese whey (*scotta)* and cassava wastewater to be used as low-cost alternative substrates to grow the microalga *Chlorella protothecoides* and the yeast *Rhodotorula glutinis*. Furthermore, culture strategies were applied in order to improve the bioactive molecules production. The results suggest that both *scotta* and cassava wastewater have a great potential to be used as culture mediums to grow the above mentioned microorganisms. *C. protothecoides* shifted to mixotrophic growth, using the organic carbon source provided when cultured in *scotta*. Moreover the stress strategy that we applied enhanced carotenogenesis, allowing the cellular accumulation of well quoted carotenoids, namely astaxanthin and lutein/zeaxanthin. The results showed that cassava wastewater can be used as a sole source of nutrients to support *R. glutinis* growth and metabolites accumulation in its cells. High growth (10.28 $g \cdot L^{-1}$) as well as high productions of carotenoids (0.98 $mg \cdot L^{-1}$) and lipids (1.34 $g \cdot L^{-1}$) were obtained when R. glutinis was cultivated in cassava wastewater as the sole source of nutrients. Moreover, the fatty acids profile from the biomass cultivated in the mediums containing cassava wastewater showed a majority (over 50 %) of unsaturated fatty acids. The agro-industrial by-products studied were efficiently used as carbon sources by the microorganisms and the results obtained for the mediums containing the alternative substrates were even better when compared with the cultivation in synthetic mediums containing similar amounts of organic carbon, which is possibly due to the adequate balance of nutrients in these by-products.

Key-words: Microalga · Red yeast · Food ingredients · Biotechnological process

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1. INTRODUÇÃO

A conscientização dos consumidores a respeito da influência da dieta sobre a saúde tem ocasionado o aumento da demanda por ingredientes alimentares de origem natural (VALDUGA et al., 2009) e pela ingestão de alimentos que contêm em sua composição moléculas bioativas e que, portanto, além de nutrir trazem benefícios adicionais à saúde (SIRO et al., 2008). Diante deste cenário, a indústria de alimentos tem buscado alternativas para a substituição de aditivos artificiais, tais como alguns corantes e conservantes, por aqueles obtidos a partir de fontes naturais (MARTINS et al., 2016).

Algumas classes de lipídeos, como os pigmentos carotenoides e determinados ácidos graxos, têm sido alvo de diversas pesquisas com foco no desenvolvimento de processos biotecnológicos para a obtenção de tais compostos, que além de suas propriedades bioativas, podem ser empregados como ingredientes naturais na indústria alimentícia (CAMPENNI' et al., 2013; MANNAZZU et al., 2015; MATA-GÓMEZ et al., 2014).

Os carotenoides são importantes pigmentos naturais, produzidos por uma grande variedade de microrganismos e plantas, sendo em sua maioria C_{40} terpenóides (MAROVA et al., 2012; PARK et al., 2005). Devido as suas propriedades como agentes antioxidantes, anticancerígenos, estimulantes de resposta imune, nutracêuticos e corantes, os carotenoides são amplamente utilizados na medicina e na indústria química, farmacêutica, de cosméticos, de alimentos e de rações (BHOSALE; GADRE, 2001; CUTZU et al., 2013). O mercado global de carotenoides cresce a uma taxa anual de 2,3% e em 2018 a expectativa é que este mercado alcance o valor de 1,4 bilhão de dólares (BCC, 2011) e, por isso, existe um interesse crescente no desenvolvimento de processos visando à produção eficiente destes pigmentos.

Os ácidos graxos poliinsaturados, tais como os ω-6 e ω-3, têm ganhado cada vez mais espaço como ingredientes funcionais na indústria de alimentos em decorrência das suas características e benefícios à saúde, podendo-se destacar a diminuição das taxas de triglicérides e colesterol total no sangue e a redução da pressão arterial de indivíduos com hipertensão leve (SARGEANT et al., 2017). Devido a crescente demanda por estas moléculas, que normalmente são obtidas a partir de fontes vegetais e animais, outras fontes alternativas têm sido investigadas tais como microrganismos capazes de

acumular elevados teores de lipídeos nas células (CLEBER BERTOLDI et al., 2006; DAVOLI; MIERAU; WEBER, 2004).

A utilização de sistemas que empregam microalgas e leveduras vermelhas para a produção de moléculas bioativas é uma área emergente e que apresenta um grande potencial para aplicações industriais (MATA-GÓMEZ et al., 2014; WICHUK; BRYNJÓLFSSON; FU, 2014). Diversos processos biotecnológicos têm demonstrado o potencial destes microrganismos para as indústrias de alimentos e rações, visando à produção pigmentos e aditivos naturais (RODRIGUES et al., 2014). Microalgas e leveduras vermelhas são reconhecidamente excelentes fontes de carotenoides e de outras classes de lipídeos, como os ácidos graxos (DUFOSSÉ et al., 2005; HERNÁNDEZ-ALMANZA et al., 2014; PEREZ-GARCIA et al., 2011).

A microalga de água doce, *Chlorella protothecoides*, é capaz de acumular elevados teores de carotenoides e ácidos graxos em suas células (CAMPENNI' et al., 2013). Esta microalga é conhecida principalmente pela sua capacidade de acumular elevados teores de luteína assim como outros carotenoides de alto valor comercial, como a astaxantina e o β-caroteno (ARAYA et al., 2014; CAMPENNI' et al., 2013; O'GRADY; MORGAN, 2011; TERCERO; SFORZA; MORANDINI, 2014). Além disto, apresenta grande versatilidade em relação ao seu cultivo, sendo capaz de crescer sob condições autotróficas, mixotróficas e heterotróficas (FERNÁNDEZ-SEVILLA; ACIÉN FERNÁNDEZ; MOLINA GRIMA, 2010).

A levedura vermelha *Rhodotorula glutinis* é um microrganismo aeróbico capaz de acumular lipídeos e pigmentos carotenoides em suas células, sintetizando o β-caroteno, o toruleno e a torularrodina como carotenoides principais (AKSU; EREN, 2007). O acúmulo celular de lipídeos pode alcançar rendimentos de até 72% em relação a biomassa seca (SCHNEIDER et al., 2013). Esta levedura oleaginosa apresenta amplo potencial para ser utilizada na indústria de alimentos por ser uma fonte de moléculas de alto valor comercial, como: carotenoides e ácidos graxos insaturados, como os ácidos oleico, linoleico e linolênico (SAENGE; CHEIRSILP, 2011).

Apesar de apresentar algumas vantagens, tais como, a rápida taxa de produção de biomassa, a possibilidade de controlar as condições do cultivo e a não dependência de condições climáticas, a produção e comercialização de moléculas bioativas a partir de microrganismos enfrenta como principal desafio o elevado custo do processo biotecnológico (MATA-GÓMEZ et al., 2014). Deste modo, para viabilizar estes processos microbianos, diversos resíduos e subprodutos da agroindústria têm sido

testados como meios de cultura de baixo custo para o cultivo (FRENGOVA; BESHKOVA, 2009). No entanto, o principal problema relacionado com a utilização de substratos alternativos como meio de cultura é encontrar um substrato com um balanço adequado de nutrientes que permita o crescimento celular e a acumulação de produto (MAKKAR; CAMEOTRA, 1999).

A manipueira é um subproduto agroindustrial rico em carboidratos e sais minerais que é gerado em grandes quantidades durante a produção de farinha de mandioca, sendo considerado um substrato muito atrativo para processos biotecnológicos (NITSCHKE; PASTORE, 2006). Do mesmo modo, o soro de queijo ricota é também um subproduto gerado em grandes quantidades e que apresenta potencial para ser empregado em processos biotecnológicos, sendo uma boa fonte de carbono, nitrogênio e minerais (PRAZERES; CARVALHO; RIVAS, 2012).

Portanto, o objetivo geral deste estudo consistiu no desenvolvimento de processos biotecnológicos para a obtenção de lipídeos e carotenoides a partir da microalga *Chlorella protothecoides*, cultivada em soro de queijo ricota (*scotta*) e da levedura vermelha *Rhodotorula glutinis*, cultivada na manipueira e em soro de queijo ricota (*scotta*). Para atingir esta meta, os subprodutos agroindustriais utilizados foram inicialmente caracterizados quanto a sua composição química e determinados parâmetros do cultivo foram avaliados quanto à influência sobre o rendimento final de biomassa e dos metabólitos de interesse.

2. REVISÃO DE LITERATURA

2.1 Uso de resíduos ou subprodutos agroindustriais em processos biotecnológicos

As agroindústrias são uma importante atividade econômica no mundo, no entanto, durante as etapas de processamento dos produtos agroindustriais diversos resíduos e subprodutos são obtidos em grande quantidade (RODRIGUES et al., 2014). Em geral estes resíduos ou subprodutos da agroindústria contêm quantidades consideráveis de carbono, nitrogênio e elementos minerais derivados da matéria-prima utilizada no processamento (XUE et al., 2010).

Os resíduos caracterizam-se como as sobras do processo que não possuem valor comercial e não são utilizadas em aplicações posteriores na planta industrial, portanto, são tratados e descartados pela indústria (BIFF; SILVA, 2016). Em contrapartida, subprodutos caracterizam-se como as sobras do processo que são aproveitadas para a elaboração de outros produtos e sendo assim apresentam um valor comercial baixo, no entanto, bem definido (JUNIOR et al., 2016). As definições de resíduos e subprodutos dependem de fatores socioeconômicos, visto que a depender da região, um resíduo pode passar a ser considerado um subproduto, como ocorre com a manipueira que é utilizada no estado do Pará para a elaboração do tucupi, mas é descartada na região nordeste.

A maioria dos resíduos/subprodutos agroindustriais apresenta elevado teor de matéria orgânica e consequentemente elevados teores de demanda química de oxigênio (DQO) e demanda bioquímica de oxigênio (DBO), necessitando assim de tratamento adequado antes do descarte correto no meio ambiente, o que gera custo para as empresas. Em muitas situações resíduos são descartados de forma inadequada, provocando sérios impactos ambientais (MARKOU; GEORGAKAKIS, 2011).

Atualmente, as alternativas de valorização de resíduos através do seu aproveitamento tem sido muito incentivadas, já que podem contribuir para a redução da poluição ambiental, bem como permitir a valorização econômica desses resíduos tornando-os subprodutos e, deste modo, agregando valor ao processo de agroindustrialização (MAROVA et al., 2012). Os resíduos agroindustriais apresentam baixo risco químico, estão disponíveis em grande quantidade e apresentam composição adequada para serem empregados em processos biotecnológicos para a obtenção de produtos de alto valor comercial (MARKOU; GEORGAKAKIS, 2011; RODRIGUES et al., 2014).

Neste sentido, diversas pesquisas vêm sendo conduzidas com o emprego de resíduos da agroindústria como substratos alternativos de baixo custo para o cultivo de diversos microrganismos com o interesse na obtenção de moléculas de alto valor comercial (Quadro 1).

Quadro 1. Resíduos agroindustriais estudados quanto a sua aplicação em processos biotecnológicos.

Resíduo	**Microrganismo**	**Produto**	**Autores**
Efluente de abatedouros	*Phormidium autumnale*	Carotenoides	(RODRIGUES et al., 2014)
Soro de queijo	*Sporobolomyces roseus*, *Rhodotorula glutinis* e *Rhodotorula mucilaginosa*	Carotenoides	(MAROVA et al., 2012)
Melaço de cana-de-açúcar	*Chlorella protothecoides*	Lipídeos	(YAN et al., 2011)
Borra de café	*Sporobolomyces roseus*, *Rhodotorula glutinis* e *Rhodotorula mucilaginosa*	Carotenoides e ergosterol	(PETRIK et al., 2014)
Glicerol	*Chlorella protothecoides*	Lipídeos	(O'GRADY; MORGAN, 2011)
Glicerol	*Spirulina platensis*	Lipídeos	(NARAYAN et al., 2005)
Silagem de milho	*Chlorella vulgaris*	Lipídeos	(MITRA; VAN LEEUWEN; LAMSAL, 2012)
Melaço de cana-de-açúcar	*Chlorella zofingiensis*	Lipídeos e astaxantina	(LIU et al., 2012)
Efluente da aquicultura	*Chlorella* sp. e *Scenedesmus quadricauda*	Clorofila, amido e lipídeos	(HALFHIDE et al., 2014)
Permeado do soro de queijo	*Scenedesmus obliquus*	Lipídeos	(GIRARD et al., 2014)
Resíduo do processamento de tomates	*Rhodotorula glutinis*	Carotenoides	(CHANDI; SINGH; GILL, 2010)
Scotta	*Kluyveromyces marxianus*	Etanol	(SANSONETTI et al., 2009)
Scotta	*Lactobacillus casei*, *Lactobacilus helveticus* e *Streptococcus thermophilus*	Ácido Lático	(SECCHI et al., 2012)
Glicerol	*Rhodotorula glutinis*	Carotenoides	(CUTZU et al., 2013)
Salmoura de rabanete fermentado	*Rhodotorula glutinis*	β-caroteno	(MALISORN; SUNTORNSUK, 2008)
Farinha da casca do feijão	*Rhodotorula glutinis*	Carotenoides	(TINOI; RAKARIYATHAM; DEMING, 2005)

2.2 Manipueira

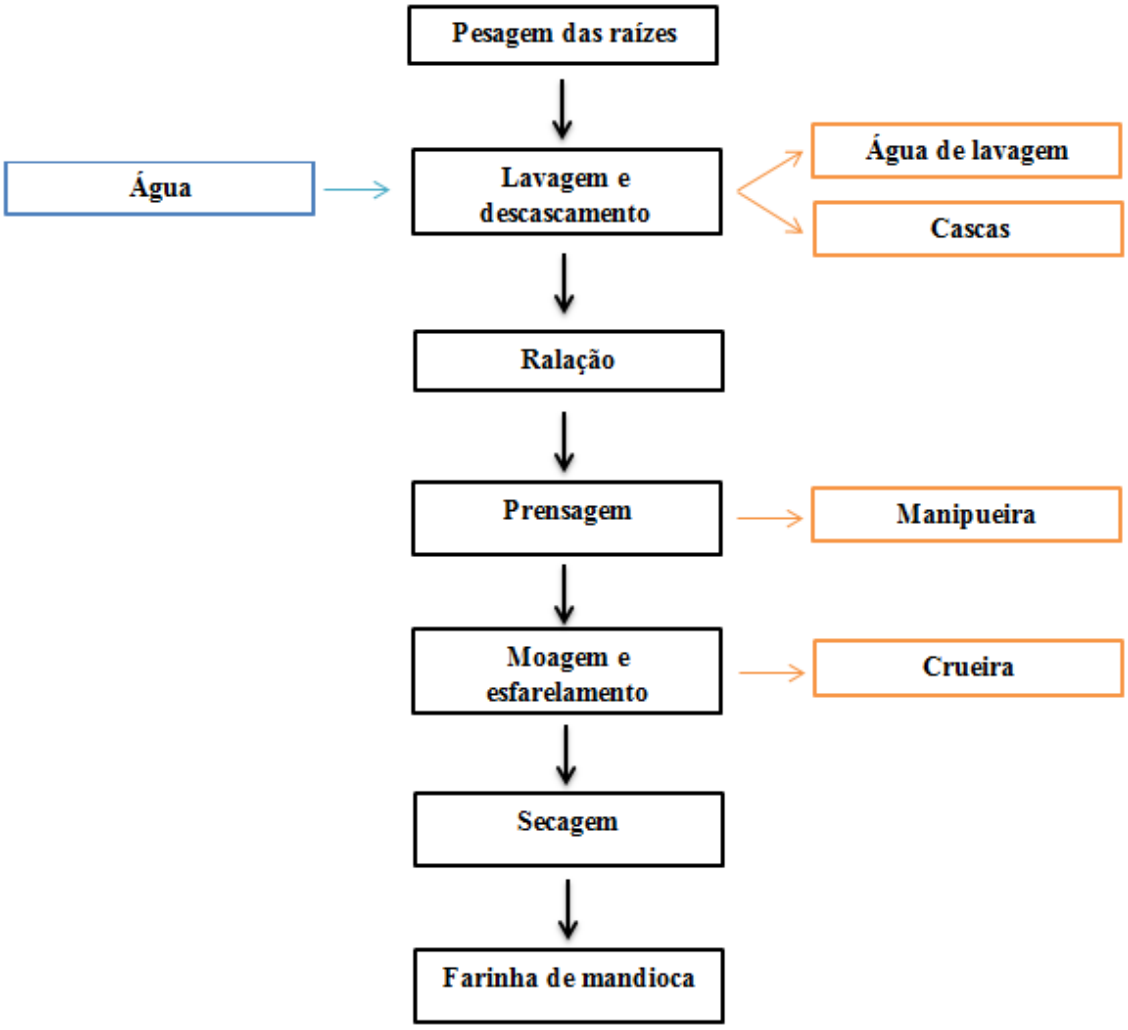
A manipueira é um subproduto agroindustrial rico em carboidratos e sais minerais que é gerado em grande quantidade durante a produção de fécula e de farinha de mandioca, sendo considerado um substrato muito atrativo para processos biotecnológicos (NITSCHKE; PASTORE, 2006). O processamento de uma tonelada de mandioca para a produção de farinha gera aproximadamente 300 litros de manipueira, que normalmente não é aproveitada pelos produtores devido à falta de conhecimento sobre o seu potencial de uso, tornando-se um dos principais problemas da cadeia produtiva da mandioca, em função do impacto ambiental ocasionado pelo descarte inadequado deste resíduo na natureza (COSTA et al., 2010).

A preocupação com este resíduo é bastante significativa, uma vez que uma indústria farinheira de médio porte chega a processar em torno de 260 t/mês de raiz de mandioca, o que pode gerar cerca de 100 mil litros de manipueira em um mês (DAMASCENO et al., 2003).

Apesar de toda a preocupação acerca da quantidade de manipueira gerada com o processamento da farinha de mandioca e do impacto ambiental provocado pelo seu descarte inadequado, a manipueira é uma excelente fonte de carbono, além de outros nutrientes, podendo ser considerada como um substrato potencial para ser empregado em processos biotecnológicos para a produção de diferentes metabólitos de interesse comercial o que simultaneamente resolveria o problema do seu tratamento e ainda recuperaria recursos na sua utilização (LEONEL; CEREDA, 1995).

No processo de produção da farinha de mandioca (Figura 1), as raízes de mandioca são inicialmente lavadas, descascadas e trituradas, obtendo-se uma massa que é em seguida prensada para a remoção de parte do teor de umidade. A parte sólida remanescente é utilizada para a produção de farinha, seguindo-se processos de moagem e secagem, enquanto a manipueira que é o líquido residual obtido após a prensagem (Figura 2) é, normalmente, estocado em reservatórios de alvenaria e posteriormente é descartado pelas casas de farinha. Neste processo é gerado também um subproduto sólido denominado crueira, que é uma farinha de alta granulometria, utilizada para a alimentação de animais.

Figura 1. Fluxo do processo de produção da farinha de mandioca.



Fonte: Própria (2018)

Figura 2. Manipueira obtida após a prensagem da mandioca para a produção de farinha.

Fonte: EMBRAPA (2015)

2.3 Soro de queijo ricota ou *scotta*

O soro de queijo ricota ou *scotta* é o subproduto obtido após a produção de queijo ricota, o qual tem sido pouco estudado e consequentemente pouco aplicado na indústria, sendo utilizado normalmente na alimentação de animais. A tecnologia para o processamento do queijo ricota consiste basicamente no aquecimento do soro de queijo e na coagulação e separação das proteínas (Figura 3). Após este processo a solução líquida de cor amarelada que permanece após a obtenção do queijo (Figura 4) é o soro de queijo ricota, conhecido como *scotta*, o qual possui reduzido teor proteico, apresentando características diferentes em relação ao soro de queijo (SECCHI et al., 2012).

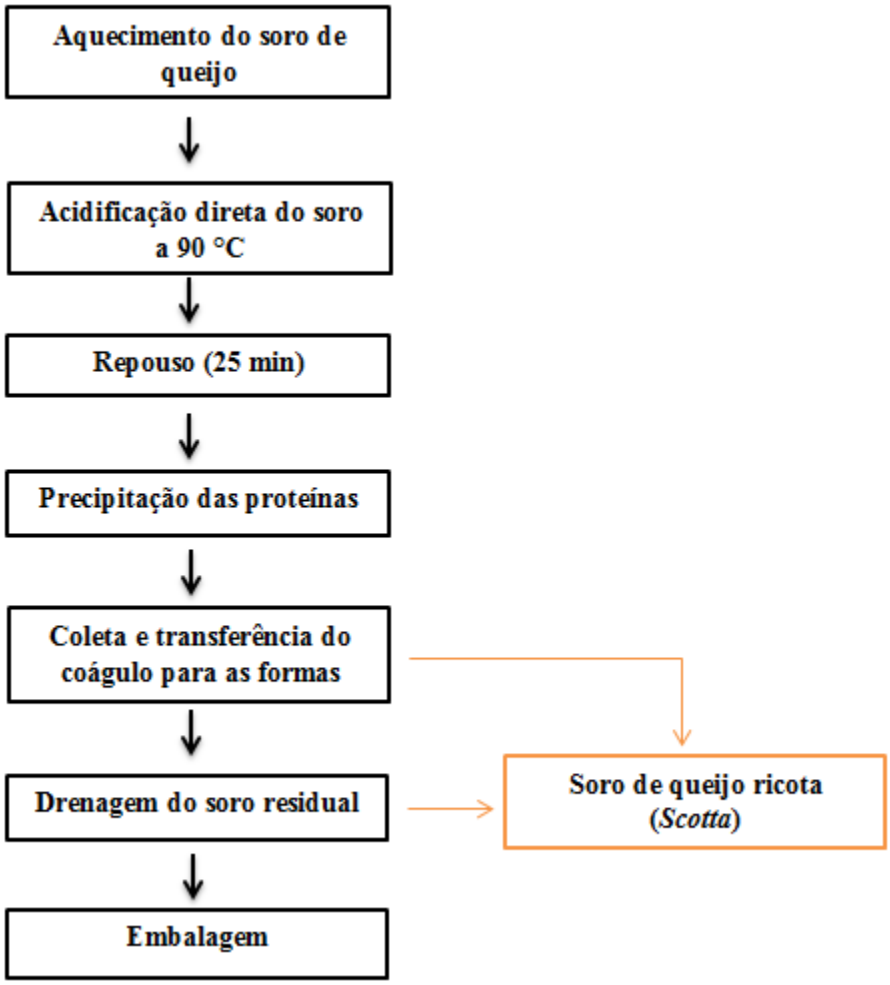
O baixo teor de proteínas neste soro de queijo ricota (*scotta*) faz com que este subproduto não seja atrativo para ser utilizado em processamentos posteriores para a obtenção de outros produtos (PISPONEN et al., 2013). O crescimento do consumo de queijo ricota no mundo, por ser um produto com baixo teor de gordura e versátil para aplicações culinárias, tem como consequência o aumento da quantidade de *scotta* produzida, abrindo assim a possibilidade de aplicação industrial deste subproduto em processos biotecnológicos para a obtenção de produtos de valor agregado.

A Itália produz uma quantidade de 10^9 kg/ano de *scotta* (SANSONETTI et al., 2009). Este subproduto da indústria de laticínios é caraterizado por valores de DBO e DQO de 50 g/L e 80 g/L, respectivamente (SECCHI et al., 2012). Portanto, devido a grande e amplamente distribuída produção de *scotta*, existe a preocupação com o seu descarte inadequado que pode causar problemas ambientais em decorrência do elevado teor de matéria orgânica e o seu tratamento biológico representa uma demanda de custos na indústria de laticínios.

A *scotta* obtida a partir do soro de queijo bovino contém proteínas (0,15–0,22%), sais minerais (1,0–1,13%), e lactose (4,8–5,0%). A *scotta* obtida a partir do soro de queijo de ovelha apresenta composição similar, mas caracteriza-se pelo teor proteico mais elevado (MUCCHETTI; CARMINATI; PIRISI, 2002). Portanto, a *scotta* apresenta-se como uma fonte de carbono interessante para ser empregada como substrato em processos biotecnológicos.

De fato, a bioconversão da *scotta* em produtos de valor agregado, além de representar uma abordagem atraente para a redução dos impactos ambientais, permitiria a exploração e valorização deste subproduto.

Figura 3. Etapas básicas do fluxo do processo de produção do queijo ricota.



Fonte: Própria (2018)

Figura 4. *Scotta* obtida após a produção de queijo ricota.

2.4 Lipídeos microbianos

Os lipídeos constituem uma classe de substâncias químicas que tem como principal característica o fato de serem hidrofóbicos, ou seja, não serem solúveis em água. Os exemplos mais conhecidos de lipídeos são os ácidos graxos e seus derivados, esteróis, ceras e carotenoides. Esses compostos tem em comum a presença de cadeias orgânicas com um elevado número de carbonos, o que lhes confere o caráter hidrofóbico, podendo apresentar apenas átomos de carbono e hidrogênio ou, ainda, grupos funcionais, como álcoois, fenóis, ácidos carboxílicos e ésteres (FAHY et al., 2005).

Entre os lipídeos, o grupo conhecido como óleos e gorduras e seus derivados teve uma importância ímpar na história da humanidade. Nos óleos e gorduras, os ácidos graxos podem ser encontrados livres ou combinados, na forma de monoacilglicerídeos, diacilglicerídeos e triacilglicerídeos (MORRIS; WHARRY, 1966).

Atualmente uma grande variedade de óleos vegetais como o óleo de soja, óleo de algodão e óleo de milho, têm sido utilizados pela indústria de alimentos, e mais recentemente para a produção de biocombustíveis. No entanto, existe uma demanda crescente pela busca de outras fontes mais eficientes e sustentáveis, com perfis de ácidos graxos mais favoráveis para a aplicação na indústria de alimentos e para a produção de biodiesel. Recentemente diversas pesquisas vêm sendo conduzidas no âmbito da obtenção de lipídeos a partir de microrganismos oleaginosos, tais como algumas linhagens de microalgas e leveduras (SUN et al., 2015; XUE et al., 2010).

Os lipídeos microbianos oferecem uma série de possibilidades, podendo ser utilizados como aditivos alimentares ou até mesmo substituir óleos e gorduras vegetais, como manteiga de cacau e óleo de palma em diversas aplicações industriais. Estes lipídeos apresentam algumas vantagens em relação àqueles obtidos a partir de fontes vegetais, tais como: curto período de produção, menor necessidade de mão de obra, a produção não sofre influência com as mudanças sazonais e é relativamente fácil ampliar a escala de produção (WANG; YANG; WANG, 2014).

2.4.1 Ácidos Graxos

Ácidos graxos são ácidos carboxílicos que em sua maioria não apresentam ramificações e contêm um número par de carbonos devido à rota bioquímica de síntese

(DAVOLI; MIERAU; WEBER, 2004). Os ácidos graxos diferem entre si pelo número de carbonos da cadeia e também pelo número de insaturações. A qualidade de uma gordura é definida com base no seu perfil de ácidos graxos, especificamente em relação às percentagens de ácidos graxos monoinsaturados (MUFAs) e poli-insaturados (PUFAs) que ela contém (FAHY et al., 2005). Portanto, lipídeos obtidos a partir de microrganismos devem ser analisados para determinar a sua composição indicando-se assim a sua aplicação com base em seu perfil de ácidos graxos (SARGEANT et al., 2014).

Lipídeos ricos em PUFAs são utilizados como aditivos pela indústria de alimentos devido à demanda crescente dos consumidores por alimentos benéficos a saúde. Lipídeos microbianos ricos em PUFAs podem ser adicionados diretamente em vários alimentos, na forma de óleo ou em emulsões (CALDER, 2015).

2.4.2 Carotenoides

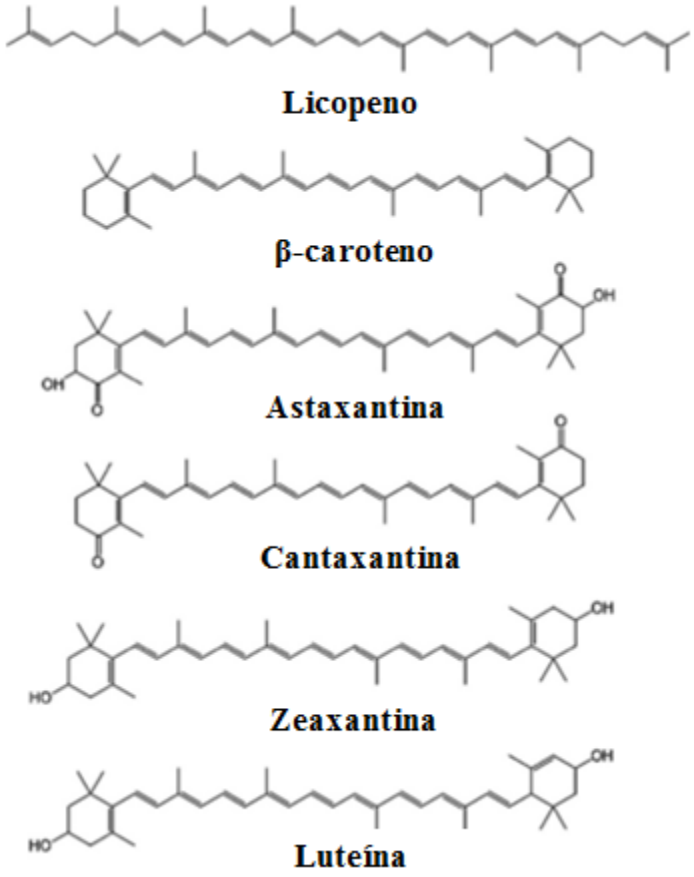
Os pigmentos carotenoides (Figura 5), responsáveis pelas cores amarelo, laranja e vermelho em vegetais, são compostos derivados do isopreno, formados por cadeias de 40 carbonos que possuem como principal característica a presença de um polieno de cadeia longa (onde a presença de duplas ligações varia de três até quinze) que é responsável pela cor percebida pelo olho humano (SQUINA; MERCADANTE, 2003).

Os carotenoides representam um grupo de moléculas valiosas para as indústrias farmacêutica, de cosméticos, química, de rações e de alimentos, não apenas por que podem agir como percussores da vitamina A, mas também por suas ações como antioxidantes, corantes e melhoradores da resposta imune, levando a prevenção de doenças e a proteção contra infecções por bactérias e fungos (AKSU; EREN, 2007; MALDONADE; RODRIGUEZ-AMAYA; SCAMPARINI, 2012).

O mercado global de carotenoides cresce a uma taxa anual de 2,3% e em 2018 a expectativa é que este mercado alcance o valor de 1,4 bilhão de dólares (BCC, 2011) e, por isso, existe um interesse no desenvolvimento de processos visando à produção eficiente destes pigmentos (MANNAZZU et al., 2015). A crescente busca da indústria de alimentos por antioxidantes e corantes de fontes naturais tem sido um dos fatores deste aumento na demanda pelos pigmentos carotenoides extraídos de fontes naturais, como plantas (WICHUK; BRYNJÓLFSSON; FU, 2014). No entanto, o custo destes

carotenoides é superior em relação aos seus análogos sintéticos e as mudanças sazonais interferem na produção destes compostos.

Figura 5. Estrutura molecular dos carotenoides



Fonte: Adaptada de Hernández-Almanza et al. (2014)

Neste sentido, diversas alternativas têm sido estudadas para a obtenção destes pigmentos naturais com custos mais reduzidos e evitando-se os problemas com as mudanças sazonais, podendo-se destacar a produção por via microbiana utilizando-se microalgas, bactérias e fungos (VALDUGA et al., 2009).

β-caroteno

O β-caroteno é um pigmento alaranjado comumente encontrado em plantas e em tecidos de animais, considerado um nutriente essencial devido as suas funções biológicas e ao seu papel como percussor da vitamina A e de hormônios (MALISORN; SUNTORNSUK, 2009). A vitamina A é essencial para o corpo humano, auxiliando o

sistema imunológico e consequentemente prevenindo algumas doenças como certos tipos de câncer e doenças nos olhos e na pele (AGARWAL; RAO, 2000).

O β-caroteno apresenta atividade antioxidante e como tal ajuda a reduzir os efeitos dos radicais livres, evitando assim o envelhecimento precoce (TÖRNWALL et al., 2004). Devido as suas propriedades como composto antioxidante juntamente com a sua função como corante o β-Caroteno é amplamente utilizado como aditivo natural em alimentos, rações, cosméticos e fármacos (MALISORN; SUNTORNSUK, 2009).

Astaxantina

A astaxantina é um carotenoide pertencente à família das xantofilas que pode ser encontrado em grande quantidade na microalga *Haematococcus pluvialis* e em microalgas do gênero *Chlorella* (PARK; LEE, 2001). Apesar de poder ser sintetizada por plantas, bactérias e alguns fungos, a microalga *H. pluvialis* possui a maior capacidade para o acúmulo de astaxantina na célula, podendo-se obter de 4 a 5% em relação a sua biomassa seca (YUAN et al., 2011). Além de poder ser biossintetizado por microalgas, representando o primeiro nível de produção no ambiente aquático, este pigmento carotenoide de coloração vermelha é encontrado em alguns pescados, como camarão, lagosta e salmão, sendo responsável pela coloração vermelho-rosada destes animais (CHEN et al., 2009).

Devido a sua capacidade antioxidante, a astaxantina é conhecida pelo seu extraordinário potencial para a proteção do organismo contra uma ampla variedade de doenças, apresentando um potencial considerável e promissor para ser utilizada na nutrição e saúde humana (YUAN et al., 2011). Diversos estudos têm demostrado os efeitos positivos da astaxantina para a prevenção e tratamento de várias doenças como, câncer, doenças crônicas inflamatórias, síndromes metabólicas, diabetes, doenças cardiovasculares, doenças neurodegenerativas e doenças nos olhos e na pele (RANGA RAO et al., 2013).

O interesse pelo uso da astaxantina como corante natural na indústria de alimentos e como aditivo em rações para a aquicultura tem crescido também, especialmente para o cultivo de salmão e truta, já que a coloração vermelha da astaxantina contribui para a formação da cor vermelho-rosada no músculo destes animais (SARADA et al., 2006).

Este carotenoide apresenta propriedades químicas únicas, baseadas em sua estrutura molecular. Sua molécula apresenta dois grupos carbonila, dois grupos

hidroxila e onze duplas ligações etilênicas conjugadas (Figura 5). Este sistema polieno confere a astaxantina a sua estrutura molecular distinta, as suas propriedades químicas, e a sua capacidade de absorção da luz (IP; CHEN, 2005). A astaxantina pode agir como um forte antioxidante através da doação de elétrons para radicais livres tornando-os produtos mais estáveis, boqueando a reação em cadeia destes radicais em uma ampla variedade de organismos vivos (YUAN et al., 2011).

Luteína e Zeaxantina

A luteína e a zeaxantina são isômeros, e estão entre os principais carotenoides presentes em microalgas de água doce, ambos apresentam interesse comercial devido ao potencial para serem aplicados em produtos nutracêuticos e também na prevenção e tratamento de doenças tais como a catarata e diferentes tipos de câncer (DEL CAMPO et al., 2004).

Tradicionalmente a luteína tem sido extraída de flores, no entanto, devido ao aumento na demanda por este carotenoide para a sua aplicação nas indústrias de alimentos, rações e fármacos, os pesquisadores tem buscado fontes alternativas mais sustentáveis, tal como as microalgas (FERNÁNDEZ-SEVILLA; ACIÉN FERNÁNDEZ; MOLINA GRIMA, 2010).

Estes carotenoides apresentam elevado poder antioxidante, inibindo a ação de radicais livres e espécies reativas do oxigênio. Estes pigmentos estão entre os constituintes do pigmento macular e apresentam um importante papel na prevenção da formação de radicais livres na retina (CHEW et al., 2013). Além destas propriedades benéficas a saúde humana, estes pigmentos apresentam uma coloração vermelho alaranjada característica, apresentando potencial para serem utilizados como corantes naturais na indústria de alimentos (ARAYA et al., 2014).

2.5 Obtenção de lipídeos a partir de microrganismos

Em comparação com óleos vegetais e gorduras de origem animal, a produção de lipídeos a partir de microrganismos apresenta diversas vantagens: curto período de produção, menor necessidade de mão-de-obra, não sofre influência de mudanças sazonais e alterações climáticas, exclui o risco das pragas e é fácil de ampliar a escala de produção (TAHER et al., 2014). Diferentes microrganismos oleaginosos têm sido explorados para a obtenção de lipídeos tais como, microalgas e leveduras vermelhas

(ARAYA et al., 2014; SCHNEIDER et al., 2013). As microalgas podem acumular de 30 – 70% de lipídeos em suas células enquanto que a produção pelas leveduras pode chegar até 72% (PRABAKARAN; RAVINDRAN, 2011; SAENGE et al., 2011).

O processo de cultivo constitui um fator chave na produção de lipídeos e no perfil de ácidos graxos destes nos microrganismos (SARGEANT et al., 2014). Para a levedura *R. glutinis* sabe-se que elevadas relações C/N favorecem o acúmulo de lipídeos nas células (BRAUNWALD, 2013), enquanto que em microalgas o processo de acúmulo de lipídeos ocorre de forma potencializada em condições de estresse nutricional, principalmente em situações de limitação do nitrogênio no meio de cultura (SUN et al., 2015).

Dentre as microalgas utilizadas para a obtenção de lipídeos destacam-se *Botryococcus braunii*, *Chlorella* sp., *Cylindrotheca* sp., *Dunaliella primolecta*, *Isochrysis* sp., *Nannochloris* sp., *Nannochloropsis* sp., *Neochloris oleoabundans*, *Nitzschia* sp., *Phaeodactylum tricornutum* e *Schizochytrium* sp. (HARUN et al., 2010). Dentre as leveduras destacam-se *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*, *Rhodotorula Rubra*, *Sporobolomyces roseus* e *Phaffia rhodozyma* (VALDUGA et al., 2009).

2.6 Obtenção de carotenoides a partir de microrganismos

Por um longo período extratos de plantas estiveram sendo utilizados como fontes de pigmentos carotenoides, particularmente β-caroteno e xantofilas (WICHUK; BRYNJÓLFSSON; FU, 2014). Embora plantas ainda sejam importantes fontes de carotenoides naturais, vários processos microbiológicos têm sido comercialmente explorados para a obtenção de tais compostos (CAMPENNI' et al., 2013). Diferentes espécies de fungos filamentosos, leveduras, bactérias e microalgas têm sido testadas em processos biotecnológicos para a obtenção de carotenoides (DRAAISMA et al., 2013; FRENGOVA; BESHKOVA, 2009).

A função mais importante dos carotenoides nas leveduras é a proteção contra a combinação prejudicial do oxigênio singleto com a luz visível ou UV (BERERA et al., 2010). A ação destas moléculas está em desativar os radicais livres produzidos durante o metabolismo normal das células, tais como o oxigênio singleto ($^1O_2$), o radical hidroxila (OH^-), peróxidos e outros oxidantes por meio de um processo no qual a energia é

transferida de altos níveis de excitação para uma molécula de carotenoide, a qual pode retornar ao estado fundamental liberando calor (BERERA et al., 2010).

Moliné et al. (2009) estudaram o papel fotoprotetor dos carotenoides em leveduras através da comparação das respostas a radiação UV-B de células pigmentadas e albinas naturais. O estudo foi realizado com diferentes tempos de exposição a radiação UV-B, em vários estágios do crescimento da levedura, da mesma forma avaliaram a produção de carotenoides por fotoindução e o efeito da radiação UV-B na sobrevivência das leveduras. Estes autores observaram que as cepas pigmentadas são mais tolerantes a radiação do que as cepas albinas e que o aumento no teor de carotenoides durante a fase de crescimento estacionário beneficia a sobrevivência das leveduras.

A produção de carotenoides pela levedura *R. glutinis* cultivada em diferentes subprodutos agroindustriais vem sendo estudada. Malisorn e Suntornsuk (2008) aplicaram um delineamento composto central rotacional para otimizar as condições de cultivo para a levedura *R. glutinis* DM28, tendo como resposta a produção de β-caroteno. O experimento foi conduzido em um tanque reator com agitação e a salmoura de rabanete fermentado foi utilizada como substrato. Os Autores observaram que as condições ótimas para a produção de β-caroneto (201 μg/L) foi de 30 °C, pH 6 e 80% de oxigênio dissolvido.

2.7 *Chlorella protothecoides*

As microalgas constituem uma ampla faixa de microrganismos autotróficos cujo crescimento através de fotossíntese é igual ao das plantas terrestres. Sua estrutura unicelular permite que elas convertam facilmente a energia solar em energia química (HARUN et al., 2010). Sistemas de cultivo de microalgas para a produção de moléculas de alto valor comercial tais como carotenoides e lipídeos, vêm sendo cada vez mais explorados em todo o mundo, apresentando uma grande perspectiva para aplicações industriais (CAMPENNI' et al., 2013).

Vários processos têm demonstrado a potencialidade do cultivo de microalgas principalmente para as indústrias de alimentos e rações, produzindo pigmentos e lipídeos, assim como para as indústrias farmacêutica e de cosméticos, produzindo compostos antioxidantes como a astaxantina (RODRIGUES et al., 2014). As microalgas são reconhecidamente uma excelente fonte de carotenoides (DUFOSSÉ et al., 2005).

A microalga de água doce *C. protothecoides* é capaz de acumular elevados teores de proteínas, clorofila, carotenoides e lipídeos em suas células, sendo conhecida principalmente pela sua capacidade de acumular elevados teores de luteína assim como outros carotenoides de alto valor comercial, como a astaxantina e o β-caroteno (GUEDES; AMARO; MALCATA, 2011). Esta microalga tem sido amplamente estudada com relação a sua potencialidade para ser empregada em processos biotecnológicos para a produção de carotenoides e lipídeos (ARAYA et al., 2014; CAMPENNI' et al., 2013; O'GRADY; MORGAN, 2011; TERCERO; SFORZA; MORANDINI, 2014), apresentando grande versatilidade em relação ao seu cultivo, sendo capaz de crescer sob condições autototróficas, mixotróficas e heterotróficas (FERNÁNDEZ-SEVILLA; ACIÉN FERNÁNDEZ; MOLINA GRIMA, 2010).

2.8 *Rhodotorula Glutinis*

A levedura vermelha *Rhodotorula glutinis* é um microrganismo aeróbico capaz de acumular lipídeos e pigmentos carotenoides em suas células, sintetizando o β-caroteno, o toruleno e a torularrodina como carotenoides principais (AKSU; EREN, 2007). O acúmulo celular de lipídeos pode alcançar rendimentos de até 72% em relação da biomassa seca (SCHNEIDER et al., 2013). A *Rhodotorula glutinis* apresenta amplo potencial para ser utilizada na indústria de alimentos por ser uma fonte de moléculas de alto valor comercial, como: proteínas, pigmentos e ácidos graxos insaturados como os ácidos oleico, linoleico e linolênico (SAENGE et al., 2011).

O emprego deste microrganismo em processos biotecnológicos voltados para a obtenção destes compostos tem sido amplamente estudado e resultados atrativos têm sido obtidos a partir destas investigações. Cutzu et al. (2013) avaliaram a produção de carotenoides por *R. glutinis* em meio de cultura contendo glicerol e obtiveram uma produção máxima de carotenoides 14 mg/L.

Esta espécie pertence ao filo *Basidiomycota*, classe *Urediniomycetes* e ordem esporídica. As células possuem um diâmetro de três a cinco micrômetros, com formato elíptico, brotação multipolar, produzindo pseudo-hifas e apresentam reprodução sexual com conexão micelial da braçadeira e teliosporos. Leveduras do gênero *Rhodotorula* são encontradas no ar, no solo e em frutas e folhas, podendo ser encontradas também em leite e derivados (HERNANDEZ-ALMANZA et al., 2014).

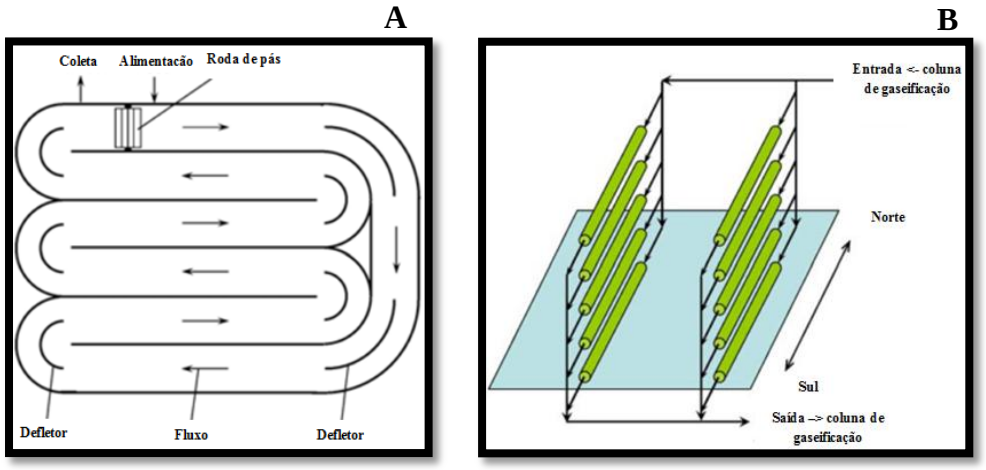
2.9 Processo de cultivo

2.9.1 *Chlorella protothecoides*

O meio de cultura para o cultivo de microalgas deve fornecer os elementos inorgânicos que constituem a célula da microalga. Os elementos essenciais incluem nitrogênio, fósforo e ferro. O requerimento nutricional mínimo pode ser estimado usando-se a fórmula molecular aproximada da biomassa de microalgas, que é $CO_{0,48}H_{1,83}N_{0,11}P_{0,01}$ (CHISTI, 2007). O crescimento das microalgas, assim como o acúmulo de metabólitos em suas células, depende significativamente das condições de cultivo (CHOJNACKA; MARQUEZ-ROCHA, 2004).

De acordo com a fonte de energia e de carbono, existem três principais modos de cultivo para as microalgas: autotrófico, heterotrófico e mixotrófico. Além disto, o cultivo pode ser realizado utilizando-se sistemas abertos (Figura 6A) ou fechados (Figura 6B).

Figura 6. Cultivo de microalgas em sistema aberto (A) e sistema fechado (B).



Fonte: Adaptada de Chisti (2007)

Como exemplo destes dois sistemas, os mais utilizados, respectivamente, são as lagoas abertas (*open raceway ponds*) e os fotobiorreatores, onde estes últimos apresentam os mais variados modelos (CHEN et al., 2011). Os sistemas abertos são menos favoráveis, pois apresentam limitações no que diz respeito ao controle de contaminações, enquanto que nos sistemas fechados as condições de cultivo são fáceis de serem controladas (temperatura, iluminação, CO_2 dissolvido e concentração de nutrientes) e os problemas de contaminação podem ser mais facilmente evitados

(UGWU; AOYAGI; UCHIYAMA, 2008). Entretanto, a grande desvantagem é que os fotobiorreatores têm custo inicial elevado e são específicos de acordo com a espécie de microalga a ser cultivada (UGWU; AOYAGI; UCHIYAMA, 2008).

Cultivo autotrófico

O modo de cultivo autotrófico ocorre quando a microalga utiliza a luz (natural ou artificial) como fonte de energia e carbono inorgânico (CO_2) como fonte de carbono para produzir energia química através da fotossíntese (LI et al., 2014). Esta condição de cultivo é a mais utilizada para o crescimento de microalgas (GOUVEIA et al., 1996) e é bem conhecida por favorecer o acúmulo celular de pigmentos, no entanto, as produções de lipídeos e de biomassa são inferiores quando esta condição é comparada com os cultivos heterotrófico e mixotrófico (ABREU et al., 2012). Devido a isto, quando se objetiva a obtenção de lipídeos condições de estresse normalmente são empregadas para os cultivos autotróficos, tais como a deficiência de nutrientes no meio de cultura (CAMPENNI' et al., 2013).

A principal vantagem em utilizar o cultivo autotrófico para o crescimento de microalgas é o consumo de CO_2 como fonte de carbono, o que pode ser interessante para estratégias de redução da emissão de gás carbônico na atmosfera e, além disto, é uma fonte de carbono de custo mais reduzido quando comparada com fontes orgânicas como a glicose (LI et al., 2014). Outra vantagem da utilização deste modo de cultivo em relação aos demais é que os problemas de contaminação são menos severos, uma vez que não há a presença de carbono orgânico nos meios de cultura. Portanto, na ampliação de escala com a utilização de sistemas abertos tais como "open ponds" e "raceway ponds" são usualmente empregadas condições de cultivo autotróficas (CHEN et al., 2011).

Cultivo heterotrófico

Algumas espécies de microalgas podem não apenas crescer sob condições autotróficas, mas também utilizando fontes de carbono orgânicas e na ausência da luz. Esta situação na qual a microalga utiliza uma fonte de carbono orgânica como fonte de energia e de carbono é conhecida como cultivo heterotrófico (PEREZ-GARCIA et al., 2011). Este tipo de tipo de cultivo pode evitar problemas devido a limitação da luz em cultivos com elevada densidade celular. Neste modo de cultivo é possível obter maiores

produções de biomassa e de lipídeos comparando-se com o cultivo autotrófico (DE LA HOZ SIEGLER et al., 2011).

Xu, Miao e Wu (2006) observaram um aumento do acúmulo celular de lipídeos de 40% na microalga *C. protothecoides* mudando-se a condição de cultivo de autotrófica para heterotrófica. As microalgas podem assimilar uma grande variedade de fontes de carbono orgânicas para o crescimento, tais como: glicose, acetato, glicerol, frutose, galactose, lactose e manose (LIANG; SARKANY; CUI, 2009). A desvantagem da utilização do cultivo heterotrófico está no custo da fonte de carbono orgânica, portanto, alguns estudos têm avaliado a utilização de fontes alternativas de baixo custo, tais como os resíduos e subprodutos agroindustriais (YEN; YANG; YU, 2012). Além disto, o cultivo heterotrófico deve ser conduzido sob condições bem controladas visto que o meio de cultura contendo a fonte de carbono orgânica é mais susceptível a contaminação (CHEN et al., 2011).

Cultivo mixotrófico

O cultivo mixotrófico ocorre quando a microalga realiza o processo de fotossíntese e utiliza ambas as fontes de carbono orgânica e inorgânica (CO_2) para o crescimento (LEE et al., 1996). Isto significa que as microalgas são capazes de viver sob condições autotróficas, heterotróficas ou em ambas (modo mixotrófico). Para algumas espécies o cultivo mixotrófico pode aumentar significativamente a produção de biomassa e além disso é um método eficiente tanto para a produção de lipídeos como para a produção de pigmentos, uma vez que este modo é a junção dos cultivos heterotrófico e mixotrófico (ABREU et al., 2012).

2.10.2 Rhodotorula glutinis

O crescimento da levedura *Rhodotorula glutinis* é influenciado por diversos parâmetros do processo de cultivo (AKSU; EREN, 2007). Para melhorar a performance e consequentemente reduzir os custos dos processos biotecnológicos, alguns estudos têm sido conduzidos para otimizar as condições de cultivo (PARK et al., 2005), incluindo parâmetros físicos e nutricionais, tais como a natureza e concentração das fontes de carbono e nitrogênio, as concentrações de minerais e vitaminas e fatores como pH, aeração, temperatura, luz, estresse, irradiação, microfiltração e agitação. Estes

parâmetros são conhecidos por exercerem influência no crescimento e no acúmulo de metabólitos da levedura *Rhodotorula glutinis* (BUZZINI, 2000; MALISORN; SUNTORNSUK, 2008).

O estresse em tecidos biológicos é conhecido por trazer respostas bioquímicas envolvendo um aumento na atividade enzimática e nos níveis de carotenoides quando as células estão crescendo sob condições desfavoráveis (SALAR et al., 2013). Kim et al. (2004) observaram que o perfil de carotenoides produzidos pela levedura *R. glutinis* foi dependente da adição de fenol no meio. O teor de β-caroteno aumentou em 35% quando o fenol foi adicionado ao meio de cultura a uma concentração de 500 ppm.

Várias fontes de carbono e nitrogênio alternativas têm sido utilizadas para o cultivo da levedura *R. glutinis* com o propósito de desenvolver um meio de cultura de baixo custo e que possua o balanço adequado de nutrientes para favorecer o crescimento e o acúmulo celular das moléculas de interesse (CUTZU et al., 2013).

2.10 Estresse induzido

A função biológica dos carotenoides é de proteger os componentes celulares contra os efeitos de radicais livres e de espécies reativas do oxigênio (IP; CHEN, 2005). Geralmente, a carotenogênese em microalgas é induzida através do estresse oxidativo (DINESHKUMAR et al., 2015). Quando radicais livres encontram outra molécula e obtém um elétron a partir da mesma para se estabilizar ocorrem danos nas células já que a molécula afetada passa a agir como um radical livre dando início a uma reação em cadeia. A síntese de carotenoides pela célula é importante pois estas moléculas têm a capacidade de doar elétrons, agindo como inibidoras dos processos oxidativos mesmo estando presentes em pequenas concentrações (JOMOVA; VALKO, 2013).

A exposição das células a alta incidência luminosa é uma das técnicas mais utilizadas para se induzir o estresse oxidativo em culturas de microalgas. O acúmulo de energia em excesso leva a formação de espécies reativas do oxigênio nas células, portanto, a síntese de carotenoides é induzida com o objetivo de proteger as células da fotodegradação. Esta indução da síntese de carotenoides pode seguir diferentes vias metabólicas a depender do tipo de luz e da espécie de microalga(GOUVEIA, 1996).

Dentre os carotenoides de maior poder antioxidante, luteína, astaxantina e β-caroteno podem ser sintetizados em excesso das células de microalgas durante a exposição à elevada incidência luminosa. Outra condição de estresse comumente

utilizada para induzir a síntese de carotenoides é a limitação de nutrientes no meio de cultura (CAMPENNI' et al., 2013). A limitação ou a falta de macro e/ou microelementos no meio é conhecida por induzir as estratégias de defesa da célula tal como a indução da síntese de alguns carotenoides (WEI et al., 2008).

Microalgas sob condições de estresse são capazes de produzir grandes quantidades de substâncias de elevado valor agregado, tais como: carotenoides, compostos fenólicos e ácidos graxos poli-insaturados (IP; CHEN, 2005; PETTI et al., 2011). Os metabólitos secundários produzidos por organismos fotossintéticos encontram numerosas aplicações nas indústrias de cosméticos, farmacêutica e de alimentos (WICHUK; BRYNJÓLFSSON; FU, 2014). Os carotenoides secundários são amplamente utilizados como antioxidantes, agindo como alvos para espécies reativas do oxigênio (IP; CHEN, 2005).

2.11 Processo de extração dos metabólitos intracelulares

A extração de carotenoides e lipídeos a partir de leveduras e microalgas apresenta um desafio adicional devido ao fato da produção destes metabólitos ser intracelular e, além disso, as características destes compostos que variam entre polares e não polares tornam difícil a escolha do solvente orgânico devido as diferentes solubilidades. Além disso, a ruptura das células destes microrganismos, antes do processo de extração, é uma tarefa fundamental para a obtenção de bons rendimentos e também muito difícil de ser realizada (PRABAKARAN; RAVINDRAN, 2011; RYCKEBOSCH; MUYLAERT; FOUBERT, 2011).

Diferentes técnicas são utilizadas para a ruptura celular, dentre as mais utilizadas pode-se citar: a ruptura química utilizando dimetilsulfóxido (DMSO) ou ácidos, a ruptura enzimática, e processos físicos utilizando calor, microondas, congelamento e descongelamento, pressões elevadas, sonicação e procedimentos mecânicos com o uso de pérolas de vidro e areia (CASTRO-PUYANA et al., 2013).

3. MATERIAL E MÉTODOS

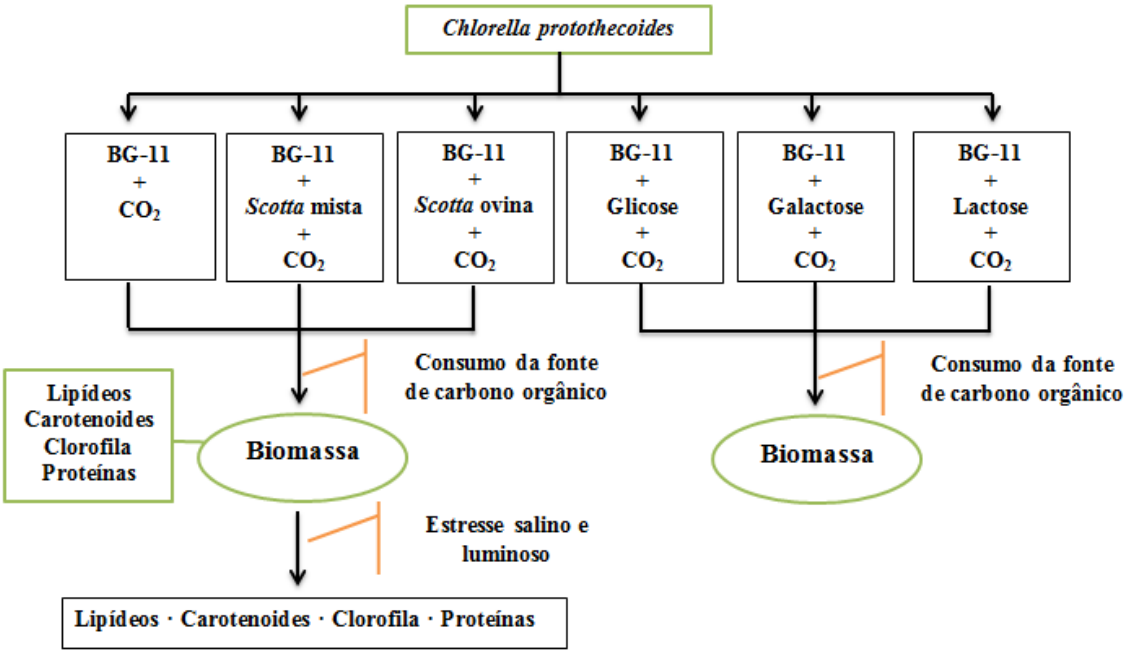
3.1 Delineamneto experimental

Em decorrência de um período de um ano de doutorado sanduíche (CAPES) realizado na Universidade de Pisa (Toscana, Itália), o desenvolvimento do estudo da presente tese foi conduzido em duas etapas denominadas: Experimento I e Experimento II.

3.1.1 Experimento I – Itália

Este estudo foi desenvolvido no Departamento de Biologia da Universidade de Pisa (Itália). A microalga *Chlorella protothecoides* e a levedura *Rodotorula glutinis* foram cultivadas em meios de cultura contendo o soro de queijo ricota, conhecido na Itália como *scotta*. No delineamento adotado para a microalga *Chlorella protothecoides* (Figura 7), foram realizados cinco experimentos com a utilização de fontes de carbono orgânico em combinação com o CO_2 (cultivos mixotróficos) e um experimento em modo autotrófico. Para todos os experimentos foi adotado o meio inorgânico BG-11 e um fotoperíodo de 16:8 (luz:escuro). Todos os experimentos foram realizados em triplicata.

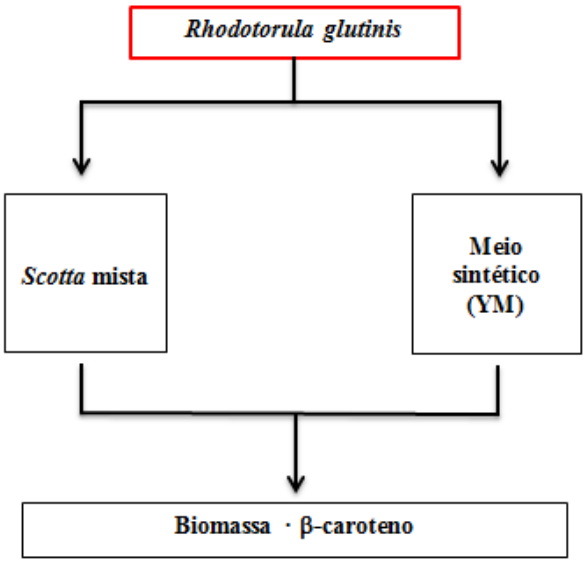
Figura 7. Esquema experimental para o cultivo da *Chlorella protothecoides*



Fonte: Própria (2018)

No delineamento adotado para o cultivo da levedura *Rodotorula glutinis* (Figura 8) foram realizados dois experimentos, um com o cultivo da levedura em meio contendo a *scotta* mista (90% de soro bovino e 10% de soro ovino) como única fonte de nutrientes e outro em meio sintético YM. Os experimentos foram realizados em triplicata e repetidos duas vezes.

Figura 8. Esquema experimental utilizado para o cultivo da *R. glutinis*



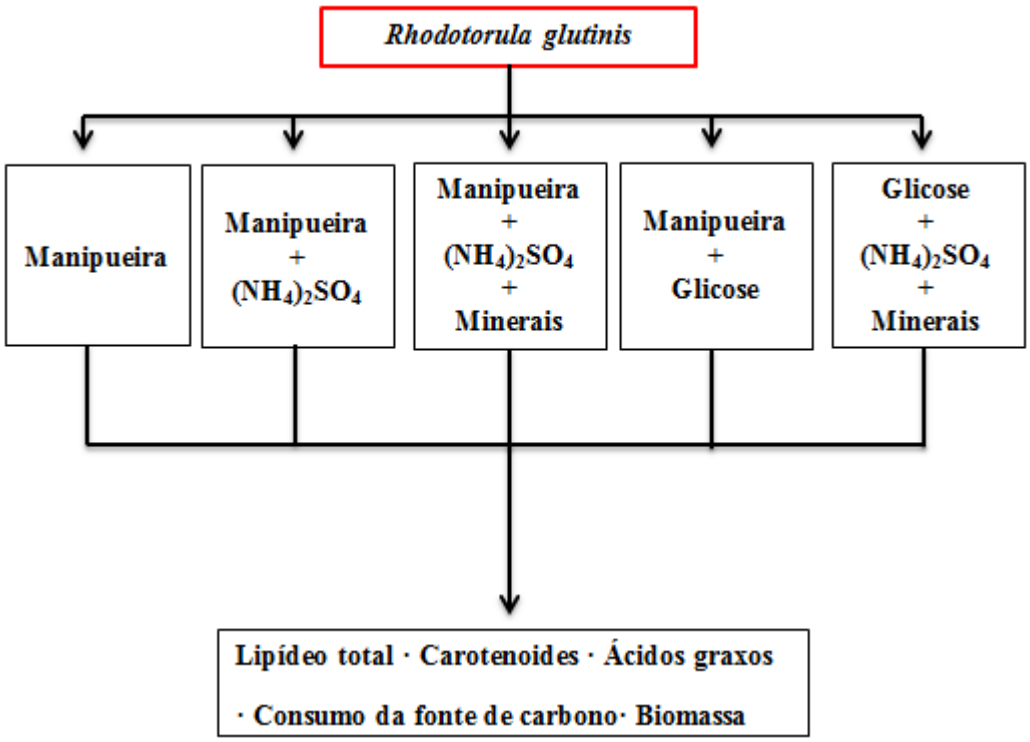
Fonte: Própria (2018)

3.1.2 Experimento II – Brasil

Este estudo foi desenvolvido no Laboratório de Bioengenharia do Departamento de Engenharia Química da Universidade Federal da Paraíba. A levedura *Rodotorula glutinis* foi cultivada em meios de cultura compostos pela manipueira.

No delineamento experimental adotado (Figura 9), foram realizados cultivos utilizando a manipueira como única fonte de nutrientes, variando-se o teor de açúcares redutores inicial nestes meios. Foram também realizados testes com a suplementação destes meios com glicose, sulfato de amônia e minerais (macros e micro elementos). Para efeito de comparação com os meios alternativos foram também conduzidos cultivos em meios sintéticos contendo glicose como única fonte de carbono. No total foram conduzidos dez experimentos. Todos os experimentos foram realizados em triplicata.

Figura 9. Esquema experimental utilizado para o cultivo da *R. glutinis*



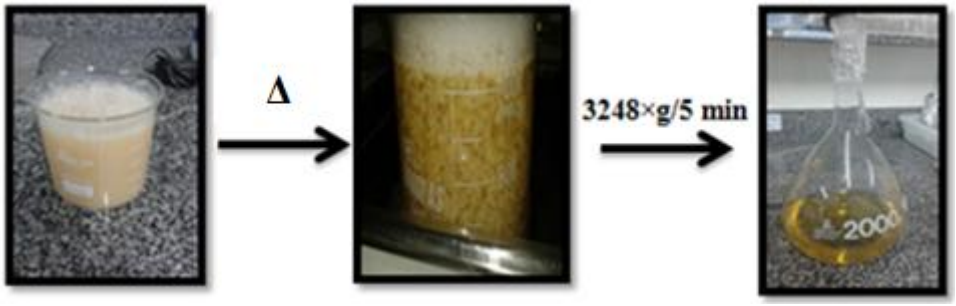
Fonte: Própria (2018)

3.2 Manipueira

A manipueira utilizada nos experimentos foi fornecida pela casa de farinha "Folha verde", localizada no município de Sobrado (07º 08' 43" S, 35º 14' 11" O), estado da Paraíba. A coleta da manipueira foi feita logo após a prensagem da massa de mandioca triturada. Para a utilização nos experimentos a manipueira foi submetida a um pré-tratamento onde foi aquecida a 100 °C por 20 minutos, resfriada e centrifugada a 3248×g por 5 minutos, coletando-se o sobrenadante e desprezando-se o resíduo sólido remanescente (Figura 10).

Esta manipueira pré-tratada foi analisada para a determinação dos seguintes parâmetros físico-químicos: pH, açúcar redutor, carboidrato total, cianeto livre, matéria seca total, nitrogênio total, sódio, cálcio, magnésio, fósforo, potássio, ferro, zinco e cobre.

Figura 10. Pré-tratamento da manipueira.



Fonte: Própria (2018)

3.3 *Scotta*

A *scotta* utilizada nos experimentos foi fornecida por laticínios localizados na região da toscana, na Itália. Dois tipos de *scotta* foram utilizados: *scotta* ovina e *scotta* mista (90% bovina e 10% ovina). Estes dois tipos de *scotta* foram analisados para a determinação dos seguintes parâmetros físico-químicos: pH, acidez, lactose, matéria seca total, matéria mineral, nitrogênio total, cálcio, magnésio, fósforo e lipídeos totais.

3.4 Microrganismos

3.4.1 Microalga – Chlorella protothecoides

Para a realização dos experimentos foi utilizada a microalga de água doce *Chlorella protothecoides* Krüger (ATCC® 30411). As células foram obtidas já reativadas em meio BG-11 contendo 2% de ágar.

3.4.2 Levedura – Rhodotorula glutinis

Para a realização do Experimento I, a cepa da levedura *Rhodotorula glutinis* foi fornecida pelo laboratório de parasitologia do Departamento de Ciências Veterinárias da Universidade de Pisa. As células de levedura foram fornecidas ativadas em placas com o meio YMA.

A levedura *Rhodotorula glutinis* (CTT 2182), utilizada na realização do Experimento II, foi gentilmente fornecida pela Fundação André Tosello (Campinas, São Paulo, Brasil). As células, obtidas na forma liofilizada, foram hidratadas por 10 dias, a uma temperatura de 30 °C, em meio YM, contendo: 3 g/L de extrato de levedura, 3 g/L

de extrato de malte, 5 g/L de peptona e 10,0 g/L de glicose. Após este período as células foram transferidas com uma alça esterilizada para placas de Petri contendo o meio YM com 2 % de ágar bacteriológico (meio YMA). As placas foram incubadas de forma invertida a 30 °C por 48 horas e armazenadas em refrigeração a 5 °C.

3.5 Manutenção das células

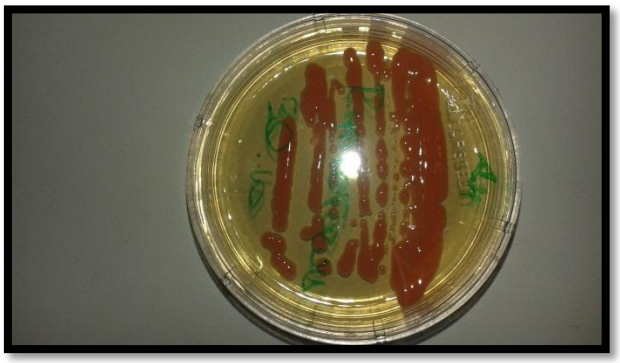
3.5.1 Chlorella protothecoides

As células foram mantidas vivas por meio de transferência periódica (a cada quatro meses) para placas de Petri contendo meio BG-11 com 2% de ágar. O meio BG-11 foi composto por: 1500 mg/L de $NaNO_3$, 3,05 mg/L de KH_2PO_4, 6 mg/L de citrato de amônio férrico, 1,81 mg/L de $MnCl_2{\cdot}4H_2O$, 75 mg/L de $MgSO_4{\cdot}7H_2O$, 0,079 mg/L de $CuSO_4{\cdot}5H_2O$, 36 mg/L de $CaCl_2{\cdot}2H_2O$, 0,222 mg/L de $ZnSO_4{\cdot}7H_2O$, 0,05 mg/L de $CoCl_2{\cdot}6H_2O$, 2,86 mg/L de H_3BO_3, 6 mg/L de ácido cítrico$\cdot 1H_2O$ e 20 mg/L de Na_2CO_3. Após o crescimento as placas foram mantidas em condição de temperatura controlada.

3.5.2 Rhodototula glutinis

As células foram mantidas vivas por meio de transferência periódica (mensalmente) para placas de Petri contendo meio YMA (0,3% de extrato de levedura, 0,3% de extrato de malte, 0,5% de peptona, 1% de dextrose e 2% de ágar) (Figura 11). Após o crescimento as placas foram mantidas sob refrigeração (5 °C).

Figura 11. Placa de Petri contendo a levedura *R. glutinis* em meio YMA.



Fonte: Própria (2018)

3.6 Preparo do inóculo

3.6.1 Chlorella protothecoides

Para o preparo do inóculo, as células foram transferidas das placas para fotobiorreatores contendo 1000 mL do meio de cultura BG11. Os fotobiorreatores foram incubados a 25 °C, durante 7 dias, com fotoperíodo de 16:8 (Luz:Escuro). A agitação do meio ocorreu por meio da injeção de ar enriquecido com CO2 (10%), a uma taxa de 0.1L/min.

3.6.2 Rhodotorula glutinis

Para o preparo do inóculo, as células foram transferidas das placas para frascos Erlenmeyer flasks de 300 mL, contendo 100 mL do meio de cultura (glicose (10 g/L), peptona (5 $g \cdot L^{-1}$), extrato de levedura (3 $g \cdot L^{-1}$), e extrato de malte (3 $g \cdot L^{-1}$)). Os frascos foram incubados a 30 °C e 200 rpm por 24 horas. Em seguida as células foram coletadas por centrifugação a 3248×g por 5 min e suspensas em água destilada esterilizada para atingir uma concentração celular de 10^9 celúlas/mL. A concentração celular da suspensão foi determinada utilizando-se uma câmara de Neubauer.

3.7 Meio e condições de crescimento

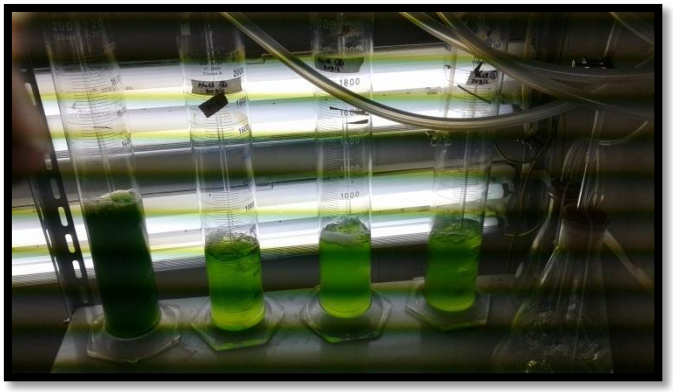
3.7.1 Chlorella protothecoides

Para o cultivo autotrófico foi utilizado o meio BG-11 (ARAYA et al., 2014). Para os cultivos mixotróficos utilizando a *scotta*, o meio inorgânico foi parcialmente substituído pelo substrato alternativo (30% v/v). Com o objetivo de avaliar a assimilação das fontes de carbono orgânico pela microalga *C. protothecoides*, nas condições de cultivo utilizadas, lactose (12 g/L), glicose (12 g/L) e galactose (12 g/L) foram adicionadas ao meio BG-11 como controles. A concentração dos açúcares foi definida com base na concentração de lactose nos meios contendo 30 % de *scotta*.

O cultivo em batelada foi realizado inoculando-se 10% (v/v) da cultura inicial (biomassa seca igual a 1.2 $g \cdot L^{-1}$) em 1 litro do meio de cultura. Antes do inóculo, todos dos meios foram esterilizados a 121 °C por 20 min. O cultivo ocorreu durante 7 dias sob

condições assépticas em fotobiorreatores de 2 L (Figura 12) e fotoperíodo de 16:8 (Luz:Escuro). Após o cultivo normal, foi realizado um estresse induzido, diluindo-se (1:5) a cultura final em uma solução esterilizada de NaCl 20 g/L.

Figura 12. Biorreatores utilizados no cultivo da microalga *C. protothecoides*.



Fonte: Própria (2018)

3.7.2 Rhodotorula glutinis

O cultivo foi conduzido em frascos Erlenmeyer de 300 mL contendo 150 mL do meio de cultura (Figura 13). Foram realizados um total de dez cultivos (Tabela 1) variando-se os nutrientes (manipueira, glicose, sulfato de amônia e elementos minerais) utilizados na composição do meio e as concentrações das fontes de carbono utilizadas (manipueira e glicose). Para a glicose foram utilizadas concentrações de 10 g/L, 30 g/L e 50 g/L. A manipueira foi utilizada nos meios com base na concentração de açúcares redutores (g de AR/L), utilizando-se concentrações de 10, 20 e 30 g de AR/L. O sulfato de amônia foi utilizado como fonte de nitrogênio na concentração de 5 g/L.

Para a suplementação dos meios com minerais foram utilizados elementos macro e traço, conforme segue: KH_2PO_4 (1 $g \cdot L^{-1}$), $MgSO_4 \cdot 7H_2O$ (0,25 $g \cdot L^{-1}$), $Na_2HPO_4 \cdot 12H_2O$ (1 $g \cdot L^{-1}$), 6 $mL \cdot L^{-1}$ de uma solução de $FeSO_4$ (4 $g \cdot L^{-1}$) e 10 $mL \cdot L^{-1}$ de uma solução de minerais traço. A solução de minerais traço utilizada contém por litro: 0,36 g de $CaCl_2 \cdot 2H_2O$, 0,075 g de $ZnSO_4 \cdot 7H_2O$, 0,013 g de $CuSO_4 \cdot 5H2O$, 0,05 g de $MnSO_4 \cdot H_2O$, 0,013 g de $CoCl_2 \cdot 6H_2O$ e 0,035 g de $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (BRAUNWALD, 2013).

Antes do inóculo todos os meios foram esterilizados a 121 °C por 15 minutos. As células foram inoculadas no meio para atingir uma concentração inicial de 10^7 cells/mL. Todos os experimentos foram conduzidos em triplicata e repetidos duas vezes.

A levedura *R. glutinis* também foi avaliada com relação a sua adaptação e crescimento em meio de cultura composto pela *scotta* mista. Para efeito de comparação foi realizado um tratamento controle com o cultivo em meio sintético YM. Estes dados foram publicados na forma de artigo (Apêndice I).

Tabela 1. Composição do meio de cultura dos ensaios realizados em frascos Erlenmeyer

	$(NH_4)_2SO_4$ (g/L)	Glicose (g/L)	Manipueira (g de AR/L)	Suplementação* mineral
Ensaio 1	5	-	30	-
Ensaio 2	5	30	-	+
Ensaio 3	5	-	30	+
Ensaio 4	-	-	30	-
Ensaio 5	-	10	30	-
Ensaio 6	5	50	-	+
Ensaio 7	-		20	-
Ensaio 8	-	-	10	-
Ensaio 9	5	-	20	+
Ensaio 10	5	-	10	+

*Com suplementação (+); Sem suplementação (-)

Figura 13. Cultivo da levedura *R. glutinis* em frascos erlenmeyer.

Fonte: Própria (2018)

3.8 Crescimento da biomassa

3.8.1 Chlorella protothecoides

A biomassa seca foi quantificada ao longo do cultivo normal e ao final da "fase de estresse". Amostras foram coletadas (50 mL) e centrifugadas a 3951×g por 5 minutos. O sobrenadante foi descartado e a biomassa fresca (Figura 14) foi desidratada em estufa a 60 °C por 24 horas. A biomassa seca foi quantificada por gravimetria.

Figura 14. Biomassa fresca coletada por centrifugação.

Fonte: Própria (2018)

3.8.2 Rhodotorula glutinis

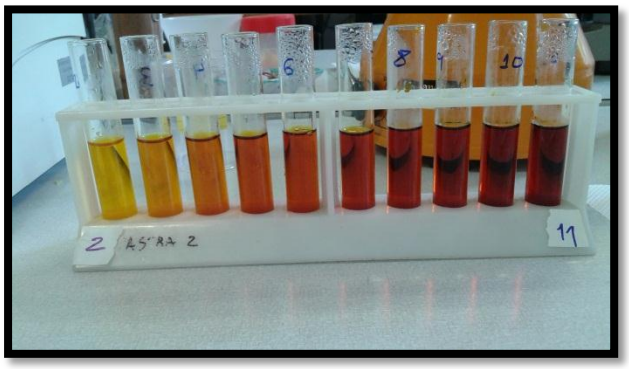
O crescimento foi monitorado regularmente através do mesuramento da turbidez do meio em espectrofotômetro modelo U2M Quimis em comprimento de onda de 600 nm. Para a quantificação da concentração de biomassa no meio foi utilizada uma curva padrão com os valores de densidade ótica em função da concentração de biomassa seca. O meio sem as células, e na mesma diluição das amostras do meio, foi utilizado como branco.

3.9 Açúcares redutores

Os açúcares redutores foram quantificados ao longo do cultivo, utilizando o reagente DNS, segundo metodologia proposta por MILLER (1959). Para a

quantificação foram preparadas curvas padrões com lactose, glicose e galactose (Figura 15), a depender do tipo de açúcar presente no meio.

Figura 15. Preparo da curva padrão de DNS para a análise de açúcares redutores.



Fonte: Própria (2018)

3.10 Carboidratos totais

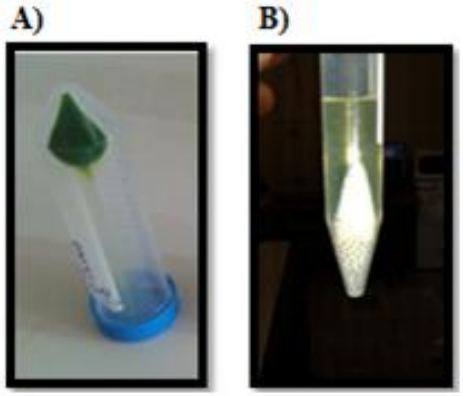
Os carboidratos totais foram quantificados ao longo do cultivo, utilizando o reagente Antrona, segundo metodologia proposta por Yemm e Willis (1954). Para a quantificação foi preparada um curva padrão com uma solução de glicose (0,1 g/L).

3.11 Extração e quantificação de pigmentos

3.11.1 Chlorella protothecoides

Clorofila e carotenoides totais foram quantificados ao longo da "fase verde" e ao final da "fase de estresse", enquanto os teores de astaxantina, luteína/zeaxantina e β-caroteno foram determinados apenas ao final de cada fase. A extração dos pigmentos foi feita a partir da biomassa fresca. Amostras de 15 mL do meio de cultura foram coletadas e centrifugadas a 3951×g por 10 minutos, então o sobrenadante foi descartado e a biomassa (Figura 16A) foi misturada com pérolas de vidro de 1,0 mm de diâmetro (Sigma-Aldrich, Italy) e 5 mL de metanol. Para a extração dos pigmentos as amostras foram colocadas no vortex por 2 minutos e após centrifugação a 3951×g por 5 minutos foi coletado o sobrenadante contendo dos pigmentos extraídos. Este procedimento foi repetido até o sobrenadante e a biomassa ficarem descoloridos (três vezes) (Figura 16B).

Figura 16. Biomassa da microalga antes da extração dos pigmentos (A) e após este processo (B)



Fonte: Própria (2018)

Os teores de clorofila e carotenoides totais foram determinados por espectrofotometria de acordo com a metodologia descrita por Lichtenthaler e Buschmann (2001). Os espectros de absorção dos extratos foram mesurados em espectrofotômetro SPECTROstar Nano (BMG Labtec, Ortenberg, Germany) em faixa de comprimento de onda variando de 380 a 700 nm. Os teores de clorofila e carotenoides totais foram expressos em base seca. O peso da biomassa seca da microalga *C. protothecoides* foi determinado após secagem em estufa a 60 °C até peso constante.

Para a quantificação dos teores de astaxantina, Luteína/Zeaxantina e β-caroteno a análise foi conduzida em um instrumento HPLC SpectraSystem (Figura 14) equipado com um detector UV-VIS (Thermo, Rodano, Italy). A coluna foi utilizada foi uma Kinetex C18, 250 x 4.6 mm ID, 5 µm particle size (Phenomenex, Torrance, CA, USA), eluição a 1 ml min^{-1} com solvente A (acetonitrile: methanol: Tris buffer 0.1 M pH 8 84:2:14) e B (methanol:ethyl acetate 68:32), de acordo com a seguinte programação: 100% solvente A por 4 minutos, em seguida um gradiente linear de 0 a 100% B em 10 minutos, e 100% B por 15 minutos.

Os picos de astaxantina, luteín/zeaxantina e β-caroteno foram identificados por meio da comparação dos tempos de retenção dos picos da amostra com aqueles dos padrões puros de β-caroteno, luteína, zeaxantina (Extransynthese, Lyon, France) e astaxantina (Sigma-Aldrich, Italy). Os teores de Astaxantina, Luteína/Zeaxantina e β-caroteno foram quantificados tomando-se curvas de calibração como referências.

3.11.2 Rhodototula glutinis

A extração dos carotenoides foi realizada de acordo com a metodologia descrita por Cutzu et al. (2013), com modificações. A biomassa fresca foi coletada por centrifugação a 2260×g por 10 minutos, o sobrenadante foi descartado e as células foram congeladas a -20 °C por 24 horas. Para a ruptura celular, as células foram descongeladas e suspendidas em 2 ml de dimetilsulfóxido (DMSO) a 60 °C, foram adicionadas pérolas de vidro de 0,1 mm de diâmetro (Sigma-Aldrich, Brasil) e esta mistura foi colocada no vortex por 2 minutos, sendo em seguida incubada a 60 °C por 15 min. Para a extração e separação dos pigmentos foram adicionados em sequência 2 mL de acetona, 2 mL de éter de petróleo e 2 mL de NaCl 20 %, posteriormente colocando-se a amostra em vortex por um tempo total de 5 min.

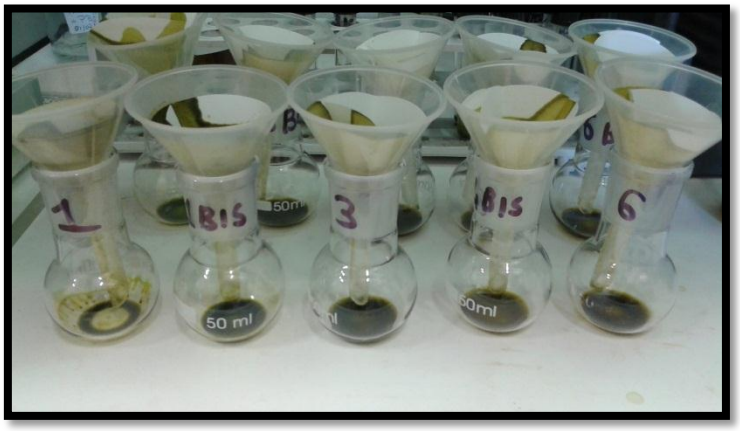
Esta mistura foi centrifugada a 2260×g por 10 min e a fase superior de éter de petróleo, contendo os carotenoides extraídos, foi coletada e os carotenoides totais foram quantificados como equivalentes em β-caroteno em espectrofotômetro com leitura de comprimento de onda em 450 nm, seguindo-se a metodologia proposta por Rodriguez-Amaya e Kimura (2004).

3.12 Extração e quantificação de lipídeos

3.12.1 Chlorella protothecoides

Os lipídeos totais foram quantificados ao final do cultivo normal e ao final da "fase de estresse". Para a determinação de lipídeos totais a biomassa foi desidratada a 60°C por 24 horas e macerada utilizando o almofariz e pistilo. Após este processo foi feita a extração e quantificação dos lipídeos de acordo com a metodologia descrita por BLIGH e DYER (1959), utilizando 200 mg da biomassa seca. Os extratos lipídicos foram coletados em balões de 50 mL (Figura 17) e evaporados em rotavapor a 70 °C, sob vácuo. Para a ruptura celular foram utilizadas pérolas de vidro de 1 mm de diâmetro (Sigma-Aldrich, Italy). Os resultados desta análise constam no Apêndice II.

Figura 17. Extração de lipídeos da microalga *Chlorella protothecoides*.



Fonte: Própria (2018)

3.12.1 Rhodotorula glutinis

Para a determinação de lipídeos a biomassa foi seca a 80 °C por 24 horas e macerada em almofariz e pistilo. Um seguida pesou-se 200 mg de amostra e foi realizada uma hidrólise acida para a ruptura celular utilizando ácido clorídrico 2 N e os lipídeos foram extraídos e quantificados seguindo-se metodologia preconizada por Bligh e Dyer (1959).

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4. RESULTADOS E DISCUSSÃO

Os resultados e discussão estão apresentados sob a forma de dois artigos científicos originais, intitulados:

ARTIGO I – "Production of *Chlorella protothecoides* biomass, chlorophyll and carotenoids using the dairy industry by-product *scotta* as a substrate". Publicado no periódico *Biocatalysis and Agricultural Biotechnology* (ISSN: 1878-8181), cujo Qualis é A2 em Ciência de Alimentos.

ARTIGO II – "Production of carotenoids and fatty acids from *Rhodotorula glutinis* by using cassava wastewater as a substrate". Submetido ao periódico *Journal of the Science of Food and Agriculture* (ISSN: 1097-0010), cujo Qualis é A2 em Ciência de Alimentos.

Solicitação de patente: A partir dos resultados obtidos (Artigo II) foi elaborado também um pedido de patente, intitulada "Processo de produção de biomassa, lipídeos e pigmentos por *Rhodotorula glutinis*, utilizando a manipueira como substrato" (Apêndice III).

ARTIGO I

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Production of *Chlorella protothecoides* biomass, chlorophyll and carotenoids using the dairy industry by-product *scotta* as a substrate

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ABSTRACT

Microalgae-based systems for the production of high value molecules are an emergent area, representing a great promise for industrial applications. The main challenge, however, is the development of high efficiency strategies for the large-scale production at low costs. The aim of this study was to evaluate the potential of ricotta cheese whey (*scotta*) to be used as a low-cost alternative substrate to grow the microalga *Chlorella protothecoides*. Furthermore, a salt and light stress condition was imposed in order to improve the carotenogenesis process. A significant reduction in lactose concentration was observed along the cultivation in the culture mediums containing *scotta*, indicating that the tested *C. protothecoides* shifted to mixotrophic growth, using the organic carbon source provided. Mixotrophic cultures presented a higher amount of biomass than the autotrophic one, however, the cellular accumulation of chlorophyll and carotenoids was higher in the latter culture. Despite this, the stress strategy that we applied enhanced carotenogenesis, allowing the cellular accumulation of well quoted carotenoids, namely astaxanthin and lutein/zeaxanthin. The results suggest that *scotta* has a great potential to be used as a culture medium to grow *C. protothecoides*. Moreover, through an adequate stress strategy it is possible to control carotenogenesis, allowing the production of high amounts of the desirable high value molecules.

1. Introduction

Microalgae may be exploited for synthesizing a range of products, including carbohydrates, proteins, essential amino acids, vitamins and pharmaceuticals, as well as bioactive molecules (Dufossé et al., 2005). In recent years, microalgae have emerged as attractive sources for many value-added molecules such as carotenoids (Dineshkumar et al., 2015; Rodrigues et al., 2014). However, the use of microalgae cultures to produce carotenoids has historically been limited, and the economic viability of algal biotechnology is hampered by processing costs and photosynthetic efficiency, as well as by productivity of algal cultures (Wichuk et al., 2014).

The most common procedure for cultivation of microalgae is autotrophic growth. Under autotrophic cultivation, the cells harvest light energy and use CO_2 as carbon source (Perez-Garcia et al., 2011). Despite several advantages, autotrophic growth leads to low biomass production. Hence, heterotrophic and mixotrophic mode of growth have been proposed as feasible alternatives to reach a higher biomass productivity (Perez-Garcia et al., 2011; Zhang et al., 2011). Under heterotrophic conditions organic molecules, such as sugars and organic acids, serve as carbon sources and the requirement for light is eliminated, whereas mixotrophic cultivation requires light and the simultaneous utilization of inorganic (CO_2) and organic compounds as carbon sources. Mixotrophic growth, for some microalgae, can significantly increase the biomass and, furthermore, compounds characteristic of both photosynthetic and heterotrophic metabolisms are synthesized at high production rates (Abreu et al., 2012). However, to make this cultivation technique feasible, a cheap organic carbon source must be used (Girard et al., 2014; Rodrigues et al., 2014). Therefore, the exploitation of cheap and easily available agro-industrial byproducts as alternative culture media has been considered to grow microalgae.

The ricotta cheese whey, also called *scotta*, is the main by-product of ricotta cheese manufacture process. The ricotta cheese is produced after the cheese making process, from raw residual cheese whey; fresh milk

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Key-words: Microalga · Mixotrophic growth · Pigments · Ricotta cheese whey · Stress phase

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The most common procedure for cultivation of microalgae is autotrophic growth. Under autotrophic cultivation, the cells harvest light energy and use CO_2 as carbon source (Perez-Garcia et al., 2011). Despite several advantages, autotrophic growth leads to low biomass production. Hence, heterotrophic and mixotrophic mode of growth have been proposed as feasible alternatives to reach a higher biomass productivity (Perez-Garcia et al., 2011; Zhang et al., 2011). Under heterotrophic conditions organic molecules, such as sugars and organic acids, serve as carbon sources and the requirement for light is eliminated, whereas mixotrophic cultivation requires light and the simultaneous utilization of inorganic (CO_2) and organic compounds as

carbon sources. Mixotrophic growth, for some microalgae, can significantly increase the biomass and, furthermore, compounds characteristic of both photosynthetic and heterotrophic metabolisms are synthesized at high production rates (Abreu et al., 2012). However, to make this cultivation technique feasible, a cheap organic carbon source must be used (Girard et al., 2014; Rodrigues et al., 2014). Therefore, the exploitation of cheap and easily available agro-industrial byproducts as alternative culture media has been considered to grow microalgae.

The ricotta cheese whey, also called *scotta*, is the main by-product of ricotta cheese manufacture process. The ricotta cheese is produced after the cheese making process, from raw residual cheese whey; fresh milk (up to 10%), milk fat and an acid solution of salts can also be added. The obtained mixture is maintained at high temperature (85–90 °C) to promote the precipitation of most of whey proteins that make ricotta cheese. The liquid solution remaining after ricotta cheese separation is the *scotta*, with different characteristics compared to cheese whey (Sansonetti et al., 2009). *Scotta* is widely produced in southern Europe and particularly in Italy where it represents a by-product that must be disposed of (Secchi et al., 2012).

Most of *scotta* is used as supplement feed for livestock. However, this by-product is rich in lactose and contains organic nitrogen, hydrosoluble vitamins and a variety of minerals that could make it a good substrate in biotechnological processes for the production of commercial high-value compounds. Although several studies have proved the viability of using *scotta* as a substrate for the production of high-value products, such as bio-ethanol and lactic acid (Sansonetti et al., 2009; Secchi et al., 2012), to the best of our knowledge, this by-product is still poorly used in biotechnological processes, despite its large availability and low cost.

Microalgae biosynthesis of metabolites is species dependent and is influenced by the stress imposed by environmental conditions, such as deviations from normal values of salinity, temperature, heavy metal concentration and, above all, nitrogen availability and light intensity (Gouveia et al., 1996). *C. protothecoides* is a freshwater microalga which can provide appreciable amounts of proteins, chlorophyll and lipids as well as an interesting profile of well quoted carotenoids for nutraceutical and/or food and feed supplements (Campenni' et al., 2013). Moreover, since this freshwater microalga is known to exhibit endogenous β-galactosidase activity (Davies et al., 1994) it could be a potential candidate to be cultivated in a lactose-containing medium.

Therefore, the aim of this study was to investigate the production of biomass, chlorophyll and carotenoids by *C. protothecoides* when cultured under mixotrophic condition in a medium containing *scotta* as organic carbon source. Furthermore, the influence of salt and light stress upon the carotenogenesis process was also evaluated.

2. Materials and methods

2.1 Microorganism and inoculum

The freshwater microalga *Chlorella protothecoides* Krüger (ATCC® 30411™) was used in all the experiments. The strain was maintained in a standard inorganic medium at 25 °C (Araya et al., 2014). The microalgal inoculum was prepared in a BG-11 medium at 25 °C in 2 L glass photobioreactors under photoautotrophic conditions. The culture was aerated with CO_2-enriched air (0.1 L min^{-1}), under a photoperiod of 16:8 (light:dark).

2.2 Ricotta cheese whey (Scotta)

Two different kinds of ricotta cheese whey (*scotta*) were used as organic carbon sources: one obtained from ricotta cheese manufactured starting from a mixture of bovine and ovine cheese whey (90% and 10%, respectively) and called mixed *scotta* (MS) and the other obtained from ricotta cheese manufactured starting from 100% ovine cheese whey and called ovine *scotta* (OS). Both *scotta* used in this study were supplied by dairy industries located in Toscana region, central Italy. The physicochemical characterization of *scotta* was carried out for the following parameters: pH, acidity (g lactic acid 100 mL^{-1}), reducing sugars (g lactose 100 mL^{-1}), dry matter, fat, ash content, total nitrogen, calcium, magnesium, phosphorous, sodium, potassium, zinc, iron and manganese. All the analysis were carried out according to the methodologies proposed by AOAC (2005). To ensure that the reducing sugar present in *scotta* was in form of lactose an HPLC analysis was also carried out following the methodology proposed by Giovannelli et al. (2011).

2.3 Media and growth conditions

Six different cultivation conditions were carried out in triplicate (Table 1). For the autotrophic cultivation (run 1) the BG-11 medium was used, containing per liter: 1500 mg $NaNO_3$, 3.05 mg KH_2PO_4, 6 mg ferric ammonium citrate, 1.81 mg $MnCl_2 \cdot 4H_2O$, 75 mg $MgSO_4 \cdot 7H_2O$, 0.079 mg $CuSO_4 \cdot 5H_2O$, 36 mg $CaCl_2 \cdot 2H_2O$, 0.222 mg $ZnSO_4 \cdot 7H_2O$, 0.05 mg $CoCl_2 \cdot 6H_2O$, 2.86 mg H_3BO_3, 6 mg citric acid$\cdot 1H_2O$ and 20 mg Na_2CO_3 (Araya et al., 2014). For the mixotrophic cultivation (runs 2-3), the inorganic medium was partially replaced by the alternative substrate (30% v/v substitution). With the aim of evaluating the assimilation of organic carbon sources by *C. protothecoides*, under the cultivation conditions provided, pure lactose (12 $g \cdot L^{-1}$), glucose (12 $g \cdot L^{-1}$) and galactose (12 $g \cdot L^{-1}$) were added to the BG-11 medium as controls (runs 4-6). The sugar concentration adopted was based on the lactose content in experiments containing 30 % *scotta*.

The batch cultivation of the microalga was performed by inoculating 10% (v/v) of starter culture (cell dry weight equal to 1.2 $g \cdot L^{-1}$) into 1 L culture medium. Before inoculation, all culture mediums were sterilized at 121 °C for 20 min. The microalga was grown under aseptic conditions in 2 L photobioreactors.

2.4 Cultivation process

2.4.1 Green phase

Experiments were carried out at 25 °C in 2 L glass photobioreactors containing 1 L of medium, under a photoperiod of 16:8 (light:dark) for 7 days. The cultures were illuminated with a photon flux density of 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (photosynthetically active radiation) supplied by fluorescent lights. Agitation during cell growth was provided by bubbling CO_2-enriched filtered air with 10% CO_2 supplied at 0.1 L min^{-1}. Microscopic observation was done during growth to check the purity of the culture. During the cultivation, reducing sugar concentration, biomass concentration and cellular pigments were determined periodically (from day 3 up to day 7). The cultivations were performed in three replicates (n = 3).

2.4.2 Stress phase

In order to lead *C. protothecoides* metabolism to the accumulation of carotenoids, after the normal cultivation process (green phase) the culture was diluted (1:5) into a 20 g L^{-1} NaCl sterilized solution, resulting in salinity and light stresses, following a procedure similar to that described by Campenni' et al. (2013). Dilution of the culture enhance light penetration, leading the microalga to a higher exposure to light (Gouveia et al., 1996). The experiments were carried out in photobioreactors (2 L) for 5 days and the culture conditions such as temperature, photoperiod, air supply and CO_2 were the same as those used for the green phase. All experiments were performed in three replicates (n = 3).

2.5 Analytical methods

2.5.1 Biomass

Samples were aseptically collected regularly. Biomass concentration was estimated by cell dry weight (cdw) after centrifugation of the sample (3951×g for 10 min) and drying at 60 °C in a hot oven until constant weight.

2.5.2 Determination of carbohydrate concentration in the culture media

Reducing sugar concentration was determined regularly, according to the dinitrosalicylic acid (DNS) method proposed by Miller (1959). For the quantification a reducing sugar standard curve was adopted. Lactose, glucose and galactose were used as pure standards.

2.5.3 Chlorophyll and total carotenoids

To extract pigments from the fresh biomass, 15 mL of the culture medium was collected in a falcon tube and centrifuged at 3951×g for 10 min, then the supernatant was discarded and the fresh biomass was mixed with 1.0 mm small glass beads (Sigma-Aldrich, Italy) and 5 mL of methanol. The mixture was vortexed for 2 min and the supernatant was collected after further centrifugation. The extraction process was repeated until both biomass and supernatant became colorless (three times). The amount of total chlorophyll and total carotenoids in the extract were determined

spectrophotometrically according to the methodology described by Lichtenthaler and Buschmann (2001). Absorption spectra of extracts were measured in a spectrophotometer (SPECTROstar Nano, BMG Labtec, Ortenberg, Germany) reading between 380 and 700 nm. Total chlorophyll and total carotenoid contents were expressed on dry weight basis. *C. protothecoides* dry weight was measured by drying the fresh biomass at 60 °C until constant weight.

2.5.4 Astaxanthin, Lutein/Zeaxanthin and β-carotene

The analysis was conducted using a SpectraSystem HPLC instrument equipped with a UV-VIS detector (Thermo, Rodano, Italy). The column was a Kinetex C18, 250 x 4.6 mm ID, 5 µm particle size (Phenomenex, Torrance, CA, USA), eluted at 1 ml min^{-1} with solvent A (acetonitrile: methanol: Tris buffer 0.1 M pH 8 84:2:14) and B (methanol:ethyl acetate 68:32), according to the following program: 100% solvent A for 4 min, then a linear gradient from 0 to 100% B in 10 min, followed by 100% B for 15 min. The astaxanthin, lutein/zeaxanthin and β-carotene peaks were identified by comparison of the retention times of sample peaks with those of pure standards of β-carotene, lutein, zeaxanthin (Extransynthese, Lyon, France) and astaxanthin (Sigma-Aldrich, Italy). Astaxanthin, Lutein/Zeaxanthin and β-carotene quantifications were carried out by reference to calibration curves.

2.6 Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA). Average values were compared using Tukey's test. Statistical analyses were performed by using software *Statistica*®, version 7.0 (Statsoft, USA). Differences were considered as significant when $p < 0.05$.

3. Results and discussion

3.1 Physicochemical characterization of mixed and ovine scotta

The physicochemical characteristics of the two types of *scotta* used are shown in Table 2. As reported in the literature the main component present in both *scotta* is

lactose (Sansonetti et al., 2009). Through the HPLC analysis (2.2) it was confirmed that the reducing sugar in *scotta* was only in form of lactose (Supplement figure). This disaccharide is a suitable carbon source that can support the growth of some freshwater microalgae strains (Girard et al., 2014). Furthermore, the organic nitrogen availability, the mild acidity and the variety of minerals found in both *scotta* support their application in biotechnological processes. Phosphorous, for example, is a macronutrient that plays a vital function in cellular metabolic processes by forming many structural and functional components required for normal growth and development of microalgae (Abreu et al., 2012). Both *scotta* were very similar in composition, although OS presented a higher amount of lactose, a milder acidity and a lower amount of sodium.

3.2 Biomass

Biomass concentration as cell dry weight (cdw) was estimated along the green phase (from day 3 up to day 7). Higher biomass productivity was achieved when the alternative substrates were used in the culture medium (runs 2 and 3). Substituting 30% (v/v) of BG-11 medium with both *scotta* led to important growth stimulation in *C. protothecoides* cultures under mixotrophic condition (Figure 1). It can be observed that the final values of 3.60 $g \cdot L^{-1}$ and 3.17 $g \cdot L^{-1}$ achieved in the mixotrophic cultures using ovine *scotta* and mixed *scotta*, respectively, resulted in 2.8- and 2.5-fold increase when compared to the values obtained in the autotrophic culture. Mixotrophic cell cultivation, by using light and both organic and inorganic carbon sources, has been considered the most efficient process for the production of microalgal biomass (Lee et al., 1996). The stimulatory effect of both types of *scotta* on biomass production is probably related to the use of lactose as an organic carbon source (Figure 2) and also to the presence of other nutrients, such as organic nitrogen and a variety of minerals (Table 1). Cheirsilp and Torpee (2012) also reported that the mixotrophic culture of freshwater *Chlorella* sp. provided a better growth than autotrophic culture. It is also important to mention that because of the faster growth of the mixotrophic cultures, no differences ($p > 0.05$) were found for the biomass concentration from day 3 up to day 7 for the medium containing mixed *scotta* and from day 4 up to day 7 for the medium containing ovine *scotta*. Therefore, in addition to the higher biomass concentration when compared with the autrotrophic mode, the use of both *scotta* as an organic carbon source could allow to shorten the time to harvest the cells, thereby increasing the biomass production for year.

Regarding to the addition of pure sugars (runs 4-6) into BG-11 medium, no growth stimulation was observed as biomass concentration was similar or lower than that observed for the autotrophic culture (run 1). Along the cultivation process the biomass concentration in the medium containing pure lactose (run 4) was lower ($p < 0.05$) when compared to the medium without sugar addition (Figure 1). These results were probably due to the lack of ability by *C. prototheco*ides to use the organic carbon sources when pure sugars were added to the inorganic medium, under the cultivation conditions used as it is known that this microalga is capable of use glucose and galactose to support cell growth (Gao et al., 2014). Girard et al. (2014) also tested the addition of pure lactose into a synthetic medium to grow *Chlorella* spp. microalgae and observed that the pure sugar did not support the growth as results were similar for the medium without lactose supplementation. Regarding to the present study as the cultivation conditions were the same for all experiments it can be suggested that the nutrients in *scotta* may be presenting a biostimulant effect toward the use of the sugar by *C. prototheco*ides to support its growth.

*3.3 Consumption of the organic carbon sources by Chlorella prototheco*ides

It was observed that under the culture system provided, *C. prototheco*ides strain used in this study, was unable to shift from autotrophic to mixotrophic mode of growth when pure sugars were added to the inorganic medium. Lactose, glucose and galactose concentrations did not present significant ($p > 0.05$) changes along the cultivation process which means that only the inorganic carbon source (CO_2) was used by the microalga (Figure 2). These results can explain the low growth achieved when the BG-11 medium was supplemented with the pure sugars (runs 4-6), which was similar to the autotrophic cultivation (Figure 1). According to some Authors the transition from autotrophic to mixotrophic mode in microalgae appears to be influenced by culture conditions such as temperature, light intensity, organic carbon source concentration and CO_2 supply (Chandra et al., 2014). Organic carbon concentration and light intensity in mixotrophic growth are for example critical factors that exhibit inhibitory effects at high doses (Li et al., 2014).

On the other side, our results demonstrated that *C. prototheco*ides was able to grow mixotrophically in the presence of lactose when *scotta* was used in the culture medium (runs 2-3). A significant decrease ($p < 0.05$) in lactose concentration was

observed during the cultivation, leading to an almost complete depletion of the organic carbon source after 7 days of cultivation (Figure 2). To assimilate lactose, living organisms have to synthesize a β-galactosidase enzyme to hydrolyze lactose into glucose and galactose and must absorb these molecules through the adequate transmembrane proteins (Girard et al., 2014). Endogenous β-galactosidase activity has already been demonstrated in some freshwater microalgae, including *C. protothecoides* (Davies et al., 1994). Girard and collaborators (2014) observed the ability of freshwater microalga *Scenedesmus obliquus* to use lactose as an organic carbon source. Along the cultivation the authors observed a decrease in lactose content followed by an increase in glucose and galactose concentrations, suggesting extracellular lactose hydrolysis by the microalgae. Previously, lactose had been shown to support *Scenedesmus* growth by Samejima and Myers (1958). However, to the best of our knowledge there are still no reports in the literature about the consumption of lactose by *C. protothecoides* cultivated under mixotrophic condition in a lactose containing medium.

It was previously reported that *C. protothecoides* cultured under heterotrophic conditions was unable to utilize lactose as an organic carbon source (Espinosa-gonzalez et al., 2014; Girard et al., 2014). However, it is well known that the culture conditions may be a limiting factor for the carbon source consumption by microalgae (Gao et al., 2014). From our results it can be suggested that the cultivation conditions used were decisive for the shift from autotrophic to mixotrophic mode of growth, allowing the consumption of lactose by *C. protothecoides*. Moreover it can also be suggested that the presence of some nutrients in *scotta* had a stimulatory effect on the growth and on the assimilation of the organic carbon source. Abreu and collaborators (2012) cultivated freshwater microalgae *C. vulgaris* under mixotrophic conditions using cheese whey as a substrate and observed that the biomass concentration at the end of the cultivation did not show significant differences by comparing a medium containing non-hydrolyzed cheese whey (10 $g \cdot L^{-1}$ of lactose) and an inorganic medium supplemented with glucose (5 $g \cdot L^{-1}$) and galactose (5 $g \cdot L^{-1}$). However, the authors did not report any data about lactose consumption.

3.4 Chlorophyll and carotenoids

Pigments production was evaluated in microalgal biomass cultured under mixotrophic (runs 2-3) and autotrophic (run 1) conditions. Total chlorophyll and total

carotenoids were estimated along the green phase (Figures 3 and 4) and after the stress treatment (Table 3). The maximum cellular accumulation of total chlorophyll (25.15 $mg \cdot g_{cdw}^{-1}$) and total carotenoids (6.38 $mg \cdot g_{cdw}^{-1}$) at the end of the green phase were obtained when the microalga was cultured under the autotrophic mode of growth. The enhancement of chlorophyll biosynthesis by autotrophic *Chlorella* sp. strains compared with that resulting from mixotrophic cells has been previously reported (Cheirsilp and Torpee, 2012). It has been suggested that the formation of the photosynthetic apparatus in *Chlorella* may be disturbed by the presence of organic substrates, resulting in a decreased production of photosynthetic pigments when compared with that obtained in autotrophic mode (Yang et al., 2000). Moreover, high organic carbon concentration inhibits the photosynthetic CO_2 fixation and also modifies the pathways of photosynthetic pigment biosynthesis (Chandra et al., 2014).

Among several species of microalgae, *Chlorella* sp. is reported to have a high amount of chlorophyll (Harun et al., 2010). Chlorophyll is used in food industry as a natural pigment in processed foods. Because of its strong green color and consumers demand for natural foods, chlorophyll is gaining importance as a food additive (Humphrey, 2004). Regarding to the biosynthesis of carotenoids, the results obtained are in agreement with those of Abreu et al. (2012) who found a lower amount of carotenoids in mixotrophic cells of *C. vulgaris* when compared to cells grown on autotrophic culture. However, as shown previously, under the mixotrophic condition a higher amount of biomass was obtained (Figure 1), therefore the differences in pigments production between autotrophic and mixotrophic mode of growth can be dampened by expressing the results as milligrams of pigments per unit volume of culture medium as in Figures 3 and 4 (right *y* axis). In that way, the highest amount of chlorophyll (42.17 $mg \cdot L^{-1}$) and carotenoids (11.98 $mg \cdot L^{-1}$) were found in the medium containing ovine *scotta* after four days of cultivation. Moreover, under mixotrophic conditions there was no increase ($p > 0.05$) in cellular accumulation ($mg \cdot g_{cdw}^{-1}$) of total chlorophyll and total carotenoids from day 3 to day 7, therefore, for the production of pigments the cells grown mixotrophically could be harvested earlier, due to the faster growth, thereby increasing the annual productivity of pigments, making the process more viable and sustainable.

After the salt and light stress a decrease in total chlorophyll content ($mg \cdot g_{cdw}^{-1}$) ($p < 0.05$) was observed for the autotrophic *C. protothecoides* (Table 3). Using a similar stress condition, also Campenni' et al. (2013) observed the degradation of chlorophyll

in autotrophic *C. protothecoides*. This behavior is usually observed when stress conditions are applied on microalgae cultures and is generally followed by the revelation of carotenoids pigments (Gouveia et al., 1996; Ip and Chen, 2005).

When compared with the green phase, no differences ($p > 0.05$) in total carotenoids content were found when *C. protothecoides* was exposed to the stress condition (Table 3). However, changes in the carotenoids profile were observed due to the stress applied. HPLC analysis revealed the presence of astaxanthin, lutein/zeaxanthin and β-carotene in the pigments extracts obtained from *C. protothecoides* samples. Astaxanthin content increased significantly ($p < 0.05$) for microalgae cultured under autotrophic mode and under mixotrophic mode when mixed *scotta* was used, whereas *C. protothecoides* cultured in the medium containing ovine *scotta* showed a significant increase ($p < 0.05$) only in Lutein/Zeaxanthin content, following the onset of the stress. Campenni' et al. (2013) observed that the dilution of the culture medium led to a higher exposure to light, therefore the enhancement of the carotenogenesis process was achieved through the dilution of the sample. The role of some carotenoid molecules in photoprotection is widely acknowledged (Müller et al. 2001). Furthermore, oxidative stress caused by high irradiance is effective for inducing accumulation of carotenoids in algae (Park and Lee, 2001). Strong light can lead to the generation of reactive oxygen species (ROS), in the presence of which antioxidative carotenoids are produced in order to protect the cells against oxidative damage (Rise et al., 1994).

Beside the increase in astaxanthin and lutein/zeaxanthin contents, after the stress, a lower amount of β-carotene was observed in all treatments, although the change was not significant ($p > 0.05$) for the medium containing mixed *scotta*. Known carotenogenic pathways show the possibilities of various oxidative transformations of β-carotene up to canthaxanthin, and of the hydroxylative pathways towards zeaxanthin/lutein. Either of these compounds may be considered as precursors of the hydroxylated/oxidized astaxanthin (Gouveia et al., 1996). The observed enhancement of the astaxanthin concentration through the stress period was an important feature as this carotenoid is a powerful antioxidant and has a high commercial value (Yuan et al., 2011), however, lutein/zeaxanthin were found as the main carotenoids present in *C. protothecoides* cells even after the stress, indicating that their oxidation to astaxanthin was low. Despite this, the relatively high amounts of astaxanthin, lutein/zeaxanthin and

β-carotene present in this microalga, represent an important result as these pigments are well quoted in the market.

There is a growing interest in the use of astaxanthin and lutein/zeaxanthin as food-coloring agents, natural feed additive for the poultry industry and for aquaculture, especially as a feed supplement in the culture of salmon, trout, and shrimp (Araya et al., 2014; Dufossé et al., 2005; Wichuk et al., 2014). Astaxanthin-rich extracts derived from *Haematococcus pluvialis* have been approved for several companies such as U.S. Nutra (USA), AstaReal AB (Sweden), Algatechnologies Ltd. (Israel), and Cyanotech (USA) (Campenni' et al., 2013). β-carotene serves as an essential nutrient (provitamin-A) and has high demand in the market as a food-coloring agent, as an additive to cosmetics and also as a health food ingredient (Raja et al., 2007). Furthermore, these carotenoids have been attributed with an extraordinary potential for promising applications in human health protection (Araya et al., 2014; Ip and Chen, 2005; Raja et al., 2007). These protective actions are likely to involve antioxidant mechanisms, including prevention of oxidative damage and cellular necrosis or apoptosis induced by oxidative stress (Yuan et al., 2011).

4. Conclusions

Chlorella protothecoides shifted to a mixotrophic growth when *scotta* was used in the medium as it was able to use lactose as a carbon source. Mixotrophic cultivation of *C. protothecoides* using *scotta* can be considered as a feasible strategy to reduce the costs of microalgal biomass production, while also contributing to solve the environmental problem caused by *scotta* disposal in dairy industries. High amounts of chlorophyll and carotenoids were found in both modes of growth, i.e. mixotrophic and autotrophic. Although the cellular accumulation of these pigments was higher in autotrophic *C. protothecoides*, their concentration per unit volume of liquid culture was greater under mixotrophic conditions and the peak value was achieved in a shorter time, thus raising interest toward this cultivation technique. Moreover, the imposition of a salt and light stress demonstrated that the carotenogenesis process can be controlled toward the accumulation of well quoted carotenoids, namely astaxanthin and lutein/zeaxanthin, thereby improving the efficiency of the pigments production by the mixotrophic *C. protothecoides*.

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Table 1. Different cultivation conditions of *C. protothecoides*

Run	Culture medium	Carbon source
1	BG-11	CO_2
2	BG-11 + MS	CO_2 + Mixed *scotta*
3	BG-11 + OS	CO_2 + Ovine *scotta*
4	BG-11 + Lactose	CO_2 + Lactose
5	BG-11 + Glucose	CO_2 + Glucose
6	BG-11 + Galactose	CO_2 + Galactose

Table 2. Composition of *scotta* utilized in this work

Parameter	**Mixed *scotta***	**Ovine *scotta***
pH	6.02 ± 0.03^{b}	6.20 ± 0.04^{a}
Acidity (g lactic acid 100 mL^{-1})	0.15 ± 0.01^{a}	0.12 ± 0.01^{b}
Reducing sugar (Lactose) (g 100 mL^{-1})	4.07 ± 0.21^{b}	4.69 ± 0.05^{a}
Dry material (g 100 g^{-1})	7.75 ± 0.13^{a}	8.24 ± 0.35^{a}
Total nitrogen (g 100 mL^{-1})	0.11 ± 0.2^{a}	0.11 ± 0.01^{a}
Fat (g 100 g^{-1})	0.18 ± 0.02^{a}	0.12 ± 0.04^{a}
Ash content (g 100 g^{-1})	0.94 ± 0.01^{a}	0.91 ± 0.02^{a}
Ca (g 100 g^{-1})	0.048 ± 0.004^{a}	0.037 ± 0.011^{a}
Mg (g 100 g^{-1})	0.030 ± 0.003^{a}	0.028 ± 0.006^{a}
P (g 100 g^{-1})	0.093 ± 0.002^{a}	0.114 ± 0.016^{a}
Na (g 100 g^{-1})	0.200 ± 0.004^{a}	0.186 ± 0.002^{b}
K (g 100 g^{-1})	0.119 ± 0.009^{a}	0.101 ± 0.002^{a}
Zn (mg 1000 g^{-1})	73.125 ± 0.884^{a}	72.500 ± 1.768^{a}
Fe (mg 1000 g^{-1})	164.375 ± 0.884^{a}	165.625 ± 13.258^{a}
Mn (mg 1000 g^{-1})	3.125 ± 0.884^{a}	4.375 ± 0.884^{a}

Data are expressed as means of three replicates ± standard deviation.

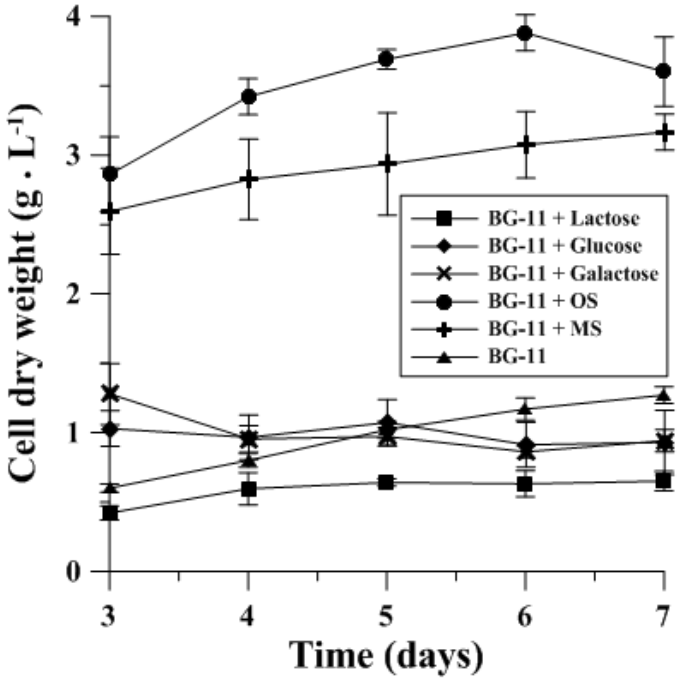
[a-b] Means in the same row with different superscripts are significantly different ($p < 0.05$).

Table 3. Pigments production in green phase and stress phase as a function of the growth condition.

Green phase					
Growth condition	Total chlorophyll ($mg \cdot g_{cdw}^{-1}$)	Total carotenoids ($mg \cdot g_{cdw}^{-1}$)	Astaxanthin ($\mu g \cdot g_{cdw}^{-1}$)	Lutein/Zeaxanthin ($\mu g \cdot g_{cdw}^{-1}$)	β-carotene ($\mu g \cdot g_{cdw}^{-1}$)
$Autotrophic_{BG-11}$	25.17 ± 1.68a	6.38 ± 0.19a	96.54 ± 5.50b	1178.48 ± 101.93a	335.71 ± 37.24a
$Mixotrophic_{BG-11+MS}$	6.85 ± 1.44a	2.31 ± 0.32a	28.85 ± 4.42b	532.49 ± 67.40a	73.61 ± 12.73a
$Mixotrophic_{BG-11+OS}$	8.56 ± 2.03a	2.70 ± 0.22a	73.73 ± 9.77a	598.29 ± 21.06b	128.24 ± 8.47a
Stressed phase					
Growth condition	Total chlorophyll ($mg \cdot g_{cdw}^{-1}$)	Total carotenoids ($mg \cdot g_{cdw}^{-1}$)	Astaxanthin ($\mu g \cdot g_{cdw}^{-1}$)	Lutein/Zeaxanthin ($\mu g \cdot g_{cdw}^{-1}$)	β-carotene ($\mu g \cdot g_{cdw}^{-1}$)
$Autotrophic_{BG-11}$	14.17 ± 1.09b	5.64 ± 0.79a	131.02 ± 6.05a	1235.60 ± 152.59a	93.95 ± 27.60b
$Mixotrophic_{BG-11+MS}$	7.77 ± 0.99a	2.87 ± 0.30a	54.71 ± 6.32a	698.25 ± 41.85a	41.98 ± 3.63a
$Mixotrophic_{BG-11+OS}$	7.26 ± 2.19a	2.83 ± 0.52a	91.84 ± 2.69a	783.98 ± 10.26a	69.98 ± 10.02b

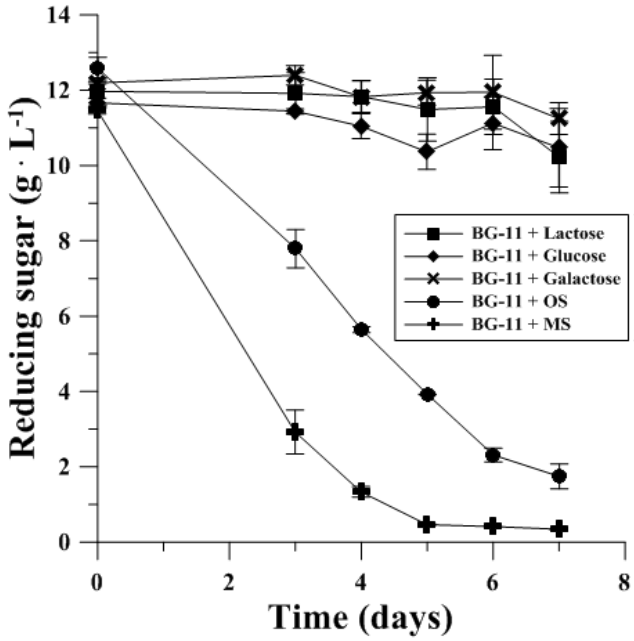
Data are expressed as means of three replicates ± standard deviation.
a-b: Different lowercase letters in the same column mean significant differences for the same growth condition by comparing the green phase and the stress phase at 5% level of probability.

Figure 1. Biomass concentration along the cultivation process (green phase).



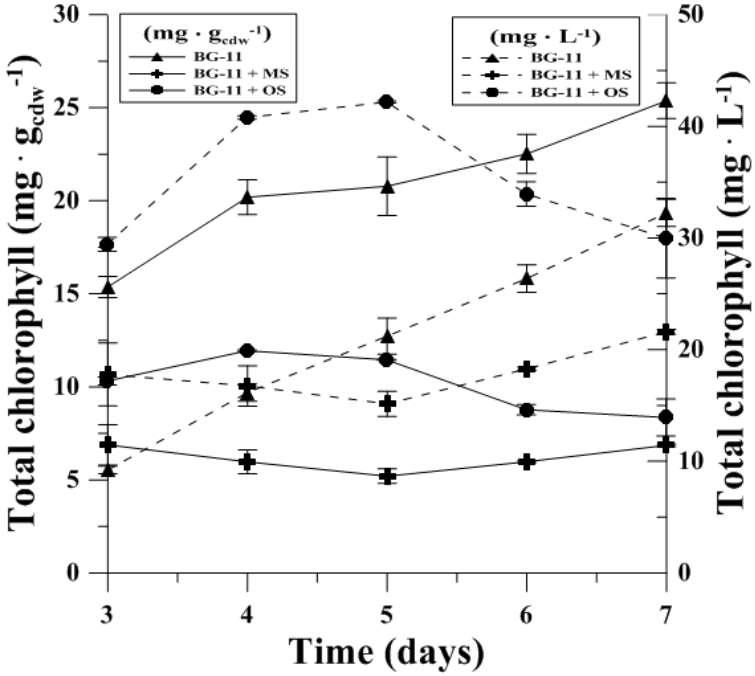
Data are expressed as means of three replicates ± standard deviation.

Figure 2. Reducing sugar concentration along the cultivation process (green phase).



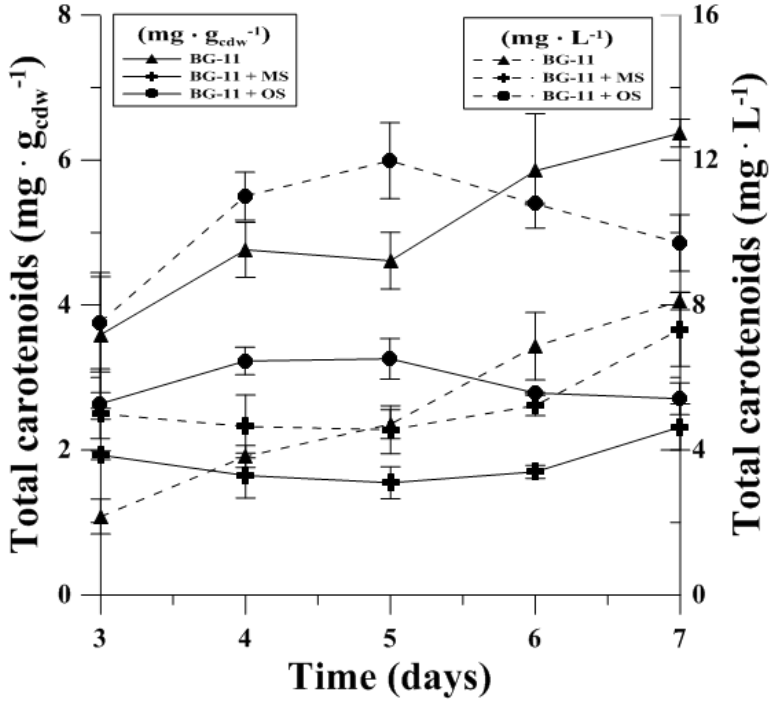
Data are expressed as means of three replicates ± standard deviation.

Figure 3. Total chlorophyll in *C. protothecoides* along the cultivation process (green phase).



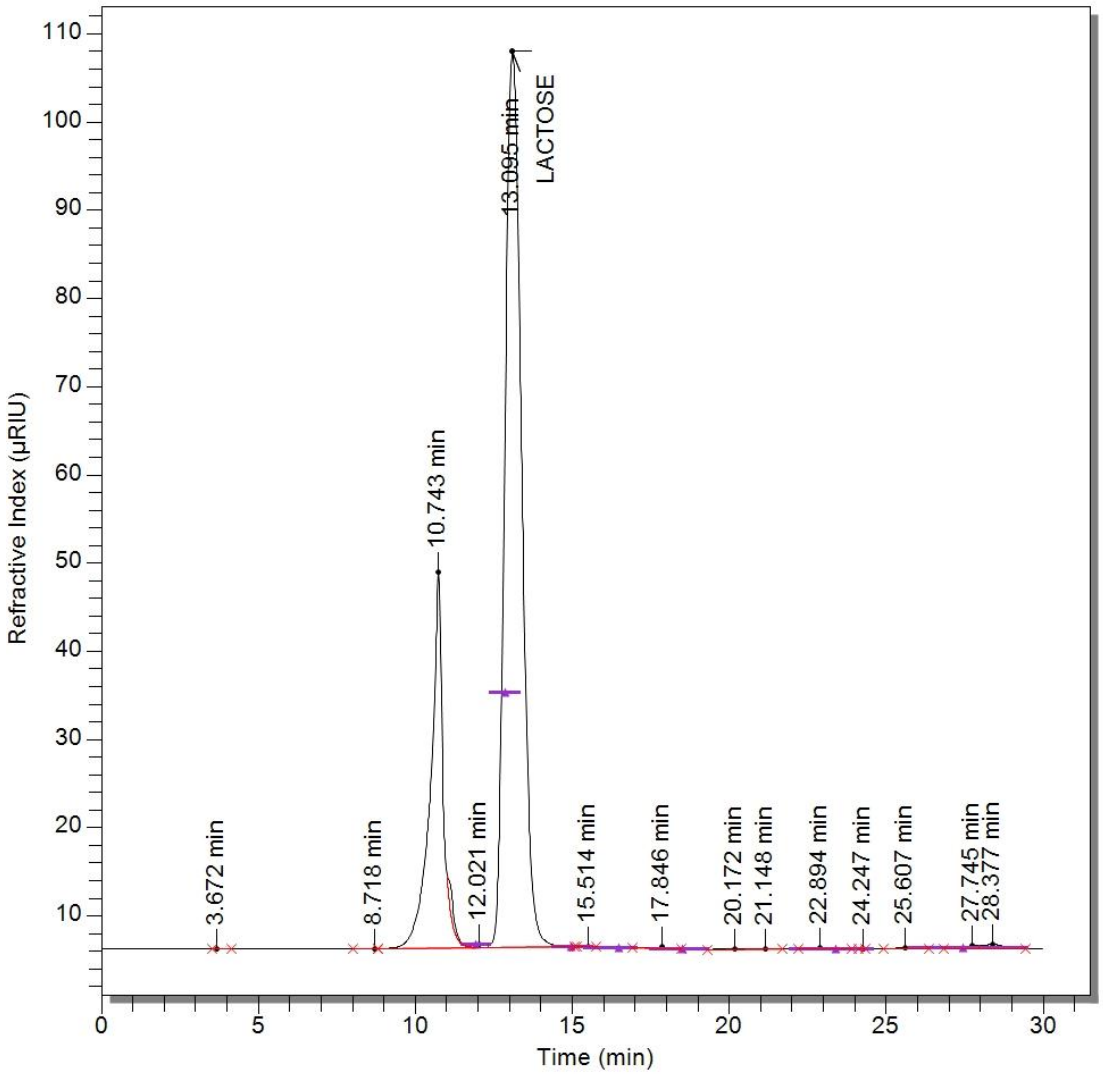
Data are expressed as means of three replicates ± standard deviation.

Figure 4. Total carotenoids in *C. protothecoides* along the cultivation process (green phase).



Data are expressed as means of three replicates ± standard deviation.

Supplement Figure. Chromatogram of the HPLC analysis of sugars present in mixed *scotta*.



ARTIGO II

Production of carotenoids and fatty acids from *Rhodotorula glutinis* by using cassava wastewater as a substrate

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Abstract

The cassava (*Manihot esculenta* Crantz) wastewater is the main by-product of cassava flour processing. This by-product is an extraordinary source of organic carbon what could make it a good substrate in biotechnological processes for the production of commercial high-value compounds. Nowadays, the demand for natural additives and health-promoting food ingredients has increased. Carotenoids present a high commercial value as they are widely used as natural colorants and antioxidants. Other compounds widely requested by the industry because of their health-promoting properties are the polyunsaturated fatty acids. Yeasts from *Rhodotorula* genus are able to accumulate large amounts of carotenoids and fatty acids in their cells. The aim of this study was to evaluate the potential of using cassava wastewater as substrate for the growth of *Rhodotorula glutinis* intended for the production of carotenoids and fatty acids. The results showed that cassava wastewater can be used as a sole source of nutrients to support *R. glutinis* growth and metabolites accumulation in its cells. High growth of the yeast (10.28 $g \cdot L^{-1}$) as well as high productions of carotenoids (0.98 $mg \cdot L^{-1}$) and lipids (1.34 $g \cdot L^{-1}$) were obtained when *R. glutinis* was cultivated in cassava wastewater as the sole source of nutrients. Moreover, the fatty acids accumulated in the yeast cells were mostly (over 50%) unsaturated.

Keywords: agro-industrial waste · lipids · biotechnology · natural pigments · bioactive molecules

INTRODUCTION

Rhodotorula species are yeasts with peculiar metabolic characteristics such as the production of great amounts of lipids and carotenoid pigments during their growth.[1] Chemical synthesis processes and the extraction from plants have been used as ways to obtain carotenoids, in particular β-carotene and xanthophylls, however, various microbiological processes have been commercially exploited for the production of such compounds.[2] Compared with the extraction from plants or chemical synthesis, the microbial production of carotenoids presents some advantages, mainly because of the seasonal problems related to the colorants from plant origin, and because of the marketing appealing as a safety and natural product in contrast to its synthetic analogues.[3]

Lipids are becoming an increasingly important chemical feedstock for the manufacture of biofuels, care-products and as a food source.[4] Due to the increasing demand for these molecules, other oil sources than crops have been investigated such as microbial oils from oleaginous yeasts. These have many advantages such as high growth rates, year-round productivity and high lipids yield.[5] However, the fatty acid profile of the lipids is extremely important in determining their eventual use. Oils high

in oleic acid are the most suitable biodiesel feedstocks, whereas to replace palm oils in the cosmetic or food industries, high levels of saturated lipids are necessary.[6] Moreover, for the food and pharmaceutical industries there is an increased demand for lipids with high concentrations of polyunsaturated fatty acids due to the health benefits of these compounds.[7] *Rhodotorula* spp. can produce high amounts of lipids and has a simple fatty acid profile, composed mainly of C_{16} and C_{18} molecules. However, by changing cultivation parameters it was shown that the fatty acid profile of *R. glutinis* could be tailored towards a desired application.[8]

One of the drawbacks to the commercialization of lipids and carotenoids from red yeasts is the high production costs involved.[9] Therefore, in an attempt to reduce processing costs, alternative substrates like agro-industrial wastes or by-products have been used as cheap sources of nutrients.[10] The main problem of using agro-industrial wastes or by-products is to find one with an adequate balance of nutrients to allow cell growth and product accumulation, as the medium composition is important not only for the growth but also for the carotenogenesis and total lipids production, that influence the accumulation of the metabolites in the cells and the chemical profiles of carotenoids and fatty acids.[5,11] Grape must, beet molasses, soybean flour extract, corn flour extract, cheese whey, fermented radish brine and sugar cane molasses are examples of agro-industrial wastes already evaluated for cultivation of *Rhodotorula* yeasts.[12,13]

Cassava wastewater is a carbohydrate-rich residue generated at large amounts during the production of cassava flour, a very common food ingredient.[14] It has been estimated that the production of 1 t of cassava flour generates 300 L of wastewater.[15] Cassava is among the ten most produced commodities in the world with an estimated production of 263 million t y^{-1} worldwide.[16] The wastewater of its processing presents high amounts of sugars and minerals which can support its utilization as a culture medium to grow microrganisms.[17] However, the low nitrogen level in cassava wastewater and the resultant high C/N ratio prompted the need for supplementation with an external source of nitrogen. A nitrogen source has long been known to promote growth of *R. glutinis*, but its high concentration also suppresses the biosynthesis of lipids and other secondary metabolites.[18]

Despite the large amount of cassava wastewater generated worldwide, it has still few applications, mainly as a fertilizer, and most of the production is disposed of in the enviroment without further treatments, which causes many problems due to its high chemical oxigen demand (COD). The aim of the present study was to assess the

suitability of cassava wastewater as a growth medium for the red yeast *Rhodotorula glutinis* in the production of carotenoids and fatty acids. A successful use of this byproduct would allow to cut the costs of production of these valuable compounds as well as to lower its impact on the environment.

MATERIAL AND METHODS

Substrate preparation and characterization

Cassava wastewater (CW) was collected from local cassava flour producers in Northeastern Brazil (07º 08' 43" S, 35º 14' 11" W) and was stored at – 20 °C. Prior to use, CW was boiled and then centrifuged at 3248×g for 5 min to eliminate the solids.[15] The supernatant was collected and analyzed for the following parameters: total nitrogen (TN), reducing sugars (RS), total carbohydrates (TC), phosphorus, calcium, sodium, iron, zinc, copper, potassium and magnesium contents, chemical oxygen demand (COD), free cyanide and pH. The analyses were performed in accordance to AOAC[19], except for minerals and free cyanide. Minerals were evaluated through flame atomic absorption/emission spectrometry using a spectrometer iCE 3000 series (Thermo Fisher Scientific, Cambridge, UK). Free cyanide was evaluated by using a Quantofix® cyanide test kit (Sigma-Aldrich, Brazil).

Microorganism

Rhodotorula glutinis (CTT 2182) was obtained from André Tosello Foundation (Campinas, São Paulo, Brazil). For the inoculum the cells were transferred to 300 mL Erlenmeyer flasks containing 100 mL of culture medium: glucose (10 $g \cdot L^{-1}$), peptone (5 $g \cdot L^{-1}$), yeast extract (3 $g \cdot L^{-1}$), and malt extract (3 $g \cdot L^{-1}$). The flasks were incubated at 30 °C and shaken at 200 rpm for 24 h. Afterwards, yeast cells were collected by centrifugation at 3248×g for 5 min. The cells were suspended in sterile distilled water to make a final cell concentration of approximately 10^9 $cells \cdot mL^{-1}$. The cell concentration of this suspension was determined in a Neubauer chamber.

Yeast cultivation in flasks

Erlenmeyer flasks (300 mL) were prepared containing 150 mL of medium. Ten different runs were performed (Table 1) by varying the nutrients (CW, glucose, ammonium sulphate and minerals) used in the mediums and the concentration of the carbon sources. Glucose was used in the concentrations of 10, 30 and 50 $g \cdot L^{-1}$ whereas CW was used based on reducing sugars (RS) concentration of 30, 20 and 10 g of $RS \cdot L^{-1}$. Ammonium sulphate (5 $g \cdot L^{-1}$) was used as nitrogen source. For all runs the C/N ratio was calculated from COD/TN.

For the supplementation of the medium with minerals, macro and trace elements were used as follows: KH_2PO_4 (1 $g \cdot L^{-1}$), $MgSO_4 \cdot 7H_2O$ (0.25 $g \cdot L^{-1}$), $Na_2HPO_4 \cdot 12H_2O$ (1 $g \cdot L^{-1}$), 6 $mL \cdot L^{-1}$ $FeSO_4$ solution (4 $g \cdot L^{-1}$) and 10 $mL \cdot L^{-1}$ trace mineral solution. Trace mineral solution contained per L: 0.36 g $CaCl_2 \cdot 2H_2O$, 0.075 g $ZnSO_4 \cdot 7H_2O$, 0.013 g $CuSO_4 \cdot 5H2O$, 0.05 g $MnSO_4 \cdot H_2O$, 0.013 g $CoCl_2 \cdot 6H_2O$ and 0.035 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.[8] Prior to inoculation all media were sterilized at 121 °C for 15 min. Cells were inoculated in the medium to reach an initial concentration of 10^7 cells mL^{-1}. The flasks were incubated at 30 °C and shaken at 200 rpm for 120 h in darkness. All experiments were carried out in triplicate.

Analytical methods

Reducing sugar concentration in the culture medium was determined regularly, according to the dinitrosalicylic acid (DNS) method proposed by Miller[20]. Total carbohydrates were quantified by using the Anthrone reagent.[21] For the quantification of the sugars a glucose standard curve was adopted. Growth was monitored regularly by turbidity measurements using a spectrophotometer (model U2M, Quimis, Brazil) with absorbance reading at 600 nm and correlated to cell dry weight using a standard curve. Medium without yeast cells was taken as blank.

Carotenoids extraction was carried out according to Cutzu et al.[3], modified as follows: aliquots of the cultures (5 mL) were centrifuged at 2260×g for 10 minutes, then the supernatant was discarded and the pellets were frozen at -20 °C for 24 hours. The thawed cell pellet was re-suspended in 2 mL DMSO pre-heated at 60 °C, added with glass beads (0.5 g), vortexed for 2 min and incubated at 60 °C for 15 min. Then, 2 mL acetone, 2 mL petroleum ether and 2 mL NaCl 20 % were sequentially added, the

mixture was vortexed for a total time of 5 minutes and centrifuged at 2260×g for 10 min. The upper petroleum ether layer containing the extracted carotenoids was collected and the total carotenoids were quantified as β-carotene equivalents by a spectrophotometer at 450 nm wavelength according to the methodology proposed by Rodriguez-Amaya and Kimura[22].

For lipids extraction the cells were harvested by centrifugation (3248×g for 5 min), dried at 80 °C for 24 hours and ground in a mortar to obtain a fine powder. The rupture of the cells was performed through acid hydrolysis using HCl 2N. The lipids content of the cells was determined according to the method described by Bligh and Dyer[23]. The transesterification into FAMEs was performed according to the procedure described by Hartman and Lago[24]. FAMEs were analyzed using a GC-FID (Trace™ 1310, Thermo Fisher Scientific, USA) equipped with a SP™-2380 capillary column (60 m×0.25 mm ID and 0.25 μm film thickness). The different peaks were identified by comparison to a FAMEs standard (Supelco® 37 Component FAME Mix, USA) and quantification was based on their respective peak areas, and normalized.

Yields and productivities

The kinetics of substrate consumption (RS and TC), cell mass and carotenoids were followed by periodic sampling of the medium (up to 120 h). The conversion factors determined as the amount of carotenoids produced per unit weight of substrate consumed (Yp/s), the amount of biomass produced per unit weight of substrate consumed (Yx/s) and the amount of carotenoids produced per unit of dry weight of cells (Yp/x) as well as the carbon source (TC) consumption rate (Q_S) and productivity in cells (Q_X) and carotenoids (Q_P) were determined in the different runs. Total lipids production ($g \cdot L^{-1}$) was determined from the final biomass after 120 h cultivation time.

Statistical analysis

The results obtained were treated by one-way analysis of variance followed by Tukey's test, using the software Statistica 7.0 (Statsoft, Tulsa, USA). Analyses were performed considering a 95 % confidence level.

RESULTS AND DISCUSSION

Physicochemical characteristics of cassava wastewater

Cassava wastewater presented high concentrations of TC and RS, what support its use as a carbon source in biotechnological processes (Table 2), according to the literature. Moreover, great amounts of macro- and micro-minerals important for microorganisms growth were found. The values of TC we found are quite higher than those found by Nitschke and Pastore[14] who observed an amount of 35.3 g · L^{-1} in CW used to grow *Bacillus subtilis* for the production of surfactants. However, the contents of TC and RS found in the present study are very close to those found by Damasceno et al.[25], who found 58.2 and 38.0 g · L^{-1} of TC and RS, respectively, in CW used to grow *Geotrichum fragrans* for the production of volatile compounds.

The value found for free cyanide (3.0 mg · L^{-1}) was low when compared with those previously reported in the literature.[26] However, Souza et al.[27] also found considerably low values of free cyanide, ranging from 5.30 to 16.60 mg L^{-1} in CW collected in cassava flour houses located in semiarid region of the state of Alagoas (Northeastern, Brazil). The concentration of free cyanide in CW depends on the plant genotype, cultivation and processing conditions, and storage time.[26]

A high COD (61.27 g O_2 L^{-1}) was found in the CW utilized in this work. The high COD is associated with the high concentration of sugars in CW and represents an environmental problem when this by-product is disposed without further treatment. The pH value we found was very close to 6.0 which is an optimum value for a culture medium intended to grow red yeasts.[28]

Biomass production

The maximum growth (12.78 g·L^{-1}) was achieved in run 3 (Figure 1), with CW (30 g of RS·L^{-1}), minerals supplementation, ammonium sulphate (5 g·L^{-1}), and 120 h of culture. The medium containing only CW (run 4), diluted to 30 g of RS·L^{-1}, as the sole source of nutrients presented also a high growth (10.28 g·L^{-1}), showing no significant differences ($p > 0.05$) when compared with the mediums containing CW (30 g of RS·L^{-1}) and supplemented with 5 g·L^{-1} of ammonium sulphate (run 1) or 10 g·L^{-1} of glucose (run 5), after 120 hours of cultivation.

By comparison with runs 3 and 4, when CW was diluted to reach 20 g of $RS \cdot L^{-1}$ (runs 7 and 9) and 10 g of $RS \cdot L^{-1}$ (runs 8 and 10) a lower growth ($p < 0.05$) was observed as a consequence of the reduction in the carbon source available in the medium. Regarding to the synthetic mediums containing glucose as the sole carbon source (runs 2 and 6) a lower growth ($p < 0.05$) was observed when compared with the treatments that used CW. After 120 h of culture, runs 2 and 6 reached a concentration of cell dry weight of 5.51 and 5.21 $g \cdot L^{-1}$, respectively. It can be suggested that the higher growth obtained in the medium with CW can be due to the presence of considerable amounts of other nutrients than sugar, which are important to support and stimulate cell growth. *R. glutinis* growth is affected by numerous parameters, such as the nature and concentration of carbon and nitrogen sources, minerals, vitamins, temperature, stress, and agitation.[5,29]

Comparing with other studies that used agro-industrial by-products as substrates, Saenge et al.[17] found a maximum biomass of 8.05 $g \cdot L^{-1}$ for *R. glutinis* cultivated in palm oil mill effluent and through a response surface analysis they observed that for cell growth a relatively high COD and low C/N ratio is required. Using fermented radish bride as a substrate, Malisorn and Suntornsuk[30] observed a biomass production of 2.6 $g \cdot L^{-1}$ for *R. glutinis* in a batch cultivation. Marova et al.[12] found a biomass growth of 9.16 $g \cdot L^{-1}$ when *R. glutinis* was cultured in deproteinized cheese whey and Park et al.[31] found a maximum biomass of 12.5 $g \cdot L^{-1}$ when *R. glutinis* was cultured in sugar cane molasses supplemented with urea and KH_2PO_4. Therefore, the biomass production we found can be considered satisfactory, as the values are higher or similar to those found in the literature when *R. glutinis* was grown in alternative substrates. Moreover it was observed that biomass production in the mediums composed by CW can be increased by the supplementation with minerals and nitrogen and by controlling the initial concentration of reducing sugars.

Consumption of the carbon sources

The high growth obtained for all the mediums containing CW suggests that this agro-industrial by-product provides a suitable carbon source to support cell yeast growth. Cassava wastewater is reported in the literature to present a sugar composition of dextrin (2.6%), maltose (1.4%), sucrose (32.1%), glucose (38.3%), and fructose (25.6%).[25] The contents of RS and TC in all treatments showed a significant decrease

along the cultivation time (Figure 2A and 2B). Sugar consumption rates were different among the runs, what can be explained by the differences in C/N ratios of these mediums (Table 1), as shown by Braunwald et al.[8] For the mediums containing CW a very low decrease in RS was observed after 24 hours of cultivation, despite the high growth rate during this period. This result may be due to the hydrolysis of non-reducing sugars, as *R. glutinis* is known to present β-glucosidase activity.[32] The consumption of TC ranged from 31.65% (run 5) to 71.62% (run 1). Runs 5 and 6 showed the lowest consumptions (%) of carbohydrates after 120 h of culture (31.65 and 33.4%, respectively), although these mediums were prepared with the highest amounts of RS. For run 5, the presence of glucose may be a reason for the lower consumption of sugars, as microbial β-glucosidases are known to be very sensitive to glucose inhibition which limits their activity toward the hydrolysis of non-reducing sugars in the medium.[33]

Production of carotenoids

The conversion of organic compounds in CW into lipids and carotenoids by using *R. glutinis* is of importance for its treatment and valorization. The accumulation of carotenoids in the yeast cells and the production of carotenoids per unit of volume are displayed in Figures 3A and 3B, respectively. The highest accumulation of carotenoids in the cells (167.23 $\mu g \cdot g^{-1}$), after 120 h, was achieved in the medium containing CW (10 g of RS $\cdot L^{-1}$) supplemented with minerals and 5 $g \cdot L^{-1}$ of ammonium sulphate (run 10). Near values (139.18 $\mu g \cdot g^{-1}$) were obtained for the same medium without minerals and nitrogen supplementation (run 8). Following these runs, great amounts of carotenoids were found also in the cells grown on synthetic mediums, with concentrations of 88.2 and 114.4 $\mu g \cdot g^{-1}$, for runs 2 and 6, respectively. By comparing the mediums with CW as the sole source of nutrients, it was observed that the cellular accumulation of carotenoids was lower when the concentration of initial RS was higher: among these mediums, run 4 showed the lowest content (56. 8 $\mu g \cdot g^{-1}$) of carotenoids in the cells at the end of the cultivation process.

Regarding the production of carotenoids per unit volume, no differences ($p > 0.05$) were found between runs 8 and 10, that reached means values of 0.98 and 1.03 $mg \cdot L^{-1}$, respectively. These runs presented the best results for the volumetric production of carotenoids after 120 h of culture. As to the other mediums, similar

results for volumetric production of carotenoids were obtained, because high cellular accumulations corresponded to low growth rates and vice versa.

It can be suggested that the high production of carotenoids in the mediums containing CW, diluted to reach 10 g of RS · L^{-1}, may be a consequence of nutrients limitation, as it can lead to oxidative stress response in the yeast cells.[34] The most important function of carotenoids in yeasts is to deactivate the free radicals produced during normal metabolism of cells, such as singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot -}$), peroxides and other oxidants.[35] Growth under unfavorable conditions induces a stress in *R. glutinis*, which brings about a bio-chemical response involving an increase in the activity of enzymes and of the levels of carotenoids.[36]

Yields and productivities

The mediums containing CW presented higher or similar values for the conversion of the substrate into biomass ($Y_{X/S}$) when compared with synthetic mediums with glucose as the only carbon source (Table 3). Among the mediums in which CW was used as the sole source of nutrients, those with a low initial RS concentration led to higher $Y_{X/S}$ values . The conversion of the biomass into carotenoids ($Y_{P/X}$) showed its highest values for the mediums containing 10 g of RS·L^{-1} (runs 8 and 10). The same behavior was observed for $Y_{P/S}$. The increase in the specific yield of carotenoids is an indication of an augmented biosynthesis of carotenoids by the yeast cells and this behavior is possibly related with the changes in metabolism and cell stress.[37] By performing an evaluation of the effect of simultaneous nitrogen and minerals supplementation in the medium with CW, it can be observed that for the medium containing 30 g of RS·L^{-1} a higher biomass productivity (Q_X) was achieved, whereas for the mediums with 20 g of RS·L^{-1} and 10 g of RS·L^{-1}, a little decrease was observed. Regarding to the productivity of carotenoids (Q_P), similar results were obtained for all experiments except for the runs 8 and 10, which showed Q_P values 1.6-2.0 times superior in comparison to the other runs. For the carbon source consumption rate (Q_S) the highest values were observed for the mediums containing only CW, diluted to 30 g of RS·L^{-1}, as the carbon source (runs 1, 3 and 4), whereas the lowest values were observed for runs 8 and 10 with CW diluted to 10 g of RS·L^{-1}.

Lipids production and fatty acids composition

A lower cellular accumulation of lipids ($p < 0.05$) was observed in the mediums containing CW when compared with the synthetic mediums (Table 4). The highest ($p < 0.05$) cellular accumulation of lipids (32.39 g·100 g_{cdw}^{-1}) was observed in run 6 which presented the highest C/N ratio. The lipid accumulation in oleaginous yeasts is only triggered when a growth-required nutrient (mostly nitrogen) is limited, and carbon is still abundantly available.[38] From this, it can be deduced that a high C/N ratio of the growth mediums will positively affect the lipid accumulation in these organisms. Since the mediums containing CW diluted to 30 g of RS·L^{-1} (runs 1, 3, 4) presented a higher growth, the productions of lipids per unit volume for these mediums were similar to those obtained for the synthetic mediums and for the medium supplemented with glucose (run 5). This is a valuable result, since the cost of the carbon (glucose) contributes a high share to the overall cost of the whole process, and any potential savings in pure glucose utilization will help to improve the economic features of the approach. The lower productivities of lipids we found in run 9 (0.82 g·L^{-1}) and 10 (0.67 g·L^{-1}) are expected since a C/N ratio of 20 is seen as a minimum condition for lipid induction.[39] In general the production of lipids per unit volume was higher according to the carbon availability in the medium. It is agreed that only under excess carbon conditions, lipids are synthesized.[11]

The fatty acids composition was generally similar in all treatments containing CW as the carbon source (Table 5). With average values ranging from 47.28 – 59.76 %, oleic acid (C 18:1 n9 *cis*) was the main fatty acid found, followed by palmitic acid (C 16:0; 13.03 - 15.66%) and stearic acid (C 18:0; 2.97 - 13.46%). According to the literature,[5] in this investigation palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) account for over 80% of the total fatty acids in lipids from *R. glutinis*.

When compared with the mediums containing CW, synthetic mediums (runs 2 and 6) presented lower values ($p < 0.05$) for oleic acid (C 18:1 n9 *cis*; 28.73 – 41.25%) and higher values ($p < 0.05$) for palmitic acid (C 16:0; 21.92 - 29.05%) and Gondoic acid (C 20:1 n9; 4.47 – 5.63%). The medium with the highest C/N ratio (Run 6) showed the lowest amount ($p < 0.05$) for oleic acid (C 18:1 n9 *cis*; 28.73%) and the highest amount ($p < 0.05$) for palmitic acid (C 16:0; 29.05%). This is also reported in the literature focusing on lipids produced by *R. glutinis*. Braunwald et al.[8] observed that

high C/N ratios led to higher contents of saturated fatty acids and lower contents of unsaturated fatty acids. Mondala et al.[40] also found increasing levels of C 16:0 and C 18:0 with increasing initial C/N ratios.

For the mediums containing CW, oleic acid is the prevailing fatty acid with a share of over 50%, except for run 4 which showed 47.28 %. Since oleic acid is often found in the lipids of cell membranes, this high proportion supports the assumption that in these treatments lipids were directly derived from cell membranes. This result is of interest for the production of biodiesel as oleic acid is seen as optimal fatty acid for improved fuel properties.[41] For all runs the unsaturated fatty acids stand out as major contributors to the overall FAME content (over 50%). Amongst these it was found considerable amounts of the polyunsaturated ω-6 fatty acids, linoleic acid (C 18:2 n6 *cis*; 1.77 – 15.20%) and arachidonic acid (C 20:4 n6; 0.33 – 1.91%), as well as ω-3 linolenic acid (C 18:3 n3 *cis*; 0.27 – 1.85%). It is known that the lipids profile of microbial oils depends upon the carbon source used in the cultivation[5] and that these fatty acids are associated to health benefits when included in human diet. Therefore, due the large presence of these fatty acids in *R. glutinis* yeast grown on CW mediums, it can be suggested that this by-product has potential to be applied in the food industry.

CONCLUSION

Rhodotorula glutinis growth was supported by the carbohydrates present in CW. Moreover, this residue can be employed as a sole source of nutrients to grow *R. glutinis*, as the supplementation with glucose, nitrogen and minerals was observed to be not necessary for the growth. For the purpose of carotenoids production the dilution of CW to reach 10 g of $RS \cdot L^{-1}$ can be considered the best choice, as satisfactory growth and maximum carotenoids production were obtained. Lipids production showed to be influenced by the availability of carbon in the medium and for this purpose the use of CW at 30 g of $RS \cdot L^{-1}$ would be recommended, since this concentration also provided a majority of unsaturated fatty acids in the lipids. The results support the viability of the use of CW alone as a substrate to be applied in a biotechnological process for the production of carotenoids and fatty acids by *R. glutinis*, helping to reduce the costs of the process and valorizing the main effluent of cassava processing industry. However, other parameters of the cultivation process such as the aeration of the growth medium

and stress conditions should be tested further in order to improve lipids and carotenoids yield in *R. glutinis* cells cultivated in CW.

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Table 1. Composition of the culture mediums in shaken flasks.

	$(NH_4)_2SO_4$ ($g \cdot L^{-1}$)	Glucose ($g \cdot L^{-1}$)	CW (g of RS$\cdot L^{-1}$)	Minerals supplementation*	C/N
Run 1	5	-	30	-	18.2
Run 2	5	30	-	+	26.5
Run 3	5	-	30	+	18.2
Run 4	-	-	30	-	31.5
Run 5	-	10	30	-	38.0
Run 6	5	50	-	+	44.1
Run 7	-		20	-	31.5
Run 8	-	-	10	-	31.5
Run 9	5	-	20	+	15.0
Run 10	5	-	10	+	9.8

* "+" means supplemented and "-"means not supplemented.

Table 2. Composition of the pre-treated cassava wastewater utilized in this work.

Parameter	**Mean ± standard deviation**
pH	5.98 ± 0.03
Free cyanide (mg L^{-1})	3.0 ± 0.0
COD (g O_2 L^{-1})	61.27 ± 5.94
Reducing sugars (g L^{-1})	40.60 ± 1.74
Non reducing sugars (g L^{-1})	17.51 ± 1.74
Total carbohydrates (g L^{-1})	58.11 ± 2.13
Total nitrogen (g L^{-1})	1.94 ± 0.08
Dry material (g 100 mL^{-1})	9.51 ± 0.29
Ca (mg L^{-1})	241.62 ± 5.56
Mg (mg L^{-1})	370.59 ± 4.35
P (mg L^{-1})	220.35 ± 3.28
Na (mg L^{-1})	147.55 ± 1.31
K (mg L^{-1})	1247.92 ± 7.36
Zn (mg L^{-1})	1.83 ± 0.16
Fe (mg L^{-1})	15.37 ± 1.75
Cu (mg L^{-1})	1.51 ± 0.02

Table 3. Effect of medium composition on the conversion factors ($Y_{X/S}$; $Y_{P/X}$; $Y_{P/S}$) and biomass and carotenoids productivities (Q_X; Q_P).

Run	$Y_{X/S}$ ($g \cdot g^{-1}$)	$Y_{P/X}$ ($mg \cdot g^{-1}$)	$Y_{P/S}$ ($mg \cdot g^{-1}$)	Q_X ($g \cdot L^{-1} \cdot h^{-1}$)	Q_P ($mg \cdot L^{-1} \cdot h^{-1}$)	Q_S ($g \cdot L^{-1} \cdot h^{-1}$)
1	0.29	0.06	0.02	0.083	0.005	0.29
2	0.22	0.09	0.02	0.043	0.004	0.19
3	0.38	0.04	0.01	0.103	0.004	0.27
4	0.30	0.06	0.02	0.082	0.005	0.27
5	0.56	0.05	0.03	0.083	0.004	0.15
6	0.30	0.12	0.04	0.041	0.005	0.14
7	0.36	0.07	0.03	0.063	0.005	0.18
8	0.59	0.14	0.08	0.056	0.008	0.10
9	0.29	0.09	0.03	0.050	0.004	0.18
10	0.53	0.17	0.09	0.048	0.008	0.09

Table 4. Lipids production after 120 h cultivation.

Assay	Total lipids	
	($g \cdot 100\ g_{cdw}^{-1}$)	($g \cdot L^{-1}$)
Run 1	11.80 ± 0.14^{cd}	1.19 ± 0.01^{c}
Run 2	28.28 ± 0.72^{b}	1.59 ± 0.04^{a}
Run 3	12.36 ± 0.10^{cd}	1.59 ± 0.01^{a}
Run 4	13.23 ± 0.16^{cd}	1.34 ± 0.02^{b}
Run 5	15.51 ± 0.70^{c}	1.64 ± 0.07^{a}
Run 6	32.39 ± 0.71^{a}	1.71 ± 0.04^{a}
Run 7	12.96 ± 0.38^{cd}	1.01 ± 0.03^{d}
Run 8	10.43 ± 0.16^{d}	0.73 ± 0.01^{ef}
Run 9	12.64 ± 0.48^{cd}	0.82 ± 0.01^{e}
Run 10	10.29 ± 0.74^{d}	0.67 ± 0.05^{f}

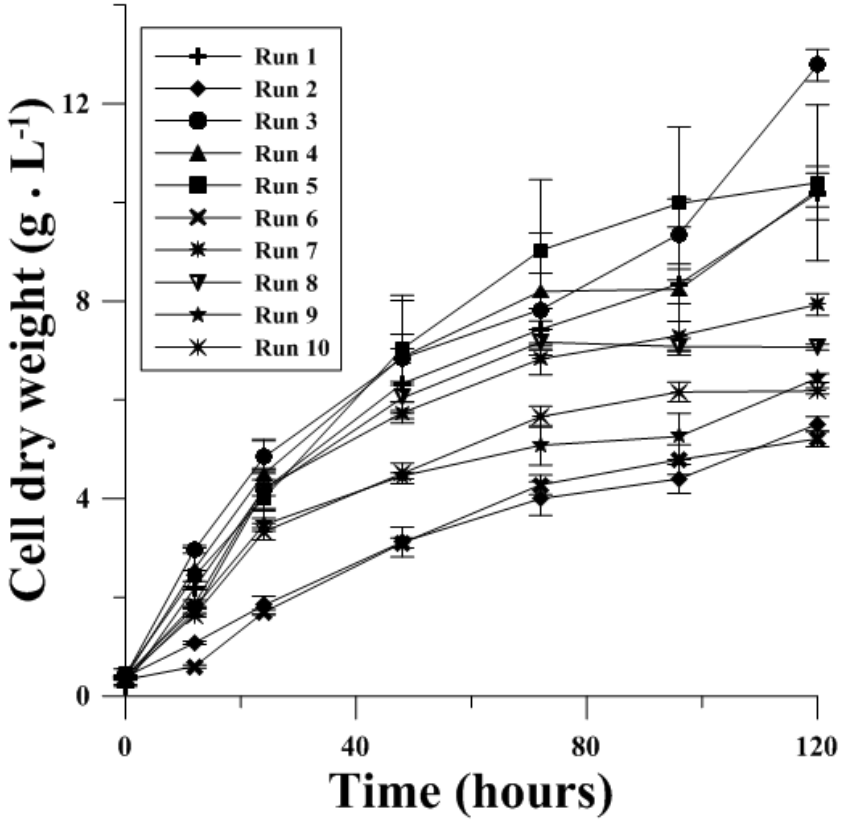
Data are expressed as means of three replicates ± standard deviation.
Values in the same column that do not share the same alphabetic superscript are significantly different at 5 % levels of probability.

Table 5. Fatty acids composition (% of total) of *Rhodotorula glutinis*.

	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Run 8	Run 9	Run 10
C14:0	1.20±0.22^{bc}	2.37±0.33^{a}	1.43±0.28^{bc}	1.69±0.45^{bc}	1.15±0.09^{bc}	2.74±0.14^{a}	0.88±0.05^{c}	1.73±0.38^{b}	0.97±0.01^{c}	1.26±0.42^{bc}
C16:0	13.03±1.16^{c}	21.92±0.20^{b}	13.29±1.23^{c}	15.66±3.75^{c}	13.45±0.52^{c}	29.05±1.64^{a}	15.54±0.12^{c}	14.07±2.11^{c}	13.68±0.07^{c}	13.45±1.09^{c}
C16:1 n7	0.49±0.17^{d}	0.87±0.06^{c}	0.67±0.09^{cd}	0.73±0.15^{cd}	0.61±0.17^{cd}	1.12±0.01^{b}	0.81±0.05^{c}	1.84±0.36^{a}	0.60±0.01^{d}	0.82±0.14^{c}
C17:0	0.84±0.14^{a}	0.21±0.02^{b}	0.94±0.11^{a}	0.97±0.21^{a}	0.80±0.24^{a}	0.19±0.01^{b}	0.77±0.06^{a}	0.39±0.13^{c}	0.99±0.11^{a}	1.12±0.36^{a}
C17:1 n10	0.71±0.04^{a}	0.09±0.03^{b}	0.88±0.22^{a}	0.87±0.26^{a}	0.71±0.15^{a}	0.05±0.00^{b}	0.64±0.08^{a}	0.58±0.10^{a}	0.70±0.07^{a}	0.85±0.35^{a}
C18:0	8.75±1.66^{a}	8.34±1.67^{a}	11.12±1.18^{a}	13.46±4.15^{a}	12.53±2.08^{a}	9.63±2.64^{a}	9.69±0.75^{a}	2.97±0.87^{b}	11.62±0.21^{a}	9.75±0.95^{a}
C18:1 n9 *cis*	59.76±0.58^{a}	41.25±2.25^{b}	55.49±5.23^{a}	47.28±4.34^{ab}	54.13±2.82^{a}	28.73±1.81^{c}	56.29±2.67^{a}	57.95±3.50^{a}	55.58±0.10^{a}	51.10±3.41^{ab}
C18:2 n6 *cis*	2.15±0.12^{c}	13.01±1.54^{a}	2.08±0.29^{c}	2.07±0.49^{c}	2.47±0.69^{c}	15.20±0.93^{a}	1.77±0.17^{c}	7.59±1.18^{b}	2.00±0.13^{c}	2.73±0.91^{c}
C18:3 n3	0.63±0.28^{b}	0.28±0.01^{c}	0.78±0.19^{b}	1.85±0.44^{a}	0.68±0.23^{b}	0.27±0.04^{c}	0.77±0.05^{b}	0.89±0.14^{b}	0.75±0.10^{b}	0.94±0.19^{b}
C20:1 n9	0.25±0.06^{c}	4.47±0.25^{a}	0.44±0.06^{c}	0.33±0.08^{c}	0.54±0.16^{c}	5.63±0.05^{a}	0.28±0.05^{c}	1.15±0.16^{b}	0.49±0.03^{c}	0.52±0.14^{c}
C20:4 n6	0.96±0.35^{b}	0.95±0.05^{b}	0.90±0.29^{b}	1.91±0.48^{a}	1.31±0.47^{ab}	1.01±0.02^{b}	1.07±0.11^{b}	0.33±0.05^{c}	1.00±0.08^{b}	1.25±0.32^{ab}
C21:0	0.12±0.00^{a}	0.04±0.00^{b}	0.13±0.01^{a}	0.12±0.04^{a}	0.09±0.02	0.03±0.01^{b}	0.06±0.02^{b}	0.14±0.01^{a}	0.14±0.06^{a}	0.16±0.04^{a}
C22:0	1.75±0.62^{ab}	1.42±0.15^{b}	2.04±0.16^{a}	2.69±0.53^{a}	2.29±0.78^{a}	1.44±0.03^{b}	2.06±0.11^{a}	0.62±0.07^{c}	2.07±0.08^{a}	2.30±0.63^{a}
C23:0	0.18±0.04^{a}	0.09±0.01^{c}	0.17±0.03^{a}	0.22±0.07^{a}	0.18±0.03^{a}	0.09±0.01^{c}	0.21±0.06^{a}	0.30±0.08^{a}	0.22±0.02^{a}	0.22±0.04^{a}
C24:0	2.44±0.95^{ab}	1.30±0.19^{b}	3.23±0.45^{a}	3.65±0.76^{a}	2.55±0.83^{ab}	1.19±0.01^{b}	2.94±0.62^{ab}	1.73±0.16^{b}	3.21±0.24^{a}	3.53±0.52^{a}
Other	6.76±1.63^{a}	3.37±0.33^{b}	6.41±0.51^{a}	6.49±0.78^{a}	6.51±0.65^{a}	3.63±0.08^{b}	6.24±0.37^{a}	7.73±1.71^{a}	5.98±0.34^{a}	9.98±3.12^{a}

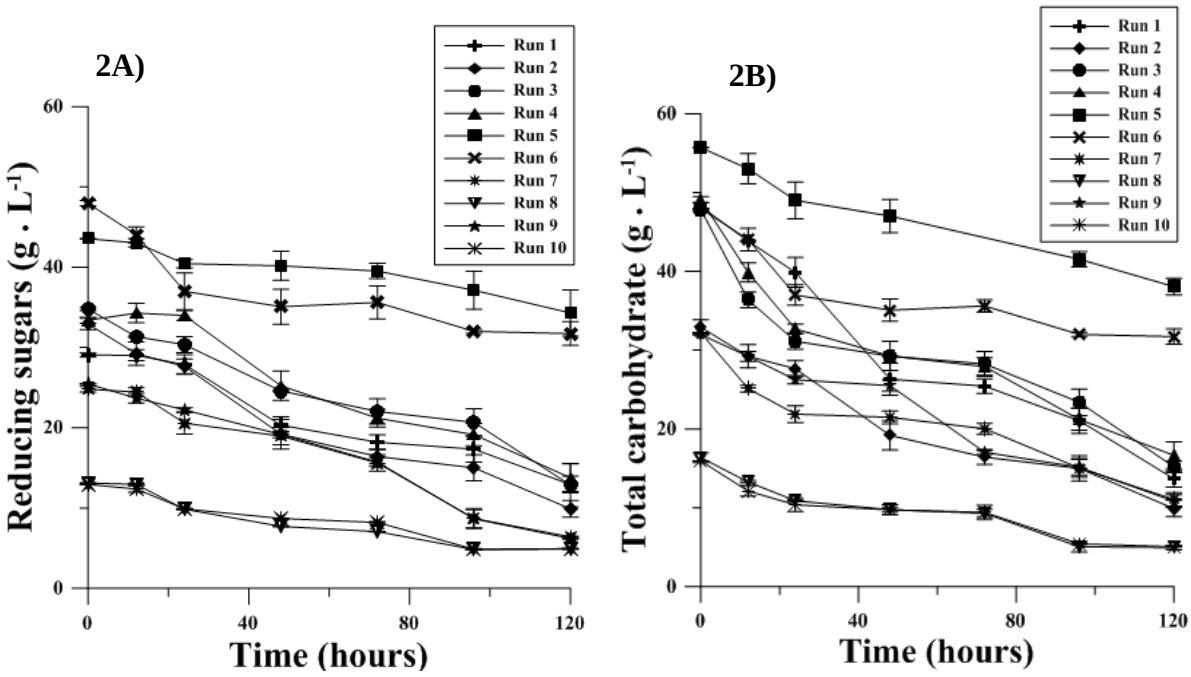
Data are expressed as means of three replicates ± standard deviation.
Values in the same line that do not share the same alphabetic superscript are significantly different at 5 % levels of probability.

Figure 1. Biomass concentration along the cultivation process.



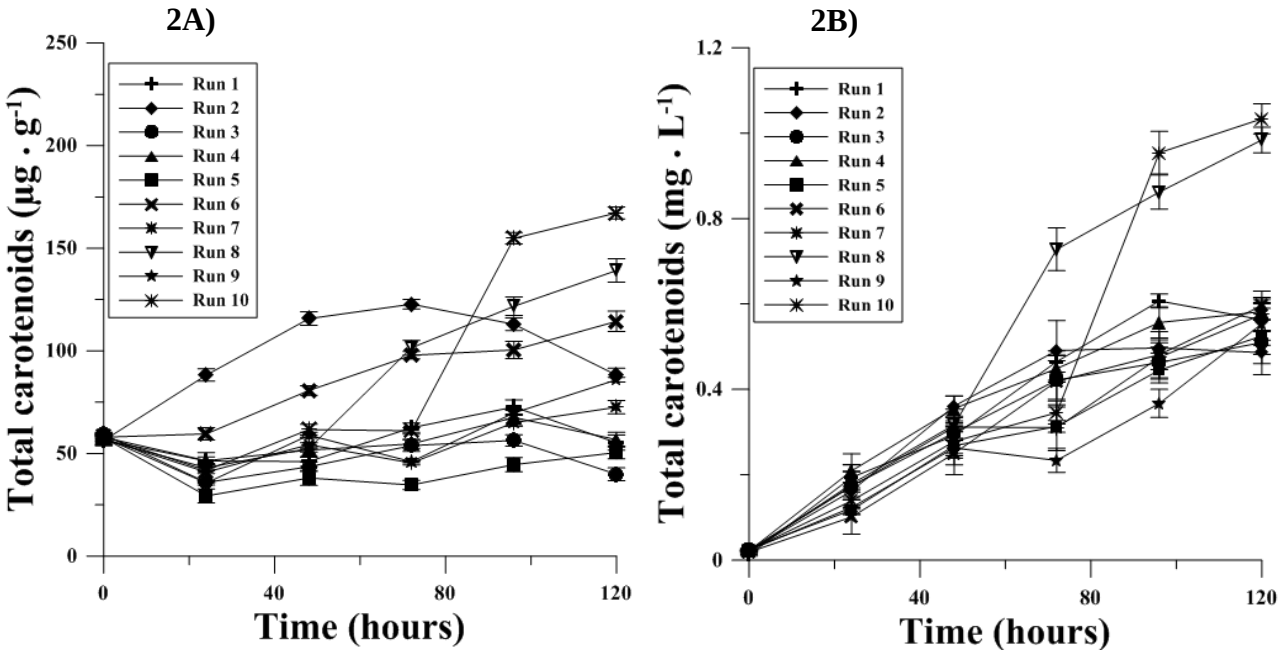
Data are expressed as means of three replicates ± standard deviation.

Figure 2. Consumption of the carbon source along the cultivation



Data are expressed as means of three replicates ± standard deviation.

Figure 3. Carotenoids production by *R. glutinis* along the cultivation period.



Data are expressed as means of three replicates ± standard deviation.

5. CONSIDERAÇÕES FINAIS

O soro de queijo ricota (*scotta*) e a manipueira, subprodutos da agroindústria, apresentaram composição química favorável para o cultivo de microrganismos, verificando-se, em ambos, elevadas concentrações de carbono e minerais. O desenvolvimento de um processo biotecnológico, com o uso destes subprodutos como substratos e com foco na obtenção de moléculas bioativas mostrou ser uma alternativa viável em função dos resultados obtidos para o rendimento de biomassa e de bioprodutos. Os microrganismos utilizados (*Chllorella protothecoides* e *Rhodotorula glutinis*) adaptaram-se bem aos substratos alternativos, utilizando de forma eficiente às fontes de carbono disponíveis e acumulando elevadas concentrações dos metabólitos de interesse em suas células.

Os objetivos propostos nesta tese foram alcançados, uma vez que por meio da utilização de subprodutos agroindustriais e de estratégias de cultivo, foi possível a obtenção de biomassas microbianas contendo elevados teores de moléculas bioativas que podem ser utilizadas como aditivos naturais na indústria de alimentos.

No entanto, como perspectiva futura, sugere-se que outros experimentos sejam realizados, com vista a aumentar a escala de produção e ao desenvolvimento de métodos eficientes e sustentáveis para a extração dos metabólitos presentes nas biomassas obtidas. Além disso, é importante também a realização de estudos para analisar os efeitos da aplicação destas moléculas bioativas em matrizes alimentares.

APÊNDICES

APÊNDICE I

Preliminary investigation of reuse of ricotta-cheese whey *(scotta)* as substrate for the growth of *Rhodotorula glutinis* intended for the production of carotenoids

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ABSTRACT

The ricotta-cheese whey, also called *scotta*, is the main by-product of ricotta-cheese manufacture process. This by-product is rich in nutrients that could make it a good substrate in biotechnological processes for the production of commercial high-value compounds. Nowadays, the demand for natural pigments and health-promoting food ingredients has increased. Carotenoids present a high commercial value as it are widely used in food, feed, pharmaceutical and cosmetic industries. A wide range of microorganisms and *Rhodotorula* genera yeast are able to produce carotenoids. The aim of this study was to evaluate the potential re -use of ricotta cheese whey (*scotta*) as substrate for the growth of *Rhodotorula glutinis* intended for the production of carotenoids. Under the cultivation conditions used, *R. glutinis* strain showed similar ability to grow and to produce carotenoids when cultivated in *scotta* compared to a semi-synthetic substrate.

Under the cultivation conditions used, *R. glutinis* strain showed similar ability to grow and to produce carotenoids when cultivated in scotta compared to the semi-synthetic substrate. Thus, the results showed that *scotta* has potential to be used in a biotechnological process to obtain a high-value molecule. Further investigations are needed to test if changes in the cultivation parameters and the hydrolysis of lactose could improve the growth and the amount of cellular β-carotene.

Keywords: dairy by-products, *scotta*, carotenoids, yeast

APÊNDICE I

INTRODUCTION

Waste management is a crucial point for dairy plants for the high organic matter and high nutrient levels contained in dairy effluents.

Ricotta-cheese whey, also called *scotta*, is the main by-product of ricotta-cheese manufacture process. Ricotta-cheese is produced after the cheese making process, from raw residual cheese whey; fresh milk (up to 10%), milk fat and an acid solution of salts can also be added. The obtained mixture is maintained at high temperature (85–90 °C) to promote the precipitation of most of whey proteins that make ricotta-cheese. The liquid solution remaining after ricotta-cheese separation is called *scotta* and has different characteristics compared to cheese whey [1]. *Scotta* is widely produced in southern Europe and particularly in Italy where it represents a by-product to be disposed of (Secchi et al., 2012). The most of *scotta* is used as supplement feed for livestock. However, this by-product is rich in lactose and contains nitrogen, hydrosoluble vitamins and a variety of minerals that could make it a good substrate in biotechnological processes for the production of commercial high-value compounds.

Carotenoids are a group of over 600 molecules which fulfill diverse functions; dietary carotenoids are converted to vitamin A, are antioxidants and scavenger of oxygen radicals. Epidemiological evidence and experimental studies suggest that carotenoids enhance the immune response, inhibit the onset of many age-related diseases in which free radicals are thought to play a role in initiation, such as arteriosclerosis, cataracts, multiple sclerosis and cancer [3]. Carotenoids and β-carotene are popularly used as natural colorants and antioxidants in food, feed, cosmetic and pharmaceutical products [4].

Scientists and consumers are interested on natural colorants due to the negative health effects of synthetic colorants [3] and because of the stringent rules and regulations applied to chemically synthesized/purified pigments.

The microbial production of carotenoids go beyond the problems of seasonal and geographic variability of the extraction from vegetables and allows economic advantages if low-cost substrates are used. Various sources of carbon and nitrogen from agro-industrial origin (grape must, beet molasses, soybean flour extract, corn flour extract, cheese whey, fermented radish brine and sugar cane molasses) were already evaluated for cultivation of yeasts, with the purpose of developing a low-cost culture medium for carotenoid production [4, 5, 6].

Although several studies have proved the viability of using *scotta* as a substrate for the production of high-value products, such as bio-ethanol and lactic acid [1, 2], to the best of our knowledge there are no reports about the use of *scotta* as a substrate to grow carotenoid-producing yeasts. Despite its large availability and low price, this by-product is still poorly used in biotechnological processes.

Rhodotorula yeasts are non-photosynthetic organisms, widely distributed in nature, which can biosynthesize characteristic carotenoids [7, 8]. *Rhodotorula glutinis* is generally recognized in the literature as a good producer of carotenoids [9].

The aim of this study was to investigate the potentiality of reuse of *scotta* as substrate for the growth of *R. glutinis* intended for the production of carotenoids and compare the results with Malt Extract Broth (MEB), the first choice substrate for its cultivation.

MATERIALS AND METHODS

Microorganism

The yeast isolate was obtained from poultry droppings immediately after being shed and transported to the laboratory. The sample was dissolved in 2 mL of sterile water added with 50 µL mL^{-1} gentamicin and seeded onto Malt Extract Agar (MEA) supplemented with biphenyl 0.01%. The plates were incubated at 25 °C and daily examined over a 7 days period. To avoid contamination from fast-growing molds and/or bacteria, subcultures were achieved from day 4 post inoculation onto MEA until pure yeast colonies were obtained. *Rhodotorula* species isolates were screened macroscopically by the aspect of texture and colonies color, and microscopically by culture on cornmeal-Tween 80 agar.

The isolates were identified via the carbohydrate assimilation pattern, using the ID32C galleries (bioMérieux Italia SpA, Roma), along with a nitrate assimilation test. Definitive identification of *R. glutinis* was achieved by amplification and sequencing of ITS region of ribosomal DNA (rDNA). Genomic DNA was extracted from single colonies using the method described by Nunes *et al.* [6]. PCR and sequencing procedures were performed with the universal primers ITS1 and ITS4 [10]. Sequences were assembled and corrected by visual analysis of the electropherogram using Bioedit v.7.0.2, then compared with those available in GenBank using the basic local alignment search tool program [11] to assign the species.

Culture media

The *scotta* used in this study was supplied by dairy industries located in Toscana region, in Italy. The *scotta* was obtained from ricotta-cheese manufactured starting from a mixture of bovine and ovine cheese whey (90% and 10%, respectively). The physicochemical characterization was carried out for the

following parameters: pH, acidity (g lactic acid 100 mL^{-1}), reducing sugars (g lactose 100 mL^{-1}), dry material, fat, ash content, total nitrogen, calcium, magnesium, phosphorous, sodium, potassium, zinc, iron and manganese. All the analysis were carried out according to the methodologies proposed by AOAC [12]. As a control, malt extract broth (MEB) was used as a semi-synthetic growth medium for the cultivation. The composition per liter was as follows: peptone (1 g), glucose (20 g) and malt extract (20 g).

Growth conditions

Yeasts cells at log phase were transferred to MEB (100%) with an inoculum concentration of 10^7 cells mL^{-1} . Yeasts were grown in Falcon tubes using 100% *scotta*; MEB was used as control. Tubes were exposed to visible light for 10 hours, maintained at room temperature and manually shaken until processed. The cultivation was carried out up to 28 days to evaluate the changes in the pigments accumulation and in the carotenoids profile along a large cultivation period. The long cultivation time was adopted due to the investigative character of the experiment. Each experiment was repeated at least three times with two replicates.

Analytical methods

Yeast biomass

The total biomass in fresh weight was quantified after 7, 14, 21 and 28 days of cultivation. A culture medium sample was collected and centrifuged at 6000 rpm for 5 min, the supernatant was discarded and the fresh biomass was weighted.

Carotenoids extraction and β-carotene quantification

Carotenoids extraction was carried out according to Cutzu *et al.* [13], modified as follows. Cultures were harvested by centrifugation at 6000 rpm for 5 minutes, then the supernatant was discarded and the pellets were frozen at -20 °C for 24 hours. The thawed cell pellet was re-suspended in 2 mL DMSO pre-heated at 60 °C, added with glass beads (0.5 g), vortexed for 2 min and incubated at 60 °C for 5 min. 2 mL acetone, 2 mL petroleum ether and 2 mL NaCl 20 % were sequentially added. Then the mixture was vortexed for a total of 5 min and centrifuged at 6000 rpm for 5 min. The upper petroleum ether layer containing the extracted carotenoids was transferred to a separatory funnel and washed five times with cold distilled water to remove all the DMSO from the petroleum ether phase.

The analysis of carotenoids was conducted using a SpectraSystem HPLC instrument equipped with a UV-VIS detector (Thermo, Rodano, Italy). The column was a Kinetex C18, 250 x 4.6 mm ID, 5 µm particle size (Phenomenex, Torrance, CA, USA), eluted at 1 ml min^{-1} with solvent A (acetonitrile: methanol: Tris buffer 0.1 M pH 8 84:2:14) and B (methanol:ethyl acetate 68:32), according to the following program: 100% solvent A for 4 min, then a linear gradient from 0 to 100% B in 10 min, followed by 100% B for 15 min. The β-carotene peak was identified by comparison of the retention time of sample peaks with that of a pure β-carotene standard (Extransynthese, Lyon, France). β-carotene quantification was carried out by reference to a standard curve.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA). Average values were compared using Tukey's test. Statistical analyses were performed using software *Statistica*®, version 7.0 (Statsoft, USA). Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Physicochemical characterization of *scotta*

The physicochemical characteristics of *scotta* used as culture medium are shown in Table 1. As reported in the literature the main component present in the *scotta* is lactose [1]. This disaccharide is a suitable carbon source for many microorganisms [7]. The amount of nitrogen was similar to the results of Sansonetti et al. [1], in *scotta* produced from bovine raw cheese whey.

The variety and the concentration of minerals found in *scotta* support its application in biotechnological processes. In fact, El-Banna *et al.* [14] observed that supplementation of culture media with mineral salts resulted in an increase in cellular carotenoids from *R. glutinis var. glutinis*. These authors also suggested that the higher production of carotenoids was due to a stimulatory effect of cations on carotenoid-synthetizing enzymes. Moreover, metals such as potassium has important functions in microorganisms [15].

APÊNDICE I

Table 2. Composition of the *scotta* utilized in this work

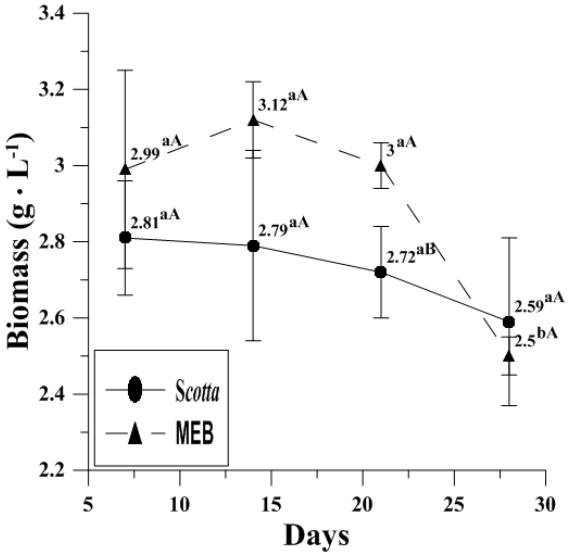
Parameter	Mean ± standard deviation
pH	6.02 ± 0.03
Acidity (g lactic acid 100 mL^{-1})	0.155 ± 0.006
Reducing sugar (Lactose) (g 100 mL^{-1})	4.07 ± 0.21
Dry material (g 100 g^{-1})	7.75 ± 0.13
Total nitrogen (g 100 mL^{-1})	0.096 ± 0.017
Fat (g 100 g^{-1})	0.19 ± 0.00
Ash content (g 100 g^{-1})	0.94 ± 0.00
Ca (g 100 g^{-1})	0.048 ± 0.004
Mg (g 100 g^{-1})	0.030 ± 0.003
P (g 100 g^{-1})	0.093 ± 0.002
Na (g 100 g^{-1})	0.200 ± 0.004
K (g 100 g^{-1})	0.119 ± 0.009
Zn (mg 1000 g^{-1})	73.125 ± 0.884
Fe (mg 1000 g^{-1})	164.375 ± 0.884
Mn (mg 1000 g^{-1})	3.125 ± 0.884

Yeast biomass

The experiments showed that the growth of *R. glutinis* strain tested was satisfying when *scotta* was used as substrate. Regarding the total biomass (Figure 1), a significant difference ($p < 0.05$) between the two media was observed only at 21 days, when the MEB medium showed a slightly higher biomass concentration than *scotta*. The total biomass was similar for the rest of the cultivation period, showing that *scotta* can be a potential substrate to grow *R. glutinis* yeast, as the results were comparable to that obtained for the semi-synthetic medium. Concerning the composition of *scotta* (Table 1), we can conclude that this cheap substrate may support the growth similarly to MEB and may be used as the sole source of nutrients. Regarding the growth along the cultivation time, the amount of biomass for the yeast cultivated in *scotta* did not change significantly, whereas a decrease ($p < 0.05$) in the biomass was observed for the yeast cultivated in MEB after 21 days of cultivation.

It must be noted that the main carbon source found in *scotta* is lactose and that lactose-assimilating yeasts are rarely found in natural conditions [16]. Previous research has demonstrated the lack of ability to assimilate lactose by *R. glutinis* [17]. Furthermore, Aksu and Eren [7] observed that *R. glutinis* has a low tendency to grow in lactose containing media. It is tempting to speculate that a pre-treatment step to hydrolyze lactose could improve the growth through a higher consumption of this carbon source, as glucose and galactose can be well assimilated by this yeast [18].

Figure 1. Biomass concentration along the cultivation time.



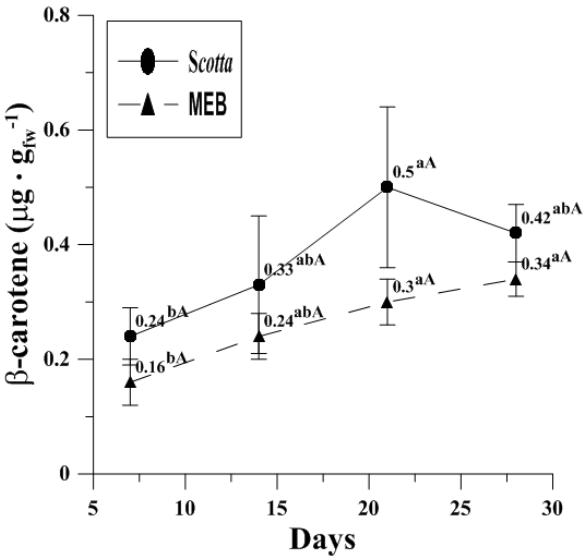
a,b: Different lowercase letters in the same plot mean significant difference along the cultivation time at 5% level of probability.

A,B: Different uppercase letters mean significant difference between the mediums for each cultivation time at 5% level of probability.
Data is expressed as mean of three replicates ± standard deviation.

Carotenoids production

The HPLC analysis enabled to identify β-carotene peak showing that the *R. glutinis* strain tested was able to produce carotenoids in the culturing conditions used. The amount of β-carotene ($\mu g \cdot g_{fw}^{-1}$) did not differ between the growth media ($p > 0.05$) (Figure 2). It was previously reported that the cultivation time affects carotenoids production in the yeast cell [15]. In agreement with this observation, an increasing trend of β-carotene concentration during the cultivation time was observed in the present study, for the yeast cultivated in both *scotta* and MEB. Furthermore, the yeast grown in *scotta* showed a significant difference ($p < 0.05$) in the concentration of β-carotene between the first and third week, when the content of the pigment duplicated (Figure 2). In the yeast cultivated in MEB a higher β-carotene amount ($p < 0.05$) was observed after 21 days of cultivation, compared to the yeast cultured for 7 days. The *R. glutinis* biomass and β-carotene production in both substrates were lower in the present study than those by other researchers [1, 4, 19]. However, it is important to note that this study is a preliminary investigation, and through the optimization of culture conditions, such as the physical environment and the nutritional supply, it is possible to enhance cell growth and carotenoid biosynthesis, allowing the improvement of the process yield (Chandi et al., 2010)

Figure 2. Cellular β-carotene content along the cultivation time



a,b: Different lowercase letters in the same plot mean significant difference along the cultivation time at 5% level of probability.
A: The same uppercase letter means no significant difference between the mediums for each cultivation time at 5% level of probability.
Data is expressed as mean of three replicates ± standard deviation.

CONCLUSIONS

This study was an exploratory investigation on the use of a by-product of a dairy plant as a substrate for a *R. glutinis* strain intended to the production of carotenoids. Ricotta-cheese whey (*scotta*) was never tested before for this purpose. Under the cultivation conditions used, *R. glutinis* strain showed similar ability to grow and to produce carotenoids when cultivated in *scotta* compared to semi-synthetic substrate. Further investigations are needed to test if changes in the cultivation parameters and the hydrolysis of lactose could improve the growth and the amount of cellular β-carotene. The present research shows also a sustainability perspective as an agro-industrial by-product is exploited in a biotechnological process to produce a commercial high-value molecule.

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APÊNDICE I

-------- Messaggio originale --------
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APÊNDICE II

Tabela 3. Teores de proteína e lipídeos na biomassa da microalga *Chlorella protothecoides*.

Ensaio	Condição	Proteína	Lipídeos
Run 1 – cultivo autotrófico	Fase verde	38,68 ± 0,42	7,97 ± 0,16
	Fase de estresse	21,65 ± 0,66	10,64 ± 0,10
Run 2 – Cultivo mixotrófico	Fase verde	29,98 ± 1,22	8,69 ± 0,72
(*scotta* mista)	Fase de estresse	21,18 ± 0,83	18,29 ± 2,42
Run 2 – Cultivo mixotrófico	Fase verde	40,83 ± 0,5	10,62 ± 0,45
(*scotta* ovina)	Fase de estresse	27,6 ± 0,03	16,43 ± 1,57

APÊNDICE III

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

01/03/2018 870180016625 11:48

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2018 004118 5

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DA PARAIBA
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APÊNDICE III

Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	PROCESSO DE PRODUÇÃO DE BIOMASSA, LIPÍDEOS E PIGMENTOS POR RHODOTORULA GLUTINIS UTILIZANDO A MANIPUEIRA COMO SUBSTRATO
Resumo:	A presente invenção trata de um processo para a produção de biomassa, pigmentos e lipídeos por Rhodotorula glutinis utilizando a manipueira como substrato. O efluente da indústria de processamento da mandioca, conhecido como manipueira, é utilizado como fonte de carbono de baixo custo para o desenvolvimento da levedura Rhodotorula glutinis. O processo, de acordo com a presente invenção, compreende as etapas básicas de preparação da manipueira, preparação do inoculo com a levedura Rhodotorula glutinis, cultivo em condições controladas de temperatura, agitação e iluminação, coleta e secagem da biomassa de levedura e extração dos metabólitos intracelulares.
Figura a publicar:	1

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