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ISOLAMENTO, SELEÇÃO E CARACTERIZAÇÃO DE MICROALGAS COM ALTA PRODUTIVIDADE DE BIOMASSA EM MEIO DE CULTIVO A BASE DE VINHAÇA E CO_2

Vitória da Conquista, BA
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RESUMO

Nos últimos anos, devido ao crescente interesse no uso de biocombustíveis, fontes alternativas de biomassa têm sido buscadas com o objetivo de reduzir os custos de produção desses combustíveis e torná-los comercialmente competitivos frente aos combustíveis derivados de petróleo. A biomassa produzida a partir de microalgas tem se mostrado promissora devido à sua maior taxa de crescimento, ao menor requerimento nutricional e a reduzida área necessária para obtenção da mesma produtividade que plantas terrestres normalmente utilizadas para a produção de biocombustíveis. No entanto, os custos envolvidos com a colheita e o processamento dessa biomassa é elevado, reduzindo assim a sua competitividade comercial. Desta maneira, o uso de sistemas de cultivos alternativos se faz necessário para tornar a produção de biocombustíveis a partir de microalgas economicamente viável. O objetivo deste trabalho foi selecionar e identificar cepas de microalgas coletadas de águas continentais brasileiras e cultivá-las em um sistema de cultivo que utiliza vinhaça de cana-de-açúcar e CO_2, resíduos da cadeia de destilação do etanol. Para isto, foram realizadas coletas de amostras de água em áreas ambientalmente conservadas em vários biomas brasileiros e em áreas de lançamento de rejeitos agroindustriais. A partir destas amostras, 52 cepas de microalgas foram isoladas e identificadas utilizando os marcadores moleculares *nu*ITS1, *nu*ITS2 e rbc*L*. Os resultados da identificação mostraram que todas as espécies isoladas pertencem à divisão Chlorophyta, no entanto apenas 6% das cepas avaliadas puderam ser identificadas a nível de espécie. Após a identificação, 40 das 52 cepas selecionadas foram triadas para crescimento em vinhaça sob diferentes condições e caracterizadas quanto ao seu crescimento e produtividade de

biomassa, assim como bioprodutos de interesse. Como resultado, foi possível selecionar, dentre as 40 avaliadas, duas cepas de microalgas, *Embrapa*|LBA#32 e *Embrapa*|LBA#40 capazes de crescer em meio à base de vinhaça. Estas cepas foram avaliadas quanto ao seu potencial de produção de biomassa neste meio alternativo, sendo possível observar que as cepas selecionadas crescem de maneira foto-heterotrófica, realizando fotossíntese e utilizando glicerol como fonte de carbono quando cultivadas em vinhaça. Além disso, quando as microalgas selecionadas foram cultivadas neste meio de cultivo, a produtividade de biomassa foi superior àquela observada em meio sintético Bold's Basal (BBM), indicando que este sistema de cultivo alternativo pode ser adequado para a produção de biomassa microalgal. A caracterização da biomassa obtida mostrou que conteúdo de proteínas presentes na biomassa aumentou quando as cepas selecionadas foram cultivadas em meio à base de vinhaça, em comparação ao observado em meio sintético BBM, indicando o potencial de utilização destas cepas de microalgas como fontes de proteínas. A cepa *Embrapa*|LBA#32 também apresentou potencial para a produção de carboidratos devido a sua produtividade de biomassa, no entanto etapas de otimização do processo de cultivo são necessários para que esta microalga possa ser aplicada na produção de bioetanol. Em conclusão, os resultados demonstram que microalgas obtidas de diversidade brasileira podem ser utilizadas para a produção de biomassa em sistemas de cultivo à base de vinhaça. Desta maneira, a microalgas selecionadas poderiam ser utilizadas em uma estratégia de biorrefinaria para a produção de biomassa ou bioprodutos em um sistema de cultivo que utiliza vinhaça e CO_2.

Palavras-chave: Microalgas. Vinhaça. Biocombustíveis. Biorrefinaria.

ABSTRACT

In the last years, due the crescent interest in biofuels utilization, alternative sources of biomass has been searched in order to reduce the production costs of these fuels, increasing its competitiveness against petroleum-derived fuels. The biomass produced from microalgae has shown promising due its high grow rate, reduced nutritional requirement and reduced area needed to obtain the same productivity as the plants crops used for biofuels production. However, the costs involved with the harvesting and processing of the microalgal biomass is high, reducing its competitiveness. Due to this, the use of alternative cultivation systems are necessary in order to increase the economic viability of biofuels production from microalgae. The objective of this work was to select and identify microalgae strains from continental Brazilian inland waters for cultivation in a system using sugarcane vinasse and CO_2, residues of the ethanol distillation industries. In order to achieve the objective of this work, water samples were collected from environmentally preserved areas in Brazilian biomes and in agroindustrial wastewater. From these samples, 52 microalgae strains were isolated and identified using the molecular markers *nu*ITS1, *nu*ITS2 and rbc*L*. The results of the identification showed that all strains were Chlorophyta species, however only 6% of the evaluated strains were identified in specie level. After the identification, 40 of the 52 strains isolated were screened for cultivation in vinasse. The isolated strains able to grow in vinasse under different conditions were characterized for their growth profile, and biomass and bioproducts productivity. As result, was possible to select among 40 evaluated, two microalgae strains, *Embrapa*|LBA#32 and E*mbrapa*|LBA#40, for growth in

vinasse. The selected strains were evaluated for their biomass production potential in this alternative medium. The results showed that these strains grow using mixotrophic metabolism in this medium, performing photosynthesis and using glycerol as carbon source when cultured in vinasse. Furthermore, the selected strains showed biomass productivity higher than that observed in Bold's Basal Medium (BBM), indicating that this alternative cultivation can be suitable for microalgal biomass production. The characterization of the biomass produced has shown that the protein content of biomass have increased when the selected strains were culture in vinasse-based media, in comparison to that obtained in BBM. In conclusion, the results of this work show that microalgae obtained from de Brazilian biodiversity can be used for biomass production in a vinasse-based cultivation system. In this way, the selected microalgae could be used in a biorefinery strategy for the production of biomass or bioproducts in a cultivation system that utilize vinasse and CO_2.

Key words: Microalgae. Vinasse. Biofuels. Biorefinery.

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1. INTRODUÇÃO

O termo microalgas se refere a um grupo de microrganismos fotossintéticos que apresentam, em geral, tamanho entre 1-10 µm. Este grupo é tipicamente composto por organismos unicelulares e eucarióticos, no entanto, as cianobactérias, microrganismos fotossintéticos procarióticos, também são frequentemente referidas como microalgas (GONG *et al.*, 2011; LARKUM *et al.*, 2012). Estima-se que em todo o mundo existam mais de 50.000 espécies de microalgas distribuídas por todos os ecossistemas terrestres, sendo encontrados em ambientes marinhos ou dulcícolas. Apresentam alta plasticidade em termos de adaptação a diversos ambientes devido à sua grande diversidade genética e o seu baixo requerimento nutricional, sendo o nitrato, o fosfato, o dióxido de carbono (CO_2) a água e luz fundamentais para o seu crescimento. Porém, outros nutrientes também são necessários em menor quantidade para que as microalgas se desenvolvam (LI, HORSMAN, WANG*, et al.*, 2008). Devido a estas características, microalgas têm sido estudadas como potencial fonte de produção de diversos produtos, como pigmentos, ácidos graxos e proteínas, o que torna esses microrganismos adequados para uso como ração animal, fertilizantes e matéria-prima para a produção de biocombustíveis (GOUVEIA *et al.*, 2006; GOUVEIA E OLIVEIRA, 2009; PEREZ-GARCIA *et al.*, 2011).

Nos últimos anos, devido à crescente demanda de biocombustíveis, fontes alternativas de biomassa têm sido buscadas de maneira a reduzir o seu custo e tornar a produção desses combustíveis sustentável a longo prazo (MÜLLER-LANGER *et al.*, 2014). A biomassa microalgal tem se mostrado como promissora devido à sua maior taxa de crescimento, menor requerimento nutricional e reduzida área de cultivo necessária para obtenção da mesma produtividade que

plantas terrestres normalmente utilizadas para a produção de biocombustíveis (CHISTI, 2007). No entanto, devido ao alto custo de colheita e processamento da biomassa microalgal, se faz necessário o uso de sistemas de cultivo alternativos para tornar a produção de biocombustíveis a partir de microalgas economicamente viável (KANG *et al.*, 2015; QUINN e DAVIS, 2015).

2. REVISÃO BIBLIOGRÁFICA

2.1. Microalgas e produção de biocombustíveis

O potencial biotecnológico e econômico das microalgas é conhecido desde o início da década de 1950, por meio de estudos que buscavam fontes alternativas de proteínas. A partir da década de 1970, com o investimento dos EUA na geração de novas tecnologias ambientais, as pesquisas envolvendo microalgas como recurso para tratamento de águas residuais, como biomassa para biocombustíveis e fertilizantes e como fonte biomoléculas receberam investimentos significativos. Isso levou ao surgimento de indústrias de biotecnologia com foco na produção de biomoléculas a partir de microalgas (DE LA NOUE e DE PAUW, 1988; SPOLAORE *et al.*, 2006). No entanto, apesar do grande interesse na geração de produtos com valor agregado, apenas uma pequena variedade de espécies tem sido estudada (Mata *et al.*, 2010; LARKUM *et al.*, 2012).

A produção de biocombustíveis a partir de microalgas é a área que mais tem atraído a atenção de governos e pesquisadores. Isto se deve principalmente a projeções sobre a demanda futura de combustíveis derivados de petróleo. Estima-se que no ano de 2025 esta demanda seja 40% maior que a atual, tornando necessária a busca por alternativas que possuam viabilidade de aplicação na matriz energética mundial (GHASEMI *et al.*, 2012). Neste panorama, as microalgas se apresentam como uma alternativa promissora para a produção de biocombustíveis. Espécies de microalgas como *Botryococcus braunii* e *Nannochloropsis sp.* produzem uma grande quantidade de lipídios de reserva em uma proporção que pode corresponder a cerca de 70% do peso seco total da biomassa, o que é consideravelmente superior a quantidade de lipídios

produzidos pelos cultivares com maior produtividade de lipídios disponíveis, como a soja e o dendê (SHARMA E SINGH, 2009; MALCATA, 2011). Com isto, estima-se que seja possível produzir 58.700 L/ha de óleo a partir de microalgas contendo 30% de óleo por peso seco, enquanto que o pinhão-manso, por exemplo, apresenta uma taxa produtiva de óleo de 1892 l/ha. Esta característica, aliada ao alto percentual de acúmulo de carboidratos em sua parede celular, os torna bons candidatos para a sua aplicação na produção de bioetanol e biodiesel (MALCATA, 2011).

Além do alto percentual de acúmulo de precursores de biocombustíveis, a utilização das microalgas ainda apresenta como vantagens: (a) a sua alta taxa de crescimento, o que torna possível satisfazer a grande demanda de biocombustíveis usando recursos de terra limitados; (b) o seu cultivo necessita de um volume de água potável inferior ao volume necessário em cultivares terrestre; (c) são matérias-primas não-baseados em alimentos e que podem ser cultivados em terras improdutivas ou não-aráveis, o que reduz a competição com alimentos; (d) podem ser cultivadas em uma grande variedade de fontes de água (fresca, água do mar e águas residuais), o que permite a sua associação a outros sistemas de produção; e (e) podem ser fontes de coprodutos de alto valor agregado. Por outro lado, uma das principais desvantagens das microalgas para a produção de biocombustíveis é a baixa concentração de da cultura microalgal devido ao limite de penetração de luz, o que, em combinação com o tamanho reduzido das células algais, torna a coleta da biomassa algal relativamente custosa. (CHISTI, 2008; LI, HORSMAN, WU*, et al.*, 2008; PARMAR *et al.*, 2011).

2.2. Sistemas de cultivo de microalgas

Para que seja realizado o cultivo das microalgas, existem basicamente dois sistemas distintos para cultivo em escala industrial: Os tanques abertos (Ex: *raceways*) e os fotobiorreatores fechados (Figura 1). Os tanques abertos são sistemas semelhantes que consistem em um lago raso, ou raceway, que apresentam agitação mecânica da água por meio de um sistema de pás. Estes sistemas possuem baixo custo produtivo e operacional, porém não apresentam produtividades altas por estarem expostos à contaminação por outros microrganismos, sejam outras algas competidoras, patógenos ou predadores, e variações no ambiente. Os fotobiorreatores fechados são uma alternativa para a produção de microalgas. Eles possuem produtividade maior que os sistemas de cultivo aberto, além de apresentarem um reduzido risco de contaminação, uma vez que o sistema não está em contato direto com o ambiente. Apesar dos benefícios, o uso dos fotobiorreatores é mais oneroso que os demais sistemas abertos (CHISTI, 2007; SCOTT *et al.*, 2010; BAHADAR e BILAL KHAN, 2013; RAZEGHIFARD, 2013).

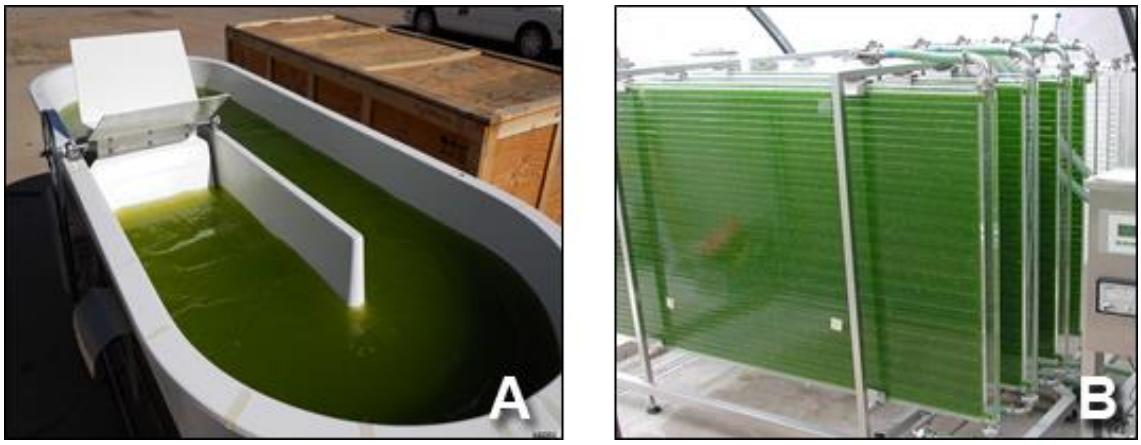

Figura 1. Sistemas de cultivos de microalgas.
(A) Tanque aberto tipo *raceway* (KHANDAN, 2010); (B) Fotobiorreator do tipo placa plana (NATIONAL RESEARCH COUNCIL, 2012).

2.3. Cultivo de microalgas em estratégia de biorrefinaria

É bem estabelecido que microalgas podem gerar uma grande quantidade de bioprodutos. Dentre esses, estão incluídos lipídios, carboidratos, pigmentos e proteínas. Devido a esta característica, o uso desses microrganismos dentro de um sistema de biorrefinaria pode ser uma importante estratégia para a redução dos custos de produção de biocombustíveis derivados de microalgas e (STEPHENS *et al.*, 2010; ADARME-VEGA *et al.*, 2012; YEN *et al.*, 2013). Além de coprodutos de alto valor, a biomassa resultante da extração de lipídios e pigmentos também pode favorecer a redução do custo produtivo final, uma vez que isto pode ser aplicado na produção de biogás ou fertilizantes. Estes derivados, podem ainda contribuir para o próprio sistema de cultivo, a exemplo do biogás, que pode ser utilizado para produzir eletricidade a própria indústria. Além disso, o CO_2 gerado a partir da queima do biogás poderia ser utilizado como suplemento para o crescimento algal, uma vez que essa substância é necessária para a realização da fotossíntese, principal rota metabólica de produção de energia nas microalgas (STEPHENS *et al.*, 2010).

Dentro desta abordagem de biorrefinaria, a utilização de águas residuais como fonte de nutrientes para a produção de biomassa é uma estratégia que pode contribuir significativamente para a redução dos custos de produção (RAZZAK *et al.*, 2013). Como se sabe, algumas microalgas são capazes de crescer tanto em um sistema de crescimento autotrófico, ou seja, através da absorção de CO_2 e da realização de fotossíntese, quanto em um sistema heterotrófico, no qual é feita a absorção de compostos orgânicos e CO_2 presentes no meio de cultivo (PEREZ-GARCIA *et al.*, 2011; PRATHIMA DEVI *et*

al., 2012). Essa característica é fundamental para que seja possível estabelecer um sistema de cultivo de microalgas que utilize resíduos industriais com alta carga orgânica. (RAZZAK *et al.*, 2013).

2.3.1. Resíduos da indústria sucro-energética

A produção de etanol pode ser realizada a partir de quatro matérias-primas principais: carboidratos fermentáveis (e.g. sacarose), disponíveis em diversos cultivares comerciais, como a beterraba (*Beta vulgaris* L.), a uva (*Vitis vinífera* L.), e a cana-de-açúcar (*Saccharum officinarum* L.); o amido, encontrado em cultivares cereais, como o milho (*Zea mays* L.) e o trigo (*Triticum aestivum*), e em tubérculos, como a mandioca (*Manihot suculenta Crantz*) e a batata (*Solanum tuberosum* L.); a celulose, encontrada em resíduos agroindustriais e florestais; e a inulina, encontrada em plantas como *Agave spp.* e banana (*Musa velutina*). As fontes de carboidratos fermentáveis, como a sacarose, são as mais simples de serem utilizadas, uma vez que podem ser fermentados diretamente durante o processo de produção do etanol. O amido, a celulose e a inulina, no entanto, são carboidratos de maior complexidade, que não podem ser diretamente fermentados, necessitando de etapas adicionais para que o etanol possa ser produzido, aumentando o custo final de produção (ESPAÑA-GAMBOA *et al.*, 2011).

No Brasil, a maior parte da produção de etanol é realizada a partir da cana-de-açúcar em uma abordagem integrada, onde é realizada a produção do açúcar e do etanol em uma mesma planta industrial (LEITE, 2009). Neste processo, a cana-de-açúcar é prensada para a obtenção do caldo que é utilizado para a produção do açúcar e para a produção de etanol, a partir da fermentação

alcóolica do caldo por leveduras. A maior parte das usinas brasileiras utiliza este processo integrado de produção do etanol e do açúcar, associando isto a uma estratégia de biorrefinaria, onde os resíduos gerados são utilizados dentro da cadeia de produção com objetivo de se reduzir o impacto ambiental e o custo final dos produtos gerados (Figura 2).

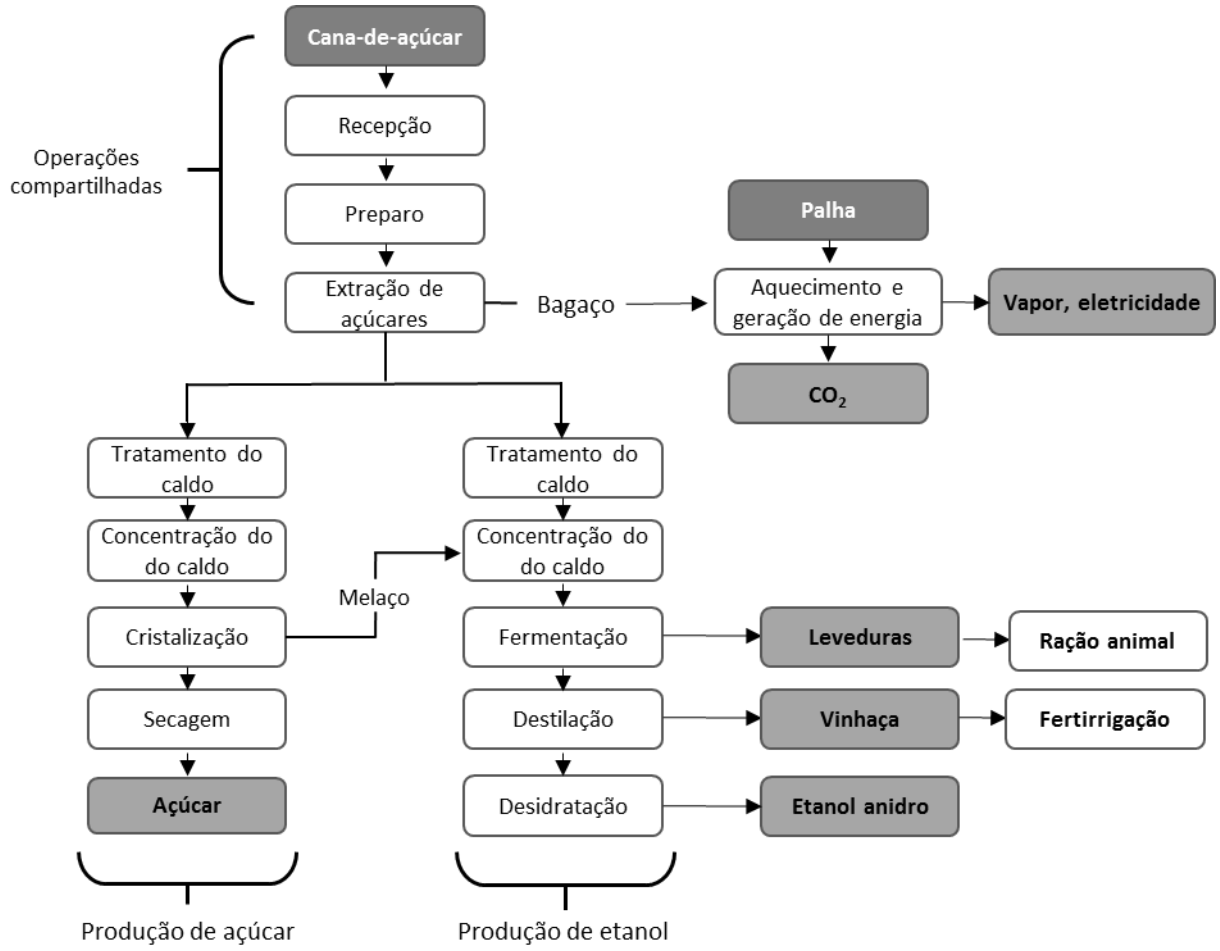

Figura 2. Esquema simplificado da produção de açúcar e etanol de primeira geração a partir da cana-de-açúcar (*Saccharum spp.*). Adaptado de Dias *et al.* (2015).

Durante o processo de produção do etanol, são gerados dois resíduos principais: a vinhaça e o CO_2. A vinhaça é o principal produto da destilação do caldo fermentado (mosto), sendo produzido em uma proporção de 12-14 L para cada litro de etanol. Este resíduo é um líquido marrom escuro, rico em

componentes orgânicos, como açúcares, glicerol e ácidos orgânicos (e.g. ácido láctico, ácido acético), nitrogenados (e.g. nitrito, nitrato) e fosforados (e.g. fosfato), além de íons como K^+, Ca^{2+} e Mg^{2+}. (PARNAUDEAU *et al.*, 2008; CAVALETT *et al.*, 2011; ESPAÑA-GAMBOA *et al.*, 2011; DIAS *et al.*, 2012; DIAS *et al.*, 2015; Ortegón *et al.*, 2016). Devido a estas características, a vinhaça é utilizada para fertirrigação do canavial, reduzindo os custos relacionados ao uso de fertilizantes químicos aplicados no solo para o cultivo da cana-de-açúcar, e minimizando o impacto ambiental causado pelo despejo em leitos aquosos. No entanto, a longo prazo a aplicação da vinhaça no solo pode levar à lixiviação do solo devido a salinização causada pela vinhaça, além de alterações na qualidade do solo, devido ao desbalanço dos componentes presentes no mesmo (CHRISTOFOLETTI *et al.*, 2013). O CO_2 é resultante da queima do bagaço da cana-de-açúcar. Este resíduo [bagaço] é produzido em uma quantidade que varia entre 240 kg e 280 kg para cada tonelada de cana moída e é utilizado nas caldeiras a vapor para geração da energia necessária ao processamento da cana-de-açúcar. Com a queima, são gerados cerca de 9 kg de CO_2 por tonelada de cana-de-açúcar processada (LEITE, 2009).

2.4. Diversidade de microalgas

O termo microalgas se refere a um grupo taxonomicamente diverso de microrganismos fotossintetizantes com aproximadamente 8000 espécies descritas, podendo ser encontrados em ambientes aquáticos diversos, como oceanos e ambientes de água doce (ex. rios e lagos) (BECKER, 2011; STENGEL, 2011; GUIRY, 2012). Esses organismos produzem uma diversidade de componentes que apresentam interesse comercial, como proteínas,

carboidratos, pigmentos e lipídios. No entanto, o teor e as características desses componentes varia entre diferentes espécies. Por exemplo, espécies como *Spirulina maxima* e *Synechococcus sp.* apresentam um alto teor de proteínas (50-71%), podendo ser utilizadas como suplemento alimentar. Estas espécies, porém, não poderiam ser aplicadas à produção de etanol pois as mesmas apresentam um baixo teor de carboidratos (10-16%). Por outro lado, espécies como *Anabaena cylindrica Porphyridium cruentum* possuem teor de carboidrato elevado (25-57%), sendo assim interessantes para a produção de etanol (SPOLAORE, 2006).

Considerando a diversidade de microalgas, a seleção de uma linhagem de microalga que apresente uma boa produção do(s) produto(s) de interesse é de primordial importância para que haja êxito no estabelecimento de um sistema de produção comercial. O grupo das microalgas compreende um número muito grande de espécies que apresentam características produtivas muito distintas, portanto um processo de prospecção e seleção deve ser realizado. Este processo deverá levar em consideração diversos fatores, tais como o produto alvo, o sistema de cultivo, a capacidade de crescimento em condições adversas, a taxa de crescimento, a temperatura ótima de crescimento, entre outras características (SINGH e GU, 2010; MALCATA, 2011; LIM *et al.*, 2012).

3. JUSTIFICATIVA

Combustíveis derivados de petróleo são uma parte fundamental para a economia global, estando diretamente envolvidos na infraestrutura de transporte e também na geração de químicos industriais e produtos comerciais (THORNLEY e GILBERT, 2013). No entanto, devido à preocupação com a ocorrência de mudanças climáticas decorrente da queima de combustíveis fósseis, a insegurança do suprimento de petróleo a longo prazo e também da volatilidade do preço do petróleo tem conduzido ao desenvolvimento de políticas e programas para o desenvolvimento de alternativas a este problema, como o Programa Nacional do Álcool (Proálcool) no Brasil, que visou explorar o potencial do país para a produção etanol a partir da cana-de-açúcar (TIMILSINA, 2014).

Com o constante crescimento da demanda por combustíveis fósseis, biocombustíveis como o bioetanol e o biodiesel, têm se apresentado como uma promissora fonte energética alternativa (DEMIRBAS, 2009). Estes combustíveis apresentam como diferencial a facilidade de obtenção da matéria-prima e por apresentarem importante papel na manutenção do ciclo do dióxido de carbono. (DEMIRBAS, 2008; THORNLEY e GILBERT, 2013).

Em todo o mundo, o processo de produção dos denominados biocombustíveis de primeira geração é bem estabelecido. Esse sistema de produção utiliza cultivares comerciais como a cana-de-açúcar (*Saccharum spp.*), a beterraba (*Beta vulgaris*), a canola (*Brassica napus*), a soja (*Glycine max*), o trigo (*Triticum spp.)* e o milho (*Zea mays*). No entanto, devido a necessidade de utilização de uma extensa área de terra cultivável e o crescente aumento na demanda por alimentos, tem existido a preocupação de que haja um direcionamento do uso dessas plantas para a produção de biocombustíveis em

detrimento a produção alimentos (STEPHENS *et al.*, 2010). Devido a isto, uma nova geração de biocombustíveis que não necessita de áreas aráveis de terra tem sido desenvolvida. Essa geração utiliza matérias-primas lignocelulósicas de plantas como o S*orghum, Myscanthus e Camelina*, que não são utilizadas para na alimentação humana ou animal, não competindo diretamente com a produção de alimentos para a produção de biocombustíveis. No entanto, além de existir uma significativa limitação logística para a obtenção da biomassa lignocelulósica, eventualmente a competição por área para produção da biomassa conduziria a mesma discussão levantada pela produção de combustíveis de primeira geração (STEPHENS *et al.*, 2010; STEPHENSON *et al.*, 2011).

Frente a este problemática, a produção de biocombustíveis a partir de microalgas tem se mostrado como uma alternativa viável para a produção de biocombustíveis. A vantagem em se utilizar a biomassa microalgal para a produção de combustíveis é a sua alta produtividade e não necessidade de extensas áreas cultiváveis, apresentando pouco ou nenhum impacto na produção de alimentos e outros produtos agrícolas (CHISTI, 2008; MALCATA, 2011; SINGH *et al.*, 2011).

Considerando as características das microalgas, tem-se afirmado que biocombustíveis derivados de microalgas serão economicamente praticáveis em menos de uma década. No entanto, é necessário que haja melhora nas condições de mercado, como a redução dos custos de insumos, para que as microalgas sejam uma alternativa completamente competitiva (MALCATA, 2011). Para tal, o desenvolvimento da tecnologia de produção se torna imperativo para que o uso de microalgas como fonte enérgica se torne viável.

Desta forma, considerando o potencial bioenergético brasileiro, a sua biodiversidade e a importância estratégica deste setor no país, a realização de pesquisas voltadas para o desenvolvimento e inovação em produção de microalgas poderá garantir domínio tecnológico dessa importante fonte de novos produtos, contribuindo assim para a economia do país.

4. OBJETIVO GERAL

Obter, isolar e caracterizar, linhagens de microalgas obtidas a partir da biodiversidade brasileira com potencial para a produção de biomassa a partir de resíduos da indústria sucroalcooleira.

4.1. OBJETIVOS ESPECÍFICOS

4.1.1. Coletar, isolar e identificar linhagens de microalgas derivadas da biodiversidade brasileira;

4.1.2. Selecionar linhagens com potencial para crescimento em um sistema foto-autotrófico que utiliza vinhaça de cana-de-açúcar e CO_2 como insumos para o crescimento;

4.1.3. Caracterizar o crescimento de linhagens de microalgas selecionadas em meios de cultivo a base de vinhaça;

4.1.4. Cultivar linhagens de microalgas selecionadas em meios de cultivo a base de vinhaça suplementado com CO_2 em fotobiorreatores do tipo placa plana;

4.1.5. Avaliar a composição da biomassa obtida com o cultivo das microalgas em meios a base de vinhaça;

4.1.6. Caracterizar a composição dos meios a base de vinhaça antes e depois do cultivo das cepas selecionadas.

5. MATERIAIS E MÉTODOS

5.1. Coleta de amostras e isolamento de microalgas

Para a obtenção de microalgas com potencial para cultivo em vinhaça de cana-de-açúcar foram realizadas coletas de amostras de água em pontos com indícios da presença de microalgas em diversos ambientes brasileiros (figura 3) utilizando tubos tipo Falcon de 50 mL estéreis.

Figura 3. Mapa indicativo dos ambientes em que foram realizadas as coletas de amostras de água para o isolamento de microalgas.

Inicialmente foram realizadas a coletas de amostras de corpos de água doce ambientalmente conservados localizadas nos biomas Cerrado, Floresta Amazônica, Pantanal, além de efluente da indústria da cana-de-açúcar, lagoas de estabilização de esgoto rural e lagoas de piscicultura. A localização dos ambientes de coleta está apresentada na tabela 1.

Tabela 1. Localização geográfica dos ambientes de coleta de amostras de água para isolamento de microalgas.

Descrição do ambiente de coleta	Localização geográfica
Cerrado	15° 33’ 35.99’’ S / 48° 6’ 23.91’’ W
Floresta amazônica	1° 25’ 59.90’’ S / 48° 26’ 24.69’’ W
Pantanal	15° 33’ 35.99’’ S / 48° 6’ 23.91’’ W
Efluente de Indústria de cana-de-açúcar	15° 12’ 40.87’’ S / 48° 59’ 7.20’’ W
Lagoa de estabilização de esgoto rural	27° 18’ 37.94’’ S / 51° 59’ 31.28’’ W
Lagoas de psicultura	15° 74’ 00.70’’ S / 48° 19’ 67.03’’ W

As amostras de água coletadas (50 mL) foram centrifugadas a 2650 RCF por 3 minutos a 25°C e avaliadas quanto à presença de um pellet de coloração esverdeada. As amostras que se apresentaram positivas para a presença desse pellet tiveram 30 mL do sobrenadante descartado e o volume restante (20 mL) foi utilizado em um processo de enriquecimento em meios sintéticos quimicamente definidos. Para este processo, foi realizado o inóculo de 1 mL de cada umas das amostras em 9 mL de meio Bold’s Basal – BBM, pH 7.1 (Tabela 2) em tubos de ensaio com tampa semiabertos. As amostras foram cultivadas por 12 dias a 28 ºC ± 1 °C, sob regime de 16 horas de iluminação (intensidade de iluminação de 50 $\mu Em^{-2}\ s^{-1}$) e 8 horas de escuro para enriquecimento da amostra.

Tabela 2. Composição do meio Bold's Basal (BBM) descrito por Nichols e Bold (1965) utilizado para o cultivo de microalgas.

Macronutrientes		Micronutrientes	
Reagente	Concentração	Reagente	Concentração
$NaNO_3$	250 mg/L	$ZnSO_4$	8,82 mg/L
$CaCl_2$	25 mg/L	$NaNO_3$	1,44 mg/L
$MgSO_4.7H_2O$	75 mg/L	$MoNaSO_4.2H_2O$	0,71 mg/L
K_2HPO_4	75 mg/L	$Cl_2C_O.6H_2O$	1,57 mg/L
KH_2PO_4	175 mg/L	$CuSO_4$	0,49 mg/L
NaCl	25 mg/L		
EDTA	50 mg/L		
KOH	31 mg/L		
$FeSO_4.7H_2O$	4,98 mg/L		
H_2SO_4	1 μL/L		
H_3BO_3	11,42 mg/L		

Após 15 dias de crescimento das amostras em meio BBM, coletou-se 10μL das amostras para semeio por esgotamento em placas de petri contendo BBM-ágar (1,5%) suplementado com ampicilina (100 μg/mL), cloranfenicol (25 μg/mL) e anfotericina B (2,5 μg/mL) para inibição do crescimento de bactérias e fungos contaminantes. O cultivo foi realizado sob as mesmas condições descritas anteriormente. Após 20 dias de cultivo, colônias isoladas nas placas de petri foram coletadas e inseridas em 10 mL de meio BBM para gerar culturas axênicas. Ao final de 7 dias, as culturas foram avaliadas para a presença de contaminantes através de microscopia. O processo foi repetido nos casos em que as amostras não se mostraram axênicas. As culturas axênicas foram adicionadas à Coleção de Microrganismos e Microalgas Aplicados em Agroenergia e Biorrefinarias da EMBRAPA (Brasília, DF). O fluxograma do processo de isolamento pode ser verificado na Figura 4.

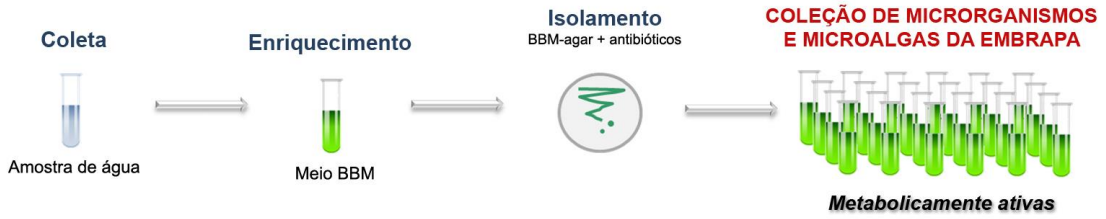

Figura 4. Fluxograma descrevendo o processo de isolamento de microalgas a partir de amostras de água coletadas utilizado neste trabalho.

Ao final do processo de isolamento, as cepas obtidas foram identificadas utilizando os marcadores moleculares Rbc*L* e *nu*ITS2, em associação à confirmação taxonômica (Hadi *et al.*, 2016).

5.2. Meios de cultivo a base de vinhaça

A vinhaça de cana-de-açúcar *in natura* foi cedida pela usina de etanol Jalles Machado (Goianésia, GO). A mesma teve o seu pH mensurado (4.5), foi autoclavada a 1 atm por 15 minutos e armazenada a 4 °C até o momento de sua utilização.

Para a realização do cultivo das microalgas, forma preparadas duas formulações para os meios a base de vinhaça: vinhaça diluída e vinhaça clarificada. Para o preparo da vinhaça diluída, a vinhaça bruta foi centrifugada a 4816 RCF por 10 minutos e o sobrenadante foi coletado e diluído em água destilada para as concentrações 25%, 50% e 75%. A vinhaça clarificada foi preparada a partir da adição de cal hidratada ($Ca(OH)_2$) à vinhaça bruta (3 g/L) (ARAÚJO, 2007). A solução foi mantida em repouso durante 40 minutos e posteriormente centrifugada a 4816 RCF por 10 minutos e o sobrenadante coletado. Para utilização em cultivo axênico, o pH dos meios a base de vinhaça

foi mantido em 4.5. Para o cultivo não-axênico, o pH foi ajustado para 8.0 com o objetivo de reduzir a taxa de crescimento de possíveis fungos filamentosos contaminantes. Ambos os meios foram esterilizados por autoclavagem durante 15 minutos a 1 atm. A transmitância de ambas as formulações da vinhaça foram analisadas por espectrofotometria (*Spectramax M3 plate analyzer*).

5.3. Seleção de microalgas para cultivo em vinhaça

As cepas isoladas Embrapa|LBA#1 a Embrapa|LBA#40 foram triadas para a sua capacidade de crescimento em vinhaça de cana-de-açúcar. Para isto, a vinhaça bruta foi diluída em água destilada nas concentrações de 25%, 50% e 75% e utilizada como meio de cultivo. Para esta seleção, todas as amostras foram inicialmente cultivadas em BBM durante 7 dias para preparo do inóculo. As amostras foram então centrifugadas a 2650 RCF por 3 minutos, o sobrenadante descartado e o pellet ressuspendido em 30 mL de água destilada estéril. Dez mililitros da amostra foram utilizados como inóculo em cada uma das diluições do meio à base de vinhaça. Ao cultivo foram adicionados ampicilina (100 μg/mL) e cloranfenicol (25 μg/mL). O cultivo foi realizado em triplicata durante 20 dias em frascos erlenmeyer estéreis aerados com injeção de ar atmosférico (5 L/h) a 26 °C ± 1 °C, sob ciclo de iluminação de 16/8 h (intensidade de luz de 106 $\mu Em^{-2} s^{-1}$) (Figura 5).

Figura 5. Frascos erlenmeyer com aeração utilizados para o cultivo de microalgas.

5.4. Cultivo de microalgas em vinhaça sob diferentes condições

As microalgas selecionadas, Embrapa|LBA32 e LBA40, foram submetidas ao cultivo e meio baseado em vinhaça sob diferentes condições. Para preparação do inóculo, as duas cepas foram cultivadas axenicamente em frascos erlenmeyer estéreis contendo BBM durante 7 dias a 26 °C ± 1 °C sob aeração com aeração (5 L/h de ar atmosférico), sob ciclo de iluminação de 12/12 h (intensidade de luz de 106 $\mu Em^{-2}\,s^{-1}$). Após o cultivo, o inóculo foi determinado como o volume necessário para que os meios a base de vinhaça apresentassem absorbância de 0,1 a 680 nm (*q.s.p..* 150 mL). As cepas Embrapa|LBA32 e LBA40 foram cultivadas em triplicata (n=3) durante 8 dias sob as seguintes condições: 1) Vinhaça bruta centrifugada, cultivo axênico, pH ajustado para 4,5, regime de iluminação de 12/12 h (intensidade de luz de 106 $\mu Em^{-2}\,s^{-1}$) e injeção de ar atmosférico (5 L/h). 2) Vinhaça bruta centrifugada, cultivo axênico, pH ajustado para 4,5, sem regime de iluminação de 12/12 h (intensidade de luz de 106 $\mu Em^{-2}\,s^{-1}$) e injeção de ar atmosférico (5 L/h). 3) Vinhaça bruta centrifugada,

cultivo não-axênico, pH ajustado para 8,0, regime de iluminação de 12/12 h (intensidade de luz de 106 $\mu Em^{-2}\ s^{-1}$) e injeção de ar atmosférico (5 L/h). 4) Vinhaça clarificada, cultivo não-axênico, pH ajustado para 8,0, regime de iluminação de 12/12 h (intensidade de luz de 106 $\mu Em^{-2}\ s^{-1}$) e injeção de ar atmosférico (5 L/h). Todos os frascos foram mantidos a 26 °C ± 1 °C.

5.5. Análise da presença de compostos orgânicos em meios à base de vinhaça após cultivo de microalgas

Para avaliar a presença de açúcares redutores totais (glicose, frutose e sacarose), glicerol, ácido lático e ácido acético na vinhaça antes e após o cultivo das cepas selecionadas, alíquotas de 10 mL das amostras cultivadas foram coletadas nos dias 0, 2, 4, 7 e 8 do cultivo. As mostras foram então centrifugadas a 10700 RCF por 10 minutos e 1 mL do sobrenadante foi utilizado para determinar açúcares redutores totais, glicerol, ácido lático e ácido acético através de cromatografia líquida de alta eficiência (Agilent 1260 Infinity Binary LC System) utilizando uma coluna adequada para a detecção de carboidratos e ácidos orgânicos (Biorad Aminex HPX-87H). A análise foi realizada com a temperatura da coluna a 45°C utilizando H_2SO_4 0.005M como fase móvel. A taxa de fluxo do eluente foi de 0,6 mL/min.

5.6. Cultivo de microalgas em fotobiorreatores de placa plana

Para realizar o preparo do inóculo para o cultivo em fotobiorreatores de placa plana, as cepas Embrapa|LBA#32 e Embrapa|LBA#40 foram cultivadas de maneira axênica em BBM durante 7 dias em frascos erlenmeyer com injeção de ar atmosférico (5 L/h), sob regime de iluminação de 12/12 h claro/escuro

(Intensidade de iluminação de 106 $\mu Em^{-2}\ s^{-1}$). Após o cultivo, o inóculo foi determinado como o volume necessário para que os meios a base de vinhaça apresentassem absorbância de 0,1 a 680 nm (*q.s.p.* 14 L). As cepas Embrapa|LBA32 e Embrapa|LBA40 foram cultivadas de maneira não-axênica em meios contendo vinhaça diluída (50%) e em vinhaça clarificada durante 3 dias em fotobiorreatores de placa plana.

O cultivo foi realizado em um sistema aerado (64 L/h de ar atmosférico) suplementado com 5% de CO_2 (3,2 L/h), sob regime de iluminação de 12/12 h claro/escuro (intensidade de iluminação de 422 $\mu Em^{-2}\ s^{-1}$) a 37 °C ±1 °C durante o período iluminado e 24 °C ±1 °C durante o período de ausência de luz. Como controle, foi realizado o cultivo das cepas selecionadas em BBM sob as mesmas condições descritas anteriormente.

5.7. Determinação do rendimento de biomassa

Para realizar a determinação do rendimento de biomassa das microalgas cultivadas, amostras de 10 mL foram coletadas em triplicata (n=3), centrifugadas por 10 minutos a 10700 RCF e o sobrenadante foi descartado. O pellet formado foi lavado através de três ciclos de ressuspensão em água destilada estéril por centrifugação a 10700 RCF por 10 minutos. O pellet lavado foi seco em estufa de secagem a 105 °C e posteriormente pesado para determinação da biomassa produzida.

5.8. Análise de composição química da vinhaça

Para analisar a vinhaça após o cultivo das cepas selecionadas em fotobiorreatores, a cultura foi centrifugada a 4816 RCF durante 10 minutos e o

sobrenadante foi analisado quanto a sua composição química. Análise da Demanda Química de Oxigênio (DQO), Demanda Bioquímica de Oxigênio (DBO), fosfato, potássio total, carbono orgânico total, pH e turbidez foram realizadas pelo Laboratório Ambientale (Maringá, PR) utilizando procedimentos padrão descritos na tabela 2. Para a determinação do nitrogênio total presente nas amostras, 10 mL do material foi seco em estufa de secagem a 105°C e o material obtido foi analisado utilizando o analisador elementar Perkin Elmer 2400 Series II CHNS/O. Como controle, foi realizada a análise dos parâmetros supracitados na vinhaça bruta, na vinhaça diluída (50%) e na vinhaça clarificada.

Tabela 3. Metodologias utilizadas para a determinação dos parâmetros químicos na vinhaça e em meios a base de vinhaça, antes e após o cultivo das microalgaas.

Parâmetro	Procedimento
DQO	QAM.IT.FQ.16A
DBO	SM 5210 B
Fosfato	SM 4500-P E
Potássio total	SM 3500-K B
Nitrato	ABNT NBR 12620:1992
Nitrito	SM 4500-NO2-B
Nitrogênio amoniacal	SM 4500-NH3 F
Carbono orgânico total	SM 4500-O/D
pH	SM 4500-H+ B
Turbidez	SM 2130 B

Water Environmental Federation and American Public Health Association (2012).
Associação Brasileira de Normas Técnicas (ABNT), Norma Brasileira (NBR).

5.9. Análise de composição da biomassa microalgal

Para caracterização da biomassa obtida com o cultivo das cepas selecionadas, o pellet resultante da centrifugação foi lavado por três vezes com água destilada utilizando centrifugação a 4861 RCF durante 10 minutos. A biomassa obtida foi mantida por 2 horas a -80°C e posteriormente foi liofilizada *overnight*. O material foi analisado quanto ao teor de cinzas, proteínas totais, carboidratos totais, carotenoides totais e ésteres metílicos de ácidos graxos. Para o teor total de cinzas, foi aplicada a metodologia proposta por Van Wychen e Laurens (2013c). Proteínas totais foram determinadas com o uso do método Kjeldahl proposto pelo AOAC (1990), utilizando o fator de conversão de nitrogênio para proteínas proposto para microalgas (LOURENÇO *et al.*, 2004). Para a determinação dos carboidratos totais, foi utilizada a metodologia proposta por Van Wychen e Laurens (2013a). Para carotenoides totais, a análise foi realizada utilizando o protocolo descrito por Porra e colaboradores (1989) e adaptado por Huang e Cheung (2011), utilizando acetona 90% como solução de extração. Os ésteres metílicos de ácidos graxos presentes na biomassa foram determinados através do protocolo descrito por Van Wychen e Laurens (2013b).

6. REFERÊNCIAS BIBLIOGRÁFICAS

ADARME-VEGA, T. C. et al. Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. **Microb Cell Fact,** v. 11, p. 96, 2012.

ARAÚJO, F. D. Processo de clarificação do caldo de cana pelo método da bicarbonatação. **Revista Ciências e Tecnologia. Ano,** v. 1, p. 1-5, 2007.

BAHADAR, A.; BILAL KHAN, M. Progress in energy from microalgae: A review. **Renewable and Sustainable Energy Reviews,** v. 27, n. 0, p. 128-148, 2013.

CAVALETT, O. et al. Environmental and economic assessment of sugarcane first generation biorefineries in Brazil. **Clean Technologies and Environmental Policy,** v. 14, n. 3, p. 399-410, 2011.

CHISTI, Y. Biodiesel from microalgae. **Biotechnology advances,** v. 25, n. 3, p. 294-306, 2007.

_____. Biodiesel from microalgae beats bioethanol. **Trends in Biotechnology,** v. 26, n. 3, p. 126-131, 2008.

CHRISTOFOLETTI, C. A. et al. Sugarcane vinasse: environmental implications of its use. **Waste management,** v. 33, n. 12, p. 2752-2761, 2013.

DE LA NOUE, J.; DE PAUW, N. The potential of microalgal biotechnology: A review of production and uses of microalgae. **Biotechnology Advances,** v. 6, n. 4, p. 725-770, 1988.

DEMIRBAS, A. Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. **Energy Conversion and Management,** v. 49, n. 8, p. 2106-2116, 2008.

_____. Biofuels securing the planet's future energy needs. **Energy Conversion and Management,** v. 50, n. 9, p. 2239-2249, 2009.

DIAS, M. O. S. et al. Improving bioethanol production - Comparison between extractive and low temperature fermentation. **Applied Energy,** v. 98, p. 548-555, 2012.

DIAS, M. O. S. et al. Sugarcane processing for ethanol and sugar in Brazil. **Environmental Development**, 2015.

ESPAÑA-GAMBOA, E. et al. Vinasses: characterization and treatments. **Waste Management & Research,** v. 29, n. 12, p. 1235-1250, 2011.

FEDERATION, W. E.; ASSOCIATION, A. P. H. **Standard methods for the examination of water and wastewater**. Washington, DC: Charles Darwin House, 2012. 1496p.

GHASEMI, Y. et al. Microalgae biofuel potentials (Review). **Applied Biochemistry and Microbiology,** v. 48, n. 2, p. 126-144, 2012.

GONG, Y. et al. Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects. **Journal of industrial microbiology & biotechnology,** v. 38, n. 12, p. 1879-1890, 2011.

GOUVEIA, L.; OLIVEIRA, A. C. Microalgae as a raw material for biofuels production. **Journal of industrial microbiology & biotechnology,** v. 36, n. 2, p. 269-274, 2009.

GOUVEIA, L. et al. Chlorella vulgaris and Haematococcus pluvialis biomass as colouring and antioxidant in food emulsions. **European Food Research and Technology,** v. 222, n. 3-4, p. 362-367, 2006.

HADI, S. I. I. A. et al. DNA Barcoding Green Microalgae Isolated from Neotropical Inland Waters. **PloS one,** v. 11, n. 2, p. e0149284, 2016.

HUANG, J. J.; CHEUNG, P. C. Enhancement of polyunsaturated fatty acids and total carotenoid production in microalgae by ultraviolet band A (UVA, 365 nm) radiation. **Journal of agricultural and food chemistry,** v. 59, n. 9, p. 4629-4636, 2011.

KANG, Z. et al. A cost analysis of microalgal biomass and biodiesel production in open raceways treating municipal wastewater and under optimum light wavelength. **Journal of microbiology and biotechnology,** v. 25, n. 1, p. 109-118, 2015.

LARKUM, A. W. D. et al. Selection, breeding and engineering of microalgae for bioenergy and biofuel production. **Trends in Biotechnology,** v. 30, n. 4, p. 198-205, 2012.

LEITE, R. **Bioetanol combustível: uma oportunidade para o Brasil**. Brasília, DF: Centro de Gestão de Estudos Estratégicos–CGEE, 2009. 536 p. ISBN 978-85-60755-15-8.

LI, Y. et al. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. **Applied microbiology and biotechnology,** v. 81, n. 4, p. 629-636, 2008.

LI, Y. et al. Biofuels from microalgae. **Biotechnology progress,** v. 24, n. 4, p. 815-820, 2008.

LIM, D. K. et al. Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters. **PloS One,** v. 7, n. 7, p. e40751, 2012.

LOURENÇO, S. O. et al. Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. **European Journal of Phycology,** v. 39, n. 1, p. 17-32, 2004.

MALCATA, F. X. Microalgae and biofuels: A promising partnership? **Trends in Biotechnology,** v. 29, n. 11, p. 542-549, 2011.

MATA, T. M.; MARTINS, A. A.; CAETANO, N. S. Microalgae for biodiesel production and other applications: A review. **Renewable and Sustainable Energy Reviews,** v. 14, n. 1, p. 217-232, 2010.

MÜLLER-LANGER, F.; MAJER, S.; O'KEEFFE, S. Benchmarking biofuels—a comparison of technical, economic and environmental indicators. **Energy, Sustainability and Society,** v. 4, n. 1, p. 1-14, 2014.

NICHOLS, H. W.; BOLD, H. C. Trichosarcina polymorpha gen. et sp. nov. **Journal of Phycology,** v. 1, n. 1, p. 34-38, 1965.

ORTEGÓN, G. P. et al. Vinasse application to sugar cane fields. Effect on the unsaturated zone and groundwater at Valle del Cauca (Colombia). **Science of The Total Environment,** v. 539, p. 410-419, 2016.

PARMAR, A. et al. Cyanobacteria and microalgae: A positive prospect for biofuels. **Bioresource Technology,** v. 102, n. 22, p. 10163-10172, 2011.

PARNAUDEAU, V. et al. Vinasse organic matter quality and mineralization potential, as influenced by raw material, fermentation and concentration processes. **Bioresource Technology,** v. 99, n. 6, p. 1553-1562, 2008.

PEREZ-GARCIA, O. et al. Heterotrophic cultures of microalgae: Metabolism and potential products. **Water research,** v. 45, n. 1, p. 11-36, 2011.

PORRA, R.; THOMPSON, W.; KRIEDEMANN, P. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. **Biochimica et Biophysica Acta (BBA)-Bioenergetics,** v. 975, n. 3, p. 384-394, 1989.

PRATHIMA DEVI, M.; VENKATA SUBHASH, G.; VENKATA MOHAN, S. Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation. **Renewable Energy,** v. 43, n. 0, p. 276-283, 2012.

QUINN, J. C.; DAVIS, R. The potentials and challenges of algae based biofuels: A review of the techno-economic, life cycle, and resource assessment modeling. **Bioresource Technology,** v. 184, p. 444-452, 2015.

RAZEGHIFARD, R. Algal biofuels. **Photosynthesis research,** v. 117, n. 1-3, p. 207-219, 2013.

RAZZAK, S. A. et al. Integrated CO2 capture, wastewater treatment and biofuel production by microalgae culturing—A review. **Renewable and Sustainable Energy Reviews,** v. 27, n. 0, p. 622-653, 2013.

SCOTT, S. A. et al. Biodiesel from algae: challenges and prospects. **Current Opinion in Biotechnology,** v. 21, n. 3, p. 277-86, 2010.

SHARMA, Y.; SINGH, B. Development of biodiesel: current scenario. **Renewable and Sustainable Energy Reviews,** v. 13, n. 6, p. 1646-1651, 2009.

SINGH, A.; NIGAM, P. S.; MURPHY, J. D. Renewable fuels from algae: an answer to debatable land based fuels. **Bioresource Technology,** v. 102, n. 1, p. 10-16, 2011.

SINGH, J.; GU, S. Commercialization potential of microalgae for biofuels production. **Renewable and Sustainable Energy Reviews,** v. 14, n. 9, p. 2596-2610, 2010.

SPOLAORE, P. et al. Commercial applications of microalgae. **Journal of Bioscience and Bioengineering,** v. 101, n. 2, p. 87-96, 2006.

STEPHENS, E. et al. Future prospects of microalgal biofuel production systems. **Trends in Plant Science,** v. 15, n. 10, p. 554-564, 2010.

STEPHENSON, P. G. et al. Improving photosynthesis for algal biofuels: toward a green revolution. **Trends in Biotechnology,** v. 29, n. 12, p. 615-623, 2011.

THORNLEY, P.; GILBERT, P. Biofuels: balancing risks and rewards. **Interface Focus,** v. 3, n. 1, p. 20120040, 2013.

TIMILSINA, G. R. Biofuels in the long-run global energy supply mix for transportation. **Philos Trans A Math Phys Eng Sci,** v. 372, n. 2006, p. 20120323, 2014.

VAN WYCHEN, S.; LAURENS, L. Determination of Total Carbohydrates in Algal Biomass. **NREL Laboratory Analytical Procedure**, 2013a. Disponível em: < http://www.nrel.gov/docs/fy14osti/60957.pdf >. Acesso em: 01-15-2016.

_____. Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification. **NREL Laboratory Analytical Procedure**, 2013b. Disponível em: < http://www.nrel.gov/docs/fy14osti/60958.pdf >. Acesso em: 01-15-2016.

_____. Determination of Total Solids and Ash in Algal Biomass. **NREL Laboratory Analytical Procedure**, 2013c. Disponível em: < http://www.nrel.gov/docs/fy14osti/60956.pdf >. Acesso em: 01-15-2016.

YEN, H.-W. et al. Microalgae-based biorefinery – From biofuels to natural products. **Bioresource Technology,** v. 135, n. 0, p. 166-174, 2013.

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[1]De acordo com a Associação Brasileira de Normas Técnicas. NBR 6023/2002.

7. CAPÍTULO I

DNA barcoding green microalgae isolated from neotropical inland waters

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Abstract

This study evaluated the feasibility of using the Ribulose Bisphosphate Carboxylase Large subunit gene (*rbc*L) and the Internal Transcribed Spacers 1 and 2 of the nuclear rDNA (*nu*ITS1 and *nu*ITS2) markers for identifying a very diverse, albeit poorly known group, of green microalgae from neotropical inland waters. Fifty-one freshwater green microalgae strains isolated from Brazil, the largest biodiversity reservoir in the neotropics, were submitted to DNA barcoding. Currently available universal primers for ITS1-5.8S-ITS2 region amplification were sufficient to successfully amplify and sequence 47 (92%) of the samples. On the other hand, new sets of primers had to be designed for *rbc*L, which allowed 96% of the samples to be sequenced. Thirty-five percent of the strains could be unambiguously identified to the species level based either on *nu*ITS1 or *nu*ITS2 sequences' using barcode gap calculations. *nu*ITS2 Compensatory Base Change (CBC) and ITS1-5.8S-ITS2 region phylogenetic analysis, together with morphological inspection, confirmed the identification accuracy. In contrast, only 6% of the strains could be assigned to the correct species based solely on *rbc*L sequences. In conclusion, the data presented here indicates that either *nu*ITS1 or *nu*ITS2 are useful markers for DNA barcoding of freshwater green microalgae, with advantage for *nu*ITS2 due to the larger availability of analytical tools and reference barcodes deposited at databases for this marker.

Key words: *nu*ITS1; *nu*ITS2; *rbc*L; Chlorophyta; tropical freshwater microalgae; compensatory base change; barcode gap.

Introduction

DNA barcoding is a method used for species identification, which identifies specimens based on DNA sequence similarity against a sequence database of *a priori* defined species. This powerful technique has brought significant improvements to applications such as taxonomy [2-4], ecology [5,6], biosecurity [7-9] and food product regulation [10-12]. DNA-based identification is particularly useful for unveiling cryptic diversity at various taxonomic levels and identifying species where there are few or difficult to observe structural characters [13-17].

The green algae, Chlorophyta, are an ancient and taxonomically diverse lineage with approximately 8,000 described species [18,19]. It is estimated that at least 5,000 species still remain undescribed, notably in tropical and subtropical areas [19]. Chlorophytes are important producers in aquatic and humid terrestrial ecosystems, which are often used as bioindicators in water monitoring and ecological studies [20,21]. In addition, there is a growing interest in using green microalgae for biotechnological applications such as the production of fuels, chemicals, food and animal feed [22,23]. The identification of green microalgae can be a difficult task and often requires careful microscopic examination of live cultured cells by a trained specialist [14,24,25]. Even so, the presence of cryptic species and phenotypic plasticity found in some species may hamper conclusive morphologic species diagnosis [26,27]. DNA barcodes could provide the means to identify green microalgae consistently and rapidly, regardless of life stage [13,28,29].

Targets for potential Chlorophyta DNA barcodes have included chloroplast (*rbc*L, *tuf*A and Cp23S), mitochondrial (COI) and nuclear genes (18S rDNA, *nu*ITS1 and *nu*ITS2) [13,28-30]. However, none of these markers were considered ideal for use across all lineages tested [13,29,31,32]. Given the complexity and heterogeneity of chlorophytes, the protist working group of the Consortium for the Barcode of Life (CBOL) recommended the use of a two-step barcoding pipeline in which a universal pre-barcode marker should be used first, followed by the use of a group-specific second barcode [29]. A dual marker barcode based on *mat*K and *rbc*L genes has been formally proposed for use in DNA barcoding embryophytes [4]. However, the *mat*K gene is absent in

chlorophytes precluding its use in this group [33]. Despite the unavailability of a universal PCR toolkit for *rbc*L amplification, this marker is considered a promising barcode for green algae [13]. Indeed, there are currently 4,449 *rbc*L sequences from chlorophyte species deposited at the Barcode of Life Data Systems (BOLD), a taxonomically curated database [3]. Apart from *rbc*L, the most promising candidates for green microalgae barcoding are the *nu*ITS1 and *nu*ITS2 markers [13,14,26,28,30,34]. The ITS1-5.8S-ITS2 region from virtually all Viridiplantae can be amplified with a single set of universal primers [35], despite these being markers of high variability [13]. Furthermore, it is possible to analyze not only the *nu*ITS1 and *nu*ITS2 primary sequence, but also their secondary structures [36]. Although there are reports indicating that *nu*ITS1 and *nu*ITS2 might be insufficiently conserved or confounded by introgression or biparental inheritance patterns, a growing body of evidence has shown that simultaneous analysis of nucleotide data and compensatory base changes (CBCs) with secondary structure information can overcome most of the limitations of this potential barcode [14,28,30]. In addition, *nu*ITS1 and *nu*ITS2 have been the molecular markers of choice in several recent taxonomic revisions of freshwater chlorophytes species that were based on integrated morphological, physiological and molecular approaches [14,26,27,34,37-42] The use of *nu*ITS1- and *nu*ITS2-based phylogenies promoted considerable changes in green microalgae taxonomy, especially in taxa with simple morphology and few ultrastructural characteristics such as coccoid chlorophytes [26,27].

This study aimed to identify neotropic green microalgae specimens isolated from Brazilian inland waters through the use of *rbc*L, *nu*ITS1 and *nu*ITS2 molecular markers as DNA barcodes. Brazilian continental waters comprise a biodiversity reservoir of enormous global significance and might contain up to 25% of the world's algae species [43]. Novel primers for neotropic specimens' *rbc*L gene amplification and sequencing are presented, as well as comparisons between *rbc*L, *nu*ITS1 and *nu*ITS2 markers variability, primers universality and databases accuracy and comprehensiveness.

Materials and Methods

Isolation and culturing

All the sample collections were made under the authorization SISBIO #39146 (09/26/2013) conceded by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) of the Brazillian Ministry of the Environment (MMA). The collections made on private land were also authorized by the owner of the land. This study did not involve endangered or protected species. Water samples were collected from the sites shown in S1 Fig. The collection environments included natural freshwater bodies within the Amazon rainforest, the Cerrado savanna and the Pantanal flooded grasslands, as well as anthropogenic wastewater deposits from the sugarcane industry (vinasse), pisciculture ponds and wastewater from swine farming. Sampling areas were delimited as being a 1 km radius centered in the geographic coordinates shown in S1 Fig. The collected environmental samples were submitted to an enrichment step through suspension in modified Bold's Basal Medium – BBM [44] and subsequent culturing at 28º C, light intensity of 50 μEm^{-2} s^{-1} and 16/8h light/dark regime. After 15 days of culture, the microalgae strains were isolated by two subsequent rounds of subculturing on BBM agar plates supplemented with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml) and amphotericin B (2,5 µg/ml) under the same conditions described above. Individualized macroscopic colonies on agar plates were collected and inoculated into liquid BBM media to derive axenic cultures. The absence of contaminants was confirmed through microscopic inspection. The isolated strains were deposited in the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries at Embrapa (Brasília/DF – Brazil).

DNA extraction, amplification and sequencing

Total genomic DNA was isolated from 30 mg of fresh algal biomass using the Cetyl Trimethylammonium Bromide (CTAB) DNA extraction protocol adapted by [45]. The *rbc*L and ITS1-5.8S-ITS2 DNA regions were submitted to PCR amplification using the primers described in Table 1. The 25 µL PCR reaction mix was composed of 14.5 µL of ultrapure water, 5 µL of GoTaq 5X PCR buffer, 1.5

µL $MgCl_2$ 25 mM, 0.75 µL BSA 10 mg/mL, 0.5 µL dNTPs 10 mM, 0.25 µL of GoTaq DNA polymerase (5 U/µL) (Promega, USA), 0.25 µL of each primer (10 µM) and 2.0 µl of DNA template (50–100 ng/µL). The PCR amplification protocol used for both markers was: 96ºC for 5 min, 40 cycles of 96º C for 1 min, (primer annealing temperature - see Table 1) for 1 min and 72ºC for 1 min, with a final extension at 72º C for 5 min. The PCR products (5 µL) were visualized on agarose gels and selected for direct sequencing. Sequences were determined bi-directionally for at least two different amplicons using the BigDye Terminator v.3.1 Cycle Sequencing Kit on the ABI 3130 automated DNA sequencer (both from Life Technologies, USA), in accordance with the manufacturer's instructions. The forward and reverse sequences were aligned and edited using Geneious 6.1 software [46], generating consensus nucleotide positions with QV ≥ 20. Sequences were deposited in GenBank under the accession numbers: *rbc*L sequences (KT307991 to KT308039); ITS1-5.8S-ITS2 sequences (KT308040 to KT308042; KT308046 to KT308076; KT308078 to KT308086; KT445859 to KT445863).

Table 1. List of primers used in this study, including the primer sequences, amplicon length, annealing temperature and the sequencing success rate for a total of 51 strains tested.

Primer pair	Molecular marker	Sequence	Amplicon length (Nucleotides span)	Annealing temperature	Sequencing success rate	Reference
Fw_ITS1/Rv_ITS4	ITS1-5.8S-ITS2	Fw_ITS1: 5’ – AGGAGAAGTCGTAACAAGGT – 3’ Rv_ITS4: 5’ – TCCTCCGCTTATTGATATGC – 3’	≈ 650 pb	52 °C	92,15%	[35]
Fw_rbcL_192/Rv_rbcL_657	rbcL	Fw_rbcL_192: 5’ – GGTACTTGGACAACWGTWTGGAC – 3’ Rv_rbcL_657: 5’ – GAAACGGTCTCKCCARCGCAT – 3’	465 pb (position 192 to 657)	52 °C	82,35%	This study
Fw_rbcL_375/Rv_rbcL_1089	rbcL	Fw_rbcL_375: 5’ – TTTGGTTTCAAAGCIYTWCGTGC – 3’ Rv_rbcL_1089: 5’ – ATACCACGRCTACGRTCTTT – 3’	714 pb (position 375 to 1089)	52 °C	50,98%	This study
Fw_rbcL_192/Rv_rbcL_1089	rbcL	Fw_rbcL_192: 5’ – GGTACTTGGACAACWGTWTGGAC – 3’ Rv_rbcL_1089: 5’ – ATACCACGRCTACGRTCTTT – 3’	897 pb (position 192 to 1089)	52 °C	37,25%	This study
Fw_rbcLa_f/Rv_rbcL_ajf634R	rbcL	Fw_rbcLa_f 5’ – ATGTCACCACAAACAGAAACTAAAGC – 3’ Rv_rbcL_ajf634R: 5’ – GAAACGGTCTCTCCAACGCAT – 3’	654 pb (position 1 to 654)	54 °C	15,69%	[4]
Fw_rbcL_109/Rv_rbcL_657	rbcL	Fw_rbcL_109: 5’ – TTCTTGCTGCITTYCGTATG – 3’ Rv_rbcL_657: 5’ – GAAACGGTCTCKCCARCGCAT – 3’	548 pb (position 109 to 657)	52 °C	13,75%	This study
Fw_rbcLa_f/rbcLA_rev	rbcL	Fw_rbcLa_f: 5’ – ATGTCACCACAAACAGAGACTAAAGC – 3’ rbcLA_rev: 5’ – GTAAAATCAAGTCCACCRCG – 3’	599 pb (position 1 to 599)	54 °C	7,84%	[4]
Fw_rbcL_109/Rv_rbcL_1089	rbcL	Fw_rbcL_109: 5’ – TTCTTGCTGCITTYCGTATG – 3’ Rv_rbcL_1089: 5’ – ATACCACGRCTACGRTCTTT – 3’	980 pb (position 109 to 1089)	52 °C	1,96%	This study
Fw_rbcL_RH1/rbcL_724R	rbcL	Fw_rbcL_RH1: 5’ – ATGTCACCACAAACAGAAACTAAAGC – 3’ rbcL_724R: 5’ – TCGCATGTACCTGCAGTAGC – 3’	743 pb (position 1 to 743)	54 °C	1,96%	[4]
Fw_rbcL_RH1/rbcL_1385R	rbcL	Fw_rbcL_RH1: 5’ – ATGTCACCACAAACAGAAACTAAAGC – 3’ rbcL_1385R: 5’ – AATTCAAATTTAATTTCTTTCC – 3’	1406 pb (position 1 to 1406)	48 °C	0%	[13]

Molecular data analysis

Sequences were aligned automatically using ClustalW [47] under default parameters using MEGA5 software [48]. The *nu*ITS1, 5.8S and *nu*ITS2 sequences were annotated using ITSx v. 1.0.11 [49]. For similarity searches, the *rbc*L sequences were submitted to the Barcode of Life Data Systems (BOLD systems) using the *Plant identification* tool, while *nu*ITS2 sequences were submitted to the Basic Local Alignment Search Tool (BLASTN) for comparisons against nucleotide sequences deposited at the Genbank. The *nu*ITS2 secondary structures were predicted by either direct fold (energy minimization) or homology modelling [50]. Subsequently, in order to locate hemi-compensatory base changes (hemi-CBCs) and compensatory base changes (CBCs), each sequence-structure along with its top match on *ITS2 Blast* tool were aligned and analyzed with 4SALE v. 1.7 [51,52].

The barcode gap was inferred based on uncorrected pair–wise (*p*) distance matrices. MEGA5 software was used for calculation. The taxon samplings used were reference *nu*ITS1, *nu*ITS2 and *rbc*L sequences derived from recent taxonomic revisions of the *Chlorella* and *Desmodesmus* genera [14,53,54] (S1-S3 Tables). The maximum intraspecific distances and minimum interspecific distances obtained were computed.

For phylogenetic tree analysis, the ITS1-5.8S-ITS2 sequences from Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-44 and #50 strains were included in the dataset together with their respectively closest sequences at GenBank. *Desmodesmus* sp., *Chlorella* sp. and *Micractinium* sp. ITS1-5.8S-ITS2 reference sequences [14,39,53-55]. The dendrograms were constructed through the maximum likelihood (ML) method using MEGA5 software. The GTR model with invariable sites (I) and gamma distribution shape parameter (G) was chosen. The neighbor-joining (NJ) algorithm was used to generate the initial tree for ML computation. A phylogenetic test using the Bootstrap method (1,000 replicates) was used.

Morphologic Identification

Microscopic morphologic identification at the genus level was performed according to Bellinger & Sigee, 2015 [56]. Further identification to species levels was accomplished by comparison with the species original descriptions that are available at the AlgaeBase [57]. In the case of the as of yet undescribed species, the morphological comparisons were made with the closest strains obtained in the molecular identification step: *Desmodesmus* sp. MAT2008c [58]; *Micractinium* sp. CCAP 211/92 [39]; *Desmodesmus* sp. GM4a [59]. A Carl Zeiss Axio Imager A2 microscope (Zeiss.co, Brazil) equipped with Differential Interference Contrast (DIC) was used for morphological analysis.

Results

Barcode markers primer universality

A total of 51 unialgal strains (named Embrapa|LBA#1 to #51) were isolated from natural water bodies within the Cerrado savanna, the Pantanal wetlands and the Amazon rainforest, as well as anthropogenic wastewater deposits (S1 Fig). Coccoid morphotypes were the most abundant among the isolated strains (51%), followed by monadoids/palmelloids morphotypes (41%) (data not shown).

The ITS1-5.8S-ITS2 region could be successfully sequenced from DNA samples extracted from 47 strains (92,15% sequencing success rate) by using the universal primers described by White and coworkers (1990) [35] (Table 1). Even though all the 51 samples could be amplified with this set of primers, the presence of multiple PCR products impaired direct sequencing of four samples. On the other hand, the sequencing success rate obtained using the *rbc*L gene universal primer sets described by Hall and coworkers (2010) [13] or the sets proposed for embryophytes by the CBOL Plant working group [4], ranged from 0% to 15,69% (Table 1). In order to circumvent this problem, new sets of primers targeting *rbc*L gene partial amplification (Table 1) were designed based on 175 *rbc*L reference sequences from distinct Chlorophyta taxa mined from BOLD Systems. The newly designed primer pairs *Fw_rbcL_192/Rv_rbcL_657* and *Fw_rbcL_357/Rv_rbcL-1089* could successfully amplify and sequence 82,35% and 50,98% of the dataset, respectively (Table 1). The combination of the sequencing results from both these *rbc*L primer pairs allowed the construction of quality consensus sequences (QV≥20) for 49 samples (96,08% sequencing success rate). A total of 18 distinct 5.8S genotypes, 23 distinct *nu*ITS1 genotypes, 23 *nu*ITS2 distinct genotypes and 26 distinct *rbc*L genotypes were obtained.

Similarity search based on *nu*ITS1, *nu*ITS2 and *rbc*L markers

In order to perform the molecular identification of Embrapa|LBA strains, the *rbc*L sequences obtained were submitted to similarity searches against the DNA

barcoding dedicated database, BOLD systems. The closest matches retrieved for *rbc*L sequences ranged from 90% to 99% of similarity (Table 2). Currently, there are very few *nu*ITS1 and *nu*ITS2 sequences from chlorophytes deposited at taxonomically curated databases such as BOLD, therefore similarity searches were performed against the GenBank. The closest matches retrieved for *nu*ITS1 sequences ranged from 70% to 100% of similarity and for *nu*ITS2 sequences ranged from 81% to 100% of similarity (Table 2). Embrapa|LBA strains retrieved matches from species that belong to the Chlorophyceae and Trebouxiophyceae classes, especially to the orders Chlamydomonadales, Chlorococcales, Sphaeropleales and Chlorellales (Table 2). Ten *nu*ITS1 sequences, 14 *nu*ITS2 sequences and 0 *rbc*L sequences retrieved matches with a 100% similarity (Table 2).

Table 2. Molecular identification of the strains used in this study, including the percentual of identity, accession number and the name of the identified species on the Barcode of Life Database (based on *rbc*L marker sequence) and GenBank (based on *nu*ITS2 marker sequence).

ITS1 (GenBank)			ITS2 (GenBank)				*rbc*L (BOLD)			
Strain	**Closest match species**	**Identity**	**GenBank access**	**Closest match species**	**Identity**	**Number of CBCs / hCBCs**	**GenBank access**	**Closest match species**	**Identity**	**GenBank access**
LBA#1	*Desmodesmus armatus*	95%	KP281288.1	*Desmodesmus bicellularis*	91%	1 / 7	AB917134.1	*Scenedesmus quadricauda*	90%	AB084332.1
LBA#2	*Desmodesmus* sp. MAT-2008c	100%	EU502836.1	*Desmodesmus* sp. MAT-2008c	100%	0 / 0	EU502836.1	*Acutodesmus obliquus*	93%	DQ396875.1
LBA#3	*Desmodesmus* sp. MAT-2008c	100%	EU502836.1	*Desmodesmus* sp. MAT-2008c	100%	0 / 0	EU502836.1	*Acutodesmus obliquus*	90%	DQ396875.1
LBA#4	*Chlamydopodium starrii*	70%	AB983644.1	*Chlorococcum oleofaciens*	91%	1 / 2	AB983633.1	*Chlorococcum ellipsoideum*	91%	EF113431.1
LBA#5	*Desmodesmus* sp. Tow 10/11 T-12W	79%	DQ417556.1	*Desmodesmus regularis*	84%	4 / 2	AM228924.1	*Desmodesmus santosii*	93%	GU192417.1
LBA#6	*Chlamydopodium starrii*	70%	AB983644.1	*Chlorococcum oleofaciens*	94%	-	AB983633.1	*Chlorococcum ellipsoideum*	91%	EF113431.1
LBA#7	*Desmodesmus* sp. Tow 10/11 T-12W	79%	DQ417556.1	*Desmodesmus regularis*	84%	4 / 2	AM228924.1	*Desmodesmus santosii*	93%	GU192417.1
LBA#8	*Chlamydomonas* sp. KU107	94%	KM061447.1	*Chlamydomonas* sp. KU107	87%	0 / 1	KM061447.1	*Chlamydomonas oblonga*	95%	EF113424.1
LBA#9	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#10	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#11	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1

LBA#12	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	_	-	-
LBA#13	*Coelastrella* sp. shy-188	96%	KP702302.1	*Scenedesmus rubescens*	95%	0 / 2	JX513884.1	*Scenedesmus quadricauda*	90%	AB084332.1
LBA#14	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#15	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#16	-	-	-	-	-	-	-	*Ecballocystopsis dichotomus*	90%	JX018187.1
LBA#17	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#18	*Chlamydopodium starrii*	90%	AB983644.1	*Chlamydopodium starrii*	93%	0 / 1	AB983644.1	*Chlorococcum ellipsoideum*	92%	KC810301.1
LBA#19	-	-	-	-	-	-	-	*Ecballocystopsis dichotomus*	90%	JX018187.1
LBA#20	*Coelastrum astroideum*	76%	GQ375093.1	*Scenedesmus arcuatus*	81%	0 / 6	AY170855.1	*Hariotina reticulata*	93%	JQ394815.1
LBA#21	*Coelastrella* sp. shy-188	96%	KP702302.1	*Scenedesmus rubescens*	95%	0 / 2	JX513884.1	*Desmodesmus costato-granulatus*	94%	GU192427.1
LBA#22	*Desmodesmus ultrasquamatus*	100%	GU192392.1	*Desmodesmus ultrasquamatus*	99%	0 / 0	GU192392.1	*Desmodesmus costato-granulatus*	93%	GU192427.1
LBA#23	*Desmodesmus ultrasquamatus*	100%	GU192392.1	*Desmodesmus ultrasquamatus*	99%	0 / 0	GU192392.1	*Desmodesmus costato-granulatus*	94%	GU192427.1
LBA#24	*Desmodesmus ultrasquamatus*	94%	GU192392.1	*Desmodesmus ultrasquamatus*	94%	0 / 3	AM228926.1	*Desmodesmus costato-granulatus*	94%	GU192427.1
LBA#25	*Desmodesmus ultrasquamatus*	94%	GU192392.1	*Desmodesmus ultrasquamatus*	94%	0 / 3	AM228926.1	*Desmodesmus costato-granulatus*	94%	GU192427.1
LBA#26	*Desmodesmus* sp. MAT-2008c	100%	EU502836.1	*Desmodesmus* sp. MAT-2008c	100%	0 / 0	EU502836.1	*Acutodesmus obliquus*	92%	DQ396875.1

LBA#27	*Chlorella sorokiniana*	100%	KM061456.1	*Chlorella sorokiniana*	100%	0 / 0	KJ676113.1	*Chlorella sorokiniana*	99%	HM101339.1
LBA#28	-	-	-	-	-	-	-	*Selenastrum* sp. KMMCC 1456	94%	JQ315488.1
LBA#29	*Chlorella* sp. MAT-2008a	92%	EU502833.1	*Chlorella* sp. MAT-2008a	91%	0 / 2	EU502833.1	*Chlorella* sp. IFRPD 1018	93%	AB260911.1
LBA#30	*Desmodesmus* sp. MAT-2008c	100%	EU502836.1	*Desmodesmus* sp. MAT-2008c	100%	0 / 0	EU502836.1	*Acutodesmus obliquus*	93%	DQ396875.1
LBA#31	*Chlorella* sp. MAT-2008a	92%	EU502833.1	*Chlorella* sp. MAT-2008ª	91%	0 / 2	EU502833.1	*Chlorella* sp. IFRPD 1018	93%	AB260911.1
LBA#32	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1
LBA#33	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1
LBA#34	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1
LBA#35	*Desmodesmus* sp. GM4a	100%	AB917128.1	*Desmodesmus* sp. GM4a	99%	0 / 1	AB917128.1	*Desmodesmus baconii*	93%	KC315289.1
LBA#36	*Desmodesmus* sp. MAT-2008c	100%	EU502836.1	*Desmodesmus* sp. MAT-2008c	100%	0 / 0	EU502836.1	*Acutodesmus obliquus*	93%	DQ396875.1
LBA#37	*Chlamydomonas* sp. YB3-2	90%	JN862852.1	*Chlamydomonas applanata*	92%	1 / 2	FR865616.1	*Ascochloris multinucleata*	94%	EF113411.1
LBA#38	*Chlamydomonas* sp. YB3-2	90%	JN862852.1	*Chlamydomonas applanata*	92%	1 / 2	FR865616.1	*Ascochloris multinucleata*	94%	EF113411.1
LBA#39	*Chlorella sorokiniana* KU207	100%	KM061456.1	*Chlorella sorokiniana*	100%	0 / 0	KJ676113.1	*Chlorella sorokiniana*	99%	HM101339.1
LBA#40	*Chlamydomonas zebra*	79%	AF033294.1	*Chlamydomonas* sp. XJU-36	95%	2 / 0	FJ572059.1	*Chlamydomonas orbicularis*	96%	AB511849.1
LBA#41	*Chlamydomonas* sp. KU107	94%	KM061447.1	*Chlamydomonas* sp. KU107	87%	0 / 3	KM061447.1	*Chlamydomonas oblonga*	95%	EF113424.1
LBA#42	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1

LBA#43	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1
LBA#44	*Micractinium* sp. CCAP 211/92	99%	FM205863.1	*Micractinium* sp. CCAP 211/92	100%	0 / 0	FM205863.1	*Chlorella pyrenoidosa*	99%	FM205863.1
LBA#45	*Chlorococcum oleofaciens*	82%	AB983633.1	*Spongiochloris spongiosa*	86%	-	U34776.1	*Protosiphon botryoides*	92%	EF113465.1
LBA#46	*Uronema* sp. AF-2012	98%	JX092263.1	*Uronema trentonense*	100%	0 / 0	HF920659.1	-	-	-
LBA#47	*Tetracystis tetraspora*	95%	KM020024.1	*Dunaliella* sp. SPMO 300-4	85%	2 / 0	DQ377118.1	*Nautococcus solutus*	91%	AB360758.1
LBA#48	-	-	-	-	-	-	-	*Gungnir* sp. NIES-1851	93%	AB603749.1
LBA#49	*Lobochlamys segnis*	83%	FR865604.1	*Chlamydomonas* sp. CCAP 11/150	90%	0 / 1	FR865545.1	*Asterococcus korschikoffii*	90%	AB175944.1
LBA#50	*Chlorella* sp. KMMCC 1468	99%	JQ315774.1	*Chlorella sorokiniana*	96%	0 / 0	LK021940.1	*Chlorella* sp. IFRPD 1014	99%	AB260910.1
LBA#51	*Chlorococcum oleofaciens*	74%	AB983630.1	*Chlorococcum* sp. CCAP 11/52	84%	2 / 1	FR865591.1	*Chlamydopodium vacuolatum*	95%	EF113426.1

The compensatory and hemi-compensatory base changes (CBCs/hemi-CBCs) between the indicated sequence and its closest match in the ITS2 Database are shown. An hyphen (-) is indicated for samples that could not be amplified and/or sequenced, and for the *nu*ITS2 sequences for which secondary structure predictions and CBCs/Hemi-CBCs analysis were not possible.

Barcode gap analysis

Similarity searches only configure the first step for DNA barcoding since they provide information about the closest matches present in reference databases, but not necessarily species-level identification. In order to establish a genetic distance threshold for species-level identification that is applicable to chlorophytes, barcode gap analyses were conducted based on reference sequences from two species-dense green microalgae genera, *Chlorella* and *Desmodesmus* (S2-S4 Figs; S1-S3 Tables).

Chlorella genus *nu*ITS1 intraspecific distances ranged from 0 to 0,014, while *nu*ITS1 interspecific distances ranged from 0,058 to 0,199 (S2A Fig). *Desmodesmus* genus *nu*ITS1 intraspecific distances ranged from 0 to 0,018, while *nu*ITS1 interspecific distances ranged from 0,029 to 0,193 (S2B Fig). The presence of a barcode gap (gap between maximum intraspecific and minimum interspecific distances) was observed for all species analyzed (S2 Fig). *Chlorella* genus *nu*ITS2 intraspecific distances ranged from 0 to 0,071, while *nu*ITS2 interspecific distances ranged from 0,076 to 0,204 (S3A Fig). *Desmodesmus* genus *nu*ITS2 intraspecific distances ranged from 0 to 0,02, while *nu*ITS2 interspecific distances ranged from 0,032 to 0,167 (S3B Fig). The presence of a barcode gap was also observed for all species analyzed (S3 Fig). *Desmodesmus rbc*L genus intraspecific distances ranged from 0 to 0,108, while *rbc*L interspecific distances ranged from 0,015 to 0,086 (S4 Fig). The presence of a barcode gap is observed for all species based on *rbc*L sequences, except for *Desmodesmus serratus* species (S4 Fig).

Distance thresholds for species-level identification were inferred for each marker based on the minimum interspecific distances observed for each marker (S2-S4 Figs), as follows: i) *nu*ITS1 sequences (< 0,029); ii) *nu*ITS2 sequences (< 0,032); ii) *rbc*L sequences (< 0,015). The application of these distance thresholds to the data presented in Table 2 suggests that species-level identification has been achieved for: i) 35% of the *nu*ITS1 sequences, namely Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-44, #46 and #50; ii) 33% of the *nu*ITS2 sequences, namely Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-

44 and #46. iii) 18% of the *rbc*L sequences, namely Embrapa|LBA#27, #32-34, #39, #42-44 and #50.

Additionally, even though *nu*ITS2 Embrapa|LBA#50 sequence presents only 96% of identity to its GenBank closest match, it can also be considered that species-level identification has been achieved, since the lowest interspecific distance calculated specifically for the *Chlorella* genus *nu*ITS2 sequences is 0,076 (S3A Fig). On the other hand, *rbc*L based identification assigned Embrapa|LBA #32-34 and #42-44 strains to *Chlorella pyrenoidosa* species, which is not currently a taxonomically accepted name [57]. Therefore, Embrapa|LBA #32-34 and #42-44 strains were excluded from the subset of strains identified to the species-level based on *rbc*L sequences.

In conclusion, the results presented so far indicate that 18, 18 and 3 Embrapa|LBA strains were identified to the species-level based on *nu*ITS1, *nu*ITS2 or *rbc*L sequences, respectively.

Morphologic, Phylogenetic and Compensatory Base Changes (CBCs) analyses

In order to confirm the species-level identification based on barcode gap calculations, the strains Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-44,#46 and #50 were identified based on morphology. The strains Embrapa|LBA#22-23 were identified as *Desmodesmus ultrasquamatus*, Embrapa|LBA#27, 39 and 50 were identified as *Chlorella sorokiniana* and Embrapa|LBA#46 was identified as *Uronema trentonense*, according to these species original descriptions [57]. The molecular identification of strains Embrapa|LBA#2-3, #26, #30, #32-36 and #42-44 (Table 2) suggest that they correspond to species still not formally described. Indeed, strains Embrapa|LBA#2-3, #26, #30 and #36 correspond to unicellular spineless coccoid *Desmodemus* species with sizes ranging from 4-6 μm (Figs 1A and 1D), similar to the description of its closest GenBank match (Table 2) the strain *Desmodesmus* sp. MAT-2008c isolated in Australia [58]. Strains Embrapa|LBA#32-34 and #42-44 correspond to coccoid bristleless *Micractinium*

species with sizes ranging from 3-5 µm (Figs 1B and 1E), which is congruent with the description reported for its closest GenBank match (Table 2) the strain *Micractinium* sp. CCAP 211/92 isolated from a soil sample collected from Mahe Island, Seychelles [39]. Strain Embrapa|LBA#35 corresponds to a two-, four- or eight-celled coenobia forming *Desmodemus* species that present few spines and dimensions of 3-6 x 8-13 µm (Figs 1C and 1F), similar to the description of its closest GenBank match (Table 2) the strain *Desmodesmus* sp. GM4a isolated from German inland waters [59].

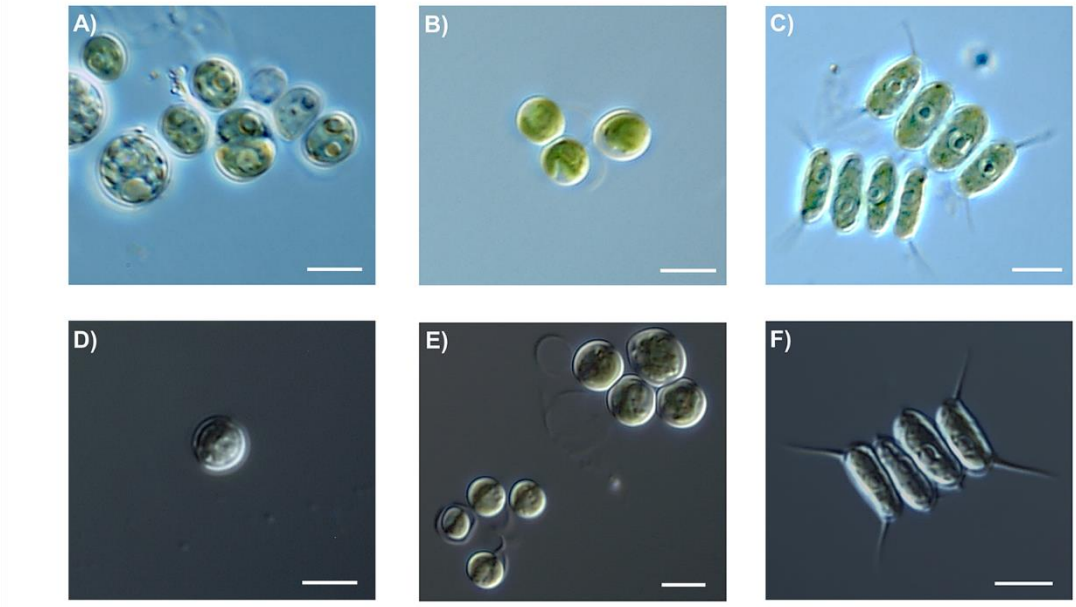

Fig 1. Representative DIC microscopic images of Embrapa|LBA strains assigned to not formally described species.

(A and D) Embrapa|LBA#36. (B and E) Embrapa|LBA#32. (C and F) Embrapa|LBA#35. Scale bars = 5 µm.

Furthermore, the species-level identification obtained for strains Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-44,#46 and #50 is corroborated by the absence of Compensatory Base Changes (CBCs) between *nu*ITS2 sequences of these strains and their closest matches at GenBank (Table 2). Additionally, phylogenetic analyses using reference ITS1-5.8S-ITS2

sequences from currently accepted *Chlorella*, *Micractinium* and *Desmodesmus* species also corroborate species-level identification of strains Embrapa|LBA#2-3, #22-23, #26-27, #30, #32-36, #39, #42-44 and #50 (Figs 2 and 3). Figs 2 and 3 clearly demonstrate that sequences from these strains group together with their closest matches from GenBank (Table 2) in monophyletic clades.

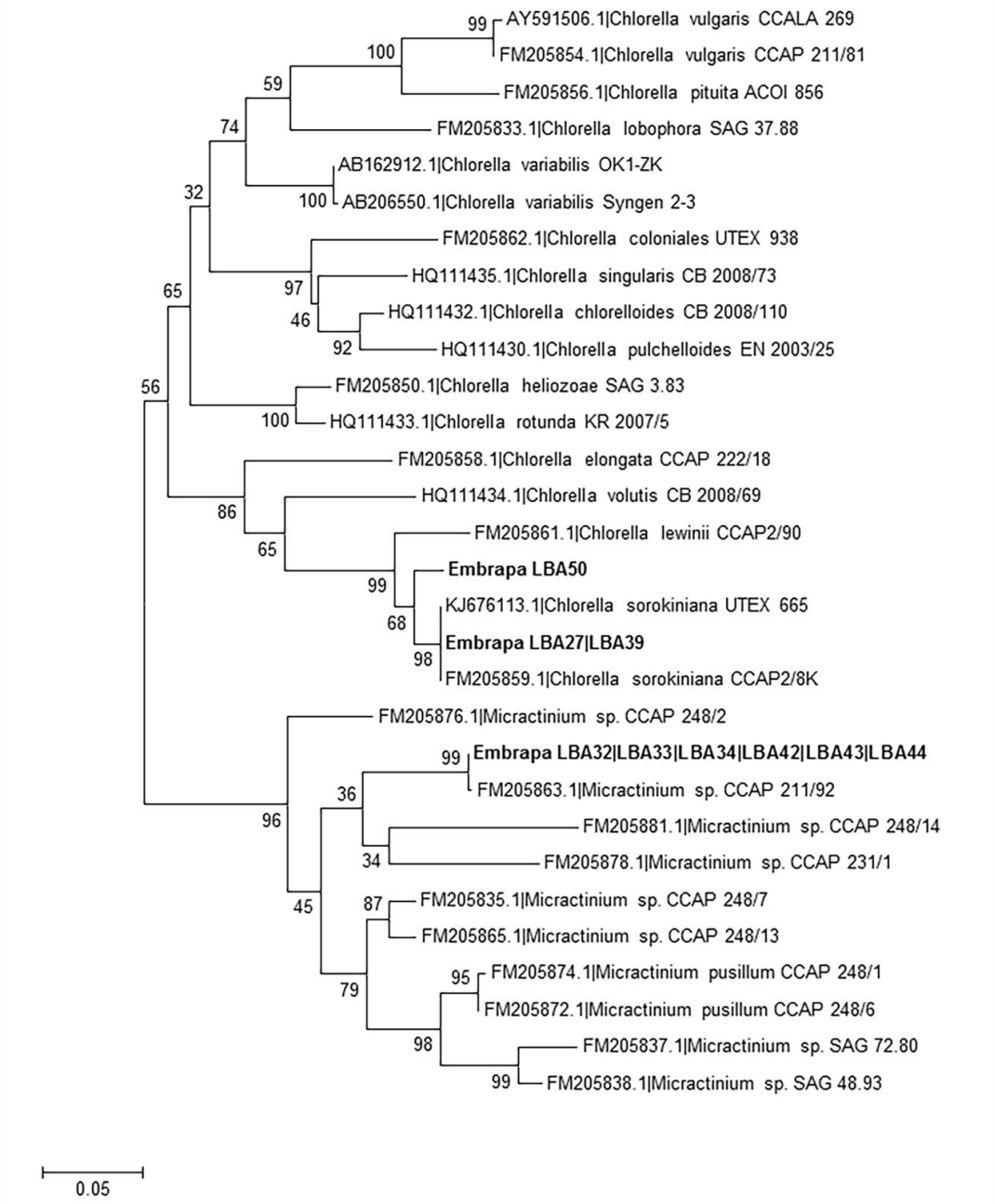

Fig 2. Phylogenetic tree for *Chlorella* and *Micractinium* genera inferred based on ITS1-5.8S-ITS2 sequences.

Chlorella sp. and *Micractinium* sp. ITS1-5.8S-ITS2 reference barcode sequences reported by Luo et al. (2010) [39] and Bock et al. (2011) [14] were included in the analysis together with Embrapa|LBA#27, #32-34, #39, #42-44 and #50 strains sequences and their respectively closest sequences at GenBank. Identical sequences were omitted for simplification. The phylogenetic tree was inferred using the Maximum Composite Likelihood method based on dataset of 472 aligned positions of 31 nucleotide sequences. For the analysis, the GTR+G+I model was chosen. For the analysis, the GTR model with invariable sites (I) and gamma distribution shape parameter (G) was chosen. The bootstrap values (1000 replicates) are shown next to the branches.

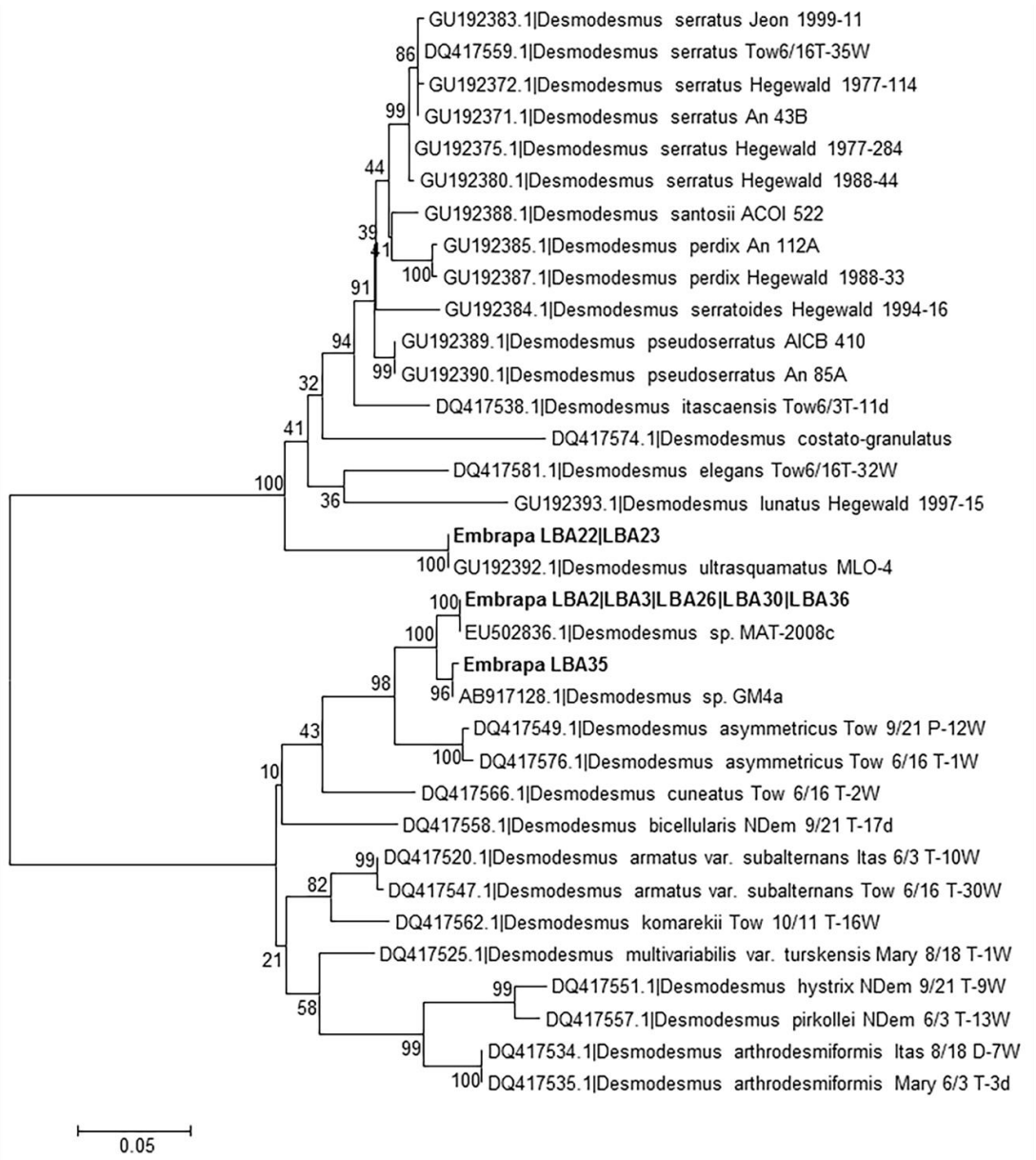

Fig 3. Phylogenetic tree for *Desmodesmus* genus inferred based on ITS1-5.8S-ITS2 sequences.

Demodesmus sp. ITS1-5.8S-ITS2 reference barcode sequences reported by Fawley et al. (2011) [53] and Gorelova et al. (2014) [54] were included in the analysis together with Embrapa|LBA#2-3, #22-23, #26, #30 and #35-36 strains sequences and their respectively closest sequences at GenBank. Identical sequences were omitted for simplification. The phylogenetic tree was inferred using the Maximum Composite Likelihood method based on a dataset of 470 aligned positions of 34 nucleotide sequences. For the analysis, the GTR+G+I

model was chosen. The bootstrap values (1000 replicates) are shown next to the branches.

Discussion

A dual marker DNA barcode system has been proposed as a potential solution to cope with the great diversity of protists, however there is no current consensus about which marker should be used [29,32]. Ideally the two chosen markers should be easily amplified/sequenced using a single set of primers and sufficiently variable to permit clear species delimitations without loss of the phylogenetic signal [29,32]. Even though *tuf*A has been reported to be a promising barcode for chlorophytes [13,31,32], the number of green algae *tuf*A sequences deposited at GenBank is three times lower than the number of deposits for the protein-coding plastid gene *rbc*L or the non-coding regions of nuclear rDNA ITS1 and ITS2 (over 6,000 sequences deposited for *rbc*L and *nu*ITS1 and over 7,000 sequences deposited for *nu*ITS2 markers up to December/2015). Furthermore, recent taxonomic revisions of green algae have been based mainly on *rbc*L, *nu*ITS1 or *nu*ITS2 sequences [14,26,27,32,34,37-42,60]. In addition, there are thousands of *rbc*L sequences from chlorophytes deposited at BOLD systems, which is the most complete taxonomically curated DNA database available [3]. Therefore, although a formal proposal for Chlorophyta DNA barcodes has not been made, a preference for *rbc*L, *nu*ITS1 and *nu*ITS2 markers by several research groups involved in green algae taxonomy can be observed.

Brazil holds the largest reservoir of algal genetic resources in the neotropical region [43,61]. In order to evaluate the applicability of *nu*ITS1, *nu*ITS2 and *rbc*L markers as DNA barcodes for neotropic freshwater chlorophytes, a subset of green microalgae strains was isolated from Brazilian inland water bodies (S1 Fig). This study, however, did not intend to perform an exhaustive sampling of all the Chlorophyta taxa present in the neotropics. Instead, it used specimens from this largely unexplored biodiversity hotspot as test case. DNA from all 51 Embrapa|LBA strains could be amplified and sequenced for at least one of the markers tested. The higher primer universality obtained for ITS1-5.8S-

ITS2 region compared to the *rbc*L marker (Table 1) is in agreement with previous studies [13,28,62]. This can be explained by the presence of highly conserved neighbor regions flanking *nu*ITS (1 and 2) markers, such as the 18S and 28S rDNA genes that function as annealing sites for the primers, described by White and coworkers (1990) [35], which are not available for the *rbc*L gene.

The levels of nucleotide diversity observed among the 5.8S, *nu*ITS1, *nu*ITS2 and *rbc*L sequences were of 0,046, 0,537, 0,321 and 0,250, respectively. Indeed, although *nu*ITS1, *nu*ITS2 and *rbc*L markers may fluctuate depending on the taxa analyzed, these markers rank among the most diverse barcode candidates for chlorophytes [13,28,31]. On the other hand, the 5.8S marker might not present sufficient resolution for species discrimination. Therefore, although other studies used the nuclear rDNA region ITS1-5.8S-ITS2 as a barcode for Chlorophyta (14, 34, 39), in this study the *nu*ITS1 and *nu*ITS2 regions were used separately to avoid genetic distance calculation bias eventually introduced by the simultaneous analysis of DNA regions with distinct evolutionary rates.

It is noteworthy that 53% of the *nu*ITS1 and 42% of the *nu*ITS2 matches retrieved from GenBank lacked the Latin binomial that characterizes the complete species name, compared to 10% of the *rbc*L matches retrieved from BOLD (Table 2). This might be due to the combination of two factors: i) CBOL's effort to preserve traditional taxonomic nomenclature; ii) The overall tendency in phycology to gradually move away from species identifiers based on Latin binomials pushed by the faster rate of genetic information discovery compared with the traditional taxonomic descriptions [24]. Importantly, species names that are not currently taxonomically accepted were found at both the BOLD and GenBank databases. That is the case, for example, of the strains Embrapa|LBA#32-34 and #42-44, which were assigned as *Chlorella pyrenoidosa* (Table 2), currently *Pseudochlorella pyrenoidosa* [26,38], at BOLD systems. Although this finding is not unexpected within GenBank, it is especially relevant in a taxonomically curated database such as BOLD. A possible explanation is that these are, actually, non-validated reference sequences mined directly from GenBank that are currently under taxonomic revision by BOLD collaborators. Indeed, it can be observed that the *Acutodesmus obliquus rbc*L reference

sequence DQ396875.1 retrieved from BOLD (Table 2) is deposited with the old species name, *Scenedesmus obliquus*, at GenBank (data not shown).

Only few sequences retrieved matches with 100% of identity from GenBank and BOLD (Table2), suggesting incomplete taxa coverage within the reference databases analyzed. This is corroborated by the fact that there are less than 500 hundred *rbc*L records from the neotropical region (only 21 from Brazil) deposited at BOLD up to July/2015. Thus, it seems that the incongruences observed between species names retrieved from *nu*ITS1, *nu*ITS2 and *rbc*L similarity searches (Table 2) are mainly due to reference databases incompleteness rather than to real conflicts derived from distinct species identification by each marker. This is important information to be considered since the possibility of biased performance, eventually leading to sample misidentification, when using search algorithms such as BLAST is increased when analyzing poorly sampled groups [63].

Barcode gap analyses can provide the means to improve the accuracy for species level identification [1,17]. A barcode gap is present when the maximum intraspecific distance is lower than the minimum interspecific distance for a certain taxon, thereby revealing a corresponding distance threshold that can be applied to delimit species [17]. However, the same distance threshold may not be applicable to every species and should be determined for each taxon analyzed [32,63,64]. Due to the unavailability of a complete set of reference sequences for most of the taxa listed in Table 2, the analyses were based on sequences *Chlorella* and *Desmodesmus* genera for *nu*ITS1 and *nu*ITS2, and for *Desmodesmus* genus for *rbc*L. These reliable reference barcode sequences are originated from recent revisions of these genera based on integrative taxonomy approaches (S2-S4 Figs; S1-S3 Tables). As expect, the barcode gap analyses based on *nu*ITS1, *nu*ITS2 and *rbc*L makers (S2-S4 Figs) indicate that it is not possible to establish a single universal distance threshold that would avoid incorrect identifications and, at the same time, include all specimens into the correct species. However, assuming that incorrect specimen identification is more problematic than simply not assigning a specimen to any species, distance thresholds were inferred for each marker based on the minimum interspecific distances observed (S2-S4 Figs) allowing species-level identification.

There are several reports suggesting that the presence of compensatory base changes (CBCs) in *nu*ITS2 secondary structures correlate with reproductive isolation [65-67]. A large-scale testing with ~300.000 *nu*ITS2 secondary structures revealed that if a CBC is present then there are two different species with a probability of ~93% [65,67]. Therefore, the detection of CBCs between the Embrapa|LBA strains *nu*ITS2 sequences and their closest matches at GenBank seems to be a reasonable predictor that species-level identification has not been achieved. In accordance, the CBCs analyses shown in Table 2 corroborate the species-level identification achieved based on barcode gap calculations. Additionally, the morphological (Fig 1) and phylogenetic analyses (Fig 2 and 3) also corroborate the species-level identification based on barcode gap calculations.

The DNA barcoding results presented here using a subset of neotropic freshwater green microalgae as a test case suggest that *nu*ITS1 and *nu*ITS2 are the most useful markers, while *rbc*L presented lower primer universality and species-level identification power. Although, both *nu*ITS1 and *nu*ITS2 precisely identified the same 18 strains to the species-level based on barcode gap calculations, *nu*ITS2 accounts with a more complete set of reference sequences deposited at databases and an automated and well developed pipeline for secondary structure analysis [50]. The S5 Fig depicts the tentative DNA barcoding workflow for green microalgae specimens based on the results presented.

Conclusions

DNA barcoding can make specimens identification to species level faster, more reliable and accessible to non-specialists. Defining of the appropriate DNA barcodes for Chlorophyta identification and the availability of taxonomically curated DNA databases are pivotal to this task. The results presented here indicate that a DNA barcoding pipeline based on *nu*ITS2 should be useful for green microalgae species identification. It is clear, however, that there is an urgent need for the deposition of more taxonomically accurate reference barcodes in curated databases (e.g.: BOLD Systems). Therefore, extensive efforts on integrative taxonomy are crucial, ideally encompassing the use of both DNA markers. These studies are especially relevant for poorly studied taxa such as tropical chlorophytes.

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References

1. Hebert PD, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc Biol Sci 270: 313-321.

2. Hebert PD, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc Biol Sci 270 Suppl 1: S96-99.

3. Ratnasingham S, Hebert PD (2007) bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 7: 355-364.

4. Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, et al. (2009) A DNA barcode for land plants. Proc Natl Acad Sci U S A 106: 12794-12797.

5. Leray M, Knowlton N (2015) DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proc Natl Acad Sci U S A 112: 2076-2081.

6. Francis RA (2011) Wall ecology: A frontier for urban biodiversity and ecological engineering. Progress in Physical Geography 35: 43-63.

7. Collins RA, Armstrong KF, Meier R, Yi Y, Brown SDJ, et al. (2012) Barcoding and border biosecurity: identifying cyprinid fishes in the aquarium trade. PLoS One 7: e28381.

8. Palhares RM, Drummond MG, Brasil BDAF, Cosenza GP, Brandao MDL, et al. (2015) Medicinal plants recommended by the World Health Organization: DNA Barcode identification associated with chemical analyses guarantees their quality. Plos One 10.

9. Palhares RM, Drummond MG, Brasil BS, Krettli AU, Oliveira GC, et al. (2014) The use of an integrated molecular-, chemical-and biological-based

approach for promoting the better use and conservation of medicinal species: A case study of Brazilian quinas. J Ethnopharmacol 155: 815-822.

10. Carvalho DC, Neto DA, Brasil BS, Oliveira DA (2011) DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil. Mitochondrial DNA 22: 97-105.

11. Carvalho DC, Palhares RM, Drummond MG, Frigo TB (2015) DNA Barcoding identification of commercialized seafood in South Brazil: A governmental regulatory forensic program. Food Control 50: 784-788.

12. Drummond MG, Brasil BSAF, Dalsecco LS, Brasil RSAF, Teixeira LV, et al. (2013) A versatile real-time PCR method to quantify bovine contamination in buffalo products. Food Control 29: 131-137.

13. Hall JD, Fucikova K, Lo C, Lewis LA, Karol KG (2010) An assessment of proposed DNA barcodes in freshwater green algae. Cryptogam, Algol 31: 529-555.

14. Bock C, Krienitz L, Proeschold T (2011) Taxonomic reassessment of the genus Chlorella (Trebouxiophyceae) using molecular signatures (barcodes), including description of seven new species. Fottea 11: 293-312.

15. Costa ES, Plastino EM, Petti R, Oliveira EC, Oliveira MC (2012) The Gracilariaceae Germplasm Bank of the University of São Paulo, Brazil—a DNA barcoding approach. J Appl Phycol 24: 1643-1653.

16. Pawlowski J, Holzmann M (2014) A plea for DNA barcoding of Foraminifera. J Foraminiferal Res 44: 62-67.

17. Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS biology 2: 1657-1663.

18. Becker B, Marin B (2009) Streptophyte algae and the origin of embryophytes. Ann Bot 103: 999-1004.

19. Guiry MD (2012) How many species of algae are there? J Phycol 48: 1057-1063.

20. Torres MA, Barros MP, Campos SCG, Pinto E, Rajamani S, et al. (2008) Biochemical biomarkers in algae and marine pollution: a review. Ecotoxicol Environ Saf 71: 1-15.

21. Amengual-Morro C, Niell GM, Martínez-Taberner A (2012) Phytoplankton as bioindicator for waste stabilization ponds. J Environ Manage 95: S71-S76.

22. Subhadra B, Grinson-George (2011) Algal biorefinery-based industry: an approach to address fuel and food insecurity for a carbon-smart world. J Sci Food Agric 91: 2-13.

23. Nascimento IA, Marques SSI, Cabanelas ITD, Pereira SA, Druzian JI, et al. (2013) Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. Bioenergy Res 6: 1-13.

24. Clerck O, Guiry MD, Leliaert F, Samyn Y, Verbruggen H (2013) Algal taxonomy: a road to nowhere? J Phycol 49: 215-225.

25. Škaloud P (2006) Variation and taxonomic significance of some morphological features in European strains of Klebsormidium (Klebsormidiophyceae, Streptophyta). Nova Hedwigia 83: 533-550.

26. Krienitz L, Bock C (2012) Present state of the systematics of planktonic coccoid green algae of inland waters. Phytoplankton responses to human impacts at different scales: Springer. pp. 295-326.

27. Krienitz L, Huss VA, Bock C (2015) Chlorella: 125 years of the green survivalist. Trends Plant Sci 20: 67-69.

28. Buchheim MA, Keller A, Koetschan C, Forster F, Merget B, et al. (2011) Internal transcribed spacer 2 (nu ITS2 rRNA) sequence-structure phylogenetics: towards an automated reconstruction of the green algal tree of life. PLoS One 6: e16931.

29. Pawlowski J, Audic S, Adl S, Bass D, Belbahri L, et al. (2012) CBOL protist working group: barcoding eukaryotic richness beyond the animal, plant, and fungal kingdoms. PLoS Biol 10: e1001419.

30. Caisová L, Marin B, Melkonian M (2011) A close-up view on ITS2 evolution and speciation - a case study in the Ulvophyceae (Chlorophyta, Viridiplantae). BMC Evol Biol 11: 262.

31. Du G, Wu F, Mao Y, Guo S, Xue H, et al. (2014) DNA barcoding assessment of green macroalgae in coastal zone around Qingdao, China. J Ocean Univ China 13: 97-103.

32. Leliaert F, Verbruggen H, Vanormelingen P, Steen F, López-Bautista JM, et al. (2014) DNA-based species delimitation in algae. Eur J Phycol 49: 179-196.

33. Pombert J-F, Otis C, Lemieux C, Turmel M (2005) The chloroplast genome sequence of the green alga Pseudendoclonium akinetum (Ulvophyceae) reveals unusual structural features and new insights into the branching order of chlorophyte lineages. Mol Biol Evol 22: 1903-1918.

34. Hegewald E, Bock C, Krienitz L (2013) A phylogenetic study on Scenedesmaceae with the description of a new species of Pectinodesmus and the new genera Verrucodesmus and Chodatodesmus (Chlorophyta, Chlorophyceae). Fottea 13: 14.

35. White TJ, Bruns T, Lee SJWT, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics: Academic Press. 315-322 p.

36. Koetschan C, Förster F, Keller A, Schleicher T, Ruderisch B, et al. (2010) The ITS2 Database III—sequences and structures for phylogeny. Nucleic Acids Res 38: D275-D279.

37. Pröschold T, Bock C, Luo W, Krienitz L (2010) Polyphyletic distribution of bristle formation in Chlorellaceae: Micractinium, Diacanthos, Didymogenes and Hegewaldia gen. nov.(Trebouxiophyceae, Chlorophyta). Phycological Res 58: 1-8.

38. Darienko T, Gustavs L, Mudimu O, Menendez CR, Schumann R, et al. (2010) Chloroidium, a common terrestrial coccoid green alga previously assigned to Chlorella (Trebouxiophyceae, Chlorophyta). Eur J Phycol 45: 79-95.

39. Luo W, Proschold T, Bock C, Krienitz L (2010) Generic concept in Chlorella-related coccoid green algae (Chlorophyta, Trebouxiophyceae). Plant Biol (Stuttg) 12: 545-553.

40. Pröschold T, Darienko T, Silva PC, Reisser W, Krienitz L (2011) The systematics of Zoochlorella revisited employing an integrative approach. Environ Microbiol 13: 350-364.

41. Krienitz L, Hegewald EH, Hepperle D, Huss VAR, Rohr T, et al. (2004) Phylogenetic relationship of Chlorella and Parachlorella gen. nov.(Chlorophyta, Trebouxiophyceae). Phycologia 43: 529-542.

42. Pöschold T, Marin B, Schlosser UG, Melkonian M (2001) Molecular phylogeny and taxonomic revision of Chlamydomonas (Chlorophyta). I. Emendation of Chlamydomonas Ehrenberg and Chloromonas Gobi, and description of Oogamochlamys gen. nov. and Lobochlamys gen. nov. Protist 152: 265-300.

43. Agostinho AA, Thomaz SM, Gomes LC (2005) Conservation of the biodiversity of Brazil's inland waters. Conserv Biol 19: 646-652.

44. Schlösser U (1997) Additions to the culture collection of algae since 1994. Bot Acta 110: 424-429.

45. Bonato ALV, do Valle CB, Jank L, Resende RMS, Leguizamon GOdC (2002) Extração de DNA genômico de Brachiaria e Panicum maximum. Embrapa Gado de Corte Comunicado técnico.

46. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647-1649.

47. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.

48. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731-2739.

49. Bengtsson-Palme J, Ryberg M, Hartmann M, Branco S, Wang Z, et al. (2013) Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods in Ecology and Evolution 4: 914-919.

50. Koetschan C, Hackl T, Müller T, Wolf M, Förster F, et al. (2012) ITS2 database IV: interactive taxon sampling for internal transcribed spacer 2 based phylogenies. Mol Phylogenet Evol 63: 585-588.

51. Seibel PN, Müller T, Dandekar T, Schultz J, Wolf M (2006) 4SALE–a tool for synchronous RNA sequence and secondary structure alignment and editing. BMC bioinformatics 7: 498.

52. Seibel PN, Müller T, Dandekar T, Wolf M (2008) Synchronous visual analysis and editing of RNA sequence and secondary structure alignments using 4SALE. BMC Res Notes 1: 91.

53. Fawley MW, Fawley KP, Hegewald E (2011) Taxonomy of Desmodesmus serratus (Chlorophyceae, Chlorophyta) and related taxa on the basis of morphological and DNA sequence data. Phycologia 50: 23-56.

54. Gorelova OA, Baulina OI, Solovchenko AE, Chekanov KA, Chivkunova OB, et al. (2015) Similarity and diversity of the Desmodesmus spp. microalgae isolated from associations with White Sea invertebrates. Protoplasma 252: 489-503.

55. Bock C, Pröschold T, Krienitz L (2010) Two new Dictyosphaerium-morphotype lineages of the Chlorellaceae (Trebouxiophyceae): Heynigia gen. nov. and Hindakia gen. nov. European Journal of Phycology 45: 267-277.

56. Bellinger EG, Sigee DC (2015) Freshwater Algae: Identification and Use as Bioindicators: Wiley.

57. Guiry MD, Guiry GM (2015) AlgaeBase. World-wide electronic publication. http://www.algaebase.org: Galway: National University of Ireland.

58. Timmins M, Thomas-Hall SR, Darling A, Zhang E, Hankamer B, et al. (2009) Phylogenetic and molecular analysis of hydrogen-producing green algae. J Exp Bot 60: 1691-1702.

59. Hoshina R (2014) DNA analyses of a private collection of microbial green algae contribute to a better understanding of microbial diversity. BMC Res Notes 7: 592.

60. Loughnane CJ, McIvor LM, Rindi F, Stengel DB, Guiry MD (2008) Morphology, rbc L phylogeny and distribution of distromatic Ulva (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. Phycologia 47: 416-429.

61. Freitas LC, Loverde-Oliveira SM (2013) Checklist of green algae (Chlorophyta) for the state of Mato Grosso, Central Brazil. Check List 9: 1471-1483.

62. Nozaki H, Krienitz L (2001) Morphology and phylogeny of Eudorina minodii (Chodat) Nozaki et Krienitz, comb. nov.(Volvocales, Chlorophyta) from Germany. Eur J Phycol 36: 23-28.

63. Hoef-Emden K (2012) Pitfalls of establishing DNA barcoding systems in protists: the cryptophyceae as a test case. PLoS One 7: e43652.

64. Collins RA, Cruickshank RH (2013) The seven deadly sins of DNA barcoding. Mol Ecol Resour 13: 969-975.

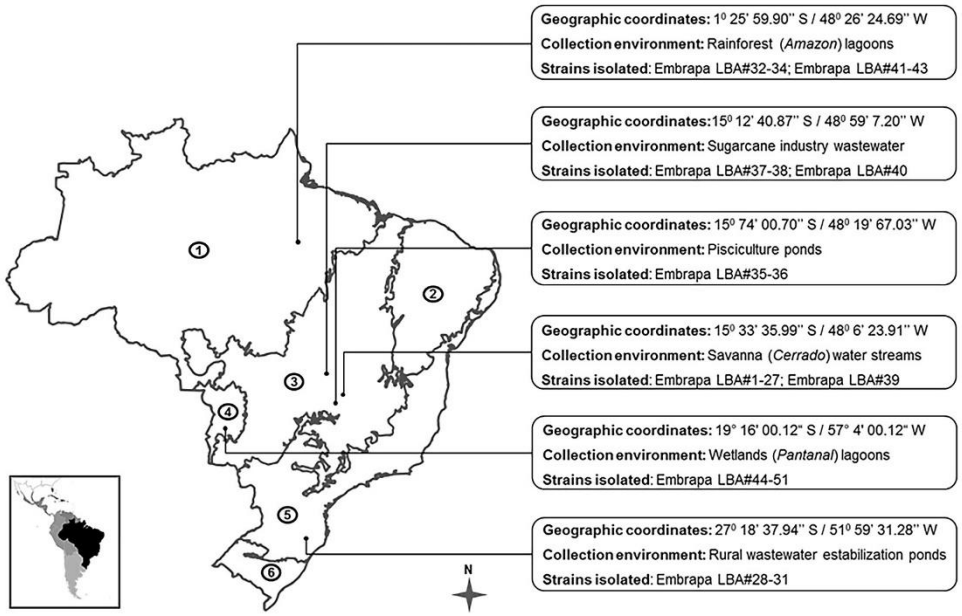
65. Wolf M, Chen S, Song J, Ankenbrand M, Muller T (2013) Compensatory base changes in ITS2 secondary structures correlate with the biological species concept despite intragenomic variability in ITS2 sequences--a proof of concept. PLoS One 8: e66726.

66. Coleman AW (2009) Is there a molecular key to the level of “biological species” in eukaryotes? A DNA guide. Mol Phylogenet Evol 50: 197-203.

67. Müller T, Philippi N, Dandekar T, Schultz J, Wolf M (2007) Distinguishing species. RNA 13: 1469-1472.

Supporting Information

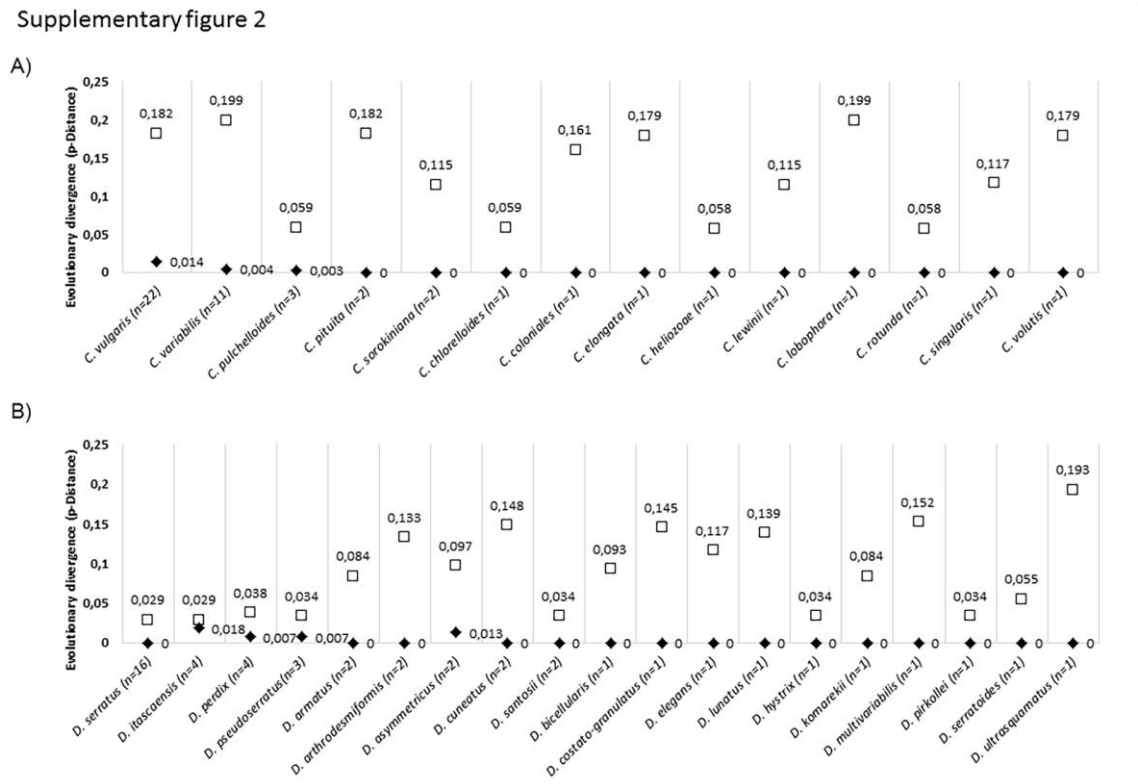
Supplementary figure 1



S1 Fig. Collection sites.

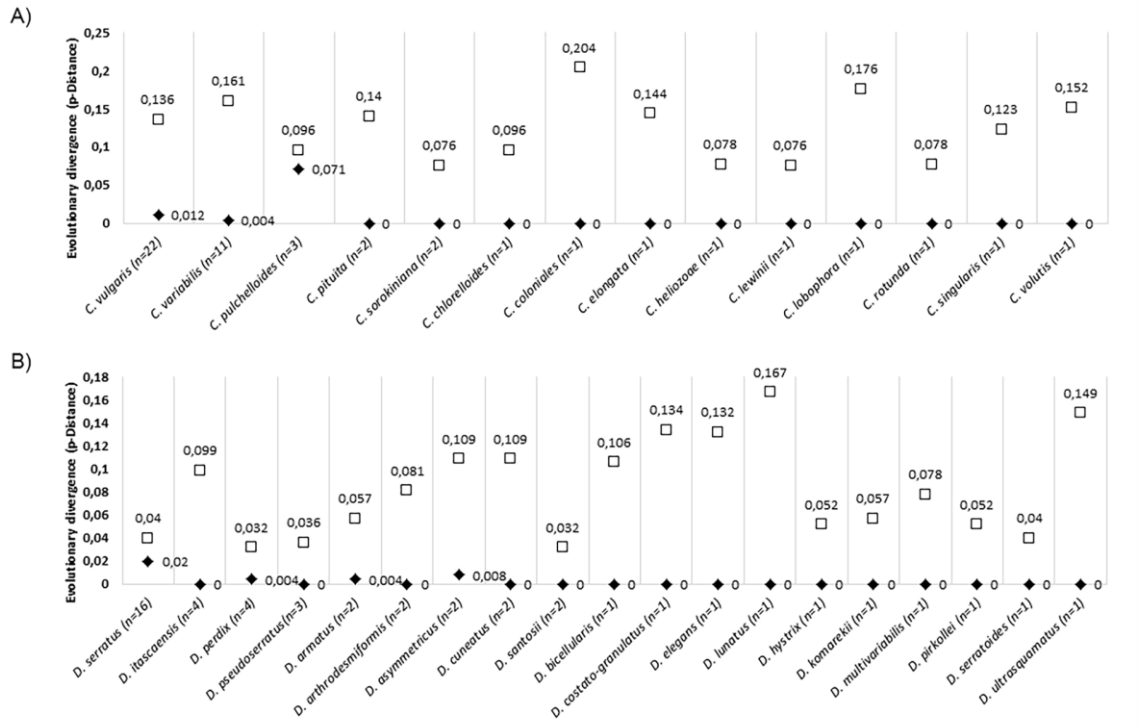
Map of Brazilian biomes, including the *Amazon* tropical rainforest (1), the *Caatinga* xeric shrublands (2), the *Cerrado* tropical Savanna (3), the *Pantanal* flooded grassland (4), the *Mata Atlântica* tropical rainforest (5) and the *Pampa* subtropical grassland (6). The geographic coordinates of the six distinct locations sampled and the respective isolated strains in each site are shown. The strains isolated were deposited in the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries at Embrapa (Brasília/DF–Brazil). The Brazilian territory is highlighted in black in the map of the neotropical region (inset).

doi:10.1371/journal.pone.0149284.s001



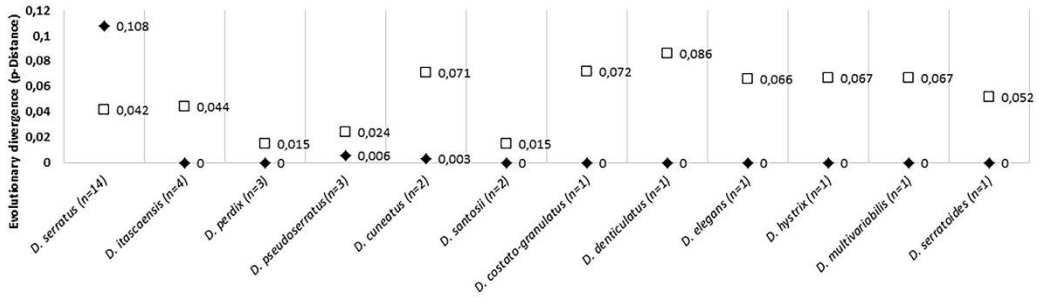
S2 Fig. *nu*ITS1-based barcode gap calculation.
The maximum intraspecific distances (◆) and minimum interspecific distances (□) based on *nu*ITS1 marker between *Chlorella* (A) and *Desmodesmus* (B) genera species are shown. The dataset was composed of reference barcode sequences reported for each genera (S1 and S2 Tables).
doi:10.1371/journal.pone.0149284.s002

Supplementary figure 3



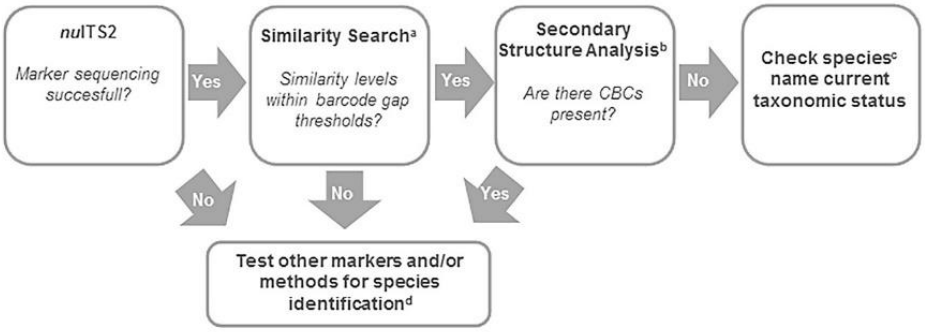
S3 Fig. *nu*ITS2-based barcode gap calculation.
The maximum intraspecific distances (◆) and minimum interspecific distances (□) based on *nu*ITS2 marker between *Chlorella* (A) and *Desmodesmus* (B) genera species are shown. The dataset was composed of reference barcode sequences reported for each genera (S1 and S2 Tables).
doi:10.1371/journal.pone.0149284.s003

Supplementary figure 4



S4 Fig. *rbc*L-based barcode gap calculation.
The maximum intraspecific distances (◆) and minimum interspecific distances (□) based on *rbc*L marker between *Desmodesmus* genus species are shown. The dataset was composed of reference barcode sequences reported this genus (S3 Table).
doi:10.1371/journal.pone.0149284.s004

Supplementary figure 5



S5 Fig. Roadmap for green microalgae DNA barcoding.
*nu*ITS2 should be primarily sequenced and submitted to similarity searches against GenBank. Similarity values obtained must be compatible with the barcode gap thresholds calculated using reference sequences for the taxon indicated (a). The absence of CBCs between the query *nu*ITS2 sequence and its closest match retrieved from similarity search is necessary to confirm species diagnosis (b). Finally, the current status of the assigned species name must be checked using a reference database (e.g.: AlgaeBase) (c). If *nu*ITS2 is not sufficient for a species diagnosis, other markers/methods should be tried (d).
doi:10.1371/journal.pone.0149284.s00

S1 Table. *nu*ITS1 and *nu*ITS2 reference sequences from *Chlorella* genus mined from GenBank used for barcode gap calculation.
doi:10.1371/journal.pone.0149284.s006

Species	Accession Number	Reference
Chlorella chlorelloides	HQ111432	[14]
Chlorella coloniales	FM205862	[39]
Chlorella elongata	FM205858	[39]
Chlorella heliozoae	FM205850	[39]
Chlorella lewinii	FM205861	[14]
Chlorella lobophora	FM205833	[39]
Chlorella pituita	FM205856, GQ176853	[39]
Chlorella pulchelloides	HQ111430, HQ111431, FM205857	[14, 39]
Chlorella rotunda	HQ111433	[14]
Chlorella singularis	HQ111435	[14]
Chlorella sorokiniana	FM205860, FM205859	[39]
Chlorella variabilis	AB162913, AB162912, AB206546, AB206550, AB162914, AB162915, AB162916, AB162917, AB219527, AB206549, FM205849	[39, 59]
Chlorella volutis	HQ111434	[14]
Chlorella vulgaris	AY591508, AY591509, AY591510, AY591511, AY591512, AY591513, AY591500, AY591501, AY591502, AY591503, AY591504, AY591505, AY591506, AY591493, AY591494, AY591495, AY591496, AY591497, AY591498, AY591499, AB162910, FM205854	[39, 59]

S2 Table. *nu*ITS1 and *nu*ITS2 reference sequences from *Desmodesmus* genus mined from GenBank used for barcode gap calculation.

doi:10.1371/journal.pone.0149284.s007

Species	Accession Number	Reference
Desmodesmus armatus var. subalternans	DQ417520, DQ417547	[54]
Desmodesmus arthrodesmiformis	DQ417534, DQ417535	[54]
Desmodesmus asymmetricus	DQ417549, DQ417576	[54]
Desmodesmus cuneatus	DQ417566, DQ417567	[54]
Desmodesmus bicellularis	DQ417558	[54]
Desmodesmus hystrix	DQ417551	[54]
Desmodesmus komarekii	DQ417562	[54]
Desmodesmus multivariabilis var. turskensis	DQ417525	[54]
Desmodesmus pirkollei	DQ417557	[54]
Desmodesmus serratus	GU192371, GU192372, GU192373, GU192374, GU192375, GU192376, GU192377, GU192378, GU192379, GU192380, GU192381, GU192382, GU192383, DQ417559, DQ417560, DQ417561	[53]
Desmodesmus itascaensis	DQ417538, DQ417539, DQ417540, DQ417541	[53]
Desmodesmus perdix	GU192385, GU192386, GU192387, DQ417573	[53]
Desmodesmus pseudoserratus	GU192389, GU192390, GU192391	[53]
Desmodesmus santosii	GU192388, DQ417524	[53]
Desmodesmus costato-granulatus	DQ417574	[53]
Desmodesmus elegans	DQ417581	[53]
Desmodesmus lunatus	GU192393	[53]
Desmodesmus serratoides	GU192384	[53]
Desmodesmus ultrasquamatus	GU192392	[53]

S3 Table. *rbc*L reference sequences from *Desmodesmus* genus mined from GenBank used for barcode gap calculation.
doi:10.1371/journal.pone.0149284.s008

Species	Accession Number	Reference
Desmodesmus cuneatus	GU192435, GU192436	[54]
Desmodesmus costato-granulatus	GU192427	[54]
Desmodesmus denticulatus var. linearis	GU192429	[54]
Desmodesmus hystrix	GU192433	[54]
Desmodesmus multivariabilis var. turskensis	GU192431	[54]
Desmodesmus serratoides	GU192413	[54]
Desmodesmus serratus	GU192399, GU192400, GU192401, GU192402, GU192403, GU192404, GU192405, GU192406, GU192407, GU192408, GU192409, GU192410, GU192411, GU192412	[53]
Desmodesmus itascaensis	GU192422, GU192423, GU192424, GU192425	[53]
Desmodesmus perdix	GU192414, GU192415, GU192416	[53]
Desmodesmus pseudoserratus	GU192419, GU192420, GU192421	[53]
Desmodesmus santosii	GU192417, GU192418	[53]
Desmodesmus elegans	GU192426	[53]

8. CAPÍTULO II

Growth, biochemical composition and nutrient uptake of microalgae with high biomass productivity in sugarcane vinasse

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Abstract

Sugarcane ethanol is produced in large scale generate wastes (vinasse and CO_2) that could be potentially used for microalgae biomass production in a biorefinery strategy. In this work, two microalgae strains, *Micractinium* sp. Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40, presented vigorous growth in sugarcane vinasse among forty (40) strains evaluated. The selected strains presented increased biomass productivity in vinasse compared to standard Bold's Basal Medium (BBM) when cultivated in air lift flat plate photobioreactors. Analysis show that both microalgae strains can uptake glycerol present in crude vinasse in a light-dependent manner, characterizing a photo-heterotrophic growth mode. Biomass composition analysis c showed that the selected strains present increased protein and carbohydrates productivities when cultivated in vinasse-based media. In conclusion, the cultivation of microalgae using residues of ethanol plants shows potential for application in industrial scale using a biorefinery strategy for the production of biofuels or valuable bioproducts from microalgae.

Key words: *Chlamydomonas*; *Micractinium*; biorefinery; photobioreactor; wastewater;

1. Introduction

Microalgae-derived biomass is recognized as an alternative source for an wide variety of bio products, such as biofuels, lipids, pigments and polymers (Perez-Garcia et al., 2011a). These photosynthetic microrganisms present high growth rate, low nutrient requirement and reduced land area requirements to obtain the same yields of terrestrial crops commonly used for biofuels production (Chisti, 2007). However, the production of microalgae biomass is still not economically viable due to the high costs required for cultivation, harvesting and processing (Quinn & Davis, 2015). Significant cost reductions can be achieved if CO_2, nutrients and water for microalgae cultivation are obtained at low cost (Slade & Bauen, 2013; Brasil et al. 2016). Indeed, the use of wastewaters and CO_2 emissions derived from industrial processes have been proposed for microalgae cultivation (Kang et al., 2015).

Several studies focusing on the cultivation of microalgae using different wastes have been performed on the last years. The results show that selected strains are capable to grow using different wastewaters. In studies using domestic wastewater, various strains were able to grow using this effluent as nutrient source (Cho et al., 2015; Kong et al., 2010; Sydney et al., 2011). In studies performed by Wang and Park (2015), *Chlorella sp.* and *Micractinium sp*. strains were successfully cultured using anaerobically digested sewage sludge as nutrient source. Dairy wastewater also were used for microalgae cultivation by Qin et al. (2016) and Woertz et al. (2009), showing potential for biodiesel production. Other studies also evaluated the feasibility of the use of CO_2 emissions from industrial plants for microalgae cultivation. The results show that the use of this effluent could contribute significantly for the reduction of biomass production costs (Benemann, 2003; Hu et al., 2012; Wilson et al., 2014).

Wastes generated during ethanol production also have the potential for microalgae cultivation. The main waste generated during this process is the vinasse, an acid dark brown liquid rich in organic compounds (e.g. glycerol, lactic acid, sugars), nitrogen and phosphorus compounds, and ions (e.g. K^+, Ca^{2+}, Mg^{2+}) (Ortegón et al., 2016; Parnaudeau et al., 2008). Vinasse is the main product of the distillation of fermented sugarcane juice, being 12-14L per liter of ethanol

produced, and is commonly applied in fertirrigation of sugarcane crops (Cavalett et al., 2011; Dias et al., 2012). However, in a long term, the application of this effluent in the soil can change its composition and reduce the productivity of the sugarcane crops (Christofoletti et al., 2013). In addition to the vinasse, considerable levels of CO_2 are produced during ethanol fermentation (Dias De Oliveira et al., 2005). Therefore, the use of the wastes generated in ethanol plants for microalgae cultivation can be a strategy to reduce the costs of biomass production (Christenson & Sims, 2011; Singh & Gu, 2010). Indeed, a report from Ramirez and coworkers (2014) described the successful cultivation of *Scenedesmus* sp. in synthetic medium supplemented with vinasse. However, successful cultivation in vinasse seems to depend on the strains/species used, since studies performed with *Spirulina maxima* and *Chlamydomonas reinhardtii* indicated that the presence of vinasse in the medium, even at low concentrations, inhibit microalgae growth (dos Santos et al., 2016; Kadioğlu & Algur, 1992). Therefore, the selection of robust microalgae strains capable of achieving and maintaining high growth rates in each specific culture condition is pivotal for the economic feasibility of the process (Brasil et al, 2016).

The aim of this study was to selected microalgae strains capable of growing in vinasse, characterizing its growth and optimizing the cultivation in this waste. In addition, analyzes of biomass production and composition using flat-plate photobioreactors were performed to evaluate the selected strains potential for the production of biofuels and bioproducts.

2. Materials and methods

2.1. Microalgae strains and inoculum preparation

In this study, axenic microalgae cultures of Embrapa|LBA#1 to Embrapa|LBA#40 strains (S1 table) derived from the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries at Embrapa (Brasília/DF – Brazil) were used. They were kept in liquid cultures of Bold's Basal Medium – BBM medium (Nichols & Bold, 1965) containing ampicillin (100 µg/mL), chloramphenicol (25 µg/mL) and amphotericin B (2,5 µg/ml), at 25º C, light intensity of 50 $\mu Em^{-2}\ s^{-1}$ and 12/12h light/dark regime. .

For the inoculum preparation, microalgae strains were axenic cultured in BBM under 12/12 h light/dark regime (light intensity of 108 $\mu Em^{-2}\ s^{-1}$) and aeration with 5 L/h of atmospheric air. During log phase of growth, these starter cultures were used to inoculate experimental units (i.e.; Erlenmeyer flasks or flat-plate photobioreactors) at an initial concentration of 0.01 absorbance at 680nm. Inoculum volumes ≤ 5% of the working load used were.

2.2. Sugarcane vinasse collection and medium preparation

Crude sugarcane vinasse was obtained from Jalles Machado (Goianésia/Brazil) ethanol plant. Crude vinasse samples, referred hereafter only as “crude vinasse”, were centrifuged at 4800 RCF during 10 minutes to remove suspended solids and debris sterilized by autoclaving at 121º C for 15 minutes and stored at 4 °C until use.

Diluted vinasse formulations, referred hereafter only as “diluted vinasse”, were prepared by addition of distilled water in the proportions indicated (i.e.: 25%, 50% or 75%).

Clarified vinasse formulation, referred hereafter only as “clarified vinasse”, was prepared as follows: hydrated lime ($Ca(OH)_2$) was added to crude vinasse (3 g/L). The solution was maintained at rest for 40 minutes, then centrifuged at 4800 RCF during 10 minutes and the supernatant was collected.

All vinasse-based media formulations were sterilized by autoclaving at 121º C for 15 minutes and stored at 4 °C until use. Transmittance was measured by spectrophotometry using Spectramax M3 plate analyzer.

2.3. Selection of microalgae for growth in vinasse

Embrapa|LBA#1 to *Embrapa*|LBA#40 microalgae strains were screened for growth in sugarcane vinasse. Starter cultures of each strain were inoculated in 250 mL of either, diluted vinasse formulations at 25%, 50% and 75% concentration in distilled water or crude vinasse. Culturing was performed in sterile 500 mL Erlenmeyer flasks aerated with 5 L/h of atmospheric air, at 26º C ± 1 °C, light intensity of 100 $\mu Em^{-2} s^{-1}$ in a 16/8h light/dark regime. Microalgae growth was monitored through microscopic inspection during 30 days of culturing.

2.4. Microalgae cultivation

Selected microalgae strains were submitted to cultivation in 250 mL of crude vinasse or BBM (control) under different conditions: I) Axenic culturing using crude vinasse at 12/12 h light/dark regime; II) Axenic culturing using crude vinasse without light (dark condition); III) Non-axenic culturing using crude vinasse (pH adjusted to 8.0) at 12/12 h light/dark regime; IV) Axenic culturing using BBM at 12/12 h light/dark regime. Culturing was performed in 500 mL Erlenmeyer flasks aerated with 5 L/h of atmospheric air, at 26º C ± 1 °C, light intensity of 100 $\mu Em^{-2} s^{-1}$ in a 12/12h light/dark regime.

2.5. Biomass dry weight determination

For biomass dry weight determination, 10 mL samples of the algal culture were collected, centrifuged during 10 minutes at 10700 RCF and the supernatant discarded. The pellet was washed through three cycles of resuspension in distilled water followed by 10 minutes centrifugation at 10700 RCF. The washed pellet was dried overnight using a dry oven at 105 °C and weighted.

2.6. Determination of organic compounds concentration in vinasse-based media

The concentration of total reducing sugars (glucose + fructose + sucrose), glycerol, lactic acid and acetic acid in vinasse-based media at the beginning (day 0) and the end (day 8) of microalgae cultivation (Section 2.4) was determined. One milliliter (1 mL) culture samples were collected, centrifuged for 10 min. at 10700 RCF and the supernatant was analyzed through High Performance Liquid Cromatography analysis (Agilent 1260 Infinity Binary LC System) using Biorad Aminex HPX-87H column (H_2SO_4 0.005M, 0.6 mL/min, 45 °C).

2.7. Microalgae cultivation in air lift flat-plate photobioreactors

Selected microalgae strains were cultivated in either diluted vinasse (50%), clarified vinasse or BBM (control) under non-axenic conditions using air lift flat-plate photobioreactors (Supplentary figure 1) at 13L of working load. Culturing was conducted for 3 days at 12h/12h light/dark regime (light intensity of 400 $\mu Em^{-2}\ s^{-1}$) and a temperature regimen of 37±1 °C during light period and 24±1 °C during dark period. Aeration with 64 L/h of atmospheric air supplemented with 5% CO_2 was provided.

2.8. Analysis of biomass biochemical composition

After cultivation, algal biomass was harvested by centrifugation at 4800 RCF during 10 minutes. The algal cell pellet was washed three times with distilled water followed by 10 minutes of centrifugation at 4800 RCF, freeze-dried and used for the determination of biochemical composition. To determine the total ash content, the methodology proposed by Van Wychen and Laurens (2013c) was used. The total protein was determined using the Kjeldahl method, as proposed by AOAC (1990), using the nitrogen – protein conversion factor proposed for microalgae (Lourenço et al., 2004). For determination of total carbohydrate was used the methodology proposed by Van Wychen and Laurens (2013a). For total carotenoids, the analysis was performed following the protocol described by Porra and coworkers (Porra et al.) adapted by Huang and Cheung (2011) using

acetone 90% as extraction solution. The Fatty Acid Methyl Esther present in the biomass was determined using the protocol described by Van Wychen and Laurens (2013b).

2.9. Determination of vinasse-based media composition

The nutrient composition of vinasse-based media at the beginning (day 0) and the end (day 3) of microalgae cultivation in air lift flat-plate photobioreactors (Section 2.7) was determined. Samples were collected, centrifuged for 10 min. at 4800 RCF and the supernatant used for analysis. The following standard methods were used. SM5210B - Biochemical Oxygen Demand (BOD); QAM.IT.FQ.16A - Chemical Oxygen demand; SM 4500-P E – Phosphate; SM 3500-K B - Total potassium; ABNT NBR 12620:1992; SM 4500-NO2-B – Nitrite; SM4500-NH3 – Ammoniacal nitrogen; SM 4500-O/D - Total organic carbon;

2.10. Statistical analysis

All the experiments were conducted in triplicates, and the results are presented as means of the three replicates expressed as mean ± error bars. Statistical analysis were performed using the software GraphPad Prism 5.

3. Results

3.1. Screening of microalgae strains for growth in vinasse

In order to select microalgae able to grow in sugarcane vinasse, forty (40) Chlorophyta microalgae strains, Embrapa|LBA#1 to Embrapa|LBA#40 (Supplementary table 1), were inoculated into atmospheric air aerated Erlenmeyer's flasks containing either crude vinasse or crude vinasse diluted in distilled water at concentrations of 25%, 50% and 75%. Cultivation was conducted under 16h/8h light/dark regimen and algal growth was monitored microscopically until 30 days of culturing. Only strains Embrapa|LBA#32 (Figura 1 A, B) and Embrapa|LBA#40 (Figure 1 C, D) presented growth in all media formulations, including undiluted crude vinasse (data not shown). Embrapa|LBA#32 belongs to a not formally described species of *Micractinium* genus isolated from a lagoon in the Amazonian rainforest (Hadi et al., 2016). Embrapa|LBA#40 was isolated from a sugarcane vinasse estabilization pond from an ethanol plant in Brazil (Hadi et al., 2016) and belongs to the *Chlamydomonas biconvexa* species.

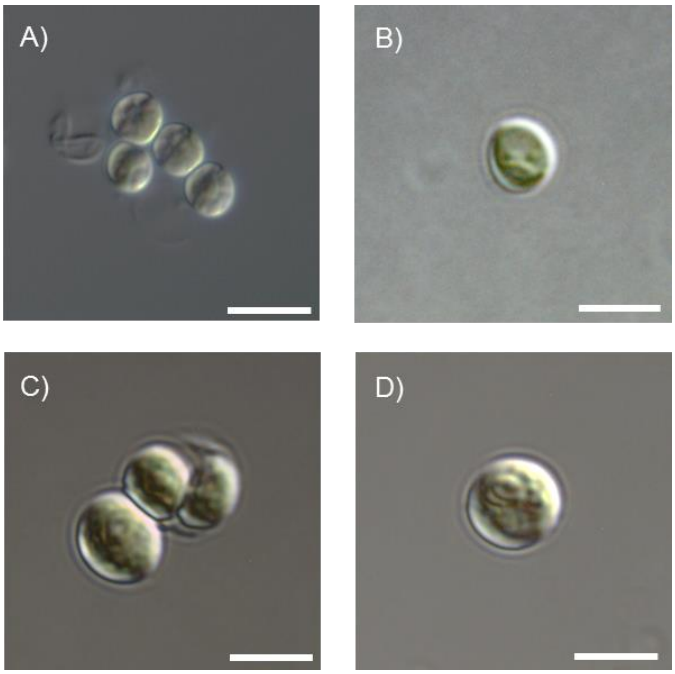

Figure 1– Representative DIC microscopic images of *Microalgae selected for growth in sugarcane vinasse. Micractinium sp.* EMBRAPA|LBA#32 (A, B) and *Chlamydomonas biconvexa* EMBRAPA|LBA#40 (C, D). Scale bars = 5μm.

3.2. Algal growth and trophic mode in vinasse under axenic and non-axenic conditions

After selection, the strains *Micractinium* sp. Embrapa|LBA#32 and *Chlamydomonas biconvexa* Embrapa|LBA#40 were cultivated in aerated Erlemeyrer's flaks containing crude vinasse under different conditions to evaluate the effect of light and microbial contaminants upon microalgae growth (Figure 2). Both strains presented similar growth in BBM and crude vinasse under axenic conditions and 12h/12h light/dark regimens. On the other hand, algal growth was impaired in the absence of light. These results indicate that both, *Micractinium* sp. Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40, are able to directly uptake crude vinasse nutrients in a light dependent manner without need of substrate metabolization by other microganisms. In non-axenic crude vinasse cultures, algal growth was outcompeted by heterotrophic contaminants, especially filamentous fungi (data not shown). In order to favor algal growth, crude vinasse p.H. was adjusted to 8,0 before inoculation, which permitted vigorous microalgae growth with minimal contamination (Figure 2).

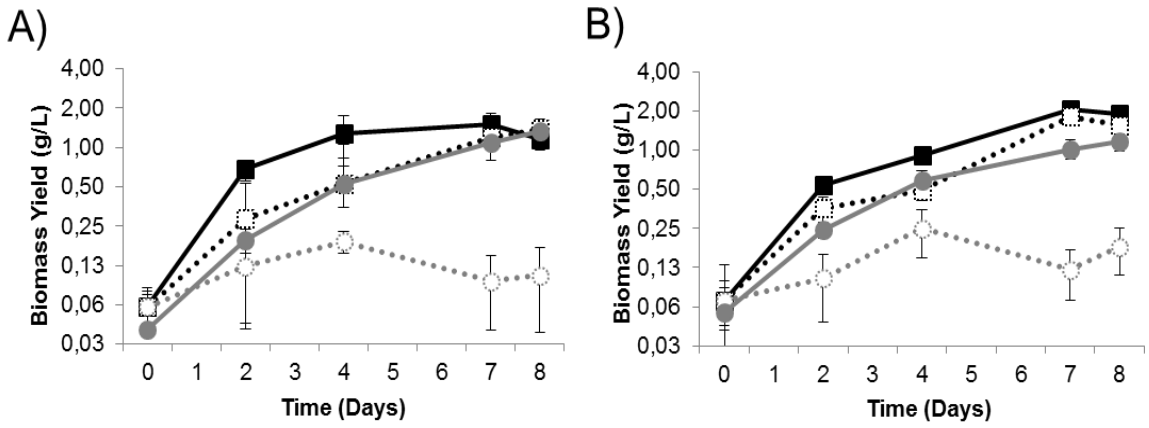

Figure 2 – *Microalgae growth in vinasse.* The strains *Micractinium* sp. Embrapa|LBA#32 (A) and *Chlamydomonas biconvexa* Embrapa|LBA#40 (B) were grown for eight days using atmospheric air aerated erlenmeyers flasks at 26±1°C under the following conditions: (—▲—) Crude vinasse, axenic culture in the dark; (┈□┈) Crude vinasse, axenic culture, 12h/12h light/dark regimen; (—■—) Crude vinasse (pH 8,0), non-axenic culture, 12h/12h light/dark regimen and (—●—) BBM, axenic culture, 12h/12h light/dark regimen. Experiments were conducted in triplicates (n=3), and the results are presented as means of the three replicates expressed as mean ± error bars.

After cultivation in crude vinasse, the concentration of glucose, fructose, sucrose, glycerol, lactic acid and acetic acid acetic acid present in *Micractinium* sp.

Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 cultures supernatants were evaluated (Figure 3). When cultivation was performed in the absence of light, no statistically significant change in the concentration of the organic compounds evaluated could be observed (Figure 3A), independent of the strains analyzed. This finding is congruent with impaired algal growth in this condition (Figure 2). When light/dark cycling is applied to axenic cultures, both strains culture supernatants presented a significant reduction in glycerol concentration, but not Total reducing sugars (TRS), lactic acid or acetic acid (Figure 3B). On the other hand, when the microalgae strains were cultured under non-axenic conditions, it was detected a reduction on TRS, lactic acid and glycerol concentrations (Figure 3C). Furthermore, a reduction in acetic acid concentration was also observed in *C. biconvexa* Embrapa|LBA#40 cultures supernatants (Figure 3C). The uptake of organic compounds observed in non-axenic cultures (Figure 3C). is probably associated with the presence of contaminants, mostly rods and cocci from airborne bacteria species and pseudo-hyphae forming yeasts (data not shown). Taken together, the results shown in Figure 2 and Figure 3 indicate that both *Micractinium* sp. Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 uptake glycerol in a light-dependent manner during growth in crude vinasse, characterizing a photo-heterotrophic metabolism.

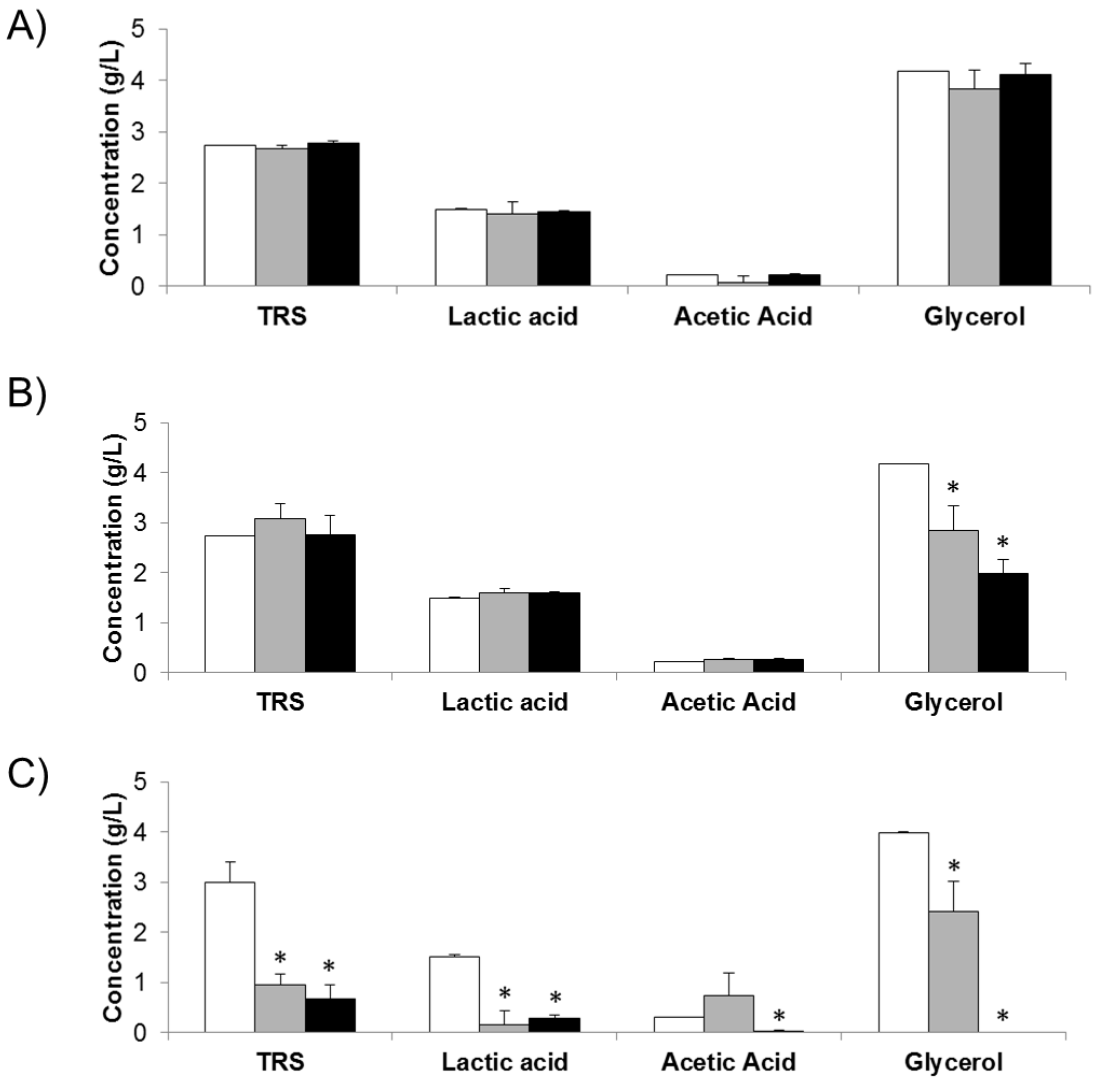

Figure 3 – O*rganic compounds concentration in crude vinasse cultures:* Microalgae strains were grown in crude vinasse for eight days using aerated erlenmeyers flasks at 26±1°C under the following conditions: (A) Axenic under dark; (B) Axenic under 12h/12h light/dark regimen; (C) Non-axenic under 12h/12h light/dark regimen. The concentration of TRS (Total Reducing Sugars: glucose + frutose + sucrose), lactic acid, acetic acid and glycerol present in culture supernatants was measured through HPLC analysis at day 0 (□) and after 8 days of *Micractinium* sp. Embrapa|LBA#32 (■) and *Chlamydomonas biconvexa* Embrapa|LBA#40 (■) cultivation. Experiments were conducted in triplicates (n=3), and the results are presented as means of the three replicates expressed as mean ± error bars. Paired t-test was performed to determinate significant variations in the samples in comparison to uncultured vinasse. Variation was considered significant (*) if p-value < 0.05 compared to the initial condition (day 0).

3.3. Cultivation of microalgae in vinasse using air lift flat plate photobioreactors

Cultivation of *Micractinium sp.* Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 was scaled-up in 13L air lift flat plate photobioreactors (Supplementary Figure 1) aerated with atmospheric air enriched with 5% CO_2 under non-axenic conditions. However, the initial attempts to perform cultivation using crude vinasse (pH 8.0) failed due to outgrowth of contaminants, especially fungi (data not shown). In order to circumvent this limitation, two strategies were employed to the increase of light transmittance and, consequently, favor algal growth in vinasse: (i) dilution in distilled water and (ii) chemical clarification. Augments up to 74% and 67,5% in light transmittance at wavelengths > 600 nm could be observed in 50% diluted vinasse and clarified vinasse, respectively (Supplementary figure 2). Figure 4 shows that the biomass productivities achieved by *Micractinium sp.* Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 using 50% diluted vinasse and clarified vinasse were higher than that observed for BBM. Microscopic inspection of cultures confirmed microalgae as the most abundant organisms present with limited contamination by other microrganisms (data not shown).

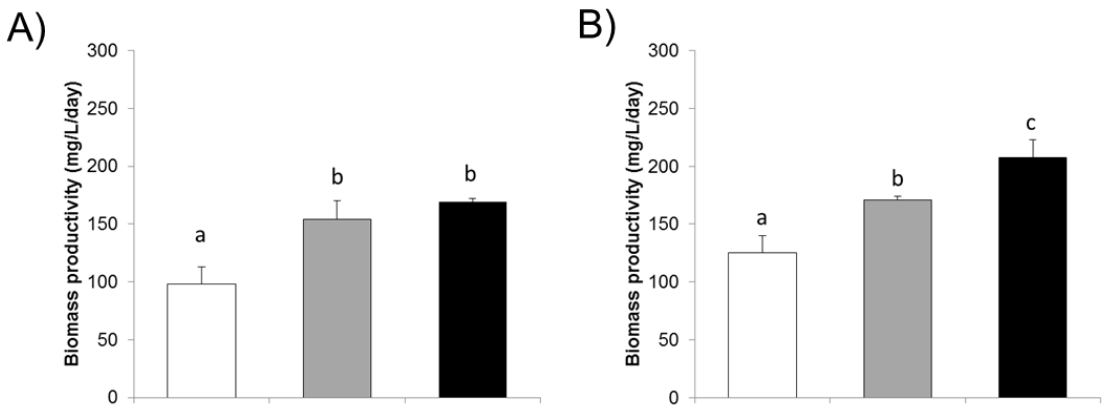

Figure 4 – *Microalgae biomass productivity in air lift flat-plate photobioreactors.* The strains *Micractinium* sp. Embrapa|LBA#32 (A) and *Chlamydomonas biconvexa* Embrapa|LBA#40 (B) were cultivated in air lift flat plate photobioreactors using BBM (Bold's Basal Medium) (□), 50% diluted vinasse (■) or clarified vinasse (■). Cultivation was conducted with aeration of atmospheric air supplemented with 5% CO_2 under a 12h/12h light/dark regimen and a temperature regimen of 37±1°C during light period and 24±1°C during dark period**.** Biomass was harvested at the end of the exponential phase (3^{rd} day), dried and weighted for productivity determination. Experiments were conducted in triplicates (n=3), and the results are presented as means of the three replicates expressed as mean ± error bars. Statistical analysis were performed with One-way ANOVA using Tukey post-test. Differences were considered significant if p-value < 0.05. Different letter (a, b, c) means statistically different.

3.4. Algal biomass biochemical composition

The biomass obtained with the cultivation of the *Embrapa*|LBA#32 and *Embrapa*|LBA#40 strains in vinasse-based media and in BBM were analyzed for the presence of bioproducts that have commercial interest in industry, such as carbohydrates, proteins, carotenoids, lipids and fatty acid methyl esters (FAME).

In this study, was possible to observe that, for both strains, the cultivation in vinasse-based media have induced the microalgae to increase its protein content (%), but not carbohydrates, carotenoids and FAME, that showed an significant reduction when compared to the cultivation in BBM. However, when the productivity of these organics compounds were analyzed, was possible to notice significant increase in its productivity, except for carotenoid and FAME (Table 1), results that are associated with the increased productivity of the biomass in the vinasse-based media.

Table 1 – P*roductivity and composition of microalgal biomass produced in a flat-plate photobioreactor. Micractinium* sp. Embrapa|LBA#32 and *Chlamydomonas biconvexa* Embrapa|LBA#40 *biomass Embrapa|LBA#32 s*train were cultivated for 3 days in BBM (Bold's Basal Medium), clarified vinasse and 50% diluted vinasse. Cultivation was conducted with atmospheric air aeration supplemented with 5% CO_2 in a 12/12h light/dark regimen and a temperature regimen of 37±1°C during light period and 24±1°C during dark period**.** Experiments were conducted in triplicates (n=3) and the results are presented as means of the three replicates expressed as mean ± error bars. Statistical analysis was performed with one-way ANOVA using Tukey post-test . Differences were considered significant if p-value < 0.05 (a, b, c). Different letter (a, b, c) means statistically different.

	***Micractinium sp.* Embrapa|LBA#32**			***Chlamydomonas biconvexa* Embrapa|LBA#40**		
	100% BBM	**100% Clarified Vinasse**	**50% Diluted Vinasse**	**100% BBM**	**100% Clarified Vinasse**	**50% Diluted Vinasse**
Carbohydrates content (%)	28.21 (± 0.24)a	21.79 (± 0.36)b	17.55 (±0.03)c	31.61 (±0.18)a	11.71 (±0.14)b	13.50 (±0.76)c
Carbohydrates productivity (mg/L/day)	28.50 (± 4.29)a	35.87 (± 3.98)a	31.20 (± 1.05)a	31.79 (± 4.88)a	26.03 (± 1.22)b	24.58 (± 2.48)b
Protein content (%)	34.03 (± 0.10)a	39.62 (± 0.09)b	39.50 (± 0.47)b	30.96 (±0.17)a	39.92 (±0.60)b	41.68 (±0.35)c
Protein productivity (mg/L/day)	34.40 (± 5.36)a	65.15 (± 6.20)b	70.20 (± 1.20)b	40.94 (±4.94)a	88.71 (±4.83)b	71.27 (±6.29)b
Carotenoid content (μg/g)	669.85 (± 10.86)a	26.34 (± 3.15)b	192.08 (± 24.66)c	805.26 (±174.46)a	12.97 (±0.01)b	165.61 (±22.58)b
Carotenoid productivity (μg/L/day)	19.10 (± 2.98)a	0.95 (± 0.18)b	5.99 (± 0.74)c	33.94 (± 9.74)a	0.33 (±0.02)b	4.05 (±0,54)b
FAME content (%)	3.23 (± 0.55)a	2.50 (± 1.30)a	2.21 (± 0.44)a	2.06 (± 0.35)a	1.26 (± 0,07)bc	1.58 (± 0.21)ac
FAME productivity (mg/L/day)	3.22 (± 0.45)a	3.99 (± 1.64)a	3.94 (± 0.90)a	2.73 (± 0.64)a	2.79 (± 0.26)a	2.88 (± 0.50)a
Calorific value (cal/g)	5037.81 (± 16.25)a	5260.21 (± 44.65)b	5184.49 (± 36.85)b	5069.56 (± 34.53)a	5114.23 (± 19.35)ac	5137.54 (± 17.86)bc
Ash content (%)	3.21 (± 0.30)a	5.83 (± 0.36)b	6.00 (± 0.15)b	5.44 (±0.28)a	6.67 (±0.31)bc	6.09 (±0.54)ac

In order to determine the potential use of the biomass as a fuel, the calorific value was also analyzed. For the *Embrapa*|LBA#32 strain, was observed an increase in the calorific value when the biomass was obtained for both vinasse-based media when compared to that observed in BBM. For the *Embrapa*|LBA#40 strain was possible to notice an increase in the calorific value when the microalgae was cultured in the diluted vinasse, but not in clarified vinasse (Table 1).

The ash content (%) of the biomass was also determined, in order to determine how much of the biomass obtained is composed by inorganic compounds. The results shows that for the *Embrapa*|LBA#32 strain, occur an increase in ash content during the cultivation in both vinasse-based media. For *Embrapa*|LBA#40 strain, however, the ash content was significantly increased only when this microalgae was cultured in clarified vinasse (Table 1).

3.5. Effect of algal growth upon vinasse composition

The concentration of major nutrients (i.e.: NO_3, NO_2, NH_4, PO_4, K, organic carbon), as well as BOD and COD, of vinasse-based media before and after *Micractinium* sp. Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 cultivation in flat plate photobioreactors (Section 3.3.) was determined (Table 2). The growth of neither microalgae strain significantly altered the BOD, COD, organic carbon or PO_4 content of vinasse-based media (Table 2). On the other hand, NO_3 and K concentrations decreased after microalgae *Micractinium* sp. Embrapa|LBA#32 and *C. biconvexa* Embrapa|LBA#40 growth in both vinasse-based media (Table 2). NH_4 concentration was also diminished to undetectable levels in the supernatant of *C. biconvexa* Embrapa|LBA#40 cultured in clarified vinasse (Table 2). Taken together, these results suggest the uptake of N and K sources by microalgae during exponential growth in vinasse.

Table 2 – *Vinasse composition after microalgae cultivation*. Analysis of the supernatant of either 50% vinasse diluted or clarified vinasse cultures of strains *Micractinium* sp. Embrapa|LBA#32 and *Chlamydomonas biconvexa* Embrapa|LBA#40 after 3 days of cultivation in air lift flat plate photobioreactors. Cultivation was conducted with atmospheric air aeration supplemented with 5% CO_2 in a 12/12h light/dark regimen and a temperature regimen of 37±1°C during light period and 24±1°C during dark period**.** Experiments were conducted in triplicates (n=3) and the results are presented as means of the three replicates expressed as mean ± error bars. Statistical analysis was performed with one-way ANOVA using Tukey post-test . Differences were considered significant if p-value < 0.05 (a, b, c). Different letter (a, b, c) means statistically different. N.D.= Not-detected (concentration < 5.0 mg/L)

	50% Diluted Vinasse			**Clarified Vinasse**		
	Initial condition	**LBA#32 culture supernatant**	**LBA#40 culture supernatant**	**Initial condition**	**LBA#32 culture supernatant**	**LBA#40 culture supernatant**
Biochemical Oxygen Demand (g/L)	3.74 (±0,20)a	4.98 (±0.21)a	5.36 (±1.45)a	9.64 (±0.69)a	11.73 (±2.09)a	12.23 (±2.50)a
Chemical Oxygen Demand (g/L)	8.93 (±1.45)a	12.08 (±0.25)a	13.38 (±2.51)a	22.65 (±2.64)a	26.85 (±0.78)a	27.98 (±0.32)a
Total organic carbon (mg/L)	13.14 (±0.68)a	12.59 (±0.29)a	10.87 (±0.97)a	25.66 (±1.85)a	23.80 (±1.95)a	24.42 (±1.47)a
Nitrate (mg/L)	21.49 (±1.24)a	11.50 (±0.00)b	13.00 (±1.41)b	39.41 (±0.99)a	21.00 (±1.41)b	27.25 (±1.06)c
Nitrite (mg/L)	0.10 (±0.01)a	0.07 (±0.01)a	0.09 (±0.01)a	0.15 (±0.02)a	0.27 (±0.06)a	0.27 (±0.04)a
Ammoniacal nitrogen (mg/L)	N.D.	N.D.	N.D.	9.25 (±2.52)a	5.63 (± 0.88)a	N.D.
Phosphate (mg/L)	12.01 (±0.35)a	11.45 (±2.05)a	21.20 (±4.53)a	18.54 (±3.29)ab	19.05 (±0.64)a	15.15 (±1.20)b
Total potassium (mg/L)	10.77 (±0.31)a	1.37 (±0.22)b	1.32 (±0.15)b	11.18 (±0.27)a	3.00 (±0.08)b	3.09 (±0.05)b

4. **Discussion**

4.1. Cultivation of microalgae in vinasse can increase biomass productivity

Using vinasse for microalgae cultivation can be a strategy to reduce the costs of biomass production, since the nutrient required for the production of microalgae biomass contributes significantly for the non-competitive prices of some the microalgae-derived products (Christenson & Sims, 2011; Singh & Gu, 2010).

In this work, was possible to select two strains of microalgae that were able to grow in sugarcane vinasse. However, not all strains cultured in this effluent are able to grow using sugarcane as the only nutrient source. In fact, during the selection of strains for growth in sugarcane vinasse, only two out of 40 strains screened were able to grow in this effluent. This result are congruent to that observed in literature, in which few strains were reported to be able to grow using vinasse as only nutrient source. In studies performed with *Spirulina maxima* and *Chlamydomonas reinhardtii*, the presence of vinasse in the medium, even at low concentrations, inhibit microalgae growth (dos Santos et al., 2016; Kadioğlu & Algur, 1992). However, there are reports on the successful cultivation of microalgae in synthetic medium supplemented with vinasse, though with a significant reduction in productivity when compared to using synthetic medium only (Ramirez et al., 2014). Besides, the problem in the use of this strategy for microalgae cultivation is that synthetic nutrients are still required, which increases the costs for microalgae cultivation.

As showed in the results, the selected strains *Embrapa*|LBA#32 and *Embrapa*|LBA#40, strains of the genera *Micractinium sp.* and *Chlamydomonas sp.*, respectively, showed a productivity similar or even higher in sugarcane vinasse, under different conditions, than that observed during the cultivation in synthetic medium. These results shows the potential of these strains for cultivation using alternative media as the only nutrient source, a required step to obtain cost reduction of biomass production in a large scale.

4.2. Selected microalgae shows mixotrophic metabolism

As well known, microalgae can be cultivated in photoautotrophic systems, where cells harvest light and use CO_2 as carbon source; in heterotrophic systems, in which the organism use organic carbon (e.g. glucose, acetate) added to the medium; and in a mixotrophic system, where microalgae can assimilate both CO_2 and organic carbons for metabolization. However, not all microalgae can be cultivated under heterotrophic conditions (Perez-Garcia et al., 2011b).

In this work, the *Embrapa*|LBA#32 and *Embrapa*|LBA#40 biomass accumulation seems to be strongly dependent of light. As showed in figure 2, both strains did not show significant increase in biomass at the end of the cultivation when there was no illumination in the cultivation system, indicating that the growth of both strains is light dependent. The results are consistent with the findings of Gris and coworkers (2014), in which microalgae exposed to more intense light source show higher productivity than those cultured under less incidence or absence of light. However, only these data cannot determinate if the strains have photoautotrophic or mixotrophic metabolism.

As can be seem in literature, some strains of the genera *Micractinium sp.* and *Chlamydomonas sp.* were able to grow in wastewaters due their mixotrophic metabolism (Bouarab et al., 2004; Kothari et al., 2013; Wang & Park, 2015) and similar results were observed in this work. During the cultivation of *Embrapa*|LBA#32 and *Embrapa*|LBA#40 strains in crude vinasse, analysis for the presence of organic compounds were performed. The data obtained showed that both strains can partly metabolize glycerol during its growth under illuminated conditions, but not total reducing sugars (TRS), lactic acid, acetic acid and glycerol, indicating that these microalgae perform mixotrophic metabolism using glycerol. This result is corroborated by the fact that was not observed significant metabolism of this organic compound when the cultivation was performed in the dark. Studies have reported that some mixotrophic microalgae strains cultured with glycerol can metabolize this organic compound, using it as primary carbon source (Ceron Garcia et al., 2006; Chi et al., 2007; Ethier et al., 2011; Pyle et al., 2008). The reduction of glycerol levels on vinasse is an indicative that the strains *Embrapa*|LBA#32 and *Embrapa*|LBA#40 could perform mixotrophic metabolism,

but further studies are required to determine the pathway involved with glycerol metabolization.

4.3. High biomass productivity during the cultivation in non-axenic photobioreactors

The scaling of the cultivation system is an essential step in order to integrate the microalgae cultivation into the ethanol industry, using a biorefinery strategy. During the cultivation of microalgae at a laboratorial scale, is possible to control various parameters that can affect the microalgae growth, as the presence of contaminants or the system temperature. In a large-scale cultivation, those parameters cannot be easily controlled, which makes it necessary to use strains that are not easily affected by those variations and that are competitive against contaminants (Christenson & Sims, 2011).

The results of the cultivation show that the productivity of the *Embrapa*|LBA#32 and *Embrapa*|LBA#40 strains are significantly higher in vinasse-media than that obtained with BBM, indicating that the selected strains have potential for biomass accumulation in this cultivation system. It is interesting to observe that these strains have shown a significant yield in a short time. In similar works, in which microalgae were cultivated in wastewaters, more time was required to achieve similar yields (Liu et al., 2012; Prandini et al., 2016; Ramirez et al., 2014). It can be explained by the different light intensity applied to the cultures on these works. As reported in several studies, the light is one of the key factors affecting the microalgae growth. Jacob-Lopes and coworkers (2009) have demonstrated that microalgae exposed to longer light regimen present higher yields in comparison to that cultured with reduced light exposition times. Others studies have also demonstrated that the intensity of the light directly affects the microalgae yield, despite the fact that too high light intensities can affect the lipid accumulation or microalgae growth on some strains (Ho et al., 2012; Qiang & Richmond, 1996; Solovchenko et al., 2008; Yeesang & Cheirsilp, 2011). Considering these data, clarification processes of the sugarcane vinasse were successful to increase the biomass productivity in the cultivation based in vinasse. Due the high turbidity of the vinasse (Data not shown), the light penetration at the chlorophyll absorption

peaks wavelengths is reduced, compromising the photosynthesis by the microalgae strains. This was observed when the cultivation was performed with crude vinasse in the photobioreactors, when small microalgae biomass productivity was observed. The clarification processes performed prior the cultivation, increased the light penetration and thus, the productivity of biomass in the vinasse-based media, indicating that both clarification can be used to increase the productivity of microalgae strains.

4.4. Excess of nitrogen induces the accumulation of protein

The biomass obtained with the cultivation of the *Embrapa*|LBA#32 and *Embrapa*|LBA#40 strains in vinasse formulations or BBM were analyzed for its composition. As well-know, microalgae can produced various bioproducts that have commercial interest in industry. Carbohydrates for bioethanol production, proteins for use as feedstock, and lipids for biodiesel, are the organic compounds with the major interest for future utilization, but algal biomass can also be used with other purposes (Benemann, 2013; Pulz & Gross, 2004). The production of these compounds depends on the microalgae strain cultured and the cultivation conditions.

In this study, was observed that in vinasse-base media, the nitrate levels reduced significantly after the cultivation of both strains. These reductions were associated with the increase in protein accumulation (%) during the cultivation of both strains tested in vinasse formulations. These data shows that the high disponibility of nitrogen in the media seems to induce the accumulation of protein instead of reserve compounds, such as carbohydrates and lipids, including FAME. In fact, results as that obtained by Wang and coworkers (Wang et al., 2013), shows that exists an inverse correlation between protein accumulation and carbohydrates and/or lipid content in the biomass. This correlation was also observed by Ho *et al* (2012; 2013)., in which cultivation of microalgae with nitrogen depletion leads to increased lipid or carbohydrate accumulation, with reduction in the protein content. However, further studies are still necessary to stablish the correlation among these factors in sugarcane vinasse.

Despite the correlation between the nitrogen levels and the carbohydrate content, when the carbohydrate productivity was evaluated in the strain *Embrapa*|LBA#32, was observed a significant increase in the productivity of this compound when the cultivation was performed in vinasse-based media, indicating an potential of use as carbohydrate source. However, the cultivation needs to be optimized in order to induce the accumulation of this compound. Nitrogen depletion, alkaline pH, and optimum CO_2 supplementation are required conditions for the accumulation of carbohydrates (Chen et al., 2013; Markou et al., 2012).

Carotenoids are pigments produced in order to contribute for light harvesting during the photosynthesis, acting as well as photoprotective agents. The excess of nitrogen in the media, however, also seems to affect the carotenoids accumulation when the microalgae were cultured in vinasse-based media. Our data showed that during the cultivation in vinasse formulations, the content (%) of total carotenoids reduced significantly for both strains when compared to the levels observed in BBM. Similar correlation was observed when cultivation of microalgae were performed with nitrogen limitation. In this condition, the accumulation of carotenoids, as the astaxanthin, increases when nitrogen limitation in the cultivation medium exists (Del Campo et al., 2007; Del Campo et al., 2000).

5. Conclusions

In this study, the results showed that the cultivation in vinasse is able to increase the biomass productivity of the cultivated biomass in comparison to the synthetic media even in non-axenic conditions. This characteristic is important during the large-scale cultivation due the fact that in this situation, microalgae needs to show good productivity in adverse situations, such the presence of biological contaminants. The biomass obtained with this cultivation has potential for its use as feedstock due the increased protein content in the biomass, but for biofuels productions, studies are required in order to increase the productivity of the compounds required for its production, such as carbohydrates. In conclusion, sugarcane vinasse can be used for microalgae cultivation using a biorefinery strategy but studies are necessary in order to improve microalgae cultivation in this effluent.

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References

Benemann, J. 2013. Microalgae for biofuels and animal feeds. *Energies*, **6**(11), 5869-5886.

Benemann, J.R. 2003. Bio-fixation of CO2 and greenhouse gas abatement with microalgae-technology roadmap.

Bouarab, L., Dauta, A., Loudiki, M. 2004. Heterotrophic and mixotrophic growth of Micractinium pusillum Fresenius in the presence of acetate and glucose:effect of light and acetate gradient concentration. *Water Research*, **38**(11), 2706-2712.

Cavalett, O., Junqueira, T.L., Dias, M.O.S., Jesus, C.D.F., Mantelatto, P.E., Cunha, M.P., Franco, H.C.J., Cardoso, T.F., Maciel Filho, R., Rossell, C.E.V., Bonomi, A. 2011. Environmental and economic assessment of sugarcane first generation biorefineries in Brazil. *Clean Technologies and Environmental Policy*, **14**(3), 399-410.

Ceron Garcia, M., Camacho, F.G., Mirón, A.S., Sevilla, J.F., Chisti, Y., Grima, E.M. 2006. Mixotrophic production of marine microalga Phaeodactylum tricornutum on various carbon sources. *Journal of microbiology and biotechnology*, **16**(5), 689.

Chen, C.-Y., Zhao, X.-Q., Yen, H.-W., Ho, S.-H., Cheng, C.-L., Lee, D.-J., Bai, F.-W., Chang, J.-S. 2013. Microalgae-based carbohydrates for biofuel production. *Biochemical Engineering Journal*, **78**, 1-10.

Chi, Z., Pyle, D., Wen, Z., Frear, C., Chen, S. 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Process Biochemistry*, **42**(11), 1537-1545.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology advances*, **25**(3), 294-306.

Cho, D.-H., Ramanan, R., Heo, J., Kang, Z., Kim, B.-H., Ahn, C.-Y., Oh, H.-M., Kim, H.-S. 2015. Organic carbon, influent microbial diversity and temperature strongly influence algal diversity and biomass in raceway ponds treating raw municipal wastewater. *Bioresource technology*, **191**, 481-487.

Christenson, L., Sims, R. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnology Advances*, **29**(6), 686-702.

Christofoletti, C.A., Escher, J.P., Correia, J.E., Marinho, J.F.U., Fontanetti, C.S. 2013. Sugarcane vinasse: environmental implications of its use. *Waste management*, **33**(12), 2752-2761.

Del Campo, J.A., García-González, M., Guerrero, M.G. 2007. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. *Applied microbiology and biotechnology*, **74**(6), 1163-1174.

Del Campo, J.A., Moreno, J., Rodríguez, H., Angeles Vargas, M., Rivas, J.n., Guerrero, M.G. 2000. Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in Muriellopsis sp. (Chlorophyta). *Journal of Biotechnology*, **76**(1), 51-59.

Dias De Oliveira, M.E., Vaughan, B.E., Rykiel, E.J. 2005. Ethanol as Fuel: Energy, Carbon Dioxide Balances, and Ecological Footprint. *BioScience*, **55**(7), 593-602.

Dias, M.O.S., Junqueira, T.L., Jesus, C.D.F., Rossell, C.E.V., Maciel, R., Bonomi, A. 2012. Improving bioethanol production - Comparison between extractive and low temperature fermentation. *Applied Energy*, **98**, 548-555.

dos Santos, R.R., Araújo, O.d.Q.F., de Medeiros, J.L., Chaloub, R.M. 2016. Cultivation of Spirulina maxima in medium supplemented with sugarcane vinasse. *Bioresource Technology*.

Ethier, S., Woisard, K., Vaughan, D., Wen, Z. 2011. Continuous culture of the microalgae Schizochytrium limacinum on biodiesel-derived crude glycerol for producing docosahexaenoic acid. *Bioresource Technology*, **102**(1), 88-93.

Gris, B., Morosinotto, T., Giacometti, G.M., Bertucco, A., Sforza, E. 2014. Cultivation of Scenedesmus obliquus in photobioreactors: effects of light intensities and light–dark cycles on growth, productivity, and biochemical composition. *Applied biochemistry and biotechnology*, **172**(5), 2377-2389.

Hadi, S.I.I.A., Santana, H., Brunale, P.P.M., Gomes, T.G., Oliveira, M.D., Matthiensen, A., Oliveira, M.E.C., Silva, F.C.P., Brasil, B.S.A.F. 2016. DNA Barcoding Green Microalgae Isolated from Neotropical Inland Waters. *PLoS ONE*, **11**(2), e0149284.

Ho, S.-H., Chen, C.-Y., Chang, J.-S. 2012. Effect of light intensity and nitrogen starvation on CO2 fixation and lipid/carbohydrate production of an indigenous microalga Scenedesmus obliquus CNW-N. *Bioresource Technology*, **113**, 244-252.

Ho, S.-H., Huang, S.-W., Chen, C.-Y., Hasunuma, T., Kondo, A., Chang, J.-S. 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource Technology*, **135**, 191-198.

Hu, B., Min, M., Zhou, W., Li, Y., Mohr, M., Cheng, Y., Lei, H., Liu, Y., Lin, X., Chen, P. 2012. Influence of exogenous CO2 on biomass and lipid accumulation of microalgae Auxenochlorella protothecoides cultivated in concentrated municipal wastewater. *Applied biochemistry and biotechnology*, **166**(7), 1661-1673.

Huang, J.J., Cheung, P.C. 2011. Enhancement of polyunsaturated fatty acids and total carotenoid production in microalgae by ultraviolet band A (UVA, 365 nm) radiation. *Journal of agricultural and food chemistry*, **59**(9), 4629-4636.

Jacob-Lopes, E., Scoparo, C.H.G., Lacerda, L.M.C.F., Franco, T.T. 2009. Effect of light cycles (night/day) on CO2 fixation and biomass production by microalgae

in photobioreactors. *Chemical Engineering and Processing: Process Intensification*, **48**(1), 306-310.

Kadioğlu, A., Algur, Ö.F. 1992. Tests of media with vinasse for Chlamydomonas reinhardii for possible reduction in vinasse pollution. *Bioresource technology*, **42**(1), 1-5.

Kang, Z., Ramanan, R., Yang, J.W., Kim, H.S. 2015. A cost analysis of microalgal biomass and biodiesel production in open raceways treating municipal wastewater and under optimum light wavelength. *Journal of microbiology and biotechnology*, **25**(1), 109-118.

Kong, Q.-x., Li, L., Martinez, B., Chen, P., Ruan, R. 2010. Culture of microalgae Chlamydomonas reinhardtii in wastewater for biomass feedstock production. *Applied biochemistry and biotechnology*, **160**(1), 9-18.

Kothari, R., Prasad, R., Kumar, V., Singh, D.P. 2013. Production of biodiesel from microalgae Chlamydomonas polypyrenoideum grown on dairy industry wastewater. *Bioresource Technology*, **144**, 499-503.

Liu, J., Huang, J., Jiang, Y., Chen, F. 2012. Molasses-based growth and production of oil and astaxanthin by Chlorella zofingiensis. *Bioresource technology*, **107**, 393-398.

Lourenço, S.O., Barbarino, E., Lavín, P.L., Lanfer Marquez, U.M., Aidar, E. 2004. Distribution of intracellular nitrogen in marine microalgae: calculation of new nitrogen-to-protein conversion factors. *European Journal of Phycology*, **39**(1), 17-32.

Markou, G., Angelidaki, I., Georgakakis, D. 2012. Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. *Applied Microbiology and Biotechnology*, **96**(3), 631-645.

Nichols, H.W., Bold, H.C. 1965. Trichosarcina polymorpha gen. et sp. nov. *Journal of Phycology*, **1**(1), 34-38.

Ortegón, G.P., Arboleda, F.M., Candela, L., Tamoh, K., Valdes-Abellan, J. 2016. Vinasse application to sugar cane fields. Effect on the unsaturated zone and groundwater at Valle del Cauca (Colombia). *Science of The Total Environment*, **539**, 410-419.

Parnaudeau, V., Condom, N., Oliver, R., Cazevieille, P., Recous, S. 2008. Vinasse organic matter quality and mineralization potential, as influenced by raw material, fermentation and concentration processes. *Bioresource technology*, **99**(6), 1553-1562.

Perez-Garcia, O., Escalante, F.M., de-Bashan, L.E., Bashan, Y. 2011a. Heterotrophic cultures of microalgae: metabolism and potential products. *Water research*, **45**(1), 11-36.

Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Bashan, Y. 2011b. Heterotrophic cultures of microalgae: Metabolism and potential products. *Water Research*, **45**(1), 11-36.

Porra, R., Thompson, W., Kriedemann, P. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, **975**(3), 384-394.

Prandini, J.M., da Silva, M.L.B., Mezzari, M.P., Pirolli, M., Michelon, W., Soares, H.M. 2016. Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae Scenedesmus spp. *Bioresource technology*, **202**, 67-75.

Pulz, O., Gross, W. 2004. Valuable products from biotechnology of microalgae. *Applied microbiology and biotechnology*, **65**(6), 635-648.

Pyle, D.J., Garcia, R.A., Wen, Z. 2008. Producing Docosahexaenoic Acid (DHA)-Rich Algae from Biodiesel-Derived Crude Glycerol: Effects of Impurities on DHA Production and Algal Biomass Composition. *Journal of Agricultural and Food Chemistry*, **56**(11), 3933-3939.

Qiang, H., Richmond, A. 1996. Productivity and photosynthetic efficiency of Spirulina platensis as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. *Journal of Applied Phycology*, **8**(2), 139-145.

Qin, L., Wang, Z., Sun, Y., Shu, Q., Feng, P., Zhu, L., Xu, J., Yuan, Z. 2016. Microalgae consortia cultivation in dairy wastewater to improve the potential of nutrient removal and biodiesel feedstock production. *Environmental Science and Pollution Research*, 1-9.

Quinn, J.C., Davis, R. 2015. The potentials and challenges of algae based biofuels: A review of the techno-economic, life cycle, and resource assessment modeling. *Bioresource Technology*, **184**, 444-452.

Ramirez, N.N.V., Farenzena, M., Trierweiler, J.O. 2014. Growth of microalgae Scenedesmus sp in ethanol vinasse. *Brazilian Archives of Biology and Technology*, **57**(5), 630-635.

Singh, J., Gu, S. 2010. Commercialization potential of microalgae for biofuels production. *Renewable and Sustainable Energy Reviews*, **14**(9), 2596-2610.

Slade, R., Bauen, A. 2013. Micro-algae cultivation for biofuels: cost, energy balance, environmental impacts and future prospects. *Biomass and Bioenergy*, **53**, 29-38.

Solovchenko, A., Khozin-Goldberg, I., Didi-Cohen, S., Cohen, Z., Merzlyak, M. 2008. Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga Parietochloris incisa. *Journal of Applied Phycology*, **20**(3), 245-251.

Sydney, E., Da Silva, T., Tokarski, A., Novak, A., De Carvalho, J., Woiciecohwski, A., Larroche, C., Soccol, C. 2011. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. *Applied Energy*, **88**(10), 3291-3294.

Van Wychen, S., Laurens, L. 2013a. Determination of Total Carbohydrates in Algal Biomass. in: *NREL Laboratory Analytical Procedure*, Vol. 2016.

Van Wychen, S., Laurens, L. 2013b. Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification. in: *NREL Laboratory Analytical Procedure*, Vol. 2016.

Van Wychen, S., Laurens, L. 2013c. Determination of Total Solids and Ash in Algal Biomass. in: *NREL Laboratory Analytical Procedure*, Vol. 2016.

Wang, L., Li, Y., Sommerfeld, M., Hu, Q. 2013. A flexible culture process for production of the green microalga Scenedesmus dimorphus rich in protein, carbohydrate or lipid. *Bioresource Technology*, **129**, 289-295.

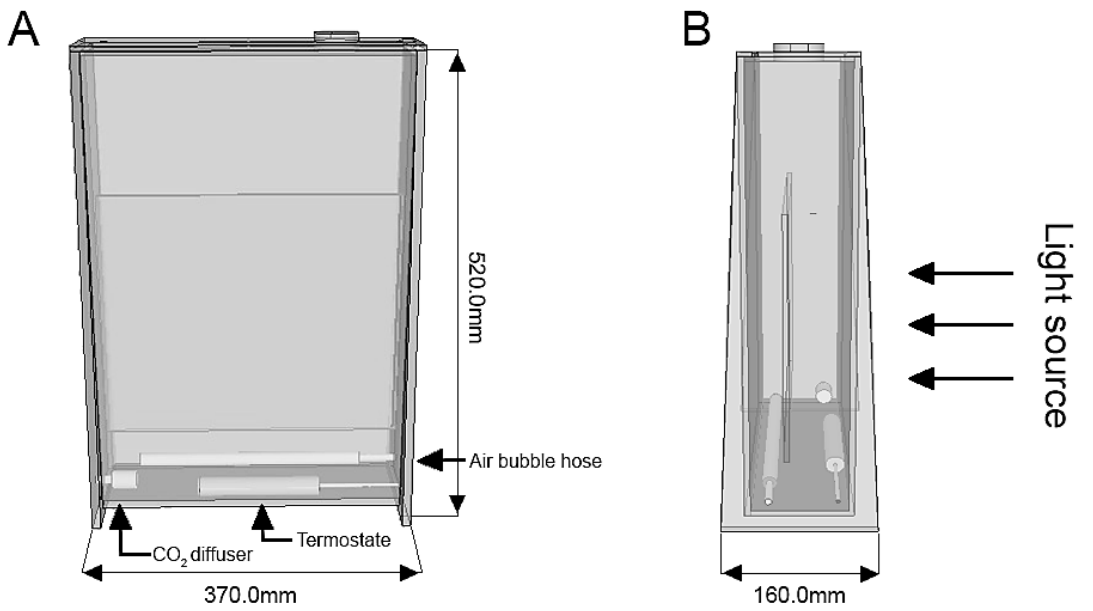
Wang, M., Park, C. 2015. Investigation of anaerobic digestion of Chlorella sp. and Micractinium sp. grown in high-nitrogen wastewater and their co-digestion with waste activated sludge. *Biomass and Bioenergy*, **80**, 30-37.

Wilson, M., Groppo, J., Placido, A., Graham, S., Morton III, S., Santillan-Jimenez, E., Shea, A., Crocker, M., Crofcheck, C., Andrews, R. 2014. CO2 recycling using microalgae for the production of fuels. *Applied Petrochemical Research*, **4**(1), 41-53.

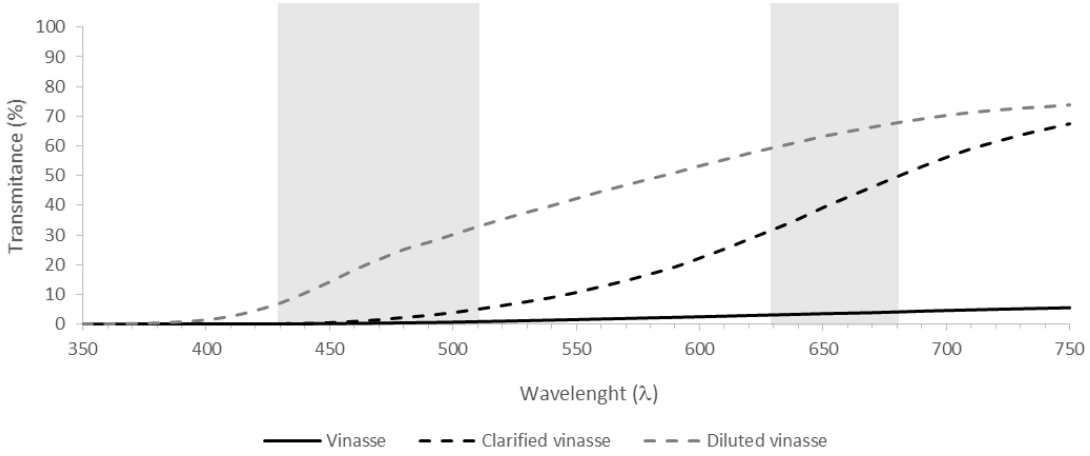
Woertz, I., Feffer, A., Lundquist, T., Nelson, Y. 2009. Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *Journal of Environmental Engineering*, **135**(11), 1115-1122.

Yeesang, C., Cheirsilp, B. 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. *Bioresource Technology*, **102**(3), 3034-3040.

Supporting Information



Supplementary figure 1 – *Schematic view of the air lift flat panel photobioreactor.* (A) Front view; (B) Lateral view.



Supplementary figure 2– *Transmittance spectrum of vinasse before and after different clarification treatments.* Shadded area represents the peaks of highest light absorbance of chlorophyll in the visible spectrum.

9. CONSIDERAÇÕES FINAIS

Microalgas são organismos fotossintetizantes que podem crescer em diversos ambientes aquosos. Muitos destes organismos podem produzir bioprodutos de interesse com carboidratos, lipídios e proteínas que podem ser utilizados para a produção de biocombustíveis ou de ração. Apesar disto, a obtenção destes produtos em larga escala ainda é cara e necessita do uso de estratégias para minimizar os custos de produção. Neste trabalho, foi realizada a avaliação do potencial de produção de biomassa microalgal utilizando vinhaça suplementada com CO_2 como meio de cultivo. Para isto, 52 cepas de microalgas isoladas de diversos biomas brasileiros e locais de lançamento de resíduos agroindustriais foram identificadas e avaliadas quanto a este potencial. Dentre 40 cepas foram avaliadas, duas se mostraram adequadas para o cultivo no sistema proposto neste trabalho, *Embrapa*|LBA#32 e *Embrapa*|LBA#40. Estas cepas se mostraram interessantes para a produção de biomassa uma vez que sua produtividade ao final do processo foi maior do que o observado em meio sintético BBM, indicando o potencial de aplicação deste sistema de cultivo a uma indústria sucro-energética utilizando uma estratégia de biorrefinaria para redução dos custos de produtividade da biomassa microalgal. A biomassa obtida, apresentou características que indicam que a biomassa poderia ser utilizada como fonte de proteínas. No entanto, a biomassa gerada nas condições de cultivo testadas não apresenta características adequadas para a produção de biocombustíveis. Sendo assim, a otimização do processo produtivo para que a biomassa gerada neste sistema de cultivo seja adequada à produção de combustíveis. Em conclusão, os resultados deste trabalho demonstram que microalgas obtidas de diversidade brasileira podem ser utilizadas para a produção de biomassa em sistemas de cultivo à base de vinhaça. Desta maneira, a microalgas selecionadas poderiam ser utilizadas em uma estratégia

de biorrefinaria para a produção de biomassa ou bioprodutos em um sistema de cultivo que utiliza vinhaça e CO_2.