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PRODUÇÃO DE BIOMASSA DE *Euglena* sp. UTILIZANDO FERTILIZANTES INORGÂNICOS TIPO NPK COMO CULTIVOS ALTERNATIVOS

Santa Maria, RS

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Dissertação apresentada ao Curso de Pós-Graduação em Agrobiologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Agrobiologia.**

Orientadora: Profª. Drª. Maria Angélica Oliveira Linton

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RESUMO

PRODUÇÃO DE BIOMASSA DE *Euglena sp.* UTILIZANDO FERTILIZANTES INORGÂNICOS TIPO NPK COMO CULTIVOS ALTERNATIVOS

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A utilização de microalgas com fins comerciais tem aumentado nos últimos anos em virtude de seus produtos apresentarem grande potencial biotecnológico e demanda comercial. Contudo, problemas no sistema de cultivo fazem com que a produtividade seja baixa e os custos de produção altos. Assim, o meio de cultivo torna-se um fator fundamental para a manutenção e o desenvolvimento de microalgas. O presente trabalho tem por objetivo avaliar a influência do uso de fertilizantes inorgânicos em diferentes proporções de nitrogênio (N) fósforo (P) e potássio (K) como meios alternativos para o cultivo da microalga *Euglena* sp. Os experimentos foram conduzidos em fotobiorreatores tubulares, sob sistema de operação descontínua, aeração contínua, reator isotérmico operando em 25 °C, intensidade luminosa de 1 klux. Parâmetros cinéticos, perfil bioquímico e de aminoácidos foram avaliados. O meio NPK 20:10:15 apresentou a maior concentração celular 687,36 mgL^{-1} e uma produtividade em biomassa de 2,74 $mgL^{-1}d^{-1}$ sendo 3 vezes maior que o controle ($p<0,05$). A biomassa de *Euglena* sp. apresentou alto conteúdo de proteínas, lipídeos e carboidratos além de todos os aminoácidos essenciais. Os meios de cultivo com base em fertilizantes inorgânicos tipo NPK foram adequados para cultivar *Euglena* sp. A biomassa de *Euglena* obtida tem o nível nutricional aceitável para se utilizar como alimento ou suplemento alimentar para humanos ou animal, com o custo de produção baixo.

Palavras-chave: Aminoácidos. Microalgas. Nutrição.

ABSTRACT

BIOMASS PRODUCTION OF Euglena sp. USING NPK INORGANIC FERTILIZERS AS ALTERNATIVE MEDIUM

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The use of commercial microalgae has increased in recent years due to its products that have great biotechnological potential and commercial demand. However, problems in the cultivation system mean that productivity is low and production costs are high. Thus, the culture medium becomes a fundamental factor for the maintenance and development of microalgae. The aim of this work is to evaluate the influence of the use of inorganic fertilizers in different proportions of NPK as alternative medium for the cultivation of *Euglena* sp. The experiments were conducted in tubular photobioreactors under a discontinuous operation system, continuous aeration, isothermal reactor at 25 °C, light intensity of 1 klux. Kinetic parameters, biochemical profile and amino acids were evaluated. The NPK 20:10:15 medium showed the highest cell concentration 687.36 mgL^{-1} and a biomass productivity of 2.74 $mgL^{-1}d^{-1}$ being 3 times greater than the control ($p < 0.05$). Biomass of *Euglena* sp showed high content of proteins, lipids and carbohydrates in addition to all the essential amino acids. The culture medium based on inorganic fertilizers NPK-type were suitable for cultivating Euglena sp. The Euglena biomass obtained has the nutritional level acceptable for use as food and feed products, with a low cost of production.

Keywords: Amino acids. Microalgae. Nutrition.

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1 INTRODUÇÃO

Nas últimas décadas, o cultivo industrial de microalgas tem aumentado devido ao interesse por aplicações comerciais. Atualmente microalgas são utilizadas para nutrição humana, cosméticos, ração para animais e princípios ativos para fármacos (STEPHENS et al., 2013). Outra importante característica das microalgas é que podem ser utilizadas para o tratamento de águas residuais, fixação de nitrogênio e mitigação de CO_2 (HARUN et al., 2010).

As microalgas são microrganismos com metabolismo fotossintético que se multiplicam rapidamente, gerando metabólitos primários e secundários valiosos que são facilmente purificados (CUELLAR-BERMUDEZ et al., 2015). O cultivo de microrganismos fotossintéticos apresenta um grande potencial na biotecnologia industrial, porém, fatores como a configuração dos biorreatores, temperatura, transferência de gases, pH, agitação e compostos nutritivos no meio de cultura devem ser otimizados para atingir produções de biomassa em grande escala e com custo competitivo no mercado (SINGH & SHARMA, 2012).

No cultivo em grande escala de microalgas são utilizados, preferencialmente, sistemas abertos tipo tanque *raceway* e circular devido a seu baixo custo em relação aos sistemas fechados (SLEGERS et al., 2013). Porém, para melhorar a produção de microalgas neste tipo de sistema, necessita-se utilizar diferentes estratégias de cultivo (contínuo ou em *batch*), meios de cultura adequados para cada espécie e a reciclagem do meio de cultura desperdiçado. Reciclar o meio de cultura tem vantagens no aproveitamento de água e nutrientes e impede a liberação de nitrato em corpos d'água, o que pode levar a alterações no equilíbrio ecológico pela formação de florações de microalgas indesejáveis (DEPRAETERE et al., 2015)

A produção e produtividade de biomassa microalgal são determinadas por vários parâmetros, entre os quais se destacam a iluminação e os nutrientes (TAN et al., 2015). Em condições de crescimento ideais, as microalgas sintetizam proteínas para manter o crescimento celular, assim como carboidratos e lipídios que proporcionam o funcionamento adequado para manter o ciclo de vida celular (BIŠOVÁ & ZACHLEDER, 2014). Por isso, a formulação ideal do meio de cultura é fundamental para garantir um suprimento de nutrientes, visado atingir a taxa de crescimento máxima e, com isso, altos rendimentos de biomassa, além de proteínas

e lipídios que presentes em algumas espécies de microalgas, agregam valor comercial na indústria alimentar ao serem classificadas como organismos com alto valor nutricional (SONI et al., 2017).

Para o desenvolvimento de cultivos autotróficos de microalgas estão descritos na literatura mais de trinta tipos de meios de cultivo (ANDERSEN, 2005). Alguns estão comercialmente disponíveis, sendo os meios conhecidos como o f/2 constituído de: $NaNO_3$ (75 g L^{-1}), $NaH_2PO_4.2H_2O$ (5,65 g L^{-1}), Na_2EDTA (4,16 g L^{-1}), $FeCl_3.6H_2O$ (3,15 g L^{-1}), $CuSO_4.5H_2O$ (0,01 g L^{-1}), $ZnSO_4.7H_2O$ (0,022 g L^{-1}), $CoCl_2.6H_2O$ (0,01 g L^{-1}), $MnCl_2.4H_2O$ (0,18 g L^{-1}), $Na_2MoO_4.2H_2O$ (0,006 g L^{-1}), cianocobalamina (0,0005 g L^{-1}), tiamina (0,1 g L^{-1}), biotina (0,0005 g L^{-1}), um meio que tem custo de aproximadamente US$ 60 por litro, de tal modo que, cultivos em grande escala estão sujeitos a problemas de alto custo (SLEGERS et al., 2013). Várias abordagens foram propostas para a redução de custos, como a substituição de nitrato de sódio ($NaNO_3$) por outras fontes de nitrogênio, tais como a ureia (CH_4N_2O) e/ou nitrato de potássio (KNO_3) (GODOY DANESI et al., 2011), uso de água do mar com e sem enriquecimento de bicarbonato de sódio ($NaHCO_3$) e $NaNO_3$ (GAMI et al., 2011), adição de substratos orgânicos (ANDRADE & COSTA, 2009) e uso de fertilizantes (EL NABRIS, 2012).

2 JUSTIFICATIVA

Os cultivos de microalgas com fins alimentícios devem apresentar baixos custos de produção para tornarem-se economicamente viáveis. Tendo em vista isso, busca-se aumentar a operabilidade nos processos da indústria microalgal. A utilização de fertilizantes inorgânicos (NPK) nos meios de cultura pode apresentar vantagens por estarem facilmente disponíveis, terem baixo custo, composição definida e pH neutro. Estes fertilizantes inorgânicos também apresentam elevadas concentrações de nitrogênio, fósforo e potássio, os quais são importantes para o metabolismo e, refletem nas características bioquímicas das células de microalgas induzindo maior acúmulo de biomassa.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a influência do uso de fertilizantes inorgânicos em diferentes proporções de NPK como meios alternativos para o cultivo de *Euglena* sp.

3.2 OBJETIVOS ESPECÍFICOS

- Analisar os dados cinéticos para a produção de biomassa de *Euglena* sp.
- Analisar a composição bioquímica da biomassa de *Euglena* sp.
- Determinar o perfil de aminoácidos presentes na biomassa de *Euglena* sp.
- Comparar os dados obtidos nas diferentes proporções de NPK em relação ao meio Kostir-Lotze.

4 REVISÃO DE LITERATURA

4.1 PROCESSO PRODUTIVO DE BIOMASSA MICROALGAL

As microalgas têm um grande potencial na geração de produtos de interesse biotecnológico e industrial. Entre estes, a produção de biocombustíveis e energia é de grande repercussão por sua natureza de fonte renovável. Igualmente, a produção de biomassa e subprodutos destinados à alimentação, suplementos alimentícios, nutracêuticos, produtos químicos e farmacêuticos tem feito com que muitos investidores vejam a indústria microalgal como um grande potencial de investimento (PRIYADARSHANI & RATH, 2012).

Os sistemas de cultivo utilizados para manter o crescimento das microalgas são de dois tipos: sistemas abertos ou fechados. Entre os diferentes tipos de design de sistema aberto, os mais populares são o tanque *raceway* e o tanque circular, enquanto os sistemas fechados populares incluem o fotobiorreator, que pode ser do tipo tubular, placa plana e híbrido (RAMÍREZ-MÉRIDA et al., 2013). A compreensão dos aspectos hidrodinâmicos e da tecnologia dos fotobiorreatores escaláveis são

aspectos que devem ser levados em consideração para obter bons rendimentos (RAMÍREZ-MÉRIDA et al., 2015).

A rápida taxa de crescimento juntamente com a alta produtividade faz com que a produção de biomassa de microalgas possua um futuro promissor. Várias pesquisas sobre o uso de microalgas para obter produtos com aplicabilidade industrial foram realizadas com sucesso (STEPHENS et al., 2013; ZHU, 2015; CHEN, et al., 2016; ZHU & HILTUNEN, 2016). O processo produtivo de biomassa microalgal pode ser dividido em duas fases: *upstream processing* (USP) e *downstream processing* (DSP). O USP corresponde aos passos prévios ao sistema de cultivo envolvendo quatro áreas: (i) a estirpe de microalgas, (ii) fornecimento de carbono, (iii) fonte de nutriente (nitrogênio/fósforo) e (iv) fonte de iluminação. O DSP inclui todos os processos envolvidos logo na saída do reator ou sistema de cultivo. Eles envolvem a colheita e técnicas de biorrefinaria para obter diferentes tipos de produtos a partir da biomassa, que podem ser comercializados (JACOB-LOPES et al., 2015).

4.2 SISTEMAS DE CULTIVO DE MICROALGAS

O cultivo bem sucedido de microrganismos fotossintéticos depende de diversos fatores como iluminação, temperatura, transferência de gases, pH, agitação e disponibilidade de nutrientes, estes fornecidos adequadamente através dos vasos de reação (RAMÍREZ-MÉRIDA et al. 2013).

O cultivo fotossintético microalgal depende essencialmente do aporte de energia luminosa às células. Fenômenos de foto-limitação e foto-inibição são frequentes em culturas iluminadas inadequadamente, ocasionando significativas perdas de desempenho cinético nos biorreatores (BRINDLEY et al., 2016). Além dos aspectos quantitativos, deve-se considerar a natureza qualitativa da luz incidente nos sistemas. Iluminação natural ou artificial pode ser utilizada em função das características requeridas nos sistemas de cultivo. Aspectos como localidade, variações sazonais, variações ao longo do fotoperíodo são as principais questões dos sistemas naturalmente iluminados (GEORGE et al., 2014).

A biomassa microalgal é composta basicamente por carbono, nitrogênio e fósforo, em proporções aproximadas de 50, 8 e 1%, respectivamente (ALASSALI et

al., 2016). Consequentemente, a disponibilidade destes nutrientes é essencial para a multiplicação celular nos biorreatores. Em nível de cultivo fotossintético, o dióxido de carbono é a principal fonte de carbono usada nos cultivos (KÖNST et al., 2017). Depois do carbono, o nitrogênio é o segundo elemento limitante dos cultivos microalgais. O nitrato é a fonte usual, embora ureia, amônia e fontes orgânicas sejam assimiladas por inúmeras espécies (SHILOVA et al., 2017). Por fim, o fósforo é o terceiro elemento de maior demanda nestes sistemas. O fósforo reativo é a especiação mais facilmente assimilável, embora fósforo ácido-hidrolisável e fósforo orgânico sejam eficientemente usados (ABDEL-RAOUF et al., 2012).

4.3 CULTIVO DE MICROALGAS E FERTILIZANTES AGRÍCOLAS

Durante as últimas décadas, vários estudos foram realizados para desenvolver formulações de meios para o cultivo de diferentes espécies de microalgas. Assim, variações nas proporções dos sais (CASTRO et al., 2015; RAJASEKARAN et al., 2016) e substituição de sais inorgânicos de pureza analítica por fertilizantes agrícolas como nutrientes (SIMENTAL & SÁNCHEZ-SAAVEDRA, 2003; ASHRAF et al., 2011; EL NABRIS, 2012; SIPAÚBA-TAVARES et al 2017) são artifícios que visam melhorar o rendimento de biomassa e produtos microalgais assim como reduzir custos no processo.

Cultivos de *Spirulina platensis* usando superfosfato simples (P_2O_5CaS), cloreto de potássio (KCl), nitrato de sódio ($NaNO_3$), cloreto de sódio (NaCl), sulfato de magnésio ($MgSO_4$), cloreto de cálcio ($CaCl_2$) e bicarbonato de sódio ($NaHCO_3$) apresentaram a mesma produção de biomassa e clorofila quando comparados com o meio Zarrouk, tradicionalmente utilizado para este gênero. Do ponto de vista da escala, o meio alternativo foi altamente econômico em relação ao meio Zarrouk (RAOOF et al., 2006).

Sob diferentes condições de cultivo, e diferentes concentrações de bicarbonato e nitrato de sódio, o crescimento e produtividade da biomassa de *Arthrospira platensis* aumentou com as maiores concentrações de bicarbonato de sódio utilizadas (16 g L^{-1}) indicando que o bicarbonato de sódio é o fator com maior influência na produção de biomassa (CASTRO et al., 2015).

Modificações feitas no meio Zarrouk com a eliminação de cinco micronutrientes (boro, manganês, cobre, zinco e molibdênio) e com a substituição do nitrato de sódio pelo nitrato de potássio, mostrou alto desempenho para o crescimento de microalgas, aumentando a taxa de crescimento específica, biomassa e clorofila–*a*. Este meio de Zarrouk modificado pode ser usado como uma variante média de baixo custo para o cultivo de microalgas (RAJASEKARAN et al., 2016).

Nwoye et al., (2017) avaliaram mediante cultivo mixotrófico o potencial de uso de fertilizantes tipo NPK como meio alternativo para *Euglena gracilis*, acrescentando etanol como fonte de carbono orgânico e peptona como fonte de nitrogênio orgânico. A concentração da biomassa atingiu 2,6 gL^{-1} nas proporções de NPK 15:15:15 concluindo que o fertilizante pode servir como um meio de cultura basal barato para a produção de biomassa de *Euglena gracilis*, mas que é preciso suplementar com outros componentes.

Ramírez-López et al., (2016) desenvolveram um novo meio para o cultivo de *Chlorella vulgaris* visando aumentar a concentração de biomassa e lipídios. A composição do meio apresentou diminuição das concentrações de nitrogênio de até 50% em relação aos meios de cultura convencionais (BBM, *Bold's Basal Medium* e HAMGM, *Highly Assimilable Minimal Growth Medium*). O novo meio formulado neste trabalho mostrou ser uma alternativa promissora para o cultivo de microalgas em grande escala, resultando no aumento na concentração de biomassa e lipídios em 40% e 85%, respectivamente.

Variações e modificações dos nutrientes no meio de cultura refletem nas características bioquímicas das células de microalgas. Assim, Chia et al. (2013) ao avaliarem três meios de cultivo comerciais (LC Oligo, Chu 10 e WC) no crescimento, conteúdo de biomassa e composição bioquímica de *Chlorella vulgaris*, verificaram maior densidade celular em meio LC Oligo, sendo mais baixo em meio Chu 10. Enquanto que, concentrações e rendimentos de carboidratos, lipídios e proteínas foram mais altos em meio Chu 10 e LC Oligo.

A limitação de nutrientes é um fator eficiente para aumentar o teor de lipídios na biomassa de microalgas. Em condições de limitações de nitrogênio (2,5 mg L^{-1}) e fósforo (0,1 mg L^{-1}) cepas de *Scenedesmus* sp acumulam 30 e 53 % de lipídios respetivamente em sua biomassa (XIN et al., 2010).

As elevadas concentrações de nitrogênio, fósforo e potássio que possuem os fertilizantes inorgânicos, faz com que possam ser utilizados como fonte alternativa na formulação de meios para o cultivo de microalgas, visando melhoras no rendimento (EL NABRIS, 2012).

4.4 EUGLENOIDES, CARACTERÍSTICAS GERAIS

Os euglenoides flagelados são um grupo parafilético de organismos unicelulares flagelados, de vida livre, relacionados a outros flagelados heterotróficos (Symbiontida, Diplonemea e Kinetoplastida), que juntos compõem a ordem dos Euglenozoa, que compartilham a presença de dois corpos basais e três raízes microtubulares dispostas assimetricamente, assim como a presença de uma haste paraxial em um ou ambos dos flagelos (BREGLIA et al., 2010).

O gênero Euglena caracteriza-se por possuir células não achatadas que sofrem movimentação dinâmica, caracterizada pela presença de pequenos paramilo e grandes cloroplastos, agrupando 164 espécies aceitas até o momento (Guiry e Guiry, 2018). A classificação taxonômica é a seguinte segundo Zakryś et al., (2017): **Filo**: Euglenozoa, **Classe**: Euglenophyceae, **Ordem**: Euglenales, **Família**: Euglenaceae, **Gênero**: Euglena.

Euglena pode ser cultivado em condições autotroficas, mixotroficas ou heterotróficas, pode crescer sobre uma ampla gama de pH e também em águas residuais contendo vários poluentes (SANTEK et al., 2012). *Euglena* é uma fonte adequada de numerosos compostos, tais como α-tocoferol, paramilo, ácidos graxos poliinsaturados, biotina e aminoácidos usados para produzir nutracêuticos, alimentos e cosméticos. Assim, este microrganismo pode ser utilizado para a biorremediação e produção biotecnológica de produtos de interesse comercial (WINTERS et al., 2016; KRAJČOVIČ et al., 2015).

A produtividade fotossintética de *Euglena* pode ser 60 vezes maior do que a planta de arroz, e a eficiência de conversão de CO_2 para O_2 é duas vezes maior do que em *Chlorella* (OGAWA et al., 2015).

A biomassa de *E. gracilis* tem uma qualidade nutricional mais elevada que, por exemplo, a biomassa de *Chlorella* ou *Spirulina* (NAKANO et al., 1995). A digestibilidade in vitro de *E. gracilis* é ligeiramente superior à da caseína, sugerindo

que pode ser utilizada como suplemento alimentar para animais (OGBONNA et al., 1999). Tomadas em conjunto, estas observações demonstram claramente a utilidade da biomassa de Euglena como matéria prima.

Inclusive, o crescimento de *Euglena* em esgoto purifica a água e produz uma biomassa a partir da qual os lipídios podem ser extraídos para biocombustíveis, sendo a biomassa restante usada como suplemento nutricional na alimentação animal e como fonte de alimento na aquicultura (GRIMM et al., 2015).

Devido a isso, a oportunidade de obter biomassa de *Euglena* visando seu uso na alimentação humana e animal, utilizando cultivos mais econômicos à base de fertilizantes inorgânicos NPK, é uma alternativa interessante para investidores e empreendedores da área de microalgas.

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6 ARTIGO

Study of *Euglena* growth based on NPK inorganic fertilizers as a culture medium for biomass production of nutritional interest

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Abstract

Microalgae production for food use has increased in recent decades. The use of cost-effective growth media is fundamental for profitable processes. The aim of this study was to evaluate the influence of the use of inorganic fertilizers based on N (nitrogen), P (phosphorous) and K (potassium) as an alternative medium for the cultivation of the photosynthetic freshwater microalga *Euglena* sp and to analyze the biochemical composition of the biomass produced. Cultures of *Euglena* sp were evaluated in four proportions of NPK: 15:11:11, 15:15:20, 20:10:15 and 10:10:10 in a tubular photobioreactor in discontinuous state with 2 L of medium, continuous aeration, temperature 25 °C, illumination intensity of 1 klux for 37 days. Kinetic parameters, biochemical and amino acid profiles were evaluated in the biomass obtained. Biomass concentration and productivity found in the alternative medium were significantly different ($p < 0.05$) to the Kostir-Lotze control medium. The highest biomass productivity was observed in NPK medium 20:10:15 (2.74 mg $L^{-1}d^{-1}$). The biochemical analysis of the *Euglena* biomass showed high percentages of protein, lipids and carbohydrates and possesses all the essential amino acids. Higher proportions of N:P in culture medium provide better performance in terms of biomass. The culture medium with NPK inorganic fertilizers was suitable for the culture of *Euglena* sp. The biomass obtained contains an acceptable nutritional level to be used in food formulations for both humans and animals with low production costs.

Key words: Amino acids, Culture, Microalgae, Nutrition

1. Introduction

The use of commercial microalgae has increased in recent years because its bioproducts have great biotechnological and industrial potential. Currently, microalgae biomass is used for human nutrition, cosmetics, animal feed and active ingredients for drugs [1]. Among the main compounds of interest are carbohydrates, proteins, lipids (primary metabolites); carotenoids, phycobiliproteins, phytosterols, phytohormones, phenolic compounds and amino acids (secondary metabolites) which have nutritional, neuroprotective, antioxidant, antimicrobial, anti-inflammatory, anticancer and anti-angiogenic proven properties [2, 3].

The genus *Euglena* is composed of single-celled flagellated freshwater organisms with photoautotrophic, photoheterotrophic or heterotrophic metabolism. Around 164 species of *Euglena* have been taxonomically accepted [4]. Some authors refer that *Euglena* species possess a high nutritional and digestibility quality. *Euglena* is an adequate source of various compounds used to produce cosmetics, nutraceuticals and foods [5]. Therefore, the commercial use of *Euglena* in the food industry, as a source of amino acids, vitamins and fatty acids, is feasible provided that there is availability of cost-effective culture medium that allows large-scale production of biomass at competitive costs in the market [6].

Microalgae large-scale cultures, open raceway and circular tank type systems are used preferentially because of their low cost in relation to closed systems [7]. However, to improve the production of microalgae in this type of system, it is necessary to use different culture strategies [8].

The production and productivity of microalgal biomass are determined by several parameters, including lighting and nutrients [9]. Under optimal growth conditions, microalgae synthesize proteins, carbohydrates and lipids to maintain cell growth, and provide adequate functioning to maintain the cellular life cycle [10]. Therefore, the ideal formulation of the culture medium is essential to guarantee a nutrient supply, aiming to reach the maximum growth rate and, with

this, high yields of biomass, besides proteins and lipids present in some species of microalgae, which add commercial value at the food industry when classified as organisms with high nutritional value [11].

For the development of autotrophic cultures of microalgae, more than thirty types of culture medium have been developed and reported in the literature (Andersen, 2005). Some, such as f/2, are commercially available and international cost about USD$ 60 - 90 per liter, such that large-scale microalgae cultures are subject to high-cost problems [7].

Therefore, efforts have been made to replace pure and expensive nutrient medium with cheaper commercial fertilizers. As with pure nutrient medium, nutrients in fertilizers are one of the principal factors that influence the growth and production of phytoplankton in aquaculture ponds [12].

The use of NPK inorganic fertilizers in the culture medium may have advantages because they are easily available, have low cost, defined composition and neutral pH. These inorganic fertilizers also contain high concentrations of nitrogen, phosphorus and potassium, which are important for metabolism and reflect on the biochemical characteristics of the microalgae cells inducing greater biomass accumulation.

This study was carried out in order to evaluate the performance of inorganic fertilizers in different NPK proportions as an alternative medium for *Euglena* sp culture as well as to analyze the chemical composition of the produced biomass, aiming at a future use of *Euglena* cultures as source of nutrient for both animals and humans.

2. Material and methods

2.1. Microorganism and culture medium

The cultures of *Euglena* sp were originally isolated from a pond on the campus of the Federal University of Santa María, Santa Maria, RS, Brazil (29°43'16.8"S 53°42'55.6"W). A sample was obtained with 25µm mesh plankton net and checked under a light microscope for

identification. Stock cultures were propagated and maintained on a Kostir-Lotze medium with the following composition: KH_3PO_4 (0,25 g L^{-1}), KCl (0,25 g L^{-1}), $NaHCO_3$ (0,25 g L^{-1}), $Ca(NO_3)_2$ (1,50 g L^{-1}), $MgSO_4$ (0,50 g L^{-1}), $FeCl_3.6H_2O$ (0,0004 g L^{-1}), $MnSO_4$ (0,0002 g L^{-1}), Thiamin HCl (0,0001 g L^{-1}) Vit B12 (0,0001 g L^{-1}) e Biotin (0,0001 g L^{-1}), pH 3,4. The incubation conditions used were 25 °C, photon flux density of 1 klux and a photoperiod of 12h.

The growth of *Euglena* sp. was conducted in the following culture medium: (K-L) control medium (Kostir-Lotze), (M1) inorganic fertilizers NPK 15:11:11, (M2) NPK inorganic fertilizers 15:15:20, (M3) inorganic fertilizers NPK 20:10:15, (M4) inorganic fertilizers NPK 10:10:10, (M5) inorganic fertilizers NPK 15:11:11 plus sodium carbonate, (M6) inorganic fertilizers NPK 15:15:20 plus sodium carbonate, (M7) inorganic fertilizers NPK 20:10:15 plus sodium carbonate, (M8) fertilizers inorganic NPK 10:10: 10 plus sodium carbonate.

The experiments were carried out in tubular photobioreactors with a maximum column height/diameter (h/D) ratio of 1.5 [13]. The photobioreactor was in discontinuous operation, fed with 2.0 L of medium, initial cell concentration 200 mg L^{-1}, isothermal reactor operating at 25 °C, continuous aeration of 3 $Lmin^{-1}$ and light intensity of 1 klux. The cell concentration and pH were monitored every 24 h during the microbial growth phases. Residence times of up to 37 days were considered for all the experiments, time it took to reach stationary phase.

2.2 Analytical methods

The cell concentration was determined by gravimetric, filtration of 10 mL of culture medium through 0.45 μm filters (Millex FG, Billerica, MA, USA) and dried at 60 °C for 24 h to constant mass. The light intensity exposed in the photobioreactor was measured with a digital luximeter (Minipa MLM 1010). The pH dynamics were monitored with a digital potentiometer (Mettler-Toledo, São Paulo-SP, Brazil). Chlorophyll-*a* analyses were

performed following Nush [14], through ethanol extraction. All parameters were determined in three replicates.

2.3 Kinetic parameters

Cell productivity (*Px*, mg $L^{-1}h^{-1}$) was calculated by varying the dry biomass (*X*, mg L^{-1}) within a collection period t (h) according to the following equation:

$$Px = \frac{X_1 - X_0}{t_1 - t_0}$$

The maximum growth rate (μ_{max}, h^{-1}) was calculated using the equation:

$$\mu_{max} = \frac{\ln(X_1/X_0)}{t_1 - t_0}$$

In which X_1 and X_0 represent biomass (mg L^{-1}) in one hour t_1 and t_o respectively.

The cell generation time (t_g, h) was calculated using the following equation:

$$t_g = \frac{\ln(2)}{\mu_{max}}$$

The dry biomass was used to quantify the cellular concentration values, and the kinetic data was based on three replicates. The maximum biomass concentration achieved was designated as X_{max} (mg L^{-1}). The maximum yield during cultivation was designated as P_{Xmax} (mg $L^{-1}h^{-1}$).

2.4 Chemical analysis and amino acid profile

The centesimal composition of the biomass was characterized according to AOAC [15], being verified levels of proteins, lipids, carbohydrates, minerals, crude fiber and humidity. The microalgae biomass was obtained weekly from the growth in medium NPK 20:10:15, centrifuged and lyophilized for further analysis. The amino acid profile, except for tryptophan, was analyzed in a gas chromatography equipped with a flame ionization detector (GC-FID) VARIAN-3400 CX (CA, USA). 1 μL of samples were introduced into injection port operating in splitless mode (splitter port off for 1 min; 30:1) at 320 °C. The separation was performed in a RTX-5MS (Restek Corporation, Bellefonte, PA, USA) (30 m × 0.25 mm

id × 0.25 μm). The programming temperature of the oven column was initially 100 °C for 2 min, then increased to 180 °C with a rate of 6 °C min^{-1}, than increased for 200 °C for 1 min and was up to 320 °C with a rate 15 °C min^{-1}, maintaining isothermal for 5 min. Hydrogen was the carrier gas used at constant pressure of 15 psi. The temperature detector was maintained at 280 °C. The identification was held in a gas chromatography coupled to a mass spectrometer (GC/MS) Shimadzu, QP-2010 Plus (Tokyo, Japan) at same chromatographic conditions described for GC/FID. GC/MS interface and ion source were held at 280 °C. Tryptophan was analyzed by high-performance liquid chromatography (HPLC, LC-20AD, Shimadzu, Co. Ltd., Japan; Column, CAPCELL PAK C18 AQ, 4.6 mm ID × 250 mm, Shiseido Co. Ltd., Japan; adetector, Flourospectro photometer, RF-20Axs, Shimadzu, Co. Ltd., Japan).

2.5 Cost Analysis

The cost of the culture medium was evaluated taking into account an average of the prices supplied by three specialized companies in southern Brazil in January 2017 (exchange rate 1USD$ = 3.255 R$). The cost analysis was based on the preparation of 1000 L of culture medium.

2.6 Statistical analyzes

The data were submitted to the normality test (Kolmogorov-Smirnov), with parametric data being compared by ANOVA followed by the Tukey test and non-parametric by the Kruskal-Wallis test using the PAST program version 3.14. Data were considered with significant variation for the 95% confidence level ($p < 0.05$).

3. Results and discussion

The effect of eight culture conditions on the growth of *Euglena* sp was evaluated under the same conditions of temperature, intensity of illumination and initial pH.

Figure 1A shows a typical growth curve with its lag, exponential and stationary phase performed during 37 days. Variation is observed between the maximum cell concentration reached, being the highest in the medium NPK 15:11:11 with 1080 mg L^{-1}. When comparing the maximum cell concentration reached in the evaluated medium, an increase of 2.7 and 2.4 times more is evidenced for the medium NPK 15:11:11 and NPK 20:10:15 respectively in relation to the Kostir-Lotze control medium. The maximum cell concentrations were observed between days 30 - 31 of growth for the NPK medium, while for the Kostir-Lotze control medium from day 10 of growth. All the evaluated media were significantly different ($p < 0.05$) to the Kostir-Lotze control medium. That difference also holds for the NPK medium M1, M2 and M3 with respect to M4. Already between the NPK medium M1, M2 and M3 there was no significant difference ($p > 0.05$).

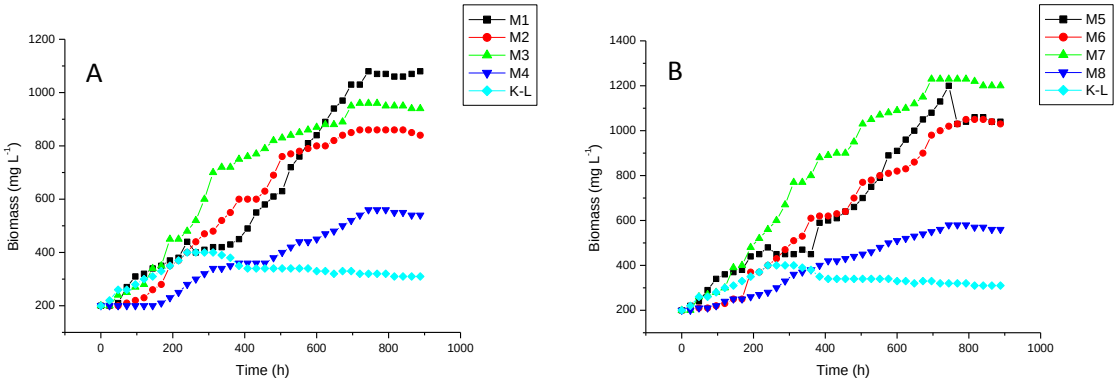

Figure 1. Growth curves for *Euglena* sp in alternative medium compared to Kostir-Lotze control medium (K-L). **A)** M1 (NPK 15:11:11), M2 (NPK 15:15:20), M3 (NPK 20:10:15). M4 (NPK 10:10:10). **B)** M5 (NPK 15:11:11 + Na_2CO_3), M6 (NPK 15:15:20 + Na_2CO_3), M7 (NPK 20:10:15 + Na_2CO_3), M8 (NPK 10:10:10 + Na_2CO_3).

The use of alternative medium based on inorganic fertilizers as a source of nitrogen, phosphorus and potassium presented better yields when compared to the commercial medium.

The continuous and constant supply of light intensity and aeration leads to biomass production being limited by the availability of macronutrients dissolved in the liquid medium, mainly nitrogen and phosphorus. This is why we observed that the medium with a low N:P ratio (M4) had the lowest cellular concentration compared to media with a higher N:P ratio (M1, M2 and M3). Similar results were observed when cultivating *Nannochloropsis* sp. in the medium of fertilizer type NPK, where an increase in cell density of approximately 30% was observed in the medium prepared with NPK 16:20:20 in relation to the preparation with NPK 14:14:14 [16].

The same pattern was observed when Na_2CO_3 was added to the culture medium (Fig. 1B). Cell concentration increased between 3.4 and 21.9% more than in medium without sodium carbonate. However, when compared statistically, no difference was observed for a level of significance of 5% ($p < 0.05$). The increase in the average cell concentration by adding sodium carbonate is due to the extra source of inorganic carbon, that makes the cell metabolize most compounds for purposes of cell parenchymal production, increasing the efficiency in biomass production. Nwoye et al. [17] when evaluating the use of basal medium composed of NPK in *Euglena gracilis* culture, showed that under mixotrophic conditions the effectiveness and efficiency in microorganism growth was superior in the NPK medium 15:15:15 in comparison with NPK 12:12:17. Similarly, when comparing the medium NPK 15:15:15 and the medium NPK 15:15:15 with an additional organic carbon source, an increase of 2.5 times more in the cell growth of the latter was recorded, so the authors recommended the use of basal medium based on fertilizer with a carbon source for the culture of *E. gracilis*. In turn, Kings et al. [18] showed that the NPK combination with coir pith compost turned out to be the best culture medium for marine microalgae species showing that the growth rate increased enormously by adding the NPK fertilizer increasing the productivity in biomass and lipids.

The highest biomass productivity was observed in the medium NPK 20:10:15. When sodium carbonate is added to this medium, productivity increases by 23.46%, presenting a significant difference ($p < 0.05$). The other medium showed no significant difference between them. *Euglena* sp presented a higher growth rate and a better generation time in the medium with a higher proportion of NPK evaluated. There was no significant difference between chlorophyll-*a* values. The pH in culture was kept acidified (Table 1).

Medium	X (mg L^{-1})	Px (mg L^{-1} d^{-1})	μ_{max} (h^{-1})	t_g (h)	Chlorophyll-*a* (mg L^{-1})	pH
M1	$637.89^{a} \pm 50.10$	$1.23^{a} \pm 0.59$	0.0019	365	$1.342^{a} \pm 0.381$	2.88 ± 1.64
M2	$593.68^{a} \pm 246.99$	$1.42^{a} \pm 0.59$	0.0024	289	$0.774^{a} \pm 0.288$	3.23 ± 1.57
M3	$687.36^{a} \pm 262.72$	$2.74^{b} \pm 1.05$	0.004	173	$1.279^{a} \pm 0.022$	3.21 ± 1.57
M4	$377.89^{b} \pm 129.30$	$0.52^{c} \pm 0.18$	0.0014	495	$0.943^{a} \pm 0.302$	2.87 ± 0.94
K-L	$328.42^{c} \pm 44.57$	$0.89^{c} \pm 0.14$	0.0027	257	$0.477^{b} \pm 0.068$	4.37 ± 1.57
M5	$699.00^{ad} \pm 306.87$	$1.32^{ad} \pm 0.58$	0.002	347	$1.042^{ac} \pm 0.221$	2.85 ± 1.65
M6	$644.73^{ad} \pm 300.47$	$1.74^{ad} \pm 0.81$	0.0027	257	$0.797^{ac} \pm 0.222$	3.18 ± 1.55
M7	$832.89^{ae} \pm 356.43$	$3.58^{e} \pm 1.53$	0.0043	161	$0.717^{ac} \pm 0.253$	3.16 ± 1.44
M8	$408.94^{bf} \pm 133.95$	$0.6^{cf} \pm 0.20$	0.0015	462	$0.876^{ac} \pm 0.142$	2.88 ± 0.95

Table 1. Analytical and kinetic parameters of *Euglena* sp during growth in alternative medium.

(K-L): Kostir-Lotze control medium. M1: NPK 15:11:11, M2: NPK 15:15:20, M3: NPK 20:10:15, M4: NPK 10:10:10, M5: NPK 15:11:11 + Na_2CO_3, M6 NPK 15:15:20 + Na_2CO_3, M7 NPK 20:10:15 + Na_2CO_3, M8 NPK 10:10:10 + Na_2CO_3, X: Dry biomass, Px: Cell productivity, μ_{max}: Maximum growth rate, t_g: Cell generation time

The different letters in the columns represent a significant difference ($p < 0.05$)

The differences in growth rate were affected by the different proportions of nitrogen and phosphorus, as well as other micronutrients essential for the activation of enzymes that participate in anabolic processes [19]. In addition to the relative concentrations of N, P and K, other factors such as their availability (solubility) and concentrations of other elements can affect the growth of *Euglena* sp. This increase in the growth rate causes greater cellular persistence in the exponential phase of cell growth, allowing that some metabolites of industrial interest can be recovered more quickly, making it more suitable for biorefinery processes [20].

The average concentration of biomass for the medium based on NPK was significantly above the Kostir-Lotze control medium, this can be attributed to the fact that the nitrogen form present in these NPK media is mainly urea, while in the control medium it is nitrate [21, 22]. Urea favors growth in various microalgae species, being more readily assimilable than nitrate [23, 24]. This may be related to the low energy expenditure required for assimilation, in view of the fact that urea crosses the cellular interior with the help of transport proteins that use the energy of a sodium gradient through the cell membrane to transport it to the cell [25]. Then, the urea in the cell can be broken down by urease into NH_4^+ and CO_2 [26].

The content of chlorophyll-*a* was proportional to the cellular concentration, this is related to intrinsic conditions of the organism. Chlorophyll is the main pigment in *Euglena* whose amount is dependent on the growth phase and physicochemical parameters of the culture [27].

The chemical analysis of the *Euglena* biomass shows high percentages of protein, lipids and carbohydrates (Table 2). *Euglena* sp. has all the essential amino acids (Table 3).

Analysis	Mean values (%)
Humidity	5,73 ± 0,2

Ashes	22,72 ± 1,9
Protein	23,03 ± 2,5
Lipids	2,86 ± 0,9
Carbohydrates	44,23 ± 3,7
Crude fiber	1,43 ± 0,4
Calcium	1,56 ± 0,1

Table 2. Chemical composition of the *Euglena* biomass

Amino acids	g of amino acids per 16 g N
Alanine	10.1
Glycine	6.4
Valine	6.6
Tyrosine	1.9
Leucine	6.3
Isoleucine	3.9
Proline	5.2
Methionine	1.7
Serine	3.8
Threonine	4.1
Phenylalanine	3.7
Aspartic acid	7.6
Cysteine	0.9
Glutamic acid	10.2

Asparagine	8.1
Histidine	2.5
Lysine	6.5
Triptophan	1.6
Total	91,1

Table 3. Amino acid profile of *Euglena* Biomass

The proportions of proteins, carbohydrates and lipids shown in the evaluated microalgal biomass are high, providing high nutritional quality when compared to conventional food plants such as soybeans and rice [28]. The amino acid profile shows the presence of essential and non-essential amino acids, which allows us to infer that this protein is highly nutritional and digestible, which would improve intestinal efficiency and regulation, as well as a better antioxidant response [29]. It has been reported that some microalgae species provide a good source of amino acids. High proportions of leucine and glutamic acid and low cysteine are a pattern found in amino acid profiles in various microalgae [30-32]. In this work we find this same pattern, which resembles the one reported in *Euglena gracilis* when used as a food supplement for ruminants [33] or when it is cultivated under conditions of darkness and anaerobiosis [34]. The amino acid content of microalgae is directly related to the culture conditions and chemical composition of the culture medium. The growth of *Euglena* sp in an acidophile range in culture makes difficult the appearance of pathogens or deteriorating microorganisms that may affect the biomass to be used for possible food. Thus, this *Euglena* biomass can be used from the nutritional point of view, as food or supplement for animal and/or human food, although cytotoxic studies must be carried out.

The cost analysis allows us to show that the use of NPK inorganic fertilizer as a medium for growing *Euglena* is 21 times cheaper than the control medium (Table 4), which can save

hundreds of dollars in its use at industrial scale. This correlates with previous studies, where fertilizer based media were more cost-effective than traditional or controlled medium [16, 17, 35, 36].

The cost of the culture medium represents a significant percentage in the productive process of microalgae and bioproducts, so it is important to use cheap medium culture for large-scale microalgae cultivation in order to reduce production costs.

	Costs USD$	
Components	**Alternative médium**	**Control médium**
NPK Inorganic fertilizer	2.99 ± 0,26	--
Analytical reagentes	--	31.52 ± 4.52
Vitamins	--	30.83 ± 1.20
TOTAL	**2.99^{a} ± 0,26**	**62.35^{b} ± 5.72**

Table 4. Comparative costing for producing 1000 L of Kostir-Lotze control medium and alternative medium

Different letters in the rows represent significant difference ($p < 0.05$) for Tukey test.

4. Conclusion

Culture media with higher proportions of N: P provided better performance in terms of biomass. The best medium based on inorganic fertilizer turned out to be the NPK 20:10:15 with a cellular productivity of 3.84 mg L^{-1} h^{-1} for *Euglena* sp.

The composition of the medium directly affected microalgae kinetics. The addition of sodium carbonate increased the biomass concentration, as well as cellular productivity.

The medium based on NPK inorganic fertilizer can be used as a minimum or base medium for the cultivation of *Euglena* sp. This medium can improve microalgae development compared to the Kostir-Lotze control medium, decreasing the cost of microalgae culture and increasing

the productivity of biomass. In addition, it is simple to prepare and is easily accessible to the population.

The biomass of *Euglena* sp obtained contains an accepted level of chemical components and essential amino acids for the formulation of foods with high nutritional value for both humans and animals. It is recommended to carry out other cytotoxic and nutraceutical assessments that help to identify and clearly explain the contribution of its elements in biological activity.

5. References

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7 CONSIDERAÇÕES FINAIS

Os meios a base de fertilizante inorgânico tipo NPK apresentam-se como alternativa para o cultivo de *Euglena* sp.

A maior concentração 678 mgL^{-1}, produtividade celular 2,74 $mgL^{-1}d^{-1}$, velocidade de crescimento 0,004 h^{-1} e tempo de geração 173 h foi obtido na proporção NPK 20:10:15 resultando ser a recomendada.

A incorporação de uma fonte de carbono no médio a base de NPK, afeta diretamente os parâmetros cinéticos, porém acrescentar carbonato de sódio melhora o rendimento e concentração de biomassa.

O custo do meio a base de fertilizantes inorgânicos é significativamente mais barato que o meio controle, pelo que a produção industrial pode ser mais rentável.

A biomassa de *Euglena* sp. obtida contém um nível aceitável de componentes bioquímicos e aminoácidos essenciais para a formulação de alimentos com alto valor nutricional para humanos e animais.