RAQUEL RODRIGUES SANTOS

OZONE APPLICATION IN THE SUPERNATANT OF MICROALGAE CULTIVATION TO RECOVER WATER, NUTRIENTS AND ORGANIC COMPOUNDS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Engenharia Agrícola, para obtenção do título de *Doctor Scientiae.*

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APROVADA: 27 de fevereiro de 2019.

Jimmy Soares	Wilfredo Milquiades Irrazabal Urruchi
André Fernando de Oliveira	Paulo Roberto Cecon (Coorientador)

Marcio Arêdes Martins
(Orientador)

"Há duas formas para viver a sua vida:
Uma é acreditar que não existe milagre.
A outra é acreditar que todas as coisas são um milagre.

(Albert Einstein)

À minha filha Lara,
Que esteve comigo em todos os momentos
e sempre acreditou que eu conseguiria.

Dedico

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BIOGRAFIA

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RESUMO

SANTOS, Raquel Rodrigues, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Application of ozone in microalgae cultivation to recovery water, nutrients and organic compounds**. Orientador: Marcio Arêdes Martins. Coorientadores: Adriano Nunes-Nesi e Paulo Roberto Cecon.

Microalgas são microrganismos com elevada eficiência fotossintética e produção de óleo e biomassa muito superior a qualquer planta vascular. A produção de biodiesel a partir de microalgas ainda apresenta limitações para que o processo seja economicamente viável, destacando-se o elevado consumo de água e nutrientes. Uma alternativa para esta situação é a utilização do gás ozônio, como agente de desinfecção, podendo viabilizar a reutilização da água e também de nutrientes dos cultivos. O ozônio possui um elevado potencial de oxidação, por isso, vem sendo utilizado em processos de tratamento de água e de efluentes. Seu elevado potencial oxidativo permite a conversão de alguns compostos orgânicos, ora não assimiláveis pelas microalgas ou até mesmo tóxicos, em nutrientes, tais como ácidos orgânicos e aminoácidos. O objetivo desta pesquisa foi investigar se o tratamento com o gás ozônio é capaz de viabilizar a reutilização da água e também de nutrientes dos cultivos. Para isso, primeiramente foi realizado uma revisão sobre os mecanismos de reação do gás ozônio e o meio de cultura de microalgas. Em seguida, foram realizados tratamentos com seguintes linhagens de microalgas: *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 e *Chlamydomonas* sp. CC503, em que o sobrenadante de cultivo foi ozonizado, com e sem a presença da biomassa desengordurada, para avaliar seu efeito sobre a água, nutrientes e compostos orgânicos. A ozonização foi capaz de descolorir o sobrenadante, o que indica a possibilidade de seu reuso pois melhora a penetração da luz para os cultivos autotróficos. As análises dos nutrientes demonstraram que houve um incremento significativo de fósforo e nitrogênio, com a biomassa desengordurada. As variações nas concentrações de carboidratos e aminoácidos solúveis totais demonstraram que houve um incremento significativo para ambos tratamentos com ozônio, acionando-se ou não a biomassa livre de lipídeos, o que sugere que o ozônio gerou carboidratos e aminoácidos solúveis a partir dos carboidratos de reserva das células e proteínas. Para verificar os resultados observados, foi realizado um novo experimento, em que as microalgas foram cultivadas no sobrenadante de cultivo, tratado com ozônio. Foram realizados os seguintes tratamentos de cultivo: tratamento controle, em que as linhagens foram cultivadas em meio de cultura, tratamentos T1 e T2 no sobrenadante do cultivo, tratamentos T3 e T4 no sobrenadante de cultivo, após tratamento

com O_3, sem adição e com adição de nutrientes, tratamentos T5 e T6 no sobrenadante de cultivo juntamente com a biomassa isenta de lipídeos e após tratamento com O_3, sem adição e com adição de nutrientes e tratamento T7 no sobrenadante do cultivo, tratado com carvão ativado e com adição de nutrientes. Foram determinadas as concentrações, em massa seca, dos cultivos e as concentrações de carboidratos, proteínas e aminoácidos solúveis totais. Concluiu-se que não houve inibição do cultivo, em nenhum dos tratamentos, e que o tratamento com ozônio do sobrenadante, adicionado de biomassa residual livre de lipídeos, apresentou maiores médias de concentração de massa seca, nas linhagens *Chlorella* sp. BR001 e *Chlamydomonas* sp. CC503. Os tratamentos do sobrenadante com ozônio, adicionado ou não de biomassa desengordurada e, adicionado ou não de nutrientes, das linhagens *Scenedesmus* sp. BR003 e *Chlamydomonas* sp. CC503, apresentaram as maiores médias de concentração de carboidratos. O tratamento com ozônio do sobrenadante, nas três linhagem testadas, apresentarou as maiores médias de concentração de proteínas. Os tratamentos do sobrenadante ozonizado com biomassa desengordurada, adicionado ou não de nutrientes, das linhagens *Scenedesmus* sp. BR003 e *Chlamydomonas* sp. CC503, apresentaram as maiores médias de concentração de aminoácidos. Os resultados obtidos sugerem que o tratamento do sobrenadante ozonizado com biomassa livre de lipídeos e com adição de nutrientes (T6-SOBN), apresentou as melhores médias em todas as análises realizadas, principalmente na linhagem *Chlamydomonas* sp. CC503. Novos estudos devem ser realizados, a fim de encontrar o melhor meio de crescimento, reaproveitando água e todos os composto de interesse, para otimizar o cultivo de microalgas vizando a produção de biocombustíveis.

ABSTRACT

SANTOS, Raquel Rodrigues, D.Sc., Universidade Federal de Viçosa, February, 2019. **Application of ozone in microalgae cultivation to recovery water, nutrients and organic compounds**. Adviser: Marcio Arêdes Martins. Co-advisers: Adriano Nunes-Nesi and Paulo Roberto Cecon.

Microalgae are microorganisms with high photosynthetic efficiency and capacity of production of oil and biomass far superior to any vascular plant. The production of biodiesel from microalgae still presents limitations to become an economically viable process, such as the high consumption of water and nutrients. Alternatively, ozone gas can be used, as a disinfection agent, which can make possible the reuse of water and nutrients of crops. Ozone has a high potential for oxidation, so it has been used in water and effluent treatment processes. Its high oxidative potential allows the conversion of some organic compounds, non-assimilable by microalgae or even toxic, into nutrients, such as organic acids and amino acids. The objective of this research was to investigate if the treatment with the ozone gas is able to make possible the reuse of water and also nutrients of the crops. For such, a review was first made on the reaction mechanisms of the ozone gas and the culture medium of microalgae. Afterwards, treatments with the following microalgae lineages were carried out: *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, in which the culture supernatant was ozonized, with and without the presence of defatted biomass, to evaluate its effect on water, nutrients and organic compounds. The ozonation was able to discolor the supernatant, which indicates the possibility of its reuse since it improves the penetration of the light for the autotrophic cultures. The analysis of the nutrients showed that there was a significant increase in phosphorus and nitrogen, with the defatted biomass. Variations in carbohydrate and total soluble amino acid concentrations demonstrated that there was a significant increase for both ozone treatments, whether or not the free lipid biomass was added, suggesting that the ozone generated carbohydrates and soluble amino acids from stored carbohydrates and proteins of the cell. To verify the observed results, a new experiment was performed, in which the microalgae were grown in the culture supernatant, treated with ozone. The following culture treatments were carried out: control treatment, in which the lineages were cultivated in culture medium; treatments T1 and T2 in the culture supernatant; treatments T3 and T4 in the culture supernatant, after treatment with O_3, without and with addition of nutrients; T5 and T6 treatments in the culture supernatant added with the lipid-free biomass and after treatment with O_3, without and with addition of

nutrients; and T7 treatment in the culture supernatant, treated with activated charcoal and with added nutrients. Concentrations, in dry mass, of the cultures and the concentrations of carbohydrates, proteins and total soluble amino acids were determined. It was concluded that there was no inhibition of the cultivation in any of the treatments and that the ozone treatment of the supernatant, added with residual lipid-free biomass, presented higher dry mass concentration in the *Chlorella* sp. BR001 and *Chlamydomonas* sp. CC503. The treatments of the supernatant with ozone, added or not of defatted biomass and, added or not of nutrients, of the lineages *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, presented the highest mean carbohydrate concentration. Ozone treatment of the supernatant in the three tested lineages presented the highest mean protein concentration. The treatments of the ozonated supernatant with defatted biomass, added or not of nutrients, of the lineages *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, showed the highest averages of amino acid concentration. The results obtained suggest that the ozonated supernatant with lipid-free biomass and nutrient addition (T6-SOBN) presented the best averages in all analyzes, especially in the *Chlamydomonas* sp CC503 lineage. New studies should be conducted in order to find the best growth medium, reusing water and all compounds of interest, to optimize the cultivation of microalgae aiming at the production of biofuels.

INTRODUÇÃO GERAL

Atualmente a população mundial é de 7,55 bilhões de pessoas (UNITED NATIONS, 2017) e estima-se que esse número seja de 8,55 bilhões de pessoas em 2030, pressionando fontes de energia, água, alimentos, uso da terra e do meio ambiente. A população mundial está crescendo a uma taxa de aproximadamente 1,1% ao ano e a variação média é estimada em cerca de 80 milhões por ano (IPEA, 2015). Esse crescimento aumenta a pressão sob os recursos críticos, como alimento e energia. A expectativa, segundo diversos autores, é de que a demanda por alimentos e energia subirá nas próximas décadas, em resposta ao rápido crescimento econômico dos países em desenvolvimento (KPMG INTERNATIONAL, 2014; U.S. EIA, 2019). Essa demanda cresce no contexto do nexo de interdependência entre água-energia-alimento. Segundo diversos estudos, haverá crescimento da demanda mundial por alimento, água e energia em 35%, 40% e 50%, respectivamente, até 2030 (UNITED NATIONS, 2017).

Segundo os dados da EIA – Energy Information Administration (U.S. EIA, 2019), mais de 85% do aumento na demanda de energia global no período de 2010-2040 ocorrerá nas nações em desenvolvimento, impulsionado pelo crescimento econômico e por populações em expansão. Nesse contexto, a participação de novas fontes de energia no consumo mundial irá crescer. A energia nuclear e as energias renováveis são as fontes de energia que apresentarão crescimento rápido, a uma taxa média anual de 2,5%. As preocupações com a segurança energética, o impacto das emissões de combustíveis fósseis no ambiente e os preços do petróleo são os principais vetores que explicam a expansão do uso da energia nuclear e das energias renováveis ao longo dessas projeções (KPMG INTERNATIONAL, 2014; U.S. EIA, 2019). Em 2017, a produção brasileira de derivados de petróleo foi de 110,2 milhões de m^3. O óleo diesel respondeu por 42% da produção total de derivados, enquanto a gasolina tipo A teve participação de 28,7%. O maior uso do diesel ocorre no transporte rodoviário, que representa 75% do total, seguido do uso agropecuário, com 14%, e 5% para geração de energia elétrica nos sistemas isolados (ANP, 2018).

No Brasil, o etanol é utilizado no setor de transportes misturado à gasolina ou na forma hidratada, produzido a partir da cana de açúcar (BAKE *et al.*, 2009) e nos Estados Unidos é produzido a partir do milho (MALLORY; HAYES; IRWIN, 2010). Contudo, a viabilidade para suprimir a demanda energética para o setor, utilizando este tipo de fonte é questionável, devido a competição por insumos e terras agricultáveis para a produção de alimentos. Uma alternativa a este cenário são os biocombustíveis, pois são os únicos com

potencial para atender qualquer tipo de demanda no setor de transporte. Desta forma, as microalgas surgem como uma excelente fonte de matéria prima para os biocombustíveis, além da possibilidade de serem cultivadas em áreas impróprias a agricultura (PARMAR *et al.*, 2011).

Microalgas são microrganismos que possuem elevada eficiência fotossintética e produção de biomassa muito superior a qualquer planta vascular (TABATABAEI *et al.*, 2011). São fonte de várias substâncias de alto valor de mercado, como pigmentos, antioxidantes, ácidos graxos poli-insaturados, vitaminas e proteínas (HARUN *et al.*, 2010). Em virtude destas características, as microalgas têm sido objeto de pesquisa em diversas áreas, destacando-se entre estas, a de biocombustíveis (FRANCO *et al.*, 2013). O interesse em se cultivar microalgas para produção de biocombustíveis provém do fato de algumas microalgas possuírem rendimento em óleo, por área de cultivo, superior a qualquer outra oleaginosa utilizada na produção de biodiesel, tem alto potencial de crescimento, e o seu cultivo não requer qualquer aplicação de pesticidas (GHORBANI *et al.*, 2018; MOFIJUR *et al.*, 2019; XUE *et al.*, 2018)

A produção de biocombustíveis a partir de microalgas não compete com a indústria de alimentos, visto que requer áreas de cultivo menos extensas e pode ser cultivada em regiões que não são atrativas para a agricultura (ADENLE; HASLAM; LEE, 2013). Por atuarem naturalmente como fixadoras de CO_2, seu cultivo pode ser associado às linhas de emissão de gás carbônico das indústrias, tais como fábricas de cimento, de papel e celulose, usinas de etanol e usinas siderúrgicas, que são grandes emissoras de gás carbônico (FULKE *et al.*, 2013; TEBBANI *et al.*, 2013).

A produção de biodiesel a partir de microalgas ainda apresenta limitações para que o processo seja economicamente viável, destacando-se o elevado consumo de nutrientes, usualmente na forma de fertilizantes. Segundo Batan *et al.*, (2010), para gerar 1,0 kg de massa seca de microalgas, são necessários 0,147 kg de nitrogênio e 0,020 kg de fósforo. Muitos trabalhos citam a importância de se reutilizar não só os nutrientes, como também a água no processo produtivo de microalgas. Esta constatação pode ser observada nos estudos reportados por Jones & Mayfield (2012), Kim *et al.* (2011), Yang *et al.* (2011) e Wu *et al.* (2012). Dzuman (2013) estudando a reciclagem repetida do meio de cultivo constatou que a reciclagem do mesmo permite reduzir os gastos com os nutrientes necessários para o cultivo de microalgas e também reduz o consumo de água.

Uma alternativa para essa lacuna no processo produtivo das microalgas é a utilização do gás ozônio como agente de desinfecção, podendo viabilizar a reutilização da água e

também de nutrientes residuais dos cultivos. O ozônio possui um elevado potencial de oxidação, por isso, vem sendo utilizado em processos de tratamento de água e de efluentes, pois oxida os compostos orgânicos e inorgânicos de diferentes formas, além de promover a eliminação de bactérias, fungos e outros organismos. Neste contexto, se fazem importantes os estudos sobre a utilização do ozônio como tecnologia para a recuperação de nutrientes do sobrenadante de cultivo de microalgas, mas também de outros compostos, de forma a complementar e auxiliar a busca por um meio de cultura adequado para produção de microalgas e, assim, reduzir os custos de produção. O elevado potencial oxidativo do ozônio permite a conversão de alguns compostos orgânicos, ora não assimiláveis pelas microalgas ou até mesmo tóxicos, em nutrientes, tais como ácidos orgânicos e aminoácidos (YANG *et al.*, 2018).

Esse trabalho se divide em três capítulos. O primeiro trata-se de uma revisão sobre os mecanismos de reação do gás O_3 e o meio de cultura de microalgas. O segundo capítulo se refere ao tratamento do sobrenadante de cultivo com O_3 e seu efeito sobre os nutrientes e compostos orgânicos. No terceiro capítulo foi analisado o efeito do sobrenadante ozonizado no cultivo das microalgas.

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ARTIGO 1

VERSATILE OZONATION-TECHNIQUE ASSOCIATED WITH CULTIVATION AND NUTRITION OF MICROALGAE CROPS FOR BIOFUELS PRODUCTION

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ABSTRACT

Microalgae are unicellular microorganisms with greater potential to convert solar energy in biomass than vascular plants. They are able to use both organic and inorganic carbon sources. Other elements, such as nitrogen and phosphorus, also contribute to higher biomass and lipids productivity. The high lipid yield and rapid growth observed in microalgae highlight the need for research and technologies to overcome the economic barriers to be exploited as feedstock for renewable energy. The cost of microalgae biomass in upstream processing can be reduced by the use of low cost alternative source of nutrients, nutrients recycling, waste water and water reusing. The culture medium once used and ozonizated, can be disinfected avoiding possible subsequent contamination, besides it promotes the conversion of cultivation leftovers into compounds, that can be assimilated by microalgae. Among the emerging technologies, the O_3 gas can be used not only for disinfection but also as a recovery agent capable to disinfect the supernatant of the crop without leaving residues, with the advantage of using smaller doses. This review addresses the challenges of O_3 use in microalgae cultivation, elucidating the mechanisms of gas and culture medium reaction. In addition, the potential of O_3 application in subsequent steps of crop microalgae production is also discussed.

Keywords: Reuse, Disinfection, Nutrient recycling, Gas reaction

1. INTRODUCTION

Microalgae are unicellular microorganisms capable of using sunlight, water and nutrients, including CO_2, as the main substrate for biomass production. They are found in almost all natural environments including saline, freshwater and humid soil environments as well as rock pools or in volcanic water that can have extreme environmental fluctuations (DUONG et al. 2012). It is estimated that they are responsible for 50% of the global photosynthetic activity (CHIU et al. 2015, MILANO et al. 2016). These organisms have simple cellular structure and large volumetric surface, which allows absorbing large amounts of nutrients. Due to their simplified structure, they are more efficient in converting solar energy than vascular plants, although their photosynthesis mechanisms are similar (PERRINE et al. 2012).

Recently, microalgae strains have been studied as potential feedstock for biofuels production (ROCHA et al. 2017) and many researches focus in the stimulation of neutral lipid accumulation, like triglycerides, for further conversion in biodiesel (DONG et al. 2016). The high biomass yield and rapid growth of microalgae called attention in both research and technology development to overcome the economic barriers related to the microalgae cultivation (FRANCO et al. 2013). The composition of algal biomass depends on the strain, the growing and stress conditions, and the cellular metabolism. A specific stress condition can shift the cell metabolism to accumulate more lipids or carbohydrate, and even pigments (NURACHMAN et al. 2015).

Biofuels based on microalgae cultivation are a promise for the bioenergy future (SASONGKO and NOGUCHI 2015). Their high oil productivity and low land requirements coupled with the ability to be cultivated using residues such as wastewater and CO_2, in non-arable land, have motivated studies using microalgae as a source for biofuel generation, such as biodiesel (SONG et al. 2016, TALEB et al. 2016). Some species stand out do to their high oil content that can reach up to 75% in mass in relation to dry biomass, such as the chlorophyceae *Botryococcus braunii* (RASHID et al. 2014). In contrast, soybean has about 18% of oil content and it is an oilseed widely used for biodiesel production. Some microalgae species present oil contents near or superior to soybean, including *Scenedesmus obliquus* (12-14%), *Scenedesmus dimorphus* (16-40%), *Chlamydomonas rheinhardtii* (21%), *Chlorella vulgaris* (14-22%), and *Dunaliella tertiolecta* (64-71%) (LAM and LEE 2012). The biomass production from microalgae can reach about 15-25 t ha^{-1} $year^{-1}$ and it is estimated that lipid production is around 4.5-7.5 t ha^{-1} $year^{-1}$. In comparison to the oil sources commonly used in this process, there is clearly a potential for biodiesel production from microalgae, since soybean, palm and jatropha produce 0.4, 3.62 and 4.14 t ha^{-1} $year^{-1}$, respectively (ÖZÇIMEN et al. 2017).

Despite the potential to make biofuel production from microalgae an economically viable activity numerous challenges still need to be overcome. In this sense, an important aspect relates to the limited efficiency of the production system, since large amounts of nitrogen and phosphorus are still required to guarantee high production of biomass and lipids (PROCHÁZKOVÁ et al. 2014). Remarkable importance has been given of reusing not only nutrients but also water in the microalgae production process (JONES and MAYFIELD 2012, Wu et al. 2012). It has been widely demonstrated that effluent water can be recycled and used for a secondary crop production, adding the essential nutrients (BOHUTSKYI et al. 2014, GONZÁLEZ-LÓPEZ et al. 2013). The recycling of the culture medium allows cost

reduction from water and nutrients, contributing to the economic feasibility of the production and optimization of the efficiency in the use of the resources (LOWREY et al. 2016).

An alternative technique for water and nutrient recycling in microalgae production system is the use of O_3 gas as a disinfection agent (REIS et al. 2013). O_3 has a high oxidation potential, being used in both water and effluents treatment processes (CARVALHO et al. 2018, RICE 2017). This gas is able to remove organic and inorganic compounds in different ways, besides promoting the removal of bacteria, fungi and other organisms that limit further use of the recycled water for microalgae cultivation (ALENCAR et al. 2012, TALBOT et al. 2012). This study aims to address a critical discussion about the challenges of the use of O_3 in microalgae cultivation, presenting the gas mechanisms of action in the culture medium to enable its reuse in subsequent algae crops.

2. METABOLISM

According to environmental conditions microalgae cultivation might exhibit different metabolisms: (i) photoautotrophic, when energy is obtained from natural or artificial light source, and carbon from an inorganic source, usually CO_2, by photosynthesis; (ii) heterotrophic, when energy and carbon are obtained from external organic substrates, usually by sugar oxidation, such as glucose and acetate; and (iii) mixotrophic, when photosynthesis and oxidation of organic compounds occur concomitantly (Bastos and Bonini 2017).

In autotrophic metabolism, microalgae perform photosynthesis using light as a source of energy and CO_2 from air as a source of carbon. Although it is efficient with the incidence of light on microalgae culture, this type of metabolism can reduce the microalgae growth, as a consequence of light limitation, due to high cell density, or the excess light (photoinhibition) (LOPES et al. 2014). Currently, it is the usual metabolism present in microalgae cultivation mainly because of the efficiency to fix atmospheric carbon. This type of cultivation has an environmental appeal, since the CO_2 emissions can be used in the biomass production for biofuels, which would lead to a favorable energy balance (GONÇALVES et al. 2015).

Photosynthesis can be expressed as a redox reaction driven by light energy where CO_2 and water are converted into carbohydrates and oxygen. This process is traditionally divided into two stages, the so-called light reactions and biochemical reactions. In the light reactions, the light energy is converted to chemical energy in bounded photosynthetic

membranes, providing a biochemical reductant NADPH and a high-energy compound ATP. The biochemical reactions occur in the stroma, where NADPH and ATP are utilized in the sequential biochemical reduction of CO_2 to trioses phosphate, the pathway named Calvin-Benson cycle. The Calvin–Benson cycle occurs in four main steps: carboxylation, reduction, regeneration and production (SHARKEY 2018).

The heterotrophic metabolism, which differs from the first one by assimilation of organic sources of carbon, mainly glycerol, glucose, and acetate, exhibits some advantages in comparison to autotrophic ones, such as better control of the cultivation process and low cost of biomass harvest (LOPES et al. 2014). In this metabolism, an organic carbon source is added to the cultivation medium, instead of fixation of CO_2 from the air. The assimilation of this carbon is faster than in autotrophy, resulting in a higher biomass productivity (FRANCISCO et al. 2014).

In mixotrophic metabolism, both autotrophic and heterotrophic metabolisms act for simultaneous assimilation of CO_2 and organic carbon sources by microalgae (LOPES et al. 2014). The mixotrophy term is defined as a metabolic process in which photosynthesis is the main source of energy, although both organic compounds and CO_2 are essential. Thus, a mixotrophic organism can assimilate organic compounds as a source of carbon while using inorganic compounds as electron donors (BONINI and BASTOS 2012). The mixotrophic regime is a variant of the heterotrophic, where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolisms operate simultaneously (LIANG 2013, YEN et al. 2013).

The mixotrophic and heterotrophic terms are well defined in literature, their difference is in the energy source required to support the growth and production of specific metabolites by microalgae. The autotrophy involves the use of light as a single source of energy, which is converted into chemical energy through photosynthetic reactions. Photosynthetic activity is directly related with the microalgae growth, and a adequate light intensity might increase the specific growth rate of several microalgae species (PRAKASH et al. 2013).

While several carbon sources have been proposed for the heterotrophic microalgae cultivation, several studies demonstrated that microorganisms can use few substrates (PEREZ-GARCIA et al. 2011b). The most frequently used carbon sources for heterotrophic and mixotrophic microalgae cultivation are glucose, acetate and glycerol (HEREDIA-ARROYO et al. 2011, PEREZ-GARCIA et al. 2011a, PEREZ-GARCIA et al. 2011b).

Glucose is the commonly used carbon source by both microalgae and other species of microorganisms. In general, when using other substrates, microalgae require an adaptation period, defined by an extended lag phase necessary for the synthesis of enzymes and the specific transport systems for nutrient assimilation and consumption. Several microalgae show a preferred consumption of glucose over other organic carbon sources. It has been shown that glucose downregulates the catabolism of other carbon sources in microalgae (ANDRULEVICIUTE et al. 2014).

3. THE ROLE OF NITROGEN AND PHOSPHORUS

Nitrogen is the second nutrient most abundant in the biomass produced from microalgae (BARROS et al. 2017). The typical responses to stress caused by nitrogen limitation are discoloration (decrease of chlorophyll content and increase of carotenoids content) and accumulation of organic carbon compounds such as polysaccharides (starch) and some lipids (polyunsaturated fatty acids) (SIAUT et al. 2011). Nitrogen has a fundamental role in cellular metabolism because it is a component of all amino acids, nucleotides, pigments and other components. The main nitrogen sources used in culture medium are nitrate salts ($NaNO_3$), ammonium salts (NH_4Cl, $(NH_4)_2SO_4$) and urea (GROBBELAAR 2013). Cells consider nitrogen deprivation as a stress condition, so they suppress growth and induce lipid accumulation (MARTINS and FERNANDES 2016). Under these conditions, changes in the metabolic pathways of the cell are usually observed, causing a redirection of the metabolism from storage of carbohydrates to the synthesis of proteins or lipids. This is usually interpreted as an attempt of the cell to adapt to the environment, since lipids have higher energy content than carbohydrates (FENG et al. 2011).

Phosphorus is essential for cellular growth and many of its processes, such as energy transfer (ATP), biosynthesis of nucleic acids, phospholipids, DNA, among others. In a simplified way, it can be assumed that phosphorus has two fundamental functions in cells: energy transfer and structural molecules constitution (ABOMOHRA et al. 2018, XIA and MURPHY 2016). Phosphorus assimilation depends on light, possibly due to energy accumulation as ATP (PRASAD et al. 2018). Inorganic orthophosphate (PO_4^{3-}) is the prime ionic form of phosphorus for use in microalgae cultures and its absorption is energy dependent (RAY et al. 2019). However, microalgae can absorb phosphorus from other sources, as dihydrogen phosphate ($H_2PO_4^-$) and hydrogen phosphate (HPO_4^{-2}) that coexist by the buffer effect, and its relative concentration is pH regulated. Although algal biomass

contains less than 1% of phosphorus, it is often one of the most important limiting factors for microalgae growth (XIA and MURPHY 2016). This is because it easily bounds to other ions, resulting in its precipitation and, consequently, unavailability for algae as essential nutrient (GROBBELAAR 2013). Microalgae can also store excess phosphorus in the polyphosphate form, known as luxury consumption. This intracellular storage suggests the phosphorous can be used when this nutrient becomes limiting (GROBBELAAR 2013). The supply of this nutrient also influences the biomass composition, especially in terms of lipids and carbohydrates content, which are affected by the external and internal sources of phosphorous (MIRANDA et al. 2015).

The nitrogen-phosphorus (N: P) ratio is also very important in the culture medium because when this ratio is high the biomass productivity becomes higher and it contributes to maintaining certain species dominance in the crop (PRASAD et al. 2018). Traditionally, a biomass C/N/P stoichiometry in accordance with the work of Redfield, termed the Redfield ratio (106C: 16N: 1P) is deemed to be optimal for microalgal growth and is widely used as a reference point for quantifying potential nutrient limitations (FLYNN et al. 2017, GROBBELAAR 2013).

4. MICROALGAE NUTRITION AND GROWTH

The composition of the culture medium for microalgae is optimized to promote the maximum growth, aiming to isolate, maintain and spread them. The solid medium prepared using agar as a solidifying agent has been used for microalgae isolation and culture maintenance (JACINAVICIUS et al. 2013). Liquid media is preferred for inoculum production, strain screening and cultivation in several scales.

It has been suggested that the culture medium formulation should consider cell composition, energy requirements and the need for specific substances (ARORA 2013). Microalgae culture conditions are essential for shaping the growth profile of each organism. Different formulations may interfere in the biochemical composition of the microalgae and change its growth rate (MATA et al. 2010). Moreover, abiotic factors as nutrient concentration, light intensity, pH, temperature, among others environmental factors, may change the metabolic pathways of these microorganisms (MATA et al. 2010).

4.1.Light intensity

The effects of light intensity are controlled by a process called photoacclimation (STRAKA and RITTMANN 2018). In this process, the composition of microalga cells changes along with biophysical and physiological alterations to increase photosynthetic activity and growth rate. In high light intensity environments, the chlorophyll and other pigments amount decrease while carbohydrates and lipids contents increase (CHENG and HE 2014).

4.2.Temperature

The effect of temperature on biochemical composition occurs according two distinct mechanisms: the dependence of the chemical and biochemical reaction rates and the transformation of the carbon fixed in the photosynthesis to the target metabolites such as proteins, lipids and carbohydrates (CHENG and HE 2014).

4.3. pH

Microalgae generally grow well in optimal pH range (6–9) and the pH is essential for the effective absorption of culture medium nutrients because it directly affects the availability of various chemical elements and enzyme activity (HUANG et al. 2016, KHALIL et al. 2010).

4.4. Aeration and agitation

Both aeration and agitation provide effective dispersion of nutrients, including the CO_2 input, which is the carbon source for photosynthetic microalgae growth, to synthesize carbohydrates and other cellular organic compounds (NELSON and COX 2017). Agitation performance strongly affects the hydrodynamic conditions and thus affects algal growth, since it increases the mass and energy transfer rates, preventing cell sedimentation and the formation of barriers. Agitation can be done with mechanical impellers that provide adequate mixing. On the other hand, excessive mixing can produce high shear conditions that may cause impaired cell growth, cell damage and cell death. As a result, it is important to determine the ideal mixing rate to satisfy the oxygen demand of microalgal cells and to prevent settling of biomass without damaging cells (ENGIN et al. 2018).

4.5.Nutrients

Variations in nutrient concentrations display considerable number of changes in the metabolic pathways and biochemical composition of microalgae. As discussed in the previous section, nitrogen has the ability to alter the metabolism of microalgae under restrained conditions. Similar to nitrogen, phosphorus deficiency reduces the amounts of chlorophyll *a* and proteins, thus increasing the relative amount of carbohydrates in the microalgae cells (SRINIVASAN et al. 2018). Other factors such as CO_2, salt concentration and trace metals may also affect cell growth and composition (CHENG and He 2014).

The culture medium should contain macronutrients (C, N, P) and micronutrients (B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, S, Zn). Some strains of microalgae also require concentrations of trace elements and vitamins (B complex) (QIANG et al. 2015). Sulfur has a fundamental role in protein structure and regulation, participating in photosynthetic and respiratory transport of electrons (COSTA et al. 2014).

Although micronutrients are added to the culture medium in small amounts, they are essential for growth as they play critical roles in various metabolic pathways and their absence may be a limiting factor. However, some of these chemical elements may be toxic when in larger amounts (COSTA et al. 2014). Not only micronutrients, but also some nitrogen sources can be toxic in high concentration, like ammonium salts (SOARES et al. 2018).

The nutritional requirement can be estimated using the approximate molecular formula of microalgal biomass, as $CH_{1.71}O_{0.42}N_{0.15}P_{0.01}$, proposed by Fortier and Sturm (2012), with higher demand in N:P ratio. However, this ratio can range from 4:1 to almost 40:1, depending on the species and nutrients availability. Under appropriate conditions, high productivity can be achieved even at relatively low N:P ratios. On the other hand, studies have suggested that nitrogen limitation reduces growth, but sometimes stimulates lipid production in microalgae (PARK et al. 2011).

Nitrogen is quantitatively the most important element after carbon. Nitrogen proportion can vary from 1-10% in dry mass. Protein, carotenoid and chlorophyll concentrations in the cells tend to vary regarding nitrogen concentration. When nitrogen is available in the culture, protein, carotenoid and chlorophyll concentrations increase, but when nitrogen concentration in the culture medium declines, the amount of these substances decrease (ÖRDÖG et al. 2012). The nitrogen depletion allowing the lipid content of the cells to increase, slowly changing the neutral lipids composition, from fatty acids to triglycerides (SURENDHIRAN et al. 2015).

5. OZONE AND MICROALGAE

O_3 is a colorless gas with a pungent odor at room temperature (25 ± 1 ºC), unstable and partially soluble in water. This gas has high oxidizing power due to the electron arrangement in its molecule and can be generated by electric discharge (5,000 V), resulting in the division of the diatomic oxygen molecule into atoms of valent oxygen (PANDISELVAM et al. 2017). The decomposition of O_3 in air occurs rapidly producing oxygen, and thus leaving the environment free of any toxic residue. As a disinfectant, O_3 has a faster and more effective action than other products with the same function (ISIKBER and ATHANASSIOU 2015, KLAWINSKI et al. 2017).

O_3 is able to react with a large number of organic compounds, mainly due to its high oxidation potential (LEE et al. 2017). Therefore, it has been demonstrated that this gas is effective in the inactivation of bacteria, molds, yeasts, protozoa, including sporulated forms and cysts, which are more resistant to biocides agents (KADIR et al. 2018, VARGA and SZIGETI 2016). Studies aiming treatment of wastewater contaminated with organic matter use O_3 as pretreatment associated with catalysts. Catalysts used with O_3 to increase the oxidation efficiency are metal ions, metals, metal oxides, hydroxides, and peroxides (ABOUZLAM et al. 2013, JEONG et al. 2014).

In microalgae cultivation, O_3 can also be used in cell harvest by flotation, using air microbubbles dispersed in water without the addition of surfactants (CHENG et al. 2011). O_3 is also employed as a pre-treatment for lipid extraction, acting on cellular disruption (NGUYEN et al. 2013). Despites the potential for application of this gas, few research papers are found in literature regarding the use of O_3 in microalgae (GAN et al. 2014, HUANG et al. 2014, KIM et al. 2014, NDIKUBWIMANA et al. 2016, OLIVEIRA et al. 2018). These studies applied O_3 to promote the rupture of the algal cell for lipid extraction (*Chlorella vulgaris*), to remove contaminants of piggery wastewater to microalgae growth (*Scenedesmus quadricauda* and *Chlorella pyrenoidosa*) and to microalgae flotation and harvesting for biofuels. The results of these studies demonstrated that there is a high potential for O_3 utilization at microalgae cultivation.

5.1. Mass transfer in the O_3-water system

The mass transfer phenomenon is the basis of many processes where one or more components migrate from one phase to another through the interface. The transfer occurs towards a decreasing chemical potential and its mechanism depends on the dynamics of the

systems involved (DAVID 2013). When a mixture contains two or more molecular compounds in different relative compositions in a system, a natural process occurs in the sense of decreasing the composition differences to reach chemical equilibrium (HAMBORG et al. 2010). In biphasic gas-liquid systems, if a gaseous component is poorly soluble in the liquid phase, the mass transfer resistance in the gas phase is negligible so the liquid phase resistance controls the transport process. When the gaseous component is highly soluble in the liquid phase, the gas phase resistance limits the mass transfer phenomenon. Thus, assuming that a component A of a gaseous phase is transferred to a liquid phase B, the mass transfer flux or absorption flux of A is given by Equation 1.

$$N_A = k_G(p_{Ab} - p_i) = k_L(C_A^* - C_{Ab}) \quad (1)$$

where N_A is the mass transfer flux (mol m^{-2} s^{-1}); k_G (mol $N^{-1}s^{-1}$) and k_L (m s^{-1}) are the mass transfer coefficients for the gas and liquid phases, respectively; p_{Ab} and p_i (Pa) are the partial pressures of gaseous component A in volume and interface, respectively; C_A* and C_{Ab} (mol m^{-3}) are the concentrations of A at the interface and within the liquid phase, respectively.

The mass transfer of O_3 in the gas phase to water is complex, not only because of its highly reactive nature but also due to its solubility in water. The efficiency of O_3 mass transfer to the liquid phase is one of the parameters that determine the economic viability of its use. In quiescent systems, the mass transfer depends on the gas diffusivity, whereas in advective processes the transport is dependent of the gas superficial velocity, liquid phase viscosity, bubble size and mainly the O_3 solubility in water (ZAPATA 2012). Some of these parameters of influence are discussed below:

- Influence of gas diffusivity in a dilute solution

A dilute solution represents the almost absence of the solute in the liquid phase where the diffusion takes place. Resistance to solute transport is controlled by the liquid phase when the gas is poorly soluble. The greater the gas ability to diffuse in the liquid medium, the better the mass transfer velocity.

- Influence of gas superficial velocity

The gas superficial velocity is the average velocity of the gas being injected into the solution. In order to increase the gas superficial velocity, in homogeneous and heterogeneous

regimes for the air-water biphasic system, the mass transfer coefficient increases (EZZI and NAJMULDEEN 2013, KANTARCI et al. 2005).

- Influence of liquid viscosity

The liquid viscosity has a great impact on the formation and coalescence of bubbles. An increase in the liquid viscosity results in the formation of larger bubbles, while decreases the mass transfer coefficient (EZZI and NAJMULDEEN 2013, KANTARCI et al. 2005).

- Influence of bubble size

The mass transfer coefficient increases as the bubble diameter decreases. Thus, the presence of small bubbles incurs an increase in mass transfer rates, since the contact area at the gas-liquid interface increases with decreasing bubble size (EZZI and NAJMULDEEN 2013, KANTARCI et al. 2005).

- O_3 solubility in water

Partially water-soluble gases dissolution, such as O_3, obeys Henry's Law, which indicates that the amount of gas in a solution at a given temperature is linearly proportional to its partial pressure. Thus, the saturation concentration of O_3 in water under ideal thermodynamic conditions is given by Equation 2.

$$C_s = \beta \, \rho \, p_g \tag{2}$$

where, C_s is the O_3 concentration in water (kg m^3); β is the volume of dissolved O_3 per unit of water volume in the O_3 presence; ρ is the O_3 density in stand condition (2.14 kg m^{-3}) and p_g is the O_3 partial pressure in the gas phase (p_a).

The O_3 dissolution in water can be expressed as a ratio of concentration in the gas (C_g) and water (C_w) phases (Equation 3), called solubility (S).

$$S = \frac{C_g}{C_w} \tag{3}$$

In addition to pressure and temperature, which directly affect the O_3 solubility in water (Table 1), other parameters exert this influence. When a solution is prepared by bubbling O_3 in water, smaller sized bubbles result in a larger contact surface, increasing the

mass transfer rate due to increased contact area. Therefore, an appropriated dissolution of O_3 in water occurs when the bubbles are 1 to 3 mm in diameter. O_3 flow and contact time also affect the mass transfer from the gas to the water. The mixing intensity increases the bubble contact time and the superficial velocity, which contribute to improve the dissolution of O_3 in water. Thus, the project of O_3 application in liquids tends to be strongly affected by the dissolution rate (KHADRE et al. 2001, REBOUÇAS and GONÇALVES 2013).

Table 1 O_3 solubility in water as a function of liquid temperature

Temperature (ºC)	Solubility (L of O_3/L of water)
0	0.640
15	0.456
27	0.270
40	0.112
60	0.000

Source: Rice et al. (1981)

The O_3 solubility in water also depends on pH. O_3 is also more stable in aqueous solutions with low pH values (GALDEANO et al. 2018). Jamil et al. (2017), observed that the influence of pH in aqueous systems on the rate of O_3 disinfection was stronger at lower pHs than at basic pH. Further, molecular O_3 was more effective in disinfection than hydroxyl radicals.

- Mechanisms of O_3 dissolution in water

In an aqueous-phase ozonation process, the mass transfer to liquid phase is limited to equilibrium condition, established by equilibrium data and quantified by partition coefficients. The O_3 mass transfer starts with the gas dispersion in the liquid phase in small bubbles. Subsequently, the O_3 incorporates into the liquid phase through the gas-liquid interface. Resistance to mass transfer in the gas phase is practically negligible, and the only resistance is during the gas absorption at the interphase. Improved absorption efficiency can be reached using countercurrent systems, where the O_3 stream is introduced into the water as bubbles in counter flow in deep columns (MIZUNO and TSUNO 2010).

The O_3 mass transfer intensity depends on the turbulence between the gas and liquid phases, the number and size of bubbles and the interfacial transfer area between the two phases of the fluids. Diffusers should produce bubbles with 3 to 5 mm diameter, which is

possible using porous diffusers with intergranular voids ranging in size from 50 to 100 μm. The larger bubbles are characterized by lower surface areas between gas and liquid, reducing ozonation efficiency (CHU et al. 2008).

Microbubbles characterized by diameter lower than 10 μm improve the O_3 dissolution efficiency. The larger interfacial area and higher bubble density contributes to increase the mass transfer rate, even with low ascent velocity in the liquid phase and high internal pressure, when compared to bubbles with a millimeter diameter (CHU et al. 2008).

5.2.Mechanisms of O_3 reaction

O_3 has different forms of reaction that can be divided into four groups: oxidation-reduction reactions, addition reactions, electrophilic substitution reactions and nucleophilic substitution reactions (BELTRÁN and REY 2017). Free radicals formed from these reactions, propagate through elemental mechanisms to produce hydroxyl radicals. These hydroxyl radicals are extremely reactive with organic compounds and some inorganic molecules present in water. For this reason, O_3 reactions in water can be classified as direct and indirect reactions. In direct reaction, the O_3 molecule reacts with any other chemical species, such as molecules, free radicals, among others. The indirect reactions occur between the hydroxyl radical, formed from the O_3 decomposition or from other O_3 direct reactions, and compounds present in water. A direct O_3 reaction is the initiation stage, leading to an indirect reaction (BELTRÁN and REY 2017, OROPESA et al. 2017).

O_3 in aqueous solution can oxidize organic and inorganic compounds by a direct reaction, predominant in acidic environment, or via hydroxyl radical, by an indirect reaction, predominant in alkaline environment (Figure 1). However, compounds oxidation does not occur in a single way. Two mechanisms occur simultaneously with their magnitude mediated by pH (GOTTSCHALK et al. 2010, SILVA et al. 2011).

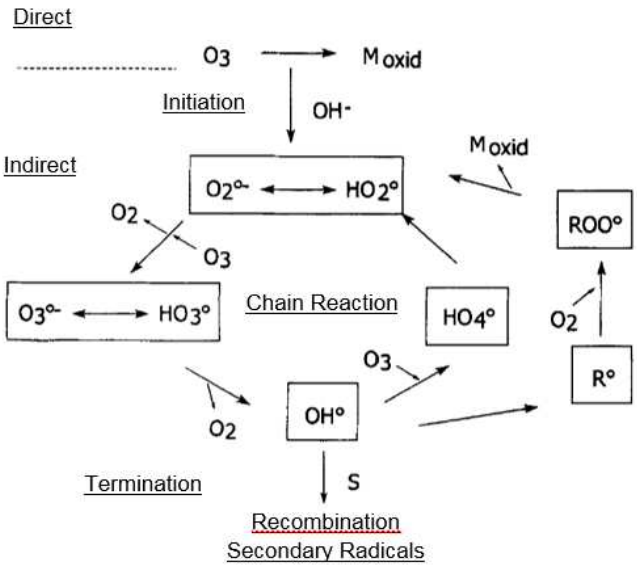

Fig. 1 Direct and indirect ozonation mechanisms (GOTTSCHALK et al. 2010)

In aqueous media, the main trigger of O_3 decomposition is the hydroxyl anion (OH^-), and the reaction between O_3 and OH^- induces a series of reactions between radicals that lead to the formation of other hydroxyl radicals. Such reaction path is quite complex and can be influenced by a number of experimental factors and the type and concentration of chemical species present. In a simplified way, the mechanism can be divided into three parts: initiation, propagation, and termination (GOTTSCHALK et al. 2010, OROPESA et al. 2017). In the initiation stage, the reaction between O_3 and hydroxyl ion occurs forming the superoxide radical anion $^{\bullet}O_2^-$ and the hydroperoxyl radical $HO_2^{\bullet}$, which has an acid-base balance (Equations 4 and 5).

$$O_3 + OH^- \longrightarrow O_2^- + HO_2^{\bullet} \tag{4}$$

$$HO_2^{\bullet} \leftrightarrow O_2^- + H^+ \tag{5}$$

In the next stage, propagation, the reaction between O_3 and superoxide radical anion occurs forming the O_3 radical anion ($^{\bullet}O_3^-$). It decomposes rapidly forming the hydroxyl radicals (Equations 6, 7 and 8). Then, the $^{\bullet}OH$ formed in Eq. 8 can react with the O_3 (Equations 9 and 10).

$$O_3 + {}^{\bullet}O_2^- \rightarrow {}^{\bullet}O_3^- + O_2 \tag{6}$$

$$HO_3^{\bullet} \leftrightarrow {}^{\bullet}O_3^- + H^+ \quad (7)$$

$$HO_3^{\bullet} \rightarrow {}^{\bullet}OH + O_2 \quad (8)$$

$${}^{\bullet}OH + O_3 \rightarrow HO_4^{\bullet} \quad (9)$$

$$HO_4^{\bullet} \rightarrow O_2 + HO_2^{\bullet} \quad (10)$$

With the formation of $HO_2^{\bullet}$ and O_2 by the $HO_4^{\bullet}$ consumption, the chain reaction may restart. Substances that convert $^{\bullet}OH$ to $^{\bullet}O_2^-$ and $HO_2^{\bullet}$ radicals promote the chain reaction and therefore they are called promoters. Organic molecules (R) may also act as promoters (Equation 11).

$$H_2R + {}^{\bullet}OH \rightarrow HR^{\bullet} + H_2O \quad (11)$$

If oxygen is present, organic peroxide radicals ($ROO^{\bullet}$) can be formed and react by eliminating $^{\bullet}O_2^-/HO_2^{\bullet}$ and entering the chain reaction (Equations 12, 13 and 14).

$$HR^{\bullet} + O_2 \rightarrow HRO_2^{\bullet} \quad (12)$$

$$HRO_2^{\bullet} \rightarrow R + HO_2^{\bullet} \quad (13)$$

$$HRO_2^{\bullet} \rightarrow RO + OH^{\bullet} \quad (14)$$

In the termination stage, some organic and inorganic compounds react with the hydroxyl radical and form secondary radicals that do not produce $^{\bullet}O_2^-$ e $HO_2^{\bullet}$, acting as inhibitors of chain reactions (Equations 15 and 16).

$${}^{\bullet}OH + CO_3^{2-} \rightarrow OH^- + {}^{\bullet}CO_3^- \quad (15)$$

$$^{\bullet}OH + HCO_3^- \rightarrow OH^- + {}^{\bullet}HCO_3 \quad (16)$$

A reaction between two radicals (Equation 17) is another possibility for termination step to occur.

$$^{\bullet}OH + {}^{\bullet}HO_2 \rightarrow O_2 + H_2O \quad (17)$$

By combining some of these reactions, three molecules of O_3 produce two radicals $^{\bullet}$OH (Equation 18).

$$3O_3 + OH^- + H^+ \rightarrow \quad 2^{\bullet}OH + 4O_2 \quad (18)$$

Thus, in the ozonation process the compounds can be oxidized by hydroxyl radicals, by molecular O_3 or by the combination of these two oxidizing agents. The reaction path will depend on the O_3 concentration, $^{\bullet}$OH radicals and reaction kinetics.

According to Gottschalk et al. (2010), in acidic environment (pH < 4) the mechanism of direct reaction, via molecular O_3, predominates. As the pH value increases, more $^{\bullet}$OH radicals are formed. For pH values above 10 the O_3 decomposition into $^{\bullet}$OH radicals is instantaneous and the indirect reaction mechanism predominates. However, at pH around 7 both direct and indirect reactions can occur, due to the two oxidizing agents presence. The direct reactions of molecular O_3 with dissolved compounds are slow and selective. Indirect reactions are very fast, however they are not selective. Although the reaction kinetics are important, the mass transfer phenomena that describe the transport of O_3 molecules from the gas to the liquid phase may limit the oxidizing action of the O_3 gas.

O_3 is the classical reagent used in organic reactions to break down carbon-carbon double bonds via the Criegee mechanism or ozonolysis (Figure 2).

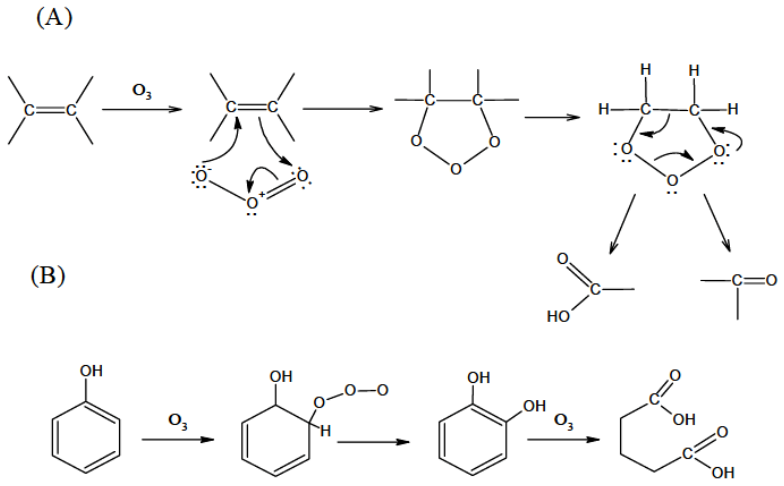

Fig. 2 Example of (A) direct reaction of O_3 with alkene by the Criegee mechanism and (B) electrophilic attack to a phenolic compound (LEE et al. 2017, OLIVEIRA and WOSCH 2012)

Aromatic compounds with deactivating substituent groups, such as chlorine, react slowly through the ozonolysis process, when compared to aromatic compounds with activating substituent groups, such as the hydroxyl group. In general, ionized or dissociated forms of organic compounds react faster with O_3 than neutral (non-dissociated) forms (LEE et al. 2017). Moreover, direct ozonolysis reactions do not usually promote the complete oxidation of the organic compounds to CO_2 and H_2O, being aldehydes, ketones, alcohols and carboxylic acids the main products of this type of reaction (GOTTSCHALK et al. 2010).

The O_3 molecule may act as a dipole, as an electrophilic agent or as a nucleophilic agent. The electrophilic reaction is restricted to molecular sites that exhibit high electron density and, in particular, to some aromatic combinations. Aromatic substituents with electron donor groups exhibit high electronic density at the *ortho* position carbons and are highly reactive in this position. In contrast, aromatic substitutes without electron donor groups poorly react O_3. Nucleophilic reactions occurs in molecular sites that show an electronic deficit, especially in carbons that do not have electron donor groups (Figure 3). In this case, the initial attack of molecular O_3 occurs mainly in the *para* position (SILVA et al. 2011).

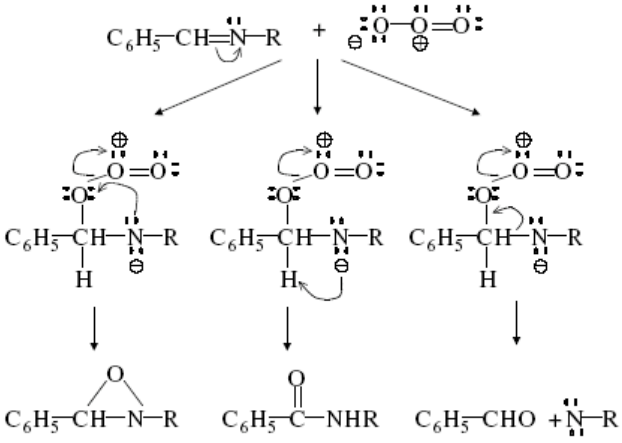

Fig. 3 O_3 nucleophilic substitution reaction (BELTRÁN 2005)

Molecular O_3 reactions are selective and limited to aromatic and aliphatic unsaturated compounds. O_3 oxidizes these compounds by cycle-addition to double bonds. Each of the oxidizing forms assumes different degrees of importance depending on the specific application used (BELTRÁN 2005, SILVA et al. 2011).

Martins and Quinta-Ferreira (2009), comparing the ozonation efficiency in the degradation of phenolic compounds in different effluents, verified that in alkaline medium (pH 9.5) the removal was 95% after 120 min of ozonation. In acid medium (pH 3.4), during the same period, there was only 84% reduction in the concentration of the same compounds. In alkaline conditions, the hydroxide ions are initiators of chain reactions, leading to the formation of reactive hydroxyl radicals that increase the reaction rate, in other words, increasing the solution pH increases the reaction efficiency.

5.3.O_3 reactions with the components of the culture medium

When collecting microalgae, the residual culture medium consists of water, contaminating microorganisms (microorganisms other than microalgae in culture), nutrients and excreta of microalgal metabolism. In order to reuse the culture medium, its disinfection is important to avoid the subsequent contamination and action of compounds that may inhibit the microalgae growth (CHEN et al. 2011, WANG et al. 2016). O_3 is a powerful oxidizing agent that not only kills both bacterial and viral pathogens, removing contaminants and also microalgae toxins (KADIR et al. 2018).

The inactivation of bacteria by O_3 is a complex process. O_3 interacts with numerous cellular components, including proteins, unsaturated lipids and respiratory enzymes in cell membranes; peptideoglycan from cellular envelopes, enzymes and nucleic acids in the

cytoplasm; proteins and peptideoglycans from spore walls and viral capsules (PIRANI 2011, SANTOS et al. 2016). O_3 mechanism of action promotes oxidation and destruction of the cytoplasmic membrane and cell wall, causing its rupture and cell death in lower contact time (LÜDDEKE et al. 2015, ZHANG et al. 2011).

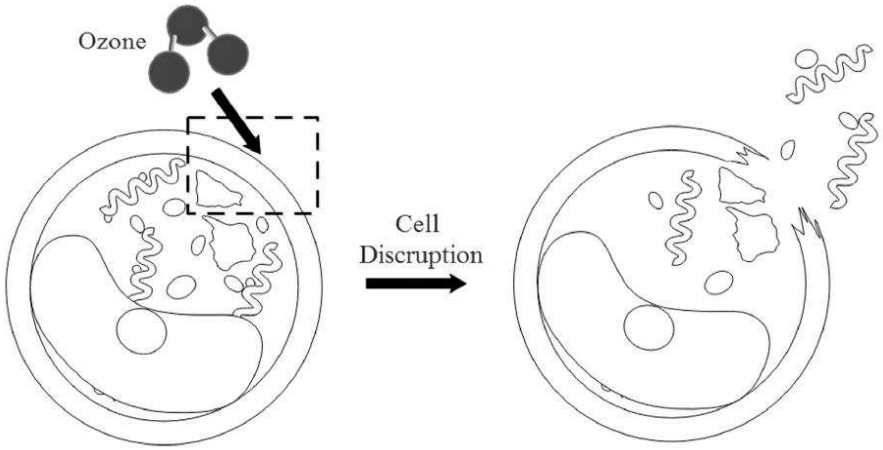

Fig. 4 Microalgae cell disruption through O_3 reaction, adapted from Kadir et al. (2018)

Ozonation was also used as pretreatment technology for increasing the lipid extraction efficiency. The O_3 microbubbles are able to lyse the microalgae extracellular wall (Figure 4), and promoting cell disruption (KADIR et al. 2018). Velásquez et al. (2017) verified that the microalgae harvest by flotation was more efficient when performed with O_3. Moreover, it has been shown that ozonation improves both lipid extraction yield from biomass and biodiesel stability, modifying the fatty acid profile by increase the saturation degree (GONZÁLEZ et al. 2016).

In addition, the ozonation process is capable of mineralizing some organic compounds or breaking them into smaller molecules, which facilitates its assimilation by microalgae (KIM et al. 2014). Reis et al. (2013) verified that increasing ozonation time, nitrogen in the inorganic form concentration also increases, for both ammonium and nitrate, with a rising trend to increase the concentration with increasing ozonation time.

According to Qiang et al. (2015), wastewater ozonation also increases phosphate concentration in the medium, due to the phospholipids entrapped in the microorganism cell membrane, however, the most abundant form of phosphorus in the medium comes from the polyphosphates, which are solubilized after the cell wall rupture. In this manner, it is possible to use these nutrients in microalgae cultivation.

6. CONCLUDING REMARKS

Microalgae production can reach a high lipid content, depending on the species, environmental conditions and cultivation system used. However, new technology for reducing production cost and reuse byproducts are key factors for microalgae-based biofuels. The O_3 injection techniques in liquid phase demonstrate that the mass transfer efficiency will be greater the lower the gas bubble size, liquid temperature and pH and the longer the contact time and system agitation. The ozonation process has the advantage of not generating residues and disinfecting and breaking compounds by different mechanisms, due to its high oxidation potential, enabling their assimilation by the microalgae cultivation. In addition, it can also be used in microalgal cell disruption, increasing the lipid extraction capacity and minimizing solvent use in order to increase the quality of the biodiesel produced. However, further research is still needed to improve each stage of the microalgae and ozonation production processes, to determine the best concentration and the best time, in order to reduce costs and disseminate the use of this technique in the production of biofuels.

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ARTIGO 2

OZONATION OF THE MICROALGAE CULTURE SUPERNATANT FOR NUTRIENTS AND ORGANIC COMPOUNDS ANALYSIS

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ABSTRACT

Microalgae cultivation consists in the usage of water and a mixture of nutrients, that is defined as the culture medium. In the context of sustainable production, there is a great concern in the reuse of wastewater from the microalgae production process. This effluent from the production process of microalgae can be treated with ozone gas, which can enable the reuse of water and nutrients. In the present work, the application of ozone gas was investigated as a strategy to allow the reuse of the culture medium. Posterior to the cultivation, the supernatant was treated with ozone gas (ozonation) with following quantification of phosphate, nitrate, nitrite, ammonium, carbohydrates, proteins and amino acids to verify the possibility of recovering water, nutrients, and organic compounds. The ozonation was able to discolor the culture supernatant, which indicates the possibility of its reuse and the results indicated that this treatment has the potential to treat microalgae wastewater by recovering some nutrients and organic compounds of interest from culture supernatants, allowing the reuse of the effluent in subsequent cultures.

Key-words: Ozone, Culture medium, Water recycling

1. INTRODUCTION

Microalgae cultivation consists in the usage of water and a mixture of nutrients, composed of salts and, eventually, organic compounds. This mixture is defined as the culture medium. In the context of sustainable production, there is a great concern in the reuse of wastewater from the microalgae production process. The knowledge of the quality parameters of these waters for their correct disposal in water courses, in addition to alternatives for the effluent reuse, aiming not only nutrients but also water, have been investigated. This finding can be observed in studies reported by Jones; Mayfield, 2012; Kim *et al.*, 2011; Wu *et al.*, 2012; Yang *et al.*, 2011. Dzuman, 2013 evaluated the successive reuse of the culture medium and found that its recycling allowed to reduce expenses with the necessary nutrients for the cultivation of microalgae and also the water consumption.

Alternatively, the effluent from the production process of microalgae can be treated with ozone gas, which can enable the reuse of water and nutrients. Ozone has a high oxidation potential and, therefore, it has been used in water treatment processes since the beginning of the 20th century. The radical forms of the ozone gas remove organic and

inorganic compounds in different ways (KASPRZYK-HORDERN; ZIÓŁEK; NAWROCKI, 2003) and also promote the elimination of bacteria, fungi and other organisms (ALENCAR *et al.*, 2011; SANTOS *et al.*, 2016). In addition to being a strong oxidizing agent, capable of oxidizing a number of organic and inorganic compounds, ozone is not an intrinsic source of pollution (SOUZA, 2010).

Research on the treatment of water contaminated with excess of organic matter uses ozone as a pretreatment or associates it with catalysts, aiming to increase the oxidation efficiency of organic and inorganic compounds (ABOUZLAM *et al.*, 2013; JEONG *et al.*, 2014). Apart from the capacity of inactivating a number of pathogenic organisms, the ozone scavenging effects are more pronounced in the removal of color and formation of smaller molecules, with higher hydrophilicity and lower toxicity, since the oxidative steps tend to fragment pollutant macromolecules (GOMES DE MORAES; SANCHES FREIRE; DURÁN, 2000). Oxidation may contribute to the transformation of these compounds into products more easily metabolized by microalgae.

In the present work, the application of ozone gas was investigated as a strategy to allow the reuse of the chlorophyllic culture medium. Posterior to the cultivation of *Chlorella* sp. BR001, *Scenedesmus obliquus* BR003, and Chlamydomonas sp. CC503, the supernatant was separated and treated with ozone gas (ozonation) with following quantification of phosphate, nitrate, nitrite, ammonium, carbohydrates, proteins and amino acids to verify the possibility of recovering water, nutrients and organic compounds to be used in subsequent cultures.

2. MATERIAL AND METHODS

The experiments were performed following four steps. (1) Three Chlorophyceae lineages were grown in a known culture medium. (2) At the end of each culture, the biomass was separated from the supernatant by centrifugation. (3) Microalgae oil was extracted from the residual biomass. (4) The ozonation of the supernatant, with and without the defatted biomass, was carried out to evaluate the nutrients.

2.1.Obtaining biological material

The Chlorophyceae lineages *Chlorella* sp. BR001, *Scenedesmus obliquus* BR003, and *Chlamydomonas* sp. CC503 were obtained from the Cyanobacteria and Microalgae

Collection of Unidade de Crescimento de Plantas (Department of Plant Biology - DBV) of the Federal University of Viçosa (UFV).

2.2.Production of inoculum

The inoculum was produced at the Biofuels Laboratory of the Department of Agricultural Engineering of UFV. *Chlorella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp. were inoculated in 250 mL Erlenmeyers using the WC, MBM and TAP medium (GORMAN; LEVINE, 1965; GUILLARD; LORENZEN, 1972; WATANABE, 1960), respectively, and cultured at pH 7.0 ± 0.1 under photoautotrophic growth conditions: temperature of 23 ± 2 °C, photoperiod of 16:8 h of light:dark, in a BOD (Thermostatic Cabinets, Logen Scienttific). After 10 days of culture, in the highest concentration (approximately 0,25 g L^{-1}; 1,1 g L^{-1} e 1,9 g L^{-1}, respectively), the lineages were transferred to higher volume Erlenmeyers, and the volume was filled with fresh culture medium until 1 L. The glassware used for inoculum production and the culture medium were autoclaved at 2 atm and 121 °C.

2.3.Culture conditions

The three chlorophytes were cultivated under photoautotrophic growth conditions: photoperiod from 12:12 h (light:dark) to simulate an outdoor cultivation condition, mean irradiance of the 134 μmols m^{-2} s^{-1} from 4 fluorescence lamps of 40 W. Cultures were carried out in 2000 mL Erlenmeyers, containing 1600 mL of culture medium and inoculum. The pH of the culture media was adjusted, once a day, at 7.0 ± 0.1 with 1 mol L^{-1} solutions of NaOH or HCl after inoculation. The temperature of the laboratory, where the cultures were growing, was monitored daily. During cultivation, the temperature was maintained at 23.5 ± 1.5 °C and the cells were kept in suspension by the injection of atmospheric air supplied by diaphragm pump (Big Air A320, Brazil) at a specific flow rate of 0.2 vvm (liters of air per minute per liter of culture). In addition to daily pH correction, crop growth was monitored daily and lasted for 11, 15 and 10 days for *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, respectively. At the end of the culture, fresh samples were collected for dry mass quantification.

2.4.Growth curves

Samples of 2 mL, for growth curves, were collected daily to evaluate growth by optical density. Samples were collected and absorption, at a wavelength of 750 nm, was measured in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

2.5.Dry mass determination

Culture samples of 15 mL were collected, at the end of the cultivation, before the biomassa was harvested. For the determination of dry mass, the samples were filtered in the vacuum filtration system with PVDF membrane (Durapore, US), 0.22 μm porosity and 47 mm diameter.

2.6.Ozonation

At the end of the cultivation period, the cultures were centrifuged at 6000 g for 10 minutes at 25 °C until all biomass was concentrated. The biomass of the three lineages was used for the extraction of lipids and, afterwards, was stored. The supernatant was separated for subsequent ozonation in two ways: (1) only the supernatant and (2) supernatant with the residual biomass after lipid extraction. Thus, it was possible to evaluate if the recovery of nutrients from the residual biomass occurred and if they were incorporated into the aqueous fraction.

Ozone gas was produced by an ozone generator (O&L 50.0 RD-CO, Brazil). The ozone concentration was adjusted both by the generator voltage variation and by the oxygen flow rate measured in a rotor. The concentration of ozone in the gas phase, in the liquid phase and the specific consumption (ozone mass per unit volume of the treated liquid phase) were also quantified.

The effectiveness of the ozonation process depends on the contact of the ozone gas with the sample, that is, the mass transfer between the liquid and gaseous phases. Thus, it is extremely important to use diffusers, capable of developing ozone microbubbles, that increase the interfacial area for mass transfer. During the treatment of the samples, the ozone gas was injected into a stirred reactor, and the residual gas was captured in a column containing 20 g L^{-1} potassium iodide (Figure 1). At predetermined time intervals, aliquots of 5 mL of KI solution were collected and the iodine content, formed by ozone oxidation of the iodide ion, was quantified by titration with sodium thiosulphate (APHA, 2017). The titration allowed the quantification of ozone mass that reacted with the sample (F, Figure 1) and the concentration of ozone in the gaseous stream.

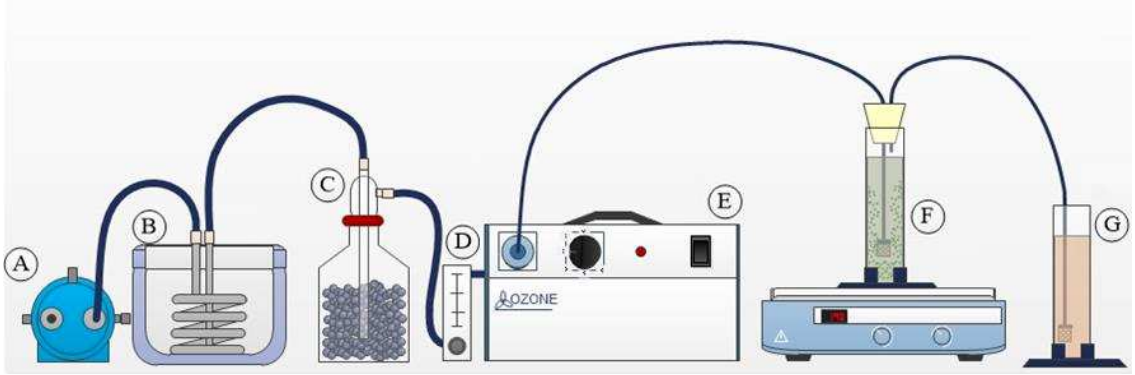

Figure 1: Illustration of the experimental ozonation assembly: (A) air compressor, (B) cooling coil, (C) silica gel for air drying, (D) rotor for quantification of gas flow, (E) ozone, (F) stirred reactor and (G) column containing KI to capture residual ozone.

2.7.Nutrient quantification

The characterization of the supernatant, after ozonation, included the quantification of nutrients (phosphorus, nitrite, nitrate, and ammonium) and organic compounds (carbohydrates, proteins, and total soluble amino acids).

2.7.1. Phosphorus (P)

2.7.1.1.Phosfate PO_4^{3-}

The quantification of PO_4^{3-} in ozonated microalgae cultures, with and without biomass after oil extraction, was performed according to the methodology proposed by Murphy; Riley (1958), with modifications.

The reactive solution was prepared by adding, in a 15 mL tube, 5.0 mL of sulfuric acid (2.5 mol L^{-1}) , 1.5 mL of ammonium molybdate (4 g 100 m L^{-1} H_2O), 3.0 mL of ascorbic acid (1.76 g 100 mL^{-1} H_2O) and 0.5 mL of antimony potassium tartrate solution (0.2743 g 100 mL^{-1} H_2O), followed by slight agitation. Afterwards, 40 µL of the reactive solution and 200 µL of the sample were pipetted into the microplate, in triplicate. The microplate was shaken for 10 min and the absorbance, at 882 nm wavelength , was measured in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

The standard curve of phosphorus was prepared by dissolving 0.018 g of KH_2PO_4 in 100 ml of deionized water in a volumetric flask. This solution contains 40 mg L^{-1} of P as PO_4^{3-}. The results were expressed as mg L^{-1} of PO_4^{3-} in the sample.

2.7.2. Nitrogen (N)

2.7.2.1.Nitrite NO_2^-

The quantification of NO_2^- in the ozonated microalgae, with and without biomass after oil extraction, was performed according to the methodology proposed by Hernández-López; Vargas-Albores (2003), with modifications.

Reactive solution 1 was prepared by adding 1 % Sulfanilamide in HCl (3 mol L^{-1}). Reactive solution 2 was prepared by adding 0.1 % ethylenediamine N-(1-naphthyl) dihydrochloride in water. Then, 180 µl of the sample and 20 µl of the reactive solution 1 were pipetted into the microplate, in triplicate. The microplate was shaken for one minute and incubated for 5 min at room temperature. Thereafter, the reactive solution 2 was added, with subsequent incubation for 10 min under the same conditions. Absorption reading was performed at a wavelength of 550 nm in spectrophotometer for microplates (Multiskan GO, Thermo Scientific, Finland).

The standard nitrite curve was prepared by dissolving 0.1232 g of $NaNO_2$ in 100 ml of deionized water in a volumetric flask. This solution contains 250 mg L^{-1} of N as NO_2^-. The results were expressed as mg L^{-1} of NO_2^- in the sample.

2.7.2.2.Nitrate NO_3^-

The quantification of NO_3^- in the extracts of ozonated microalgae, with and without biomass after oil extraction, was performed according to the methodology proposed by APHA (2017), with modifications.

Samples of 200 µL were pipetted into the quartz microplate and the absorbance was read at the wavelengths of 210, 220 and 230 nm, in the UV region, in a spectrophotometer for microplates (Multiskan GO, Thermo Scientific, Finland). The nitrate concentration is proportional to the second derivative of the absorption spectrum between 210 and 230 nm, calculated by Equation 1:

$$SD = (Abs\ 210 - Abs\ 220) - (Abs\ 220 - Abs\ 230) \quad (1)$$

The standard nitrate curve was prepared by dissolving 0.07218 g of KNO_3 in 100 ml of deionized water in a volumetric flask. This solution contains 100 mg L^{-1} of N in the form of NO_3^-. The results were expressed as mg L^{-1} of NO_3^- in the sample.

2.7.2.3. Ammonium NH_4^+

The quantification of NH_4^+ in the extracts of ozonated microalgae, with and without biomass after oil extraction, was performed according to the methodology proposed by Strickland; Parsons (1972), with modifications.

Four reagent solutions were prepared: reagent 1 - phenol solution in 95% ethyl alcohol (10 g 100 mL^{-1} CH_3CH_2OH), reagent 2 - sodium nitroprusside solution (0.5 g 100 mL^{-1} H_2O), reagent 3 - sodium citrate solution and sodium hydroxide (20 g $C_6H_5Na_3O_7$ + 1 g NaOH 100 mL^{-1} H_2O) and – reagent 4 - sodium hypochlorite reagent (1.5 mol L^{-1}). The analysis was performed by pipetting 150 µL of the sample, 15 µL of the phenol solution, 15 µL of the sodium nitroprusside solution and 25 µL of the oxidation solution (reagent 3 and reagent 4 in a ratio of 1:4) in triplicate into the microplate. The microplate was kept at room temperature for 1.0 h, then stirred for 2 min and the absorbance, at the wavelength of 640 nm, in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland) was measured.

The standard ammonium curve was prepared by dissolving 0.04714 g of $(NH_4)_2SO_4$ in 100 ml of deionized water in a volumetric flask. This solution contains 100 mg L^{-1} of N as NH_4^+. The results were expressed as mg L^{-1} of NH_4^+ in the sample.

2.8. Organic compounds quantification

2.8.1. Total Soluble Carbohydrates

The quantification of carbohydrates was perfomed using the phenol-sulfuric acid method proposed by Dubois *et al.*, (1956) and adapted for microplates by Masuko *et al.*, (2005). The total neutral carbohydrates, which correspond to oligosaccharides, proteoglycans, glycoproteins and glycolipids, were quantified using glucose as standard.

Firstly, the samples were hydrolyzed and then, the supernatands were used to the quantification. In an Eppendorf tube, 500 µL of the sample was hydrolyzed with 500 µL of 3 mol L^{-1} sulfuric acid, in a thermostated bath, at 90 °C for 90 minutes. After the solution reached room temperature, an aliquot of 400 µL was pippeted in an Eppendorf tube, along with 1.2 mL of concentrated sulfuric acid and 240 µL of the 5% phenol solution (50 g L^{-1} H_2O). The sample was then taken to the thermostated bath at 90 ° C for 5 minutes. Samples of 200 µL was pipetted into the microplate and the reading performed at the wavelength of 490 nm in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

The standard glucose curve was prepared by dissolving 0.040 g of glucose in 100 mL of deionized water in a volumetric flask. This solution contains 400 mg L^{-1} of glucose.

2.8.2. Total Soluble Proteins

Soluble protein analysis of the ozonated extract was determined according to the Bradford method Bradford (1976). Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95 % ethanol and then adding 100 ml of 85 % phosphoric acid. The solution obtained completed with deionized water until 1.0 L. After filtration on quantitative filter paper (Whatman nº 1), the solution was kept in the refrigerator. The analysis was performed by pipetting in an Eppendorf tube, 100 μL of the sample and 1.0 mL of the Bradford reagent, with subsequent stirring for 10 minutes at room temperature. Then 200 μL of this solution was added to the microplate. The reading was performed at the wavelength of 595 nm in a spectrophotometer for microplates (Multiskan GO, Thermo Scientific, Finland).

The standard curve was prepared by dissolving 0.2 g of BSA in 50 mL of saline solution (0.15 mol L^{-1} NaCl) in a volumetric flask. This solution contains 400 μg mL^{-1} protein as BSA.

2.8.3. Total Soluble Amino Acids

In the quantification of amino acids, the ninhydrin method was used (MOORE; STEIN, 1954). Working solutions were prepared as follows: reagent 1 – 1 mol L^{-1} Na-Citrate buffer (21 - 29 g 100 mL^{-1} H_2O). Then, 0.2 g of ascorbic acid was added to the buffer. Reagent 2 - ninhydrin in 70 % alcohol (1 g 100 mL^{-1} EtOH 70%).

The analysis was performed in threaded glass tubes, in which 250 μL of the Na-Citrate buffer, 500 μL of the ninhydrin solution and 250 μL of the sample were pipetted. The tubes were sealed and placed in an ultrathermostatic bath at 95 °C for 20 min, then the samples were pipetted into the microplate and the reading was performed at a wavelength of 570 nm in a spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

The standard curve was prepared by dissolving 0.013 g of leucine in 100 mL of 70% alcohol in a volumetric flask. This solution contains 1 mg mL^{-1} of leucine.

2.9.Experimental design

The experiments were carried out in a completely randomized design with three replicates. The results from the experimental work were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test and the Dunnett test at the 5% probability level using the SAEG 9.1 software.

3. RESULTS AND DISCUSSION

Figure 2 shows the growth curves of the three studied microalgae *(Chlorella* sp. BR001, *Scenedesmus* sp. BR003, and *Chlamydomonas s*p. CC503) cultivated in WC, MBM and TAP culture media. The lineages showed similar growth among the three replicates. *Chlorella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp. Cultures were interrupted on the 12th, 15th and 10th day, respectively, when they reached the stationary phase.

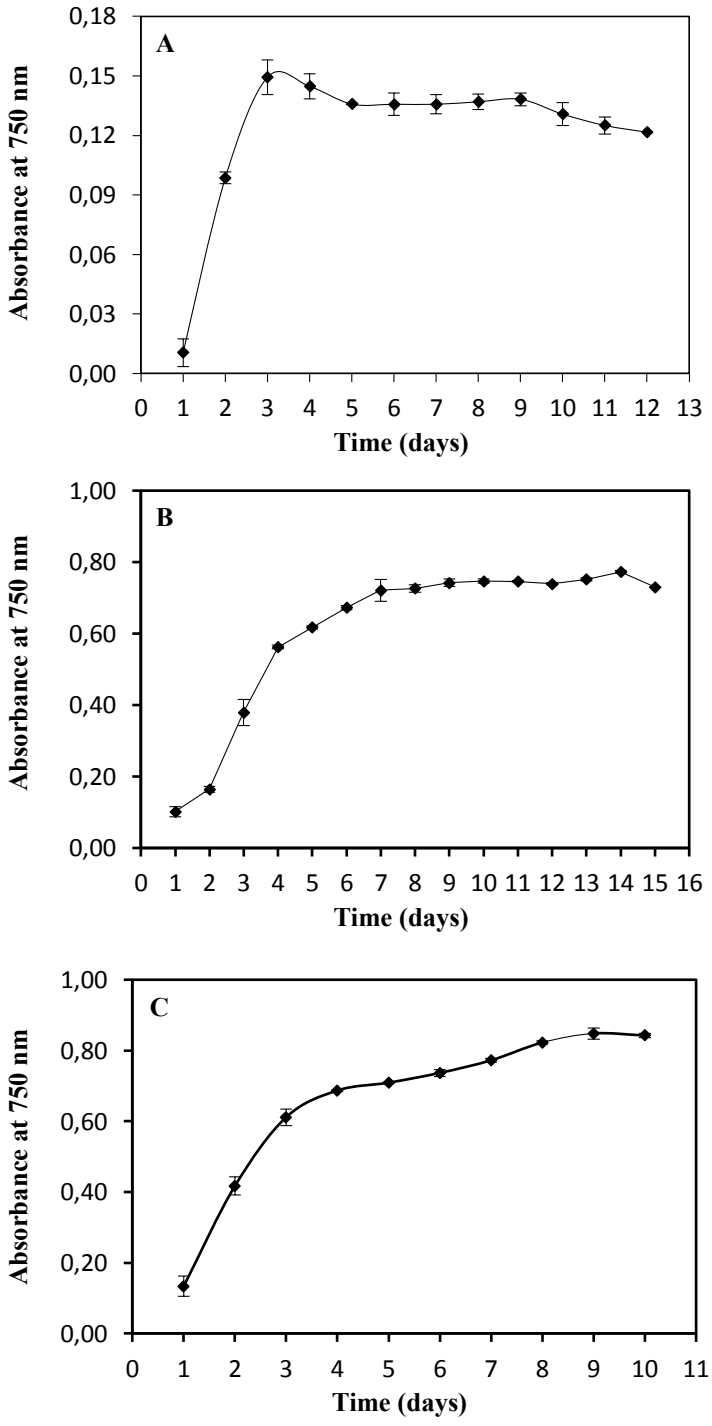

Figure 1: Growth curves (A) *Chlorella* sp. BR001; (B) *Scenedesmus* sp. BR003 and (C) *Chlamydomonas* sp. CC503.

The biomass concentration (dry mass) of *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503 was 0.30, 1.32 and 2.13 g L^{-1}, respectively.

Figure 2 shows the O_3 consumption during the ozonation of the culture supernatant of the three lineages, with and without the addition of the residual defatted biomass.

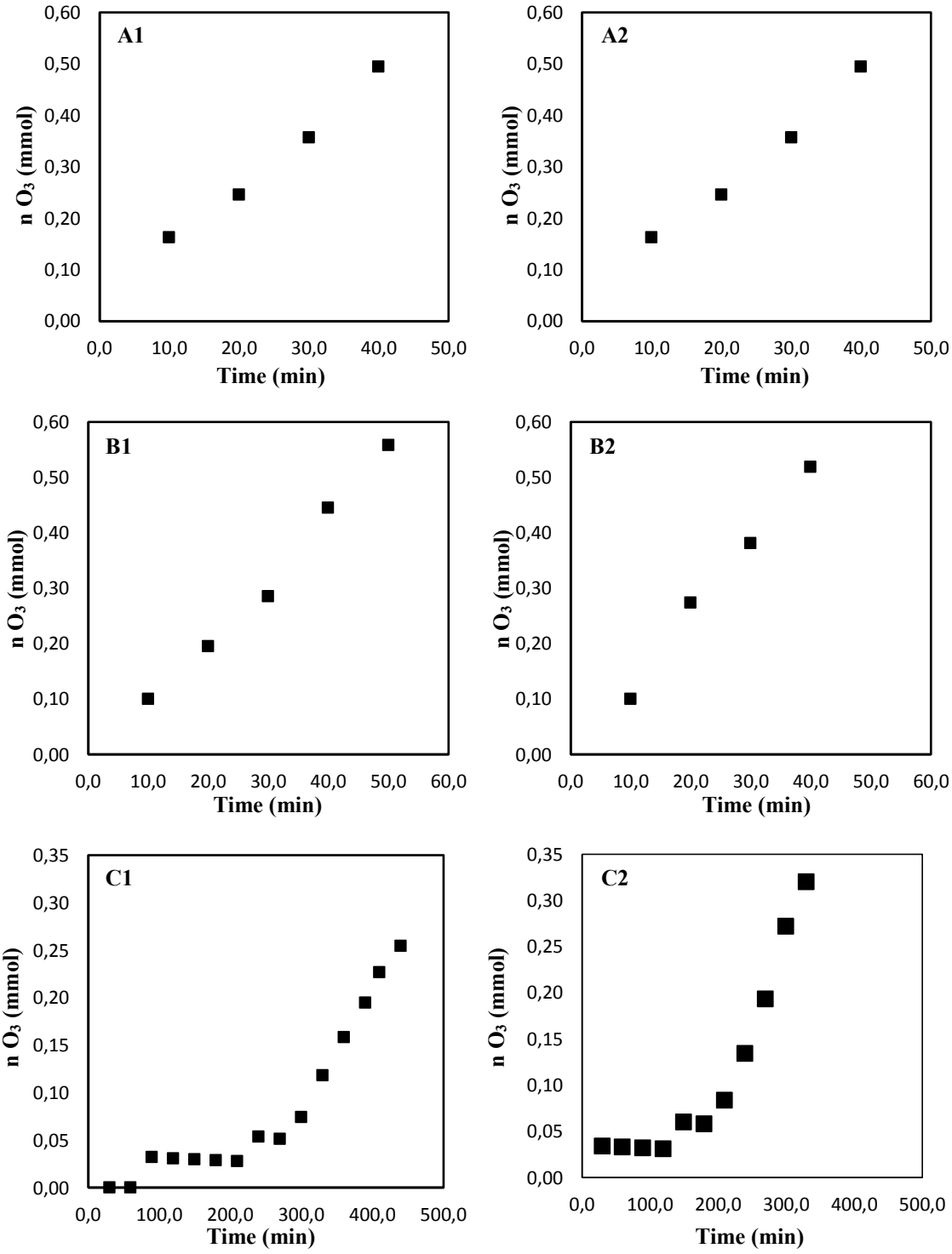

Figure 2: Ozone consumption in the reactor: (A) *Chlorella* sp. BR001 – (A1) Supernatant + defatted biomass and (A2) Supernatant; (B) *Scenedesmus* sp. BR003 – (B1) Supernatant + defatted biomass and (B2) Supernatant, (C) *Chlamydomonas* sp. – (C1) Supernatant + defatted biomass and (C2) Supernatant.

The ozonation presented different behavior for each of the studied lineages, as it is evidenced in Figure 2. O_3 can react with organic and inorganic components in two distinct forms: direct or molecular and indirect or radical. Direct reactions are more selective and slower than the indirect ones, which are not selective and faster (BELTRÁN, F. J.; REY, 2018; BELTRÁN, FERNANDO J.; REY, 2017). For that reason, the different compounds presented in the treated samples can, consequently, explain the variation observed in Figure 2.

Direct oxidation is a selective reaction, which often presents kinetic constants of low magnitude and occurs predominantly in acidic medium. This reaction is called ozonolysis. However, direct ozonolysis reactions generally do not promote the complete oxidation of organic compounds to CO_2 and H_2O, being aldehydes, ketones, alcohols and carboxylic acids the main products of this type of reaction (MAHMOUD; FREIRE, 2007).

Indirect reactions (predominant in an alkaline medium) present high oxidation potential and fast reaction kinetics, being more efficient to promote more effective oxidation of organic compounds (MAHMOUD; FREIRE, 2007). The ozone reaction rate constants, with various organic compounds, have been identified and are described in the literature (BELTRÁN, FERNANDO J.; AGUINACO; GARCÍA-ARAYA, 2010; JIN; PELDSZUS; HUCK, 2012; SHIN; HIDAYAT; LEE, 2016).

The reaction kinetics of ozone with organic compounds are typical of second order. In the process of ozonization, the oxidation of a compound is characterized by the action of two oxidants (ozone and hydroxyl radicals). Organic compounds that do not react directly with the ozone can be removed by reactions with · OH radicals (BILA *et al.*, 2007).

The ozonation process was also very effective in the discoloration of the culture supernatant (Figure 3), suggesting the possibility of reuse of the aqueous fraction. Tripathi; Tripathi (2011) also verified the color reduction of the sewage secondary effluent after treatment with O_3.

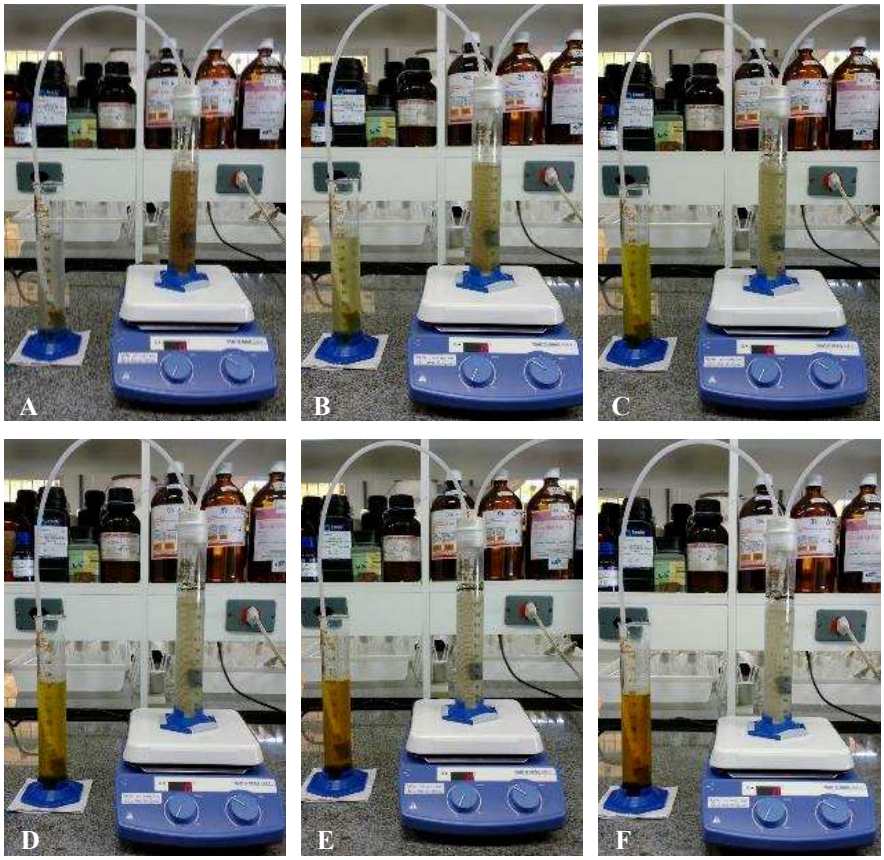

Figure 3: Ozonation process of culture supernatant: (A) 0 min, (B) 10 min, (C) 20 min, (D) 30 min, (E) 40 min and (F) 50 min.

Several studies have demonstrated the efficiency of ozone treatment in wastewater of the pharmaceutical industry (EBRAHIM; HULLE; SHEIKHA, 2018), dairy wastewater (KAYA *et al.*, 2018), food processing industry, drinking water disinfection and surface decontamination of fruits and vegetables (PANDISELVAM *et al.*, 2018), among others. These results demonstrated that, under optimal conditions of O_3 concentration, temperature, pH and time of contact with the effluent, it was possible to achieve satisfactory results in the degradation of compounds, besides the reduction of the microbial and fungal load of wastewater, before its return to hydric bodies.

After ozonization, nutrients and organic compounds were quantified. The standard curves of nutrient analyzes are linear, as expected and demonstrated in Figure 4.

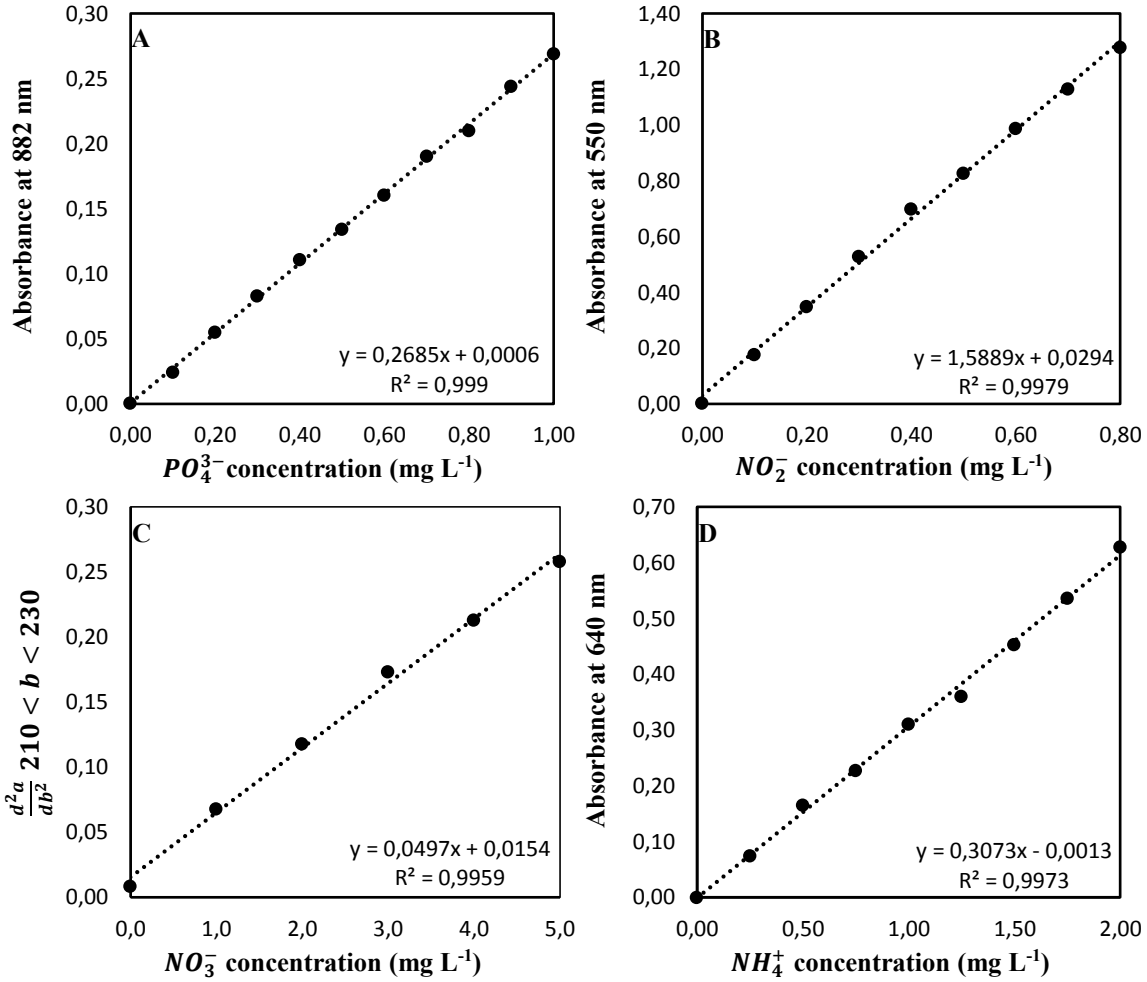

Figure 4: Analytical curves: (A) Phosphate; (B) Nitrite; (C) Nitrate and (D) Ammonium.

The concentrations of phosphate, nitrate, nitrite, and ammonium are shown in Table 1 and the total mean values of nutrients and organic compounds are given in Tables 2 and 3 for the respective combinations of lineages and treatment.

It was possible to observe that ozonation promoted the availability of phosphorus and nitrogen in both treatments, for the three lineages studied. It is verified that there is a greater recovery in the treatments in which the deffated biomass (lipid free) was added. The other nutrients showed similar behavior, confirming that the ozonation of the culture supernatant allows the reuse for subsequent cultures, as it can be verified in Figure 3, through a visual analysis of the supernatant.

The nutrients P and N and their interactions were evaluated through an analysis of variance (ANOVA), for each lineages. Two mean tests were then performed: Dunnet (5%) and Tukey (5%). The statistical analysis demonstrated that the means of the treatments with ozone (O_3) and ozone with defatted biomass (O_3+BM) were different from each other and

also, they both were different from the control treatment, with exception of the variable P (phosphorus) for *Chlorella* sp., which presented no difference (Table 2).

There was a significant increase in P and N in all treatments in which the oil-free biomass was added, reaching concentrations of 0.021; 1.473 and 0.547 mg L^{-1} of P and 0.377; 0.104 and 0.478 mg L^{-1} of N in the lineages in *Chlorella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp., respectively (Table 2). The increase in the nutrients, when the ozonation of the culture supernatant happens with the deffated biomass, reveals that the nutrients, once absorbed by the microalgae, are extracted by the O_3 and dissolved in the aqueous phase, making them available for a new crop.

Yang *et al.* (2018) verified an increase of P (3 mg L^{-1}) and N (8 mg L^{-1}) after the ozonation of PHWW (post-hydrothermal liquefaction wastewater). The ozonized material was used in the cultivation of Chlorella vulgaris and due to the N:P ratio, it was possible to reach a good growth of the microalga (approximately 2.0 g L^{-1}).

Table 1: Concentrations of PO_4^{3-}, NO_2^-, NO_3^-, NH_4^+ and total mean values of P and N for the respective combinations of species and treatment.

Nutrients (mg L^{-1})	*Chorella* sp.			*Scenedesmus* sp.			*Chlamydomonas* sp.		
	Controle	O_3	O_3+BM	Controle	O_3	O_3+BM	Controle	O_3	O_3+BM
PO_4^{3-}	0,020	0,029	0,090	54,171	50,226	58,688	22,033	19,059	23,709
P Total	0,008	0,009	0,029	17,667	16,381	19,140	7,186	6,216	7,733
NO_2^-	0,003	0,090	0,105	0,006	0,001	0,043	0,066	0,951	0,440
NO_3^-	ND	ND	ND	ND	ND	ND	ND	ND	ND
NH_4^+	0,494	0,574	0,939	0,492	0,423	0,607	0,768	1,409	1,237
N Total	0,384	0,473	0,761	0,384	0,329	0,484	0,616	1,384	1,094

ND: Values below the device detection limit. BM: Residual crop biomass after oil extraction.

Table 2: Total phosphorus (P) and nitrogen (N) concentration for the respective lineages and treatments.

Lineages	P (mg L^{-1})			N (mg L^{-1})		
	Controle	O_3	O_3+BM	Controle	O_3	O_3+BM
Chorella sp.	0,008±0,0003a	0,009±0,0003a	0,029±0,001a	0,384±0,002a	0,473±0,001*b	0,761±*0,003c
Scenedesmus sp.	17,667±0,03a	16,381±0,02*b	19,140±*0,09c	0,384±0,003a	0,329±0,001*b	0,484±0,002*c
Chlamydomonas sp.	7,186±0,05a	6,216±0,009*b	7,733±*0,04c	0,616±0,005a	1,384±*0,008b	1,094±0,006*c

(1)Averages with * in the line, for each lineage and nutrient, differ from the control by the Dunnett test at 5% probability.
(2)Averages followed by the same letter in the line, for each lineage and nutrient, do not differ among themselves by the Tukey test at 5% probability.
BM: Residual crop biomass after oil extraction.

The standard curves used in cellular content analyzes were also linear, as expected (Figure 5). The carbohydrate, protein and amino acid concentrations are found in Table 3, for the respective lineage and treatment combinations.

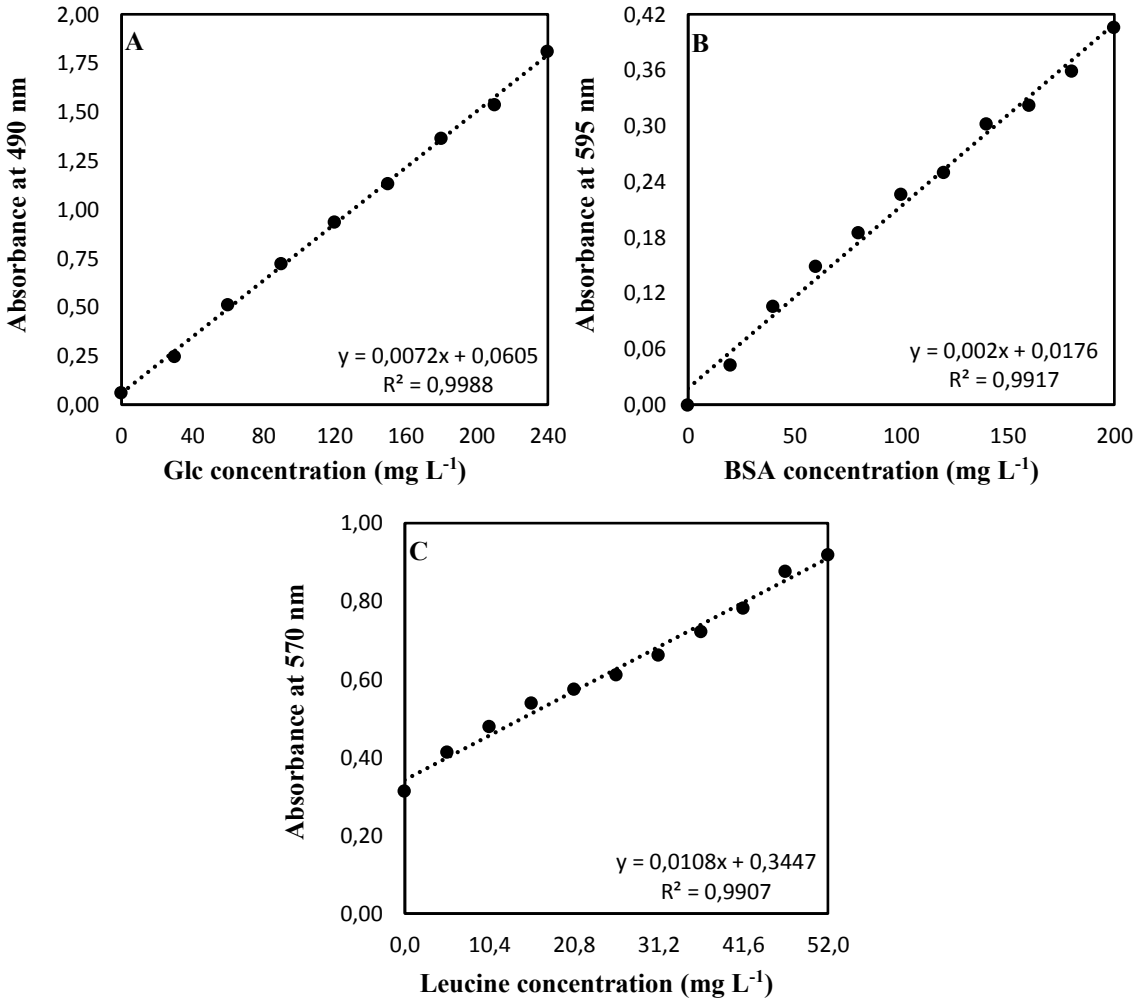

Figura 5: Analytical curves: (A) Carbohydrates; (B) Proteins; (C) Amino Acids.

The analysis of variance (ANOVA) and the average tests of Dunnet (5%) and Tukey (5%) were performed with the results obtained in the quantification of organic compounds.

In Table 3, it is observed an increase in total carbohydrate and amino acid concentration in both treatments (O_3 and O_3+BM) for Chlamydomonas sp., with significant differences between treatments and between treatments and control. There was also an increase in total soluble amino acid content, in both treatments, for Chlorella sp. and in the O_3 treatment for Scenedesmus sp., as also shown in Table 3.

Tabela 3: Carbohydrate, protein and amino acid concentrations for the respective lineage and treatment combinations.

Lineage	Controle	O_3	O_3+BM
	Carboidratos (mg L^{-1})		
Chorella sp.	36,914±0,1a	32,816±0,1a	36,816±0,1a
Scenedesmus sp.	63,624±0,04a	24,809±0,06*b	50,560±0,08ab
Chlamydomonas sp.	113,430±0,2a	1275,235±0,1*b	1102,432±0,2*c
	Proteínas (µg mL^{-1})		
Chorella sp.	161,183±0,1a	153,894±0,1*b	159,2444±0,2a
Scenedesmus sp.	162,383±0,3a	145,017±0,3*b	156,4389±0,2*c
Chlamydomonas sp.	233,483±0,2a	167,467±0,2*b	143,1556±0,3*c
	Aminoácidos (mg L^{-1})		
Chorella sp.	7,543±0,009a	11,728±0,1ab	36,202±0,1*b
Scenedesmus sp.	16,117±0,1a	6,372±0,2a	61,681±0,1*b
Chlamydomonas sp.	118,580±0,2a	514,763±0,2*b	823,848±0,2*c

(1)Averages with * in the line, for each lineage and treatment, differ from the control by the Dunnett test at 5% probability.
(2)Averages followed by the same letter in the line, for each lineage and treatment, do not differ among themselves by the Tukey test at 5% probability.
BM: Residual crop biomass after oil extraction.

Total soluble protein concentrations showed opposite behavior, with a decrease in both treatments for the three lineages studied. The O_3 probably disrupted the proteins into peptides and amino acids, which may have caused an increase of 28.7, 45.6 and 705.3 mg L^{-1} in the total soluble amino acid content in the lineages *Chlorella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp., respectively.

4. CONCLUSION

- The ozonation was able to discolor the culture supernatant of the three microalgae lineages: *Chlorella* sp. BR001, *Scenedesmus obliquus* BR003, and *Chlamydomonas* sp. CC503, which indicates the possibility of its reuse as it improves light penetration for autotrophic cultures.
- The nutrient analysis showed that there was a significant increase of phosphorus and nitrogen, for the three lineages, in the ozonation with defatted biomass;
- Variations in carbohydrate and total soluble amino acid concentrations showed that there was a significant increase for both ozone treatments, whether or not the defatted

biomass was present, in the *Chlamydomonas* sp. CC503, suggesting that ozone was effective in releasing monossacharides and soluble amino acids from the stored carbohydrates and proteins of the microalgae cell. The lines of *Chlorella* sp. BR001 and *Scenedesmus* sp. BR003 were also susceptible to degradation of proteins in total soluble amino acids by ozone.

- It is possible to conclude that ozone treatment has the potential to treat microalgae wastewater by recovering some organic compounds of interest from culture supernatants, such as amino acids, allowing the reuse of the effluent in subsequent cultures.

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ARTIGO 3

REUSE OF CULTIVATION SUPERNATANT TREATED WITH OZONE FOR MICROALGAE GROWTH

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ABSTRACT

For the economic sustainability of the process, the costs reduction with the raw material for the production of biofuel derived from microalgae is fundamental. The use of nutrients from agroindustrial and wastewater residues is an alternative to reduce crop costs. The extract obtained by the oxidation of microalgae with ozone is a promising way of reusing the nutrients assimilated by microalgae during their cultivation. In the present study, three microalgae lineages were grown: *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503 in the O_3 treated supernatant from the previous crop to check whether water and nutrients recovered can influence the production of biomass and organic compounds of interest. The cultivations of the lineages of *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503 were performed on the ozonized supernatant from the previous culture without inhibition. The obtained results suggest that ozonized supernatant with lipid-free biomass and with nutrients addition, presented the best averages in all analyzes, but new studies must be performed in order to find the best growth medium, reusing water and all the compounds of interest for microalgae, to optimize their use as raw material for biofuel production.

Key-words: Ozonized supernatant, Nutrients addition, Wastewater.

1. INTRODUCTION

Microalgae have oils with physicochemical characteristics similar to vegetable oils used in the biodiesel production chain, thus the interest in cultivating microalgae for the production of biofuels has extended (FAO, 1997). The yield of microalgae oil per crop area is higher than any other oilseed used in biodiesel production (GHORBANI *et al.*, 2018). Due to the high microalgae capacity of accumulating lipids, carbohydrates, proteins, amino acids, and other bioactive compounds, it is possible to produce ethanol, biogas, aviation fuels and biodiesel from these metabolites (ADENLE; HASLAM; LEE, 2013; PARMAR *et al.*, 2011).

The costs reduction with the raw material for the production of biofuel derived from microalgae is fundamental for the economic sustainability of the process. The use of nutrients from agroindustrial and wastewater residues is an alternative to reduce crop costs (ZHANG *et al.*, 2018).

Several organic effluents can be used as sources of nutrients for the production of microalgae (HUY *et al.*, 2018), such as effluents from the crop itself. According to Santos et al. (Data not published), water, most of the nutrients and organic compounds from microalgae cultivation can be recovered by oxidation by the O_3 of the aqueous culture residue (supernatant) after the flocculation of the biomass.

The extract obtained by the oxidation of microalgae with ozone is a promising way of reusing the nutrients assimilated by microalgae during their cultivation. According to Santos et al. (Data not published), the treatment with ozone is able to recover water, nutrients (PO_4^{3-}, NO_2^- and NH_4^+) and organic compounds (carbohydrates, proteins, and amino acids) of the microalgae culture supernatant, allowing their reuse in subsequent cultures.

In the present study, three microalgae lineages were grown: *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503 in the O_3 treated supernatant from the previous crop to check whether water and nutrients recovered can influence the production of biomass and organic compounds of interest.

2. MATERIAL AND METHODS

2.1.Obtaining the crop material

The cultures of three Chlorophyceae (*Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503) were grown under photoautotrophic growth conditions: photoperiod of 12:12 h (light:dark), mean irradiance of 134 μmols m^{-2} s^{-1} from 440 W fluorescent lamps, in 2000 mL Erlenmeyer flasks containing 1600 mL culture medium and inoculum.

Cultures were monitored daily by absorption measurements at the wavelength of 680 and 750 nm in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland). Time-absorption data were used for the growth curves construction. Upon reaching the stationary phase, the cultures were centrifuged at 6000 g in a refrigerated centrifuge at 25 °C for 10 min. The residual biomass was separated for extraction of the oil, and the supernatant was stored at 4 °C for subsequent ozonation.

The ozonation was carried out by adding the culture supernatant in a stirred reactor, in which the O_3 gas was injected until the bleaching and saturation of the liquid (Figure 1).

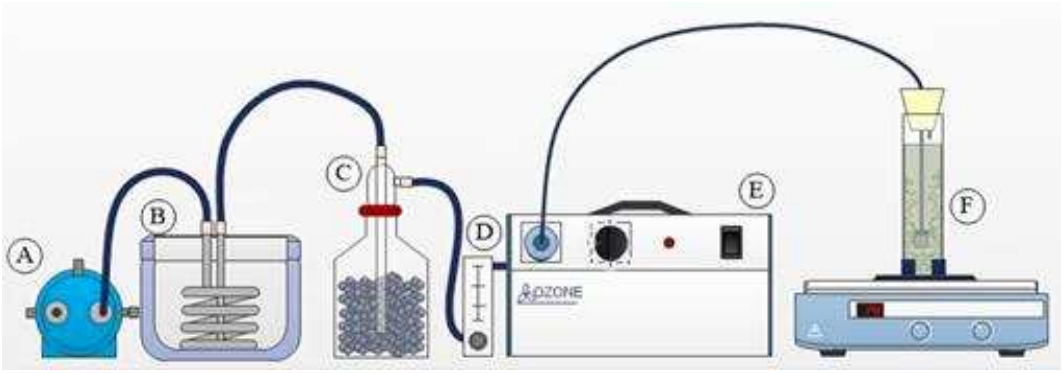


Figure 1: Illustration of the experimental ozonation assembly: (A) air compressor, (B) cooling coil, (C) silica gel for air drying, (D) rotor for quantification of gas flow, (E) ozone, (F) stirred reactor.

2.2.Culture conditions

The lineages obtained in the Collection of Cyanobacteria and Microalgae of the Unidade de Crescimento de Plantas (Department of Plant Biology - DBV) of the Federal University of Viçosa (UFV) were used for the following cultures.

The previous culture supernatant of each lineage was used, in this experimental phase, as a culture medium for the microalgae in order to evaluate if the treatment with ozone was effective in making it reusable for new cultures. That being so, the recovered supernatant, with and without the presence of defatted biomass and nutrients, were used to grow new crops.

The control treatment consisted of the WC, MBM and TAP medium (GORMAN; LEVINE, 1965; GUILLARD; LORENZEN, 1972; WATANABE, 1960) for the *Chlorella* sp. BR001, *Scenedesmus obliquus* BR003 and *Chlamydomonas* sp. CC503, respectively. The T1 and T2 treatments consisted of the culture supernatant, after centrifugation and separation of the microalgae biomass. The T3 and T4 treatments were performed with the culture supernatant, after O_3 treatment, without and with addition of nutrients of the respective culture media of each lineage. The T5 and T6 treatments were carried out with the culture supernatant together with the lipid-free biomass and after O_3 treatment, without and with addition of nutrients, respectively. The T7 treatment consisted of the culture supernatant after centrifugation and separation of the microalgae biomass, with addition of nutrients and treated with activated charcoal (HARRISON; BERGES, 2005) aiming at the adsorption of compounds that may be toxic to the lineages. Table 1 summarizes the treatments, which were performed in triplicate.

Table 1: Description of cultivation treatments for each lineage, *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503.

Tratamentos	Descrição
Controle	Culture medium WC, MBM e TAP
T1 – S	Supernatant
T2 – SN	Supernatant + nutrients
T3 – SO	Ozonized supernatant
T4 – SON	Ozonized supernatant + nutrients
T5 – SOB	Ozonized supernatant + defatted biomass
T6 – SOBN	Ozonized supernatant + defatted biomass + nutrients
T7 – SCN	Supernatant + activated charcoal + nutrients

The culture media presented in Table 1 was performed with the addition of 90 mL of medium and 10 mL of inoculum in 250 mL Erlenmeyer flasks. The cultures were maintained under photoautotrophic growth conditions: photoperiod of 12:12 h (light:dark) in Shaker Incubator (430-RFDPE - Ethik), mean irradiance of 77 μmols m^{-2} s^{-1} from 6 fluorescent lamps of 14 W, under agitation of 120 rpm and temperature of 24 ± 2 °C, (Figure 2).

The cell growth of the cultures was monitored daily by absorption measurements at the wavelength of 680 and 750 nm, measured in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland). 0.07 g $NaHCO_3$ was added daily to the *Chlorella* sp. BR001 and *Scenedesmus* sp. BR003 and 0.05 g of CH_3COOK for the lineage of *Chlamydomonas* sp. CC503, as a carbon source. After the addition of the carbon source, the pH was adjusted to pH 7.0, by adding 1 mol L^{-1} solutions of HCl or NaOH.

Figure 2: Microalgae culture in 250 mL Erlenmeyer flasks, containing 100 mL of culture medium + inoculum, with a photoperiod of 12:12 h (light:dark), shaking of 120 rpm and temperature of 24 ± 2 ºC.

2.3.Dry mass determination

Culture samples of 15 mL were collected, at the end of the cultivation, before the biomass was harvested. For the determination of dry mass, the samples were filtered in the vacuum filtration system with PVDF membrane (Durapore, US), 0.22 μm porosity and 47 mm diameter.

2.4.Organic compounds quantification

2.4.1. Total Soluble Carbohydrates

The phenol-sulfuric acid method proposed by Dubois *et al.*, (1956) and adapted for microplates by Masuko *et al.*, (2005) were used to quantify total carbohydrates. The total neutral carbohydrates, which correspond to oligosaccharides, proteoglycans, glycoproteins and glycolipids, were quantified using glucose as standard.

Firstly, the samples were hydrolyzed and then, the supernatants were used to the quantification. In an Eppendorf tube, 500 μL of the sample was hydrolyzed with 500 μL of 3 mol L^{-1} sulfuric acid, in a thermostatic bath, at 90 °C for 90 minutes. After the solution reached room temperature, an aliquot of 400 μL was pippeted in an Eppendorf tube, along with 1.2 mL of concentrated sulfuric acid and 240 μL of 5% phenol solution (50 g L^{-1} H_2O). The sample was then taken to the thermostatic bath at 90 ° C for 5 minutes. Afterwards, samples of 200 μL were pipetted into the microplate and the absorbance was read at the

wavelength of 490 nm in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

The standard glucose curve was prepared by dissolving 0.040 g of glucose in 100 mL of deionized water in a volumetric flask. This solution contains 400 mg L^{-1} of glucose.

2.4.2. Total Soluble Proteins

Soluble protein analysis of the ozonated extract was determined according to the Bradford method (BRADFORD, 1976). Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95 % ethanol and then adding 100 ml of 85 % phosphoric acid. The obtained solution was completed with deionized water until 1.0 L. After filtration on quantitative filter paper (Whatman nº 1), the solution was kept in the refrigerator. The analysis was performed by pipetting in an Eppendorf tube, 100 µL of the sample and 1.0 mL of the Bradford reagent, with subsequent stirring for 10 minutes at room temperature. Then 200 µL of this solution was added to the microplate. The reading was performed at the wavelength of 595 nm in a spectrophotometer for microplates (Multiskan GO, Thermo Scientific, Finland).

The standard curve was prepared by dissolving 0.2 g of BSA in 50 mL of saline solution (0.15 mol L^{-1} NaCl) in a volumetric flask. This solution contains 400 µg mL^{-1} protein as BSA.

2.4.3. Total Soluble Amino Acids

In the quantification of amino acids, the ninhydrin method was used (MOORE; STEIN, 1954). Working solutions were prepared as follows: reagent 1 – 1 mol L^{-1} Na-Citrate buffer (21 - 29 g 100 mL^{-1} H_2O). Then, 0.2 g of ascorbic acid was added to the buffer. Reagent 2 - ninhydrin in 70 % alcohol (1 g 100 mL^{-1} EtOH 70%).

The analysis was performed in threaded glass tubes, in which 250 µL of the Na-Citrate buffer, 500 µL of the ninhydrin solution and 250 µL of the sample were pipetted. The tubes were sealed and placed in an ultrathermostatic bath at 95 °C for 20 min, then the samples were pipetted into the microplate and the reading was performed at a wavelength of 570 nm in a spectrophotometer (Multiskan GO, Thermo Scientific, Finland).

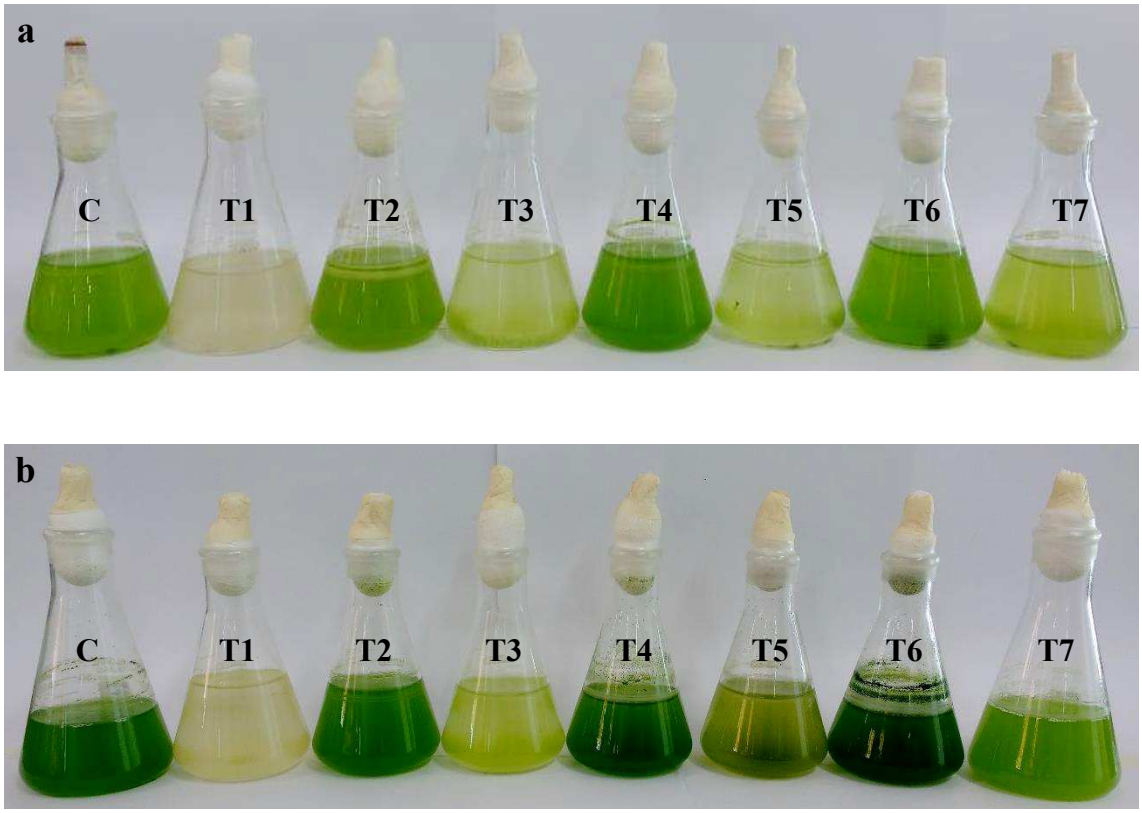
The standard curve was prepared by dissolving 0.013 g of leucine in 100 mL of 70% alcohol in a volumetric flask. This solution contains 1 mg mL^{-1} of leucine.

2.5.Experimental design

The experiments were carried out in a completely randomized design with three replicates. The results from the experimental work were submitted to analysis of variance (ANOVA) and the means were compared by the Tukey test and the Dunnett test at the 5% probability level using the SAEG 9.1 software.

3. RESULTS AND DISCUSSION

The culture treatments are presented in Figure 3 (C: Control, T1: Treatment 1, T2: Treatment 2, T3: Treatment 3, T4: Treatment 4, T5: Treatment 5, T6: Treatment 6 and T7: Treatment 7) for the lineages *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503.



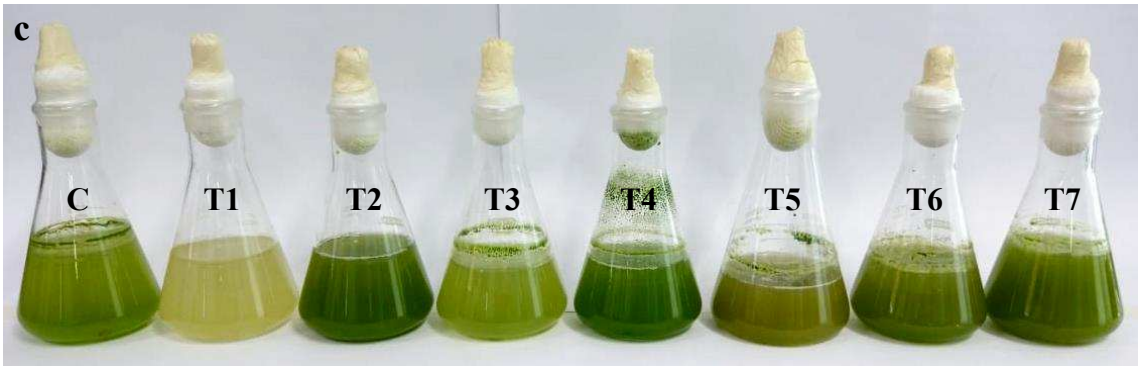


Figura 3: Crops at the end of the stationary phase for lineages (a) *Chlorella* sp. BR001, (b) *Scenedesmus* sp. BR003 and (c) *Chlamydomonas* sp. CC503.

The mean dry mass concentration of each treatment, for each lineage, is shown in Table 2. It is possible to observe that the biomass concentration showed significant differences between the treatments and also between the treatments and the control.

Table 2: The mean dry mass concentration of each treatment for each lineage, *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503.

Treatments	**Dry mass (g L^{-1})**		
	Chlorella sp. BR001	*Scenedesmus* sp. BR003	*Chlamydomonas* sp. CC503
C	0,21b	1,35e	2,20f
T1 – S	0,07*a	0,18*d	0,97*d
T2 – SN	0,23b	0,90*ab	2,78*ab
T3 – SO	0,10*a	0,51*c	1,83*e
T4 – SON	0,21b	0,91*ab	2,67*ac
T5 – SOB	0,11*a	0,75*ac	2,53*c
T6 - SOBN	0,44*d	0,95*ab	2,84*b
T7 - SCN	0,32*c	1,01*b	2,73*ab

(1) Averages with * in the column, for each lineage, differ from the control by the Dunnett test at 5% probability.
(2) Averages followed by the same letter in the column, for each lineage, do not differ by Tukey test at 5% probability.

Chlorella sp.BR001 presented statistically equal dry mass values between the control and T2 and T4 treatments. The treatments T1, T3, and T5 presented equal biomass yields, but differed from the control, with lower mean dry mass concentrations. The treatments T6 and T7 also differed from the control, presenting higher values of dry mass at the end of the

crop. The T6 treatment highlighted, resulting in higher dry mass yield (0.44 g L^{-1}), surpassing the control in 52%. This yield was higher than the results observed by Kong *et al.* (2011), in the treatments adding sodium bicarbonate and by Lu *et al.* (2015), when cultivating this genus in municipal wastewater and the textile industry. It is suggested that this greater accumulation of dry mass is a result of the microalga cultivation conditions since it was grown in the ozonated supernatant of the crop with the addition of free defatted biomass and with added nutrients, that is, the ozonated extract may have influenced biomass production.

The dry mass yield of the treatments for *Scenedesmus* sp. BR003 was statistically different from the control, but some treatments presented similarities among themselves (Table 2). The control had a mean dry mass concentration higher, similar to results reported in the literature, under the same conditions (PANCHA *et al.*, 2015). It is suggested that the use of the recovered residue, treated with O_3, did not influence the yield of dry biomass for this lineage, since the T2 treatment was not cultivated in the ozonated supernatant and resulted in a significantly similar dry mass value to T4 and T6 treatments. The addition of nutrients may have influenced the production of biomass, since T1, T3 and T5 treatments, which did not receive nutrients, presented lower averages than the other treatments.

The dry mass production of *Chlamydomonas* sp. (Table 2), in almost all treatments, were higher than the TAP medium, found in the literature (LI *et al.*, 2010). The averages were statistically different between the control and the other treatments. The treatments that received nutrient addition obtained higher means, that is, this factor influenced the dry mass yield. However, the T6 treatment presented the highest concentration in dry mass, 22.5% higher than the control, which is believed to have occurred not only by the addition of nutrients but mainly by the use of the ozonized extract in the cultivation.

Tables 3 to 5 present the average total carbohydrate, protein and amino acid values for the respective combinations of lineages and treatment.

There were significant differences between the control and the other treatments, in the three lineages (Table 3). In the cultivation of *Chlorella* sp. BR001, with the exception of the T4 and T5 treatments, which obtained carbohydrate concentration means significantly equal, the treatments presented different means between them. The treatments T1 and T2 obtained averages of the concentration of carbohydrates superior to the control whilst the others treatments presented lower means than the control.

Table 3: Total mean total soluble carbohydrate values for the respective lineage and treatment combinations.

Treatments	Total soluble carbohydrates (mg L^{-1})		
	Chlorella sp. BR001	*Scenedesmus* sp. BR003	*Chlamydomonas* sp. CC503
Controle	38,89e	63,91b	140,77d
T1 – S	48,25*f	19,31*a	77,35*c
T2 – SN	68,18*g	32,24*c	210,50*f
T3 – SO	0,32*b	20,71*a	190,83*e
T4 – SON	5,84*a	38,33*d	325,62*g
T5 – SOB	5,74*a	68,53*f	269,75*b
T6 - SOBN	25,17*d	49,82*e	260,23*ab
T7 - SCN	16,00*c	63,65b	249,78*a

(1) Averages with * in the column, for each lineage, differ from the control by the Dunnett test at 5% probability.
(2) Averages followed by the same letter in the column, for each lineage, do not differ by Tukey test at 5% probability.

In the lineage *Scenedesmus* sp. BR003, with the exception of the T7 treatment, the other treatments had mean values of carbohydrate concentration significantly different from the control (Table 3). The T5 treatment had a mean protein concentration higher than the control.

Total carbohydrate concentration of *Chlamydomonas* sp. CC503 presented only one treatment (T2) lower than the control while the other treatments presented higher concentration averages. The treatments that had received ozonized supernatant in their culture (T4, T5 and T6) stood out as the treatments with higher averages of carbohydrate concentration (Table 3). Thus, there is evidence that the use of the residue of the previous crop, treated with O_3, can stimulate the carbohydrate production of *Chlamydomonas* sp. CC503.

The three studied microalgae lineages showed similar behavior regarding protein concentration. The treatments presented significant differences when compared to the control, and, except for the T1 treatment, all treatments showed a mean protein concentration higher than the control (Table 4).

Table 04: Total mean values of total soluble proteins for the respective lineage and treatment combinations.

Treatments	Total soluble proteins (µg mL^{-1})		
	Chlorella sp. BR001	*Scenedesmus* sp. BR003	*Chlamydomonas* sp. CC503
Controle	177,59e	198,27a	245,72c
T1 – S	164,27*d	179,65*e	204,57*b
T2 – SN	187,12*b	238,69*d	459,91*e
T3 – SO	204,96*c	205,71*bc	354,50*d
T4 – SON	184,77*b	210,11*c	524,50*f
T5 – SOB	200,99*ac	200,56ab	613,98*a
T6 - SOBN	196,97*a	222,86*f	619,67*a
T7 - SCN	197,74*a	240,05*d	540,09*g

(1) Averages with * in the column, for each lineage, differ from the control by the Dunnett test at 5% probability.
(2) Averages followed by the same letter in the column, for each lineage, do not differ by Tukey test at 5% probability.

Chlorella sp BR001 showed the highest concentration of proteins in the T3 treatment, which contained residue of the previous culture, treated with O_3, in its cultivation (Table 4). The treatments with the highest averages for *Scenedesmus* sp. BR003 were T2 and T7, with statistically equal means, and also T6, which presented a high and significantly different mean from the others.

The behavior of the experiments with *Chlamydomonas* sp. CC503 demonstrated that almost all treatments reached significantly different means. The only exception was the T5 and T6 treatments, which obtained the highest protein concentration averages, with values that were significantly the same (Table 4). This suggests that the O_3 treated supernatant, from the previous culture, may have positively influenced the accumulation of proteins for this lineage, since they both were ozonated and the T5 treatment did not receive the addition of nutrients.

For amino acid concentrations, *Chlorella* sp. BR001 and *Chlamydomonas* sp. CC503 showed statistically different means from the control (Table 5). The averages of all treatments of *Chlorella* sp. BR001 were superior to the control, and the T5 treatment stood out with an average concentration higher than the others.

Tabela 05: Total mean values of total soluble amino acids for the respective lineage and treatment combinations.

Treatments	Total soluble amino acids (mg L^{-1})		
	Chlorella sp. BR001	*Scenedesmus* sp. BR003	*Chlamydomonas* sp. CC503
Controle	9,90d	13,08b	128,30d
T1 – S	12,96*ab	10,90*a	73,21*b
T2 – SN	12,86*ab	10,92*a	202,63*a
T3 – SO	13,17*b	10,32*c	99,23*c
T4 – SON	12,15*c	11,01*a	162,79*f
T5 – SOB	14,46*e	13,13b	197,52*a
T6 - SOBN	12,88*ab	13,21b	342,12*g
T7 - SCN	12,48*ac	12,44*d	148,56*e

(1) Averages with * in the column, for each lineage, differ from the control by the Dunnett test at 5% probability.
(2) Averages followed by the same letter in the column, for each lineage, do not differ by Tukey test at 5% probability.

Scenedesmus sp. BR003 presented mean amino acid concentrations lower than the control, except for the T5 and T6 treatments. Although these treatments obtained higher means, they did not differ significantly from the control (Table 5).

For *Chlamydomonas* sp. CC503, only treatments T1 and T3 presented mean concentrations of amino acids inferior to the control. All other treatments had higher means, and the T6 treatment presented a mean almost three times higher than the control (Table 5). This result may suggest that the use of the supernatant, treated with O3, influenced, individually, the concentration of amino acids in this lineage.

The results found in the present study may suggest that the use of the residue of the previous microalgae culture, treated with O3, acts differently in each of the lineages (*Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp.). The use of the ozonated supernatant in subsequent cultures may positively influence the growth and concentration of compounds of interest in microalgae cultivation. With the results of accumulation of dry mass and cellular content (Table 2), it is possible to assure that there was no growth inhibition by the excreta of the previous cultures, which can be verified when comparing the treatments T1 and T2, differentiated by the addition of nutrients in the reuse water of the cultures. Likewise, the supernatants did not produce growth inhibitory compounds, as can be seen when comparing the treatments treated with ozone gas and with

the addition of activated carbon (T2, T4, and T7). Thus, it is verified that, in fact, it is possible to reuse the wastewater from the biomass crops, as long as the nutrient supplementation is correctly used.

The results showed that nutrient supplementation is necessary, since the recovered nutrients were not enough to supply the crop. However, as noted, the ozone action is different for each sample, that is, the efficiency of the treatment will depend on the constitution of the effluent. According to the components, ozone may be able to release and make available various cellular contents of the waste biomass for the new crop.

It is important to emphasize that the inocula of the lineages used in this study are monoalgaes, but not axenic. Therefore, future studies should be conducted to evaluate the population of microorganisms present at the end of the crops. It is expected that treatment with ozone will reduce the microbial population of the wastewater, as verified in Gomes *et al.* (2019); Hajiali; Pirumyan (2018) and Wu *et al.* (2018). Corroborating this idea, numerous studies have confirmed the bactericidal effect of ozone, including Gram-positive and Gram-negative bacteria, as well as spore forms and vegetative cells, fungicidal activity and yeast inactivation (BRODOWSKA; NOWAK; ŚMIGIELSKI, 2018; SOUZA *et al.*, 2019).

4. CONCLUSION

- The cultivations of the lineages of *Chlorella* sp. BR001, *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503 were performed on the ozonizated supernatant from the previous culture without inhibition;
- The treatment with ozone of the supernatant with lipid-free residual biomass showed higher dry mass concentration in the Chlorella sp. BR001 and *Chlamydomonas* sp. CC503 cultures, surpassing the averages obtained in the control treatment that consisted of the culture in WC and TAP culture medium, respectively.
- The ozone treated supernatant with and without residual lipid-free biomass and added or not of nutrients, of the lineages *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, presented the highest averages of carbohydrate concentration, exceeding the averages obtained in the control treatment, grown in culture medium;
- The highest protein concentration means were the ozone treated supernatant of *Chlorella* sp. BR001, the ozone treated supernatant added of defatted biomass and nutrients, in the lineage Scenedesmus sp. BR003 and the ozonated supernatant with

defatted biomass, added or not of nutrients, in the lineage *Chlamydomonas* sp. CC503;

- Treatments of the ozonated supernatant with defatted biomass, added or not of nutrients, of the lineages *Scenedesmus* sp. BR003 and *Chlamydomonas* sp. CC503, respectively, presented the highest averages of amino acid concentration, and the latter reached a mean value almost three times higher than that observed in the control treatment, grown in TAP medium;
- The obtained results suggest that ozonated supernatant with lipid-free biomass and with nutrients addition, presented the best averages in all analyzes, mainly in the *Chlamydomonas* sp CC503 lineage;
- Each species of microalgae achieved different results, however, in all the studied lineages, it was possible to observe positive results, being the ozone gas used or not. So, this demonstrates the potential of reuse of the wastewater of the crops without generation of inhibitory compounds to the growth;
- In summary, new studies must be performed in order to find the best growth medium, reusing water and all the compounds of interest for microalgae, to optimize their use as raw material for biofuels production.

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