**Biorefinery process intensification by ultrasound and ozone for phosphorus
and biocompounds recovery from microalgae.**

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Abstract

This study applies individual and combined application of ultrasound and ozone pretreatments for the tandem recovery of microalgae biocompounds (lipids, proteins, and, carbohydrates) and phosphorus from *Scenedesmus obliquus* cultivated in wastewater. Results of individual application showed that ultrasound released protein to the aqueous phase ($99 \pm 3\%$), and increased microalgal lipid viability from 11 to $90 \pm 2\%$. Whilst ozone pretreatment induced carbohydrate release to the aqueous phase $28 \pm 1\%$. Combined ultrasound and ozone pretreatment were studied in sequence mode. Ultrasound was first applied to release proteins and expose lipids, followed by ozone to induce carbohydrates and phosphorus release. The maximum yields of recovered lipids, proteins, carbohydrates and phosphorus by the combined pretreatment were $89 \pm 1\%$, $91 \pm 8\%$, $63 \pm 1\%$, and $75 \pm 1\%$ respectively. In the dual pretreatment best experimental parameters applied for ultrasound were: biomass concentration of 25 g/L and applied energy of 200 kWh/kg dry biomass. Ozone pretreatment used 27 mg O_3 /L, contact time 25 min and pH of 11. The combined pretreatment intensified microalgae fractionation achieving high biocompounds and phosphorus recovery yields; reducing solvent waste by 95% and extraction time by 90%, in comparison to biocompounds extraction without pretreatment.

44 **Keywords**

45 Combined pretreatment

46 Microalgae

47 Ultrasonication

48 Ozonation

49 Biocompounds fractionation

50 **Abbreviations**

51 PI _____ Process intensification

52 SPE _____ Solid phase extraction

53 TLC _____ Thin-layer Chromatography

54 FAME _____ Fatty Acid Methyl ester

55 GC-MS _____ Gas Chromatography-Mass Spectrometry

56 ANOVA _____ Analysis of variance

57 Ortho- PO_4^{3-} _____ Orthophosphate

58 COD _____ Chemical Oxygen Demand

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1. Introduction

A microalgae biorefinery can be defined as a facility that integrates microalgae fractionation and conversion processes to transform biomass into a spectrum of bio-based products like food, feed, chemicals, and bio-energy (fuels, power, heat) [1]. The main goal of the biorefinery is to optimise the use of the microalgae; reduce waste production, and maximise profitability and benefits [2]. Cell disruption is an essential pretreatment to increase the bioavailability of microalgae constituents and maximize product recovery in the downstream processes of the microalgae biorefinery [3]. Since downstream processing is responsible for a large part of the operational costs in the whole production chain, cell disruption technologies should be low cost and result preferably in high product quality. Pretreatment should increase the valorisation of high-value compounds (lipids, proteins, and carbohydrates) and residual biomass [3-5].

A variety of cell disruption methods is currently available, and new approaches are being developed in parallel. Recently, a combination of pretreatment techniques have been proposed as a way to increase the efficiency of downstream processes [3]. Combined pretreatment should attain maximum biocompounds recovery at minimum energy consumption to achieved energy-efficient biofuel production in a sustainable way. The combination of ultrasound pretreatment with other disruption methods is interesting in a microalgae biorefinery context [6]. Ultrasound pretreatment is effective in disrupting wet microalgae for the extraction of valuable intracellular materials and thus increasing the concentration of extracellular organic microalgal material in suspension [6-8]. Ultrasound has been combined with: 1) dilute acid pretreatment to improve fermentative hydrogen production, 2) ozone for microalgae cell disruption, lipids and

carbohydrate extraction, or for stabilization of raw activated sludge [9-11]. In all the cases, combined pretreatment increased by more than 50% the efficiency of the processes [9-11]. Applying ozone to microalgae releases stored carbohydrates to the aqueous phase, an attractive raw material for biofuels [8, 12]. Ozone pretreatment is more successful in extracting higher glucose yields, for ethanol production, than acid and alkaline methods [13]. Ozone combined with microwave pretreatment, can achieve waste activated sludge solubilisation increase by 3-fold the concentration of phosphorus in the aqueous phase [14].

To ensure that pretreatments methods can be applied in a sustainable and effective way when combined, it is necessary to first consider the individual effect of each pretreatment on the microalgae cell wall and the recovery efficiency of biocompounds. This study examined the individual and combined application of ultrasound and ozone pretreatment to recover microalgae biocompounds (lipids, proteins, and carbohydrates) and phosphorus. First, ultrasound or ozone pretreatments were studied individually to identify their pretreatment effect on the tandem recovery of *Scenedesmus obliquus* biocompounds. After, their combined application was studied in sequence. The sequential pretreatment process consisted first of ultrasound to induce cell disruption, recover proteins and lipids followed by ozone to recover carbohydrates and phosphorus. Finally, intensification of the sequential pretreatment process was studied for biorefinery purposes.

2. Materials and Methods

2.1 Microalgae samples

Microalgae, *Scenedesmus obliquus* (Turpin) Kützing, used in this study was provided by Instituto Politecnico Nacional, Mexico City, Mexico. The *Scenedesmus obliquus* biomass was

grown in 10 L reactors at room temperature (20 ± 3 °C) using raw municipal wastewater as medium (85 ± 16 mg/L of $\text{NH}_4^+\text{-N}$, 544 ± 5.5 mg/L of COD, 46 ± 4.5 mg/L of ortho- PO_4^{3-} , and 47 ± 1.5 mg/L of total phosphorus). The light was provided by LED lamps (1620 lumen), under light/dark periods of 12:12 h. After 27 days of cultivation, the biomass was harvested by sedimentation; oven dried at 40 °C for 12 h and stored at 4 °C until used. The microalgae on the culture at the end of cultivation process were characterized considering their morphology via light microscopy observations with the aid of identification manuals. Species *Scenedesmus obliquus*, *Cyanobacteria* and *Mychonastes* sp. were identified. The approximate composition of the microalgae culture was determined by cell counts using a Neubauer counting chamber and microscope (AX10 Lab A1, Zeiss, Jena, Deutschland). This showed microalgae species composed of 90% *Scenedesmus obliquus*, 5% *Cyanobacteria* sp., and 5% *Mychonastes* sp. The content of biocompounds and phosphorus in the microalgae biomass on a per gram basis was determined based on the microalgae mixture. The content of lipids was determined by a gravimetric method as reported by Valeriano-González et al. [15], the content of proteins by a chemical method as reported by Safi et al. [16]. Carbohydrates were determined by colorimetric method using phenol as reported by Mirsiaghi et al. [17], and total phosphorus was obtained by following the USEPA PhosVer 3 method [18]. Microalgae was composed of: 118 ± 16 , 680 ± 12 , 125 ± 10 , and 12 ± 1 mg/g dry biomass of lipids, proteins, carbohydrates, and phosphorus, respectively. All experiments were done using the same batch of microalgal biomass.

2.2 Experimental design of individual and combined application of ultrasound and ozone pretreatments

The design creation and subsequent statistical analysis were performed using statistical software (Statgraphics Centurion XVI, Statgraphics Technologies, The Plains, VA). A design of

experiments based on a response surface methodology was used. The design of the study was divided into two stages, the objective of the first stage was to evaluate the individual influence of ultrasound and ozone pretreatment on the tandem recovery of microalgae biocompounds. Two different parameters for ultrasound pretreatment were considered: applied energy at three levels (50, 125, and 200 kWh/kg biomass) and biomass concentration at three levels (25, 50, and 75 g/L), resulting in 9 experiments. At this stage for ozone pretreatment, three different parameters at three different levels were considered: ozone concentration (3, 6, and 9 mg O₃/L), biomass concentration (25, 50, and 75 g/L), and contact time (5, 10, and 15 min) involving 27 treatments. The sum of the yield of released biocompounds in the bulk liquid during pretreatment and the yield of extracted biocompounds after pretreatment was expressed as the yield of recovered biocompounds. Ultrasound and ozone pretreatment showed a diverse effect on the recovery efficiency of each microalgae biocompound. Also, neither pretreatment achieved the simultaneous recovery of microalgae biocompounds, therefore, in the second stage of the study, a combined ultrasound and ozone pretreatment was designed. The combined pretreatment applied was designed based on the preliminary results found on the individual effect of each pretreatment about each biocompounds. These allows the effective application of each pretreatment to recover a biocompound or several biocompounds in particular at specific experimental parameters. The combined pretreatment may also intensify the tandem recovery of microalgae lipids, proteins, carbohydrates, and phosphorus due to both pretreatments induce solvent waste and extraction time reduction. Additionally, both pretreatments increase the quality of the biocompounds recovered for biofuels production. In the individual application ultrasound pretreatment achieved high yields of released proteins in the bulk liquid and recovered lipids. In the dual pretreatment, ultrasound was applied to simultaneously release proteins to the aqueous

phase and expose lipids. Desirability function was applied to find out a combination of the experimental parameters that provided the maximum efficiency for both proteins and lipids recovery. In this method, each set of responses obtained by applying an experimental design is transformed into dimensionless values called individual desirabilities, which are then aggregated into a single response called overall desirability. Desirability values can range within a scale from 0 (undesirable response) to 1 (completely desirable response). Intermediate values of desirability indicate more or less desirable responses, depending on their approximation to the unit value. Obtaining this scale makes it possible to combine the various responses of different orders of magnitude into a single response, without running the risk of overlapping the effect of another one [19]. For ozone, a new experiment for carbohydrates and phosphorus recovery was designed, since in the individual application of ozone only $28 \pm 0.2\%$ of the carbohydrates were released in the bulk liquid. The design creation and subsequent statistical analysis were performed using statistical software (Statgraphics Centurion XVI, Statgraphics Technologies, The Plains, VA). The experimental parameters evaluated for ozone in the combined pretreatment were: ozone concentration at four levels (9, 15, 21 and 27 mg O₃/L), contact time at three levels (15, 25 and 35 min) and pH at two levels (8 and 11) involving 24 experiments. In the combined pretreatment ozone desirability function was applied to find out a combination of the experimental parameters that provided the maximum efficiency for both carbohydrates and phosphorus recovery. All the experiments were performed in triplicate. Analysis of variance (ANOVA) was also performed to identify significant operating parameters at a 95% level ($p < 0.05$).

2.3 Ultrasound and ozone pretreatments

Ultrasound pretreatment was carried using a 100 W, 42 kHz with a tank capacity of 2.81 L ultrasound bath (2510-MT, Branson, Hampton, NH, US). All experiments were performed in 100 mL Erlenmeyer flasks, containing 25 mL of a microalgae suspension, immersed in the ultrasound bath, under controlled temperature of 3 ± 1.5 °C using ice-water. The dissipated acoustic power applied to the solution during this study was calculated by determining the temperature increase during the first 30s of the experiment. Based on this, the ultrasonic intensity was 0.32 W/mL.

Ozonation was conducted using 250 mL Erlenmeyer flasks containing 25 mL of microalgae suspension. Ozone was provided using an ozone generator (Labo76, Emery Trailgaz, Wayne, NJ, US) with a production capacity of 19 g O₃/h. Ozone was injected at a flow rate of 0.5 L/min. The ozone concentration in the gas phase was determined using the Iodometric method [20].

2.4 Quantification of biocompounds released into the aqueous phase

After each pretreatment, lipids, proteins, carbohydrates, orto-PO₄⁻³, and total phosphorus released into the aqueous phase were quantified separately. Lipids were determined by the sulfo-phospho-vanillin assay [21], proteins by the Biuret method [22], carbohydrates by using the phenol-sulphuric acid method [23], orthophosphate by the Amino Acid Method and total phosphorus by the USEPA PhosVer 3 method [18]. At least triplicate measurements were performed.

2.5 Extraction of the remaining microalgal biocompounds after ultrasound or ozone

After ultrasound or ozone pretreatment, the remaining microalgal biocompounds were extracted from the pretreated cell pellet in the following order: lipids, proteins, and

carbohydrates. For this purpose, microalgae suspensions were centrifuged at 2500 g, 20 °C for 15 min.

2.5.1 Lipids extraction and quantification

Lipids were extracted and quantified using a modification of the procedure reported by Valeriano-González et al. [15]. Pellet-suspension ratio and extraction time were reduced and applied for lipid extraction. A homogeneous solution of chloroform-methanol (2:1 v/v) and the cell pellet was mixed at 150 rpm for 30 min with a pellet-suspension ratio of 1:15 (w/v). The resulting mixture was centrifuged at 1500 g, 20 °C for 15 min. The supernatant was filtered through a Whatman glass microfiber filter paper, and the pellet was preserved for protein extraction. The filtrates used were collected into pre-weighed 25 mL Erlenmeyer flasks and left to dry in the incubator at 40 °C and 50 rpm. Amount of microalgal lipids extracted were calculated by subtracting the Erlenmeyer flask weight from the constant final weight. At least triplicate measurements were performed.

2.5.2 Protein extraction and quantification

After lipid extraction, the defatted algae biomass was dried in the incubator at 40 °C for 1 h to eliminate the solvents residue. Protein extraction was conducted using the procedure reported by Safi et al. [16] consisting on modifications of the pellet-suspension ratio and extraction time. Mother solution for protein extraction was prepared as follows: 500 mL of distilled water was adjusted to pH 11 with 2N NaOH for maximum protein solubility. The dried biomass was suspended in the mother solution using a 1:10 (w/v) ratio and was stirred for 15 min at 40 °C. Separation of the supernatant from the pellet was conducted by centrifugation at 1500 g, 20 °C for 15 min. The supernatant was used for protein quantification using the Biuret method

[22], and the residual biomass was preserved for carbohydrate hydrolysis. At least triplicate measurements were performed.

2.5.3 Carbohydrate hydrolysis and quantification

Carbohydrate extraction was performed by hydrolysis according with the procedure reported by Mirsiaghi et al. [17] where the pellet-suspension ratio and extraction time were reduced. The cell pellet was suspended in distilled water with a biomass concentration of 50 g/L, then the suspension was mixed with 10% (v/v) of concentrated sulphuric acid (98%). Hydrolysis was carried out at 100 °C for 1 h. The suspension was centrifuged at 1500 g, 20 °C for 15 min. The supernatant was preserved for carbohydrate quantification using the phenol-sulphuric acid method [23]. At least triplicate measurements were performed.

2.6 Recovery of the microalgal biocompounds after dual ultrasound and ozone

After ultrasound pretreatment, proteins were recovered from the aqueous phase. For that the microalgae suspension was centrifuged at 2500 g, 20 °C for 15 min. Afterwards lipid extraction from the pretreated cell pellet was performed using the procedure reported by Valeriano-González et al. [15] with some modifications (section 2.5.1). After lipid extraction, carbohydrates and phosphorus recovery from the ultrasound pretreated cell pellet was performed using 250 mL Erlenmeyer flasks containing 25 mL of microalgae suspension. Ozone was injected at a flow rate of 0.5 L/min. The ozone concentration in the gas phase was determined using the Iodometric method [20]. At least triplicate measurements were performed.

2.7 Composition analysis of recovered lipids

Fractionation and analysis of the total recovered lipids were performed on the solid phase extraction (SPE). Crude lipids (10 mg) were dissolved in 100 µL of chloroform and loaded on

the Agilent Bond Elut NH₂ cartridge (100 mg), previously conditioned with 4mL of hexane. All of the neutral lipids except polar lipids and phospholipids were eluted using 4mL of elution A (chloroform:2-propanol; 2:1, v/v). The polar lipids were eluted using 4mL of elution B (2% acetic acid in diethyl ether, v/v) and phospholipids were finally eluted with 4mL of elution C (methanol). The SPE lipid fractionation results were confirmed by thin-layer chromatography (TLC). The developing solvent for TLC was hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Spots were visualized by spraying with 10% sulphuric acid ethanol solution and charring in an oven for 10 min at 105 °C [24]. At least triplicate measurements were performed.

3. Results and Discussion

3.1 Individual application of ultrasound and ozone for the tandem recovery of microalgae biocompounds

Maximum yields of recovered lipids, proteins and carbohydrates were achieved at diverse experimental parameters as per biocompounds disposition in the microalgae cell. In ultrasound pretreatment the interaction of biomass concentration with applied energy showed a significant effect on the yield of recovered lipids, with a p-value of 0.007. The maximum yield of recovered lipids ($90 \pm 1.5\%$) was achieved at the lowest biomass concentration of 25 g/L and the highest applied energy of 200 kWh/kg dry biomass, Figure 1a. Hence, lipid release required the most intense experimental parameters (highest energy demand). In comparison with protein and carbohydrate recovery. This is attributed to lipid release requiring cell wall and substructure (cytosol and chloroplasts) disruption. Prior lipid release, improved contact between lipids and solvent during lipid extraction.

Biomass concentration and applied energy interaction showed a significant effect on the yield of recovered proteins, with a p-value of 0.034. A high biomass concentration helped increase cell disruption, resulting in a high yield of recovered proteins, as ultrasound energy has negligible attenuation in the liquid phase and it is more effective when in contact with solid matter such as microalgae cells [25]. However, when both parameters, applied energy and biomass concentration, increased the yield of recovered proteins decreased. This is because high cell disruption released proteins to the aqueous phase but also induced protein degradation due to the ultrasound waves and hydroxyl radicals. The maximum yield of recovered proteins ($99 \pm 2.5\%$) was achieved at the highest biomass concentration of 75 g/L and the lowest applied energy of 50 kWh/kg dry biomass, Figure 1b. As mentioned, at these experimental conditions, ultrasound pretreatment achieved high cell disruption and microalgal proteins were released to the aqueous phase. Therefore, no extraction process was necessary. Finally, interactions of biomass concentration and applied energy showed a significant effect on the yield of recovered carbohydrates. The maximum yield of recovered carbohydrates ($97 \pm 2.5\%$) was achieved at the middle experimental parameters of biomass concentration of 50 g/L and applied energy of 125 kWh/kg dry biomass, Figure 1c. This is because, shock waves generated during ultrasound cavitation cannot break covalent bonds found in the carbohydrates matrix, despite carbohydrates being stored in the microalgae cell wall [26]. Therefore, to recover carbohydrates fraction, applied energy should be higher than energy needed for protein recovery. Also, to achieve this yield of recovered carbohydrates ultrasound pretreatment and an additional hydrolysis process were both necessary.

Figure 1. Biocompounds recovery using ultrasound pretreatment. Response surface of yield of recovered (a) lipids, (b) proteins, and (c) carbohydrates as a function of applied energy and

biomass concentration (0.32 W/L ultrasonic intensity). Triplicate measurements (n=3) are shown for each data set.

In ozone pretreatment, only biomass concentration had a significant effect on the yield of recovered lipids, with a p-value of 0.001. The yield of recovered lipids decreased as biomass concentration raised. The highest yield of recovered lipids ($59 \pm 0.5\%$) was achieved at the experimental parameters of biomass concentration of 50 g/L, ozone concentration of 9 mg O₃/L, and contact time of 10 min, Figure 2a. Biomass concentration and its interaction with ozone concentration showed a significant effect on the yield of recovered proteins, p-values of 0.028 and 0.01, respectively. Biomass concentration showed a negative effect on the yield of recovered proteins; while its interaction with ozone concentration showed a positive effect. The highest yield of recovered proteins ($56 \pm 4\%$) was achieved at a biomass concentration of 50 g/L, ozone concentration of 6 mg O₃/L, and contact time of 10 min, Figure 2b. Finally, just biomass concentration showed a significant effect on the yield of recovered carbohydrates, with a p-value of 0.05. The highest yield of recovered carbohydrates ($92 \pm 2\%$) was achieved at the experimental parameters of biomass concentration of 50 g/L, ozone concentration on 6 mg O₃/L, and contact time of 10 min, Figure 2c. Experimental parameters that showed a significant effect on the yield of recovered biocompounds after ozone pretreatment (biomass concentration and ozone concentration) are closely linked to ozone dose. Ozone dose decreases when biomass concentration raises and increases as ozone concentration raises. By comparing the yields of recovered lipids and proteins between ultrasound and ozone, it can be hypothesised that ozone pretreatment reached low cell disruption in comparison with ultrasound pretreatment. As both, lipids and proteins, are mainly intracellular components cell disruption is required. Hence, results imply that the low cell disruption obtained was linked to the ozone dose (mg O₃/g biomass) and

this had a direct effect on the microalgae biocompounds recovery. Also, only biomass concentration had a significant effect on lipid recovery efficiency, this is because in order to intensify the biocompounds extraction process high biomass concentrations were applied. This implies an ozone dose reduction of 3-folds when biomass concentration increase from 25 to 75 g/L. Additionally, as was mentioned lipid extraction requiring cell wall and substructure (cytosol and chloroplasts) disruption. On the other hand, identical experimental conditions were needed to maximise recovered proteins and carbohydrates. This is because ozone pretreatment had a direct effect on the carbohydrates stored in the cell wall, which may have produced a slim down cell wall and improved protein extraction. Ozone, in water, generates reactive hydroxyl radicals through the formation of superoxide, which reacts with carbohydrates stored on the microalgae cell wall. The reaction between ozone or hydroxyl radicals and carbohydrates result in random cleavage of β -glucosidic bonds [26]. This cleavage begins with a hydrogen abstraction followed by oxidation of the subsequent carbon-centered radical, allowing the introduction of carbonyl groups to form carbonyl and carboxyl groups as well as lactones and hydroperoxides [27]. This reaction induces carbohydrates depolymerisation and their release into the aqueous phase. Due to the low cell disruption achieved by ozone pretreatment, the recovery of lipids, proteins, and carbohydrates required and additional extraction process besides ozonation. By comparing the yields of recovered carbohydrates between ultrasound and ozone, it can be concluded that the decisive step for carbohydrates recovery was the hydrolysis process. Both pretreatments, ultrasound and ozone, reached high yields of recovered carbohydrates. However to achieve those yields of recovered carbohydrates, at the experimental parameters studied, the pretreatment process (ultrasound or ozone) and an additional hydrolysis process were necessary. Therefore, to

331 intensify the carbohydrates recovery a pretreatment process that avoids or reduces the
332 employment of chemicals, such as H_2SO_4 , should be found.

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Figure 2. Biocompounds recovery using ozone pretreatment. Response surface of yield of recovered (a) lipids, (b) proteins, and (c) carbohydrates. Triplicate measurements (n=3) are shown for each data set. Within the variables tested for biocompounds recovery (biomass

concentration, ozone concentration, and contact time) only the significant ones are presented in the response surface plots (10 min contact time).

3.2 Combined application of ultrasound and ozone for the tandem recovery of lipids, proteins, carbohydrates and phosphorus

The main objective of the combined ultrasound and ozone pretreatment was to achieve the tandem recovery of microalgae biocompounds and intensify the extraction process for biorefinery purposes. During the study of the individual effect of each pretreatment, ultrasound was shown to be effective in cell disruption, significantly improved lipid and protein recovery, reduced solvent waste and obviated protein extraction process as previously shown [8]. On the other hand, ozone pretreatment resulted in low cell disruption efficiency at the experimental parameters applied, due to the low yields of recovered lipids and proteins. However, it was effective on carbohydrates depolymerisation and release to the aqueous phase ($28 \pm 1\%$ of total carbohydrates). Ozone pretreatment for carbohydrates recovery could intensify the biocompounds recovery process since it could obviate carbohydrates hydrolysis. Additionally, it has been described as an effective pretreatment to recover phosphorus from a variety of biomass as waste sludge, biogas sludge, pig manure, and microbial biofuel residual [28, 14]. It has been shown that inorganic elements such as phosphorus, nitrogen, and metals are released during ozone pretreatment [28, 14]. Therefore, application of ozone pretreatment for carbohydrates recovery besides intensifying the recovery process, could also add additional value to microalgae. In this context, the combined pretreatment consisted first of ultrasound to induce cell disruption, release proteins to the aqueous phase and extract lipids from the microalgae. Afterwards, ozone pretreatment was applied to release carbohydrates and phosphorus in the bulk liquid.

Desirability function was applied to find the experimental parameters for ultrasound that provide good response for cell disruption, proteins release to the aqueous phase and lipids extraction. It was found that the maximum yield of released proteins to the aqueous phase ($91 \pm 8\%$) and maximum yield of extracted lipids ($89 \pm 1\%$) were achieved at the biomass concentration of 25 g/L and applied energy of 200 kWh/kg dry biomass, the desirability of this experiment was 0.9, Figure 3.

Figure 3. Response surface for desirability function of proteins release and lipid extraction as a function of applied energy and biomass concentration for ultrasound pretreatment. Triplicate measurements ($n=3$) are shown for each data set.

In the combined pretreatment, ozone was applied to induce carbohydrates and phosphorus release to the aqueous phase. During individual ozone pretreatment, it was shown that ozone dose had an important effect. Therefore, experimental parameters for ozone in the combined pretreatment were adjusted to increase the ozone dose applied. Ozone concentration and pH were the experimental parameters that showed a significant effect on the yield of released carbohydrates, with p-values of 0.005 and 0.026, respectively. The highest yield of released

carbohydrates ($85 \pm 2\%$) was achieved at the ozone concentration of 21 mg O₃/L, contact time of 25 min and pH of 11, Figure 4a. Ozone concentration had a positive effect on the yield of released carbohydrates in the bulk liquid since the increase in ozone concentration increased the ozone dose. pH also had a positive effect, the yield of released carbohydrates increased as pH raised. Ozone pretreatment was more effective at high pH because a basic condition increases the formation of hydroxyl radicals which enhances the reaction kinetics and efficiency. Similar results were found by Herrera-Adarme et al. [29], they reported a maximum solubilisation of 45% of lignin, 48% of hemicelluloses and 40% of cellulose during sugarcane bagasse ozonation at pH 11. They found that the mechanism of hemicelluloses release was pH-dependent. When an acid condition was used there was a lower formation of hydroxyl radical and the reaction occurred primarily by direct attack of ozone. The direct attack of ozone required a high flow rate of ozone to ensure a medium with a high amount of hydroxyl radicals [29]. Additionally, ultrasound pretreatment could improve ozone pretreatment efficiency. Since ultrasound induced cell disruption enhancing the surface area and accessibility of ozone or hydroxyl radicals molecules.

Phosphorus recovery was studied as a function of the yield of released total phosphorus and orthophosphate (ortho-PO₄⁻³) in the bulk liquid. As per the yield of released carbohydrates, pH showed a significant effect on the yield of released total phosphorus and ortho-PO₄⁻³, with p values of 0.020 and 0.031, respectively. The yield of released total phosphorus and ortho-PO₄⁻³ increased as pH raised. The highest yields of released total phosphorus ($85 \pm 3\%$) and ortho-PO₄⁻³ ($51 \pm 1\%$) were achieved at the ozone concentration of 9 mg O₃/L, contact time of 25 min and pH of 11, Figures 4b and 4c. On the other hand, contact time had an important effect on the yield of released total phosphorus. The yield of released total phosphorus increased as contact time

raised while the yield of released ortho- PO_4^{3-} remained almost constant as contact time increased. This effect can be explained by polyphosphate release. Ozone pretreatment promoted internal polyphosphate release due to the high cell disruption achieved by ultrasound pretreatment. Similar results were found by Cosgun and Semerci, they studied the pretreatment of waste sludge for nutrients release by using a combined ozone/microwave pretreatment [29]. Their results showed up to 96% of total phosphorus on sewage sludge solubilized into the solution, since microwave pretreatment release the polyphosphates due to cell lysis. They also observed that ozonation of alkaline-pretreated waste sludge enhanced total phosphorus solubilisation. In contrast, the ozonation of raw sludge and acid pretreated sludge resulted in uncontrollable phosphorus precipitation during ozonation [28]. Both phosphorus forms release, ortho- PO_4^{3-} and polyphosphate, is desirable since both forms of phosphorus can be valorised as fertilizers. Polyphosphates are molecules formed by two or more phosphate groups via phosphoanhydride linkages and can be linear or cyclic in structure. Polyphosphates are a group of important phosphorus species besides orthophosphate [30]. A large number of polyphosphates are used in detergents and fertilizers production, mainly in the form of sodium triphosphate (30-35%), the most common fertilizers are based on ammonium polyphosphate [31].

Figure 4. Carbohydrates and phosphorus recovery using ozone pretreatment after ultrasound.

Response surface of yield of recovered (a) carbohydrates (25 contact time), (b) total phosphorus,

and (c) ortho-PO₄³⁻ (18 mg/O₃L ozone concentration). Triplicate measurements (n=3) are shown

for each data set. Ultrasound fixed conditions were: (25 g/L, 200 kWh/kg, 0.32 W/L). Within the variables tested for carbohydrates and phosphorus recovery (ozone concentration, pH, and contact time) only the significant ones are presented in the response surface plots.

On the other hand, phosphorus release (9 mg O₃/L) showed lower ozone consumption in comparison to carbohydrates release (21 mg O₃/L). This can be explained by carbohydrates structure and disposition in the microalgae cell wall. The two layers of the microalgae cell walls contain a polysaccharide matrix. The external layer contains carbohydrates such as pectin, agar, alginate, and algaenan. The internal layer contains hemicellulose, pectin, and glycoprotein [29]. These components are linked together and form a complex network, known as cellulose matrix which provide recalcitrance to the microalgae. Extensive hydrogen bonding among the hydroxyl groups in the cellulose molecules produces a crystalline structure that is called microfibril. Microfibrils make cellulose molecules more strength and rigid, increasing ozone consumption for carbohydrates release [32]. On the contrary, phosphorus on microalgae may be located within adenosine triphosphate, lipids, and nucleic acid, which are intracellular material [33, 34]. However, adenosine triphosphate, lipids, and nucleic acid are more prone to be attacked by ozone or hydroxyl radicals due to double bonds on their structures. Additionally, the high cell disruption achieved by ultrasound pretreatment increased interaction between these structures and ozone or hydroxyl radicals, inducing phosphorus release.

The design of the combined pretreatment was completed applying desirability function for ozone pretreatment to find the experimental parameters that provide good response for carbohydrates and phosphorus recovery. The maximum yields of released carbohydrates (62 ± 1%) and phosphorus (75 ± 1%) were achieved at the biomass concentration of 25 g/L, ozone

concentration of 27 mg O₃/L, contact time of 35 min and pH of 11, the desirability of this experiment was 0.75, Figure 5.

Figure 5. Response surface for desirability function of carbohydrates and phosphorus recovery as a function of contact time and ozone concentration for ozone pretreatment. Triplicate measurements (n=3) are shown for each data set.

Results for the combined ultrasound and ozone pretreatment for the tandem recovery of lipids, proteins, carbohydrates and phosphorus are shown in Figure 6. In addition to protein release during ultrasound pretreatment, $22 \pm 2\%$ of the total phosphorus and $12 \pm 1\%$ of the carbohydrates in the microalgae were released to the aqueous phase. After ultrasound pretreatment, lipid extraction recovered $89 \pm 1\%$ of lipids. Due to the high cell disruption achieved, the biomass-solvent ratio was reduced from 1:45 to 1:15 (w/v) and extraction time from 12 h to 30 min in comparison to biocompounds extraction alone. Since lipids were extracted with organic solvent phosphorus was not released at this stage of the tandem recovery.

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482 **Figure 6.** Results found for the tandem recovery of microalgae biocompounds and
 483 phosphorus using the combined ultrasound and ozone pretreatment.

484 The initial content of carbohydrates and total phosphorus on the residual microalgae
 485 biomass after proteins and lipids recovery was $78 \pm 2\%$ and $88 \pm 1\%$, respectively. This residual
 486 biomass was ozone pretreated. According to the desirability function, these were the
 487 experimental parameters that provided good response for both carbohydrates and phosphorus
 488 released. The maximum yields of carbohydrates and total phosphorus achieved were $63 \pm 1\%$

and $75 \pm 1\%$, respectively, $47 \pm 4\%$ of the total phosphorus released were release as ortho- PO_4^{-3} .

Phosphorus content was normalized to 100 mg of total phosphorus in the initial biomass.

Experimental parameters for ultrasound pretreatment were biomass concentration of 25 g/L and

applied energy of 200 kWh/kg dry biomass. Experimental parameters for ozone pretreatment

were biomass concentration of 25 g/L, ozone concentration of 27 mg O_3 /L, contact time of 25

min and pH of 11. Table 1 shows a comparison between the results found for individual and

combined pretreatments.

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Pretreatment	Experimental parameters	Biocompound recovered (%)
Ultrasound	25 g/L, 200 kWh/kg, and 0.32 W/L	90 ± 1.5 lipids
	75 g/L, 50 kWh/kg, and 0.32 W/L	99 ± 2.5 proteins
	50 g/L, 125 kWh/kg, and 0.32 W/L	97 ± 2.5 carbohydrates
Ozone	50 g/L, 9 mg O ₃ /L, and 10 min.	59 ± 0.5 lipids
	50 g/L, 6 mg O ₃ /L, and 10 min.	56 ± 4 proteins
	50 g/L, 6 mg O ₃ /L, and 10 min.	92 ± 2 carbohydrates
Combined ultrasound and ozone pretreatment	Ultrasound 25 g/L, 200 kWh/kg, and 0.32 W/L	89 ± 1 lipids, 91 ± 8 proteins,
	Ozone 27 mg O ₃ /L, 35 min, and pH 11	85 ± 2 carbohydrates, and 75 ± 1% phosphorus.

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514 **Table 1.** Tandem recovery of microalgae biocompounds after ultrasound, ozone and combined ultrasound and ozone pretreatment.

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3.3 Intensification of the tandem recovery of microalgae biocompounds and phosphorus for biorefinery purposes

The intensification of the microalgae biocompounds and phosphorus extraction process was evaluated as a function of improvement in process efficiency, product quality and reducing waste streams. As can be seen in Figure 7, ultrasound pretreatment increased considerably both the yield of recovered lipids and the total recovered lipids that can be converted to FAME (neutral lipids). The yield of recovered lipids, increased from 67% to 90% and the yield of neutral lipids increased from 57 % to 78% when applied energy raised from 50 to 200 kWh/kg dry biomass, using a biomass concentration of 25 g/L. At the same time, the fraction of polar lipids and phospholipids decreased from 28% to 18% and from 33% to 3%, respectively. This effect can be explained from the high cell disruption achieved by ultrasound pretreatment. Also, the high reactivity of lipids with strong oxidizing agents induces polar lipids and phospholipids oxidation to neutral lipids by hydroxyl radicals produced during water thermolysis [35]. Therefore, it can be concluded that ultrasound pretreatment increased lipids quality for biodiesel production. Additionally, no extra chemicals were used during proteins recovery.

Figure 7. Effect of ultrasound pretreatment on recovered lipids composition as function of applied energy. Error bars represent the standard deviation of triplicate measurements (n=3).

Ozone pretreatment also intensified the carbohydrates and phosphorus recovery process. The employment of chemicals, such as H_2SO_4 and H_2O_2 for carbohydrate hydrolysis and phosphorus recovery was obviated. Avoiding the use of H_2SO_4 potentially prevents vinasse production during an alcoholic fermentation process. It has also been found that ozone pretreatment improves the glucose yield by about 5-fold compared to enzymatic saccharification and can produce 18% higher glucose yield than alkali pretreatments [13, 36]. Additionally, the most common inhibitors referenced for biomass pretreatments such as furfural and 5-hydroxymethylfurfural are normally not detected after ozone pretreatment [12]. Other advantages of ozone pretreatment include the process being conducted at ambient temperature and pressure, as opposed to high temperature and pressure needed for pretreatment methods usually used for carbohydrates and phosphorus recovery [13].

3.4 Comparison with other studies

As far as we know, this is the first study focused on the tandem recovery of microalgae biocompounds and phosphorus through a combined ultrasound and ozone pretreatment. The combined pretreatment applied in this study achieved high yields of recovered microalgae biocompounds in comparison to the previous studies presented in Table 1. In particular, Keris-Sen et al. [10], applied a similar combined pretreatment consisting first of ozone pretreatment followed by ultrasound for lipids and carbohydrates recovery. They achieved lipids and carbohydrates recovery of 59% and 81%, respectively in comparison to the yield of recovered lipids and carbohydrates on this study of 92% and 85%, respectively. The major difference found in the studies was the order in which the pretreatments were applied and the level at which the experimental parameters were applied. As was described, in this study ultrasound pretreatment increased the efficiency of ozone pretreatment due to high cell disruption achieved, while ozone pretreatment achieved low cell disruption. This is more evident in the study reported by Keris-Sen et al. [10], since lower ozone doses were used (0.03-0.27 g O₃/g dry biomass) in comparison to the doses applied in this work (0.108-0.756 g O₃/g dry biomass) for individual pretreatments. It is important to highlight that ultrasonic intensity applied in this study (0.32 W/L) was three orders of magnitude lower than the applied on Keris-Sen et al. [10] study (300 W/L). Biomass concentration was two orders of magnitude higher for this study which emphasises the importance of biomass concentration during ultrasound pretreatment. On the other hand, the combined pretreatment presented also achieved high yields of recovered phosphorus in comparison to previous studies, Table 2. This considerably intensifies phosphorus recovery process in comparison to previous studies focused just on the recovery of phosphorus.

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Process	Specie	Biocompound recovered	Result	Reference
Ultrasound	<i>Nannochloropsis oculata</i> and <i>Chlorella vulgaris</i>	Protein	38% protein	[16]
Ultrasound	<i>Chlorella vulgaris</i>	Lipid	93% lipid	[37]
Ozone	Mixed microalgal culture	Harvesting and lipid extraction	16 % lipid	[15]
Ozone and ultrasound	Mixed microalgal consortium	Lipid and carbohydrate	59% lipid and 81% carbohydrate	[10]
O ₃ /H ₂ O ₂ /MW	Activated sludge	Phosphorus	4 g P/kg sludge	[38]
Hydrothermal digestion	<i>Scenedesmus dimorphus</i>	Phosphorus	9.7 g P/g biomass	[39]
Ultrasound and ozone	<i>Scenedesmus obliquus</i>	Lipids, proteins, carbohydrates, and phosphorus	91% protein, 89% lipid, 62% carbohydrate, and 75% TP. 8.9 g P/kg biomass.	This study

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579 **Table 2** Summary and comparison of case studies on the recovery of microalgae biocompounds and phosphorus with this study.

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4. Conclusions

The study of the individual effect of ultrasound and ozone pretreatment on the *Scenedesmus obliquus* biomass enabled the adequate application of each pretreatment in a dual mode. The combined pretreatment intensified microalgae fraction recovery process since it achieved high yield of recovered biocompounds and phosphorus. Also, it reduced solvent waste by 95% and extraction time by 90 % in comparison with lipid extraction alone. For proteins, carbohydrates, and phosphorus recovery the use of chemicals such as NaOH, H₂SO₄, and H₂O₂ was obviated. The maximum yields of recovered lipids, proteins, carbohydrates and phosphorus achieved by the combined pretreatment were $89 \pm 1\%$, $91 \pm 8\%$, $63 \pm 1\%$ and $75 \pm 0.3\%$, respectively. Best experimental conditions for ultrasound pretreatment were 25 g/L biomass concentration and an applied energy of 200 kWh/kg dry biomass. For ozone pretreatment the best conditions applied were ozone concentration of 27 mg O₃/L, contact time of 25 min and pH of 11.

603 **Acknowledgements**

604 The authors thank Msc. IsauraYañez-Noguez and Dr. Ignacio Monje-Ramirez for their
605 valuable help in laboratory and ozonation processes. The experimental work was carried out in
606 the Laboratorio de Ingeniería Ambiental, Instituto de Ingeniería, UNAM.

607 **Funding**

608 This work was supported by the Fondo Sectorial CONACYT-SENER-Sustentabilidad
609 Energética (project 220704) and by Consejo Nacional de Ciencia y Tecnología (CONACYT
610 grant 385966/253075).

611 **Conflict of interest statement**

612 The authors declare no conflicts, informed consent, human or animal rights applicable.

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