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Article

Metabolic Pathway Analysis for Nutrient Removal of the Consortium between *C. vulgaris* and *P. aeruginosa*

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Abstract: Anthropogenic activities have increased the amount of urban wastewater discharged into natural aquatic reservoirs confining in them a high amount of nutrients and organics contaminants. Several studies have reported that an alternative to reduce those contaminants is using consortiums of microalgae and endogenous bacteria. In this research, a genome-scale biochemical reaction network is reconstructed for the co-culture between the microalga *Chlorella vulgaris* and the bacterium *Pesudomonas aeruginosa*. Metabolic Pathway Analysis (MPA), is applied to understand the metabolic capabilities of the co-culture and to elucidate the best conditions in removing nutrients such as Phosphorus (inorganic phosphorous and phosphate) and Nitrogen (nitrates and ammonia). Theoretical yields for Phosphorus removal under photoheterotrophic conditions are calculated, determining their values as 0.042 mmol of PO_4/ g DW of *C. vulgaris*, 19.53 mmol of inorganic Phosphorus /g DW of *C. vulgaris* and 4.90 mmol of inorganic Phosphorus/ g DW of *P. aeruginosa*. Similarly, according to the genome-scale biochemical reaction network the theoretical yields for Nitrogen removal are 10.3 mmol of NH_3/g DW of *P. aeruginosa* and 7.19 mmol of NO_3 /g DW of *C. vulgaris*. Thus, this research proves the metabolic capacity of these microorganisms in removing nutrients and their theoretical yields are calculated.

Keywords: Extreme Pathways; Nutrients Removal; *C. vulgaris*; *P. aeruginosa*

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1. Introduction

Diverse human activities have increased the amount of urban wastewater effluents discharged into natural aquatic reservoirs, confining in them a high amount of nutrients and organics contaminants such as NH_4^+, NO_3^- and PO_4^{3-}. These compounds have been identified as the main cause leading to eutrophication in natural aquatic reservoirs. Therefore, finding new strategies for secondary wastewater treatments have received an important attention to decrease the amount of these compounds before being discharged into the water bodies [1].

Microalga offers a promising approach to remove and to re-use nutrients such as Nitrogen (N) and Phosphorus (P), because they can be assimilated into its biomass [2]. It has been reported that the microalga *Chlorella* accumulates concentrations ranged between 5.0 to 10.1 % for N and between

0.5 to 1.3 % for P [2]. The advantages of using microalgae for this purpose include the low cost for the growing process by using solar energy; the metabolic capability of microalgae which can use endogenous Carbon sources and, the possibility of recycling those assimilated nutrients as a fertilizer, avoiding a sludge handling problem [3]. In addition to the wastewater effluent treatments, microalgae can be also used for biodiesel production [4] and even as a food source [5]. Thus, making the secondary wastewater treatment more affordable and sustainable [6,7].

Nevertheless, a pure culture of microalgae is not always maintained. Microalgae always coexist with endogenous bacteria which are able to thriving in natural aquatic systems [8]. Hence, it is natural that some simultaneous interactions must exist between these microorganisms; on one hand, bacteria are benefited from the exudates of microalgae, like oxygen and starch and, on the other hand, the growth of microalgae is promoted by bacterial products such as Carbon dioxide (CO_2), inorganic substances and some growth factors [9,10]. Therefore, natural interactions between microalgae and bacteria are considered as an innovative technology to improve the wastewater nutrient removal.

There are some experimental studies which have been working with different species of microalgae and bacteria for urban and industrial wastewater treatments [3,11]. The consortium of *C. vulgaris* and *P. putida*, has demonstrated a good simultaneous nutrients removal (ammonium and phosphate) and organic contaminants in synthetic municipal wastewater, compared with the axenic cultures [12,13]. Lananan, et al. 2014 [14], reported a removal up to 99.15 % of the total Phosphorus concentration in domestic wastewater treatment, using the co-culture of *Chlorella* with an effective microorganism (EM-1). Moreover, it also has been reported the co-culture of *C. vulgaris* with *Azospirillum brasilense* in cellular immobilization increases the ammonia and the Phosphorus removal [9]. Again, the co-culture of *Chlorella* with other bacteria removed up to 80 % of total N presents in animal feed wastewater production. However, studies with pure cultures have not effect on Nitrogen or Phosphorus removal in industrial wastewater [15]. The above studies prove that the consortium of microalgae-bacteria is a better biological system to remove nutrients than pure cultures of these microorganisms. *Pseudomonas* is a common bacteria present in wastewater and mentioned in many studies [16,17]. However the metabolic activity and capability of these microorganisms can be altered by varying the culture conditions in the wastewater processes, including those associated with the microflora, particularly the α-Proteobacteria group, as *Pseudomonas*. To the knowledge of the authors, there is a lack of studies regarding the interaction between these microorganisms - [*C. Vulgaris* - *P. aeruginosa*]-, their metabolisms, the upper and lower bounds for nutrients removal according to their biochemical network and the possible metabolic phenotypes.

Currently, most of the genomic information from one specific microorganism is available from biological databases which is collect from high-throughput technologies, describing the metabolisms and components such as genes, proteins and metabolites. From those databases, it is possible to reconstruct genome-scale biochemical reaction networks for microorganisms and then analyze them using metabolic engineering tools [18]. Varma *et al.* (1993) [19], were the first authors in modeling a metabolic network from an entire organism (*E. coli*), obtaining the optimal carbon flux distribution using Flux Balance Analysis (FBA). Metabolic Pathway Analysis (MPA), is another technique used to analyze genome-scale metabolic networks, to find their phenotypic capabilities calculating a set of systematically independent and unique Extreme Pathways (ExPas), [20]. Extreme pathways (ExPas) are mathematically derived vectors that can be used to characterize the phenotypic potential of a defined metabolic network [20,21]. ExPas describe the conversion of substrates into products, while creating all byproducts needed to maintain the systemic elemental balance and the cofactor pools at steady state [18,20]. By calculating the ExPas from a metabolic network, it is possible to explain the active metabolisms in a particular pathway and the theoretical yields of products with respect to the sources of carbon or nutrients. Thus, calculating and analyzing ExPas from the metabolic network of the consortium between *C. vulgaris* and *P. aeruginosa*, it is possible to estimate their phenotypic potential under different schemes. Our research group has been working in the evaluation of nutrients

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removal by using different microorganisms and the consortium between them [13]. Consequently, this project provides a fundamental approach to enhance our understanding of biological system where microalgae and bacteria coexist as it occurs in most wastewater treatment.

2. Results

2.1. Stoichiometric matrix S

The stoichiometric matrix S has a dimension of 286×293, representing the metabolites and the set of internal fluxes and exchange fluxes, such as photons (Pho), external glucose (GLUext), sulfate (SO4ext), Magnesium (Mg), Potassium (K), iron (Fe), Calcium (Ca), Zinc (Zn), Copper (Cu), Manganese (Mn) and more important the nutrients which are studied in this article (PO4ext, Piext NO3ext and NH3ext). The outputs fluxes were biomass from each microorganism, polyhydroxyalkanoates, maltose, Carbon dioxide (COext) and oxygen production (O2ext).

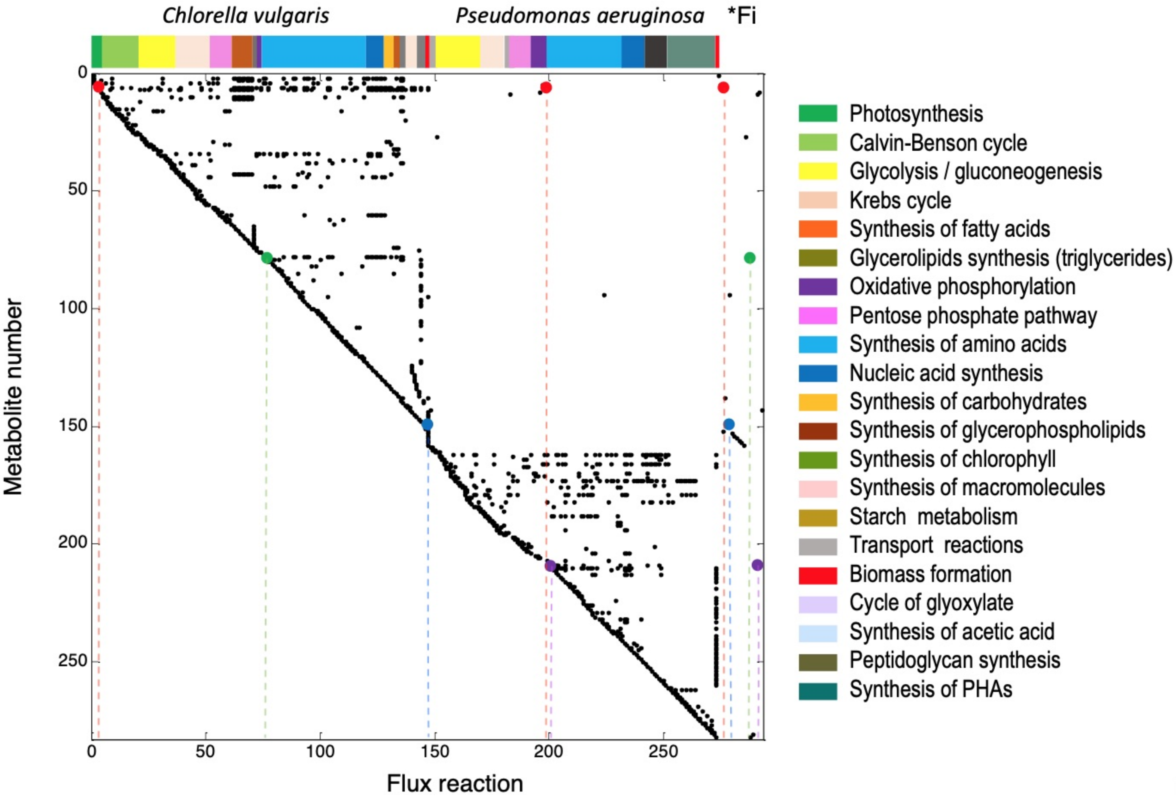

Figure 1. Estequiometric matrix S, of co-culture of *C. vulgaris* and *P. aeruginosa*. Main metabolisms of microorganisms are show at the top of the figure; *Fi represents the exchange fluxes considered in the metabolic network -biochemical reactions from 273-293, see Appendix A- Piext is the inorganic phosphorous (●), phosphate, PO4ext (●); nitrate, NO3ext (●) and ammonia, NH3ext (●).

The Figure 1 represents a novel way to describe graphically the obtained matrix S from the reconstructed metabolic model at genomic scale of this particular consortium of microalgae-bacteria. The abscissas axis represents the internal and exchange fluxes and the ordinates axis denotes the metabolites in order of appearance in the stoichiometric model. It is also represented in Figure 1 the different metabolism for each microorganisms. Hence, in Figure 1 can be noticed in which biochemical fluxes, the external nutrients are incorporated and, also it is possible to relate those fluxes with a metabolism belonging to a particular microorganism.

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2.2. Extreme pathways analysis for phosphorous species removal

Phosphorous nutrient is presented in two forms; inorganic phosphorous (Piext) and phosphate (PO4ext). The inorganic phosphorous (red circle in Figure 1,(●)) is related with both microorganisms. First, it could enter as an external flux into endogenous inorganic Phosphorus (Pi_Cv) as part of the requirements for photosynthesis metabolism in the microalgae; subsequently, Pi_Cv takes part of other fifty-two biochemical reactions which are mainly related with glycolysis, Krebs cycle and oxidative phosphorylation. Therefore, Pi_Cv is one of the metabolites that shows a greater connectivity between the fluxes in the matrix, because it is mostly required by microalga as part of its anabolism as it can observed in the Figure 2. For instance, the flux number 2, in red circle (Piext→Pi_Cv), is activated in 2844 (100 %) of the ExPas obtained. However, only 2572 ExPas correspond to an assimilation by microalga towards biomass generation. The rest, 273 ExPas are related with maltose production which is an endogenous organic compound destined for the growth of the *P. aeruginosa*. These last ExPas could suggest a commensalism interaction where microalgae metabolic machinery, works for bacteria supply Carbon in a photoautotrophic scheme. Until now, the active fluxes and their belonged metabolisms are elucidated. However, it is also possible to find theoretical yields and having a quantitative result like inorganic phosphorous removal with respect to biomass of each microorganism. For instance, the highest yield was 180.23 mmol Pi_Cv/ g DW of *C. vulgaris*, and it was discerned from 2572 calculated yields. The major ExPa removal corresponds with a pure culture of *C. vulgaris* in a photoheterotrophic scheme and it needs a requirement of 50.79 mmol of glucose/ g DW of *C. vulgaris* and 1252.06 mmol of photons/ g DW of *C. vulgaris* to be held.

Second, in the metabolism of *P. aeruginosa* (from reaction 148 to 273), the Piext (●) enter as part of the oxidative phosphorylation for ATP synthesis and after it is incorporated in twenty-eight biochemical reactions belonging to the metabolisms of glycolysis, Krebs cycle and synthesis of acetic acid (Figure 1). In 33 % of the total calculated ExPas, it was found the maximum theoretical inorganic phosphorous removal by the bacteria which was 4.90 mmol Pi_Pa/ g DW of *P. aeruginosa*. Even so, this maximum yield by the bacteria can occur either in a photoautotrophic or photoheterotrophic scheme of the consortium, its value is less than the one obtained by the microalgae.

The phosphate specie (PO4ext) which is in green circle in Figure 1 (●), is assimilated by *C. vulgaris* (PO4ext→PO4_Cv) as a part of the substrate for biomass synthesis (flux number 147), meaning a removal of this nutrient in 90.43% of the ExPas, when there is microalgae biomass production 59 PO4ext m mol / g DW of *C. vulgaris* (Figure 2).

2.3. Extreme pathways analysis for Nitrogen species removal

Nitrogen nutrient is represented in two nitrogenous species in the metabolic network, as nitrate (NO3ext) and ammonia (NH3ext). The Nitrate must be first converted into endogenous ammonia (NH3_Cv), in order to be used by the microalgae. The anterior condition is contemplated in flux number 75 (●) and it is active in the 90.43 % of the total ExPas (Figure 2) having a maximum removal of 7.19 mmol of NO3/g DW of *C. vulgaris*. NH3_Cv can be assimilated and incorporated into other twenty-two fluxes inside of the metabolisms of *C. vulgaris*. These fluxes go to the amino acids synthesis (glutamate, glutamine, glycine, proline, arginine, histidine, isoleucine, leucine, methionine, phenylalanine, chorismate and valine), which are part of the protein synthesis (number flux reaction 148) and nucleic acids (number flux reactions 140 and 141).
On the other hand, the removal of external ammonia (NH3ext, (●)) by bacteria in Figure 2, can be seen in purple circle (●)) in the number flux reaction 200, which represents the 33.33 % of the ExPas. The anterior flux is active in the proportion as biomass formation (number flux reaction 273), and it has a maximum removal of 10.30 mmol of NH3/g DW of *P. aeruginosa* either under a photoautotrophic or photoheterotrophic scheme.

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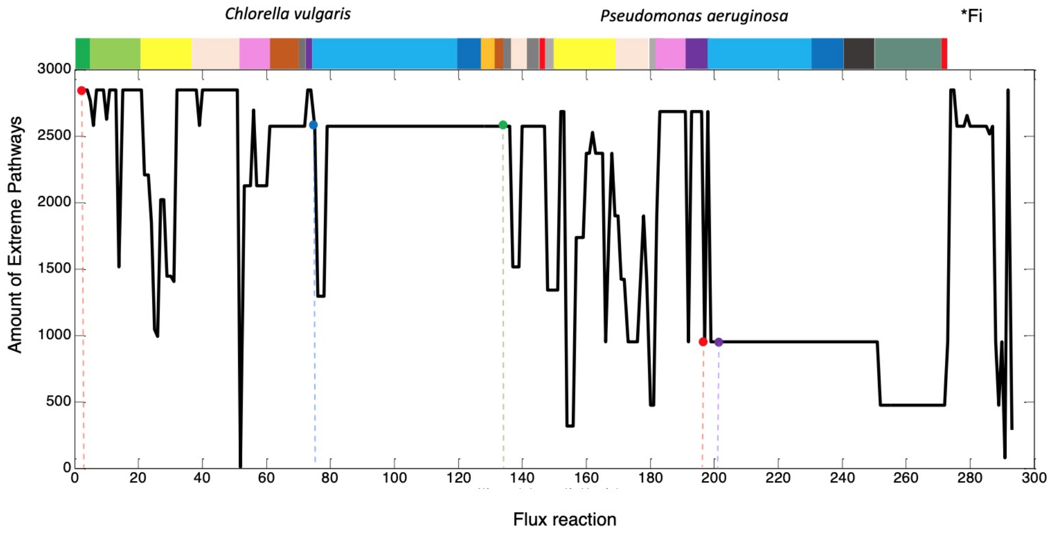

Figure 2. Participation of flux reaction in the extreme pathways. * Fi: exchange fluxes. Piext (●), PO4ext (●), NO3ext ((●)) and NH3ext ((●)).

2.4. *Analysis of the best Extreme pathways analysis for nutrients removal*

Considering only the ExPas that showed removal for the four nutrients species, there were obtained 864 feasible ExPa, 96 were for a photoautotrophic scheme and 768 for photoheterotrophic scheme. Nevertheless, with the announced ExPas it was necessary to reduce them even more. Therefore, another parameter to consider in reducing the amount of ExPas, was the degradation of organic carbon presented in wastewater, in this case was represented as an external glucose (GLUext) in the model. This last criterion reduced to 336 feasible extremes pathways, considered as the best ExPas for nutrient removal. Thus, one of the best ExPa for nutrient removal by the co-culture is schematized in Figure 3; and it accounts for 246 biochemical flux reactions, 84 % of the total metabolic network. The above means that for the nutrient removal purpose, is not needed the entire metabolism machinery of this co-culture.

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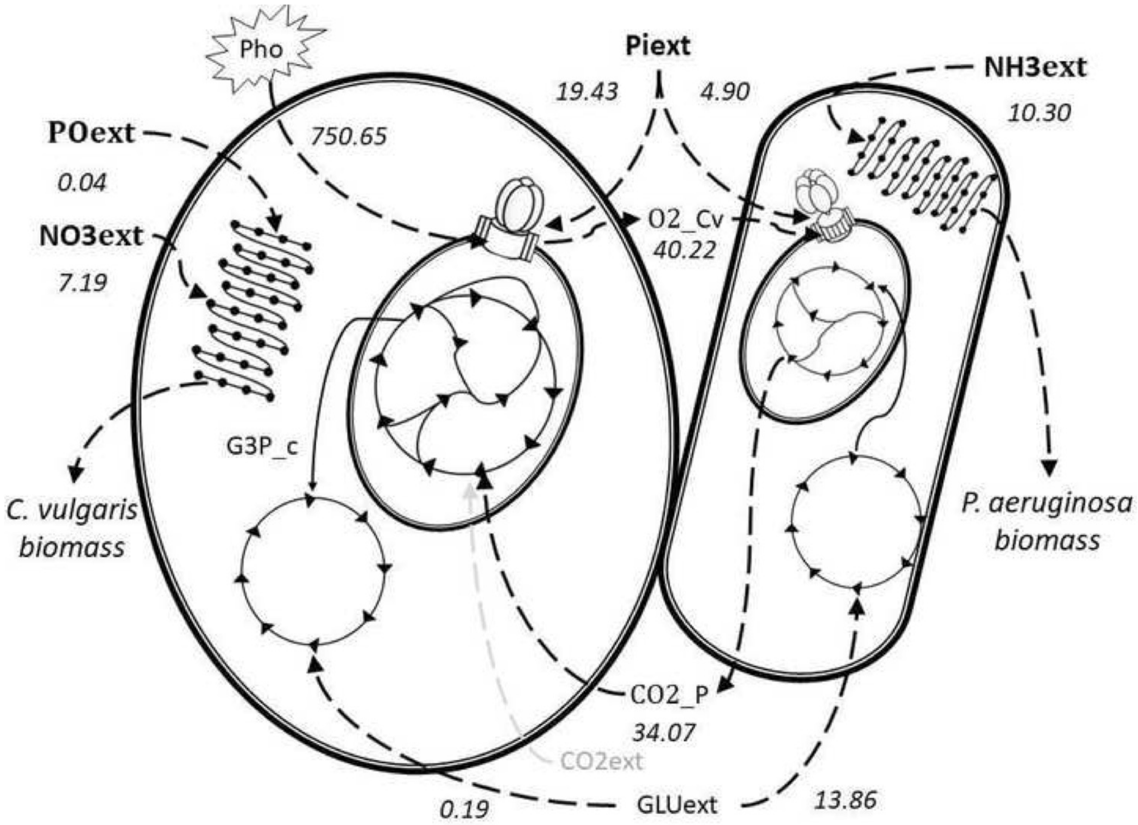

Figure 3. Schematic representation of the best ExPa for nutrients removal under a photoheterotrophic condition for the consortium of *C. vulgaris* and *P. aeruginosa*. The numbers represent the yields for consuming or producing the indicated compounds with units of mmol of nutrient / g DW of microorganism, respectively.

The mentioned ExPa corresponds to a photoheterotrophic scheme, where there is organic carbon source as glucose (GLUext), and the inorganic carbon source is the endogenous product of bacterial respiration (CO2_P); this behavior could represent a decrease in operation costs due to aeration or external Carbon dioxide supply in common wastewater treatment.

The glucose entry goes for both microorganisms, having a maximum removal of 0.93 mmol of glucose/ g DW of *C. vulgaris* and 13.85 mmol of glucose/ g DW of *P. aeruginosa*. Even so, most of the organic source is directed towards the bacteria at the glycolysis metabolism. This agree with experimental reports where the growth of a bacterium is related with glucose uptake at the expense of microalgae development [22]. While the inorganic Carbon source comes from the internal respiration of bacteria, the total yield 34.07 mmol of CO_2 goes to the Calvin cycle in the microalga metabolism to be fixed into the triose glyceraldehyde 3 phosphate (G3P_*c*). This last metabolite goes for chlorophyll synthesis and it is also incorporated into the five step of glycolysis. In this particular ExPa, G3P_c is not needed as substrate to produce starch or maltose. The mentioned metabolites are only used as an important energy compound when there is no carbon dioxide or nutrients. The last idea can be reinforced because there is simultaneous *C. vulgaris* and *P. aeruginosa* biomass synthesis, so either microorganism is not in competence with each other. Therefore, the best obtained yields for phosphorous and Nitrogen removals with microalgae were 19.43 mmol of inorganic phosphorous/ g DW of *C. vulgaris*, 0.04 mmol of phosphate/ g DW of *C. vulgaris* and 7.19 mmol of nitrate/ g of DW of *C. vulgaris*. For bacteria nutrient removals, yields of 4.90 mmol of inorganic phosphorous/ g DW of *P. aeruginosa* and 10.30 mmol of ammonia / g DW of *P. aeruginosa* were obtained. The last results indicated a more efficient removal of inorganic phosphorous from microalgae than bacteria,

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due to its requirement during photosynthesys metabolism according to Figure 1, in fact it is important to mention that all the ExPas (2844) no matter the scheme, they presented inorganic phosphorous removal. On the other hand, bacteria only exhibited a phosphorous removal in 33.3 % of the total ExPas.

3. Methods

3.1. Reconstruction of a genome-scale biochemical reaction networks

The first step to rebuild a genome-scale metabolic network for the consortium of *C. vulgaris* and *P. aeruginosa* was to assemble the stoichiometric reactions base on their genome annotation. Different metabolic databases exist to match the biochemical reactions with the specific genes for each microorganism. In this research, the reconstruction of the metabolic networks was made manually using the databases of BRENDA (BRaunschweig ENzyme DAtabase), NCBI (National Center of Biotechnology Information), MetaCyc, KEGG (Kyoto Encyclopedia of referenced literature as [23,24] and [5]. These references contain genomic, genetic, enzymatic, taxonomic and biochemical information, available for a large number of microorganisms including *Chlorella* and *Pseudomonas* genres.
The considered metabolisms for microalgae were those related with the autotrophy and photoheterotrophy schemes such as photosynthesis, chlorophyll synthesis (*Chla* and *Chlb*), Calvin-Benson cycle, starch metabolism, glycolysis/gluconeogenesis and finally, the basic metabolism for biomass formation such as TCA cycle, fatty acids synthesis, triglycerides synthesis, oxidative phosphorylation, pentose phosphate pathway, protein synthesis (18 amino acids), nucleic acids synthesis, carbohydrate synthesis, glycerophospholipids and maintenance. These metabolisms were represented in the first 147 biochemicals reactions (Appendix A).
Otherwise, biochemicals reactions from 148 to 273 denoted the metabolisms for *P. aeruginosa*. This bacterium has a huge metabolic capacity, therefore for the purpose of this project, it was considered the metabolisms related with central metabolism in a prokaryotic cell such as: starch metabolism, glycolysis, TCA cycle, glyoxylate cycle, pentose phosphate pathway, oxidative phosphorylation, amino acid synthesis, nucleic acid synthesis, peptidoglycan synthesis, synthesis of fatty acids and biomass formation. In the same way metabolisms related with synthesis of acetic acid and polyhydroxyalkanoates. Moreover, it was included the transport and exchange fluxes at the end of these metabolic networks.

Additionally, to the information from the databases, our research group obtained experimentally the elementary composition of *P. aeruginosa* using an elemental analyzer (Fisons model 1108) [25]. The results were employed to establish the biochemical reaction for the production of biomass (biochemical reaction 273, Appendix A). Metabolites such as CoA, NAD, NADP, FAD, ADP, and H_2O, were omitted because of they are present in the same concentration as their analogous pairs such as AcCoA, NADH, NADPH, FADH, and ATP [26]. All the stoichiometric coefficients have the units of mmol unless they were specified as grams. The nomenclature of compounds are given in Appendix B.

3.2. Extreme Pathway Analysis (ExPas)

After rebuilding the genome-scale metabolic network for the consortium, the biochemical reactions were ordered in a matrix S with dimension $m \times n$, whose rows (m) represent the mass balance for each metabolite (X), and n is the number of internal and exchange fluxes (v) participating in each mass balance [21,27,28], respectively.

$$\frac{dX}{dt} = S \cdot v, \tag{1}$$

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The ExPas were calculated base on the explained and analyzed principles elsewhere [20,21,27], satisfying the constraints of steady state

$$S \cdot v = 0, \tag{2}$$

the restrictions of non-negative internal fluxes -satisfying the thermodynamics of the biochemical reactions-

$$v \geq 0, \tag{3}$$

and the appropriate low and up boundaries α and β for the exchange fluxes b, such as the nutrient fluxes

$$\alpha_j \leq b \leq \beta_j. \tag{4}$$

An algorithm was developed in the MATLAB platform (The Mathworks, Inc., USA). From every single ExPa, the theoretical yield of nutrient removal was calculated with respect to the produced biomass of each microorganism. Then, a rigorous analysis was carried out to select the ExPas with the highest theoretical yield for nutrient removal.

4. Conclusions

For our knowledge, there is not reported a metabolic genomic reconstruction of the co-culture of microalgae and bacteria working together to study their interaction for nutrient removal. Thereby, the contributions of this work were the reconstruction of a genome-scale metabolic network for the consortium of the co-culture of the microalgae *C. vulgaris* and the symbiotic bacteria *P. aeruginosa*, and also the obtaining results of theoretical yields for the maximum metabolic capacity of nutrients such as Nitrogen and Phosphorus removal under a photoheterotrophic scheme, which is considered as the most related with practical wastewater treatment. The last contributions were important because of they could mean an improvement of biologic systems designs such as photon intensity, oxygen, and dioxide Carbon requirements among others to ensure the demands of microalgae and bacteria for achieving the maximal nutrients removal.

The in silico removal of Nitrogen and Phosphorous by this consortium were carried out only when there was biomass formation for both microorganisms. Therefore, that means that the elimination of these nutrients was only by assimilating them into biomass and hence, an interaction of symbiosis is critical to obtain the best removal yields. The above results indicated that there was no formation of others byproducts containing Nitrogen or phosphorous compounds with this particular co-culture and all are directed to microalgae and bacteria growth.

Even with a lack of detailed experimental information, like kinetics constants and flux limitations for most reactions, we have been able to show a closed behavior that could happen in a biologic system as a quaternary wastewater treatment. Finally, it should be noted that the developed genomic model of microalgae and bacteria is not limited to only one purpose.

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Conflicts of Interest: The authors declare NO conflict of interest with the publication of this paper

Abbreviations

The following abbreviations are used in this manuscript:

CO2-Cv	Carbon dioxide in *C. vulgaris*
CO2-P	Carbon dioxide in P. aeruginosa
Glu-Cv	L-Glutamate in *C. vulgaris*
Glu-P	L-Glutamate in P. aeruginosa
NH3ext	External ammonia
NO3ext	External nitrate
O2-Cv	Oxygen in *C. vulgaris*
O2-P	Oxygen in *P. aeruginosa*
Piext	External phosphorus
Pho	Photon
PO4ext	External phosphate
ExPas	Extreme Pathways

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