Metabolic interaction analysis between microalgae and bacteria for phenol degradation. A phenomenological approach.

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ABSTRACT Recalcitrant pollution of soil and water fonts by aromatic compounds, as consequence of industrial activity and extraction and refining process of oil, has became a global high importance issue because the adverse effects in health and environment caused by these. Bioremediation, a technical of recovery of polluted areas that explodes the microorganisms' potential to degrade pollutants, can be modeled to design decontamination sceneries applying coupled microorganism communities through *in silico* and experimental approaches.

Lineal evaluation of the microorganism consortium model using FBA reports phenol degradation in a simulated media with other carbon sources – as acetate and glucose – and reports biomass growth with only microalgae biomass input of 10,83 h^{-1}. The model can be considered as feasible approximation of a bioremediation case, which requires be adjusted and cured from experimental data and future information about proposed microorganisms' consortium and phenol degradation models reported on literature; improvements that would allow applying the researched consortia model to design decontamination sceneries and formulate optimal bioremediation conditions on real situations.

By the experimental path, was found a two-fold increase in growth rate when utilizing tris-acetate-phosphate medium compared when growing bacteria separately. Moreover, the inoculum ratio 2:1 (bacteria-microalgae) was the most effective when evaluating the growth of both organisms. The proposed consortium displays a higher phenol degradation at low initial concentrations of contaminant (50 $mg \cdot L^{-1}$), being the consortium *Microbacterium* spp.*-Chlamydomonas reinhardtii* the most effective, reaching 49,89% phenol removal.

Key words: Bioremediation, Microorganism consortium, Phenol degradation.

INTRODUCTION

Environmental pollution by aromatics compounds – such as phenol – results from the production and use of these in different industry sectors (pharmaceuticals, oil and gas, plastics) (Ahmaruzzaman, 2008; Banerjee & Ghoshal, 2011; dos Santos, Monteiro, Braga, & Santoro, 2009; Edalatmanesh, Mehrvar, & Dhib, 2008; El-Naas, Al-Muhtaseb, & Makhlouf, 2009; Huang, Huang, Tsai, & Chen, 2010; Shourian et al., 2009). Because physicochemical treatment processes of phenol pollution would be expensive, bioremediation can be a sustainable technical to recovery polluted areas (Edalatmanesh et al., 2008; Shourian et al., 2009).

This decontamination technical which use microorganism to remove organic pollutants from water and soil, explodes the capacity of several organisms such as bacteria, fungi and plant eukaryotes to suit contaminated media and use aromatic hydrocarbons and compounds xenobiotics as carbon source (dos Santos et al., 2009; Frutos et al., 2012; Scragg, 2006).

The microorganism consortium is a bioremediation technique, that causes interest in applications of organic pollutants mineralization because represents more approximation to environmental situations as microorganisms which interact between theirs and several carbon sources (Gonzalez-Bashan, Lebsky, Hernandez, Bustillos, & Bashan, 2000; L.E., 2000; Lima, Raposo, Castro, & Morais, 2004; Valderrama, Del Campo, Rodriguez, de-Bashan, & Bashan, 2002; Zamudio Pérez, 2010).

From the above exposed background, it proposed a microalgae-bacteria consortium which is capable to degrade phenol (Abdel-Raouf, Al-Homaidan, & Ibraheem, 2012; Kumar, Kumar, & Kumar, 2005; Lim, Chu, & Phang, 2010; Semple, Cain, & Schmidt, 1999; Song et al., 2009; Wang et al., 2011), based on that performance of phenol degradation depends of the microalgae-bacteria proportion, among other variables as temperature, pH, salinity, oxygen and nutrients availability, etc.

This consortium was proposed based on that microalgae-bacterium consortia can be more efficient in detoxification of organic and inorganic pollutants, and removal of nutrients from wastewaters, compared to the individual microorganisms (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2011). This efficient can be supported on the supposed symbiotic relationship between an autotrophic eukaryotic as the microalgae and a heterotrophic bacterium in which, microalgae photosynthesis provides oxygen – a key electron acceptor to the pollutant-degrading heterotrophic bacteria –, and bacterium supports photoautotrophic growth of the partners by providing carbon dioxide and other stimulatory means for continuing the photosynthetic cycle (Subashchandrabose et al., 2011). The proposed consortium using the exchange relationship O_2/CO_2 as axis of the microalgae-bacteria interaction, as is illustrated in the Figure 1.

Through experimental and *in silico* methods, the proposed consortium was evaluated at phenomenological and qualitative form. Experimental method used cultures – with phenol availability in the culture media – made from strains of *Chlamydomonas reinhardtii* and the following osmotolerant phenol resistant bacterial strains isolated from Cartagena Bay (Colombia): *Stenotrophomonas* spp*., Microbacterium* spp*. and Paenibacillus* spp.

Meanwhile, the *in silico* researched method was evaluated through a lineal application of Community Flux Analysis Balance – cFBA –. This novel application, which is used to the study of ecological populations and biotechnology and medical analysis, could a) predict the maximum population growth through the maximizing the biomass growth rate of all microorganism of the community; and b) identify the biochemical and environmental limitations of the each microorganism with the purpose of implement strategies to adjust to the effects of interaction between microorganisms aimed at greater efficiency of the consortium (Bucci & Xavier; Adam M. Feist, Herrgård, Thiele, Reed, & Palsson, 2009; A. M. Feist & Palsson, 2008; García Sánchez & Torres Sáez, 2014; Khandelwal, Olivier, Röling, Teusink, & Bruggeman, 2013; Zomorrodi & Maranas, 2012).

The consortium model evaluated through FBA was built from the genome-scale models of *Chlamydomonas reinhardtii* and *Pseudomonas aeruginosa*. These models, developed and reported by Chang (Chang et al., 2011) and Papin (Oberhardt, Puchałka, Fryer, Martins dos Santos, & Papin, 2008), are available from the latest researched from the Grupo de Diseño de Productos y Procesos – GDPP – of the Andes University (Castillo et al., 2014; Mejía, 2013).

MATERIALS AND METHODS

In silico MODEL

Genomic-scale consortium model

Current *Chlamydomonas reinhardtii* genome-scale model has 2893 reactions and 1706 metabolites and is used into the GDPP to analyze the behavior of its metabolism when

varying the CO_2 concentration on its growth environment as testing stage to research the design a reactor of biofuel production based on microalgae culture (Castillo et al., 2014).

In turn, the base *Pseudomonas aeruginosa* genome-scale model has 1754 reactions and 883 metabolites; the model lacks of the related information about aromatics degradation. This model, has been used previously to analyze optimal conditions to which bacteria can produce precursor metabolites to obtaining biofuel (Mejía, 2013).

Phenol degradation pathway

As first step, the *P. aeruginosa* model was cured with the aromatics degradation pathway through the catechol-muconate metabolic pathway implementation (meta-fission of aromatic ring illustrated at Figure 3). Also, this implementation includes linking the existing metabolites and reactions in the model with new metabolites and reactions based on information reported in KEGG and literature (Kanehisa et al., 2006; Prieto Jiménez, 1995).

After the pathway implementation, were identified 20 reactions of the aromatics degradation metabolic pathway of *P. aeruginosa*. Of these reactions, 13 correspond with the phenol degradation metabolic pathway. As a result of the implementation, were added 8 reactions and 3 metabolites to the model, which has now 1763 reactions and 886 metabolites. Model now resembles the bacteria in a growth medium with phenol: the *P. aeruginosa* model takes phenol from growth media and is metabolized to produce acetyl-CoA and succinyl-CoA, two key metabolites of the central metabolism of any cell.

Consortium genome-scale model

The microalgae-bacteria consortium's metabolic model, Built from the *C. reinhardtii* genome-scale model and the latter *P. aeruginosa* genome-scale model, has 4408 reactions and 2592 metabolites, arranged on 13 compartments, which each correspond to the happening reactions inside the organelles and cytosol of microalgae, the cytosol of bacterium and the exchange reactions space between both microorganism.

To each compartment, it has assigned an identification tag, consisting of a letter between brackets, agree the location of the each metabolite into the metabolic network (organelle, cytosol or extracellular space). The model was arranged in compartments as follows;

C. reinhardtii microalgae (reactions 1 to 2893; 1706 metabolites and 10 compartments).

[c]: Cytosol

[h]: Chloroplast

[m]: Mitochondria

[x]: Glyoxysome

[f]: Swipe

[e]: extracellular metabolite of microalgae

[n]: Core

[s]: Eyespot

[g]: Golgi apparatus

[u]: Lumen thylakoid

P. aeruginosa bacteria (reactions 2894 to 4356; 886 metabolites and two compartments).

[p]: Cytosol of bacteria

[pe]:Metabolite extracellular bacteria

Reactions of the microalgae-bacteria interaction. It is a pseudo-compartment corresponding to the intercellular medium and consisting of the set of exchange reactions between microalgae and bacteria, through which, were coupled both microorganisms to form the consortium. This set of reactions correspond to the model reactions from 4357 to 4408 – 52 reactions based on 26 common exchange metabolites as water, oxygen, carbon dioxide, salts and amino acids, between others – are represented follows as:

$$Metabolite[e] \leftrightarrows Metabolite[pe]$$

In this expression, identification tag [e] means that, the metabolite corresponds to extracellular space of *C. reinhardtii* and the tag [pe] means that, the metabolite corresponds to extracellular space of *P. aeruginosa*.

Mathematic model

Based on FBA fundamentals (Orth, Thiele, & Palsson, 2010), the metabolic network of the consortium model is arranged in stoichiometric factor matrix or $\mathbf{S}$ matrix, which is of size $n*m$. In this matrix, m is the number of metabolites arranged in the matrix rows and n, the number of reactions arranged on the matrix columns. Alike, the $\mathbf{S}$ matrix can be represented as math equation as shown below, in which, each position of the matrix – denoted as a_{ij} – is a representation of the stoichiometric coefficient of a unique metabolite j in a unique reaction i happened in a compartment k. Its sign indicates that the metabolite j is a product (positive) or reactant (negative) in a i reaction.

$$\boldsymbol{S} \in R^{m \times n} ; R_i = \sum_{j=1}^{m} a_{ij} * M_j[k]$$

The flux of all reactions of the model will maximize through the biomass growth flux, using a ν – flux vector – of n size which optimization case is the maximization of biomass production by the consortium in steady state. Based from the cFBA concept (Zomorrodi & Maranas, 2012), global consortium biomass production was proposed as a linear combination of biomass growth flux of bacteria and biomass growth flux of microalgae; manner of that it shown below:

$$\nu_{Biomass} = \nu_{Microalgae} + \nu_{Bacteria}$$

These fluxes are linked to the biomass growth forms of each microorganism; bacteria have unique growth form and microalgae have three growth forms: autotrophic, mixotrophic and heterotrophic. From this definition of the growth forms, a discrete representation of the global biomass growth is shown below:

$$\nu_{Biomass} = \nu_{Autotrophic} + \nu_{Mixotrophic} + \nu_{Heterotrophic} + \nu_{Bacteria}$$

Each growth form corresponds to a unique biomass growth reaction, reaction in which, the biomass growth is represented as the transformation of ATP, water and some metabolites – as acetyl-CoA and succinyl-CoA – into ADP, H^+ and phosphates.

From the FBA concept (Khandelwal et al., 2013; Zomorrodi & Maranas, 2012), the FBA application to analyze the microorganism consortiums and communities, it used

combination of the biomass growth rate of the microorganisms as objective function. To maximize the biomass growth flux vector, it defines an objective function $Z = c^T v$; in where c^T, is a matrix of size n, derived from the column vector – c –, which defines the flux to maximize. The defined linear programming model is shown below:

$$\max_Z c^T v$$

The consortium model is subject to next constraints;

Mass balance. From the approach of this research, the consortium modeling through FBA is conducted in steady state. For this reason, the mass balance is defined by the vector product $\mathbf{S}v=0$, definition whereby it is ensured that all reaction, the consumption rate of a metabolite a, is equal to the production of a metabolite b.

Thermodynamic constraints. These restrictions are defined as upper and lower limits of the fluxes of each reaction and represent the reaction rates for each reaction of the model. These limits expressed below are two matrices of size n represented as LB (lower limit) and UB (upper limit). Values of the limits, are expressed in mmol·gDW^{-1}·h^{-1}.

$$LB \leq \boldsymbol{v} \leq UB$$

Where;

$$\boldsymbol{v} \in R^n \wedge \boldsymbol{v} = \{v_{\boldsymbol{R1}}, v_{\boldsymbol{R2}}, v_{\boldsymbol{R3}}, \dots, v_{\boldsymbol{R4408}}\}$$

$$LB \wedge UB \in R^n$$

The next thermodynamic constraints applied to model are shown below:

Microalgae growth. Based on the previously researched *Chlamydomonas reinhardtii* model, it's defined the growth of the microalgae compartment of the model in autotrophic conditions and restricting the mixotrophic and heterotrophic growth reactions.

Assuming that, the autotrophic growth is the biomass generation from an inorganic substrate, within the model is stimulated the consumption of carbon dioxide by the microalgae, whose flux values were set in a medium with excess CO_2 regarding limits reported by Jay-Pang et al. (Castillo et al., 2014).

Likewise, the input of carbon sources different to carbon dioxide, are restricted. The main constrained metabolite is the acetate, whose extracellular fluxes (from the extracellular medium to microalgae and from the cell to extracellular medium) are restricted to cero.

The remaining limits of *C. reinhardtii* fluxes model compartment remain according to approach by Jay-Pang et al. (Castillo et al., 2014).

Bacteria growth. The growth of *P. aeruginosa* compartment of the model has three interest topics: (a) growth medium and availability of carbon sources, (b) phenol input and (c) the respiration of the microorganism.

(a) *Growth medium and carbon sources.* Based on previously research of the *P. aeruginosa* model (Mejía, 2013), the growth medium of *P. aeruginosa* is simulated in minimal salts medium (MSM) with availability of nutrients, amino acids and carbon sources.

Considering that, there are metabolites which can stimulate biomass growth (bacteria population) and the phenol degradation and others that, can interfere the phenol consumption; at the last bacteria compartment are restricted the input flux of substrates with aromatic origin, as the muconate and the hydroxybenzoate.

On the other hand, acetate and glucose, the main sources of carbon available to the cell will not have any restriction. The limits of acetate input fluxes are modified to maximize it consumption and the limits of acetate output are restricted, in order to ensure that all acetate is consumed enters the microorganism. In addition to consider maximize of acetate consumption, the limits of input and output fluxes of glucose keep the same values of limits of the original model of *P. aeruginosa* Papin et al. (Oberhardt et al., 2008).

(b) *Phenol input.* In standard conditions (reported model by Oberhart and Papin et. al. (Oberhardt et al., 2008)), the model of *P. aeruginosa* can't degrade phenol. So, after the implementation of aromatics degradation pathway from phenol and hydroxybenzoate to acetyl-CoA and succinyl-CoA, was stimulated the phenol consumption on the bacteria compartment.

This stimulation involves the manipulation of the input limits of phenol in the bacteria compartment, in order, to aid the bacteria to consume phenol. So that, while the phenol input is set between infinite to minus infinite as form to provide phenol source in the growth medium, the phenol input limits to bacteria compartment is set between 10 $mmol \cdot gDW^{-1} \cdot h^{-1}$ to infinite, stimulating the phenol consumption by the compartment.

(c) *Respiration of the microorganism.* The fluxes of the bacteria breathing involves the modification of transport reactions of oxygen and dioxide carbon.

First, the oxygen input fluxes were set between 1000 to infinite mmol·gDW^{-1}·h^{-1} and the oxygen output fluxes were restricted, to simulate an aerated medium which allows breath the bacteria and it reduces the oxygen leaks.

On the other hand, the transport fluxes from the medium to the bacteria compartment, it keeps the input and output limits of oxygen as the original *P. aeruginosa* model (Oberhardt et al., 2008).

Meanwhile, the input and output fluxes of carbon dioxide in the model remain the same values of the original *P. aeruginosa* model. Not, the input and output exchange fluxes of CO_2 between the bacterium and the growth medium are changed to favor the generation of carbon dioxide.

Bacteria-microalgae interaction reactions. Four of 52 reactions which form part of the microalgae-bacteria interaction zone, the exchange O_2/CO_2 reactions, were set so that, there is a ideal imitation of symbiotic relation between bacteria and microalgae: the flux of oxygen is restricted to only supply this metabolite to bacteria, and carbon dioxide is restricted to only supply its to the microalgae.

On the other hand, the limits of the 48 remaining microalgae-bacteria interaction reactions, raised from the 26 common exchange metabolites between the microalgae and bacteria –among others: amino-acids, nutrients (N,P,K), salts, water–, were restricted based from the exchange and transport reactions of the *P. aeruginosa* model(Oberhardt et al., 2008).

Programming package

The consortium proposed model was evaluated using a script implemented in linear programming optimization platform FICO Xpress IVE available from the Andes University.

EXPERIMENTAL

Microorganisms and media

Strains were isolated in mineral medium with phenol as the only source of carbon and energy. Strains were identified by 16 S rDNA gene, using the 1492r (5'-TACCTTG TTACGACTT) and 27F (5'-AGAGTTTGATCMTGGCTCAG) primers as previously described (Frank et al., 2008). Sequencing of PCR products was conducted by Genewiz (South Plainfield, NJ). Isolates were identified as *Stenotrophomonas* spp. (C2), *Microbacterium* spp. (C7A), and *Paenibacillus* spp. (C7B), with 99% identity to *Stenotrophomonas maltophilia* strain KW 98 16S ribosomal RNA gene (Accession number JX262398.1), 99% identity to *Microbacterium* spp. BAB-4119 16S ribosomal RNA gene, (Accession number KJ778662.1), and 99% identity to *Paenibacillus lactis* strain NASCB-5 16S ribosomal RNA gene (Accession number KP100165.1), respectively.

The growth medium was Tris-Acetate-Phosphate (TAP) medium ("TAP - Medium (Tris-Acetate-Phosphate)," 2014), which containing phosphate solution 1 mL (K_2HPO_4 28,8 g·100 mL^{-1}, KH_2PO_4 14,4 g·100 mL^{-1}), salts 25 mL (NH_4Cl 15·g L^{-1}, $MgSO_4 \cdot 7H_2O$ 4 g·L^{-1}, $CaCl_2 \cdot 2H_2O$ 2 g·L^{-1}), trace elements 1 mL (H_3BO_3 1,14 g·100 mL^{-1}, $ZnSO_4 \cdot 7H_2O$ 2,2 g·100 mL^{-1}, $MnCl_2 \cdot 4H_2O$ 0,5 g·100 mL^{-1}, $FeSO_4 \cdot 7H_2O$ 0,5 g·100 mL^{-1}, $CoCl_2 \cdot 6H_2O$ 0,16 g·100 mL^{-1}, $CuSO_4 \cdot 5H_2O$ 0,16 g·100 mL^{-1}, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0,11 g·100 mL^{-1}, EDTA 5 g·100 mL^{-1}), acetic acid 1 mL·L^{-1} and tris 2,42 g·L^{-1}. This medium was utilized for all cultures,

which included pure cultures, bacteria-alga consortia, pure cultures with phenol, and consortia with phenol (50, 100, and 150 $mg \cdot L^{-1}$).

This growth medium was selected taking into account that the C. reinhardtii growth is facilitated by saline medium and also to keep a similar growth medium to the ecosystem on which growth were isolated the phenol-degrading strains.

Growth monitoring and bacteria-algae ratio selection for the inoculum

The growth rate was evaluated aimed to identify the best conditions for degradation. Individual and consortium growth curves in TAP medium were obtained and compared in order to assess culture conditions. Optical density measurements (620 nm for bacteria and 685 for microalgae) were carried out in a UV-vis spectrophotometer (Agilent, CA, USA) to determine the growth rates for approximately 150 hours. Additionally, cell counts for microalgae were conducted utilizing the Neubauer chamber. Inoculum ratios: 1:1, 2:1 and 1:2 (for microalgae and bacteria respectively) were evaluated. Each culture was monitored by light microscopy for the purpose of verifying the symbiosis between the microalga and the bacteria.

Phenol degradation evaluation

In order to evaluate the potential for phenol degradation of the consortia we induced an acclimation of the microorganisms to phenol at 50 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$ and 150 $mg \cdot L^{-1}$ of phenol concentration. For pure cultures and consortia, in each inoculum ratio evaluated in TAP medium, different phenol concentrations were added. The evaluation of the potential for phenol degradation was carried out at 48 and 96 hours of incubation at 27°C with light,

by liquid chromatography (HPLC Aligent 1260 Infinity); column Zorbax Eclipse Plus C18, detector 61315D 1260 DAD VL, mobile phase acetonitrile-water (40/60), the injection volumes are from 0.1 to 100 µl, the temperature range was from 10°C below ambient to 80°C. 1,5 mL of growth medium from each culture was filtrated and analyzed by HPLC. Percentage of degradation was calculated by comparing the final concentration of the different cultures and the abiotic control.

RESULTS AND DISCUSSION

In silico MODEL

In silico model of the microalgae-bacteria consortium can considered feasible as a linear approximation under the evaluated conditions and constraints.

Biomass growth

The microalgae-bacteria consortium model submitted a global biomass growth rate of 10,83 h-1, value which keep constant for the all of the input testing of phenol (table 1). Of this combined biomass growth rate between microalgae and bacteria, the biomass growth rate of the microalgae was 10,83 h-1. Bacteria did not have growth.

If is considered that phenol presence at environment influences the biomass growth rate of the microorganisms, it is considered that these growth biomass rates may lack of logical meaning. Alike, the values of these growth biomass rates aren't logical and don't agree to physical magnitude of the reported growth rates by each microorganism, both in genome scale evaluations as in vivo cultures of microalgae and bacteria cultures.

Likewise, these results allow infer that, the current genome-scale models submitted limitations when were evaluated as coupled model under proposed growth conditions.

On the other hand, these limitations can be overcome by curing the biomass growth reaction from information obtained after reconstruction metabolism strains are grown culture medium with phenol.

From the above considerations, current results are a research base to future modeling of consortium in environmental sceneries. It requires adjust and cure the metabolic networks of microalgae and bacteria from data-taking derived by experimental cultures.

Phenol consumption

Under above considered constraints, the consortium model submitted a phenol consumption which varies between 10 and 600 $mmol \cdot gDW^{-1} \cdot h^{-1}$ (table 1). Also, all of the consumed phenol flux by the bacteria compartment was transformed into acetyl-CoA and succinyl-CoA through the catechol-muconate metabolic pathway.

As seen in Table 1, phenol consumption remains constant at majority of evaluated cases, wherein each case corresponds to an input value of phenol to the consortium model. Likewise to the biomass growth rate results submitted when was applied FBA optimization, the behavior of the phenol degradation results shows possible limitations of the researched model, because, the phenol consumption didn't vary respect the phenol input. Also, the corresponding phenol consumption value to the phenol input of 1000 $mmol \cdot gDW^{-}$

$^{1}\cdot h^{-1}$ may be a math solution without physical meaning, which can be associated to the set value of the phenol input limits.

Given that it is forcing the phenol consumption in the model, at future research it must be adjusted from values obtained from experience in order to validate and improve the proposed model.

It should be note that, this phenol degradation rate is a theoretical and qualitative approach. A quantitative validity of these fluxes of phenol degradation requires of the contrast and comparison between these submitted results with experimental data, which may determine an order of magnitude of the flux of phenol degradation expressed in units concentration, $mg\cdot L^{-1}$. This order of magnitude of the phenol degradation is calculated using the principle of resistance to mass transfer phenomenon that occurs from the intracellular medium, a parameter that is obtained through experimental determination.

Exchange reactions

An approximate form of exchange of oxygen-carbon dioxide was submitted at the microalgae-bacteria consortium model as indicator of feasibility of the consortium (table 1). This result is called "approximate", because the results is an adjustment of the fluxes during the optimization but not, a logical (as a physical magnitude) or real metabolite exchange of oxygen and carbon dioxide between microalgae and bacteria.

Alike to the submitted limits of the model when was evaluated the biomass growth rate, microalgae and bacteria current models should be modified to adjust the exchange of oxygen and carbon dioxide between both microorganism. At the future, this adjustment in

the ratio of O_2/CO_2 exchange will establish an approach to the microalgae proportion bacteria.

On the other hand, from the remaining exchange reactions between microalgae and bacteria, have been identified additional fluxes among microorganisms of the consortium with biological and ecological importance (table 2), which may be relevancy object at future.

From these resulting fluxes, could be consider that bacteria promote the nitrogen fixation within the consortium model. That is an approximation to model the *P. aeruginosa* behavior as rhizobacteria of promoting vegetal growth, which is a result with value added of ecological importance.

EXPERIMENTAL

First, it was analyzed the behavior of each bacterial strain and microalgae individually. In general, bacterial strains and microalgae *Chlamydomonas reinhardtii* displayed growth after 4 days of incubation at 30 °C and after 6 days of incubation at 27 °C respectively. The behavior of microalgae and bacterial strains was found to be similar as the lag phase for all strains was around 50 hours possibly indicating that the selected medium could be appropriate for consortia growth (Figure 4).

The lag phase observed in all cultures was typical for cultures that have phenol biodegradation, as the time for adaptation was remarkably longer compared to when other les toxic energy or carbon sources are used. It consider that this observation should be taken into account in order to improve the efficiency of phenol removal when scaling-up of

the system, by establishing and better stratagem for acclimation to phenol for each strain. It also found a time reduction in the exponential phase for bacterial strains compared to microalgae possibly indicating a faster depletion and better adaption for bacterial strains.

Light microscopy evaluations allowed us to evaluate the presence of potential symbiosis in the consortia (Figure 5 and figure 6). In all cases, was observed contact between the microalgae and the bacteria, possibly indicating the interaction between both. Nevertheless, the 2:1 inoculum ratio displayed greater cellular aggregations in the cultures. Also was found more contact between the species and the formation of greater quantities of cell aggregations (Figure 5). When it was evaluated the growth curves for 2:1 ratio, was found a faster growth of the consortia compared to individual cultures; there was a reduction in the lag phase to 10 hours (80% reduction), and in the time elapsed to reach stationary phase, which was half of the time compared to individual cultures.

A visual comparison of growth of different cultures, suggests that there are interesting differences between them. It appears that the agglomeration between the *Microbacterium* spp. and the microalgae *C. reinhardtii* is more clearly compared to other strains agglomeration. Moreover, the morphology of the algae does not seem to be affected when this bacterial strain is present. The cell number also displays significant differences when comparing with the pure microalgae culture counterpart as the cell number obtained for pure culture corresponds to $1,35*10^7$ cells·mL^{-1}, compared to and an average of $3,09*10^7$ cells·mL^{-1} for 2:1 inoculum ratio. Growth curves were also obtained for each strain in TAP medium (Figure 4). Generally, it seems that the growth rate of the microalgae and the bacterial strains is independent of the consortia utilized, possibly indicating that several factors such as metabolism associated with the species or growth rate are not significantly affecting the symbiosis evolution.

Phenol degradation was evaluated at different phenol concentrations (50, 100 and 150 $mg \cdot L^{-1}$) by measuring phenol at 48 and 96 hours for different inoculum ratios for each bacterial strain (Figure 7) either in pure culture or consortia. The initial phenol concentration has a significant effect on phenol removal; at 50 $mg \cdot L^{-1}$ initial phenol concentration, we observed the highest contaminant removal in the *Microbacterium* spp-*C. reinhardtii* consortium.

The efficiency of phenol removal was greatly affected by the algae-bacteria consortium; phenol degradation by *Paenibacillus* spp. was negatively affected by the presence of *C. reinhardtii*. On the other hand, phenol removal by *Stenotrophomonas* spp. was facilitated by the presence of the microalgae but was not as high as that observed in the *Microbacterium spp*.*-C. reinhardtii* consortium. In general, it seems that high phenol concentrations negatively affect the synergism between the bacteria and the algae. This was supported by the fact that in all cases phenol removal with initial phenol concentrations of 100 and 150 $mg \cdot L^{-1}$ was equivalent or higher in pure cultures than in consortia with *C. reinhardtii*. Regarding the algae-bacteria ratio, the degradation results were consistent with growth observations (without phenol), where the 2:1 bacteria-microalgae ratio displayed the most interesting removal capacity and highest growth rate, especially in the *Microbacterium* spp-*C. reinhardtii* consortium.

There are some reports of phenol bioremediation that evaluated concentrations between 250 and 500 $mg \cdot L^{-1}$ in the treatment system and fell to less than 5 $mg \cdot L^{-1}$ by some bacterial communities (Whiteley & Bailey, 2000). In the same way it has been reported that *Chlamydomonas reinhardtii* and *Chlorella vulgaris Beyerinck* have the ability for utilizing phenol as carbon source in agar salt medium (Samanthakamani & Thangaraju, 2015). Another report describes the ability of simultaneous degradation of organophosphates and

phenol by *Stenotrophomonas* species; they found a relation between the concentration of phenol and the optical density of the culture (Liu et al., 2009). Likewise there are reports of biodegradation of phenol in batch cultures by pure strains of *Paenibacillus sp.;* authors reported the evaluation of phenol degradation at different initial concentrations of phenol (100 $mg \cdot L^{-1}$ to 600 $mg \cdot L^{-1}$) in mineral salt agar medium, and observed 53,86% of phenol degradation at 500 $mg \cdot L^{-1}$ (Singh, Bahadur, & Chandra, 2009). The relation of metabolisms in both species for degradation of phenol consist in the capacity for using phenol as carbon source and for displaying proteins on the cell surface by the microalga and the bacterial strains (Liu et al., 2009; Samanthakamani & Thangaraju, 2015).

Phenol removal was finally evaluated using modified TAP medium (without acetic acid with phenol 100 $mg \cdot L^{-1}$ as solely carbon source). None of the bacterial species displayed growth. However, the microalgae grew in modified medium, although the culture coloration was less green that in medium containing acetic acid. In the first measurement it can be inferred that the microorganisms require adapting to the medium which has a carbon source that ensures growth, but is limited and then they start the consumption from another carbon source which in this case is phenol (pollutant degradation).

Previous studies about associations of microalgae-bacteria have shown in axenic cultures (only one species) smaller cell aggregations compared to those in xenic cultures (various species-consortia) and that cell formations called floccules take place due to the stimulation in the growth that generates the interaction between the different microorganisms (Lee et al., 2012).

The interactions between microalgae and bacteria are complex, including competition for resources, production of extracellular polymeric substances and exudates (Rivas &

Riquelme, 2012). Likewise, bacteria tend to be effective competitors for resources because they have a rapid growth rate, a greater volume ratio per surface area and rapid rates of nutrient intake (Rivas & Riquelme, 2012). Some authors have reported competitive interactions where inhibition of bacterial growth (*Vibrio alginolyticus*) is observed in alga cultures (*Skletonema costatum*) as a result of competitive exclusion (Natrah, Bossier, Sorgeloos, Yusoff, & Defoirdt, 2013). It is necessary to take into account the dynamics of interaction between populations in mixed cultures. There are many types of binary interactions between organisms: the first one is the negative relationships where the species involved are affected by the presence of another, such as competition, antagonism, parasitism and predation (Shuler, 1992). The negative effects are caused by the removal of resources, production of toxins or inhibitors, lytic agents, competition for space and substrate, among others (Shuler, 1992). Similarly there are relationships with positive effect such as mutualism, symbiosis, protocooperation and commensalism (Shuler, 1992). The positive effects can result from the required presence of both species for growth, physical contact, and highly specific interactions that stimulate growth, cooperation between the two organisms producing growth stimulus (presence of both populations is unnecessary for generating growth), and finally in the case of commensalism, the positive effect occurs in one of the species involved while for the other organism the effect is null (Shuler, 1992).

CONLUSIONS

It was proposed a microorganism consortium between microalgae and bacteria to degrade phenol, which were evaluated phenomenologycally through *in-silico* and experimental methods. These results allow established the bases of optimal conditions to model and

scale bioremediation sceneries, extending the knowledge about the feasible microalgae-bacteria symbiotic relationship.

Through *in-silico* method, it gets the genomic scale model of a coupled microorganisms' consortium. This model, which was built from genomic scale model of *Chlamydomonas reinhardtii* and *Pseudomonas aeruginosa*, consists of 2592 metabolites and 4408 reactions from microalgae compartment (1706 metabolites and 2893 reactions), bacteria compartment (886 metabolites and 1463 reactions) and intercellular medium (52 exchange reactions from 26 exchange common metabolites).

The model of the consortium was submitted theoretical phenol consumption rates between of 10 and 600 $mmol \cdot gDW^{-1} \cdot h^{-1}$ (Table 1) and theoretical biomass growth rate of 10,83 h^{-1}, of which, the microalgae provides 10,83 h^{-1} and the bacteria do not make any contribution of biomass. These results indicates the following possibilities: (i) the model must be adjusted from experimental data so that it can be applied throughout the supply range phenol; (ii) results represent a possible inhibition by excess substrate, the result obtained when the flux of phenol is 1000 $mmol \cdot gDW^{-1} \cdot h^{-1}$, a potential adjustment arrangement mathematic optimization performed; and (iii) the equivalent of flux concentration of 10 $mmol \cdot gDW^{-1} \cdot h^{-1}$ supplied phenol, could correspond to the maximum values of phenol degradation of the bacterium *P. aeruginosa* (Edalatmanesh et al., 2008).

Although the submitted results may not be satisfactory regarding the growth of biomass by bacteria compartment, CO_2 production in the bacteria and oxygen production the microalgae; the model can simulate the behavior of a microalgae-bacteria consortium in a growth medium with phenol and acetate as carbon source as a symbiotic relationship between an eukaryote and promoter of plant growth bacteria. Model requires to be

adjusted based on analysis of material flows to present each microorganism within the consortium, literature and the results obtained experimentally in order to obtain satisfactory results.

Experimentally, it was found that the simultaneous growth of bacteria and microalgae was favored among the studied microorganisms. A much faster growth was found for the consortia compared to pure cultures, which indicates that in the consortia stimulates growth. Furthermore, observations by light microscopy showed cell aggregations due to the tight contact between the microalgae and the different bacterial species, which is consistent with a symbiotic relationship between the two organisms. It believes that the formation of the cell aggregations occurs as a result of the activation of defense mechanisms given the presence of other microorganisms at the same medium.

Regarding the potential degradation of phenol, it could be inferred that the built consortium presents a better performance at low concentrations of the pollutant, therefore facilitating the elimination of the pollutant at low initial concentrations, which is on of the current bottlenecks in phenol remediation technologies.

The results of phenol degradation and performed microscopy indicate that the pre-inoculum ratio 2:1 (bacteria-microalgae) is the most appropriate for the development of the consortia; likewise it was found that the most efficient consortium in terms of phenol removal at 50 $mg \cdot L^{-1}$ is the one involving *Microbacterium* spp. which achieved a removal of approximately 50% (Figure 7). Our results also indicate that for these consortia, higher initial phenol concentrations (100 and 150 $mg \cdot L^{-1}$) induce resistance mechanism and reduce the biodegradation metabolism.

The observed growth of the consortia possibly indicates that there is a symbiotic relationship between the species due to the cellular aggregations. Nevertheless, is possible also that the cellular aggregations occurs by resistance mechanisms activated by microalga. Also, agree with *in silico* results, where the bacteria contributes phosphate to the microalgae and may even contribute to nitrogen fixation, converting urea supplied by the microalgae allantoin, ammonium and amino acids; experimental results indicate that submit a symbiotic relationship between the microalga and the bacteria.

According to Khandelwal and Bucci studies about community FBA (Bucci & Xavier; Khandelwal et al., 2013), future results from the consortium evaluation, it should be the result of an adjustment to the limits of the fluxes based on conditions evaluated at laboratory scale and the use of tools and platforms optimization increased robustness to the employee, as GAMS and applications Optcom, that will generate results from nonlinear models, which can provide more information to that obtained from a linear approximation.

Finally, current results contribute to the expansion of information about microalgae-bacteria relationship and provides information which help to understand from other point of view, the decontamination mechanisms at ecosystems. These are base to future researches, which will describe with more details the relationship between both microorganisms, as well as the possibility of applied to bioremediation sceneries with previous modeling that may establish the optimal conditions of microorganisms growth aimed to the higher pollutants remotion rate.

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ANEXXES

FIGURES

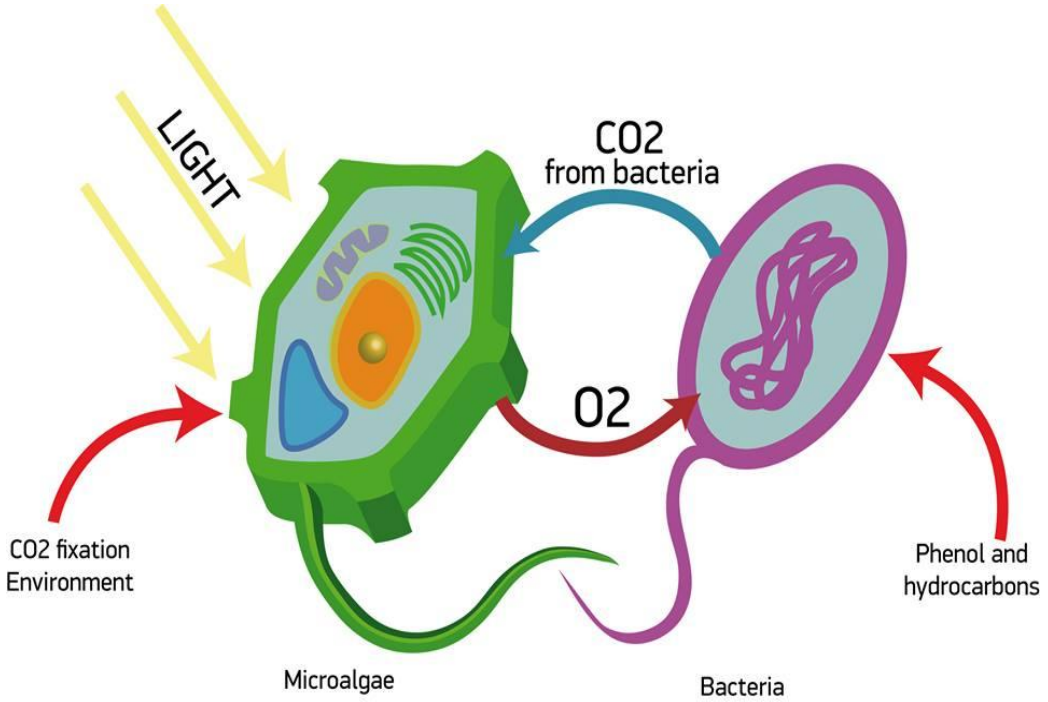

Figure 1. Microalgae-bacteria consortium proposed to phenol degradation. Source: Author.

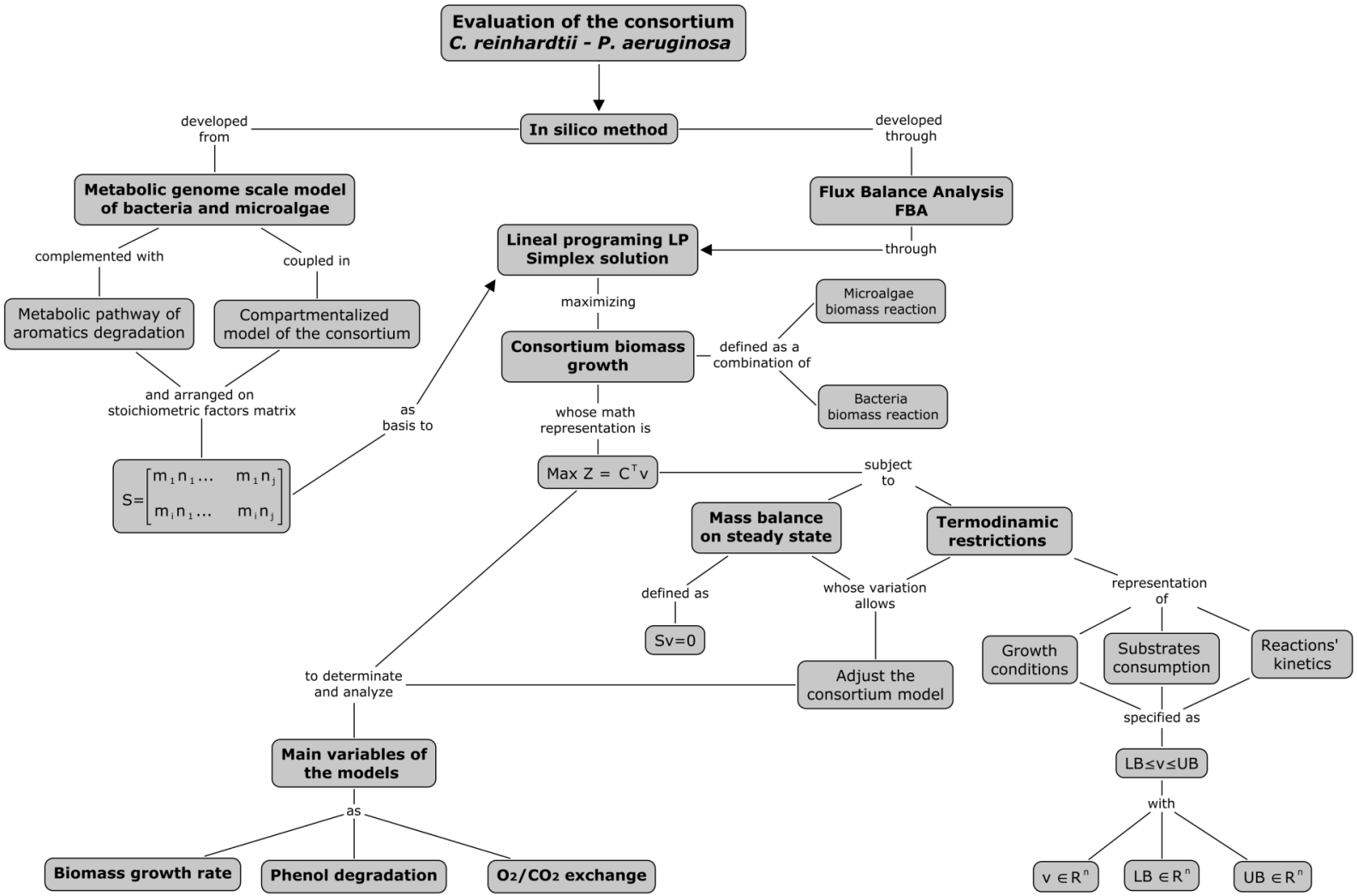

Figure 2. Research method of the microalgae-bacteria consortium model proposed to evaluate through FBA the phenol degradation. Source: Author.

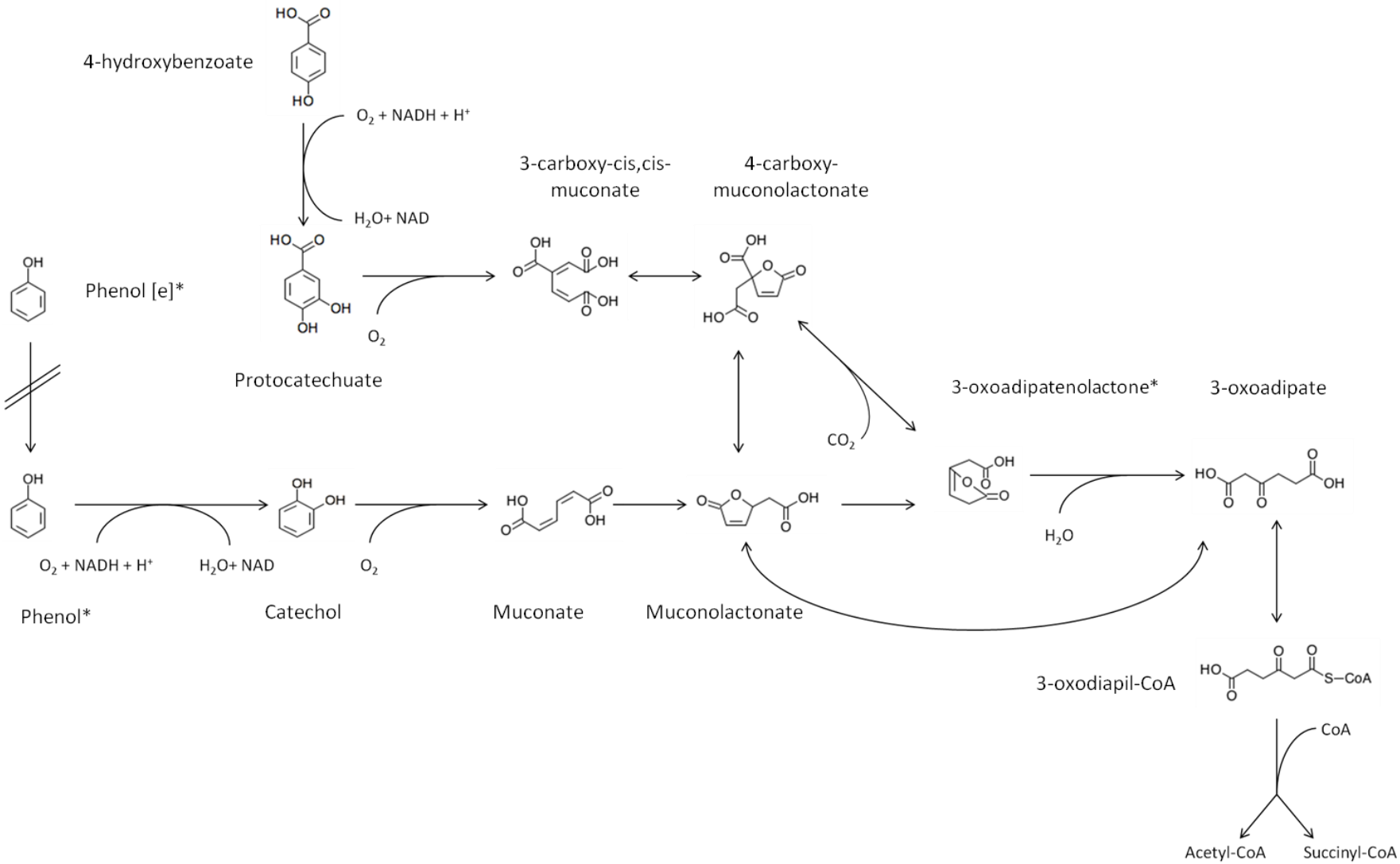

Figure 3. Metabolic pathway of aromatics degradation of the *P. aeruginosa* (Kanehisa et al., 2006; Prieto Jiménez, 1995).

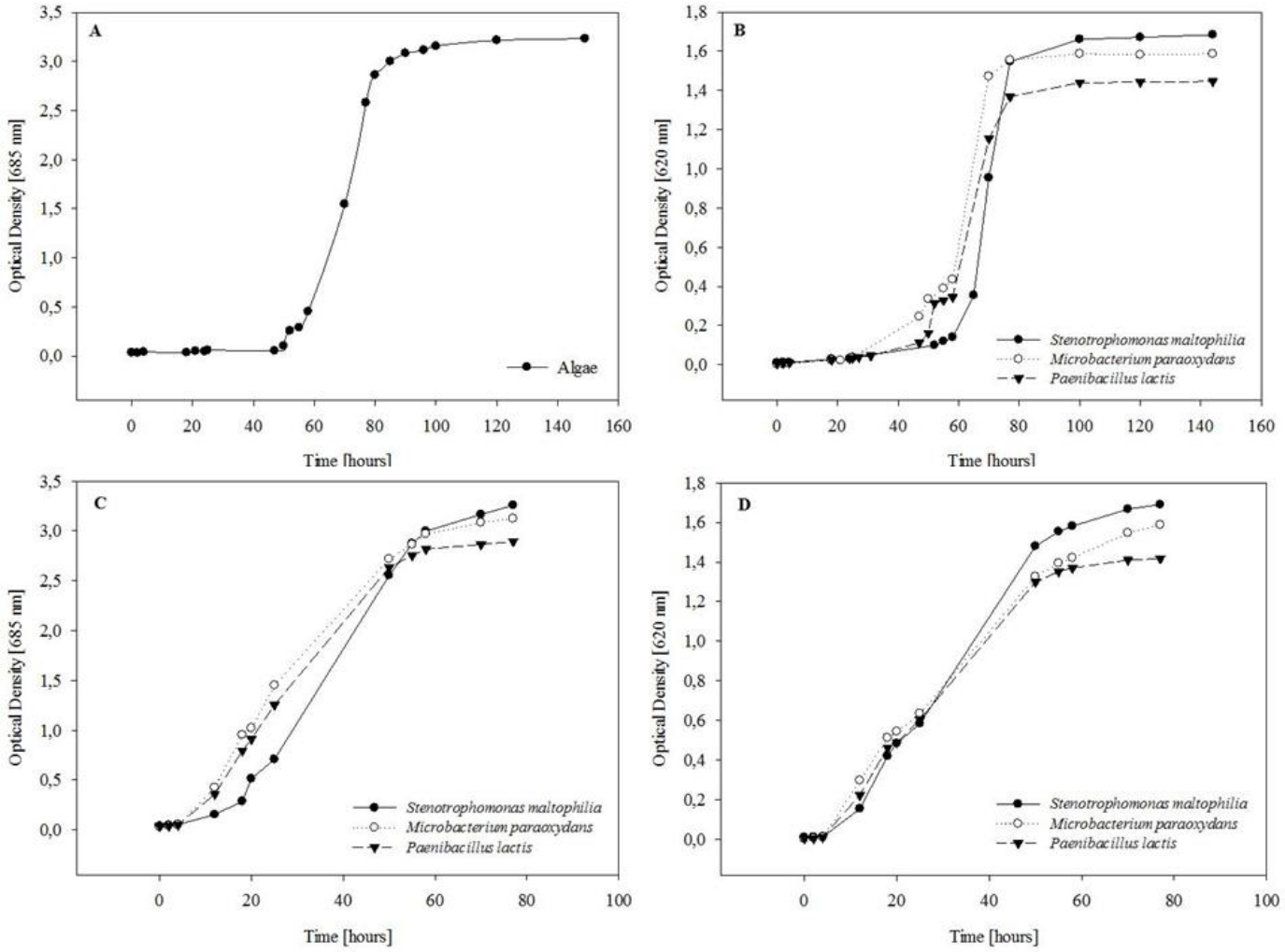

Figure 4. Growth curves in medium TAP. (A) Pure culture of microalgae *Chlamydomonas reinhardtii*, (B) Pure cultures of bacterial strains, (C) Microalgae *Chlamydomonas reinhardtii* in consortia ratio 2:1 (bacteria-microalgae), (D) Bacterial strains in consortium ratio 2:1 (bacteria-microalgae).

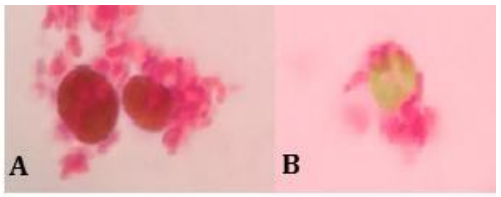

Figure 5. Optical microscopy x100 consortium C7A-microalgae, fuchsine stained samples (A) Pre inoculum ratio 2:1, (B) pre inoculum ratio 1:1.

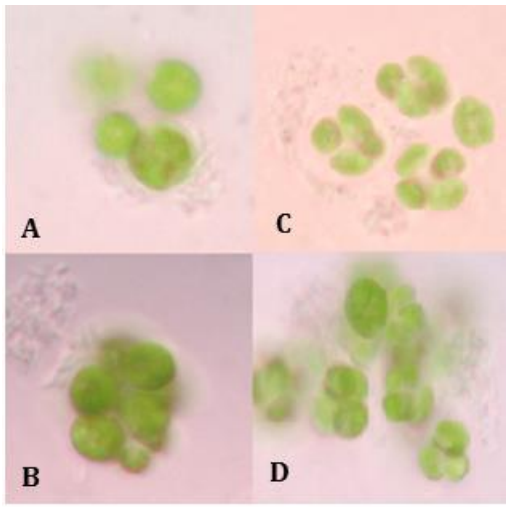

Figure 6. Optical microscopy x100 of the consortia in ratio 2:1 (bacteria-microalgae) (A) Consortium C2-microalgae, (B) Consortium C7B-microagla, (C) and (D) Consortium C7A-microalgae.

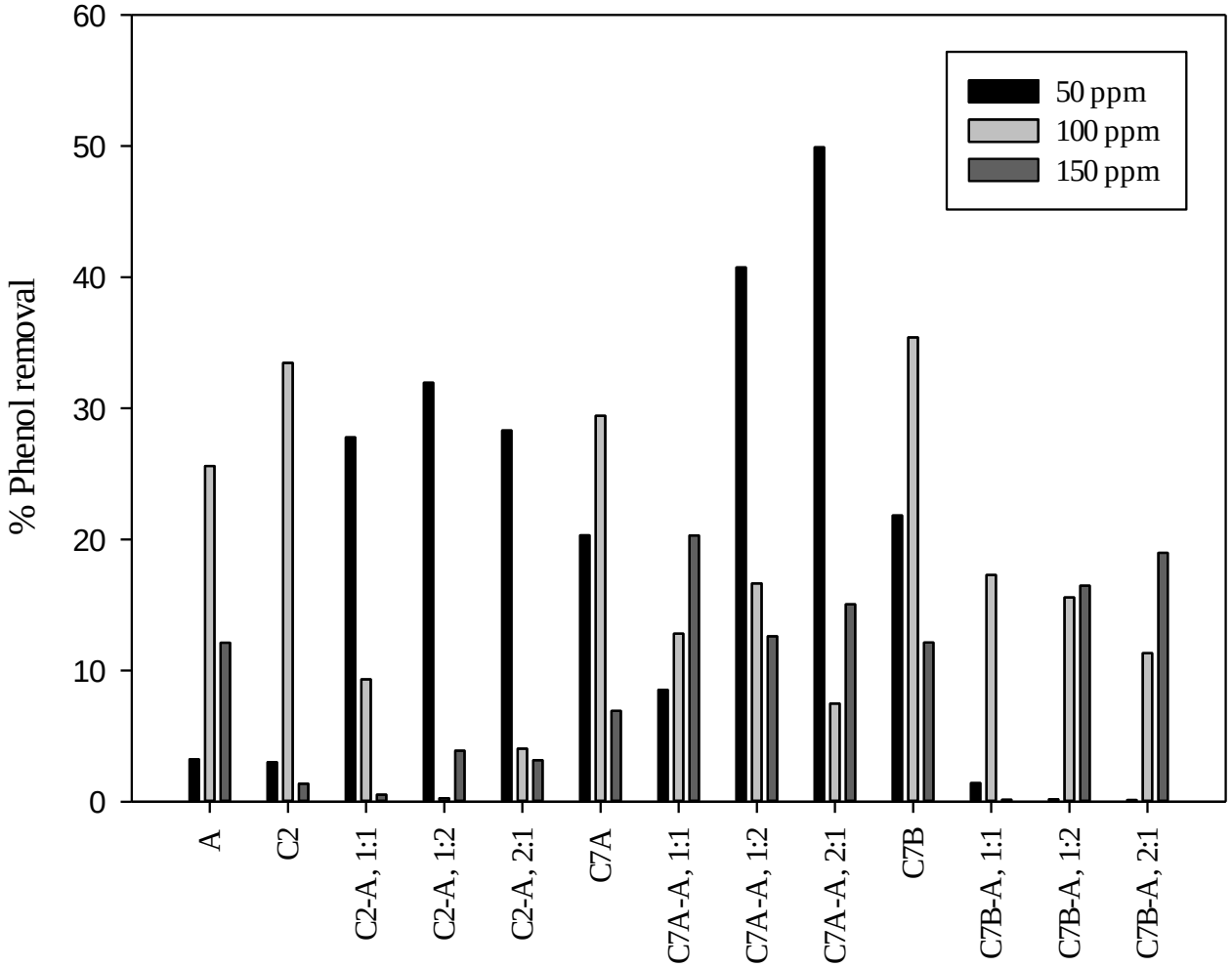

Figure 7. Percentages of phenol removal in each culture at the evaluated concentrations. Abberviations: A is microalga *Chlamydomonas reinhardtii*, C2 is *Stenotrophomonas maltóphilia*¸ C7A is *Microbacterium paraoxydans*¸ C7B is *Paenibacillus lactis*¸ 1:1, 1:2 and 2:1 correspond to inoculum ratios in the consortia.

TABLES

Table 1. Behavior of the evaluated consortium model by FBA.

Case	Phenol input ($mmol \cdot gDW^{-1} \cdot h^{-1}$)	Consumed phenol ($mmol \cdot gDW^{-1} \cdot h^{-1}$)	Microalgae biomass growth (h^{-1})	Bacteria biomass growth (h^{-1})	Combined biomass growth (h^{-1})	CO_2 from bacteria to microalgae ($mmol \cdot gDW^{-1} \cdot h^{-1}$)	CO_2 from microalgae to bacteria ($mmol \cdot gDW^{-1} \cdot h^{-1}$)	O_2 from microalgae to bacteria ($mmol \cdot gDW^{-1} \cdot h^{-1}$)	O_2 from bacteria to microalgae ($mmol \cdot gDW^{-1} \cdot h^{-1}$)
1	10	10	10,83	0	10,83	332,29	100	100	100
2	100	10	10,83	0	10,83	101,7	100	100	100
3	325	10	10,83	0	10,83	101,7	100	100	100
4	550	10	10,83	0	10,83	101,7	100	100	100
5	775	10	10,83	0	10,83	101,7	100	100	100
6	1000	600	10,83	0	10,83	313,08	100	100	100

Table 2. Behavior of the exchange metabolites between microalgae and bacteria.

From bacteria to microalgae	Shared between microorganisms	From microalgae to bacteria
• Phosphate • Ammonium • Allantoin • Amino-acids	• Water • Salts • Ethanol • Succinate • Nitrite • Amino-acids • Nitrogeus base	• Acetate • Urea • Sodium • Magnesium