Productivity enhancement of microalgae by co-cultivation with plant-growth-promoting-bacteria in airlift photobioreactor

Pedro Reynolds Brandão¹,²
¹Instituto Superior Técnico, ²Universidade Federal de Santa Catarina

Abstract

Microalgal biotechnology not only provides high-value products but also has the potential to avert a scarcity of nutrients and fuel that is likely to happen to the growing world population in a near future. However, its large-scale cultivation is limited by its rather high production costs. This work shows that the biomass production of microalgae Chlorella protothecoides (UTEX25) in a 5 L external-loop airlift photobioreactor can be increased considerably when co-culturing with plant-growth-promoting-bacteria (PGPB) of the Pseudomonas genus. Among several possible syntrophic mechanisms for this microalgal biomass enhancement, the results obtained from autotrophic cultures performed in aerated flasks with wild type and mutant PGPB suggest one in particular: stress alleviation and nitrogen recycle by the activity of the PGPB produced ACC-deaminase. In addition, it was concluded that the main limitation of the autotrophic cultures performed is nitrogen source depletion. Furthermore, although outside the scope of this work’s thesis, other interesting observations were made, such as the obtainment of an axenic culture of UTEX25 without resorting to antibiotics, the flocculation of microalgae induced by yeast, and the suppression of a contamination in an originally axenic microalgae culture through inoculation of the PGPB. This study may serve as a paradigm for future research on optimization of microalgae large-scale production in autotrophic culture systems.

Introduction

Microalgae are diverse group of photosynthetic microorganisms that have been gaining much scientific interest in the past 50 years. They have been identified as efficient biological systems for the production of high-value chemicals and nutrients, such as pigments, polyunsaturated fatty acids and antioxidants [1]. Also, they are considered a very promising third generation biofuel because of the high lipid content of several species [2], and potentially the crops of the future because of their high nutritional value, superior productivity and arable land independency [1, 3, 4]. Microalgae are, then, a good alternative to current unsustainable exploitation of natural resources, with potential to become a solution to the environmental dilemma of food and energy.

Throughout those years, several systems have been developed for large-scale cultivation of microalgae. To date, however, those systems are only financially viable for the production of high-value products, since their sale price tag is rather high; the large-scale cultivation of microalgae has very high costs of investment (i.e. technology) and operation (i.e. energy) [1, 5], being no means for the production of inexpensive commodities like food or fuel. Although these costs have been decreasing with time, due to technology development and process optimization, there are still no commercially viable microalgae plants for food or biofuel production yet [1, 5]. For these systems to become financially viable, one must find a way to push the biomass productivity of microalgae beyond the attainable, while using the same inputs.

Plant-growth-promoting-bacteria (PGPB) are, as the name suggests, bacteria that stimulate growth in plants and protect them from abiotic stresses and disease. The symbiosis phenomena between rhizobia and legumes is one of the most studied in biology and it shows the potential of bacteria as sustainable stimulators for agricultural productivity [6]. Taking into consideration that most of the microalgae that are nowadays worked with as a biotechnology are microphytes, that is, microscopic plants, it seems only obvious to try and apply these PGPB in their context. In fact, the positive influence of bacteria on the growth of microalgae has already been verified by some studies [7, 8, 9, 10, 11, 12, 13, 14, 15, 16] [17] [18] but none at a bioreactor scale. It is important, then, to study this potential productivity enhancement of microalgae by interaction with PGPB at a scale that can be paradigmatic for industrial production of microalgae, providing a new hope for the financially unviable microalgal biofuels and food.

The main purposes of this work are to verify if the presence of PGPB in relatively large microalgae culture systems (i.e. 1 L illuminated aerated flasks and 5 L photobioreactors) improves microalgal growth kinetics and/or biomass production and, also, to verify if the acdS gene (which codes for ACC-deaminase) is involved in the eventual improvement. In practical terms, this work consisted in four stages: the isolation of microalgae from a xenic culture by differential centrifugation and sub-culturing techniques; the production of axenic inculcums (microalgae and PGPB) in dark heterotrophic conditions; the autotrophic culturing and co-culturing of the microalgae in illuminated aerated flasks and in photobioreactors; and the analysis of the microalgal biomass obtained from the cultures.

Materials and Methods

Microorganisms

The microorganisms used in this work are the microalga Chlorella protothecoides, a.k.a.
UTEX25, the PGPB *Pseudomonas palleroniana*, a.k.a. 2D3, and the PGPB *Pseudomonas migulae*, a.k.a. 8R6 (wild type and acdS elimination mutant).

### Culture Media

Regarding UTEX25, two types of media were prepared and used for culturing: the medium for microalgae isolation and inoculum production, medium I, and the medium for PBR cultivation, medium II. Medium I is modified version of the “Algae Culture Broth”, being supplemented with glucose and with yeast extract. Medium I was also supplemented with agar (20 g/L) for use in plate cultures. Medium II is known as “Chlorella Broth”.

Regarding the PGPB, the media used for was King’s medium in the case of the plates, and Tryptic Soy Broth (TSB) in the case of the liquid media.

### Isolation of the microalgae from a xenic culture

UTEX25 was provided in xenic conditions, having several bacteria present in its suspension. The process of isolation consisted in two physical separation methods: differential centrifugation and solid media sub-culturing.

Regarding the differential centrifugation, a model based on the Stoke’s law was established to estimate the time and rotation speed required. Integrating the Stokes Law equation for spherical particles in a centrifugal field travelling a distance \([R_i, R_f]\) for a time interval \([0, t]\), the following expression is obtained:

\[
R/R_i = e^{\omega t^2}
\]  

Being \(s\) the sedimentation coefficient of the cells and \(\omega\) the angular velocity applied. \(R/R_i\) is thus the relative distance particles travel in the tube during the centrifugation. According to the model’s results (see Results section), it was decided that the falcon tubes containing xenic microalgae would be centrifuged for 120 seconds at a rotation speed of 6000 rpm for three times.

Regarding sub-culturing, it basically consisted in the following steps: streaking samples of the microalgae suspension onto plates with solid medium I; incubation for 7 days in mixotrophic conditions (1030 \(\mu\)mol.s\(^{-1}\).m\(^{-2}\) light intensity and 27°C); picking up of grown up colonies that appeared axenic for re-suspension in 5 mL of sterile medium I and then streaking on new solid media I. The process was repeated until an axenic culture was obtained on plate.

### Heterotrophic production of microalgae inoculums

The production of microalgae inoculum utilized liquid medium I and it was performed in a rotary shaker at 25°C and 180 rpm in dark conditions. A scale-up strategy was used: growth was monitored by measuring the optical density at 775nm (OD\(_{775}\)) to have knowledge on when microalgal growth would reach stationary phase; when it did, culture would be re-suspended in a bigger volume and then incubated in the same conditions. After obtaining the inoculum (100 mL and OD\(_{775}\) = 0.5 for aerated flask cultures or 500 mL and OD\(_{775}\) ≈ 0.2 for PBR cultures), the volume would be centrifuged to remove the remaining medium I, so the culture would start in strict autotrophic conditions. The inoculum optical density would then be adjusted if necessary (OD\(_{775}\) = 0.5 for aerated flask cultures and OD\(_{775}\) = 0.2 for PBR cultures).

### Production of PGPB inoculums

The strategy used for the production of PGPB inoculums was analogous to the one used for microalgae inoculums, only the medium used was TSB and the monitoring was performed in a different wavelength (i.e. 600 nm, OD\(_{600}\)). The quantity of PGPB inoculum density used was the one needed for achieving a ratio of UTEX25’s OD\(_{775}\) to PGPB’s OD\(_{600}\) of approximately 30:1 in every culture.

### Aerated Flask Cultures (AFCs)

Small-scale culture experiments were carried out in four 1 L glass flasks in photoautotrophic conditions. The flasks were connected in parallel to a diaphragm vacuum pump, which provided a 0.22 vvm sterilized airflow to the cultures. The light was provided by four LED lamps (1547 \(\mu\)mol.s\(^{-1}\).m\(^{-2}\)). Each one of the flasks was assigned for a different culture experiment, but all with the same culture volume of 600 mL.

### Photobioreactor Cultures (PBRCs)

In this work, two airlift photobioreactors were used for UTEX25 culturing and co-culturing: an external-loop photobioreactor, PBR A, and an external-loop photobioreactor, PBR B.

PBR A is a 5 L stainless steel external-loop airlift photobioreactor with a glass-made riser covered with LED lamps (86 - 1030 \(\mu\)mol.s\(^{-1}\).m\(^{-2}\)). It is fitted with pH and temperature sensors and controllers and it has a special window where an optical density sensor may monitor the microalgal biomass. The set-points appointed for the controllers were 25°C for temperature and 5.0±0.5 for pH. The flow of air provided was generated by a 24 L compressor and regulated to 0.22 vvm by a mass flow meter.

PBR B is a 5 L glass internal-loop airlift photobioreactor, also covered with LED lamps (1030 \(\mu\)mol.s\(^{-1}\).m\(^{-2}\)). It is fitted with pH, temperature and foam controllers. The set-points appointed were the same as PBR A. The flow of air provided was by the same compressor as PBR A but regulated by a rotameter.

For both PBRs, the signals of pH, temperature and air flux are registered by computers whose software allows their monitoring and calibration.
Sterilization of equipment and aseptic environment

All sterilization of equipment was done inside either a 25 L or a 50 L autoclave, with the sole exception of PBR A, which was done in situ with autoclave-generated steam. All aseptic transfers and sampling were made under a laminar hood.

Biomass dynamics monitoring

The methods used to monitor the microalgae biomass throughout the cultures were optical density (OD), cell counting by hemocytometry and dry weight (DW) by microfiltration (1.2 μm pore size).

The values of biomass concentration (in g/L) corresponding to each measure of OD and cell count were obtained by making a correlation with the DW values. For this purpose, ten solutions were prepared by parallel dilution of a sample of axenic UTEX25 suspension. Then, those solutions were measured for OD, cell count and DW. Finally, the values of OD and cell number were plotted against the values of dry biomass concentration (i.e. DW values divided by the filtrate volume), obtaining the correlations Biomass vs. OD and Biomass vs. Cell count.

The measures of cell count and DW also allowed the computation of the average individual cell mass of UTEX25.

Biomass dynamics and productivity: parameters and modeling

The UTEX25 cultures were evaluated by studying their biomass dynamics and productivity. For this purpose, graphical plots of biomass vs. time were made and also 5 parameters were computed for each culture: the increase in biomass concentration obtained in the culture, \( X - X_o \) (g/L), the apparent nitrogen yield on biomass, \( Y_{N/B} \) (g of biomass/g of nitrogen in the medium), the exponential growth phase time, \( t_{exp} \) (h), the average specific growth rate during exponential growth phase, \( \mu_{med} \) (h\(^{-1}\)), and the biomass productivity, \( P_X \) (g.L\(^{-1}\).h\(^{-1}\)).

Moreover, values for instant specific growth rate, \( \mu \) (h\(^{-1}\)), were calculated from the experimental data points; the expression for their calculation was deduced from a biomass mass balance to the cultures. In the case of a batch culture, the expression is

\[
X_f = X_i e^{\mu(t_f-t_i)}
\]

where \( X_f \) and \( X_i \) (g/L) are the values of biomass for an initial time, \( t_i \), and a final time, \( t_f \), respectively. The specific growth rate for each pair of experimental data points is thus obtained by:

\[
\mu (h^{-1}) = \frac{ln \left( \frac{X_f}{X_i} \right)}{t_f - t_i}
\]

The same way, for a continuous culture, those expressions are

\[
X_f = X_i e^{(\mu-D)(t_f-t_i)} \tag{4}
\]

\[
\mu (h^{-1}) = \frac{ln \left( \frac{X_f}{X_i} \right)}{t_f - t_i} + D \tag{5}
\]

Where \( D \) (h\(^{-1}\)) is the culture dilution rate.

It should be added that equations 2 and 4 were used to model the biomass data and that the \( \mu_{med} \) for each culture was obtained by exponential regression of the biomass data (a R\(^2\) of 0.950 was used as criteria).

The biomass productivity was calculated by the following expression:

\[
P_X = \frac{X - X_o}{t_{exp}} \tag{6}
\]

Monitoring of UTEX25 and PGPB populations

In the case of the aerated flask cultures, the UTEX25 and PGPB populations were also monitored by flow cytometry.

Physical properties of UTEX25 cells

Three physical properties of the UTEX25 cells were determined in this work: the absorbance spectrum, the average cell diameter and the average individual cell density. The absorbance spectrum was determined by making a wavelength scan (i.e. 400-800nm) of a sample taken from an axenic UTEX25 suspension. The average cell diameter was determined with the help of the ImageJ software by analyzing the pixel width of the diameter of 350 UTEX25 cells in a microscopic photography. The average individual cell density was determined by using the computed value of average individual cell mass of UTEX25 and dividing it by the individual cell volume of UTEX25 (estimated by the computed cell average diameter).

Cultures performed

A summary of the cultures performed during this work is presented in table 1.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Description</th>
<th>System</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFC-1</td>
<td>Axenic</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>AFC-2</td>
<td>2D3 co-culture</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>AFC-3</td>
<td>8R6 co-culture</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>AFC-4</td>
<td>8R6acdS co-culture</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>PBRC-1</td>
<td>Yeast contaminant co-culture</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>PBRC-2</td>
<td>Contaminant suppression by 2D3 co-culture</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>PBRC-3</td>
<td>2D3 co-culture 2</td>
<td>PBR A</td>
<td>800</td>
</tr>
<tr>
<td>PBRC-4</td>
<td>Axenic</td>
<td>PBR B</td>
<td>800</td>
</tr>
<tr>
<td>PBRC-5</td>
<td>Axenic</td>
<td>PBR A</td>
<td>800</td>
</tr>
</tbody>
</table>
Results and discussion

Isolation of the microalga from a xenic culture

The centrifugation model’s results for a rotation speed of 6000 rpm and a time of 120 seconds are presented in figure 1 (these were the conditions used in this work). Although the centrifugation process did not completely isolate the microalgae from the contaminant bacteria, it did help in the following sub-culturing procedure; when it was implemented before the sub-culturing, isolated microalgae colonies started to appear in the sub-cultures. After three sub-cultures, it was possible to obtain an axenic plate culture of UTEX25.

Calibration for biomass monitoring

Equations and represent the two correlations obtained for UTEX25 biomass quantification; they have a $R^2$ of 0.9962 and 0.9885, respectively.

\[
\text{Biomass (g/L)} = 0.5509 \times OD_{775} + 0.0014 \quad (8)
\]

\[
\text{Biomass (g/L)} = 0.0081 + 0.0282 \times \text{Cell Count} \left(10^6 \text{cells/mL}\right) \quad (9)
\]

The data from which the correlations were obtained was also used for estimating the average mass of individual UTEX25 cells: $2.97 \times 10^{-8}$ mg.

Physical properties of UTEX25 cells

The results of the wavelength scan to a sample taken from an axenic culture of UTEX25 are shown in figure 2.

Comparing these results with the ones found in the literature, one may conclude that absorbance peaks 1, 2 and 3 are due to pigment activity. It was decided, thus, that the safest wavelength zone to monitor UTEX25 biomass by OD is the near infrared one (750-800 nm), since the 500-680 regions seemed quite unstable; the wavelength chosen was 775 nm.

The diameter distribution of UTEX25 cells is presented in figure 3.

Heterotrophic production of axenic microalgae inoculums

The amount of biomass that was obtained at the end of the heterotrophic production was determined by measuring the OD$_{775}$ of samples and converting it to values of biomass concentration by using equation 7. Moreover, the apparent yield of the medium carbon source on biomass, $Y_{X/C}$ (g of UTEX25 biomass/g of glucose) was calculated. All these values are presented in table 2.

<table>
<thead>
<tr>
<th>Inoculum for</th>
<th>OD$_{775}$ obtained</th>
<th>Biomass obtained (g/L)</th>
<th>$Y_{X/C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFCs</td>
<td>4.350</td>
<td>2.40±0.06</td>
<td>0.228</td>
</tr>
<tr>
<td>PBRC-1</td>
<td>1.813</td>
<td>1.00±0.02</td>
<td>0.198</td>
</tr>
<tr>
<td>PBRC-2</td>
<td>1.912</td>
<td>1.05±0.02</td>
<td>0.209</td>
</tr>
<tr>
<td>PBRC-3</td>
<td>1.737</td>
<td>0.96±0.02</td>
<td>0.190</td>
</tr>
<tr>
<td>PBRC-4</td>
<td>1.452</td>
<td>0.80±0.02</td>
<td>0.159</td>
</tr>
<tr>
<td>PBRC-5</td>
<td>1.536</td>
<td>0.85±0.02</td>
<td>0.168</td>
</tr>
</tbody>
</table>
As one may notice, the values of $Y_{XC}$ obtained for the heterotrophic cultures for inoculum production had some variability (±12%). This was probably due to differential caramelization of the medium because of slightly different sterilization conditions (e.g. different heating/refrigeration times, different temperature profiles, etc.), which resulted in different glucose contents.

**Aerated flask cultures (AFCs)**

The biomass dynamics results for the four AFCs, which operated for 208 h, are presented in figure 4; their biomass production parameters are presented in table 3.

Table 3. Parameter results obtained for the four aerated flask cultures

<table>
<thead>
<tr>
<th>AFC</th>
<th>$X - X_0$ (g/L)</th>
<th>$Y_{XN}$</th>
<th>$\mu_{med}$ (h⁻¹)</th>
<th>$t_{exp}$ (h)</th>
<th>$P_x$ (g L⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.64</td>
<td>0.255</td>
<td>0.012</td>
<td>92</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>0.82</td>
<td>0.327</td>
<td>0.011</td>
<td>123</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>0.81</td>
<td>0.324</td>
<td>0.011</td>
<td>123</td>
<td>6.6</td>
</tr>
<tr>
<td>4</td>
<td>0.54</td>
<td>0.215</td>
<td>0.010</td>
<td>92</td>
<td>5.8</td>
</tr>
</tbody>
</table>

These results suggest that the PGPB’s presence in the UTEX25 culture induces an increase in biomass production and that the acdS gene is essential for that increase.

In the case of the AFCs, a flow cytometry analysis was performed. It was concluded that the flow cytometer signals which are best for distinguishing between the microalga and PGPB populations are front scattered light (FSC) and a fluorescence signal (PerCP); these two signals should account for cell diameter and pigmentation, respectively. The results are presented in table 4; the color profiles indicate population density: the higher the temperature of the color, the higher the population density.

Those results suggest that the acdS gene elimination impairs the biomass production enhancement and, also, that for AFCs-2 and 3, the microalga population benefits more in terms of growth from the co-culture than the PGPB population does, while for AFC-4 it occurs the opposite. Furthermore, one may notice that the increase in UTEX25 population in the AFCs is accompanied by an increase in the average and in the standard deviation of the cells’ diameter.

Table 4. Flow cytometry results of PerCP vs FSC for the for beginning and final culture times of the AFCs; the plots show signal density, which increases with the temperature of the color

<table>
<thead>
<tr>
<th>AFC</th>
<th>t = 0 h</th>
<th>t = 208 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UTEX25: 95% PGPB:0%</td>
<td>UTEX25: 95% PGPB:0%</td>
</tr>
<tr>
<td>2</td>
<td>UTEX25: 71% PGPB:28%</td>
<td>UTEX25: 73% PGPB:26%</td>
</tr>
<tr>
<td>3</td>
<td>UTEX25: 72% PGPB:27%</td>
<td>UTEX25: 73% PGPB:25%</td>
</tr>
<tr>
<td>4</td>
<td>UTEX25: 69% PGPB:27%</td>
<td>UTEX25: 69% PGPB:29%</td>
</tr>
</tbody>
</table>

**Overall photobioreactor cultures**

The results for the biomass dynamics of all the performed PBRCs are presented in figure 5; in table 5 one may see the biomass dynamics parameters obtained for each one of the PBRCs. One may notice peculiar dynamics in the case of PBRCs 3 and 4; this is because they were used for further purposes: PBRC-3 was used for a continuous operation trial; PBRC-4 was used for testing nitrogen source limitation.

Just like with the AFCs, the PBRCs’ results suggest that PGPB (i.e. 2D3) presence in culture induces an improvement in biomass production, only they further suggest improved growth kinetics. Strangely, the PBRC’s presented decreased biomass yields when compared to the AFCs, even though they had a higher $\mu_{med}$.
Table 5. Biomass parameter results obtained for the five PBR cultures

<table>
<thead>
<tr>
<th>PBRC</th>
<th>X̄ - XN (g/L)</th>
<th>YXMN</th>
<th>μmed (h⁻¹)</th>
<th>texp (h)</th>
<th>PX (g/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.17</td>
<td>0.069</td>
<td>0.009</td>
<td>94</td>
<td>1.8</td>
</tr>
<tr>
<td>2</td>
<td>0.33</td>
<td>0.132</td>
<td>0.016</td>
<td>97</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td>0.47</td>
<td>0.190</td>
<td>0.021</td>
<td>84</td>
<td>5.6</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>0.078</td>
<td>0.018</td>
<td>60</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>0.17</td>
<td>0.068</td>
<td>0.015</td>
<td>73</td>
<td>2.3</td>
</tr>
</tbody>
</table>

In PBRC-1, a contamination occurred: a yeast contaminant was detected at the 24th culture hour; yet, the culture was kept going since it was the first ever done. Both the biomasses of UTEX25 and the yeast were monitored by cell counting (figure 6).

PBRC-1's results

The UTEX25 exponential growth phase had a considerably low μmed, suggesting that the microalgae growth is being restricted, most likely because of the yeast presence. Surprisingly, the yeast contaminant thrived in PBRC-1, outgrowing UTEX25 in number of cells per mL. One possible explanation for what happened is the potential usage of the carbohydrate content of lysed UTEX25 cells by the yeast contaminant. Another explanation can be simply that PBRC-1 was not actually in strict autotrophic conditions; possibly, part of the glucose from the inoculums still remained and was transferred into the culture.

Furthermore, a unique event in this work was observed in samples of PBRC-1: microalgae flocculation. The sampled biomass tended to form aggregates that would settle at the bottom of the falcon tubes.

PBRC-2's results

In PBRC-2 another contaminant was observed, this time at the 12th culture hour. Having at the time a 2D3 inoculum prepared, 2D3 was aseptically inoculated in PBRC-2 at hour 22. At hour 73, UTEX25 growth was observed and 2D3 cells were detected, while contaminant cells decayed; at hour 88 the contaminant disappeared from the culture. The biomass dynamics (monitored by cell counting) is shown in figure 7.

PBRC-3’s results

Analogous to AFC-2, PBRC-3 presented the highest UTEX25 biomass production and growth kinetics of the PBRCs. It was also used for a continuous operation trial at the 214th culture hour, where its content was renewed by 5L of medium II for a 15 h period (D = 0.067 h⁻¹). The biomass dynamics during batch and continuous mode is presented in figure 8.
During the continuous operation, the biomass concentration decreased substantially; this is due to the washout phenomena caused by the unavoidable excessive dilution rate (because the pump was restricted).

Furthermore, two models were for biomass dynamics data during the continuous mode of operation based on equation 4: model 1, where the specific growth rate assigned for the biomass, \( \mu_1 \) (h\(^{-1}\)), is constant and equal to the \( \mu_{med} \) computed from the exponential curve fitted during the batch mode of PBRC-3 (i.e. 0.021 h\(^{-1}\)); and model 2, where the specific growth rate, \( \mu_2 \) (h\(^{-1}\)), is variable and its dynamics was obtained directly from computing a linear regression from \( \mu \) values calculated during continuous mode. The model results, along with the experimental data, are presented in figure 9.

The results from the models show that even though a constant \( \mu \) explains the data more accurately, a constant \( \mu \) with the value of the culture’s calculated \( \mu_{med} \) is a good approximation for predicting the verified biomass wash-out. Thus, one may affirm that the use of a dilution rate that is equal to the value of the culture’s \( \mu_{med} \) is likely to provide a chemostate, not needing any further calculations.

**PBRC-4’s results**

PBRC-4 was the first successful axenic culture and it was performed in PBR B. Its biomass dynamics is illustrated in figure 10.

**PBRC-5’s results**

PBRC-5 was the second successful axenic culture, being the control culture. It was also used to test if the nitrogen source was in fact a limiting nutrient for UTEX25’s growth. For that purpose, a solution of potassium nitrate (deionized water + \( \text{KNO}_3 \) 20 g/L, 50 mL) was added to the culture after the stationary phase of growth was apparently achieved. The biomass dynamics of PBRC-5 is presented in figure 11.
Indeed, new UTEX25 growth was observed in the culture after the mentioned adding of KN03. The results suggest thus that nitrogen is in fact restricting UTEX25 from further growth in the culture. Moreover, it was observed that when comparing to the first growth, the second growth curve kinetics is lower and less biomass was produced, suggesting that the culture’s performance is being restricted also by other factors (e.g. light).

**Further discussion**

This work’s results on the AFCs suggest a particular mechanism behind this improvement: the ACC eventually produced by microalgae was decomposed by PGPB-produced ACC-deaminase to alpha-ketobutyrate and ammonia; this prevents ACC from oxidizing to the stressful ethylene and, instead, provides microalgae with a new nitrogen source: ammonia (see scheme 1). This is not an unreasonable suggestion since microalgae from the *Chlorella* genus were already shown as producers of ethylene [19], just like higher plants are. The results obtained from PBRC-5 also support this explanation, as they suggest that nitrogen is a limiting factor for the culture growth.

![Scheme 1](image)

**Scheme 1. Ethylene biosynthesis mechanism and its deviation by ACC deaminase activity; adapted scheme from two bibliographic references [20, 21]**

Another interesting observation that can be made from the results is when one compares PBRC-1 with PBRC-5, since the latter is the axenic control: the yeast contaminant did affect considerably the biomass growth kinetics, but not its yield (the biomass production obtained for both was close to 0.17 g/L). This suggests that the yeast may potentially be used in co-culture with the microalgae (e.g. as a flocculation agent) without apparently compromising the final biomass yield.

Unfortunately, no conclusions regarding the matter of culture scale and its effect on the microalgae-PGPB interaction can be safely made. That is because the PBRCs’ biomass yield is unexpectedly lower than the AFCs, although higher kinetics was observed (tables 3 and 5). The higher kinetics in the PBRS is expected, as these culture systems provide better growth conditions for several reasons, such as better nutrient mass transfer through effective mixing and aeration, control of the culture temperature and pH, etc. The lower biomass yield, however, is unexpected, especially when higher kinetics was observed. Taking into account the characteristics and possible limitations of the two culture systems, two conjectures that may explain this difference in growth kinetics and final biomass produced are: the inferior light dilution of PBRs and the excessive movement their airlift flow induces in the microalgae cells.

**Conclusions and future work**

The main objective of this work was to verify if PGPB enhance microalgae biomass production. Indeed, the results support this hypothesis, since the co-cultures with PGPB showed a higher biomass production than the respective control axenic cultures. Moreover, it was concluded that it is possible to successfully isolate microalgae by using physical methods only, where a calculated differential centrifugation prior to sub-culturing is of crucial importance, and that dark heterotrophic conditions are quite adequate for cultivation of UTEX25, rendering much higher cell concentrations.

Studies like this one are a step forward towards making large-scale production of microalgae-based commodities financially viable and, thus, creating a potential solution for the world’s current environmental dilemma of food and fuel. And that is not only due to the enhancement of biomass production or biochemical content that was mentioned: the quest for finding a bacterial growth promoter for microalgae has a relevance that goes beyond. Axenic microalgae cultures do not exist in nature and that is why they are quite vulnerable to contamination; the engineering of a robust phycosphere, where pre-selected bacteria establish a strong and specific syntrophic relation with the microalgae of interest, would greatly decrease contamination chances (since virtually there would be no free biochemical pathway from which contaminants would thrive) and relief microalgae production from much of its labor and expense on anti-contamination agents and strategies.

It should be mentioned, however, that in regard to microalgal food commodities some extra marketing effort will be required for its financial viability, since the public must be educated to view microalgae as a food. Also, using bacteria for food growth promotion can be quite polemic: the common sense is thinking of bacteria only as contaminants and agents of disease; this misconception must be proven wrong before the public can accept the presence of beneficial bacteria in microalgae based food.

As for future work it would be interesting to further analyze the microalgae biomass so one could verify potential effects of the co-culture with PGPB on its biochemical composition, such as content in lipids or high-value compounds (e.g. lutein). Such analyses would also allow verification of the potential presence of ethylene or other phytohormones.
References


