Technical-Economical Approach for PHB Production by Ralstonia Eutropha Strain Using Concentrated Vinasse as Carbon Source and Other Biotechnological Applications

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Title: Technical-economical approach for PHB production by *Ralstonia eutropha* strain using concentrated vinasse as carbon source and other biotechnological applications

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Abstract

The Brazilian ethanol industry is one of the most important in the global market, however these important industrial activities have been generating significant amounts of vinasse and its management has become costly for distilleries. In this study, the aim was to evaluate concentrated and *in natura* vinasse as basal culture media for biotechnological processes. Different bacteria and processes were assessed: L-threonine production by *E. coli* THR14, with glucose as carbon source; PHB production by halophilic strain *Halomonas* sp. HG03, with sucrose as carbon source; and PHB biosynthesis by *R. eutropha* L359PCJ, which used glycerol from vinasse as carbon source. Strains were evaluated firstly in shake flasks cultivations using vinasse-based media. *E. coli* THR14 had no statistical difference for biomass and L-threonine concentrations among control and vinasse-based treatments (up to 50% v v⁻¹ of *in natura* vinasse). *Halomonas* sp. HG03 and *R. eutropha* L359PCJ were cultivated in mineral media diluted by *in natura* (50% and 75% v v⁻¹) and concentrated (50% and 75% v v⁻¹) vinasses. Higher vinasse concentrations resulted in higher cellular growth rather than PHB accumulation for both bacteria. In vinasse-based treatments, *Halomonas* sp. HG03 had PHB content between 19.6 – 75.2% and *R. eutropha* L359PCJ, 48.4 – 68.5%. 50% (v v⁻¹) of concentrated vinasse was the most attractive condition for PHB production by both bacteria. Further experiments in CSTR bioreactors used this nutritional condition and *R. eutropha* L359PCJ had PHB content of 66.3%, concentrations of residual cell dry weight (rCDW) = 9.4 g L⁻¹ and PHB = 18.6 g L⁻¹, with $Y_{X/S} = 0.16 \text{ g g}^{-1} \text{GLYCEROL-1}$, $Y_{P/S} = 0.32 \text{ g g}^{-1} \text{GLYCEROL-1}$ and 0.25 gPHB Lh⁻¹. *Halomonas* sp. HG03 had PHB content of 45.7%, rCDW = 9.8 g L⁻¹, PHB = 8.3 g L⁻¹ and $Y_{X/S} = 0.18 \text{ g g}^{-1} \text{SUCROSE-1}$, $Y_{P/S} = 0.16 \text{ g g}^{-1} \text{SUCROSE-1}$ and 0.12 gPHB Lh⁻¹. Finally, cost reductions of PHB production by *R. eutropha* L359PCJ with concentrated vinasse-based medium were evaluated *in silico* by using SuperPro Designer.

As a partial source of glycerol and other nutrients for PHB production by *R. eutropha* L359PCJ, vinasse reduced overall production costs by 13%. Simulated processes that used concentrated vinasse-based media combined with improvements of PHB productivity and higher cellular densities had production costs between US$ 3.9 – 7.5/kgPHB and 2.6 – 7.3 years of payback time.

**Keywords:** *in natura* vinasse; concentrated vinasse; amino acids; L-threonine; polyhydroxyalkanoates; P(3-hydroxybutyrate); SuperPro Designer.
Introduction

Global demand for green fuels has increased in recent years and it is expected to increase even further. Bioethanol is one of the most important among these, along with biodiesel, biogas and others. Together, the United States and Brazil account for about 85% of the global ethanol supply. Brazil has a sucrose-based industry, established on sugarcane exploit by using juice, molasse and, more recently, lignocellulosic material as carbon source for alcoholic fermentation and other industrial systems make use of resources from sugarcane: sugar, bioenergy and derivatives (OECD, 2021; Salles-Filho et al., 2017).

Vinasse is the main wastewater produced by Sucroenergetic activities as it is the remaining fermented broth after distillation. All residual organic matter, metabolites and nutrients from fermented broth are found in vinasse, except for ethanol, which is efficiently recovered. About 10-15 L of vinasse are generated along with 1 L of ethanol, which implies an annual vinasse supply of over 300 billion L. Its composition is dependent on the ethanol process, so different sorts and quality of feedstocks, as well as process conditions and operations affect vinasse characteristics (Cortez, 2010).

In general, vinasses have very high polluting potential (COD between 21,000 - 34,000 mgO₂ L⁻¹) and they are a source of carbon compounds and salts: glycerol has been frequently described to be found in concentrations about 10.0 g L⁻¹ (Ortiz-Muniz et al., 2010; Souza et al., 2015); organic acids such as lactic acid and acetic acid have been reported in concentrations up to 7.0 g L⁻¹ and 2.2 g L⁻¹, respectively (Dowd, Johansen & Cantarella, 1994; Lopes et al., 2016); potassium, sodium and calcium salts, in concentrations of 13.0 g L⁻¹, 31.3 g L⁻¹ and 5.2 g L⁻¹, respectively (Coelho et al., 2018; España-Gamboa et al., 2011; España-Gamboa et al., 2012; Ferreira et al., 2011; Moraes, Zaiat & Bonomi, 2015; Pedro-Escher, Maziviero & Fontanetti, 2014; Souza et al., 2015).

Vinasses are frequently used as fertilizers in sugarcane crops, but such application has shown limitations and concerns. Researchers have reported environmental issues such as soil salinization; superficial and groundwaters contamination by sulphates, nitrates, metals; and greenhouse gases emissions by degradation of carbon and nitrogen compounds found in vinasse (Cassman et al., 2018; Pedro-Escher, Maziviero & Fontanetti, 2014; Soto et al. 2015).
Vinasse application as a fertilizer for sugarcane crops in Brazil emerged during the 80’s, when a governmental program favored the bioethanol industry in Brazil. By that time, the Brazilian production increased by three fold in less than ten years, while vinasse generation is estimated to have increased about three to five fold as well. However, technologies for vinasse treatment did not receive the same investments as ethanol production did. Before the aforementioned program, vinasses were disposed of in rivers and bodies of water. Thus, applying vinasse as a soil fertilizer was an alternative and immediate solution for increasing volumes of wastewaters in such a short period of time (Ribeiro, Novais & Bahia-Filho 1983; UNICA, 2021).

Nowadays, the increasing ethanol production has made it necessary to find innovative solutions for increasing volumes of vinasse. Such solutions may be integrated with value-added bioproducts, bioenergy and biological waste treatment.

Amino acids are among the largest biocommodities markets. These bioproducts are largely used in animal nutrition, in chemical and other bio-industries. Global market of amino acids is estimated to reach about US$ 29-35 billions between 2022 and 2024 (Grand View Research, 2015; Global Industry Analysts Inc., 2018).

Poly(3-hydroxybutyrate) (PHB) is one of the main polyhydroxyalkanoates (PHAs), which are precursors of bioplastics, a growing market that has received increasing investments as a result of expanding policies on sustainability around the world. By 2025, global PHA market is expected to grow about 14% and become one of the main sectors in bioplastics market, along with polylactates (European Bioplastics, 2020; Sreedevi et al., 2014).

These bioproducts are synthesized by very robust microorganisms, which are advantageous for bioprocesses because they have sets of molecular tools available for metabolic engineering, they can metabolize a wide range of carbon sources and be cultured in low cost culture media.

In this study we aimed to evaluate technological applications for vinasse by cultivating bacterial strains in vinasse-based culture media. We cultivated an *E. coli* L-threonine-producing strain in mineral medium with volumetric dilutions of *in natura* vinasse. PHB-producing strains of *R. eutropha* and *Halomonas* sp. were cultivated in concentrated and *in natura* vinasse-based media.

Vinasse was used as basal culture media and a source of water, salts and carbon. Shake flasks and bioreactor fermentations were carried out and, finally, experimental data were used to
simulate large scale bioprocesses in SuperPro Designer. Technical and economical feasibility were assessed and the potential of using vinasse as nutrients source was evaluated.

Material and Methods

Vinasse

Vinasse was obtained from a distillery in São Paulo State, Brazil, which produces ethanol from sugarcane molasses. In our laboratory, vinasse was concentrated to 34.4ºBrix and kept at 4ºC. In order to apply vinasse in chemical analyses and experiments, the highly concentrated material was diluted in distilled water (m m⁻¹) so final concentrations could be obtained. In this study, we named in natura vinasse the material diluted to 3.4ºBrix, simulating the concentration found in the output stream of bioethanol process.

Before all analytical procedures and shake flasks experiments, vinasses were filtered in vacuum filtration system, which consisted of a Kitassato flask, Büchner funnel and qualitative paper filter. Prior to all experiments, vinasses had pH set to 6.8 < pH < 7.8 with NaOH 10 M or NH₄OH 4 M solutions, followed by autoclave sterilization, and then vinasses were added to sterile culture media. In natura vinasse had pH = 4.3 and its chemical composition is presented in Table 1.

Table 1. In natura vinasse chemical composition (3.4ºBrix)

| Microorganisms |
|----------------|
| For L-threonine production experiments, it was utilized the strain E. coli MG1655 ΔmetJIQ ΔlysA pBBR1MCS2::rhtC-thrB, a L-lysine auxotroph, which was conveniently named as E. coli THR14 in this study. For PHB production experiments, two strains were used. We employed the halophilic strain Halomonas sp. HG03, which is derived from Halomonas HG01, a PHB-producing bacteria. Halomonas sp. HG01 was first isolated from salines in Peru and it is naturally capable of consuming glucose and sucrose. Halomonas sp. HG03 was submitted to evolutionary engineering so a glycerol-consuming strain could be obtained (Moreno, 2015; Cardinali-Rezende et al., 2016). An efficient glycerol-consuming strain was also employed for PHB production experiments: Ralstonia eutropha PHB-4 LFM035-9 pBBR1MCS-2::phaPCJ, which was conveniently named as R. eutropha L359PCJ in this study. |
All microorganisms were kept in glycerol solution (20% v v\(^{-1}\)) at -80ºC. Before experiments, they were all cultivated in their respective solid culture media, kept at 30ºC for 24-36 h and then a loopful from a single colony was inoculated into fresh liquid medium for inoculum preparation.

**Shake flasks fermentations**

**L-threonine production by *E. coli* THR14**

**Culture conditions**

Luria-Bertani (LB) medium was used for *E. coli* THR14. Inoculum preparation was carried out by inoculating a single colony into fresh liquid medium, followed by a 12 h incubation at 30ºC and 180 rpm. The culture was then used as inoculum for L-threonine biosynthesis. The mineral medium MM\(_{Ec}\) was adapted from Lee et al. (2006) (L\(^{-1}\)):

- glucose, 25.0 g
- \((NH_4)_2SO_4\), 5.0 g
- KH\(_2\)PO\(_4\), 8.5 g
- MgSO\(_4\), 1.0 g
- sodium citrate, 0.5 g
- L-lysine, 0.5 g
- CaCO\(_3\), 10.0 g
- and trace elements solution, 1.0 mL, with the following composition (L\(^{-1}\)):
  - CaCl\(_2\), 13.2 g
  - FeSO\(_4\), 8.4 g
  - MnSO\(_4\), 2.4 g
  - ZnSO\(_4\), 2.4 g
  - CuSO\(_4\), 0.48 g
  - CoCl\(_2\), 0.48 g
  - Na\(_2\)MoO\(_4\), 0.24 g
  - K\(_2\)B\(_4\)O\(_7\), 0.06 g
- diluted in HCl 1 N and sterilized by ultrafiltration.

**Evaluation of *E. coli* cellular growth and L-threonine biosynthesis in vinasse-based culture media**

Vinasse-based culture media consisted of volumetric dilutions of *in natura* vinasse (3.4ºBrix) in MM\(_{Ec}\). Four treatments were evaluated: 25% (v v\(^{-1}\)) of vinasse in MM\(_{Ec}\) (MM\(_{Ec}\)V253); 50% (v v\(^{-1}\)) of vinasse in MM\(_{Ec}\) (MM\(_{Ec}\)V503); and 75% (v v\(^{-1}\)) of vinasse in MM\(_{Ec}\) (MM\(_{Ec}\)V753); control treatment was 100% (v v\(^{-1}\)) composed of MM\(_{Ec}\). In order to supply the proper nutritional conditions for *E. coli* THR14 growth, all treatments had carbon (glucose), nitrogen (ammonium sulphate), phosphate (KH\(_2\)PO\(_4\)) sources and L-lysine set to the same concentrations, as those described above for MM\(_{Ec}\), and pH was adjusted to 6.8 < pH < 7.8 with NH\(_4\)OH 4 M. All treatments for L-threonine production were carried on with inoculum ratio of 10% v v\(^{-1}\), 50 mL of culture medium in 250 mL flasks, kept at 30ºC, under agitation of 180 rpm for 24 h. All steps of *E. coli* THR14 cultivation had a final concentration of kanamycin sulphate 0.25 μg L\(^{-1}\).

Before cultivation started, culture media samples were collected for determination of initial substrates concentration. By the end of 24 h incubation, final substrates concentrations were quantified and cellular dry weight was determined by gravimetric method.
PHB production by *Halomonas* sp. HG03

**Culture conditions**

Halophilic nutrient broth was used for *Halomonas* sp. HG03 culture in solid medium and for inoculum preparation (L⁻¹): beef extract, 3.0 g; peptone, 5.0 g; NaCl, 100 g. A single colony from solid culture was inoculated into fresh liquid medium and kept at 30°C, under 150 rpm agitation for 18 h. Culture was then used as inoculum for experiments of PHB production in mineral medium (MM₉₆) adapted from Rocha et al. (2008) (L⁻¹): sucrose, 15.0 g; Na₂HPO₄, 3.5 g; KH₂PO₄, 1.5 g; (NH₄)₂SO₄, 1.0 g; MgSO₄.7H₂O, 0.2 g; CaCl₂.2H₂O, 0.01 g; ammonium ferric citrate, 0.06 g; NaCl, 80.0 g; and trace elements solution with the following composition (L⁻¹) - H₃BO₃ 0.3 g; CoCl₂.6H₂O, 0.2 g; ZnSO₄.7H₂O, 0.1 g; MnCl₂.4H₂O, 0.03 g; NaMoO₄.2H₂O, 0.03 g; NiCl₂.6H₂O, 0.02 g; CuSO₄.5H₂O, 0.01 g.

Evaluation of *Halomonas* sp. HG03 cellular growth and PHB biosynthesis in vinasse-based culture media

Control treatment consisted of culture medium composed of 100% (v.v⁻¹) MM₉₆. Experiments with *Halomonas* sp. HG03 used both in natura (3.4ºBrix) and concentrated (7ºBrix) vinasses. The following volumetric dilutions of vinasses were evaluated: 50% (v.v⁻¹) of vinasse 3.4ºBrix in MM₉₆ (MM₉₆/V503); 75% (v.v⁻¹) of vinasse 3.4ºBrix in MM₉₆ (MM₉₆/V753); 50% (v.v⁻¹) of vinasse 7ºBrix in MM₉₆ (MM₉₆/V507); and 75% (v.v⁻¹) of vinasse 7ºBrix in MM₉₆ (MM₉₆/V757). Carbon (sucrose), nitrogen (ammonium sulphate), phosphate (Na₂HPO₄, KH₂PO₄) sources and NaCl were set to the same concentrations for all treatments. Experiments were carried out with initial 6.8 < pH < 7.8, adjusted with NaOH 10 M, inoculum ratio of 5% (v.v⁻¹) in 125 mL flasks with 25 mL of culture medium, incubated at 30°C, under 150 rpm agitation for 48 h.

At time t = 0 h, culture media samples were collected in order to determine initial concentration of substrates. By the end of 48 h, processes were interrupted, cultures were submitted to centrifugation at 4500 g, 15 min, 4°C and cells were separated from supernatant. Pellets with cells were further used for determination of residual cell dry weight (rCDW) and PHB content (% m m⁻¹); supernatant was used for quantification of residual substrates and residual concentration of ammonium.
**PHB production by Ralstonia eutropha L359PCJ**

**Culture conditions**

LB culture medium was used for *R. eutropha* L359PCJ culture in solid medium and for inoculum preparation, which was carried with the same procedures described for *Halomonas* sp. HG03.

The mineral medium MM$_{Re}$ was adapted from Rocha et al. (2008) and it had the same composition as the medium used for *Halomonas* sp. HG03, except it did not have NaCl added and glycerol was the carbon source (15.0 g L$^{-1}$).

**Evaluation of *R. eutropha* L359PCJ cellular growth and PHB biosynthesis in vinasse-based culture media**

Both *in natura* (3.4ºBrix) and concentrated (7ºBrix) vinasses were evaluated for PHB biosynthesis by *R. eutropha* L359PCJ. The control treatment consisted of 100% (v v$^{-1}$) mineral medium MM$_{Re}$, in which glycerol was fully provided by a concentrated sterile solution of 150 g L$^{-1}$ glycerol.

Vinasse-based treatments had glycerol partially or fully provided by vinasse, according to the volumetric dilutions: 50% (v v$^{-1}$) of vinasse 3.4ºBrix in MM$_{Re}$ (MM$_{Re}$V503) had 2.5 g L$^{-1}$ of glycerol; 75% (v v$^{-1}$) of vinasse 3.4ºBrix in MM$_{Re}$ (MM$_{Re}$V753) had 5.0 g L$^{-1}$ of glycerol; 50% (v v$^{-1}$) of vinasse 7ºBrix in MM$_{Re}$ (MM$_{Re}$V507) had 7.5 g L$^{-1}$ of glycerol; 75% (v v$^{-1}$) of vinasse 7ºBrix in MM$_{Re}$ (MM$_{Re}$V757) had 15.0 g L$^{-1}$ of glycerol. Finally, a concentrated glycerol solution (150 g L$^{-1}$) was used to supplement glycerol in vinasse-based treatments MM$_{Re}$V503, MM$_{Re}$V753 and MM$_{Re}$V507, so all treatments were set to the same initial concentration of 15.0 g L$^{-1}$ glycerol. No external carbon source was added in MM$_{Re}$V757 treatment.

Carbon (glycerol), nitrogen (ammonium sulphate) and phosphate (Na$_2$HPO$_4$ and KH$_2$HPO$_4$) sources were set to the same concentrations in all treatments for *R. eutropha* L359PCJ cultivations. All steps of *R. eutropha* L359PCJ cultivations had a final concentration of 0.25 μg L$^{-1}$ of kanamycin sulphate.

Experiments were carried out at 30ºC, under 150 rpm agitation for 48 h, and both culture conditions and samples treatment were the same as those described above for *Halomonas* sp. HG03.
All experiments in shake flasks had treatments conducted in triplicates and analytical procedures were carried out in duplicates. Data were analyzed using ANOVA and means were compared with Tukey's test (5% probability), by using ExpDes.pt package in RStudio software (Ferreira, Cavalcanti & Nogueira 2014).

Bioreactor fermentations

PHB-producing strains *R. eutropha* L359PCJ and *Halomonas* HG03 were further cultivated in CSTR bioreactors, model Biostat® Cplus, Sartorius Stedim Biotech, with a 15 L vessel.

Bioprocesses were carried on in consecutive batches: two initial batches had growth-inducing conditions, followed by batches that induced nutritional conditions for PHB accumulation. The aim was to achieve higher concentration of rCDW before imposing nitrogen limitation for PHB accumulation. Besides, we aimed to minimize any osmotic stress in growth phases that could be a result of higher concentrations of carbon source, salts and vinasse.

Inoculum preparation followed the same procedures described above for shake flasks fermentations. Both *R. eutropha* L359PCJ and *Halomonas* HG03 strains were cultivated with initial volume of 6 L, inoculum ratio of 10% (v/v⁻¹) and nutritional conditions from MMᵣₑV507 and MMᵣᵥV507 treatments were chosen for PHB production in bioreactors.

Bacteria were cultivated in culture media composed of mineral medium MMB, adapted from Rocha et al. (2008), and a volumetric dilution of 50% of concentrated vinasse (7ºBrix) was used (Table 2).

| Table 2. Culture medium composition for bioprocesses of PHB production in CSTR bioreactors. |
|________________________________________________________________________________________|
|Similarly to flask cultivations with *R. eutropha* L359PCJ, glycerol was partially provided by vinasse (7.5 g L⁻¹), and a concentrated solution of pure glycerol (300 g L⁻¹) was used to set a final concentration of 15 g L⁻¹ in all batch cultivations. |
Once the carbon source reached limiting concentration, below 5 g L\(^{-1}\), the first batch was considered finished. The second batch was initiated by adding a concentrated solution (0.7 L), so nutrients from mineral medium could be set to concentrations described in Table 2. A concentrated solution of pure glycerol and highly concentrated vinasse (34.4ºBrix) provided the final concentration of 15 g L\(^{-1}\) of glycerol (1:1). Finally, working volume was 6.7 L.

Once carbon source in the second batch became limiting, further batches aiming to PHB accumulation were initiated by adding concentrated vinasse (34.4ºBrix) and pure glycerol solution (300 g L\(^{-1}\)) as the sole nutrients source. These materials were added in variable volumes, so the final concentrations of glycerol from vinasse (7.5 g L\(^{-1}\)) and pure glycerol (7.5 g L\(^{-1}\)) were kept the same for all batches. PHB accumulation phase was ceased once the culture reached stationary growth phase.

*Halomonas* sp. HG03 used sucrose as carbon source, which was fully provided by adding a concentrated solution (600 g L\(^{-1}\)) so final concentration could be set to 15 g L\(^{-1}\).

Once sucrose reached limiting concentration, below 5 g L\(^{-1}\), the first batch was finished and the second batch was carried on similarly to the procedure described for *R. eutropha* L359PCJ. A concentrated solution (0.7 L) containing nutrients from mineral medium (Table 2), a concentrated solution of sucrose (600 g L\(^{-1}\)) and highly concentrated vinasse (34.4ºBrix) were added. Glycerol from vinasse was not the preferable carbon source of *Halomonas* sp. HG03, but its concentration in culture medium (7.5 g L\(^{-1}\)) was efficiently used as an indicator of vinasse dilution in bioreactor, so the volumetric dilution of 50% could be kept the same for all batches.

After the second batch was finished, further batches aiming to PHB accumulation were initiated by adding concentrated vinasse (34.4ºBrix) and sucrose solution (600 g L\(^{-1}\)) as the sole nutrients source, aiming to keep the volumetric dilution of 50% (v v\(^{-1}\)) of concentrated vinasse and 15 g L\(^{-1}\) of sucrose for all batches. Consecutive batches with *Halomonas* sp. HG03 were carried on until stationary growth phase was observed.

Bioprocesses with both *R. eutropha* L359PCJ and *Halomonas* sp. HG03 were carried on at 30ºC and pH was automatically controlled at 6.8 < pH < 7.8 by addition of H\(_2\)SO\(_4\) 1 M and NaOH 2 M solutions. The air inflow was set to 3 Lpm (0.5 vvm) and agitation speed was initially set to 300
rpm. Bioreactors were set to keep saturation of dissolved oxygen at 40% by controlling both air
inflow and agitation.

Bioprocess data were further used for calculation of instantaneous velocities (g L h\(^{-1}\)) of product
formation (\(r_P\)) and cellular growth (\(r_X\)):

\[
r_P = \frac{d(P_f - P_i)}{d(t_f - t_i)}
\]

eq. (1)

\[
r_X = \frac{d(X_f - X_i)}{d(t_f - t_i)}
\]

eq. (2)

Specific velocities of residual biomass formation (\(\mu_X\) h\(^{-1}\)) and product formation (\(\mu_P\) g gh\(^{-1}\)) were
also determined:

\[
\mu_P = \frac{1}{X} \cdot r_P
\]

eq. (3)

\[
\mu_X = \frac{1}{X} \cdot r_X
\]

eq. (4)

Maximum growth velocity (\(\mu_{max}\) h\(^{-1}\)) was determined using rCDW concentration, as described by
the equation below:

\[
\ln \frac{X_f}{X_i} = \mu_{max} \cdot (t_f - t_i)
\]

eq. (5)

PHB productivity (\(Pp_{PHB}\) g Lh\(^{-1}\)) was calculated for a given variation of product concentration within
a time range:
Finally, product \( Y_{P/S} \text{ g g}^{-1} \) and biomass (rCDW) \( Y_{X/S} \text{ g g}^{-1} \) yields were determined for a variation of product and biomass concentrations given the substrate consumption:

\[
P_{P\text{PHB}} = \frac{(P_f - P_i)}{(t_f - t_i)}
\]

\text{eq. (6)}

\[
Y_{X/S} = \frac{(X_f - X_i)}{(S_i - S_f)}
\]

\text{eq. (7)}

\[
Y_{P/S} = \frac{(P_f - P_i)}{(S_i - S_f)}
\]

\text{eq. (8)}

\textbf{Analytical methods}

Vinasse refractive index was determined as Brix Degrees (ºBrix), by using digital refractometer.

Organic acids (acetic acid, lactic acid) and carbohydrates (glucose, fructose, sucrose, glycerol) were analyzed by liquid chromatography (HPLC) in a Dionex® system (Ultimate 3000, Thermo Fisher Scientific Inc, Waltham, MA, USA) with Aminex HPX-87H Bio-Rad column and RefractoMax 520 detector. Ionic forms of calcium, potassium, sodium and ammonium in vinasse were determined by ionic chromatography using Metrohm 930 Compact system, with Metrosep C4 250/4.0 column. Amino acids were quantified by using the Phenomenex "EZ: Faast (™) Family of Amino Acids Analysis Kit", for GC-FID determination (Badawy, 2012).

In \textit{E. coli} THR14 cultures, cell dry weight was determined by gravimetric method. Culture media were centrifuged at 10,000 g, for 10 minutes, at 10ºC. Pellets were then resuspended in distilled water, cells were harvested with filtration membranes (0.22 μm), and finally submitted to 105ºC until constant dry weight (Quillaguamán et al., 2007).

Residual ammonium concentration in PHB production experiments was determined by using Orion™ Ion-Specific Electrode kit for ammonia analysis (Thermo Fisher Scientific Inc, Waltham, MA, USA). Sample volumes of 3.0 mL were used and NaOH 10 M solution was used to alkalize samples and make ammonium available for detection, as procedure recommended by the manufacturer.
In *Halomonas* sp. HG03 and *R. eutropha* L359PCJ cultivations, cell dry weight was determined by lyophilization of cells, so PHB extraction could be performed next. Sample volumes of 10 mL were centrifuged, resuspended in saline solution (0.85% m v⁻¹), next washed in the same solution and centrifuged. Remaining pellets were then submitted to lyophilization overnight. Resulting dry mass was measured and cell dry weight was calculated in order to determine total biomass concentration (CDW g L⁻¹).

The same dry mass was used for PHB extraction method by propanolysis (Riis & Mai, 1988). Propyl esters were next quantified by gas chromatography Agilent 7890A with HP-5 column (5% diphenyl-95%-dimethyl-polisiloxane, 30 m, 320 mm, 0.25 μm film thickness). Volume samples of 1.0 μL were analyzed after split injection (1:20), using a flame-ionization detector. Injector and detector temperatures were, respectively, 250°C and 300°C. Helium (0.8 mL min⁻¹) was the carrier gas and a quantification program was set to 100°C 1', 210°C 1' (8°C min⁻¹), up to 240°C 1'. Benzoic acid (40 mg mL⁻¹) was used as internal standard.

PHB cellular content was determined as the ratio of PHB concentration in total CDW. Residual cell dry weight concentration (rCDW) was then obtained by subtracting PHB mass from CDW. Even though vinasses were previously filtrated, specific analyses were performed in order to make sure that vinasse would not overestimate dry weight measures, due to any insoluble solids from vinasse. Before bacterial inoculation all vinasse-based culture media were submitted to dry cell weight analyses, following the same procedures described above. Vinasse-based culture media were analyzed in triplicates, the resulting dry mass was determined and next subtracted from dry cell weight measures in samples from bioprocesses.

**In silico study of PHB production process by *R. eutropha* L359PCJ with concentrated vinasse-based medium**

**In silico** analyses were performed with SuperPro Designer software, v. 9.5 (Intelligen, Inc., Scotch Palins, NJ, USA). The software was used in all technical and economical evaluations in this study. A base scenario was designed and named SC1. The bioprocess flowsheet is detailed in Figure 1.
The stoichiometric model for simulation of PHB bioprocess was designed based on process conditions previously reported in literature and in silico analyses were performed according to methods described elsewhere (Akiyama et al., 2003; Canizales et al., 2020; Choi & Lee, 1997; Homenschuh et al., 2014; Leong et al., 2017; Petrides, 2015).

Economical evaluations aimed to estimate production costs that could be reduced by using vinasse as a partial source of glycerol and salts for PHB production by *R. eutropha* L359PCJ.

Technical-economical scenarios were simulated in order to analyze how PHB productivity and cell density might impact on production costs and profitability indicators.

Experimental data were previously converted into input data for SuperPro Designer simulation model as described below.

Experimental yields of glycerol conversion into biomass (*r*CDW) and PHB (*Y*_{X/S} g g^{-1}; *Y*_{P/S} g g^{-1}) were used in global stoichiometric equations used in both unit procedures seed fermenter SFR-101 (R1) and production bioreactor FR-101 (R2). All mineral medium components were normalized to glycerol (Table 3).

| Table 3. Global stoichiometric equations used in base scenario SC1. |
|---|

PHB productivities were estimated from experimental data. Simulated global productivities (*P*_{P_{SC}}) aimed to simulate PHB production by high cell density cultures. Time ranges for growth phase (*t*_{X_{SC}}) and PHB accumulation phase (*t*_{PHB_{SC}}) were calculated.
Equation 5 was used to calculate the term \((t_f - t_i)\). Experimental data of \(\mu_{\text{max}}\) (h\(^{-1}\)) and \(X_i\) (g L\(^{-1}\)) were used. The term \(t_i\) was set to \(t = 0\) h; the term \(X_f\) simulated high cell densities, named as \(X_{f-Si}\). Scenario SC1 used \(X_{f-Si} = 30\) g L\(^{-1}\). Finally, \(t_f = t_{X-Si}\), the time range required for high cell density.

Equation 3 was used to calculate the term \(d(t_f - t_i)\) and finally to calculate the time range required for PHB accumulation phase and the resulting PHB titer. Experimental data of \(\mu_P\) (g gh\(^{-1}\)) was used; \(X = X_{f-Si}\) and \(t_i\) was set to \(t = 0\) h. The term \(d(P_f - P_i)\) was calculated based on experimental data of PHB content (% m m\(^{-1}\)). The term \(\text{PHBcontent}_{f}\) was the experimental data of PHB content at the end of the growth phase. The term \(P_i\) was the calculated PHB titer at the end of growth phase for a high cell density culture \((X_{f-Si})\). Thus, the term \(\text{PHBcontent}_{f}\) was the PHB content experimentally determined at the end of the accumulation phase and \(P_f\) was consequently the calculated PHB titer:

\[
\begin{align*}
\frac{P_{f-Si}}{g L^{-1}} &= \frac{X_{f-Si}}{1 - (\text{PHBcontent}_{f}/100)} & \text{eq. (9)} \\
\frac{P_{i-Si}}{g L^{-1}} &= \frac{X_{f-Si}}{1 - (\text{PHBcontent}_{f}/100)} & \text{eq. (10)}
\end{align*}
\]

The term \(t_f = t_{PHB-Si}\).

Finally, the simulated global productivities \((P_{p-Si}, g Lh^{-1})\) were calculated as it follows:

\[
P_{p-Si} = \frac{P_{f-Si}}{t_{X-Si} + t_{PHB-Si}} & \text{eq. (11)}
\]

The base scenario SC1 was designed by considering specificities of integrating the PHB bioprocess with ethanol bioprocess. The aim was to reproduce the main nutritional conditions of PHB production by \(R.\ eutropha\ L359\)PCJ in concentrated vinasse-based medium (50% v v\(^{-1}\) of vinasse 7ºBrix).
Firstly, the annual production capacity was 10,000 tons of PHB. By taking into consideration that vinasse would provide 50% of carbon source, such production capacity would be compatible to vinasse production by a distillery that produces 295,245 - 443,000 m³ of ethanol annually (10 - 15 L of vinasse for 1 L of ethanol), providing 44,300 tons of glycerol annually (in natura vinasse with 10 g L⁻¹ of glycerol, Table 1).

Secondly, the annual campaign had 5760 operating hours, which is compatible to the operating hours in bioethanol campaign and sugarcane harvest (240 days year⁻¹) so vinasse supply would be guaranteed.

These two requirements defined major aspects of model design, however the base scenario SC1 did not include vinasse use as nutrients source and such factor was assessed in scenarios of technical-economical evaluation, as detailed further. The scenario SC1 used medium composition from Rocha et al. (2008) (Table 2), which means that nutrients for culture medium would need to be completely purchased from external suppliers.

The simulation model was set to batch mode and it consisted of three sections: Upstream, Bioprocess and Downstream (Figure 1). The Upstream section included culture medium preparation and seed fermentation for inoculum production.

A blending tank was set to be charged with medium components, followed by in situ sterilization. Afterwards, sterile medium was split between SFR-101 and FR-101 (1:9). Inoculum preparation was set to take place in a 40 m³ seed fermenter, operated at 30°C, 13 h.

The Bioprocess section consisted of the biotransformation process in production reactors FR-101 (315 m³). Six units of production reactors were set to be charged with culture medium and inoculum from SFR-101. Biotransformation was ruled by the global stoichiometric reaction R2 and the base scenario SC1 simulated a calculated time reaction \((t_{X_{Si}} + t_{PHB_{Si}})\) of 51.7 h, \(X_{Si} = 30\) g L⁻¹, \(P_{X_{Si}} = 59\) g L⁻¹ and \(P_{PHB_{Si}} = 1.1\) g Lh⁻¹. Finally, PHB-rich medium was set to be transferred out to a storage tank and downstream process could be initiated.

The Downstream section was based on operations described by Akiyama et al., 2003; Choi & Lee, 1997; and Leong et al., 2017. According to these authors, recovery of PHB by surfactant-
hypochlorite digestion is economically interesting and it has low environmental impacts in
comparison to other PHB extraction methods.

Cells are separated from broth by centrifugation, next they are collected in a storage tank where
surfactant solution (1% m v⁻¹) is charged and the mixture is kept at 25°C, 1 h. Hypochlorite
digestion is performed with addition of hypochlorite solution (30% m v⁻¹) in flow-through manner.
Aqueous phase with residual cell debris and PHB are separated by centrifugation, washed with
water (1:1), concentrated by centrifugation and finally spray-dried to a final form of ≥ 99% (m m⁻¹)
PHB and ≤ 1% (m m⁻¹) water. As described by Akiyama et al., 2003; Choi & Lee, 1997; and
Leong et al., 2017, the overall yield of downstream process for recovering PHB was set to 95%.

Economical evaluations were performed for a 15-year period at an annual interest rate of 6% and
selling price of US$ 10 kg⁻¹ PHB. SuperPro Designer was used in all calculations of capital costs,
production costs, annual operating costs and profitability analyses. Prices configurations were:

US$ ton⁻¹ - glycerol, 400; (NH₄)₂SO₄, 107; KH₂PO₄, 980; MgSO₄, 120; CaCl₂, 130; ammonium
iron citrate, 285; H₃BO₃, 780; CoCl₂, 10,000; ZnSO₄, 560; MnSO₄, 1,900; Na₂MoO₄, 9,750; NiCl₂,
4,700; CuSO₄, 3,800; surfactant, 800; NaOCl, 200; (US$ MT⁻¹) – steam, 12; high pressure steam,
20; chilled water, 0.5; (US$ kW-h⁻¹) power, 0.07.

Sensitivity analyses

Firstly, the base scenario SC1 was evaluated for variations of glycerol prices and reduction of
production costs due to vinasse as a partial source of nutrients for culture medium. According to
Stracke et al. (2018), crude glycerol from biodiesel processes in Brazil might have prices ranging
from US$ 200 - 400 ton⁻¹, depending on location, purity and other factors. Thus, three scenarios
simulated glycerol prices at US$ 400 ton⁻¹ (SC1), US$ 300 ton⁻¹ (SC2) and US$ 200 ton⁻¹ (SC3).
Next, each glycerol price was evaluated combined with cost reductions by vinasse. Since vinasse
contributed with 50% of carbon source in our experiments, scenarios SC4, SC5 and SC6
simulated, respectively, glycerol prices of US$ 400 ton⁻¹, US$ 300 ton⁻¹ and US$ 200 ton⁻¹ as well,
however, due to vinasse use, their final cost with glycerol in culture media were, respectively,
US$ 200 ton⁻¹, US$ 150 ton⁻¹ and US$ 100 ton⁻¹.
Due to vinasse use, scenarios SC4, SC5 and SC6 also had reduction costs with other nutrients in culture medium. *In silico*, we reproduced the experimental conditions of fully supplementing phosphate and nitrogen sources, while other mineral nutrients in medium were diluted by vinasse. Thus, these scenarios had their costs with mineral nutrients reduced by 50%, except for KH₂PO₄ and (NH₄)₂SO₄.

A second set of analyses investigated bioprocesses with high cell densities and improvements in PHB productivity. All scenarios considered vinasse as a partial source of nutrients. Glycerol price was set to US$ 400 ton⁻¹, but its final cost in culture medium was US$ 200 ton⁻¹ and other mineral nutrients had costs reduced by 50%, except for KH₂PO₄ and (NH₄)₂SO₄.

Three cell densities were evaluated, Xᵢₛᵢ = 30 g L⁻¹, Xᵢₛᵢ = 40 g L⁻¹ and Xᵢₛᵢ = 50 g L⁻¹. Each value of Xᵢₛᵢ was combined with three experimental values of μₚ (g gh⁻¹). Thus, different values of simulated PHB productivity were calculated. Scenarios SC7-SC15 were characterized as optimistic, realistic and pessimistic according to values of μₚ and their resulting PHB productivity (Table 4). All scenarios kept the same glycerol conversion into PHB (Yₚ/s g g⁻¹), biomass (rCDW) (Yₓ/s g g⁻¹) and PHB contents (% m m⁻¹).

**Table 4. Technical-economical scenarios for evaluation of high cell density cultures and PHB productivities.**

**Results and Discussion**

*L-threonine production by E. coli THR14*

*E. coli* THR14 had cellular growth in all vinasse based conditions we evaluated in this study. Volumetric dilutions of 25% and 50% of *in natura* vinasse in mineral medium increased biomass concentration by 15% and 30% in comparison to control treatment, with a maximum value of 10.7 ± 2.1 g L⁻¹ (MMₑᵥV₅₀₃), although no statistical difference was found. Vinasse dilution of 75% (v v⁻¹) resulted in the least satisfactory cellular growth, which was 4.9 ± 0.5 g L⁻¹, statistically the lowest biomass concentration (Figure 2).
Culture medium acidification is highly associated with *E. coli* glucose metabolism, so acetic acid concentration was determined after processes were interrupted. Although CDW concentrations in MM<sub>Ec</sub>, MM<sub>EcV253</sub> and MM<sub>EcV503</sub> showed no significant difference, acetic acid concentrations in MM<sub>EcV253</sub> (0.6 ± 0.1 g L<sup>-1</sup>) and MM<sub>EcV503</sub> (0.5 ± 0.2 g L<sup>-1</sup>) were significantly lower than those in MM<sub>Ec</sub> (2.3 ± 0.8 g L<sup>-1</sup>) and MM<sub>EcV753</sub> (1.9 ± 0.04 g L<sup>-1</sup>). In MM<sub>EcV253</sub> and MM<sub>EcV503</sub>, *E. coli* THR14 synthesized 1.2 (± 0.7) g L<sup>-1</sup> and 1.2 (± 0.1) g L<sup>-1</sup> of L-threonine, respectively. No statistical difference was found between L-threonine production among these vinasse-based treatments and control treatment MM<sub>Ec</sub>, which had 0.8 (± 1.1) g L<sup>-1</sup> of L-threonine. No detectable L-threonine production was determined in MM<sub>EcV753</sub>. Both L-threonine (Y<sub>P/S</sub> g g<sup>-1</sup>) and biomass yields from glucose (Y<sub>X/S</sub> g g<sup>-1</sup>) showed no significant difference between MM<sub>Ec</sub>, MM<sub>EcV253</sub> and MM<sub>EcV503</sub>. MM<sub>EcV753</sub> showed the lowest biomass production, but glucose consumption was consistently low (9.7 g L<sup>-1</sup> of residual glucose) and resulting biomass yield did not differ from those in other treatments (Table 5).

Table 5. Biomass and L-threonine yields from glucose by *E. coli* THR14

Amino acids are primary bioproducts, meaning their biosynthesis is growth related. For that reason, biomass yields are fundamentally important for amino acid bioprocesses (Li et al., 2017; Hermann, 2003).

*E. coli* THR14 cellular growth was limited by low oxygen transfer and the batch operation mode but CDW concentrations were consistent with those described by previous studies. Chen et al. (2009) cultivated an *E. coli* L-threonine producing strain. After 48 h incubation the authors reported biomass concentration of 7.5 g L<sup>-1</sup>.
In this study, biomass yields were consistent with those previously reported on *E. coli* yield on glucose (0.5 g$_{\text{biomass}}$ g$_{\text{glucose}}^{-1}$) (Shiloach & Fas, 2005), and no statistical difference was found among treatments. Furthermore, MM$_{\text{Ec}}$V253 and MM$_{\text{Ec}}$V503 treatments had significantly lower acetic acid production than control, which made these vinasse-based treatments interesting for *E. coli* THR14 cultivation.

In bioprocesses with *E. coli* strains using glucose as carbon source, acetic acid synthesis may lead to longer processes and consequently lower productivities. Glucose is assimilated and catabolized into acetyl-CoA. Conditions such as oxygen limitation and glucose concentration above saturation constant may shift acetyl-coa flux from tricarboxylic acid cycle towards ethanol, lactate and/or acetate fermentation. These pathways are advantageous because they contribute for ATP balance and once favorable conditions are restored, acetic acid might be re-assimilated whether glucose becomes limiting (Soini, Ukkonen & Neubauer 2008).

There is a large concern about acetate production in bioprocesses by *E. coli* strains. In our study, acetic acid production was expected and the final acetic acid concentration of 2.3 ± 0.8 g L$^{-1}$ in MM$_{\text{Ec}}$ was acceptable, meaning a production of 0.3 g$_{\text{acetate}}$ g$_{\text{biomass}}^{-1}$. Ying et al. (2014) described 0.95 g$_{\text{acetate}}$ g$_{\text{biomass}}^{-1}$ (biomass concentration was 7.1 g L$^{-1}$) in fed-batch bioprocesses. The final acetic acid concentrations in vinasse based treatments MM$_{\text{Ec}}$V253 (0.6 ± 0.2 g L$^{-1}$) and MM$_{\text{Ec}}$V503 (0.5 ± 0.2 g L$^{-1}$) were, respectively, 75% and 79% lower than that in control treatment. These results indicate that those treatments provided balanced nutritional conditions for *E. coli* THR14 metabolism.

As for MM$_{\text{Ec}}$V753 treatment, results indicate that low efficiency was most likely due to nutritional limitation rather than inhibition. As stated in Material and Methods section, our experiments aimed to supply all macronutrients that are known to limit cellular growth. However, micronutrients from the mineral medium were diluted by vinasse.

Since biomass yield in MM$_{\text{Ec}}$V753 treatment was satisfactory, it is possible that as long as cells had favorable nutritional conditions for growth, they were able to consume glucose for biomass production. Once the culture reached some possible nutritional limitation, cellular growth ceased and L-threonine was not favored. Acetic acid concentration in MM$_{\text{Ec}}$V753 supports the hypothesis of limitation rather than inhibition. In MM$_{\text{Ec}}$V753 treatment, glucose uptake by *E. coli* THR14 did
occur and acetyl-CoA was synthesized and driven to acetic acid pathway. The metabolic switch from cellular growth to acetic acid synthesis might have occurred once nutritional conditions started to limit biomass synthesis due to lacking (micro) nutrients, so available acetyl-CoA was driven towards acetic acid pathway.

In this study, L-threonine production was below some results described by other authors. In literature, *E. coli* producing strains have been reported to achieve production yields ranging from 0.33 to 0.57 g\textsubscript{L-threonine \text{g}^{-1}}\textsubscript{GLUCOSE}, and final concentrations between 9.7 and 17 g L\textsuperscript{-1} in shake flasks (Wang et al., 2019; Zhao et al., 2018).

Ours results indicate that much can improved in the L-threonine-producing strain *E. coli* THR14.

Previous studies have described L-threonine production by *E. coli* strains around 34.06 - 42.25 mg L\textsuperscript{-1} and further approaches in order to improve production rates required multiple strategies (Lee et al., 2009).

This study, however, aimed to investigate the potential of using vinasse for L-threonine production. To our knowledge, there is no available study about amino acids production using vinasse-based culture media, or *E. coli* strains cultivation in sugarcane vinasse aiming to bioproducts synthesis.

Suhailli et al. (2018) used vinasse from sugar beet processes to evaluate an *E. coli* BL21 strain. The authors evaluated cellular growth and biosynthesis of CV2025 ω-transaminase, an enzyme that is widely used in pharmaceutical industry. Ethanol process from sugar beet molasse yielded a very different composition of vinasse (glycerol, 183.6 - 187.6 g L\textsuperscript{-1}; acetic acid, 1.7 - 7.2 g L\textsuperscript{-1}; residual sugars, 20 g L\textsuperscript{-1}). The authors evaluated volumetric dilutions of vinasse and results of both CDW concentration and enzyme specific activity were comparable to those obtained in control treatment (without vinasse addition). Finally, authors reported sugar beet vinasse as a fit feedstock for *E. coli* BL21 cultivation and its potential for CV2025 ω-transaminase production was very attractive.

In L-threonine production by *E. coli* THR14, vinasse was a source of water and mineral salts, such as the ionic forms of calcium, sodium and potassium salts. Besides, vinasse contributed with low amounts of amino acids that might have been assimilated either as nitrogen sources or building blocks.
No previous literature was found about vinasse composition of amino acids. Most likely, the main source of amino acids in vinasse are yeast cells that lyse along the fermentation process, releasing cellular components in culture medium. L-threonine is a L-aspartate derived product and interestingly, in our analyses L-aspartate was the most important amino acid in vinasse (Table 1). By the end of processes by \textit{E. coli} THR14, L-aspartate concentrations had been depleted in all treatments.

As for glycerol, \textit{E. coli} MG1655 strains are capable of using it as carbon source. However, in the presence of highly available glucose, glycerol was neglected by \textit{E. coli} THR14, similarly to results reported by Suhaili et al. (2018).

\textit{PHB production in vinasse-based culture media by \textit{Halomonas} sp. HG03 and \textit{R. eutropha} L359PCJ}

Increasing volumetric dilutions of both \textit{in natura} and concentrated vinasse had a positive effect on bacteria cellular growth.

In \textit{Halomonas} sp. HG03 cultivations, rDW concentration in MM\textsubscript{HaV503} (1.1 ± 0.2 g L\textsuperscript{-1}) did not differ from that in control treatment (MM\textsubscript{Ha}) (0.74 ± 0.1 g L\textsuperscript{-1}). By increasing vinasse in culture media, in both treatments MM\textsubscript{HaV753} and MM\textsubscript{HaV507}, rCDW concentration increased significantly by three fold, with final values of 2.2 ± 0.1 g L\textsuperscript{-1} and 2.3 ± 0.4 g L\textsuperscript{-1}, respectively. In MM\textsubscript{HaV757} a further significant increase was achieved and residual biomass concentration improved by four fold (3.0 ± 0.6 g L\textsuperscript{-1}) (Figure 3).

\textit{Figure 3. Halomonas} sp. HG03 cellular growth and PHB biosynthesis in mineral medium, \textit{in natura} and concentrated vinasse-based treatments, after 48 h incubation.

In \textit{R. eutropha} L359PCJ cultivations, all vinasse-based treatments showed a significant increase in cellular growth. \textit{In natura} vinasse-based treatments MM\textsubscript{ReV503} and MM\textsubscript{ReV753} had rCDW
concentrations of $2.2 \pm 0.3 \, \text{g L}^{-1}$ and $2.3 \pm 0.1 \, \text{g L}^{-1}$, respectively, which were statistically different from control treatment ($1.1 \pm 0.2 \, \text{g L}^{-1}$) (Figure 4).

Figure 4. *R. eutropha* L359PCJ cellular growth and PHB biosynthesis in mineral medium, *in natura* and concentrated vinasse-based treatments, after 48 h incubation.

The use of concentrated vinasse in MM$_{ReV507}$ treatment did not contribute significantly to increase rCDW ($2.7 \pm 0.4 \, \text{g L}^{-1}$) in comparison to *in natura* vinasse treatments. MM$_{ReV757}$, however, resulted in the statistically highest concentration of rCDW, $4.1 \pm 0.2 \, \text{g L}^{-1}$.

Quantification of residual ammonium showed that no detectable concentration was found in any treatment. Both *Halomonas* sp. HG03 and *R. eutropha* L359PCJ depleted the main nitrogen source in control and vinasse-based treatments, so unbalanced nutritional conditions for PHB accumulation were met.

PHB cellular content decreased as vinasse concentration in culture media increased.

In *Halomonas* sp. HG03 cultures, the highest PHB cellular content, $81.7 \pm 3.8\%$ of CDW, and final PHB concentration of $3.45 \pm 0.3 \, \text{g L}^{-1}$ were determined in control treatment MM$_{Ha}$- MM$_{HaV503}$ treatment showed the best PHB production by *Halomonas* sp. HG03 among vinasse-based treatments: PHB content ($75.2 \pm 2.7\%$ of CDW) and final PHB concentration ($3.5 \pm 0.4 \, \text{g L}^{-1}$) were comparable to those in control treatment. PHB production decreased significantly in MM$_{HaV753}$: cellular content dropped to $56.3 \pm 3.1\%$ of CDW and PHB final concentration of $2.9 \pm 0.3 \, \text{g L}^{-1}$.

In *R. eutropha* L359PCJ cultivations, PHB cellular content in control treatment MM$_{Re}$ ($84.1 \pm 3.6\%$) was significantly higher than both volumetric dilutions of *in natura* vinasse MM$_{ReV503}$ ($68.5 \pm 4.9\%$) and MM$_{ReV753}$ ($65.1 \pm 2.3\%$). Consequently, PHB final concentration in MM$_{ReV503}$ ($4.8 \pm 0.6 \, \text{g L}^{-1}$) dropped 21% in comparison to MM$_{Re}$ ($5.8 \pm 0.3 \, \text{g L}^{-1}$), and MM$_{ReV753}$ ($4.4 \pm 0.4 \, \text{g L}^{-1}$), 33%.

The use of concentrated vinasse resulted in further decrease of PHB cellular content. *Halomonas* sp. HG03 had $50.7 \pm 6.9\%$ PBH cellular content in MM$_{HaV507}$ treatment and final PHB
concentration (2.3 ± 0.2 g L\(^{-1}\)) dropped significantly. MM\(_{Ha}\)V757 had PHB content as low as 19.6 ± 3.7% and its PHB final concentration was statically the lowest among \textit{Halomonas} sp. HG03 treatments (0.8 ± 0.3 g L\(^{-1}\)).

Our results for PHB cellular content in treatments MM\(_{Ha}\), MM\(_{Ha}\)V503, MM\(_{Ha}\)V753 and MM\(_{Ha}\)V507 were consistent to those reported by other authors for \textit{Halomonas} sp. strains. According to literature, different culture conditions and carbon sources might result in PHB cellular contents between 44 - 90% by \textit{Halomonas} sp. PHA-producing strains (Kucera et al., 2018; Quillaguamán et al., 2007; Rivera-Terceros et al., 2015).

Other authors have cultivated \textit{Halomonas} sp. strains in shake flasks with sucrose as carbon source. PHB contents between 44 – 75% and rCDW concentrations between 1.1 – 1.6 g L\(^{-1}\) have been described (Kucera et al., 2018; Rathi et al., 2012; Stanley et al., 2017). In our study, in \textit{natura} and concentrated vinasse-based treatments (MM\(_{Ha}\)V503, MM\(_{Ha}\)V753 and MM\(_{Ha}\)V507) also had PHB content comparable to those found in literature, but residual biomass values were notably higher than those reported elsewhere.

Similarly, PHB cellular content in \textit{R. eutropha} L359PCJ dropped significantly in concentrated vinasse treatments in comparison to control. In MM\(_{Re}\)V507, PHB cellular content (60.8 ± 4.0%) differed from control treatment and MM\(_{Re}\)V503, but it was comparable to that in MM\(_{Re}\)V753. The same was observed for its final PHB concentration (4.2 ± 0.3 g L\(^{-1}\)). In MM\(_{Re}\)V757, PHB cellular content (48.4 ± 1.3%) was statistically the lowest among \textit{R. eutropha} L359PCJ treatments. However, higher rCDW concentration in MM\(_{Re}\)V757 contributed to its final PHB concentration (3.9 ± 0.3 g L\(^{-1}\)), which differed only from control treatment and MM\(_{Re}\)V503.

In shake flasks cultures, using glycerol as carbon source, \textit{R. eutropha} strains were described by other authors with residual biomass production between 0.66 - 2.2 g L\(^{-1}\), and PHB cellular content from 57% to 65% (Fukui et al., 2014; Cavalheiro et al., 2009; Volova et al., 2018). Similarly to our results with \textit{Halomonas} sp. HG03, increasing vinasse in culture media improved \textit{R. eutropha} L359PCJ cellular growth and rCDW concentrations were higher than those reported by other authors.
Since PHB biosynthesis is not growth related, as substrate conversion into biomass increased, substrate conversion into product decreased. Table 6 presents conversion yields of sucrose into rCDW and PHB by *Halomonas* sp. HG03.

**Table 6. Biomass and PHB yields from sucrose by *Halomonas* sp. HG03.**

*MM*<sub>Ha</sub>V753 treatment had the best PHB production among vinasse-based treatments with *Halomonas* sp. HG03. Biomass and PHB yields in both treatments *MM*<sub>Ha</sub>V753 and *MM*<sub>Ha</sub>V507 were interestingly balanced, indicating that such conditions have good potential for high cell density cultures for PHB production. *MM*<sub>Ha</sub>V757, on the other hand, did not show a satisfactory PHB yield, which dropped 72% in comparison to control treatment.

Table 7 presents conversion yields of glycerol into PHB and biomass (rCDW) by *R. eutropha* L359PCJ. Biomass yield in *MM*<sub>Re</sub>V503 treatment was over twice higher than that in control treatment *MM*<sub>Re</sub>. As vinasse concentration in culture media increased, biomass yields increased as well, with significant difference in treatments that used concentrated vinasse. Biomass yield in *MM*<sub>Re</sub>V757 was over four times higher than biomass yield in control treatment.

In *MM*<sub>Re</sub>V757 product yield dropped 34.5% when compared to control treatment. Lower vinasse concentrations in *MM*<sub>Re</sub>V503, *MM*<sub>Re</sub>V753 and *MM*<sub>Re</sub>V507 treatments decreased product yields in 11.4 - 18.2%.

Even though PHB biosynthesis is not growth related, high cellular densities are very important for large volumetric production. Considering vinasse-based treatments, it was particularly interesting that *in natura* vinasse and concentrated vinasse at 50% (v v<sup>-1</sup>) improved biomass production for both *Halomonas* sp. HG03 and *R. eutropha* L359PCJ cultivations, while keeping PHB cellular content competitive with those obtained in mineral media, as well as those reported by other authors.

Concerning treatments with 75% (v v<sup>-1</sup>) of concentrated vinasse, the unsatisfactory results for PHB production suggest that nutritional conditions favored cellular growth and they were not
unbalanced enough for PHB accumulation, instead, these treatments provided the most efficient cellular growth conditions in our study.

Similarly to L-threonine production by *E. coli* THR14, processes with *Halomonas* sp. HG03 exploited vinasse mostly as a source of water, salts, residual amino acids and an external carbon source had to be supplemented.

Other authors have investigated the combined use of vinasses and molasses in culture media for polyhydroxyalkanoates production. Paula et al. (2021) cultivated *Burkholderia glumae* MA13 in shake flasks with basal mineral medium; they evaluated volumetric dilutions of 25% and 50% of *in natura* vinasse and sugarcane molasse was used as carbon source (20 g L⁻¹). PHA cellular content was between 14.07 - 29.4% and rCDW concentrations between 0.52 - 1.31 g L⁻¹ were determined. Acosta-Cárdenas, Alcaraz-Zapata & Cardona-Betancur (2018) cultivated *R. eutropha* ATCC17699 in culture medium with a volumetric ratio of sugarcane molasse/vinasse of 25/75. They determined final PHA concentrations between 1.9 - 3.8 g L⁻¹ and product yields ranged from 0.13 - 0.21 g g⁻¹.

Cheaper carbon sources are a major interest in large scale bioprocesses in general. Biotechnological processes might have carbon sources accounting for up to 50% of total operational costs (Eggeling & Bott, 2015; Poltronieri & Kumar, 2017). Zanfonatto et al. (2018), Bhattacharyya et al. (2012) and Pramanik et al. (2012) have investigated PHAs production by *Cupriavidus necator* DSM545, *Haloferax mediterranei* and *Haloarcula marismortuii* MTCC 1596, respectively, by using vinasse as carbon source.

Bhattacharyya et al. (2012) used mineral medium with 50% (v v⁻¹) vinasse and described the production of 17.4 g L⁻¹ of P(3HB-co-HV) (14.09 mol % HV) after 120 h cultivation. Pramanik et al. (2012) cultivated *Haloarcula marismortuii* MTCC1596 in culture medium composed of 100% vinasse and total biomass concentration was 15 g L⁻¹, with 30% of PHB, after 216 h cultivation. Zanfonatto et al. (2018) used *in natura* vinasse as glycerol source (4.5 g L⁻¹) for cultivating *C. necator* DSM545. Final concentrations of rCDW and PHB were, respectively, 3.7 g L⁻¹ and 1.3 g L⁻¹ (PHB cellular content of 26%).

In our study we also had interest on vinasse as carbon source, so the glycerol-consuming *R. eutropha* L359PCJ was cultivated. As vinasse concentration in culture media increased, demand
for external glycerol supplementation decreased, and MMReV757 finally had glycerol fully provided by concentrated vinasse (15 g L\(^{-1}\)).

Despite lower PHB production by \(R.\) eutropha L359PCJ in MMReV757 treatment, it is important to emphasize that such process had zero cost with carbon source acquisition. MMReV507 was particularly interest because PHB production was competitive with those in our control treatment and those reported by previous studies. Besides, costs with carbon source were reduced in half, since vinasse supplied 7.5 g L\(^{-1}\) of glycerol. To our knowledge, no previous study described the use of concentrated vinasse as carbon source for PHB bioprocesses.

**Bioreactor fermentations**

Because *Halomonas* sp. HG03 and *R. eutropha* L359PCJ had satisfactory cellular growth and product biosynthesis in vinasse-based treatments, these strains were chosen for further investigation of vinasse as basal medium in bioreactor bioprocesses.

Both bacteria showed satisfactory cellular growth in bioreactor cultivations. *R. eutropha* L359PCJ had a lag phase of 13 h, while lag phase in *Halomonas* sp. HG03 cultivation lasted 37 h, which suggests that inoculum adaptation to concentrated vinasse-based medium could be necessary, especially for *Halomonas* sp. HG03.

*R. eutropha* L359PCJ showed consistent cellular growth for 74 h, according to optical density monitoring during cultivation (OD\(_{600}\)). Maximum growth velocity \(\mu_{\text{max}} = 0.255\) h\(^{-1}\) was observed between \(t = 0\) h and \(t = 7.15\) h and exponential growth phase was observed until \(t = 30\) h. At \(t = 55\) h the culture reached stationary growth phase.

At \(t = 26\) h the second batch was initiated, which supplied all nutrients from culture medium and at \(t = 32\) h the batch was finished. At \(t = 34\) h the third batch supplied pure glycerol and concentrated vinasse (34.4ºBrix) as the sole nutrients source, which initiated the PHB accumulation phase. In total, seven batches were carried on with concentrated vinasse, including the initial batch (Figure 5).
Figure 5. Cellular growth by *R. eutropha* L359PCJ in bioprocess operated by consecutive batches mode in bioreactor with concentrated vinasse-based medium. (•-•): start of the second batch. (---): start of third to seventh batches.

*Halomonas* sp. HG03 was cultivated for 67 h, with increasing optical density (OD$_{600}$) until t = 53 h. Maximum growth velocity was $\mu_{\text{max}} = 0.091$ h$^{-1}$, observed between t = 20 h and t = 26.7 h. The culture reached stationary growth phase at t = 44 h (Figure 6).

Figure 6. Cellular growth by *Halomonas* sp. HG03 in bioprocess operated by consecutive batches mode in bioreactor with concentrated vinasse-based medium. (•-•): start of the second batch. (---): start of third to sixth batches.

At t = 20 h the second batch providing all nutrients to *Halomonas* sp. HG03 was initiated. At t = 26 h the third batch initiated the cultivation providing sucrose and concentrated vinasse as the sole nutrients source. Despite the fact that after t = 20 h no specific nitrogen source was supplied ([(NH$_4$)$_2$SO$_4$], *Halomonas* sp. HG03 showed increasing concentration of rCDW until t = 44 h, which suggests that amino acids from vinasse might have been used as building blocks and enabled *Halomonas* sp. HG03 to grow. In total, *Halomonas* sp. HG03 cultivation had six batches, including the initial batch.

By using vinasse as a partial source of glycerol, *R. eutropha* L359PCJ showed maximum specific growth velocity ($\mu_{\text{max}}$) higher than those previously reported by other authors that cultivated *R. eutropha* strains with residual sources of glycerol. Cavalheiro et al. (2009) and Volova et al. (2019) described $\mu_{\text{max}}$ values between 0.11 and 0.15 h$^{-1}$ for cultivations with crude glycerol as carbon source. Zanfonatto et al. (2018) evaluated the PHB production by *Cupriavidus necator* (also known as *R. eutropha*) DSM 545 with *in natura* vinasse as glycerol source (4.5 g L$^{-1}$), and $\mu_{\text{max}} = 0.21$ h$^{-1}$ was determined.
Halomonas sp. HG03 maximum specific growth velocity was not satisfactory, which might have been negatively affected by the prolonged lag phase. Moreno (2015) had previously cultivated the parental strain Halomonas sp. HG01 in glucose and described $\mu_{\text{max}} = 0.16 \, \text{h}^{-1}$.

Both bioprocesses were thoroughly monitored by their process conditions. In R. eutropha L359PCJ cultivation, nitrogen source as in the form of NH$_4^+$ had been depleted at $t = 34$ h. Glycerol was consumed by bacteria and dissolved oxygen was efficiently kept at a saturation value around 40% as well. In order to keep the established oxygen saturation, agitation speed and air inflow were highly responsive to batches durations, which are represented in Figure 7 by glycerol content (g L$^{-1}$).

Figure 7. R. eutropha L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: monitoring of agitation speed, dissolved oxygen, aeration, glycerol and ammonium concentrations.

Agitation speed was gradually increasing during first and second batches. From the third batch on, which had only pure glycerol and vinasse as the sole nutrients source, air inflow was also increasing so the minimum saturation of dissolved oxygen could be kept. Altogether, these data suggested that R. eutropha L359PCJ could efficiently consume glycerol in order to have cellular growth and PHB biosynthesis in concentrated vinasse-based medium throughout the entire process and no inhibitory effects were observed.

In Halomonas sp. HG03 cultivation, despite the increasing concentration of rCDW until $t = 44$ h (Figure 6), nitrogen as in the form of NH$_4^+$ had been depleted in culture medium at $t = 33$ h. Sucrose was satisfactorily consumed until $t = 48$ h, but sucrose accumulation was observed later. Still, it was expected that glycerol would not be significantly consumed by Halomonas sp. HG03 (Figure 8).
Saturation of dissolved oxygen was efficiently kept around 40% during the entire process, although agitation speed and air inflow responses were remarkably different from those observed in *R. eutropha* L359PCJ cultivation. Agitation speed was increasing during first and second batches, in which all nutrients from culture medium were provided. From the third batch on, agitation speed showed short responses, associated to batches initiation. Air inflow, on its turn, was mostly constant during the process. These data suggest that microbial respiration was relatively low and short periods of time with increasing agitation could easily keep dissolved oxygen around 40%.

Since the third to sixth batches had concentrated vinasse and sucrose as the sole nutrients source, it is unlikely that sucrose concentrations were related to the little increase in microbial respiration at the beginning of batches because total sugars eventually accumulated in bioreactor. On the other hand, vinasse partially replaced minerals in all vinasse-based treatments we evaluated in this study. Thus, it is possible that concentrated vinasse carried limiting nutrients for *Halomonas* sp. HG03: once batches were initiated with sucrose and concentrated vinasse supply, microbial respiration increased and higher agitation speed was needed in order to keep dissolved saturation around 40%. Possibly, once mineral nutrients from vinasse reached limiting concentrations, microbial respiration decreased, as well agitation speed, and sucrose was not efficiently metabolized. Further studies with detailed investigation on *Halomonas* sp. HG03 nutritional requirements are necessary, but such hypothesis would be consistent with the results we obtained in shake flasks cultivations that showed increasing concentrations of vinasse in culture media being related to increasing cellular growth.

Regarding PHB content, *R. eutropha* L359PCJ showed little PHB accumulation during the first two batches, which supplied nitrogen source (\((\text{NH}_4)_2\text{SO}_4\)) and other nutrients from mineral medium. By the end of the second batch, at \(t = 32\) h, PHB content reached 21.4% (\(\text{mm}^{-1}\)). Previous studies also reported PHB content around 20% by *R. eutropha* strains at the end of
growth phases (Kim et al., 1994). Finally, at t = 74 h, *R. eutropha* L359PCJ reached PHB content of 66.3% (m m\(^{-1}\)) and CDW concentration was 28.0 g L\(^{-1}\) (Figure 9).

**Figure 9.** *R. eutropha* L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: volume variation, PHB content, CDW, rCDW, consumed glycerol and PHB concentrations.

Cavalheiro et al. (2009) obtained PHB contents between 38 – 62% by cultivating *C. necator* DSM 545 in bioreactors with crude glycerol as carbon source. Bormann & Roth (1999) cultivated *R. eutropha* DSM 11348 with pure glycerol and PHB content of 42 – 65% was reported. As for in natura vinasse as glycerol source, Zanfonatto et al. (2018) described PHB content of 26% by *C. necator* DSM 545.

By the end of the growing phase (t = 32 h), conversion yield of glycerol into rCDW was \(Y_{X/S} = 0.52\) g g\(^{-1}\), and into PHB, \(Y_{P/S} = 0.14\) g g\(^{-1}\). At the end of the seventh batch the global yields were \(Y_{X/S} = 0.16\) g g\(^{-1}\), \(Y_{P/S} = 0.32\) g g\(^{-1}\) and the final concentration of 18.6 g L\(^{-1}\) PHB was reached after 74 h, which resulted into PHB productivity of 0.25 g Lh\(^{-1}\).

Shake flasks cultivations of *R. eutropha* L359PCJ had PHB content of 60.6 ± 4.0% and global conversion yields were \(Y_{X/S} = 0.23 ± 0.05\) g g\(^{-1}\) and \(Y_{P/S} = 0.34 ± 0.03\) g g\(^{-1}\) (MM\(_{Rev} V507\) treatment).

Thus, the PHB production by *R. eutropha* L359PCJ with concentrated vinasse-based medium in bioreactor had conversion yields consistent with those determined in shake flasks fermentations and PHB content satisfactorily improved.

The production results were also consistent with those described by other authors with glycerol as carbon source, which supports the use of concentrated vinasse as glycerol source for PHB biosynthesis. Sharma et al. (2021) described values between 2.1 – 11.3 g L\(^{-1}\) of CDW and 6.7 – 13.1 g L\(^{-1}\) of PHB in bioprocesses with *C. necator* DSM 545 and pure glycerol as carbon source. Productivities ranged from 0.16 to 0.27 g Lh\(^{-1}\). Kachrimanidou et al. (2014) reported productivity of 0.31 g Lh\(^{-1}\), concentrations of CDW = 24.6 g L\(^{-1}\) and PHB = 7.6 g L\(^{-1}\), with conversion yield \(Y_{P/S} = 0.30\) g g\(^{-1}\) by *C. necator* DSM 7237 with purified crude glycerol as carbon source. Cavalheiro et
al. (2009) described a fed-batch bioprocess with *C. necator* DSM 545 and crude glycerol was evaluated as carbon source. The authors obtained a PHB productivity of 0.84 g L\(^{-1}\)h\(^{-1}\) in a high cell density culture, with final rCDW concentration of 42.8 g L\(^{-1}\) and PHB content of 38%, which resulted in PHB concentration of 26 g L\(^{-1}\). Conversion yields were \(Y_{XS} = 0.45\) g g\(^{-1}\) and \(Y_{PS} = 0.34\) g g\(^{-1}\).

PHB content during growth phase in *Halomonas* sp. HG03 cultivation was more important than that observed for *R. eutropha* L359PCJ. During the first batch, at \(t = 17.7\) h, PHB content was determined at 28.5%, and it showed little variation until the end of the second batch at \(t = 26\) h, with 28.8%. By the end of the sixth batch, at \(t = 67\) h, PHB content finally reached 45.7% and CDW concentration was 18.1 g L\(^{-1}\), meaning a final concentration of PHB = 8.3 g L\(^{-1}\). Globally, conversion yields of sucrose into PHB and rCDW were, respectively, \(Y_{PS} = 0.16\) g g\(^{-1}\) and \(Y_{XS} = 0.18\) g g\(^{-1}\), and PHB productivity was 0.12 g Lh\(^{-1}\) (Figure 10).

**Figure 10.** *Halomonas* sp. HG03 cultivation in bioreactor with concentrated vinasse-based culture medium: volume variation, PHB content, CDW, rCDW, consumed total sugars and PHB concentrations.

In comparison to results obtained in shake flasks (MM\(_{Ha}\)V507 treatment), the consecutive batches operation mode was not successful in achieving higher PHB content by *Halomonas* sp. HG03, which had been 50.7 ± 6.9%. As mentioned above, further investigation on *Halomonas* sp. HG03 nutritional requirement is needed and such information could be valuable for improving bioprocess strategies for PHB production in bioreactors with concentrated-vinasse medium.

PHB production by *Halomonas* sp. HG03 indeed was less attractive than that showed by *R. eutropha* L359PCJ, however, it was still consistent with results reported by other authors for *Halomonas* sp. strains cultivated in bioreactors.

Ortiz-Veizán et al. (2020) reported PHB content of 52% by *Halomonas boliviensis* DSM 15516 in bioprocess with glucose (5 g L\(^{-1}\)) and molasse (15 g L\(^{-1}\)) as carbon sources. Quillaguamán et al. (2006) described a batch process in bioreactor with *Halomonas boliviensis* LC1\(^{T}\) with sucrose as
carbon source. PHB content was 55% and final concentrations were rCDW = 0.54 g L\(^{-1}\) and PHB = 0.66 g L\(^{-1}\). In further studies, Quillagúamán et al. (2007) described PHB production of \(H. \ boliviensis\) LC1\(^{T}\) with sucrose in bioreactors and productivities were between 0.14 – 0.16 g Lh\(^{-1}\), PHB content was 52% and final concentrations were rCDW = 6.9 g L\(^{-1}\) and PHB = 7.5 g L\(^{-1}\).

Other authors have also reported relatively important PHB production by \(Halomonas\) sp. strains during growth phases. Besides, in bioprocesses that used complex media, such as hydrolysates, the authors have also described that rCDW continued to increase despite the depletion of the main nitrogen source (Rivera-Terceros et al., 2015). Quillagúamán et al. (2008) have demonstrated that amino acids such as L-aspartate, L-glutamate and L-glycine have, individually, an important effect on \(Halomonas \ boliviensis\) cellular growth and PHB biosynthesis.

\(Halomonas\) sp. strains are highly attractive for bioprocesses that aim to biocommodities manufacturing, especially because they grow in cheap culture media and non-sterile conditions. For this reason an important set of strains and bioprocesses have been studied in recent years (Kshirsagat et al., 2012; Quillagúamán et al., 2005; Van-Thuoc et al., 2007; Yue et al., 2014).

As strategies that aimed to improve PHB production by \(Halomonas\) sp. strains, limitation by oxygen and controlled supply of inorganic and/or complex nitrogen sources have been previously evaluated by other authors and could be as well applied in bioprocesses with vinasse-based media (Kshirsagat et al., 2012; Quillagúamán et al., 2007; Quillagúamán et al., 2008).

**In silico study of PHB production process by \(R. \ eutropha\) L359PCJ with concentrated vinasse-based culture medium**

The model process considered a seed fermenter with reaction described reaction R1 (Table 3) and the glycerol conversion yields determined experimentally during growth phase were used \(Y_{X/S} = 0.52 \text{ g g}^{-1}\), \(Y_{P/S} = 0.14 \text{ g g}^{-1}\).

Settings in the production bioreactor considered initial biomass (rCDW) of 0.3 g L\(^{-1}\); PHB accumulation phase would require the increase from 21.4% PHB up to 66.3% (m m\(^{-1}\)); and glycerol conversion yields applied in R2 (Table 3) were \(Y_{X/S} = 0.16 \text{ g g}^{-1}\) and \(Y_{P/S} = 0.32 \text{ g g}^{-1}\), as obtained experimentally. In order to determine the time needed for growth phase and PHB
accumulation phase, the experimental data $\mu_{\text{max}} = 0.255 \, \text{h}^{-1}$ and $\mu_P = 0.05 \, \text{g gh}^{-1}$ ($t = 57 \, \text{h}$) were used. These assumptions about growth phase and PHB accumulation phase were considered during calculations of simulation parameters, however, settings in SuperPro Designer treated these parameters globally, as detailed in Table 8.

Table 8. Simulation parameters in base scenario SC1 – input, output data and settings in SuperPro Designer model.

The model had batch time of 78.3 h and the reaction in Bioprocess was the bottleneck procedure, which had duration of 52.3 h, including bioreactor chargings and reaction times. The bioreactor occupancy time is directly affected by PHB productivity, so improving productivity would reduce the minimum cycle time and optimize equipment usage (Figure 11).

Figure 11. Gantt chart of base scenario SC1: Unit procedure (P-)/ equipment – operation. (✶) Bottleneck unit procedure. (✷) Operations in bottleneck unit procedure.

Leong et al. (2017) also analyzed in silico the PHB production process with pure glycerol as the carbon source. The authors did not specifically relate the study to experimental data and the simulated process was described by PHB productivity of $2.86 \, \text{g Lh}^{-1}$. Some simulation parameters used by Leong et al. (2017) are compared to those applied in this study, as detailed in Table 9.

Table 9. Simulation parameters and output data – Base scenario SC1 (this study) and Leong et al. (2017).

Experimentally, using pure and crude glycerol from biodiesel process, high cell density cultures were described by Cavalheiro et al. (2009) ($r_{\text{CDW}} = 31 \, \text{g L}^{-1}$ and $r_{\text{CDW}} = 42.8 \, \text{g L}^{-1}$, respectively).
The authors achieved maximum PHB content of 62%, which resulted in PHB titer of 51.1 g L\(^{-1}\) and productivities ranged from 0.84 to 1.5 g Lh\(^{-1}\).

In comparison to the study described by Leong et al. (2009), the simulated productivity we calculated in this study, P\(_{pSi}\) = 1.1 g Lh\(^{-1}\) was low, although comparable to those experimentally described by Cavalheiro et al. (2009) that used glycerol from agroindustrial wastes as carbon source.

In regards to facility use in this study, putting as an objective the integration of PHB and bioethanol bioprocesses through vinasse imposed an additional challenge. A shorter annual campaign was considered (5760 operating hours) so PHB process would be synchronized to sugarcane harvesting period in Brazil. Consequently, facility use becomes more important, inefficient or sub-optimal use could be limiting and add to production costs.

Because PHB productivity simulated by Leong et al. (2009) was higher than P\(_{pSi}\) we calculated in this study, the PHB production per batch (ton batch\(^{-1}\)) was lower and the number of batches per year was larger. The authors described an effective and optimized use of facility, especially regarding bioreactors occupancy. Due to higher productivity, bioprocess duration is shorter and less production units are needed to achieve an annual demand of PHB production. As a result, a smaller number of bioreactor units operate in a larger number of batches during the annual campaign.

As detailed in Table 9, scenario SC1 had lower production costs related to raw materials (37.4%). However, facility-dependent production costs and investments were very important (56.9%) and remarkably disadvantageous in comparison to simulated results by Leong et al. (2017). As a consequence, despite lower costs with raw material, unit production cost in SC1 (US$ 6.2 kg\(^{PHB-1}\)) was not more competitive than that described by Leong et al. (2017) (US$ 6.1 kg\(^{PHB-1}\)) (US$ 500 ton\(^{-1}\)). Moreover, SC1 had higher investments on direct fixed capital, which made financial indicators ROI, IRR, NPV and payback less attractive.

Production costs were also analyzed by process section. The Upstream section, which included culture medium and inoculum preparation, had the most important share in costs, 39.5%. Next, Bioprocess accounted for 37.6%, which included mostly the operation of FR-101 units. Lastly, the Downstream section accounted for 22.9% of total production costs. These results implied that
optimizing bioreactor use was imperative for reduction of production costs, but cheaper culture medium were also needed.

Firstly, different prices of glycerol were evaluated, as well as the use of vinasse as a partial source of nutrients for culture medium. As expected, higher costs with glycerol resulted in higher production costs in general. Scenarios that did not consider the use of vinasse (SC1, SC2 and SC3) had annual production costs reduced by 6% due to the lower glycerol prices. In scenarios SC4, SC5 and SC6, the higher the glycerol price, more important were production costs due to vinasse use as nutrients source. Both scenarios SC1 and SC4 had glycerol price at US$ 400 ton⁻¹, and costs in SC4 were 13.1% lower. As for SC2 and SC5 (glycerol at US$ 300 ton⁻¹), SC5 had production costs reduced by 10.8%; between SC3 and SC6 (glycerol at US$ 200 ton⁻¹), costs in SC6 were 8.2% lower (Figure 12).

These results suggest that economical scenarios with more expensive carbon source, or possible increase of prices, could have lower impact on production costs by using vinasse as a partial source of carbon source and making operation less vulnerable to such external factors.

**Figure 12. Unit production costs and annual production costs discriminated by raw materials, facility and others in scenarios with mineral medium (SC1, SC2, SC3) and scenarios with concentrated vinasse as partial source of nutrients in culture medium (SC4, SC5, SC6).**

Vinasse was also a partial source of mineral components in culture medium. Considering final costs with glycerol in culture medium, scenario SC3 had the same costs with external carbon source as SC4 because the later used vinasse as a partial source of carbon source. However, due to reduced costs with mineral components for medium, total production costs in SC4 were 2%, or US$ 972,825.00 year⁻¹, lower than those in SC3. That was about the annual cost with utilities (US$ 951,802.00 year⁻¹).

The decrease of glycerol price from US$ 400 ton⁻¹ (SC1) to US$ 200 ton⁻¹ (SC3) reduced unit production cost from US$ 6.2 kg_{PHB}⁻¹ to US$ 5.5 kg_{PHB}⁻¹. Scenarios that combined decrease of
glycerol price with the use of vinasse had unit production costs of US$ 5.4 kg_{PHB}^{-1} \text{ SC4, US$ 400 ton}^{-1}) \text{ and US$ 5.1 kg}_{PHB}^{-1} \text{ SC6, US$ 200 ton}^{-1}).

Despite reduction of production costs by decreasing costs with culture medium, facility-dependent costs were significant and invariable (Figure 12). So in order to decrease process costs even further, improvements in productivity are needed.

The second set of *in silico* analyses aimed to investigate bioprocess improvements on productivity and cell density, and evaluate their impacts on production costs and profitability indicators.

As detailed in the Material and Methods section, experimental values of specific velocity of product biosynthesis ($\mu_p$) were used to simulate productivities ($P_{p_S}$). The highest $\mu_p$ value was 0.09 g gh$^{-1}$ at $t = 39$ h. So $\mu_p$ values determined between $t = 55$ h and $t = 74$ h were further used in simulations of optimistic ($\mu_p = 0.07$ g gh$^{-1}$), realistic ($\mu_p = 0.05$ g gh$^{-1}$) and pessimistic ($\mu_p = 0.02$ g gh$^{-1}$) scenarios (Figure 13).

*Figure 13. R. eutropha L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: specific velocities of product biosynthesis ($\mu_p$ g gh$^{-1}$). ( ) start of the second batch. (---): start of third to seventh batches.*

Table 10 presents calculated productivities for each scenario of optimistic, realistic or pessimistic values of $\mu_p$.

*Table 10. Optimistic, realistic and pessimistic scenarios for simulation of productivities, cell density and PHB titers.*

Previous studies described growth phases that lasted between 20 h to 26 h in high cell density bioprocesses that produced PHA from glycerol (Cavalheiro et al., 2009; Volova et al., 2018). As for PHB accumulation phase duration, bacterial bioprocesses are very rarely described with accumulation phases longer than 50 h, which would be more compatible with archaeal
bioprocesses (Bhattacharyya et al., 2012; Pramanik et al., 2012). Thus, our calculated process
times between 88.2 h and 90.3 h were appropriately pessimistic.

The realistic scenarios had total process times consistent with those experimentally described by
other authors (Acosta-Cárdenas et al., 2018; Bormann & Roth, 1999; Lenczak et al., 2011;
Kachrimanidou et al., 2014; Rodríguez-Contreras et al., 2015). These authors described
bioprocesses with *R. eutropha* strains that used glycerol as carbon source and fermentation times
ranged from 45 h to 60 h. Thus, high cell density bioprocess with duration times below 45 h could
indeed be considered optimistic scenarios.

All simulated scenarios considered PHB content of 66.3%, as we determined experimentally, so
cell densities ($X_{f-Si}$) of 30 g L$^{-1}$, 40 g L$^{-1}$ and 50 g L$^{-1}$ resulted in estimated CDW concentrations of
89 g L$^{-1}$, 118.7 g L$^{-1}$ and 148.4 g L$^{-1}$, respectively. Estimated PHB titers ($P_{f-Si}$) are presented in
Table 10. Finally, the simulated productivities ($P_{p-Si}$) ranged between 0.7 – 2.3 g Lh$^{-1}$, which were
consistent with those experimentally described elsewhere (Blunt et al., 2018; Cavalheiro et al.,
2009; Volova et al., 2018).

Scenarios from SC7 to SC15 were firstly analyzed by facility-dependent costs. Figure 14 depicts
total annual production costs specifically related to glycerol and facility-dependent costs. The
remaining production costs such as other raw materials, utilities, labor, etc., were generally
treated as “Other production costs”.

**Figure 14. Total annual production costs.** (o) optimistic scenarios; (r) realistic scenarios;
(p) pessimistic scenarios; (30) $X_{f-Si} = 30$ g L$^{-1}$; 30) $X_{f-Si} = 40$ g L$^{-1}$; 30) $X_{f-Si} = 50$ g L$^{-1}$.

In comparison to scenario SC1, optimistic and realistic scenarios for all cell densities had lower
production costs. Among pessimistic scenarios, SC9 had production costs 13.4% higher than
those in SC1, but in scenarios SC12 and SC15 these costs were, respectively, 3.4% and 13%
lower.

In order to reduce production costs, results in Figure 14 indicated that both productivity and PHB
titers were important. The later was a result of cell density, given that PHB content was invariable
among scenarios. As stated above, higher cell densities became especially important in scenarios for pessimistic productivities.

Higher cell densities also had greater impact on reduction of facility-dependent costs once productivity was improved. Comparing to pessimistic productivities, realistic scenarios decreased facility-dependent costs by 56.5%, 55.6% and 63.2% for $X_{f, Si} = 30 \text{ g L}^{-1}$, $X_{f, Si} = 40 \text{ g L}^{-1}$ and $X_{f, Si} = 50 \text{ g L}^{-1}$, respectively. By comparing realistic and optimistic scenarios, these reduction costs were 5.3% for $X_{f, Si} = 30 \text{ g L}^{-1}$, 19.6% and 19.4% for $X_{f, Si} = 40 \text{ g L}^{-1}$, and $X_{f, Si} = 50 \text{ g L}^{-1}$, respectively.

According to these results, efforts and resources dedicated to achieving higher cell densities are justifiable in order to optimize PHB bioprocesses. Even though our study estimated growth phase two hours longer in order to increase $X_{f, Si} = 30 \text{ g L}^{-1}$ up to $X_{f, Si} = 50 \text{ g L}^{-1}$, production costs were not negatively affected. Improving cell density and consequently PHB titers would also be an strategy for optimizing facility occupancy and the PHB production (tons$_{PHB}$ batch$^{-1}$) could be met by a lower number of bioreactor units (Figure 15A and B).

Higher productivities were expected to reduce unit production costs. Scenarios from SC7 to SC15 evaluated improvements on productivity and PHB titers combined with the use of vinasse as a partial source of nutrients and unit production costs ranged from US$ 3.9 kg$_{PHB}$\textsuperscript{-1} to US$ 7.5 kg$_{PHB}$\textsuperscript{-1} (Figure 15C).

**Figure 15.** Effect of productivity on production costs. A. Number of units of production bioreactor. B. PHB production as tons per batch. C. Unit production cost.
sources, production capacity, and others. Feedstocks such as sucrose, waste glycerol, whey, methane, soy oil and others have been previously evaluated by other authors and unit production costs between US$ 1.9 kg\textsubscript{PHB}\textsuperscript{-1} and US$ 18.7 kg\textsubscript{PHB}\textsuperscript{-1} were determined, and studies most commonly estimated costs between US$ 4.1 kg\textsubscript{PHB}\textsuperscript{-1} and US$ 6.8 kg\textsubscript{PHB}\textsuperscript{-1} (Akiyama et al., 2003; Choi & Lee, 1997; Koller et al., 2013; Leong et al., 2017; Levett et al., 2016; Listewnik et al., 2007; Mudliar et al., 2008; Posada et al., 2011; Van-Wegen et al., 1998). According to our study, sugarcane vinasse has great potential as feedstock for PHB bioproduction.

The best results were determined for scenario SC13, which had $X_{f,Si} = 50$ g L\textsuperscript{-1}, PHB productivity of 2.3 g L h\textsuperscript{-1}, PHB titer of 98.4 g L\textsuperscript{-1} and bioprocess time of 42.8 h. The unit production cost was US$ 3.9 kg\textsubscript{PHB}\textsuperscript{-1} and payback time was 2.6 years (Table 11).

Table 11. Unit production costs and profitability indicators in scenarios of optimistic, realistic and pessimistic productivities.

| Scenario | PHB Productivity | PHB Titer | Bioprocess Time | Unit Production Cost | Payback Time |
|----------|------------------|-----------|-----------------|----------------------|--------------|
| SC7      | 2.0 g L h\textsuperscript{-1} | 95.2 g L\textsuperscript{-1} | 41.1 h | US$ 4.5 kg\textsubscript{PHB}\textsuperscript{-1} | 3.1 years |
| SC13     | 2.3 g L h\textsuperscript{-1} | 98.4 g L\textsuperscript{-1} | 42.8 h | US$ 3.9 kg\textsubscript{PHB}\textsuperscript{-1} | 2.6 years |
| SC15     | 2.6 g L h\textsuperscript{-1} | 102.6 g L\textsuperscript{-1} | 44.5 h | US$ 4.0 kg\textsubscript{PHB}\textsuperscript{-1} | 3.2 years |

Indicators of economical feasibility and profitability are highly variable among projects, depending on their technical specificities, geographic location and markets they aim to. The payback time is one of the most general indicators and, at some level, allows comparison among a variety of projects. Considering bioprocesses that aimed to exploit agroindustrial wastes for biocommodities manufacturing, other authors have applied the SuperPro Designer software to estimate payback times of 3 – 5.5 years for valorization of whey wastes and syrups by food products (Gómez et al., 2020). The biotechnological process of L-lysine synthesis from sugarcane molasses was estimated to have payback time of 3.8 years (Anaya-Reza & Lopez-Arenas, 2017). And the integration of Brazilian sugar mills to single cell oil production had estimated payback times between 5.4 – 13.1 years (Vieira et al., 2016). The payback times determined for scenarios SC7 to SC15 ranged from 2.6 – 7.3 years and may be considered competitive with other bioprocesses of agroindustrial wastes valorization.
Our results were obtained while assuming glycerol price of US$ 400 ton\(^{-1}\), which could be considered as an expensive price in Brazil (Stracke et al., 2018). Evaluating lower prices could result in more attractive scenarios for any of the conditions presented in this study. Scenarios SC9 and SC13, which had respectively the worst and best unit production costs and profitability indicators, were further analyzed for glycerol price of US$ 200 ton\(^{-1}\). Scenario SC9 had production cost reduced to US$ 7.2 kg\(\text{PHB}^{-1}\) and profitability indicators were ROI = 14.3%, IRR 7.6%, NPV = US$ 32 million and payback time of 7.0 years. As for SC13, unit production cost was US$ 3.6 kg\(\text{PHB}^{-1}\) and ROI, IRR, NPV were respectively 40.8%, 31% and US$ 258.5 million, with a payback time of 2.5 years.

In this study, despite the proposal of integrating bioethanol and PHB processes, our simulation model in SuperPro Designer considered a production facility entirely dedicated to the PHB process. No facility sharing between processes was simulated in this study. Including facility sharing between PHB and bioethanol process would require very specific information from the Brazilian sugarcane mills to be inserted into our simulation model. Because this study employed a theoretical approach, it was decided not to assume such reduction costs due to facility sharing. However, with future perspectives for such integration, these factors should be considered and initial investments could become more attractive.

### Conclusion

In this study we were able to demonstrate that vinasse has potential for cultivating *E. coli* THR14, *Halomonas* sp. HG03 and *R. eutropha* L359PCJ. L-threonine is a growth-related bioproduct and its biosynthesis by *E. coli* THR14 was not negatively affected in vinasse-based media with *in natura* vinasse up to 50% (v v\(^{-1}\)). Further improvements in L-threonine production rates by *E. coli* THR14 could bring perspectives for use vinasse application as water and mineral source for culture medium. As for PHB biosynthesis by *Halomonas* sp. HG03 and *R. eutropha* L359PCJ, both *in natura* and concentrated forms of vinasse provided results comparable to those previously reported in
literature, for shake flasks and bioreactors fermentations. That makes vinasse a competitive feedstock for PHB production processes, especially for glycerol-consuming strains.

*R. eutropha* L359PHB could efficiently consume glycerol from vinasse and biosynthesize PHB. No inhibitory effects were observed in CSTR bioreactor cultivation, production yields, PHB content and final titer were comparable to those reported for PHB production from other glycerol sources.

Moreover, in silico analyses were determinant for assessing the potential of using vinasse in large scale bioprocesses. Important reductions of production costs could be possible due to vinasse as a partial source of glycerol and other mineral components in culture medium.

**Abbreviations**

**PHB**: poly(3-hydroxybutyrate)

**PHA**: polyhydroxyalkanoate

**COD**: Chemical Oxygen Demand

**CDW**: Cell Dry Weight

**rCDW**: residual Cell Dry Weight

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All the needed data are provided in the manuscript.
Competing interests

The authors declare they have no competing interests.

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Author’s Contributions

MSS, experimental execution, formal analysis, data curation, writing; RAMP, conceptualization, supervision of the study, manuscript review; JLMSR, experimental execution; JGCG, conceptualization, manuscript review; ASB, conceptualization, manuscript review. All authors approved the final manuscript.

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Table 1. *In natura* vinasse chemical composition (3.4ºBrix)

| Category          | Component   | Concentration | Unit      |
|-------------------|-------------|---------------|-----------|
| Organic acids     | Acetic acid | 0.3 ± 0.01    | g L⁻¹     |
|                   | Lactic acid | 0.7 ± 0.04    | g L⁻¹     |
| Carbohydrates     | Sucrose     | ND            | g L⁻¹     |
|                   | Glucose     | 0.2 ± 0.04    | g L⁻¹     |
|                   | Fructose    | 0.6 ± 0.02    | g L⁻¹     |
|                   | Glycerol    | 10.0 ± 0.1    | g L⁻¹     |
| Salts             | Calcium (Ca²⁺) | 1,586.6 ± 315.3 | mg L⁻¹ |
|                   | Potassium (K⁺) | 4,188.8 ± 41.3    | mg L⁻¹ |
|                   | Ammonium (NH₄⁺) | 113.8 ± 2.9   | mg L⁻¹   |
|                   | Sodium (Na⁺)  | 775.0 ± 4.8   | mg L⁻¹   |
| Amino acids       | L-alanine   | 22.4 ± 2.0    | mg L⁻¹   |
|                   | L-glycine   | 6.7 ± 0.4     | mg L⁻¹   |
|                   | L-valine    | 12.2 ± 0.5    | mg L⁻¹   |
|                   | L-leucine   | 4.9 ± 0.1     | mg L⁻¹   |
|                   | L-isoleucine| 5.3 ± 0.4     | mg L⁻¹   |
|                   | L-threonine | 4.0 ± 1.3     | mg L⁻¹   |
|                   | L-serine    | 22.1 ± 0.7    | mg L⁻¹   |
|                   | L-proline   | 9.8 ± 0.2     | mg L⁻¹   |
|                   | L-asparagine| 59.5 ± 34.1   | mg L⁻¹   |
|                   | L-aspartate | 133.4 ± 27.6  | mg L⁻¹   |
|                   | L-methionine| 3.0 ± 0.2     | mg L⁻¹   |
|                   | L-glutamate | 5.0 ± 0.03    | mg L⁻¹   |
|                   | L-phenilalanine | 3.9 ± 0.2 | mg L⁻¹ |
|                   | L-glutamine | ND            | mg L⁻¹   |
|                   | L-ornithine | 1.8 ± 0.5     | mg L⁻¹   |
|                   | L-lysine    | 4.9 ± 0.6     | mg L⁻¹   |
|                   | L-histidine | 3.8 ± 0.1     | mg L⁻¹   |
|                   | L-tyrosine  | 3.8 ± 0.4     | mg L⁻¹   |
|                   | L-triptophan| ND            | mg L⁻¹   |
|                   | L-cystine   | ND            | mg L⁻¹   |

ND: not detectable.

Table 2. Culture medium composition for bioprocesses of PHB production in CSTR bioreactors.
| Component                      | Rocha et al. (2008) | Final composition in bioreactor | Vinasse 7ºBrix |
|--------------------------------|---------------------|---------------------------------|----------------|
| Carbon source (g L⁻¹) †         | 15.0                | 15.0                            | 7.5 *          |
| KH₂PO₄ (g L⁻¹)                  | 0.65                | 0.65                            | -              |
| (NH₄)₂SO₄ (g L⁻¹)               | 3.6                 | 3.6                             | -              |
| MgSO₄.7H₂O (g L⁻¹)              | 0.31                | 0.155                           | -              |
| CaCl₂.2H₂O (g L⁻¹)              | 0.01                | 0.005                           | -              |
| Ammonium ferric citrate (g L⁻¹) | 0.15                | 0.075                           | -              |
| Trace elements solution (mL L⁻¹ ) | 2.0                 | 1.0                             | -              |
| Initial volume (L)              | -                   | 3.0                             | 3.0            |

* Halomonas sp. HG03 had NaCl 80.0 g L⁻¹ added to MMB medium in the first batch.
† Halomonas sp. HG03: sucrose as the main carbon source; R. eutropha L359PCJ: glycerol as carbon source
* For both bacteria, the volumetric dilution of 50% (v v⁻¹) of concentrated vinasse supplied 7.5 g L⁻¹ of glycerol.
Figure 1. Bioprocess of PHB production by R. eutropha L359PCJ in SuperPro Designer v. 9.5.
Table 3. Global stoichiometric equations used in base scenario SC1

| Section      | Reaction | Limiting component | Global stoichiometric equation (mass coefficient)                                                                                                                                 |
|--------------|----------|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Upstream     | R1       | Glycerol           | 1 glycerol + 0.24 (NH₄)₂SO₄ + 0.18 KH₂PO₄ + 0.021 MgSO₄ + 0.0007 CaCl₂ + 0.01 Ammoniacal iron citrate + 4x10⁻⁵ H₃BO₃ + 3x10⁻⁵ CoCl₂ + 1.3x10⁻⁵ ZnSO₄ + 4x10⁻⁶ MnSO₄ + 4x10⁻⁶ Na₂MoO₄ + 3x10⁻⁶ NiCl₂ + 1.3x10⁻⁶ CuSO₄ => Yₓ/S † + Yₚ/S † |
| Bioprocess   | R2       | Glycerol           | 1 glycerol + 0.24 (NH₄)₂SO₄ + 0.18 KH₂PO₄ + 0.021 MgSO₄ + 0.0007 CaCl₂ + 0.01 Ammoniacal iron citrate + 4x10⁻⁵ H₃BO₃ + 3x10⁻⁵ CoCl₂ + 1.3x10⁻⁵ ZnSO₄ + 4x10⁻⁶ MnSO₄ + 4x10⁻⁶ Na₂MoO₄ + 3x10⁻⁶ NiCl₂ + 1.3x10⁻⁶ CuSO₄ => Yₓ/S ‡ + Yₚ/S ‡ |

†Experimentally determined during growth phase. ‡ Global yields experimentally determined for the whole process.
Table 4. Technical-economical scenarios for evaluation of high cell density cultures and PHB productivities.

| Scenario | $X_{S_{Si}}$ (g L$^{-1}$) | $\mu_{P}$ (g gh$^{-1}$) |
|----------|--------------------------|-------------------------|
| SC7      | 30                       | optimistic              |
| SC8      | 30                       | realistic               |
| SC9      | 30                       | pessimistic             |
| SC10     | 40                       | optimistic              |
| SC11     | 40                       | realistic               |
| SC12     | 40                       | pessimistic             |
| SC13     | 50                       | optimistic              |
| SC14     | 50                       | realistic               |
| SC15     | 50                       | pessimistic             |
Figure 2. CDW, L-threonine and acetic acid concentrations by *E. coli* THR14 in control and vinasse-based treatments after 24 h incubation in shake flasks.
Table 5. Biomass and L-threonine yields from glucose by *E. coli* THR14

|                | $Y_{X/S}$ (g g\(^{-1}\)) | $Y_{P/S}$ (g g\(^{-1}\)) |
|----------------|---------------------------|---------------------------|
| MM\(_{Ec}\)    | 0.53 ± 0.1                | 0.03 ± 0.05               |
| MM\(_{Ec}\) V253 | 0.44 ± 0.04               | 0.06 ± 0.02               |
| MM\(_{Ec}\) V503 | 0.46 ± 0.1                | 0.05 ± 0.01               |
| MM\(_{Ec}\) V753 | 0.51 ± 0.04               | NA                        |

No statistical difference was found according to F test ($p < 0.05$).

NA: not applicable.
Figure 3. *Halomonas* sp. HG03 cellular growth and PHB biosynthesis in mineral medium, *in natura* and concentrated vinasse-based treatments, after 48 h incubation.
Figure 4. *R. eutropha* L359PCJ cellular growth and PHB biosynthesis in mineral medium, *in natura* and concentrated vinasse-based treatments, after 48 h incubation.
Table 6. Biomass and PHB yields from sucrose by *Halomonas* sp. HG03

|                | $Y_{XS}$ (g g$^{-1}$) | $Y_{PS}$ (g g$^{-1}$) |
|----------------|------------------------|------------------------|
| MM$_{His}$     | 0.07 ± 0.01 c          | 0.32 ± 0.02 a          |
| MM$_{His}$V503 | 0.09 ± 0.02 c          | 0.27 ± 0.04 bc         |
| MM$_{His}$V753 | 0.24 ± 0.02 b          | 0.31 ± 0.04 ab         |
| MM$_{His}$V507 | 0.23 ± 0.08 b          | 0.23 ± 0.02 c          |
| MM$_{His}$V757 | 0.36 ± 0.08 a          | 0.09 ± 0.04 d          |

Letters *abcd* differ according to Tukey test with 5% of significance.
Table 7. Biomass and PHB yields from glycerol by *R. eutropha* L359PCJ

|                | $Y_{XS}$ (g g$^{-1}$) | $Y_{PS}$ (g g$^{-1}$) |
|----------------|------------------------|------------------------|
| $MM_{Re}$      | 0.07 ± 0.02 $d$        | 0.39 ± 0.02 $a$        |
| $MM_{ReV503}$  | 0.16 ± 0.03 $c$        | 0.35 ± 0.03 $b$        |
| $MM_{ReV753}$  | 0.18 ± 0.01 $bc$       | 0.33 ± 0.01 $b$        |
| $MM_{ReV507}$  | 0.23 ± 0.05 $b$        | 0.34 ± 0.03 $b$        |
| $MM_{ReV757}$  | 0.31 ± 0.02 $a$        | 0.29 ± 0.03 $c$        |

Letters *abcd* differ according to Tukey test with 5% of significance.
Figure 5. Cellular growth by *R. eutropha* L359PCJ in bioprocess operated by consecutive batches mode in bioreactor with concentrated vinasse-based medium. (---): start of the second batch. (-----): start of third to seventh batches. (x) ln(CDW); (●) ln(rCDW); (▲) $\mu_{\text{max}}$ h$^{-1}$; (◆) optical density (absorbance 600 nm).
**Figure 6.** Cellular growth by *Halomonas* sp. HG03 in bioprocess operated by consecutive batches mode in bioreactor with concentrated vinasse-based medium. (...) start of the second batch. (---) start of third to sixth batches. (×) ln(CDW); (●) ln(rCDW); (▲) $\mu_{\text{max}}$ h$^{-1}$; (◆) optical density (absorbance 600 nm).
Figure 7. *R. eutropha* L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: monitoring of agitation speed, dissolved oxygen, aeration, glycerol and ammonium concentrations.
Figure 8. *Halomonas* sp. HG03 cultivation in bioreactor with concentrated vinasse-based culture medium: monitoring of agitation speed, dissolved oxygen, aeration, glycerol and ammonium concentrations.
Figure 9. *R. eutropha* L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: volume variation, PHB content, CDW, rCDW, consumed glycerol and PHB concentrations.
**Figure 10.** *Halomonas* sp. HG03 cultivation in bioreactor with concentrated vinasse-based culture medium: volume variation, PHB content, CDW, rCDW, consumed total sugars and PHB concentrations.
Table 8. Simulation parameters in base scenario SC1 – input, output data and settings in SuperPro Designer model.

| Unit procedure                  | Value | Unit |
|---------------------------------|-------|------|
| **Seed fermenter**              |       |      |
| rCDW₀                           | 0     | g L⁻¹ |
| \(Y_{X/S}\)                     | 0.52⁺ | g g⁻¹ |
| \(Y_{P/S}\)                     | 0.14⁺ | g g⁻¹ |
| rCDWᵣ                           | 0.565 * | ton batch⁻¹ |
| PHBᵣ                            | 0.152 * | ton batch⁻¹ |
| Glycerol input                  | 1.09 * | ton batch⁻¹ |
| **Production fermenter * (Growth phase)** |       |      |
| \(\mu_{\text{max}}\)           | 0.255⁺ | h⁻¹ |
| rCDW₀                           | 0.3⁺  | g L⁻¹ |
| rCDWᵣ                           | 30    | g L⁻¹ |
| PHB content                     | 21.4⁺ | % m m⁻¹ |
| PHB titerᵣ                     | 8.2 § | g L⁻¹ |
| CDW                             | 38.2 § | g L⁻¹ |
| Time                            | 18 §  | h    |
| **(PHB accumulation phase)**    |       |      |
| \(\mu_p\)                      | 0.05⁺ | h⁻¹ |
| rCDW₀ = rCDWᵣ                   | 54 *  | ton batch⁻¹ |
| PHB content₀                   | 21.4⁺ | % m m⁻¹ |
| PHB contentᵣ                   | 66.3⁺ | % m m⁻¹ |
| PHB titer₀                     | 8.2 § | g L⁻¹ |
| PHB titerᵣ                     | 59 §  | g L⁻¹ |
|                                 | 108 * | ton batch⁻¹ |
| CDWᵣ                            | 89 §  | g L⁻¹ |
| Time                            | 33.8 § | h    |
| **(Global)**                    |       |      |
| \(Y_{X/S} \) (g g⁻¹)            | 0.16⁺ | g g⁻¹ |
| \(Y_{P/S} \) (g g⁻¹)            | 0.32⁺ | g g⁻¹ |
| Productivity                    | 1.1 § | g Lh⁻¹ |
| Time                            | 51.7 § | h    |
| Glycerol input                  | 337.3 * | ton batch⁻¹ |

*Six units of working volume = 300 m³. ⁺Determined experimentally. *SuperPro Designer simulation data. §Calculated using experimental data.
Figure 11. Gantt chart of base scenario SC1: Unit procedure (P-) / equipment – operation. (●)

Bottleneck unit procedure. (✩) Operations in bottleneck unit procedure.
| Parameter                                                                 | Scenario SC1   | Leong et al. (2017) |
|---------------------------------------------------------------------------|----------------|---------------------|
| PHB productivity (g L h\(^{-1}\))                                        | 1.1\(\)       | 2.86                |
| Operating hours year\(^{-1}\) (h)                                        | 5760           | 7920                |
| Bioprocess time (h)                                                      | 51.7\(\)      | 42                  |
| Purified PHB batch\(^{-1}\) (ton)                                        | 93.9           | 56.3                |
| Number of batches year\(^{-1}\)                                         | 109            | 146                 |
| Units of production bioreactors                                          | 6              | 3                   |
| PHB content (% m m\(^{-1}\))                                            | 66.3\(\)      | 49                  |
| Glycerol batch\(^{-1}\) (ton)                                           | 307.5          | 190.3               |
| Total annual production cost (US$)                                       | 63,991,002     | 57,883,913          |
|   Raw materials (%)                                                      | 37.4           | 39.0                |
|   Glycerol (%)                                                           | 23.1           | 27.6                |
|   Facility (%)                                                           | 56.9           | 51.0                |
|   Labor (%)                                                              | 3.6            | 8.0                 |
|   Utilities (%)                                                          | 1.5            | 2.0                 |
|   Laboratory and others (%)                                              | 0.5            | < 1                 |
| Glycerol price (US$ ton\(^{-1}\))                                       | 400            | 500                 |
| Annual production (PHB tons)                                             | 10,000         | 9,000               |
| Total investment (US$)                                                   | 206,597,000    | 170,658,000         |
| Direct fixed cost (US$)                                                  | 193,501,000    | 160,098,000         |
| Equipment purchase cost (US$)                                           | 32,281,250     | 27,535,000          |
| Unit production cost (US$ kg\(\text{PHB}^{-1}\))                        | 6.2            | 6.1                 |
| Return on investment (ROI) (%)                                           | 20.05          | 21.8                |
| Internal rate of return (IRR) (%)                                        | 14.0           | 18.0                |
| Net present value (NPV) (US$)                                            | 112,135,000    | 117,213,000         |
| Payback (years)                                                          | 5.0            | 4.6                 |
Figure 12. Unit production costs and annual production costs discriminated by raw materials, facility and others in scenarios with mineral medium (SC1, SC2, SC3) and scenarios with concentrated vinasse as partial source of nutrients in culture medium (SC4, SC5, SC6).
Figure 13. *R. eutropha* L359PCJ cultivation in bioreactor with concentrated vinasse-based culture medium: specific velocities of product biosynthesis ($\mu_{P} \text{ g gh}^{-1}$). (---): start of the second batch. (-----): start of third to seventh batches.
Table 10. Optimistic, realistic and pessimistic scenarios for simulation of productivities, cell density and PHB titers.

| Scenario | $X_{fSi}$ (g L^{-1}) | $\mu_P$ (g gh^{-1}) | $t_{X_{fSi}}$ (h) | $t_{PHB_{Si}}$ (h) | Total process time (h) | $P_{f_{Si}}$ (g L^{-1}) | $P_{p_{Si}}$ (g Lh^{-1}) |
|----------|---------------------|---------------------|------------------|------------------|-----------------------|------------------------|------------------------|
| SC7$^o$  | 30                  | 0.07                | 18               | 23.4             | 40.8                  | 59                     | 1.4                    |
| SC8$^r$  | 30                  | 0.05                | 18               | 33.8             | 51.7                  | 59                     | 1.1                    |
| SC9$^p$  | 30                  | 0.02                | 18               | 70.4             | 88.2                  | 59                     | 0.7                    |
| SC10$^o$ | 40                  | 0.07                | 19               | 23.4             | 41.9                  | 78.7                   | 1.9                    |
| SC11$^r$ | 40                  | 0.05                | 19               | 33.8             | 52.8                  | 78.7                   | 1.5                    |
| SC12$^p$ | 40                  | 0.02                | 19               | 70.4             | 89.4                  | 78.7                   | 0.9                    |
| SC13$^o$ | 50                  | 0.07                | 20               | 23.4             | 42.8                  | 98.4                   | 2.3                    |
| SC14$^r$ | 50                  | 0.05                | 20               | 33.8             | 53.7                  | 98.4                   | 1.8                    |
| SC15$^p$ | 50                  | 0.02                | 20               | 70.4             | 90.3                  | 98.4                   | 1.1                    |

$^o$: optimistic scenarios; $^r$: realistic scenarios; $^p$: pessimistic scenarios.
Figure 14. Total annual production costs. (o) optimistic scenarios; (r) realistic scenarios; (p) pessimistic scenarios; (30) $X_{FSi} = 30 \text{ g L}^{-1}$; (40) $X_{FSi} = 40 \text{ g L}^{-1}$; (50) $X_{FSi} = 50 \text{ g L}^{-1}$.
Figure 15. Effect of productivity on production costs. A. Number of units of production bioreactor. B. PHB production as tons per batch. C. Unit production cost.
Table 11. Unit production costs and profitability indicators in scenarios of optimistic, realistic and pessimistic productivities.

| Scenario | Unit production cost (US$ kg<sub>PHB</sub><sup>-1</sup>) | ROI (%) | Payback (years) | IRR (%) | NPV (million) (US$) |
|----------|-------------------------------------------------|---------|-----------------|---------|---------------------|
| SC7<sup>o</sup> (30) | 5.4 | 23.2 | 4.3 | 17.0 | 152.3 |
| SC8<sup>o</sup> (30) | 5.4 | 22.6 | 4.4 | 16.2 | 151.3 |
| SC9<sup>p</sup> (30) | 7.5 | 13.6 | 7.3 | 6.8 | 15.1 |
| SC10<sup>o</sup> (40) | 4.3 | 32.8 | 3.1 | 25.1 | 217.6 |
| SC11<sup>r</sup> (40) | 4.8 | 27.2 | 3.7 | 20.6 | 186.0 |
| SC12<sup>p</sup> (40) | 6.5 | 17.0 | 5.9 | 10.7 | 80.5 |
| SC13<sup>o</sup> (50) | 3.9 | 28.9 | 2.6 | 29.8 | 241.6 |
| SC14<sup>r</sup> (50) | 4.3 | 32.6 | 3.1 | 24.9 | 217.2 |
| SC15<sup>p</sup> (50) | 5.9 | 19.5 | 5.2 | 13.2 | 115.1 |

<sup>o</sup>: optimistic; <sup>r</sup>: realistic; <sup>p</sup>: pessimistic; (30) $X_{f-Si} = 30$ g L<sup>-1</sup>; (40) $X_{f-Si} = 40$ g L<sup>-1</sup>; (50) $X_{f-Si} = 50$ g L<sup>-1</sup>.
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