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

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Keywords: in natura vinasse, concentrated vinasse, amino acids, L-threonine, polyhydroxyalkanoates, P(3-hydroxybutyrate), SuperPro Designer

Posted Date: October 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-942291/v1>

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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 $Y_{X/S} = 0.16 \text{ g g}_{\text{GLYCEROL}}^{-1}$, $Y_{P/S} = 0.32 \text{ g g}_{\text{GLYCEROL}}^{-1}$ and $0.25 \text{ g}_{\text{PHB}} \text{ Lh}^{-1}$. *Halomonas* sp. HG03 had PHB content of 45.7%, rCDW = 9.8 g L⁻¹, PHB = 8.3 g L⁻¹ and $Y_{X/S} = 0.18 \text{ g g}_{\text{SUCROSE}}^{-1}$, $Y_{P/S} = 0.16 \text{ g g}_{\text{SUCROSE}}^{-1}$ and $0.12 \text{ g}_{\text{PHB}} \text{ Lh}^{-1}$. 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/kg_{PHB} 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 Sucreoenergetic 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 34.4°Brix and kept at 4°C. 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 3.4°Brix, 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 $6.8 < \text{pH} < 7.8$ with NaOH 10 M or NH_4OH 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 (3.4°Brix)

Microrganisms

For L-threonine production experiments, it was utilized the strain *E. coli* MG1655 $\Delta\text{metJIQ } \Delta\text{lysA}$ 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 -80°C. Before experiments, they were all cultivated in their respective solid culture media, kept at 30°C 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 30°C 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; (NH₄)₂SO₄, 5.0 g; KH₂PO₄, 8.5 g; MgSO₄, 1.0 g; sodium citrate, 0.5 g; L-lysine, 0.5 g; CaCO₃, 10.0 g; and trace elements solution, 1.0 mL, with the following composition (L⁻¹) - CaCl₂, 13.2 g; FeSO₄, 8.4 g; MnSO₄, 2.4 g; ZnSO₄, 2.4 g; CuSO₄, 0.48 g; CoCl₂, 0.48 g; Na₂MoO₄, 0.24 g; K₂B₄O₇, 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 (3.4°Brix) 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 (KH₂PO₄) 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 NH₄OH 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 30°C, 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 (MM_{Ha}) adapted from Rocha et al. (2008) (L^{-1}): sucrose, 15.0 g; Na_2HPO_4 , 3.5 g; KH_2HPO_4 , 1.5 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; ammonium ferric citrate, 0.06 g; NaCl, 80.0 g; and trace elements solution with the following composition (L^{-1}) - H_3BO_3 0.3 g; $CoCl_2 \cdot 6H_2O$, 0.2 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.03 g; $NaMoO_4 \cdot 2H_2O$, 0.03 g; $NiCl_2 \cdot 6H_2O$, 0.02 g; $CuSO_4 \cdot 5H_2O$, 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% ($v v^{-1}$) MM_{Ha} . Experiments with *Halomonas* sp. HG03 used both *in natura* (3.4°Brix) and concentrated (7°Brix) vinasses.

The following volumetric dilutions of vinasses were evaluated: 50% ($v v^{-1}$) of vinasse 3.4°Brix in MM_{Ha} ($MM_{Ha}V503$); 75% ($v v^{-1}$) of vinasse 3.4°Brix in MM_{Ha} ($MM_{Ha}V753$); 50% ($v v^{-1}$) of vinasse 7°Brix in MM_{Ha} ($MM_{Ha}V507$); and 75% ($v v^{-1}$) of vinasse 7°Brix in MM_{Ha} ($MM_{Ha}V757$).

Carbon (sucrose), nitrogen (ammonium sulphate), phosphate (Na_2HPO_4 , KH_2HPO_4) 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* (3.4°Brix) and concentrated (7°Brix) 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 3.4°Brix in MM_{Re} (MM_{Re}V503) had 2.5 g L⁻¹ of glycerol; 75% (v v⁻¹) of vinasse 3.4°Brix in MM_{Re} (MM_{Re}V753) had 5.0 g L⁻¹ of glycerol; 50% (v v⁻¹) of vinasse 7°Brix in MM_{Re} (MM_{Re}V507) had 7.5 g L⁻¹ of glycerol; 75% (v v⁻¹) of vinasse 7°Brix 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 (Na₂HPO₄ and KH₂HPO₄) 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 30°C, 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 MM_{Re}V507 and MM_{Ha}V507 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 (7°Brix) 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 (34.4°Brix) 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 (34.4°Brix) 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 (34.4°Brix) 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 (34.4°Brix) 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 30°C and pH was automatically controlled at 6.8 < pH < 7.8 by addition of H₂SO₄ 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 ($g\ Lh^{-1}$) 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:

$$P_{phb} = \frac{(P_f - P_i)}{(t_f - t_i)}$$

eq. (6)

Finally, product ($Y_{P/S}$ g g⁻¹) and biomass (r_{CDW}) ($Y_{X/S}$ g g⁻¹) yields were determined for a variation of product and biomass concentrations given the substrate consumption:

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

eq. (7)

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

eq. (8)

Analytical methods

Vinasse refractive index was determined as Brix Degrees (°Brix), 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 *E. coli* THR14 cultures, cell dry weight was determined by gravimetric method. Culture media were centrifuged at 10,000 g, for 10 minutes, at 10°C. Pellets were then resuspended in distilled water, cells were harvested with filtration membranes (0.22 µm), and finally submitted to 105°C 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 15' (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.

Figure 1. Bioprocess of PHB production by *R. eutropha* L359PCJ in SuperPro Designer v. 9.5.

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 ($P_{p_{Si}}$) aimed to simulate PHB production by high cell density cultures. Time ranges for growth phase (t_{X-Si}) and PHB accumulation phase (t_{PHB-Si}) were calculated.

Equation 5 was used to calculate the term $(t_f - t_i)$. Experimental data of μ_{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 μ_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 $PHBcontent_i$ 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 $PHBcontent_f$ was the PHB content experimentally determined at the end of the accumulation phase and P_f was consequently the calculated PHB titer:

$$P_{i-Si} (g L^{-1}) = \frac{X_{f-Si}}{1 - (PHBcontent_i/100)} \quad \text{eq. (9)}$$

$$P_{f-Si} (g L^{-1}) = \frac{X_{f-Si}}{1 - (PHBcontent_f/100)} \quad \text{eq. (10)}$$

The term $t_f = t_{PHB-Si}$.

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

$$P_{pSi} = \frac{P_{f-Si}}{t_{X-Si} + t_{PHB-Si}} \quad \text{eq. (11)}$$

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* L359PCJ in concentrated vinasse-based medium (50% v v⁻¹ of vinasse 7°Brix).

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, $X_{f-Si} = 30 \text{ g L}^{-1}$, $P_{f-Si} = 59 \text{ g L}^{-1}$ and $P_{p-Si} = 1.1 \text{ g Lh}^{-1}$. 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_{PHB}⁻¹. 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 KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$.

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 KH_2PO_4 and $(\text{NH}_4)_2\text{SO}_4$.

Three cell densities were evaluated, $X_{f-Si} = 30 \text{ g L}^{-1}$, $X_{f-Si} = 40 \text{ g L}^{-1}$ and $X_{f-Si} = 50 \text{ g L}^{-1}$. Each value of X_{f-Si} was combined with three experimental values of μ_P (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 μ_P and their resulting PHB productivity (Table 4). All scenarios kept the same glycerol conversion into PHB ($Y_{P/S} \text{ g g}^{-1}$), biomass (rCDW) ($Y_{X/S} \text{ g g}^{-1}$) 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 $10.7 \pm 2.1 \text{ g L}^{-1}$ (MM_{EcV503}), although no statistical difference was found. Vinasse dilution of 75% (v^{-1}) resulted in the least satisfactory cellular growth, which was $4.9 \pm 0.5 \text{ g L}^{-1}$, statistically the lowest biomass concentration (Figure 2).

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.

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_{Ec}, MM_{Ec}V253 and MM_{Ec}V503 showed no significant difference, acetic acid concentrations in MM_{Ec}V253 (0.6 ± 0.1 g L⁻¹) and MM_{Ec}V503 (0.5 ± 0.2 g L⁻¹) were significantly lower than those in MM_{Ec} (2.3 ± 0.8 g L⁻¹) and MM_{Ec}V753 (1.9 ± 0.04 g L⁻¹).

In MM_{Ec}V253 and MM_{Ec}V503, *E. coli* THR14 synthesized $1.2 (\pm 0.7)$ g L⁻¹ and $1.2 (\pm 0.1)$ g L⁻¹ of L-threonine, respectively. No statistical difference was found between L-threonine production among these vinasse-based treatments and control treatment MM_{Ec}, which had $0.8 (\pm 1.1)$ g L⁻¹ of L-threonine. No detectable L-threonine production was determined in MM_{Ec}V753.

Both L-threonine ($Y_{P/S}$ g g⁻¹) and biomass yields from glucose ($Y_{X/S}$ g g⁻¹) showed no significant difference between MM_{Ec}, MM_{Ec}V253 and MM_{Ec}V503. MM_{Ec}V753 showed the lowest biomass production, but glucose consumption was consistently low (9.7 g L⁻¹ 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⁻¹.

In this study, biomass yields were consistent with those previously reported on *E. coli* yield on glucose ($0.5 \text{ g}_{\text{biomass}} \text{ g}_{\text{glucose}}^{-1}$) (Shiloach & Fas, 2005), and no statistical difference was found among treatments. Furthermore, MM_{Ec}V253 and MM_{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 $2.3 \pm 0.8 \text{ g L}^{-1}$ in MM_{Ec} was acceptable, meaning a production of $0.3 \text{ g}_{\text{acetate}} \text{ g}_{\text{biomass}}^{-1}$. Ying et al. (2014) described $0.95 \text{ g}_{\text{acetate}} \text{ g}_{\text{biomass}}^{-1}$ (biomass concentration was 7.1 g L^{-1}) in fed-batch bioprocesses. The final acetic acid concentrations in vinasse based treatments MM_{Ec}V253 ($0.6 \pm 0.2 \text{ g L}^{-1}$) and MM_{Ec}V503 ($0.5 \pm 0.2 \text{ g L}^{-1}$) 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_{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_{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_{Ec}V753 supports the hypothesis of limitation rather than inhibition. In MM_{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_{L-threonine} g_{GLUCOSE}⁻¹, and final concentrations between 9.7 and 17 g L⁻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⁻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.

Suhaili 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⁻¹; acetic acid, 1.7- 7.2 g L⁻¹; residual sugars, 20 g L⁻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 *E. coli* THR14, L-aspartate concentrations had been depleted in all treatments.

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

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

Increasing volumetric dilutions of both *in natura* and concentrated vinasse had a positive effect on bacteria cellular growth.

In *Halomonas* sp. HG03 cultivations, rDW concentration in MM_{Ha}V503 ($1.1 \pm 0.2 \text{ g L}^{-1}$) did not differ from that in control treatment (MM_{Ha}) ($0.74 \pm 0.1 \text{ g L}^{-1}$). By increasing vinasse in culture media, in both treatments MM_{Ha}V753 and MM_{Ha}V507, rCDW concentration increased significantly by three fold, with final values of $2.2 \pm 0.1 \text{ g L}^{-1}$ and $2.3 \pm 0.4 \text{ g L}^{-1}$, respectively. In MM_{Ha}V757 a further significant increase was achieved and residual biomass concentration improved by four fold ($3.0 \pm 0.6 \text{ g L}^{-1}$) (Figure 3).

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

In *R. eutropha* L359PCJ cultivations, all vinasse-based treatments showed a significant increase in cellular growth. *In natura* vinasse-based treatments MM_{Re}V503 and MM_{Re}V753 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 $\text{MM}_{Re}\text{V507}$ treatment did not contribute significantly to increase rCDW ($2.7 \pm 0.4 \text{ g L}^{-1}$) in comparison to *in natura* vinasse treatments. $\text{MM}_{Re}\text{V757}$, 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} . $\text{MM}_{Ha}\text{V503}$ 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 $\text{MM}_{Ha}\text{V753}$: 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 $\text{MM}_{Re}\text{V503}$ ($68.5 \pm 4.9\%$) and $\text{MM}_{Re}\text{V753}$ ($65.1 \pm 2.3\%$). Consequently, PHB final concentration in $\text{MM}_{Re}\text{V503}$ ($4.8 \pm 0.6 \text{ g L}^{-1}$) dropped 21% in comparison to MM_{Re} ($5.8 \pm 0.3 \text{ g L}^{-1}$), and $\text{MM}_{Re}\text{V753}$ ($4.4 \pm 0.4 \text{ g L}^{-1}$), 33%.

The use of concentrated vinasse resulted in further decrease of PHB cellular content. *Halomonas* sp. HG03 had $50.7 \pm 6.9\%$ PHB cellular content in $\text{MM}_{Ha}\text{V507}$ treatment and final PHB

concentration ($2.3 \pm 0.2 \text{ g L}^{-1}$) dropped significantly. *MM_{Ha}V757* had PHB content as low as $19.6 \pm 3.7\%$ and its PHB final concentration was statically the lowest among *Halomonas* sp. HG03 treatments ($0.8 \pm 0.3 \text{ g L}^{-1}$).

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 *Halomonas* sp. strains. According to literature, different culture conditions and carbon sources might result in PHB cellular contents between 44 - 90% by *Halomonas* sp. PHA-producing strains (Kucera et al., 2018; Quillaguamán et al., 2007; Rivera-Terceros et al., 2015).

Other authors have cultivated *Halomonas* sp. strains in shake flasks with sucrose as carbon source. PHB contents between 44 – 75% and rCDW concentrations between $1.1 - 1.6 \text{ g L}^{-1}$ have been described (Kucera et al., 2018; Rath et al., 2012; Stanley et al., 2017). In our study, *in 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 *R. eutropha* L359PCJ dropped significantly in concentrated vinasse treatments in comparison to control. In *MM_{Re}V507*, PHB cellular content ($60.8 \pm 4.0\%$) 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 ($4.2 \pm 0.3 \text{ g L}^{-1}$). In *MM_{Re}V757*, PHB cellular content ($48.4 \pm 1.3\%$) was statistically the lowest among *R. eutropha* L359PCJ treatments. However, higher rCDW concentration in *MM_{Re}V757* contributed to its final PHB concentration ($3.9 \pm 0.3 \text{ g L}^{-1}$), which differed only from control treatment and *MM_{Re}V503*.

In shake flasks cultures, using glycerol as carbon source, *R. eutropha* strains were described by other authors with residual biomass production between $0.66 - 2.2 \text{ g L}^{-1}$, and PHB cellular content from 57% to 65% (Fukui et al., 2014; Cavaleiro et al., 2009; Volova et al., 2018). Similarly to our results with *Halomonas* sp. HG03, increasing vinasse in culture media improved *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_{Ha}V753 treatment had the best PHB production among vinasse-based treatments with *Halomonas* sp. HG03. Biomass and PHB yields in both treatments MM_{Ha}V753 and MM_{Ha}V507 were interestingly balanced, indicating that such conditions have good potential for high cell density cultures for PHB production. MM_{Ha}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_{Re}V503 treatment was over twice higher than that in control treatment MM_{Re}. 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_{Re}V757 was over four times higher than biomass yield in control treatment.

In MM_{Re}V757 product yield dropped 34.5% when compared to control treatment. Lower vinasse concentrations in MM_{Re}V503, MM_{Re}V753 and MM_{Re}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⁻¹) 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⁻¹) 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 gluma* 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). Zanonatto et al. (2018), Bhattacharyya et al. (2012) and Pramanik et al. (2012) have investigated PHAs production by *Cupriavidus necator* DSM545, *Haloferax mediterranei* and *Haloarcula marismortui* 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 marismortui* 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. Zanonatto 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 MM_{Re}V757 finally had glycerol fully provided by concentrated vinasse (15 g L⁻¹).

Despite lower PHB production by *R. eutropha* L359PCJ in MM_{Re}V757 treatment, it is important to emphasize that such process had zero cost with carbon source acquisition. MM_{Re}V507 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 (OD₆₀₀). Maximum growth velocity $\mu_{\max} = 0.255 \text{ h}^{-1}$ was observed between $t = 0 \text{ h}$ and $t = 7.15 \text{ h}$ and exponential growth phase was observed until $t = 30 \text{ h}$. At $t = 55 \text{ h}$ the culture reached stationary growth phase.

At $t = 26 \text{ h}$ the second batch was initiated, which supplied all nutrients from culture medium and at $t = 32 \text{ h}$ the batch was finished. At $t = 34 \text{ h}$ the third batch supplied pure glycerol and concentrated vinasse (34.4°Brix) 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 (OD₆₀₀) until t = 53 h. Maximum growth velocity was $\mu_{\max} = 0.091 \text{ h}^{-1}$, 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 ((NH₄)₂SO₄), *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 ($\mu_{\max}$) 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 $\mu_{\max}$ values between 0.11 and 0.15 h⁻¹ for cultivations with crude glycerol as carbon source. Zanfatto 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 $\mu_{\max} = 0.21 \text{ h}^{-1}$ 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 $\mu_{\max} = 0.16 \text{ h}^{-1}$.

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 $t = 34 \text{ h}$. 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 $t = 44 \text{ h}$ (Figure 6), nitrogen as in the form of NH_4^+ had been depleted in culture medium at $t = 33 \text{ h}$. Sucrose was satisfactorily consumed until $t = 48 \text{ h}$, but sucrose accumulation was observed later. Still, it was expected that glycerol would not be significantly consumed by *Halomonas* sp. HG03 (Figure 8).

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.

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 ((NH₄)₂SO₄) and other nutrients from mineral medium. By the end of the second batch, at t = 32 h, PHB content reached 21.4% (m m⁻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 $Y_{X/S} = 0.52 \text{ g g}^{-1}$, and into PHB, $Y_{P/S} = 0.14 \text{ g g}^{-1}$. At the end of the seventh batch the global yields were $Y_{X/S} = 0.16 \text{ g g}^{-1}$, $Y_{P/S} = 0.32 \text{ g g}^{-1}$ 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 $60.6 \pm 4.0\%$ and global conversion yields were $Y_{X/S} = 0.23 \pm 0.05 \text{ g g}^{-1}$ and $Y_{P/S} = 0.34 \pm 0.03 \text{ g g}^{-1}$ (MM_{Re}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 $2.1 - 11.3 \text{ g L}^{-1}$ of CDW and $6.7 - 13.1 \text{ g L}^{-1}$ 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 $Y_{P/S} = 0.30 \text{ g g}^{-1}$ 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 Lh⁻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 $Y_{X/S} = 0.45 \text{ g g}^{-1}$ and $Y_{P/S} = 0.34 \text{ g g}^{-1}$.

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 $t = 17.7 \text{ h}$, PHB content was determined at 28.5%, and it showed little variation until the end of the second batch at $t = 26 \text{ h}$, with 28.8%. By the end of the sixth batch, at $t = 67 \text{ h}$, 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, $Y_{P/S} = 0.16 \text{ g g}^{-1}$ and $Y_{X/S} = 0.18 \text{ g g}^{-1}$, 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 $50.7 \pm 6.9\%$. 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 $rCDW = 0.54 \text{ g L}^{-1}$ and $PHB = 0.66 \text{ g L}^{-1}$. In further studies, Quillaguamán et al. (2007) described PHB production *H. boliviensis* LC1^T with sucrose in bioreactors and productivities were between $0.14 - 0.16 \text{ g Lh}^{-1}$, PHB content was 52% and final concentrations were $rCDW = 6.9 \text{ g L}^{-1}$ and $PHB = 7.5 \text{ g L}^{-1}$.

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). Quillaguamán 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 (Kshirsagar et al., 2012; Quillaguamán 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 (Kshirsagar et al., 2012; Quillaguamán et al., 2007; Quillaguamán 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 reation 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 $\mu_{\max} = 0.255 \text{ h}^{-1}$ 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 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 Cavaleiro et al. (2009) ($r_{CDW} = 31 \text{ g L}^{-1}$ and $r_{CDW} = 42.8 \text{ g L}^{-1}$, respectively).

918 The authors achieved maximum PHB content of 62%, which resulted in PHB titer of 51.1 g L⁻¹
 919 and productivities ranged from 0.84 to 1.5 g Lh⁻¹.

920 In comparison to the study described by Leong et al. (2009), the simulated productivity we
 921 calculated in this study, $Pp_{Si} = 1.1 \text{ g Lh}^{-1}$ was low, although comparable to those experimentally
 922 described by Cavaleiro et al. (2009) that used glycerol from agroindustrial wastes as carbon
 923 source.

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

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

936 As detailed in Table 9, scenario SC1 had lower production costs related to raw materials (37.4%).
 937 However, facility-dependent production costs and investments were very important (56.9%) and
 938 remarkably disadvantageous in comparison to simulated results by Leong et al. (2017). As a
 939 consequence, despite lower costs with raw material, unit production cost in SC1 (US\$ 6.2 kg_{PHB}⁻¹
 940 ¹) was not more competitive than that described by Leong et al. (2017) (US\$ 6.1 kg_{PHB}⁻¹) (US\$
 941 500 ton⁻¹). Moreover, SC1 had higher investments on direct fixed capital, which made financial
 942 indicators ROI, IRR, NPV and payback less attractive.

943 Production costs were also analyzed by process section. The Upstream section, which included
 944 culture medium and inoculum preparation, had the most important share in costs, 39.5%. Next,
 945 Bioprocess accounted for 37.6%, which included mostly the operation of FR-101 units. Lastly, the
 946 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 (SC4, US\$ 400 ton⁻¹) and US\$ 5.1 kg_{PHB}⁻¹ (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 (μ_P) were used to simulate productivities (P_{PS}). The highest μ_P value was 0.09 g gh⁻¹ at t = 39 h. So μ_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 (μ_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 μ_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; Ienczak 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; Cavaleiro 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; (40) $X_{f-Si} = 40$ g L⁻¹; (50) $X_{f-Si} = 50$ 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 ($\text{tons}_{\text{PHB}} \text{ batch}^{-1}$) 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 $\text{kg}_{\text{PHB}}^{-1}$ to US\$ 7.5 $\text{kg}_{\text{PHB}}^{-1}$ (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.

Unit production costs determined in pessimistic scenarios were less competitive than those determined in SC1 (US\$ 6.2 $\text{kg}_{\text{PHB}}^{-1}$) and by Leong et al. (2017) (US\$ 6.1 $\text{kg}_{\text{PHB}}^{-1}$). On the other hand, all realistic and optimistic scenarios had more competitive unit production costs and profitability indicators ROI, IRR, NPV and payback as well.

Unit production costs determined in scenarios from SC1 to SC15, that ranged between US\$ 3.9 $\text{kg}_{\text{PHB}}^{-1}$ and US\$ 7.5 $\text{kg}_{\text{PHB}}^{-1}$ were consistent with previous studies. Many factors might affect PHB production costs, such as microbial strain, PHB content, type, quality and location of carbon

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_{PHB}⁻¹ and US\$ 18.7 kg_{PHB}⁻¹ were determined, and studies most commonly estimated costs between US\$ 4.1 kg_{PHB}⁻¹ and US\$ 6.8 kg_{PHB}⁻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⁻¹, PHB productivity of 2.3 g Lh⁻¹, PHB titer of 98.4 g L⁻¹ and bioprocess time of 42.8 h. The unit production cost was US\$ 3.9 kg_{PHB}⁻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.

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_{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_{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.

1135 **Competing interests**

1136 The authors declare they have no competing interests.

1137 **Funding**

1138 This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
1139 - Brasil (CAPES), Finance Code 001.

1140 **Author's Contributions**

1141 MSS, experimental execution, formal analysis, data curation, writing; RAMP, conceptualization,
1142 supervision of the study, manuscript review; JLMSR, experimental execution; JGCG,
1143 conceptualization, manuscript review; ASB, conceptualization, manuscript review. All authors
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1146

1147 **References**

1148 Acosta-Cárdenas A, Alcaraz-Zapata W, Cardona-Betancur M (2018) Sugarcane molasses and
1149 vinasse as a substrate for polyhydroxyalkanoates (PHA) production. Dyna. 85:220-225. doi:
1150 10.15446/dyna.v85n206.68279.

1151 Akiyama M, Tsuge T, Doi Y (2003) Environmental life cycle comparison of polyhydroxyalkanoates
1152 produced from renewable carbon resources by bacterial fermentation. Polymer Degradation and
1153 Stability, 80:183–194. doi: 10.1016/S0141-3910(02)00400-7.

1154 Anaya-Reza O, Lopez-Arenas T (2017) Comprehensive assessment of the l-lysine production
1155 process from fermentation of sugarcane molasses. Bioprocess and Biosystems Engineering,
1156 40:7. doi: 10.1007/s00449-017-1766-2.

1157 Badawy AAB (2012) The EZ:Faast family of amino acids analysis kits: application of the GC-FID
1158 kit for rapid determination of plasma tryptophan and other amino acids. Methods Mol Biol.
1159 828:153-164. doi: 10.1007/978-1-61779-445-2_14.

1160 Bhattacharyya A, Pramanik A, Maji SK, Haldar S, Mukhopadhyay UK, Mukherjee J (2012)
 1161 Utilization of vinasse for production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) by *Haloferax*
 1162 *mediterranei*. Dig AMB Express. doi: 10.1186/2191-0855-2-34.

1163 Blunt W, Levin D, Cicek N (2018) Bioreactor Operating Strategies for Improved
 1164 Polyhydroxyalkanoate (PHA) Productivity. Polymers, 10:11. doi: 10.3390/polym10111197.

1165 Bormann EJ, Roth M (1999) The production of polyhydroxybutyrate by *Methylobacterium*
 1166 *rhodesianum* and *Ralstonia eutropha* in media containing glycerol and casein hydrolysates.
 1167 Biotechnology Letters, 21:1059–1063.

1168 Canizales L, Rojas F, Pizarro CA, Caicedo-Ortega NH, Villegas-Torres MF (2020) SuperPro
 1169 Designer®, User-Oriented Software Used for Analyzing the Techno-Economic Feasibility of
 1170 Electrical Energy Generation from Sugarcane Vinasse in Colombia. Processes, 8:9. doi:
 1171 10.3390/pr8091180.

1172 Cardinali-Rezende J, Nahat RATPS, Moreno CWG, Farfán CRC, Silva LF, Taciro MK, Gomez
 1173 JGC (2016) Draft Genome Sequence of *Halomonas* sp. HG01, a Polyhydroxyalkanoate-
 1174 Accumulating Strain Isolate from Peru. Dig Genome Announcements.
 1175 doi:10.1128/genomeA.01598-15.

1176 Cassman NA, Lourenço KS, Carmo JB, Cantarella H, Kuramae EE (2018) Genome-resolved
 1177 metagenomics of sugarcane vinasse bacteria. Bioenergy for Biofuels. 11:48. doi:10.1186/s13068-
 1178 018-1036-9.

1179 Cavalleiro, JMBT, Almeida MCMD, Grandfils C, Fonseca MMR (2009) Poly(3-hydroxybutyrate)
 1180 production by *Cupriavidus necator* using waste glycerol. Process Biochemistry. 44:509-515. doi:
 1181 10.1016/j.procbio.2009.01.008.

1182 Chen N, Huang J, Feng ZB, Yu L, Xu QY, Wen TY (2009) Optimization of Fermentation
 1183 Conditions for the Biosynthesis of L-Threonine by *Escherichia coli*. Appl Biochem Biotechnol.
 1184 158:595-604. doi: 10.1007/s12010-008-8385-y.

1185 Choi J, Lee SY (1997) Process analysis and economic evaluation for Poly(3-hydroxybutyrate)
 1186 production by fermentation. *Bioprocess Engineering*, 17:6. doi: 10.1007/s004490050394.

1187 Coelho MPM, Correia JE, Vasques LI, Marcato ACC, Guedes TA, Soto MA, Basso JB, Kiang C,
 1188 Fontanetti CS (2018) Toxicity evaluation of leached of sugarcane vinasse: Histopathology and
 1189 immunostaining of cellular stress protein. *Ecology and Environmental Safety*. 165:367-375.
 1190 doi:10.1016/j.ecoenv.2018.08.099.

1191 Cortez LAB (2010) Sugarcane Bioethanol: R&D for productivity and sustainability. Editora Edgard
 1192 Blucher Ltda., São Paulo. ISBN-10: 8521205309.

1193 Dowd MK, Johansen SL, Cantarella L (1994) Low molecular weight organic composition of
 1194 ethanol stillage from sugarcane molasses, citrus waste, and sweet whey. *J. Agri. Food Chem*.
 1195 42:283-288. doi:10.1021/jf00038a011.

1196 Eggeling L, Bott M (2015) A giant Market and a powerful metabolism: L-lysine provided by
 1197 *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol*. 99:3387-3394. doi: 10.1007/s00253-
 1198 015-6508-2.

1199 España-Gamboa E, Mijangos-Cortes J, Barahona-Perez L, Dominguez-Maldonado J,
 1200 Hernández-Zarate G, Alzate-Gaviria L (2011) Vinasses: characterization and treatments. *Waste*
 1201 *Management and Research*. 29:1235-1250. doi:10.1177/0734242X10387313.

1202 España-Gamboa E, Mijangos-Cortes J, Hernández-Zarate G, Dominguez-Maldonado J, Alzate-
 1203 Gaviria L (2012) Methane production by treating vinasses from hydrous ethanol using a modified
 1204 UASB reactor. *Biotechnology for Biofuels*. 5:82-90. doi:10.1186/1754-6834-5-82.

1205 European Bioplastics (2020) Dynamic growth: global production capacities of bioplastics 2020-
 1206 2025. [https://docs.european-](https://docs.european-bioplastics.org/conference/Report_Bioplastics_Market_Data_2020_short_version.pdf)
 1207 [bioplastics.org/conference/Report_Bioplastics_Market_Data_2020_short_version.pdf](https://docs.european-bioplastics.org/conference/Report_Bioplastics_Market_Data_2020_short_version.pdf). Accessed
 1208 24 May 2021.

1209 Ferreira LFR, Aguiar MM, Messias TG.; Pompeu GB, Lopez AMQ, Sila DP, Monteiro RT (2011)
 1210 Evaluation of sugar-cane vinasse treated with *Pleurotus sajor-caju* utilizing aquatic organisms as

1211 toxicological indicators. *Ecotoxicology and Environmental Safety*. 74:132-137.
 1212 doi:10.1016/j.ecoenv.2010.08.042.

1213 Ferreira E, Cavalcanti P, Nogueira D (2014) ExpDes: Na R Package for ANOVA and Experimental
 1214 Designs. *Applied Mathematics*. 5:2952-2958. doi:10.4236/am.2014.519280.

1215 Fukui T, Mukoyama M, Orita I, Nakamura S (2014) Enhancement of glycerol utilization ability of
 1216 *Ralstonia eutropha* H16 for production of polyhydroxyalkanoates. *Appl Microbiol Biotechnol*.
 1217 98:7559-7568. doi: 10.1007/s00253-014-5831-3.

1218 Global Industry Analysts Inc. (2018) Healthy Market for meat and nutraceuticals drive the global
 1219 amino acids market. 2018. [https://www.strategyr.com/MarketResearch/market-report-](https://www.strategyr.com/MarketResearch/market-report-infographic-amino-acids-forecasts-global-industry-analysts-inc.asp)
 1220 [infographic-amino-acids-forecasts-global-industry-analysts-inc.asp](https://www.strategyr.com/MarketResearch/market-report-infographic-amino-acids-forecasts-global-industry-analysts-inc.asp). Accessed 22 Feb 2020.

1221 Gómez JA, Sánchez ÓJ, Correa LF (2020) Techno-economic and Environmental Evaluation of
 1222 Cheesemaking Waste Valorization Through Process Simulation Using SuperPro Designer. *Waste*
 1223 *and Biomass Valorization*, 11:11. doi: 10.1007/s12649-019-00833-4.

1224 Grand View Research (2015) Amino Acids Market Analysis By Product (L-Glutamate, Lysine,
 1225 Methionine, Threonine, Tryptophan, Leucine, Iso-Leucine, Valine, Glutamine, Arginine, Glycine,
 1226 Phenylalanine, Tyrosine, Citrulline, Creatine, Proline, Serine), By Source (Plant-Based & Animal-
 1227 Based), By Application (Animal Feed, Food & Dietary Supplements & Pharmaceuticals) And
 1228 Segment Forecasts To 2022. [https://www.grandviewresearch.com/industry-analysis/amino-](https://www.grandviewresearch.com/industry-analysis/amino-acids-market)
 1229 [acids-market](https://www.grandviewresearch.com/industry-analysis/amino-acids-market). Accessed 22 Feb 2020.

1230 Hermann (2003) Industrial production of amino acids by coryneform bacteria *Journal of*
 1231 *Biotechnology*. 104:155-172. doi: 10.1016/S0168-1656(03)00149-4.

1232 Hohenschuh W, Kumar D, Murthy GS (2014) Economic and cradle-to-gate life cycle assessment
 1233 of poly-3-hydroxybutyrate production from plastic producing, genetically modified hybrid poplar
 1234 leaves. *Journal of Renewable and Sustainable Energy*, 6:6. doi: 10.1063/1.4901870.

1235 Ienczak JL, Quines LK, Melo AA, Brandellero M, Mendes CR, Schmidell W, Aragão GMF (2011)
 1236 High cell density strategy for poly(3-hydroxybutyrate) production by *Cupriavidus necator*. Brazilian
 1237 Journal of Chemical Engineering, 28:4. doi: 10.1590/S0104-66322011000400004.

1238 Kachrimanidou V, Kopsahelis N, Papanikolaou S, Kookos IK, Bruyn M, Clark JH, Koutinas AA
 1239 (2014) Sunflower-based biorefinery: Poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-
 1240 hydroxyvalerate) production from crude glycerol, sunflower meal and levulinic acid. Bioresource
 1241 Technology, 172:121–130. doi: 10.1016/j.biortech.2014.08.044.

1242 Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo, SI (1994) Production of poly(3-
 1243 hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration
 1244 control. Biotechnology and Bioengineering, 43:9. doi: 10.1002/bit.260430908.

1245 Koller M, Niebelschütz H, Braunegg G (2013) Strategies for recovery and purification of poly[(R
 1246)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. Engineering in Life
 1247 Sciences 13:6. doi: 10.1002/elsc.201300021.

1248 Kshirsagar P, Suttar R, Nilegaonkar S, Kulkarni S, Kanekar P (2012) Scale up production of
 1249 polyhydroxyalkanoate (PHA) at different aeration, agitation and controlled dissolved oxygen
 1250 levels in fermenter using *Halomonas campisalis* MCM B-1027. J Biochem Tech, 41:512–517.

1251 Kucera D, Pernicová I, Kovalcik A, Koller M, Mullerova L, Sedlacek P, Mravec F, Nebesarova J,
 1252 Kalina M, Marova I, Krzyzanek V, Obruca S (2018) Characterization of the promising poly(3-
 1253 hydroxybutyrate) producing halophilic bacterium *Halomonas halophila*. Bioresource Technology.
 1254 256:552-556. doi: 10.1016/j.biortech.2018.02.062.

1255 Lee MH, Lee HW, Park JH, Ahn JO, Jung JK, Hwang YI (2006) Improved L-Threonine Production
 1256 of *Escherichia coli* Mutant by Optimization of Culture Conditions. Journal of Bioscience and
 1257 Bioengineering. 101:127-130. doi: 10.1263/jbb.101.127.

1258 Lee JH, Sung BH, Kim MS, Blattner FR, Yoon BH, Kim JH, Kim SC (2009) Metabolic engineering
 1259 of a reduced-genome strain of *Escherichia coli* for L-threonine production. Dig Microbial Cell
 1260 Factories. doi: 10.1186/1475-2859-8-2.

1261 Leong YK, Show PL, Lan JCW, Loh HS, Lam HL, Ling TC (2017) Economic and environmental
 1262 analysis of PHAs production process. *Clean Technologies and Environmental Policy*, 19:1941–
 1263 1953. doi: 10.1007/s10098-017-1377-2.

1264 Levett I, Birkett G, Davies N, Bell A, Langford A, Laycock B, Lant P, Pratt S (2016) Techno-
 1265 economic assessment of poly-3-hydroxybutyrate (PHB) production from methane—The case for
 1266 thermophilic bioprocessing. *Journal of Environmental Chemical Engineering* 4:4. doi:
 1267 10.1016/j.jece.2016.07.033.

1268 Li Y, Wei H, Wang T, Xu Q, Zhang C, Fan X, Ma Q, Chen N, Xie X (2017) Current status on
 1269 metabolic engineering for the production of L-aspartate family amino acids and derivatives.
 1270 *Bioresource Technology*. 245:1588-1602. doi: 10.1016/j.biortech.2017.05.145.

1271 Listewnik HF, Wendlandt KD, Jechorek M, Mirschel G (2007) Process Design for the Microbial
 1272 Synthesis of Poly- β -hydroxybutyrate (PHB) from Natural Gas. *Engineering in Life Sciences* 7:3.
 1273 doi: 10.1002/elsc.200620193.

1274 Lopes ML., Paulillo SCL, Godoy A, Cherubin RA, Lorenzi MS, Giometti FHC (2016) Ethanol
 1275 production in Brazil: a bridge between science and industry. *Brazilian Journal of Microbiology*.
 1276 47:64-76. doi:10.1016/j.bjm.2016.10.003.

1277 Moraes B. S, Zaiat M, Bonomi A (2015) Anaerobic digestion of vinasse from sugarcane ethanol
 1278 production in Brazil: Challenges and perspectives. *Renewable and Sustainable Energy Reviews*.
 1279 44:888-903. doi:10.1016/j.rser.2015.01.023.

1280 Moreno CWG (2015) Produção de Polihidroxialcanoatos (PHA) por *Halomonas* sp. HG01.
 1281 Dissertation, University of São Paulo.

1282 Mudliar SN, Vaidya AN, Suresh Kumar M, Dahikar S, Chakrabarti T (2008) Techno-economic
 1283 evaluation of PHB production from activated sludge. *Clean Technologies and Environmental*
 1284 *Policy* 10:3. doi: 10.1007/s10098-007-0100-0.

1285 OECD/FAO. (2021) OECD-FAO Agricultural Outlook 2021-2030. OECD Publishing, Paris.
 1286 doi.org/10.1787/19428846-en.

1287 Ortiz-Muniz B, Carvajal-Zarrabal O, Torrestiana-Sanchez B, Aguilar-Uscanga MG (2010) Kinetic
 1288 study on ethanol production using *Saccharomyces cerevisiae* ITV-01 yeast isolated from sugar
 1289 cane molasses. J Chem Technol Biotechnol. 85:1361–1367. doi:10.1002/jctb.2441.

1290 Ortiz-Veizán N, Daga-Quisbert J, Perez-Zabaleta M, Guevara-Martínez M, Larsson G,
 1291 Quillaguamán J (2020) Improvements of poly(3-hydroxybutyrate) production in an air-lift reactor
 1292 using simple production media. Bioresources and Bioprocessing, 7:1. doi: 10.1186/s40643-020-
 1293 00308-8.

1294 Paula CBC, Paula-Elias FC, Rodrigues MN, Coelho LF, Oliveira NML, Almeida AF, Contiero J
 1295 (2021) Polyhydroxyalkanoate Synthesis by *Burkholderia glumae* into a Sustainable Sugarcane
 1296 Biorefinery Concept. Frontiers in Bioengineering and Biotechnology. doi:
 1297 10.3389/fbioe.2020.631284.

1298 Pedro-Escher J, Maziviero GT, Fontanetti CS (2014) Mutagenic action of sugarcane vinasse in
 1299 the *Tradescantia pallida* Test System. J Ecosys Econograph. doi:10.4172/2157-7625.1000145.

1300 Petrides, D (2015). Bioprocess Design and Economics. In Harrison RG, Todd PW, Rudge SR, &
 1301 Petrides D (Eds.), *Bioseparations Science and Engineering* (pp. 11-1-11–83). Oxford University
 1302 Press.

1303 Poltronieri P, Kumar P (2017) Polyhydroxyalkanoates (PHAs) in Industrial Applications. In:
 1304 Martínez L, Kharissova O, Kharisov B (eds) Handbook of Ecomaterials. Springer, Cham. doi:
 1305 10.1007/978-3-319-48281-1_70-1.

1306 Posada JA, Naranjo JM, López JA, Higueta JC, Cardona CA (2011) Design and analysis of poly-
 1307 3-hydroxybutyrate production processes from crude glycerol. Process Biochemistry 46:1. doi:
 1308 10.1016/j.procbio.2010.09.003.

1309 Pramanik A, Mitra A, Arumugam M, Bhattacharyya A, Sadhukhan S, Ray A, Haldar S,
 1310 Mukhopadhyay UK, Mukherjee J (2012) Utilization of vinasse for the production of
 1311 polyhydroxybutyrate by *Haloarchula marismortui*. Folia Microbiol. 57:71-79. doi: 10.1007/s12223-
 1312 011-0092-3.

1313 Quillaguamán J, Delgado O, Mattiasson B, Hatti-Kaul R (2006) Poly(β -hydroxybutyrate)
 1314 production by a moderate halophile, *Halomonas boliviensis* LC1. Enzyme and Microbial
 1315 Technology, 38:1. doi: 10.1016/j.enzmictec.2005.05.013.

1316 Quillaguamán J, Doan-Van T, Guzmán H, Guzmán D, Martín J, Everest A, Hatti-Kaul R (2008).
 1317 Poly(3-hydroxybutyrate) production by *Halomonas boliviensis* in fed-batch culture. Applied
 1318 Microbiology and Biotechnology, 78:2. doi: 10.1007/s00253-007-1297-x.

1319 Quillaguamán J, Hashim S, Bento F, Mattiasson B, Hatti-Kaul R (2005) Poly(β -hydroxybutyrate)
 1320 production by a moderate halophile, *Halomonas boliviensis* LC1 using starch hydrolysate as
 1321 substrate. Journal of Applied Microbiology, 99:1. doi: 10.1111/j.1365-2672.2005.02589.x.

1322 Quillaguamán J, Muñoz M, Mattiasson B, Hatti-Kaul R (2007) Optimizing conditions for poly(β -
 1323 hydroxybutyrate) production by *Halomonas boliviensis* LC1 in batch culture with sucrose as
 1324 carbon soucr. Appl Microbiol Biotechnol. 74:981-986. doi: 10.1007/s00253-006-0754-2.

1325 Rath DN, Amir HG, Abed RMM, Kosugi A, Arai T, Sulaiman O, Hashim R, Sudesh K (2012)
 1326 Polyhydroxyalkanoate biosynthesis and simplified polymer recovery by a novel moderately
 1327 halophilic bacterium isolated from hypersaline microbial mats. Journal of Applied Microbiology.
 1328 114:384-395. doi: 10.1111/jam.12083.

1329 Ribeiro AC, Novais RF, Bahia-Filho AFC (1983) Efeitos da vinhaça sobre a dispersão de argila
 1330 de amostras de latossolos. Revista Ceres 30. 167:12-18.

1331 Riis V, Mai W (1988) Gas chromatography determination of poly- β -hydroxybutyric acid in
 1332 microbial biomass-ester hydrochloric acid propanolysis. J Chromatogr. 445:285-289.

1333 Rivera-Terceros P, Tito-Claros E, Torrico S, Carballo S, Van-Thuoc D, Quillaguamán J (2015)
 1334 Production of poly(3-hydroxybutyrate) by *Halomonas boliviensis* in na air-lift reactor. Dig Journal
 1335 of Biological Research-Thessaloniki. doi: 10.1186/s40709-015-0031-6.

1336 Rocha RCS, Silva LF, Taciro MK, Pradella JGC (2008) Production of poly(3-hydroxybutyrate-co-
 1337 3-hydroxyvalerate) P(3HB-co-3HV) with a broad range of 3HV content at high yields by

1338 *Burkholderia sacchari* IPT 189. World. J. Microbiol. Biotechnol. 24:427-431. doi: 10.1007/s11274-
1339 007-9480-x.

1340 Rodríguez-Contreras A, Koller M, Dias MMS, Calafell-Monfort M, Braunegg G, Marqués-Calvo
1341 MS (2015) Influence of glycerol on poly(3-hydroxybutyrate) production by *Cupriavidus necator*
1342 and *Burkholderia sacchari*. Dig Biochemical Engineering Journal. doi: 10.1016/j.bej.2014.11.007.

1343 Salles-Filho SL, Castro PFD, Bin A, Edquist C, Ferro AFP, Corder S (2017) Perspectives for the
1344 Brazilian bioethanol sector: The innovation driver. Energy Policy 108:70-77.
1345 doi:10.1016/j.enpol.2017.05.037.

1346 Sharma K, Srivastava AK, Sreekrishnan TR (2021) Model-based optimization of biopolymer
1347 production from glycerol. Chemical and Biochemical Engineering Quarterly. 1:65–80. doi:
1348 10.15255/CABEQ.2020.1864.

1349 Shiloach J, Fass R (2005) Growing *E. coli* to high cell density – a historical perspective on method
1350 development. Biotechnol Adv. 23:345-357. doi: 10.1016/j.biotechadv.2005.04.004.

1351 Soini J, Ukkonen K, Neubauer P (2008) High cell density media for *Escherichia coli* are generally
1352 designed for aerobic cultivation – consequences for large-scale bioprocesses and shake flasks
1353 cultures. Dig Microbial Cell Factories. doi: 10.1186/1475-2859-7-26.

1354 Soto MAA, Basso JB, Kiang CH, Genuchten MT (2015) Simulação de fluxo e transporte de íons
1355 de vinhaça através da vertente da formação Rio Claro. Águas Subterrâneas. 29:162-
1356 174. doi:10.14295/ras.v29i2.28239.

1357 Souza JKC, Mesquita FO, Dantas-Neto J, Souza MMA, Farias CHA, Mendes HC, Nunes RMA
1358 (2015) Fertirrigação com vinhaça na produção de cana-de-açúcar. Agropecuária Científica no
1359 Seminária. 11:7-12.

1360 Sreedevi S, Unni KN, Sajith S, Priji P, Josh MS, Benjamin S (2015) Bioplastics: Advances in
1361 Polyhydroxybutyrate Research. In: Advances in Polymer Science. doi:10.1007/12_2014_297.

1362 Stanley A, Kumar HNP, Mutturi S, Vijayendra SVN (2017) Fed-Batch Strategies for Production of
 1363 PHA Using a Native Isolate of *Halomonas venusta* KT832796 Strain. Appl Biochem Biotechnol.
 1364 184:935-952. doi: 10.1007/s12010-017-2601-6.

1365 Stracke MP, Wbatuba BBR, Padilha G, Seibert RM, Schrekker HS (2018) Biodiesel Production
 1366 Process: Valorization of the Crude Glycerol Constituents. Brazilian Journal of Development,
 1367 4:3401–3416.

1368 Suhaili N, Cárdenas-Fernández M, Ward JM, Lye GJ (2018) Potential of sugar beet vinasse as a
 1369 feedstock for biocatalyst production within an integrated biorefinery context. Journal of Chemical
 1370 Technology and Biotechnology. 94:739-751. doi: 10.1002/jctb.5819.

1371 UNICA (2021) Histórico de produção e moagem. [https://observatoriodacana.com.br/historico-de-](https://observatoriodacana.com.br/historico-de-producao-e-moagem.php?idMn=31&tipoHistorico=2)
 1372 [producao-e-moagem.php?idMn=31&tipoHistorico=2](https://observatoriodacana.com.br/historico-de-producao-e-moagem.php?idMn=31&tipoHistorico=2). Accessed 04 Jun 2021.

1373 Van-Thuoc D, Quillaguamán J, Mamo G, Mattiasson B (2007) Utilization of agricultural residues
 1374 for poly(3-hydroxybutyrate) production by *Halomonas boliviensis* LC1. Dig Journal of Applied
 1375 Microbiology. doi: 10.1111/j.1365-2672.2007.03553.x.

1376 Van-Wegen RJ, Ling Y, Middelberg APJ (1998) Industrial Production of Polyhydroxyalkanoates
 1377 Using *Escherichia Coll*: An Economic Analysis. Chemical Engineering Research and Design 76:3.
 1378 doi: 10.1205/026387698524848.

1379 Vieira JPF, Ienczak JL, Costa PS, Rossell CEV, Franco TT, Pradella JGC (2016) Single cell oil
 1380 production integrated to a sugarcane-mill: Conceptual design, process specifications and
 1381 economic analysis using molasses as raw material. Dig Industrial Crops and Products. doi:
 1382 10.1016/j.indcrop.2016.05.046.

1383 Volova T, Demidenko A, Kiselev E, Baranovskiy S, Shishatskaya E, Zhila N (2018)
 1384 Polyhydroxyalkanoate synthesis based on glycerol and implementation of the process under
 1385 conditions of pilot production. Appl Microbiology and Biotechnology. 103:225-237. doi:
 1386 10.1007/s00253-018-9460-0.

1387 Wang J, Ma W, Yang J, Chen S, Wang X (2019) Increasing L-threonine production in *Escherichia*
 1388 *coli* by overexpressing the gene cluster *phaCAB*. Journal of Industrial Microbiology &
 1389 Biotechnology. 46:1557-1568. doi: 10.1007/s10295-019-02215-0.

1390 Ying H, He X, Chen K, Ouyang P (2014) Optimization of Culture Conditions for Enhanced Lysine
 1391 Production Using Engineered *Escherichia coli*. Appl Biochem Biotechnol. 172:3835-3843. doi:
 1392 10.1007/s12010-014-0820-7.

1393 Yue H, Ling C, Yang T, Chen X, Chen Y, Deng H, Wu Q, Chen J, Chen GQ (2014) A seawater-
 1394 based open and continuous process for polyhydroxyalkanoates production by recombinant
 1395 *Halomonas campaniensis* LS21 grown in mixed substrates. Biotechnology for Biofuels, 7:1. doi:
 1396 10.1186/1754-6834-7-108.

1397 Zanfonato K, Schmidt M, Quines LK, Gai CS, Schmidell W, Aragão GMF (2018) Can vinasse be
 1398 used as carbon source for poly(3-hydroxybutyrate) production by *Cupriavidus necator* DSM545?
 1399 Brazilian Journal of Chemical Engineering. 35:901-908. doi: 10.1590/0104-
 1400 6632.20180353s20170265.

1401 Zhao H, Fang Y, Wang X, Zhao L, Wang J, Li Y (2018) Increasing L-threonine production in
 1402 *Escherichia coli* by engineering the glyoxylate shunt and the L-threonine biosynthesis pathway.
 1403 Applied Microbiology and Biotechnology. 102:5505-5518. doi: 0.1007/s00253-018-9024-3.

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

		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 (Ca ²⁺)	$1,586.6 \pm 315.3$	mg L ⁻¹
	Potassium (K ⁺)	$4,188.8 \pm 41.3$	mg L ⁻¹
	Ammonium (NH ₄ ⁺)	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-phenylalanine	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-tryptophan	ND	mg L ⁻¹
	L-cystine	ND	mg L ⁻¹

ND: not detectable.

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1422 Table 2. Culture medium composition for bioprocesses of PHB production in CSTR bioreactors.

	Mineral medium MMB		
	Rocha et al. (2008)	Final composition in bioreactor *	Vinasse 7°Brix
Carbon source (g L ⁻¹) †	15.0	15.0	7.5 ‡
KH ₂ PO ₄ (g L ⁻¹)	0.65	0.65	-
(NH ₄) ₂ SO ₄ (g L ⁻¹)	3.6	3.6	-
MgSO ₄ ·7H ₂ O (g L ⁻¹)	0.31	0.155	-
CaCl ₂ ·2H ₂ O (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.

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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	$1 \text{ glycerol} + 0.24 \text{ (NH}_4\text{)}_2\text{SO}_4 + 0.18 \text{ KH}_2\text{PO}_4 + 0.021 \text{ MgSO}_4 + 0.0007 \text{ CaCl}_2 + 0.01 \text{ Ammoniacal iron citrate} + 4 \times 10^{-5} \text{ H}_3\text{BO}_3 + 3 \times 10^{-5} \text{ CoCl}_2 + 1.3 \times 10^{-5} \text{ ZnSO}_4 + 4 \times 10^{-6} \text{ MnSO}_4 + 4 \times 10^{-6} \text{ Na}_2\text{MoO}_4 + 3 \times 10^{-6} \text{ NiCl}_2 + 1.3 \times 10^{-6} \text{ CuSO}_4$ $\Rightarrow Y_{X/S}^{\dagger} + Y_{P/S}^{\dagger}$
Bioprocess	R2	Glycerol	$1 \text{ glycerol} + 0.24 \text{ (NH}_4\text{)}_2\text{SO}_4 + 0.18 \text{ KH}_2\text{PO}_4 + 0.021 \text{ MgSO}_4 + 0.0007 \text{ CaCl}_2 + 0.01 \text{ Ammoniacal iron citrate} + 4 \times 10^{-5} \text{ H}_3\text{BO}_3 + 3 \times 10^{-5} \text{ CoCl}_2 + 1.3 \times 10^{-5} \text{ ZnSO}_4 + 4 \times 10^{-6} \text{ MnSO}_4 + 4 \times 10^{-6} \text{ Na}_2\text{MoO}_4 + 3 \times 10^{-6} \text{ NiCl}_2 + 1.3 \times 10^{-6} \text{ CuSO}_4$ $\Rightarrow Y_{X/S}^{*} + Y_{P/S}^{*}$

[†]Experimentally determined during growth phase. * Global yields experimentally determined for the whole process.

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

Scenario	X_{f-Si} (g L ⁻¹)	μ_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

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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.

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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

	$Y_{X/S}$ (g g ⁻¹)	$Y_{P/S}$ (g g ⁻¹)
MM _{Ha}	0.07 ± 0.01 <i>c</i>	0.32 ± 0.02 <i>a</i>
MM _{Ha} V503	0.09 ± 0.02 <i>c</i>	0.27 ± 0.04 <i>bc</i>
MM _{Ha} V753	0.24 ± 0.02 <i>b</i>	0.31 ± 0.04 <i>ab</i>
MM _{Ha} V507	0.23 ± 0.08 <i>b</i>	0.23 ± 0.02 <i>c</i>
MM _{Ha} V757	0.36 ± 0.08 <i>a</i>	0.09 ± 0.04 <i>d</i>

Letters *abcd* differ according to Tukey test with 5% of significance.

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Table 7. Biomass and PHB yields from glycerol by *R. eutropha* L359PCJ

	$Y_{X/S}$ (g g ⁻¹)	$Y_{P/S}$ (g g ⁻¹)
MM _{Re}	0.07 ± 0.02 <i>d</i>	0.39 ± 0.02 <i>a</i>
MM _{Re} V503	0.16 ± 0.03 <i>c</i>	0.35 ± 0.03 <i>b</i>
MM _{Re} V753	0.18 ± 0.01 <i>bc</i>	0.33 ± 0.01 <i>b</i>
MM _{Re} V507	0.23 ± 0.05 <i>b</i>	0.34 ± 0.03 <i>b</i>
MM _{Re} V757	0.31 ± 0.02 <i>a</i>	0.29 ± 0.03 <i>c</i>

Letters *abcd* differ according to Tukey test with 5% of significance.

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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) $\ln(\text{CDW})$; (•) $\ln(r\text{CDW})$; (▲) $\mu_{\text{max}} \text{ h}^{-1}$; (◆) 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. (x) $\ln(CDW)$; (•) $\ln(rCDW)$; ($\blacktriangle$) μ_{max} h^{-1} ; ($\blacklozenge$) 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		
$rCDW_0$	0	$g\ L^{-1}$
$Y_{X/S}$	$0.52^{\ddagger}$	$g\ g^{-1}$
$Y_{P/S}$	$0.14^{\ddagger}$	$g\ g^{-1}$
$rCDW_f$	0.565^*	$ton\ batch^{-1}$
	14.1	$g\ L^{-1}$
PHB_f	0.152^*	$ton\ batch^{-1}$
	3.8	$g\ L^{-1}$
Glycerol input	1.09^*	$ton\ batch^{-1}$
Production fermenter ^a		
(Growth phase)		
μ_{max}	$0.255^{\ddagger}$	h^{-1}
$rCDW_0$	$0.3^{\ddagger}$	$g\ L^{-1}$
$rCDW_f$	30	$g\ L^{-1}$
PHB content	$21.4^{\ddagger}$	$\% m\ m^{-1}$
PHB titer _f	$8.2^{\S}$	$g\ L^{-1}$
CDW	$38.2^{\S}$	$g\ L^{-1}$
Time	$18^{\S}$	h
(PHB accumulation phase)		
μ_P	$0.05^{\ddagger}$	h^{-1}
$rCDW_0 = rCDW_f$	54^*	$ton\ batch^{-1}$
	30	$g\ L^{-1}$
PHB content ₀	$21.4^{\ddagger}$	$\% m\ m^{-1}$
PHB content _f	$66.3^{\ddagger}$	$\% m\ m^{-1}$
PHB titer ₀	$8.2^{\S}$	$g\ L^{-1}$
PHB titer _f	$59^{\S}$	$g\ L^{-1}$
	108^*	$ton\ batch^{-1}$
CDW _f	$89^{\S}$	$g\ L^{-1}$
Time	$33.8^{\S}$	h
(Global)		
$Y_{X/S}\ (g\ g^{-1})$	$0.16^{\ddagger}$	$g\ g^{-1}$
$Y_{P/S}\ (g\ g^{-1})$	$0.32^{\ddagger}$	$g\ g^{-1}$
Productivity	$1.1^{\S}$	$g\ Lh^{-1}$
Time	$51.7^{\S}$	h
Glycerol input	337.3^*	$ton\ batch^{-1}$

^aSix 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.

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

	Scenario SC1	Leong et al. (2017)
PHB productivity (g Lh ⁻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 _{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

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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 (μ_P 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_{f-Si} (g L ⁻¹)	μP (g gh ⁻¹)	t_{X-Si} (h)	t_{PHB-Si} (h)	Total process time (h)	P_{f-Si} (g L ⁻¹)	Pp_{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.

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

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 _{PHB} ⁻¹)	ROI (%)	Payback (years)	IRR (%)	NPV (million) (US\$)
SC7 ^o (30)	5.4	23.2	4.3	17.0	152.3
SC8 ^r (30)	5.4	22.6	4.4	16.2	151.3
SC9 ^p (30)	7.5	13.6	7.3	6.8	15.1
SC10 ^o (40)	4.3	32.8	3.1	25.1	217.6
SC11 ^r (40)	4.8	27.2	3.7	20.6	186.0
SC12 ^p (40)	6.5	17.0	5.9	10.7	80.5
SC13 ^o (50)	3.9	28.9	2.6	29.8	241.6
SC14 ^r (50)	4.3	32.6	3.1	24.9	217.2
SC15 ^p (50)	5.9	19.5	5.2	13.2	115.1

^o: optimistic; ^r: realistic; ^p: pessimistic; (30) $X_{f-Si} = 30 \text{ g L}^{-1}$; (40) $X_{f-Si} = 40 \text{ g L}^{-1}$; (50) $X_{f-Si} = 50 \text{ g L}^{-1}$.

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