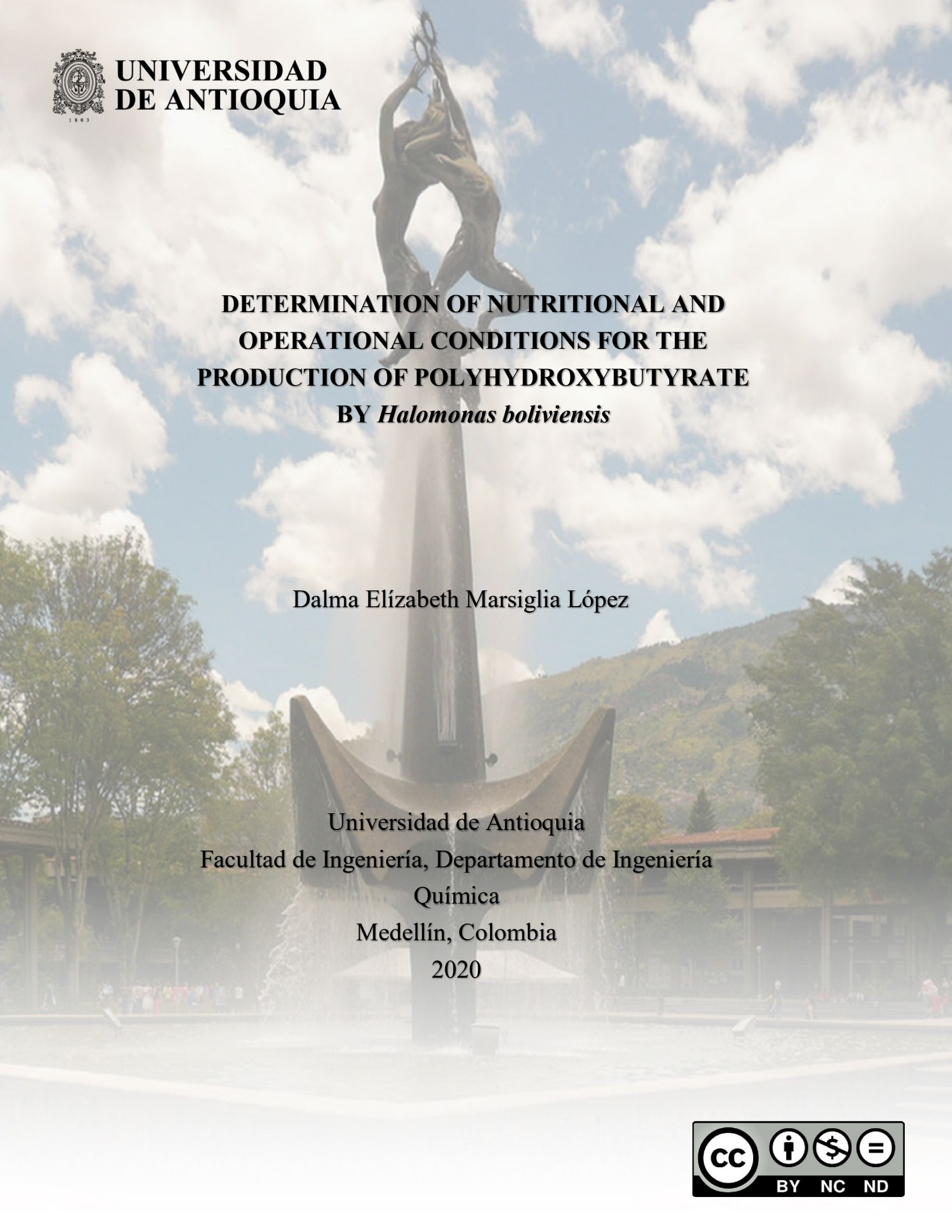

UNIVERSIDAD DE ANTIOQUIA

DETERMINATION OF NUTRITIONAL AND OPERATIONAL CONDITIONS FOR THE PRODUCTION OF POLYHYDROXYBUTYRATE BY *Halomonas boliviensis*

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Abstract

The production of biopolymers is being supported worldwide given the environmental impacts and consciousness regarding the abusive use of conventional synthetic plastics. Polyhydroxyalcanoates (PHAs) are biobased and biodegradable biopolymers that are of interest given their outstanding properties and carbon footprint, that enable them to be a suitable replacement to plastics. The most studied PHA is the polyhydroxybutyrate (PHB), a short chain length PHA with a broad application field due to its physical and thermal properties alike polyethylene, making it useful in the packaging industry and biocompatible non-toxic properties availing its use in biomedical applications.

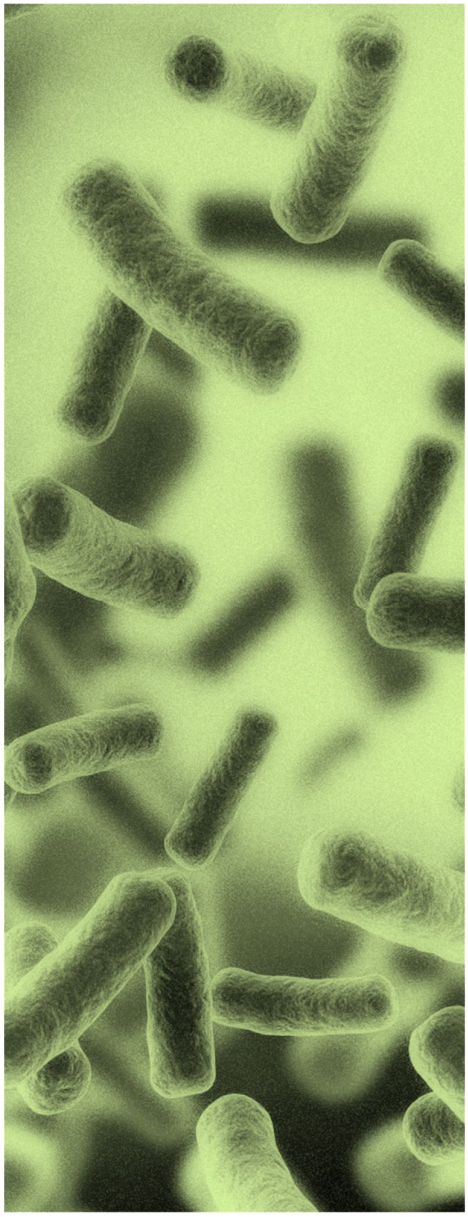
Despite the benefits provided by this biopolymer, its production at industrial scale is expensive making them uncompetitive or unfeasible respect to petrochemical plastics. Improvements and optimizations must be made to the fermentation process to reduce the costs associated with the production. In this work, the study of nutritional and operational conditions to favor the production of PHB by *Halomonas boliviensis* using an inexpensive substrate was carried out.

The development of this work is presented in four chapters that comprise the overall objective of the research. The first chapter covers the introduction to the topic from the research problem, to the theoretical frame and state of the art leading to understanding what took us to perform this research work. The second chapter contains the first objective, which was to evaluate three inexpensive alternative nitrogen sources on cell growth, PHB production using *Halomonas boliviensis* considering that is the most expensive constituent of culture media per Kg, and that its cost affects the production economics. In the third chapter, the effect of dissolved oxygen concentrations at bioreactor scale on cell growth and PHB content attained by *Halomonas boliviensis* was studied to fulfill the second objective. The fourth chapter shows the construction of an unstructured model and parameter estimation of polyhydroxybutyrate production by *Halomonas boliviensis*.

Keywords: polyhydroxybutyrate, *Halomonas boliviensis*, production costs.

1

Introduction



1.1. Environmental problem of plastics

Since ancient times, human communities have been dependent on the environment to suit their fundamental needs, namely food, energy, and materials, performing adaptations or modifications in it to obtain their benefits (Naranjo, 2014). Energy sources and materials that we develop and use according to the conditions created and established by civilizations across time have shaped human history. Interestingly, however, human progress has also lead to the collapse of communities, often attributed to disasters in the environment and ecology (Mortazavi and Negari, 2010).

This is the case of the polymer industry that has experienced an explosion in its progress, initially substituting naturally-occurring products in clothing, decoration, shelter, tools, weapons, and other requirements (Larso, 2015), and currently providing high-quality options over other materials given their outstanding properties, versatility, and technical advantages, becoming essential materials in every scenario of our lives (Namazi, 2017). Nevertheless, widely used polymers, synthetic plastics, are derived from fossil feedstock, and its increasing use is related to consumerism and large production at industrial scale, due to low costs, availability, and manufacturing processes. As the manufacture of plastics also requires energy, its production is responsible for the consumption of a similar additional quantity of fossil fuels (Hopewell, Dvorak and Kosior, 2009).

Despite all their benefits, synthetic plastics are prompting a mounting environmental crisis, not only because of the pollution from processing but also due to their accumulation in landfills and oceans in large volumes (Yates and Barlow, 2013). According to data reported for 2016, the world plastic production was 335 million tonnes (PlasticsEurope and EPRO, 2018), from which estimations state that only around 20 % was recycled, 26 % was incinerated for energy recovery, and the rest were discarded in sanitary landfills, open dumps or released directly into the natural environment (Geyer, Jambeck and Law, 2017).

Plastics are typically chemically resistant, i.e. they degrade only slowly and thus its accumulation in the environment, harming living organisms in ocean environments by entangling or ingesting plastic wastes, and causing various health problems by direct physical action or from the release of chemicals contained within the plastics that interfere with physiological processes both in animals and humans (Rhodes, 2018).

Considering all the reasons above, the global market of plastic is gradually changing towards a sustainable production that looks for new technologies to enhance the use of renewable resources and biotechnological processes. This tendency is held on the rise of the environmental awareness, reflected in the increase of new strict environmental regulations around the world, and the introduction of biopolymers as a potential alternative to replace synthetic plastics in the market (Song *et al.*, 2009; Babu, O'Connor and Seeram, 2013).

1.2. Biopolymers

The term "biopolymer" is currently used for polymers produced from natural sources either chemically synthesized from biological material or entirely biosynthesized by living organisms (Smith, Moxon and Morris, 2016). According to the previous definition, the generally accepted categorization covers polymers that are biobased, biodegradable, or a mixture of both (Imre and Pukánszky, 2013; Enriquez, Kumar and Misra, 2016).

Biobased polymers are commonly defined as polymers produced from renewable resources, biomass in general, and include three different categories: (1) natural polymers, which are from biomass such as the agro-polymers from agro-resources, e.g., starch, cellulose, protein, lipids; (3) synthetic biopolymers, which are chemically synthesized using monomers obtained from agro-resources, e.g., poly(lactic acid); and (2) polymers produced by microorganisms, e.g., polyhydroxyalkanoates (Degruson, 2016).

According to ASTM, the term "biodegradable" refers to the capability of decomposition into carbon dioxide, methane, water, inorganic compounds, or biomass by the enzymatic action of microorganisms (Avérous, 2004). Therefore, we can only refer to biodegradable polymers as polymers that can be easily decomposed in the environment by microorganisms losing their initial properties, and those that are more resilient are attributed to as durable (Rhodes, 2018), and if it requires the use of light sun, heat, temperature, mechanical stress, or oxygen to reduce the polymeric chains of the polymer are referred to as oxo-degradable (Mora-Sanjuán, 2010).

With the aim of substituting the functionality of plastics from a petrochemical origin, as well as encourage the use of biobased and biodegradable polymers, polyhydroxyalkanoates (PHAs) are perceived as an attractive biomaterial because of their great versatility and range in their properties, from thermoplastic to elastomeric (Steinwandter, 2014).

1.3. Polyhydroxybutyrate

Polyhydroxybutyrate (PHB) is the most widely studied member of the PHA family and the first one that has been produced at industrial scale (Naranjo, 2014). PHB is a short chain length biopolymer synthesized by bacteria, as insoluble granules accumulated in the cytoplasm as a way to store excess carbon under limited or lack of essential nutrients, namely nitrogen, phosphorous, sulfur, and oxygen (Van-Thuoc *et al.*, 2008; Getachew and Woldesenbet, 2016; Marudkla *et al.*, 2018). Bacteria are capable of synthesizing hydroxyacyl-CoA from carbon sources incorporating the PHA constituent monomer to the polymer through the PHA synthase enzyme β-ketothiolase, which is responsible for the regulation of the PHB synthesis metabolic pathway, and it is inhibited by the free CoA, which is highly present in balanced conditions necessary for the cell multiplication stage (Trigueros *et al.*, 2017).

The first bacterium reported to accumulate PHB was the Gram-positive bacteria *Bacillus megaterium*, thenceforth the ability of several microorganisms to synthesize PHB has attracted the attention of scientists (Quillaguaman, Mattiasson and Hatti-Kaul, 2005). PHB is currently industrially produced by recombinant Escherichia coli strains by fed-batch cultivation (Philip, Keshavarz and Roy, 2007). High cell densities are achieved in the first stage; and in the second stage of the process, the PHB is accumulated inside cells by nutrient limitation (Tanadchangsaeng and Yu, 2012). After extraction from the cells, PHAs possess common features of non-toxic, biocompatible, biodegradable, and recyclable thermoplastics. Thus, they are crystalline, optically active, piezoelectric, and insoluble in water; and the wide diversity of monomers found in PHAs provides a broad spectrum of polymers with varying physical properties. These features render them highly competitive with polypropylene or other petroleum-derived plastics (Van-Thuoc *et al.*, 2008).

There are different alternatives to improve the PHB fermentative processes, standardization, and selection of microorganisms of interest, main requirements of raw materials used, operation regime and the technological configuration for the production of PHB production (Yamin *et al.*, 2016).

1.4. Polyhydroxybutyrate production by *Halomonas boliviensis*

Since the last decade, microorganisms from the lake Laguna Colorada in the Andean region of Bolivia have been studied; one of those has been the halophilic microorganism *Halomonas boliviensis* (*H. boliviensis*) (Quillaguamán *et al.*, 2004; Van-Thuoc, 2009).

H. boliviensis is a moderately halophilic bacterium, aerobic, motile, Gram-negative, with rod-shaped cells that tolerate a wide range of NaCl concentrations (0-25 % w/v), temperature (0-45 °C), and pH (6-11) in its culture medium. The optimal concentration of NaCl for cell growth is between 4-5 % (w/v). The colonies are circular with undulate margins, convex and have a cream pigmentation enhanced in old cultures. *H. boliviensis* belongs to genus *Halomonas*, family *Halomonadaceae*, order *Oceanospirillales*, and class *Gammaproteobacteria* (Quillaguaman, Mattiasson and Hatti-Kaul, 2005; Van-Thuoc *et al.*, 2008).

Quillaguamán and co-workers were the first to establish that *H. boliviensis* can grow intracellular granules of PHB in a medium containing an excess of carbon, and a limitation of nitrogen source provided by a low amount of yeast extract. They found that NaCl concentration of 4.5 % (w/v) provide high PHB accumulation (54 wt.%), and higher salt concentrations decrease the growth rate of microorganism, which retards PHB accumulation. They found that the combination of 0.8 % (v/v) butyric acid and sodium acetate 0.8 % (w/v) as carbon source lead to a maximum value of 88 wt.% of accumulated PHB, which is the highest obtained thus far (Quillaguamán *et al.*, 2006).

Quillaguamán et al. performed a study for the PHB production by *H. boliviensis* using an alternative carbon source: 0.8 % w/v hydrolyzed starch. According to the effect of nutrients, they found that: the addition of peptone decreased the PHB and cell mass concentration after 21 h of cultivation, the increase of phosphate concentration gave low PHB accumulation but high cell growth, and the use of glucose prevented the PHB degradation. Moreover, the complete restriction of air supply at the cultivation stage leads to an inhibition on the metabolism of the cells resulting in low PHB formation. However, greater enhancements were attained when oxygen limitation was induced in the fermenter, but it is necessary to supply high air inflow and adequate oxygen transfer rate for optimal growth of *H. boliviensis* until PHB accumulation is initiated (Quillaguamán *et al.*, 2005).

In 2007, Quillaguamán and colleagues studied the production of PHB by *H. boliviensis* in batch cultures at high cell concentrations using sucrose as carbon source. They found that a high concentration of yeast extract is necessary for high cell growth but needs to be combined with a low phosphate concentration to increase PHB formation. They concluded that the crucial factors for optimal cell growth and polymer production by *H. boliviensis* are: adequate carbon and complex nitrogen sources, and oxygen depletion during the process (Quillaguamán *et al.*, 2007).

Van-Thouc and collaborators used hydrolyzed wheat bran as the carbon source for the PHB production by *H. boliviensis*, but they found that it led to a low PHB content (34 wt.%) after 30 h. In addition, PHB accumulation was improved by reducing the amount of yeast extract in the medium, although this effect was influenced by the high protein concentration from the hydrolysate, which provided, besides carbon, nitrogen for cells (Van-Thuoc *et al.*, 2008).

In 2008, Quillaguamán et al. carried out a study for the PHB production by *H. boliviensis* in fed-batch culture. In this research, they found that only three amino acids, i.e., aspartic acid, glycine, and glutamine induce *H. boliviensis* growth, obtaining high results on cell dry weight (5.3 g/L) using glutamine at concentration of 0.2 % (w/v). The effect of initial concentrations of NH_4Cl and K_2HPO_4 on cell growth and PHB accumulation by *H. boliviensis* was then analyzed using a fed-batch fermentation system, resulting best conditions 0.4 % (w/v) NH_4Cl and 0.22 % (w/v) K_2HPO_4, and adding monosodium glutamate intermittently to the fermenter. PHB and cell content reached 90 wt.% and 23 g/L, respectively, after 18 h of cultivation. They indicate that phosphate salts do not have a determining role in the PHB accumulation (Quillaguamán *et al.*, 2008).

When Nitrogen is limited in the culture media of *H. boliviensis* has led to a high PHB content; however, it is necessary to consider that in many cases depletion of nitrogen may decrease the ability to store PHB or even lose this ability almost completely (Van-Thuoc, Guzmán, Thi-Hang, *et al.*, 2010) (Johnson et al., 2010). According to this, nitrogen limitation plays a pivotal role in the PHB production, as has been stated by several researchers.

In 2012, Gnanasekhar studied the production of PHB from *Halomonas boliviensi*s, where they corroborate the PHB accumulation by nitrogen-limited cultivation. They carried out fed-batch cultivation to determine the possibility of growth phase PHB accumulation in *H. boliviensis* in a rich and minimum medium with yeast extract, obtaining higher amounts of PHB accumulation in the rich cultivation (Gnanasekhar, 2012).

In 2015, Rivera-Terceros and collaborators studied the production of PHB by *Halomonas boliviensis* in an airlift reactor. This paper reports the production of PHB in shake flasks by *H. boliviensis* using different combinations of carbohydrates and partially hydrolyzed starch as carbon sources. Highest PHB yields were in the range of 56 and 61 wt.% when either starch hydrolysate or a mixture of glucose and xylose were used as carbon sources, the highest amount of PHB, 41 wt.%, was attained after 24 h of cultivation (Rivera-Terceros *et al.*, 2015).

In 2016, García-Torreiro et al. studied the effect of nitrogen and oxygen concentrations on PHB accumulation by *H. boliviensis* and examined their behavior during growth in fed-batch culture. Single limitations of nitrogen and oxygen provoked PHB accumulations of 45 and 37 wt.%, respectively, while N limitation with low O_2 supply caused the highest PHB accumulation of 73 wt.%. Oxygen starvation regulates PHA metabolism by the inhibition of the tricarboxylic acid cycle (TCA), which is the most important competing pathway for PHB route (García-Torreiro, Lu-Chau and Lema, 2016).

1.5. Polyhydroxybutyrate production in Colombia

Despite the environmental problems and the world tendency to replace conventional plastics with eco-friendly polymers, in Colombia, the production of plastics with biodegradable properties is not been held. However, the government has made some efforts to encourage the defense of nature and changes in the production and consumption patterns of the Colombian society. In 2017, an excise tax by the Decree 2198 was declared to discourage and reduce the consumption of plastic bags and encouraging the production of biodegradable and reusable plastic bags (Ministerio de hacienda y crédito público, 2017). Having the support of Colombia government is necessary to propel the production of biopolymers at industrial scalene considering all the advances that have been developed thus far.

1.5.1. Identification of PHB producing strains

The study of the production of polyhydroxybutyrate in Colombia started in 1997 when the *Instituto de Biotecnología* from *Universidad Nacional* (IBUN) began with the identification of potential bacteria to produce biopolymers, highlighting three species of *Pseudomonas putida*. Other studies performed by researchers of this institution have focused on the bioprospecting of high producer microorganisms in Colombian soils by isolation and selection of native strains able to accumulate PHA (Moreno *et al.*, 2007; Becerra-Jiménez, 2013; Montoya-Castaño *et al.*, 2017).

The research group of *Producción, Estructura y Aplicación de Biomoléculas* (PROBIOM) from Universidad Nacional de Colombia, Medellín campus, have studied the isolation of bacteria as an environmentally sustainable alternative for the management of agro-industrial wastes and their potential income source by the production of PHB. Soils contaminated with fique industry wastes in the municipality of Guarne (Antioquia) led to the isolation of two bacterial morphotypes identified as *Bacillus megaterium* with the potential to produce PHB

with levels between 63.8 mg/g and 95.3 mg/g in the fermentation test when using glucose as carbon source (Sánchez *et al.*, 2012).

1.5.2. Genetic modifications

In the University of the Andes, studies have focused on the production of PHB with genetic modifications of the widely known PHB producer strain *Cupriavidus necator* (Bojacá-Bautista, 2012; López-Tamayo, 2014; Zuñiga-Burgos, 2016).

IBUN has performed different advances regarding to different techniques to increase PHA accumulation (Montoya-Castaño *et al.*, 2017). In 2007, developed a most effective method for selecting a broad range of PHB producing microorganisms (Revelo-Romo *et al.*, 2007).

1.5.3. Evaluation of alternative carbon sources

In 2005, Barbosa et al. studied the influence of initial concentrations of commercial fructose as carbon source on wild strain *Ralstonia eutropha* ATCC 17697 to produce PHB, using a two-stage fed-batch fermentation system in a 3L bioreactor at 30°C and 300 rpm. The best results for producing PHB were obtained when using 5 g/l of initial fructose concentration, from which an accumulation of 66.2% PHB and a 0.1245 g PHB/ l h productivity (Barbosa *et al.*, 2005).

In 2010, Naranjo et al. studied the production of PHB using a commercial strain *Cupriavidus necator* NCIMB 11842 with industrial glycerol as carbon source reaching up to 55.99% of PHB accumulation, 8.73 g/L of PHB concentration, PHB productivities of 0.024-0.1068 g/Lh variating initial concentrations of glycerol at 200 rpm for 96 hours. Higher initial concentrations of glycerol (50 g/L) and higher temperatures increased PHB productivity for both microorganisms (Naranjo-Vasco, 2010).

In 2011, Cardona-Betancur studied the production of PHB using *Cupriavidus necator* ATCC 17699 using hydrolysates of cassava flour of the "copiblanca" variety from Mutatá (Antioquia) and rejected banana of the "gran enana" variety from Apartadó (Antioquia). Carbon/Nitrogen ratios were evaluated at flask scale to establish the proper conditions for the obtaining of PHB, and at 3L bioreactor scale was validated the production. Results indicated higher concentrations of biopolymer using C/N=20 with rejected banana obtaining up to 5.96 g/L of PHB at bioreactor scale, at flask scale 3.43 and 2.58 g/L of PHB were obtained using rejected banana and cassava flour, respectively (Cardona-Betancur, 2011).

In 2013, Becerra-Jiménez studied the production of polymer PHA type by *Burkholderia cenocepacia* 2G-57 using wastes from biodiesel industry, after performing characterization results showed that this strain only accumulates PHB when using this type of carbon source (Becerra-Jiménez, 2013).

In 2014, Salazar et al. studied carob pulp and fique juice using a *Bacillus megaterium* for the PHB production. Results confirmed the synthesis of PHB using carob pulp (from *Hymenaea courbaril*) and suggested that PHB production could be as high as with sugar cane molasses. Characterization confirmed the presence of hydroxybutyric (HB) monomers in all samples except in those from fique juice indicating that this carbon source is not suitable for the PHB production, although enhanced pretreatments should be considered to increase the efficiency of fique juice as a substrate (Salazar *et al.*, 2014).

Rojas-Betancourt studied at Erlenmeyer-flask the production of PHB using wastewaters from a juice industry after anaerobic digestion, obtaining 1.3 g/L of PHB and 65% accumulation at 72 hours, 30 °C, 150 rpm using *Cupriavidus necator* (Rojas-Betancourt, 2014).

In 2015, Álvarez performed the analysis of PHB production using glycerol, lactose and milk whey from Colombian agribusiness. Results regarding PHB accumulation showed 62, 76, and 70% percentages of intracellular PHB by glycerol, lactose and milk whey, respectively. A Chlorella vulgaris cake was proposed as an alternative for PHB production due to the economic feasibility regarding to the production costs of $ 8.8 USD/kg for PHB (Álvarez, 2015).

In 2016, Rojas et al. synthesized PHB by bacterial fermentation with *Ralstonia eutropha* using hydrolyzed HMC1 cassava flour from Santander de Quilichao considering the food production chain of the Cauca region and their low cost as raw material. Fermentation was performed at 30° C, 150 rpm for 36 hours, using ammonium sulfate as the nitrogen source, which was considered the limiting nutrient. Results showed a carbon/nitrogen ratio of 20 is the most favorable condition for the production of PHB with a concentration of 0.62 g/L (Rojas-Fernández, Hoyos-Concha and Mosquera-Sánches, 2016).

In 2018, Acosta-Cárdenas et al. studied the production of PHB by *Ralstonia eutropha* ATCC 17699 using different ratios of a mixture consisting of cane molasses and residual vinasse from the alcohol industry as substrates. Results showed the potential of the mixture as a culture medium, after reaching polymer concentrations of 2.71 g/L with a molasses/vinasse ratio of 25/75, and a biopolymer accumulation of 97.8% with respect to the biomass produced,

characterization confirmed a correlation with the standard polyhydroxybutyrate sample - PHB of 99.25% (Acosta-Cárdenas, Alcaraz-Zapata and Cardona-Betancur, 2018).

1.5.4. Optimization of operating conditions

In 2002, Barbosa implemented a fermentation strategy by fed-batch system using *Ralstonia eutropha H16* in order to increase the productivity of the biopolymer (Barbosa, 2002). IBUN has performed biopolymer production in fermenters with volumes of 3, 5, 7.5, and 100 L, to establish protocols for the handle and evaluation of PHA producing microorganisms (Mora-Sanjuán, 2010). In association with Biopolab Company, have developed a pilot semi-industrial plant consisting of a 2000 L bioreactor and a separation and purification system, evaluating physical and thermal properties of the obtained biopolymer (Montoya-Castaño *et al.*, 2017).

Naranjo-Vasco studied the efficient process design and analysis of the potential implementation of the PHB production from agro-industrial wastes in Colombia. Simulation procedures based on experimental experiences were used to evaluate the yield of different technologies for the pretreatment of raw materials, fermentation and extraction processes to reduce the costs related to the production of PHB and the environmental impacts. A decrease in the PHB production costs was reached when these microorganisms were adapted to non-conventional substrates and mass and energy integrations into a biorefinery. Interestingly, a high-energy demand during the PHB separation stage was increasing the production costs (Naranjo-Vasco, 2010).

1.5.5. Recovery methods

Studies performed by IBUN have focused on recovery methods and characterization of synthesized biopolymer (Becerra-Jiménez, 2013; Montoya-Castaño *et al.*, 2017). In 2001, were evaluated different recovery methods for the synthetized PHA's from *Pseudomonas* (Malagón & Cortazar, 2001).

1.5.6. Applications

In 2008, the use of PHB granules led to the novel process of toxins liberation using a Bacillus thuringiensis strain, giving another important application to environmental solutions, in this case to the control of plagues (Salazar-Martínez, 2010).

1.5.7. Legal aspects

A remarkable study related to the production of PHB and the judicial and legal limitations of the commercial application for the biopolymer in the agricultural area in Colombia was published. There are factors blocking the business development of the biopolymer, related to the processing time and the forms and information requested by the correspondent ministry, due to the use and manipulation of microorganisms. However, the Colombian government promotes and gives tax benefits for the development of companies and sustainable products using biotechnology-based applications (Mora-Sanjuán, 2010).

1.6. Aims and Scope

The purpose of this research is to determine the optimized nutritional and operational conditions for polyhydroxybutyrate production by *Halomonas boliviensis* considering its economic feasibility in Colombia. To achieve this, the effect of alternative nitrogen sources and oxygen supply on the cell growth and PHB accumulation, and an unstructured model to predict the behavior of the microorganism at different conditions will be evaluated.

1.6.1. Overall Objective

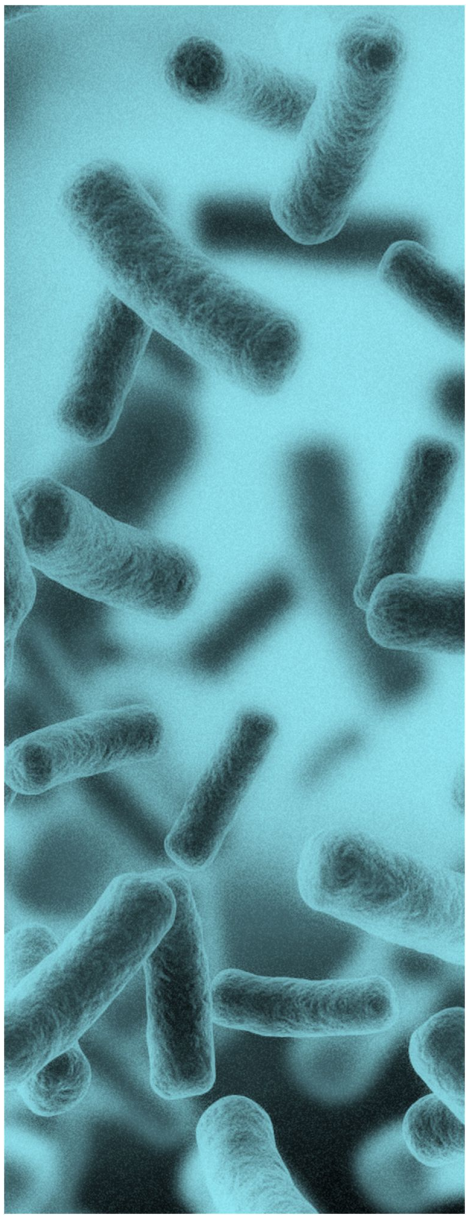
Determine nutritional and operational conditions for polyhydroxybutyrate production by *Halomonas boliviensis* towards the modelling of the process.

1.6.2. Specific Objectives

- Evaluate the effect of alternative nitrogen sources on cell growth and PHB production using *Halomonas boliviensis*.
- Determine the effect of dissolved oxygen concentrations at bioreactor scale on cell growth and PHB content attained by *Halomonas boliviensis*.
- Adjust an unstructured model for *Halomonas boliviensis* according to the oxygen supply in a bioreactor in terms of PHB productivity (g PHB/Lh).

2

Evaluation of inexpensive complex nitrogen sources on cell growth and PHB production by *Halomonas boliviensis*



Abstract

Biodegradable polymers have shown to be an excellent alternative to replace petrochemical plastics considering their exceptional mechanical and thermal properties, taking advantage of the ability of some microorganisms to synthesize biopolymers like PHB. However, the major drawback for PHB is its high production costs; therefore, the purpose of this research was to use an effective microorganism supplied with an inexpensive nutrient source available in the Colombian market to induce higher amounts of biomass and biopolymer to accomplish a competitive commercial cost.

The moderately halophilic bacteria *H. boliviensis* is an efficient microorganism capable of accumulating PHB in high amounts, and its growth is induced by three amino acids, i.e., aspartic acid, glycine, and glutamine (Quillaguamán *et al.*, 2008). Considering this, the effect on cell growth and PHB production by *H. boliviensis* using three proteins: Soluble Soy Protein Isolate (SISP), Wheat Gluten Protein (WGP), and Whey Protein (WP), was evaluated in this research. The enzymatic hydrolysis of protein was performed using a protease (Savinase 16L) to break peptide bonds and solubilize the culture media. PHB production was carried out at flask scale, 30 °C, 200 rpm during 48 h.

Results showed biomass concentrations of 1.8, 4.9, and 1.5 g/L, and biopolymer production of 0.6, 0.9, and 0.6 g/L, by SISP, WGP, and WP, respectively. These results are promising as the addition of cheap nutrient sources in culture media can make the production of PHB economically feasible at industrial scale and thus become the substitute for conventional plastics.

2.1. Introduction

Bacterial growth is affected by several conditions such as temperature, aeration, salinity, and pH. However, the availability of a suitable nutrient source in the medium is a must, primarily needing a carbon, nitrogen, and phosphorus source to favor cell building and growth (Bhattacharya, Dev and Das, 2018).

At laboratory scale, the components of the culture media come from commercial houses that provide pure preparations but are very expensive in the local market (Andualem and Gessesse, 2013), therefore, when scaling up the process these preparations represent a high impact on expenses for companies. In the case of PHA production, the carbon source is the major

component affecting the production costs, about 40 % depending on the requirements and yields of microorganisms, but also other factors affecting fermentation economics are oxygen and complex nitrogen sources (Choi and Lee, 1999). The bulk of researchers have focus their attention on reducing the cost of production using different waste materials as carbon source (Raza, Abid and Banat, 2018), but few authors have turned the spotlight on the nitrogen source (Page and Cornish, 1993; Lee and Chang, 1994; Purushothaman *et al.*, 2001; Koller *et al.*, 2005(a); Koller *et al.*, 2005(b); Vijayendra *et al.*, 2007). The nitrogen source is, actually, the most expensive medium constituent (per kg) of bacterial growth substrates (Taskin and Kurbanoglu, 2011; Andualem and Gessesse, 2013). Hence, besides the carbon source, the availability of cheap complex nitrogen sources for effective and fast biomass production is advantageous (Koller *et al.*, 2010).

In the production of PHB, different sources of nitrogen can be utilized by bacteria for cellular growth, from defined reactants and amino acids to complex nitrogen sources (Steinwandter, 2014). The selection of the nitrogen sources might depend on the requirement of the microorganism and the needs of the production process, and possibly other factors as purity, economy, solubility, quality, yields, and productivity.

The aerobic bacteria *H. boliviensis* has a high accumulation percentage of PHB (up to 80 wt.%) in response to physiological stress by the nitrogen and oxygen supply (García-Torreiro, Lu-Chau, & Lema, 2016). The conventional nitrogen sources used by this bacterium are yeast extract, peptone, and NH_4Cl; however, the essential nitrogen source for *H. boliviensis* that would allow an optimal PHB production, are the ones that have a high content in three amino acids: aspartic acid, glycine, and glutamine that induce *H. boliviensis* growth (Quillaguamán *et al.*, 2008). Among all, glutamine has the highest impact on cell growth, but its use as reactant is limited due to its poor solubility and high purchase costs (Quillaguamán *et al.*, 2008). For that reason, monosodium glutamate (MSG) came up as an economical replacement solution, because the enzyme glutamine synthetase can use MSG and ammonia to synthesize glutamine, and there are no significant differences on cell growth by using it (Balderrama-Subieta and Quillaguamán, 2013).

Although MSG has been reported as five times cheaper than yeast extract (Quillaguamán *et al.*, 2008), in the Colombian market costs are quite similar, comparison on the purchase costs of several nitrogen sources in the country is shown in Table 1.

Table 1. Purchase costs of different nitrogen sources in the Colombian market

Nitrogen source	Cost (COP/Kg)
Yeast extract	$ 630,700
Peptone	$ 867,200
NH_4Cl	$ 261,800
Monosodium glutamate	$471,200
Aspartic acid	$1'085,300
Glycine	$1'038,100
Glutamine	$ 1'220,600
$(NH_4)_2SO_4$	$219,292

Source: elaborated by the author from prices provided by commercial houses, suppliers of Universidad de Antioquia (Importecnical S.A.S., Laboratorios Medellín LTDA, BLAMIS S.A.S., Filtración y Análisis S.A.S.)

Complex nitrogen sources such as yeast extract and peptone are quite expensive in the Colombian market, therefore they are not recommended for the production of bulk products, but are employed because they can lead to fast and good PHA accumulation due to its amino acid and peptide content (Steinwandter, 2014). Even though NH_4Cl is the cheapest nitrogen source from Table 1, cell growth of *H. boliviensis* with only NH_4Cl is limited, and the presence of soluble amino acids are a must in the culture medium (Balderrama-Subieta and Quillaguamán, 2013). Considering the above, this study evaluated three inexpensive complex nitrogen sources: SISP, WGP, and WP, that have high amino acid content (Table 2) that promotes cell growth and PHB production by *H. boliviensis.* Commercial costs of these proteins are $20,000 and $28,000 COP/kg for SISP and WGP, respectively, WP is a waste from the dairy industry without commercial cost nowadays.

Table 2. Amino acid composition of proposed nitrogen sources

Amino acids	**SISP (%)** (Kalman, 2014)	**WGP (%)** (FAO, 2013)	**WPC (%)** (Kalman, 2014)
Alanine	3.6	1.6	4.9
Arginine	6.7	1.9	2.1
Aspartic acid	***10.2***	***1.9***	***10.8***
Cystine	1.0	1.3	2.1
Glutamic acid	***17.5***	***23.4***	***16.7***
Glutamine[1]	***17-20***	***36***	***5-10***
Glycine	***3.6***	***2.0***	**1.8**
Histidine	2.3	1.4	2.2
Isoleucine	4.3	2.6	5.8
Leucine	6.8	4.3	10.2
Lysine	5.3	0.9	9.6
Methionine	1.1	1.0	1.9
Phenylalanine	4.6	3.2	3.3
Proline	5.0	8.3	5.8
Serine	4.6	3.1	4.7
Threonine	3.1	1.6	7.2
Tyrosine	3.2	2.3	1.8
Valine	4.1	2.7	5.8

[1] Tomado de (Flambeau, Redl and Respondek, 2016)

2.2. Materials and Methods

2.2.1. Microorganism and culture media

Inoculum was prepared by suspending 3 colonies in a 500 mL-flask with 100 mL of liquid HM medium, containing (per liter): NaCl, 45 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.09 g; KCl, 0.5 g; NaBr, 0.06 g; K_2HPO_4, 0.55 g; peptone, 5 g; yeast extract, 10 g; and glucose, 1 g (Quillaguamán *et al.*, 2008). The seed culture was incubated at 33 °C for 12 h and 200 rpm in a rotary shaker incubator (Thermo Scientific MaxQ 6000).

Grown cells were suspended in 300 mL of culture medium in 500 mL-flasks. Culture media containing (per liter): NaCl, 45 g; $MgSO_4 \cdot 7H_2O$, 0.38 g; $CaCl_2 \cdot 2H_2O$, 0.13 g; NaBr, 0.2 g; KCl, 0.75 g; an initial glucose concentration of 10 g/L and C/N ratio of 20:1 g/L for each nitrogen source. Growth conditions were 33 °C with a rotary shaking of 200 rpm for 72 h.

2.2.2. Preparation of nitrogen sources

Soluble isolated soy and wheat gluten proteins were provided by INTAL (Institución de Ciencia y Tecnología Alimentaria) (Itagüí, Colombia), and whey protein by the dairy plant of Universidad Nacional (Medellín campus, Colombia). Proteins were solubilized separately by adding 1L of water and 1 mL of protease enzyme to 7.0, 8.5, and 9.0 g of SISP, WGP, and WP, respectively, at 70 °C, 400 rpm, and 12 h. The hydrolyzed protein mixtures were centrifuged at 5000 rpm for 10 min, filtered with 0.2 µm cellulose filter and stored at -70 °C.

2.2.3. Analytical Methods

Determination of cell growth - Biomass Quantification

The concentration of biomass was determined by absorbance at 600 nm using a BioTek™ Synergy™ H1 Microplate Reader. The spectrophotometric absorbance was converted to biomass concentration using an established calibration curve of bacterial biomass applying the Cell Dry Weight (CDW) method (Wang, 2007).

Measurement of sugars

To determine the amount of carbon substrate in the media, reducing sugars in samples were quantified by applying the dinitro-salicylic acid (DNS method) (Miller, 1959). The specific

glucose consumption was measured by the glucose oxidase method using a Biosystem S.A. kit, the concentration of glucose was correlated with absorbance at 500 nm using BioTek™ Synergy™ H1 Microplate Reader. Measurements by DNS and glucose oxidase methods were verified by HPLC system (Agilent Technologies 1200 series) using a CARBOSep COREGEL-87P sugar column and refractive index detector (Turhan, 2014).

Protein Content

Biuret test was used to determine the protein content of the different nitrogen sources at the pretreatment stage, supernatant samples were measured at 540 nm after 20 min of reaction with biuret reactant.

Extraction and quantification of polyhydroxybutyrate

The PHB extraction from centrifuged cells was made by an established solvent methodology (Salmiati *et al.*, 2009), applying a 1:2 ratio of chloroform and sodium hypochlorite at 5 % during 180 min, 40 °C, and 200 rpm. After the treatment, a three-phase suspension was obtained by centrifugation at 15000 rpm for 10 minutes. PHB was precipitated using three volumes of methanol, then washed with distilled water and dried at room temperature (Aramvash, Moazzeni Zavareh and Gholami Banadkuki, 2018).

PHB from *H. boliviensis* was quantified by spectrophotometry, applying the crotonic acid method by Slepecky & Law. The UV absorbance was read at 235 nm and contrasted to a calibration curve made with commercial PHB from Sigma-Aldrich (Gnanasekhar, 2012).

2.2.4. Statistical analysis

Analysis of Variance (ANOVA) and analysis of multiple comparisons by the Honestly Significant Difference (HSD) method proposed by Tukey was employed to analyze the results. Calculations were made using the statistical software STATGRAPHICS Centurion XVII v.17.0.16 (Statpoint Technologies, Inc, Virginia, USA).

2.3. Results and discussion

2.3.1. Preparation of culture media

Conventional nitrogen sources, as peptone, yeast extract, chloride ammonium, among others, have a high solubility in water; therefore, its addition to the culture media is no different than

that of the other nutrient components, giving a clear-transparent media. However, working with alternative nitrogen sources that do not have a reactant grade, implies an additional step to obtain an adequate and work-full culture media.

The proposed nitrogen sources are proteins with characteristics that affect their addition to the media. Originally, nitrogen sources were supplied without any pretreatment, but the solubility of the complex proteins was low and affected the biomass measurement by spectrophotometry, due to the incidence of turbidity in the media. The solubility of proteins is affected by different factors such as pH, salinity, temperature and the presence of other chemical compounds. Table 3 shows the conditions to increase the solubility of proposed proteins in the culture media. These conditions were tested with no significant improvements and remaining insoluble protein in the media.

Table 3. Conditions for solubility of nitrogen sources

Solubility conditions	**Wheat gluten** (Ortolan and Steel, 2017)	**Soy protein** (Lee, Ryu and Rhee, 2003)	**Whey protein** (Wijayanti, Bansal and Deeth, 2014)
pH	↓6,45	↑6 (7)	↑6 (6)
Temperature (°C)	↓75 (55)	↓80 (50)	↓85 (70)
Salinity	↑0.5 M	↓0.1 M (0)	↓0.1 M (0)

↓ Values below increase the solubility ↑ Higher values increase the solubility
Values in parenthesis are optimum conditions favoring solubility of the protein.

A pre-treatment was developed to obtain a clear-transparent culture media and easier availability of the nitrogen source for the microorganism. A protease enzyme (Savinase 16L) was supplied to break peptide bonds and solubilize amino acids, peptides, and proteins in the solution, and then the protein content was measured. Results of protein concentration with and without enzymatic hydrolysis after they were centrifuged and filtrated are shown in Figure 1.

Figure 1. The concentration of protein of nitrogen sources with and without enzyme

2.3.2. Evaluation of nitrogen sources on cell growth and PHB production

To decrease costs related to raw materials required for PHB production, three inexpensive complex nitrogen sources were evaluated to induce cell growth and PHB productivity by *H. boliviensis*. The remaining components of culture media were equally supplied for each trial, the C/N ratio was established to initiate at 5.0±0.6 g/L as shown in figure 2 in order to favor the biomass growth in the early hours, and it was not controlled in order to let the protein act freely and thus know the behavior of the microorganism throughout the kinetic.

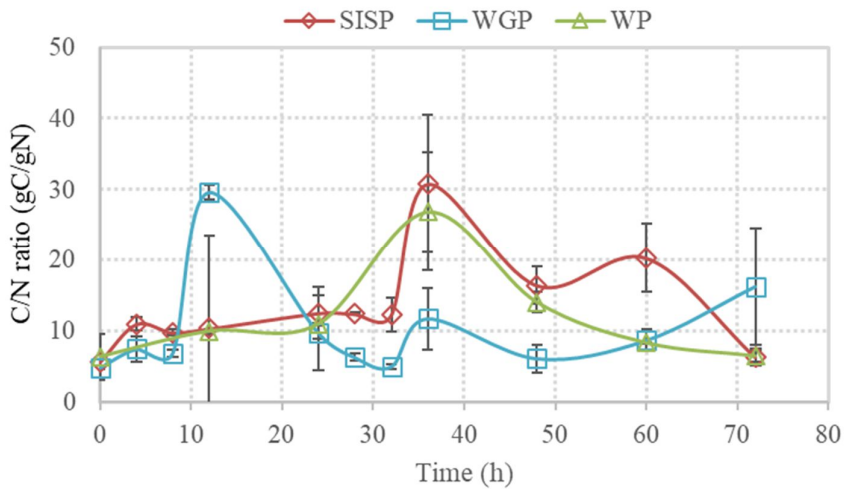


Figure 2. C/N ratio during PHB production using SISP, WGP and WP.

Figure 3 represents the kinetics of biomass growth, glucose consumption, and PHB production for A) soluble isolated soy protein, B) wheat gluten protein, and C) whey protein. Cell growth shows similar behavior for SISP and WP, reaching up to 1.82 and 1.45 g/L respectively, and then reaching a stationary phase at 24 h. However, during almost all fermentation times, WGP shows an exponential growth phase, reaching a maximum cell growth of 4.96 g/L. From the three proteins evaluated, WGP seems to be the one that favors the most *H. boliviensis* growth, and this could be related to the high content of glutamic acid in the protein, the precursor of glutamine, which is the main amino acid that induces its growth.

The glucose consumption showed similar tendencies for the three proteins, even an increase between the 28-32 hours of fermentation, which were confirmed by HPLC sugar analysis showed in annexes. In the case of WP, the presence of remaining sugars as lactose could also affect *H. boliviensis* growth (Quillaguaman, Mattiasson and Hatti-Kaul, 2005) as it cannot be used as a carbon source and perhaps can cause inhibition for its growth.

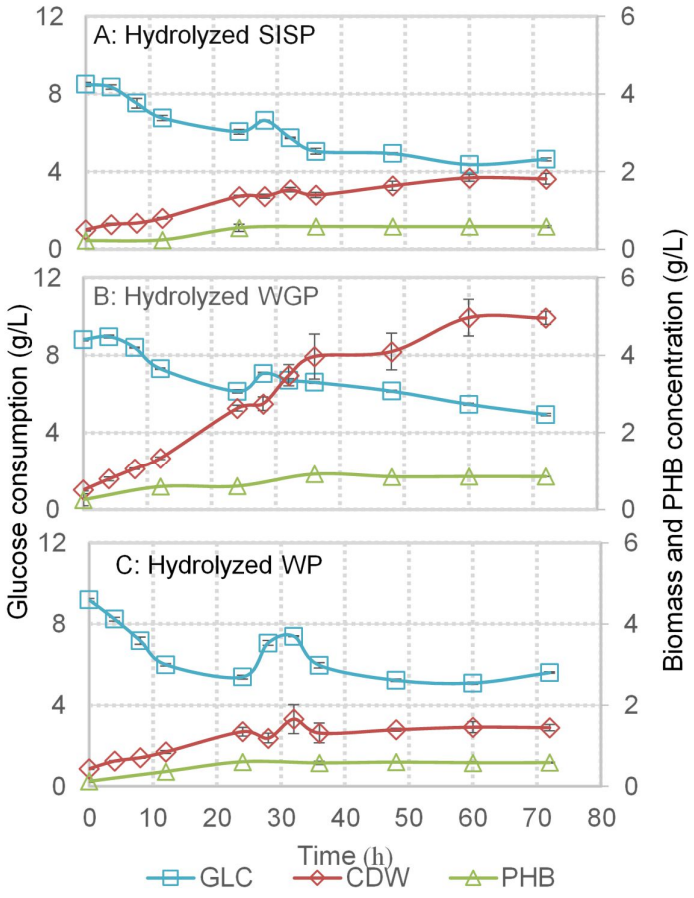


Figure 3. Kinetics of PHB production by *Halomonas boliviensis* at 33°C, 200 rpm, 72 h.

PHB production showed a similar trend for all proteins, as a secondary metabolite, it did not follow biomass growth; concentrations of 0.58±0.02, 0.86±0.00, and 0.59±0.01 g/L were obtained for SISP, WGP, and WP at 72 h. Even though WGP shows a higher concentration at the end of the fermentation time, it seems that *H. boliviensis* was focused on growing instead of producing PHB, which can be related to the amount of inducing amino acids still available in the culture media.

2.3.3. Kinetic parameters

Table 4 summarizes the kinetic parameters of nitrogen sources evaluated at 48 h, and the time when maximum concentrations of biomass and PHB were reached are indicated in parenthesis. The highest values of biomass parameters were obtained using WGP as the nitrogen source, even the best for PHB productivity and maximum concentration, but whey was found to be the most suitable nitrogen source to obtain a high yield and accumulation of PHB.

Table 4. Yield and productivity of biomass by *Halomonas boliviensi*s using alternative nitrogen sources

Kinetic parameters	SISP	WGP	WP
Max biomass concentration (g/L)	1.64±0.12	4.10±0.47	1.40±0.03
Biomass yield on substrate Y_{xs} (g/g)	0.42±0.10	0.97±0.24	0.30±0.03
Biomass productivity (g/Lh)	0.03±0.00	0.09±0.01	0.03±0.00
Max PHB concentration (g/L)	0.59±0.00	0.86±0.10	0.60±0.00
PHB yield on biomass Y_{px} (g/g)	0.31±0.01	0.15±0.05	0.44±0.03
PHB productivity (mg/Lh)	12.19±0.09	17.80±0.14	12.52±0.01
Max PHB accumulation (wt.%)	43.29±0.01 (36 h)	44.23±0.01 (12 h)	47.35±0.01 (24 h)

The numbers in brackets show the time at which those values were attained.

At flask scale, maximum biomass concentration for SISP and WP are similar to those reported by (Quillaguamán *et al.*, 2005, 2006), these authors used 1.0 g/L of yeast extract and 1.0/0.2 g/L of yeast extract/$(NH_4)_2SO_4$, respectively, as nitrogen sources. In the case of WGP, the highest cell mass obtained in this evaluation resulted to be to comparable to those obtained by (Quillaguamán *et al.*, 2008; García-Torreiro, Lu-Chau and Lema, 2016) who attained 5.1 and 5.3 g/L respectively, and used 1:20 g/L of NH_4Cl:MSG, and 2.0 g/L of Glutamine. Our results corroborate the importance of the addition of components with a high content of glutamic acid for *H. boliviensis* growth.

Regarding the maximum concentration of PHB obtained in this work, we can find similarities at flask scale in values reported by J. Quillaguamán et al., 2005; and Jorge Quillaguamán et al., 2008 between a range of 0.55-1.08 g/L, but it disparities for values between 3.5-5.8 g/L (Guzmán *et al.*, 2009, 2012; Rivera-Terceros *et al.*, 2015; García-Torreiro, Lu-Chau and Lema, 2016) using NH_4Cl and MSG as nitrogen sources. These results can suggest that glucose consumption was mainly used for biomass growth and not for producing PHB in the case of WGP, and in other conditions for maintenance.

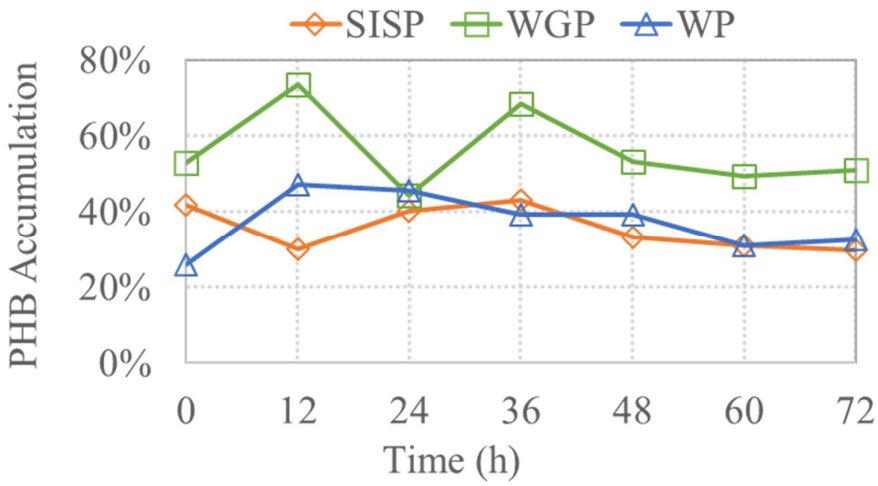


Fig 4. PHB accumulation by *Halomonas boliviensis* using SISP, WGP and WP hydrolysates.

Looking at the PHB accumulation percentage, the values obtained in this work are similar to those obtained by D. Guzmán et al., 2012; and D. Van-Thuoc et al., 2008 for a range of 44.9-54.0 wt.% using a concentration of 2 g/L of MSG and yeast extract, respectively, but distant with respect to 58.8-78 wt.% attained by (Quillaguamán *et al.*, 2005, 2007, 2008; Guzmán *et al.*, 2009; Rivera-Terceros *et al.*, 2015; García-Torreiro, Lu-Chau and Lema, 2016).

2.3.4. Statistical analysis

The results of ANOVA for the response variable of the final concentration of biomass (48 hours) showed that there is a statistically significant difference between the three nitrogen sources evaluated (P-value< 0.05). In addition, multiple range test by the Tukey HSD method showed that there is no statistically significant difference between SISP and WP, but it does with WGP. Therefore, the best nitrogen source evaluated in this study for *H. boliviensis* growth is hydrolyzed WGP.

ANOVA for PHB productivity (48 hours) showed that there is a statistically significant difference between the three nitrogen sources evaluated (P-value< 0.05). The Tukey HSD method showed that there is no statistically significant difference between using SISP or WP, but there is an impact when using WGP. Therefore, the best nitrogen source evaluated in this study for PHB production by *H. boliviensis* is hydrolyzed WGP.

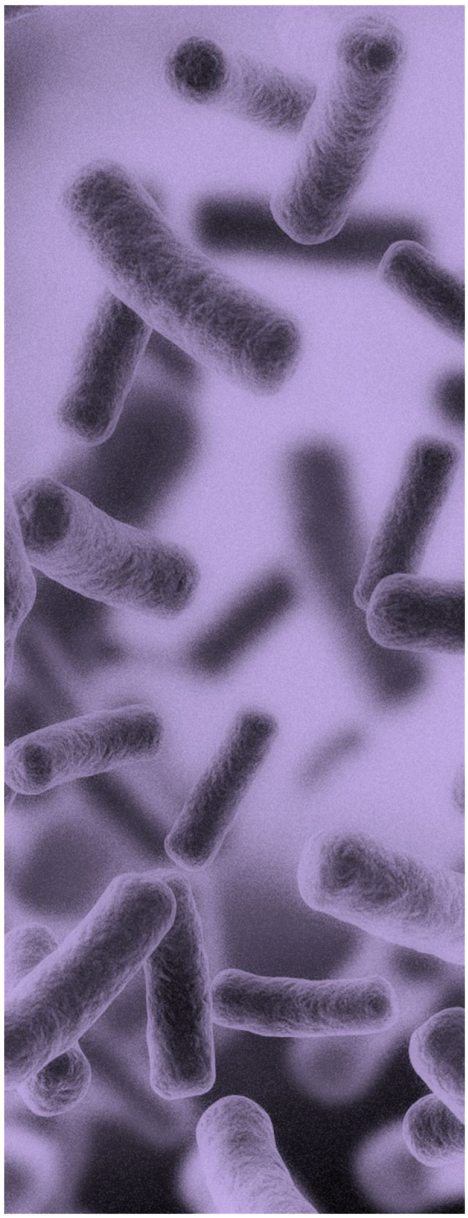
2.4. Conclusions

Results obtained in this chapter show the advantages of using alternative nitrogen sources for the cell growth and PHB production by *H. boliviensis*. From the three inexpensive nitrogen sources evaluated, hydrolyzed wheat gluten protein showed the best results for final concentrations of biomass and PHB productivity at 48 hours of fermentation time. Notwithstanding *H. boliviensis* grew instead of producing when using WGP, this study was carried out at flask scale where cell growth rate is low, therefore, supplied protein did not reach limiting values, hence PHB synthesis was not substantially induced. However, when working at bioreactor scale the velocity of cell growth is faster, thus, there is no need to consider a reduction in the protein amount supplied at this scale, this study will be developed in the next chapter.

Although the nitrogen source was thought to be supplied without any pretreatment, results indicated there is a need for using a protease enzyme to have a workable media. The additional cost of this enzyme it is considered and hydrolyzed WGP serves as an inexpensive nitrogen source indeed, reducing costs from the nitrogen source from 44 % up to 96 % comparing with the composition of culture media for *H. boliviensis*.

3

Evaluation of dissolved oxygen on cell growth and PHB content by *Halomonas boliviensis* using Wheat Gluten hydrolysate



Abstract

In aerobic bioprocesses, oxygen is an essential substrate; the oxygen transfer rate must be known, and if possible, predicted to achieve an optimum design operation and scale-up of bioreactors. The dissolved oxygen concentration in a suspension of aerobic microorganisms depends on the rate of oxygen transfer from the gas phase to the liquid, on the rate at which oxygen is transported into the cells, and on the oxygen uptake rate by the microorganism for growth, maintenance, and production (García-Ochoa and Gómez, 2009).

Results showed biomass concentrations of 10.74±0.37, 7.15±0.03, and 17.83±0.57 g/L and biopolymer production of 2.46±0.05, 2.00±0.11, and 4.49±0.06 g/L by k_La of 0.002, 0.001, and 0.006 s^{-1}, respectively at 72 h of fermentation time. These results are promising as the addition of cheap nutrient sources in culture media can make the production of PHB economically feasible at industrial scale and thus become the substitute for conventional plastics.

3.1. Introduction

For efficient large-scale production, produced organisms have to be cultivated under controlled conditions in closed bioreactors, where stability of process parameters (pH-value, temperature, oxygen supply, substrate concentration) can be warranted, and aseptic conditions are guaranteed by excluding microbial competitors (Koller and Braunegg, 2015). A bioreactor or fermenter is a vessel where biological reactions occur to obtain a higher production of metabolites or products of interest, depending on geometry, capacity and operating mode a variety of bioreactors are offered, from stirred tanks, airlift reactor, membrane bioreactors, fluidized bed reactors among others (Bobadilla-Cuberos and Ponce-Patiño, 2009).

Among operating factors, agitation and aeration are pivotal in aerobic fermentation bioreactors since they are of prime importance in industrial bioprocess and scale-up of aerobic biosynthesis systems because these factors provide a suitable environment for the growth of the host microorganism and obtain the optimal amount of the desired product (Saad *et al.*, 2014). Aeration has a significant effect in aerobic fermentations due to the low solubility of oxygen in the medium, affecting the oxygen supply to the broths because it could be not enough to meet the demand of the microorganisms growth rate (Karimi *et al.*, 2013). Agitation of culture is required to maintain uniform conditions throughout the bioreactors contents because bacterial cells aggregate near the base given that cell mass is heavier than liquid medium, and nutrients are limited (Ali *et al.*, 2015).

Oxygen supply plays a significant role in the scale-up and economy of aerobic biosynthesis systems. The volumetric oxygen mass transfer coefficient (k_La) is an important parameter since it is related to the oxygen transfer rate and is commonly applied as criteria for scale-up (Luvizetto-Faccin *et al.*, 2013). The oxygen supply in the production of PHB by *H. boliviensis* has been studied given the relevant role in the regulation of the PHB metabolism by the inhibition of the TCA cycle (García-Torreiro, Lu-Chau and Lema, 2016).

H. boliviensis are aerobic bacteria (Quillaguamán *et al.*, 2006), and like other microorganisms are entirely dependent on atmospheric oxygen for growth, therefore, for optimal growth, it is necessary to provide extensive aeration (Saiz-Güemes, 2015). However, it has been found that oxygen limitation enhances PHA production (Singh-Saharan, Grewal and Kumar, 2014). For that reason, different strategies have been tested to determine suitable conditions to produce PHB by *H. boliviensis*.

Quillaguamán et al. studied the influence of oxygen concentration by variations of air inflows and agitation speeds in a 2 L fermenter. The first strategy was to provide air inflow and agitation speed higher than 0.8 L/min and 700 rpm to avoid acidification of the medium and for optimal growth, later the air supply was restricted when oxygen uptake was not detected. Secondly, air inflow and mixing speed was increased and decreased gradually. Finally, air inflow and agitation speed were maintained constant. Results indicated that high air inflow and adequate oxygen transfer rate are required for optimal growth of *H. boliviensis* until the nitrogen source becomes limiting, at which PHB accumulation was initiated (Quillaguamán *et al.*, 2005).

Several authors working with the PHB production with *H. boliviensis* usually set agitation speed at 700 rpm which is the highest speed that can be provided by many commercial fermenters, air inflow was set to 0.5 or 1 L/min and increases of air inflow up to 4 L/min have been made when the percentage of dissolved oxygen was below the initial value (Quillaguamán *et al.*, 2006; Van-Thuoc *et al.*, 2008; Van-Thuoc, 2009; Van-Thuoc, Guzmán, Quillaguamán, *et al.*, 2010). Increasing of agitation speed of previously mentioned values, up to 900 and 1100 rpm was set by (Quillaguamán *et al.*, 2007; Van-Thuoc *et al.*, 2008).

In this context, the aim of this chapter is to evaluate the influence of dissolved oxygen on the cell growth and PHB production by *H. boliviensis* using wheat gluten hydrolysate through the comparison of cultures performed under different k_La obtained by variation of stirrer speed at bioreactor scale.

3.2. Materials and Methods

3.2.1. Microorganism and culture media

For PHB production in the bioreactor, inoculum was prepared as detailed in chapter 2, maintaining the initial biomass concentration of flask scale experiments. Grown cells were centrifuged using 50 mL Falcon tubes at 5000 rpm during 10 min to add concentrated cells without standard media, and thus not modifying the selected culture media with the alternative nitrogen source, wheat gluten hydrolysate.

Initially, hydrolysis of wheat gluten protein was done, adding 26 g of WGP to 1L of water and 1 mL of protease enzyme at 70 °C, 400 rpm, and 12 h to obtain a higher concentration of nitrogen and thus later dissolve on glucose solution, because these nutrients have to sterilize by separate to avoid Maillard reactions.

Fermentation was performed in a bioreactor (Bioflo 110, New Brunswick Scientific, United States) equipped with pH, dissolved oxygen, temperature, and foam probes. Temperature was maintained at 33 °C for 72 hours using a vessel jacket. 3 L bioreactor vessel with a working volume of 2 L was inoculated to start PHB fermentation. Antifoam was added before the beginning of fermentation and pH was maintained at 8.0 by using 0.3 M HCl/NaOH (Quillaguamán *et al.*, 2005).

3.2.2. Strategies to the evaluation of dissolved oxygen concentration

Oxygen supply strategies variated in accordance to the air inflow rate and agitation speed, obtaining different oxygen mass-transfer coefficients (k_La). Dissolved oxygen (pO2) was monitored online as the percentage of the maximum oxygen saturation attained at the initial conditions of aeration, agitation, and temperature. The dissolved oxygen percentage was measured with an oxygen electrode, Mettler Toledo DO sensor INPRO 6000, 12 mm x 220 mm.

Table 5. Aeration and agitation speed related to k_La at bioreactor scale.

Factor	Agitation speed	Aeration	k_La (s^{-1})
pO_2 % (1)	300 rpm	3 vvm	0.002
pO_2 % (2)	300 rpm	1 vvm	0.001
pO_2 % (3)	300 rpm	5 vvm	0.006

3.2.3. Quantitative analysis

Samples of 10 mL were collected at defined time intervals during the cultivation of *H. boliviensis* and were analyzed for CDW, PHB content, reducing sugars and glucose concentration, and protein content. All analytical method for the measurements of mentioned variables were performed as detailed in chapter 2.

3.2.4. Statistical analysis

Analysis of Variance (ANOVA) and analysis of multiple comparisons by the Honestly Significant Difference (HSD) method proposed by Tukey was employed to analyze the results. Calculations were made using the statistical software STATGRAPHICS Centurion XVII v.17.0.16 (Statpoint Technologies, Inc, Virginia, USA).

3.3. Results and discussion

3.3.1. Evaluation of oxygen transfer on cell growth and PHB production

To determine the proper operational conditions for PHB production by *H. boliviensis* using wheat gluten as the nitrogen source, the effect of dissolved oxygen on cell growth and PHB productivity was evaluated. For these experiments, the fermentation process was divided into two stages, the first one, when cell growth was induced by supplying air inflow and agitation speed at maximum default settings (5 vvm and 300 rpm) to avoid oxygen limitation for 24 hours. In the second stage, agitation speed was maintained constant at 300 rpm, and airflow rate was decreased, increased or maintained constant according to each trial, this stage was evaluated during 48 h.

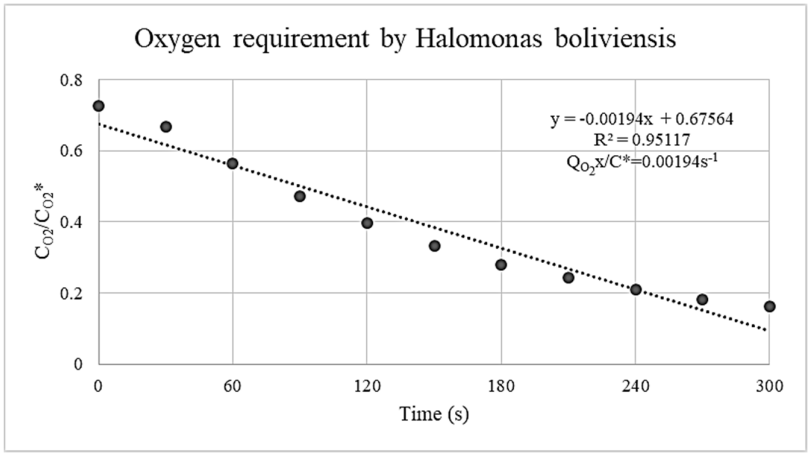


Figure 4. Oxygen consumption rate OD (%) of *Halomonas boliviensis*

The kinetics under different k_La conditions of cell growth, glucose consumption and PHB production are shown in Fig. 4.

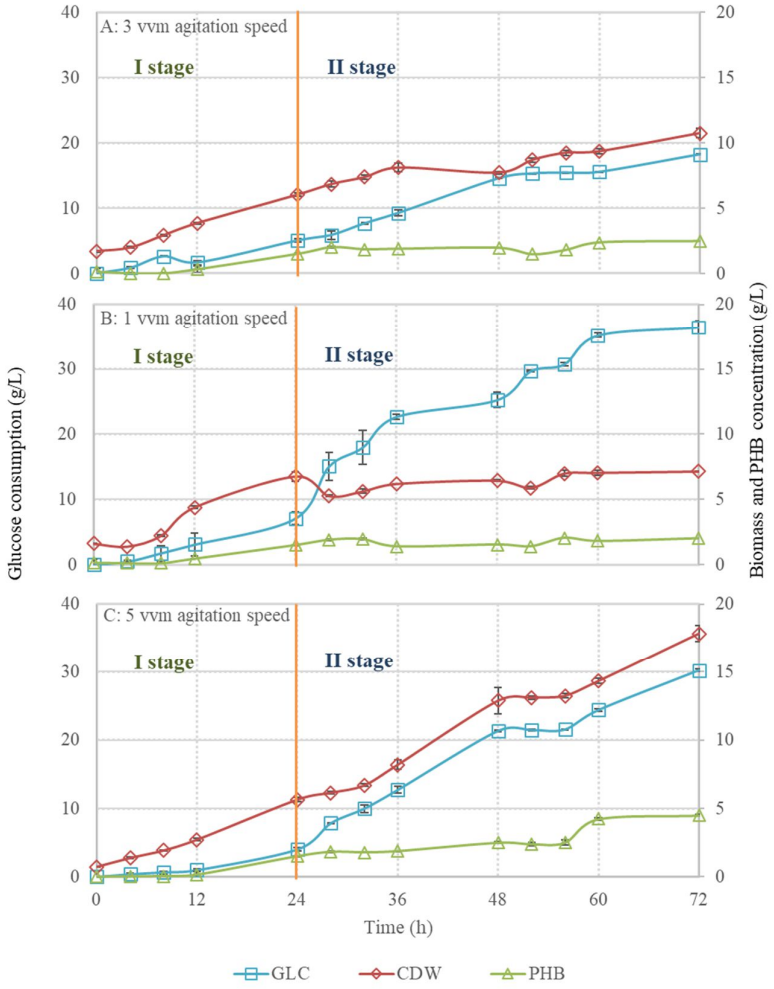


Figure 5. Kinetics of PHB production by *Halomonas boliviensis* at bioreactor scale.

The PHB production started at exponential growth phase and continued until the beginning of the stationary phase, suggesting that has growth-associated and non-growth associated components. The highest PHB concentration, 4.49±0.06 g/L, was attained under k_La of 0.006 s^{-1}. The length of exponential growth phase decreased proportionally with k_La, suggesting growth limitation under low oxygen supply, since *H. boliviensis* is an aerobic bacterium.

The effect of applying limitations of an essential nutrient, such as nitrogen, and the influence of different O_2 concentrations PHB production during the accumulation phase of *H. boliviensis* was studied by (García-Torreiro, Lu-Chau and Lema, 2016). They determined that single limitations of nitrogen and oxygen provoke PHB accumulations of 45 and 37 % (w/w),

respectively, while N limitation with low O_2 supply causes the highest PHB accumulation of 73 wt.%.

Oxygen assimilation by *H. boliviensis* was studied by (Quillaguamán *et al.*, 2005) varying the air-flow rate and agitation speed according to the requirements of cells during cultivation in the medium supplemented with peptone. The first strategy started under standard conditions of air inflow (0.8 L/min) and agitation speed (700 rpm) until oxygen uptake was no longer noted, which led to the restriction of the air supply completely at this stage and inhibited the metabolism of the cells resulting in only 6.0 ±1.0 wt.% of PHB. Subsequently, a maximum air supplementation of 1.7 L/min and a mixing speed of 800 rpm was employed for 6 hours, and subsequently the oxygen uptake was gradually lowered until there was no need for the air supply, whereupon PHB accumulation continued for 21 h to 41 wt.%, equivalent to that obtained when peptone and glucose were added to the medium. In another experiment, the aeration rate and agitation speed were maintained constant during the cultivation. The PHB accumulation under these conditions was 49 wt.% accumulation, which was an improvement over the cultivations with no oxygen limitation.

3.3.2. Kinetic parameters

Table 6 summarizes the kinetic parameters of nitrogen sources evaluated at 60 h, and the time when maximum concentrations of biomass and PHB were reached are indicated in parenthesis. The highest values of biomass parameters were obtained using WGP as the nitrogen source, even the best for PHB productivity and maximum concentration, but whey was found to be the most suitable nitrogen source to obtain a high yield and accumulation of PHB.

Table 6. Yield and productivity of biomass by *Halomonas boliviensi*s using different aeration rates

Kinetic parameters	**3 vvm**	**1 vvm**	**5 vvm**
Max biomass concentration (g/L)	10.74±0.37 (72 h)	7.15±0.03 (72 h)	17.83±0.57 (72 h)
Biomass yield on substrate Y_{xs} (g/g)	0.49±0.01	0.15±0.01	0.56±0.01
Biomass productivity (g/Lh)	0.16±0.00	0.12±0.00	0.24±0.00
Max PHB concentration (g/L)	2.46±0.05 (72 h)	2.05±0.11 (56 h)	4.49±0.06 (72 h)
PHB yield on biomass Y_{px} (g/g)	0.29±0.03	0.30±0.26	0.31±0.33
PHB productivity (g/Lh)	0.04±0.00	0.03±0.00	0.07±0.02
Max PHB accumulation (wt.%)	29.25±0.98 (28 h)	35.96±1.89 (28 h)	29.87±0.13 (28 h)

The numbers in brackets show the time at which those values were attained.

The highest PHB accumulation, 35.96±1.89 wt.%, was achieved for k_La of 0.006 s^{-1} at 28h. Lower values of the PHB accumulations were obtained for k_La of 0.002 $s^{-1}$and 0.013 s^{-1}, with no significant difference between the values obtained at these two conditions at the same hour.

3.3.3. Statistical analysis

The results of ANOVA for the response variable of the final concentration of biomass (60 hours) showed that there is a statistically significant difference between the three air inflows evaluated (P-value< 0.05). In addition, multiple range test by the Tukey HSD method showed that there is statistically significant difference between each oxygen transfer rate.

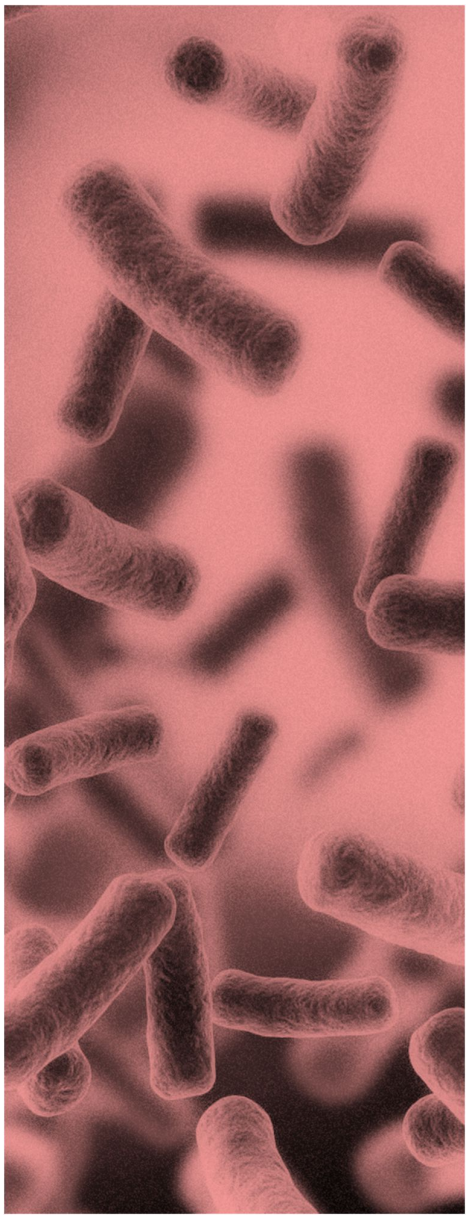
ANOVA for PHB productivity (60 hours) showed that there is a statistically significant difference between means of oxygen transfer rates evaluated (P-value< 0.05). To know which means were different between each other, the multiple range test was evaluated by the Tukey HSD method; results showed that there is statistically significant difference between using each of the air inflows.

3.4. Conclusions

Results obtained in this chapter show the influence of oxygen supply for cell growth and PHB production by *H. boliviensis*. From the three oxygen transfer rates evaluated, k_La=0,006 s^{-1} showed the best results for final concentrations of biomass and PHB productivity at 60 hours of fermentation time. Notwithstanding *H. boliviensis* showed great results at bioreactor scale, further work involving different cultivation strategies to improve the cell growth and polymer productivity by *H. boliviensis* should be studied.

4

Unstructured model and parameter estimation of polyhydroxybutyrate by *Halomonas boliviensis*



Abstract

In the biotechnological engineering field, strategies are being implemented to describe, improve and establish optimal operational production conditions. One of the tools used is the mathematical modeling through the development of predictive models to facilitate the industrial scaling using results obtained from laboratory and pilot plant scale.

In this work, an unstructured model for the prediction of dynamic behavior of cell growth and polyhydroxybutyrate (PHB) accumulation by *H. boliviensis* is presented. The proposed model is the first model to be implemented to produce PHB by *H. boliviensis*, which accounts for the dynamics of cell growth, carbon, nitrogen and oxygen uptake, and biopolymer accumulation produced under nitrogen and oxygen limitation.

4.1. Introduction

The global tendency to replace petrochemical plastics with sustainable products has open the market to the bioplastics industry, and bio-based and biodegradable polymers like polyhydroxybutyrate (PHB) are driving this growth thanks to their wide array of physical and mechanical properties (European Bioplastics, 2018b). Although, PHB has finally entered the market at commercial scale, its global production capacity still remains behind from other bioplastics (1.4 % of total bioplastics produced in 2018) due to its major drawback: high production costs (Muhammadi, Muhammad and Hameed, 2015; European Bioplastics, 2018a). Therefore, studies are focusing on reducing the production cost of PHB by implementing strategies to optimize and control the fermentation process aiming high yields and productivity (Marudkla *et al.*, 2018).

A bioprocess engineering approach to tackle the cost-effective PHB production problem is to develop mathematical models that help in the understanding of the microbiological system predicting their behavior in different scenarios (Gahlawat and Srivastava, 2013). Supporting on mathematical modeling reduces the need to evaluate experimentally multiple operational and nutritional conditions to determine the best response of the microorganism to such variations. At industrial scale, the information provided by the kinetic parameters from mathematical models can be used to develop new process configurations, control and optimize operating conditions (López *et al.*, 2013; Mendez, 2016).

The confidence of mathematical modeling relies on the accurate understanding and analysis of the system, which is why the selection or the proposal of possible models that could describe the process behavior and the underlying mechanisms is a critical step in the model development. Novak *et al.* (2015) has elucidated all classifications found in literature to differentiate types of mathematical models and has reviewed models previously applied for the microbial synthesis of the polyhydroxyalkanoate (PHA) family. Recent papers related to mathematical modelling applied to PHB production include unstructured and non-segregated models based on formal kinetic also known as mechanistic models, putting their attention to the widely studied *Cupriavidus necator* (Mozumder *et al.*, 2015; Mozumder, Garcia-Gonzalez, De Wever and Eveline I.P. Volcke, 2016; Pérez Rivero *et al.*, 2016; Yousuf and Winterburn, 2016; Trigueros *et al.*, 2017; Das and Grover, 2018; Marudkla *et al.*, 2018). Some works have also proposed mechanistic models considering different microorganisms: *Alcaligenes latus* (Gahlawat and Srivastava, 2017) and *Synechocystis* PCC6803 (Carpine *et al.*, 2018). Structured modeling based on metabolic models has been embraced using *Azohydromonas lata* (Penloglou *et al.*, 2017). Hybrid cybernetic models considering non-segregated (Carius *et al.*, 2018) and segregated (Franz, Dürr and Kienle, 2014) population has been developed for *C. necator*.

On the other hand, the reliability of a proposed model is recognized when routines of model calibration or validation are performed. Common parameter estimation routines do not consider the non-linear interaction of systems or the higher influence of some parameters on the model, therefore, do not consider that there is not a unique solution for several parameters, that are called non-identifiable. Here comes the concept of identifiability, a necessary prerequisite for mathematical analysis of a model (Hengl *et al.*, 2007), where the model structure may be changed by removing or adding new parameters, or by setting a confidence interval value of parameters.

Even though several models have been proposed thus far there is still a need of expressing all the characteristics of producing strains and/or features of industrial-scale plants. The high promising gram-negative bacteria *H. boliviensis* have shown significant results for high cell growth and PHB production, i.e., cell dry weight (CDW) of 62.9 g/L (Guzmán *et al.*, 2009), maximum PHB content of 90 wt.% and PHB concentration of 35.4 g/L (Quillaguamán *et al.*, 2008), and PHB volumetric productivity of 1.32 g/L/h (García-Torreiro, Lu-Chau and Lema, 2016). To the best of our knowledge, a mathematical model that describes cell growth, nutrients uptake, and biopolymer accumulation by *H. boliviensis* has not been developed.

In this work, a non-segregated and unstructured model for the production of PHB by *H. boliviensis* is proposed, i.e., the model considers a homogeneous cell population, and takes into account macroscopic variables and conditions in the bioreactor containing the main important dynamics of *H. boliviensis* growth, consumption of glucose, nitrogen, dissolved oxygen, and PHB production. Identifiability analysis was included to determine the most sensitive and linearly independent parameters, and the model was validated by using a new set of experimental data and calculating the confidence intervals for the subset of identifiable parameters.

4.2. Material and methods

The strategy followed in this study for formulating the model includes the following steps: gathering of experimental data, kinetic model development, parameter estimation, identifiability analysis, and model validation (Villegas *et al.*, 2017b).

4.2.1. Gathering of experimental data

Experimental data was taken from two datasets, the first set was used to build the mathematical model, it was taken from a previous study from (García-Torreiro, Lu-Chau and Lema, 2016), in which fed-batch cultures with *H. boliviensis* were carried out applying limitations of nitrogen and oxygen for PHB production and cell growth. Although data was taken from the fed-batch cultivation where the glucose concentration was maintained at 20 g/L by adding a feed solution, a calculation of the glucose consumption was made to model it as a batch fermentation system that is aim of this work.

The second dataset was used to validate the model and obtain a kinetic parameter set, taken from experimental results obtained from chapter 3, where dissolved oxygen concentrations were evaluated on cell growth and PHB production by *H. boliviensis* using WGP hydrolysate. In the fermentation system, glucose concentration was maintained above 5 g/L to not limit PHB production by glucose concentration; therefore, the glucose consumption was also used for this dataset.

The model describes residual cell mass concentration, PHB production, glucose and nitrogen consumption, and dissolved oxygen concentration by *H. boliviensis*.

4.2.2. Model development

The developed model is based on previous works by Villegas *et al.* (2017a) and Mozumder *et al.* (2015, 2016). The unstructured and non-segregated model proposed in this work is derived from the mass balances of the extracellular dynamics for cell growth, substrate uptake and metabolite production without considering metabolic prevailing elements or mechanisms, and the cell population was considered physiological and morphologically homogeneously distributed (Villegas *et al.*, 2017a), this type of model in general terms can be described as:

$$\frac{dC_X}{dt} = \mu C_X \qquad \text{Eq. 1}$$

$$\frac{dC_S}{dt} = -\left[\frac{1}{Y_{X/S}}\mu + \frac{1}{Y_{P/S}}q_P + m_S\right]C_X \qquad \text{Eq. 2}$$

$$\frac{dC_P}{dt} = [\propto \mu + \beta]C_X \qquad \text{Eq. 3}$$

$$\mu = \mu_{max}\frac{C_S}{K_S + C_S} \qquad \text{Eq. 4}$$

where C_X, C_S and C_P are the biomass, substrate and product concentration, respectively; μ is the specific growth rate; q_P is the specific rate of product formation; m_S is the maintenance coefficient; $Y_{X/S}$ and $Y_{P/S}$ are the yield coefficients for substrate and product, respectively; α and β are the growth-associated and non-growth associated product formation constants, respectively; μ_{max} is the maximum specific growth rate and K_S is the saturation constant based on substrate (Villegas *et al.*, 2017a).

Considering the above, the mathematical model proposed in this work consists of 5 differential mass balance equations (Equation 5) which are the main output variables: residual biomass (X_R), PHB (P), Glucose (S_G), Nitrogen (S_N), and Oxygen (O_2) concentrations, all expressed in g L^{-1}.

$$Y = g(X, t, \theta) = \left[\frac{dX_R}{dt}, \frac{dP}{dt}, \frac{dS_G}{dt}, \frac{dS_N}{dt}, \frac{dO_2}{dt}\right]^T \qquad \text{Eq. 5}$$

where *X* indicates the state variables vector, *t* is the time, *Θ* is the vector of model parameters, and *Y* corresponds to the model output vector (Villegas *et al.*, 2017b).

To define the model, the following assumptions were considered:

- The model comprises two main processes: a) biomass growth and b) PHB production. The biomass is composed of: i) active biomass (residual cell concentration, X_R) which is the catalytically active fraction responsible of the metabolic activity of cells, and ii) the secondary metabolite that is the biopolymer PHB (P) (Novak *et al.*, 2015).

- Cell growth and PHB production by *H. boliviensis* is mainly affected by the availability of an adequate carbon source (in this case glucose), a complex nitrogen source, and oxygen depletion (Quillaguamán *et al.*, 2007). The stoichiometry of each process is described in table 1.

Table 1. Stoichiometry of the PHB production model

Process	Glucose	Nitrogen	Oxygen	Biomass	PHB
Cell growth	$-1/Y_{x/G}$	$-1/Y_{x/N}$	$-1/Y_{x/O}$	1	
PHB production	$-1/Y_{P/G}$	-	$-1/Y_{P/O}$		1

- An excess of the carbon source in the culture medium of *H. boliviensis* is a prerequisite for optimum production of the polymer (Rivera-Terceros *et al.*, 2015), therefore low concentrations of glucose (<5 g/L (Pleissner *et al.*, 2014)) during stationary phase limits PHB accumulation.

- During biomass growth phase, nitrogen exhaustion inhibits bacterial growth since the machinery needed for bacterial division cannot be built (García-Torreiro, Lu-Chau and Lema, 2016), therefore absence of nitrogen limits cell growth.

- Nevertheless, when nitrogen and/or oxygen concentrations decrease provokes an imbalance in the culture media triggering the PHB synthesis (García-Torreiro, Lu-Chau and Lema, 2016), thus high concentrations hinder PHB production. PHB accumulation is initiated when nitrogen becomes limiting (Quillaguamán *et al.*, 2005), however so far those key and limiting values have not been defined yet.

- High oxygen concentrations (air inflow and adequate oxygen transfer rate) stimulate cell growth but inhibits PHB production. At an intermediate oxygen concentration, both biomass growth and PHB production take place (Mozumder *et al.*, 2015). Complete restriction of oxygen supply inhibits cells metabolism resulting in low PHB formation, but oxygen limitation (low concentration) limits biomass growth and induces PHB production (Quillaguamán *et al.*, 2005).

- The effects of temperature, pH, NaCl and phosphorus concentrations were not considered and remained constant during the process.

The model is presented in equations (6-12), where the dynamics equations of residual cell growth, product formation, and substrates uptake are given.

$$\frac{dx_R}{dt} = \mu_x x_R \quad \text{Eq. 6}$$

$$\frac{dP}{dt} = R_P x_R \quad \text{Eq. 7}$$

$$\frac{dS_G}{dt} = S_{Gi} - \left[\frac{\mu_x}{Y_{x/G}} + \frac{R_P}{Y_{P/G}} + m_G\right] x_R \quad \text{Eq. 8}$$

$$\frac{dS_N}{dt} = S_{Ni} - \left[\frac{\mu_x}{Y_{x/N}} + \frac{R_P}{Y_{P/N}}\right] x_R \quad \text{Eq.9}$$

$$\frac{dO_2}{dt} = k_{laO}\left(O_{eq} - O_i\right) - \left[\frac{\mu_x}{Y_{x/O}} + \frac{R_P}{Y_{P/O}}\right] x_R \quad \text{Eq. 10}$$

$$\mu_x = \mu_{xm}\left[\frac{S_G}{S_G + K_{xG} + \frac{{S_G}^2}{K_{xiG}}}\right]\left[\frac{S_N}{S_N + K_{xN} + \frac{{S_N}^2}{K_{xiN}}}\right]\left[\frac{O_2}{O_2 + K_{xO} + \frac{{O_2}^2}{K_{xiO}}}\right] \quad \text{Eq. 11}$$

$$R_P = R_{Pm}\left[\frac{S_G}{S_G + K_{pG} + \frac{{S_G}^2}{K_{piG}}}\right]\left[\frac{K_{piN}}{S_N + K_{piN}}\right]\left[\frac{O_2}{O_2 + K_{pO} + \frac{{O_2}^2}{K_{piO}}}\right] \quad \text{Eq. 12}$$

Two specific rates were taken into consideration: one related to cell growth and the other to product formation in equations (4) and (5), respectively. The specific growth rate (μ_x) relies on a multiplicative model, considering three limiting nutrients: glucose, nitrogen and dissolved oxygen, because the absence of any of these substrates inhibits cell growth. The product formation includes nitrogen and dissolved oxygen

The limitation by glucose and oxygen was described as Monod kinetics, and the combined limitation and inhibition effect of nitrogen was modeled through Haldane kinetics (Mozumder *et al.*, 2015). The product formation rate (R_p) is limited by glucose and nitrogen concentration modeled as Monod and is inhibited by high concentration of oxygen described by Haldane.

Residual biomass and product formation profiles were obtained from their respective mass

balances considering the biochemical conversion to residual biomass and PHB:

Mass balances were set up for glucose, nitrogen and oxygen concentrations in the fermenter. Equation (8) was used to describe glucose consumption which is associated to cell growth and PHB formation, and equation (10) for oxygen concentration, where O_2 supply is needed for both processes as explained before in assumptions. Residual nitrogen in equation (9) was determined by assuming that the consumption of this nutrient is used only for cell growth. S_{Gi}, S_{Ni} and O_{2i} correspond to the concentrations of glucose, nitrogen and oxygen in the feed.

Oeq represents the equilibrium liquid phase concentration corresponding with the gas phase composition of O2 as expressed by Henry's law in equation (11).

$$O_{eq} = P_O / k_H \qquad \text{Eq. 11}$$

where PO is the partial pressure of the gas (atm), and kH the Henry's constant (atm/g/L), that is calculated at the fermentation conditions (30°C and 1 atm).

The proposed model includes 5 output variables, comprises 5 differential equations, and 15 parameters. The model was implemented in M ATLAB (version 8.1.0.604, R2013a; MathWorks, USA) software.

4.2.3. Parameter estimation

In the case of bioprocesses, models tend to have a dynamic and non-linear character, and the estimation of the parameters is usually considered as the minimization of an objective function that measures the quality of the fit of the model with respect to a given set of experimental data. Mathematically, this problem is formulated as a nonlinear programming (NLP) problem with possible algebraic constraints (Villegas *et al.*, 2017a).

The MATLAB software was employed to estimate the values of constant parameters. An objective function was defined for solving the parameter estimation problem, expressed as the global minimization of the sum of squared errors (MSE_Y) (Equation 12) in order to obtain the best possible fit between the model predictions and experimental data.

$$minJ(\theta) = \sum_{i=1}^{n} MSE_Y \qquad \text{Eq. 12}$$

The MSEY for each variable is defined by Equation 13:

$$MSE_Y = \frac{1}{n}\sum_{i=1}^{n}\left(Y_{\exp,i} - Y_i\right)^2 \qquad \text{Eq. 13}$$

where $Y_{exp,i}$ corresponds to the experimental points, and Yi denotes the model predictions corresponding to parameter values. The initial parameter values (θ_0) to solve the optimization problem were taken from previous studies that worked with microorganisms different than *H. boliviensis* to produce PHB (*Ralstonia eutropha* (Khanna and Srivastava, 2005), *Hydrogenophaga pseudoflava* (Mahmoudi *et al.*, 2010), *Burkholderia cepacia B27* (Mendez *et al.*, 2016), *Pseudomonas fluorescens* (Vanegas and Ramírez, 2016)), because there is no available information of mathematical modeling regarding to the production of PHB by *H. boliviensis*.

During the parameter estimation, to avoid a local optima solution, two optimization algorithms were used non-simultaneously for solving the objective function, the deterministic 'Nonlinear least-squares' and the stochastic 'Simulated Annealing' optimization algorithms, and an initial pseudo-optimal set of parameters values θ^* was obtained, this set is presented in Table 3.

Table 3. Description of the kinetic parameters of the unstructured model

Parameter	Unit	Definition
μ_x	h^{-1}	Specific growth rate
μ_{xm}	h^{-1}	Maximum specific growth rate
α	$g\ g^{-1}$	Constant related to product formation
β	$g\ g^{-1}$	Constant related to product formation
K_{SN}	$g\ L^{-1}$	Affinity constant to nitrogen source
K_{SO}	$g\ L^{-1}$	Affinity constant to oxygen source
$Y_{x/SG}$	$g\ L^{-1}$	Biomass yield with respect to glucose
$Y_{x/SN}$	$g\ g^{-1}$	Biomass yield with respect to nitrogen
m_N	$g\ g^{-1}h^{-1}$	Maintenance by nitrogen source
m_G	$g\ g^{-1}h^{-1}$	Maintenance by glucose source

The stoichiometric and kinetic parameter values applied in this study are summarized in Table S.1.; the values for the operating parameters are given in Table S.2.

Two independent experimental datasets were used to estimate the parameters, whereas one different independent data set was used to validate the model. Each experimental data set was obtained at different limitations of nitrogen and oxygen supply.

To overcome the nonlinear nature of the model, and the strong correlation among the effects of the parameters, identifiability analysis was carried out for establishing a new subset if identifiable parameters ($\theta_k \subset \theta^*$) that presents a high effect in the objective function, and low linear dependency. The remaining parameters (i.e., those non-identifiable) are fixed at their pseudo-optimal values, forming the subset θ_{fixed}. A new optimization routine is performed where the decision variables are the parameters in the subset, until a new optimal subset of parameters is obtained. The new set of optimal parameters is where will be takes as the final set when it presents a good fit.

To determine the subset of identifiable parameters, a local sensitivity analysis was performed based on the sensitivity matrix. This matrix denotes the relationship between the derivatives of the state variables of the model x_i with respect to the parameter θ_j as it the following equation:

$$Z = \frac{\partial X}{\partial \theta} = \begin{bmatrix} \frac{\partial x_1}{\partial \theta_1} & \cdots & \frac{\partial x_1}{\partial \theta_m} \\ \vdots & \ddots & \vdots \\ \frac{\partial x_n}{\partial \theta_1} & \cdots & \frac{\partial x_n}{\partial \theta_m} \end{bmatrix} \quad \text{Eq. 14}$$

4.2.4. Model validation

Finally, cross validation is important to determine the predictive capabilities of models (Villegas *et al.*, 2017a). The proposed model was validated by using an independent set of experimental data from the experiments developed in this study, and the calculation of the confidence intervals for the subset of identifiable parameters was established (Villegas *et al.*, 2017b).

4.3. Results and discussion

The optimal set of parameters of the model are presented in Table 9.

Table 7. Optimal set of parameters of the model.

Parameter	Value
μxm	0.3429
Rpm	0.2380
Kx_G	0.4525
Kx_n	0.0004
y_{xG}	0.9999
y_{xN}	0.9367
y_{xO}	0.0038

m_g	0.1390
k_{La}	0.8512
So_{eq}	0.4705
k_d	0.2695
Kpin	0.0735
Kpio	0.4292
$K x_o$	0.1350

The results of the model adjustment using the optimal parameters are shown in Figure 6.

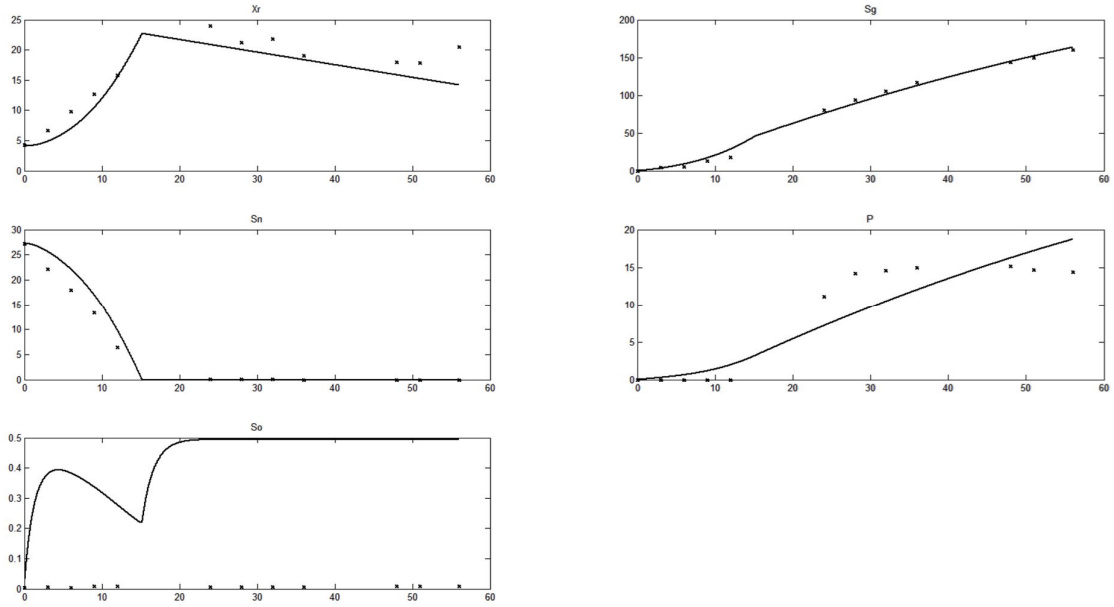


Figure 6. Representation of the model fist for a) residual biomass (Xr), b) glucose consumption (Sg), c) nitrogen consumption (Sn), d) product formation (P), and e) oxygen consumption (So)

These results show a good fit for residual biomass (X_R), glucose consumption (S_g) and nitrogen consumption (S_n). However, to get a better fit of oxygen consumption and product formation, the identifiability analysis was carried out and a new subset of identifiable parameters was obtained which are showed in Figure 7. Parameters 1, 6, 8 and 11 corresponding to specific rates of cell growth (μ_x), biomass yield with respect to the nitrogen substrate (Yx/S_N), maintenance by glucose source (m_g), and dead rate (Kd) showed higher effects in the objective function.

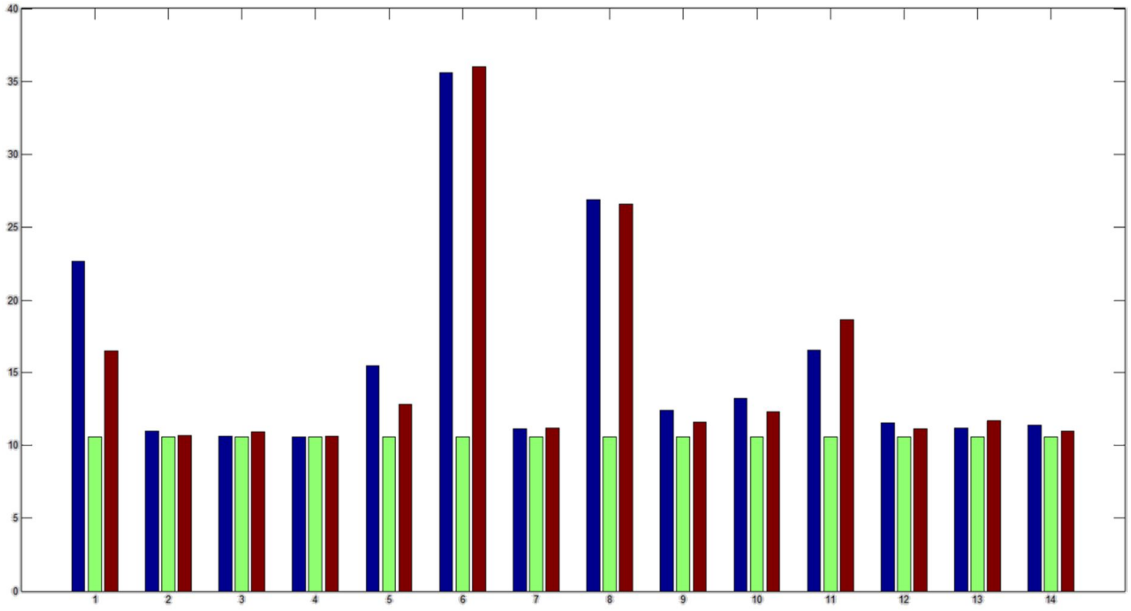


Figure 7. Identifiable parameters from identifiability analysis.

The model predictions calculated using validation data obtained from an experiment differ from the used for estimating the model parameters are presented in Figure 7. The validation results indicate that the model presents good predictive capability in the range of the initial conditions used for the model construction.

Although the model proposed in this study for the production of PHB by *H. boliviensis* is based on similar models as presented for different authors using several microorganisms mentioned before, there are differences in the values of the parameters due to variations in the behavior of each microorganism.

4.4. Conclusions

In this study, a novel unstructured model for cell growth and PHB production by *H. boliviensis* was developed, and validated for two different experimental datasets, namely the one developed by (García-Torreiro, Lu-Chau and Lema, 2016) and from this work. The identifiability analysis indicated that only two parameters of the model are identifiable parameters, which evidences the high correlations among parameter effects for this type of model. This model is the base for the development of future studies in the determination of optimal nutritional and operational conditions to produce PHB by *H. boliviensis*. Future work regarding the metabolic pathway, and the dynamic of intracellular and extracellular compounds

of PHB production by *H. boliviensis* should be conducted to improve the predictive capabilities of the model.

However, to get a better fit of biomass and product it is necessary to perform additional studies to determine the effects of intracellular and extracellular components to obtain a more accurate representation of the process.

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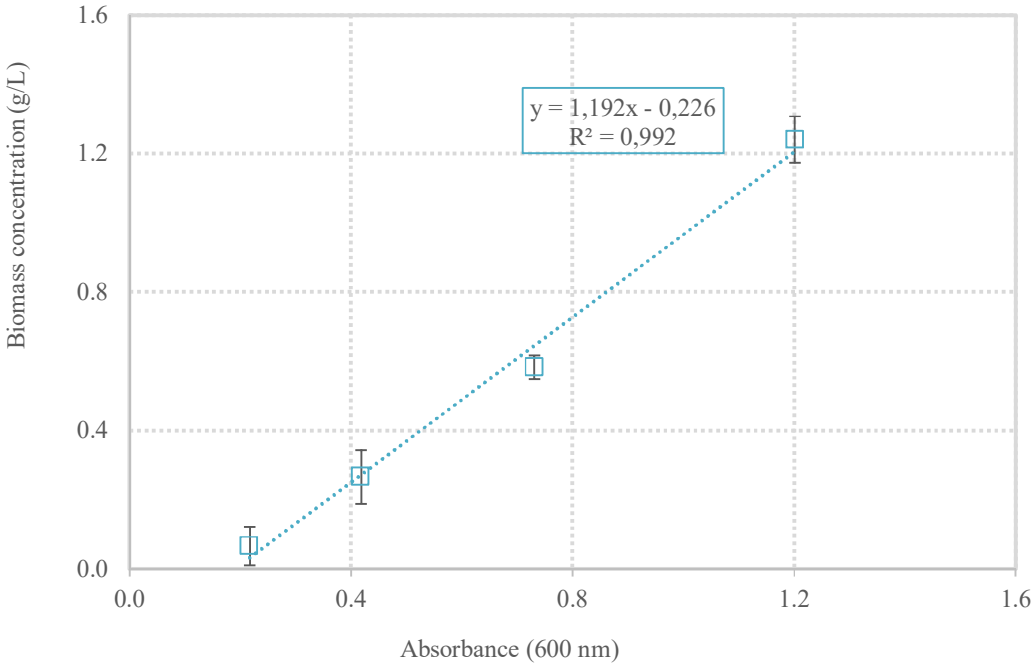
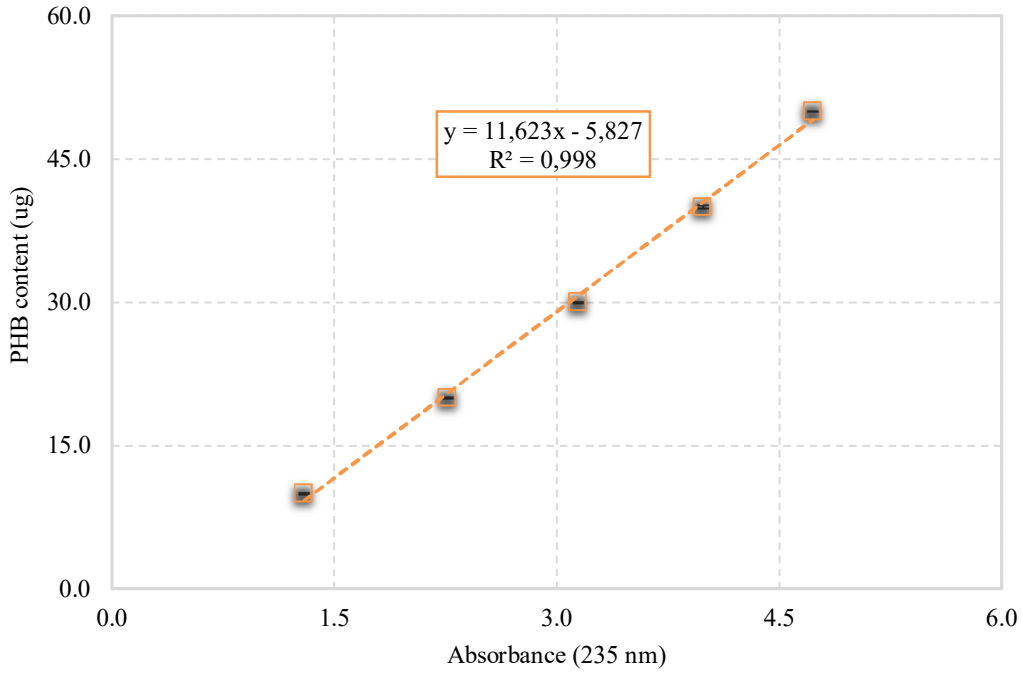
Annexes

1.1. Calibration curves

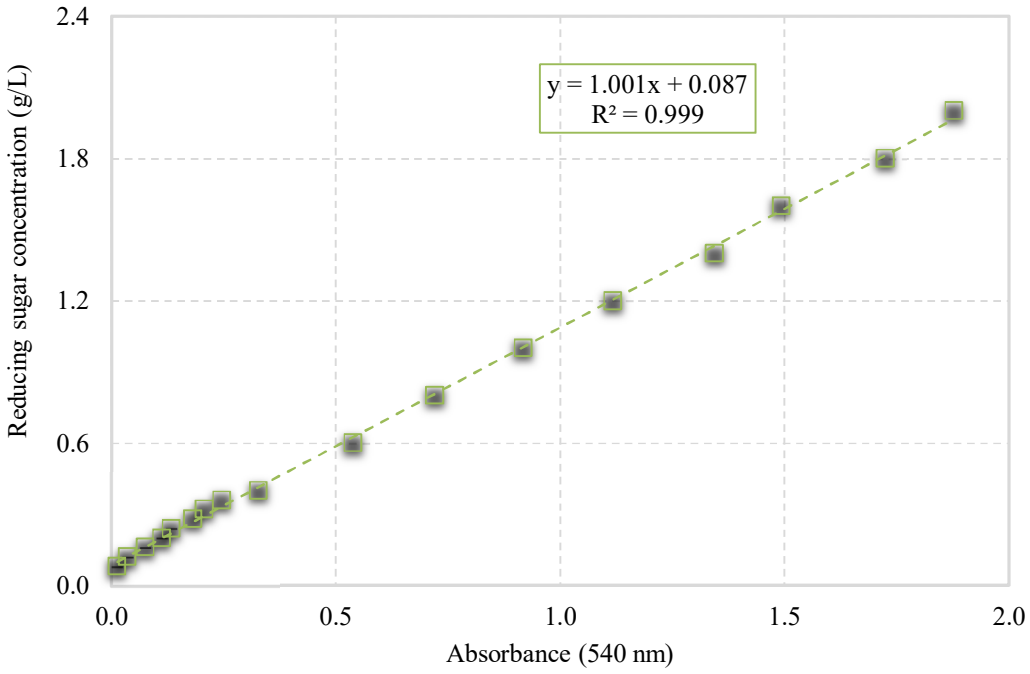
1.1.1. Biomass concentration of *Halomonas boliviensis*



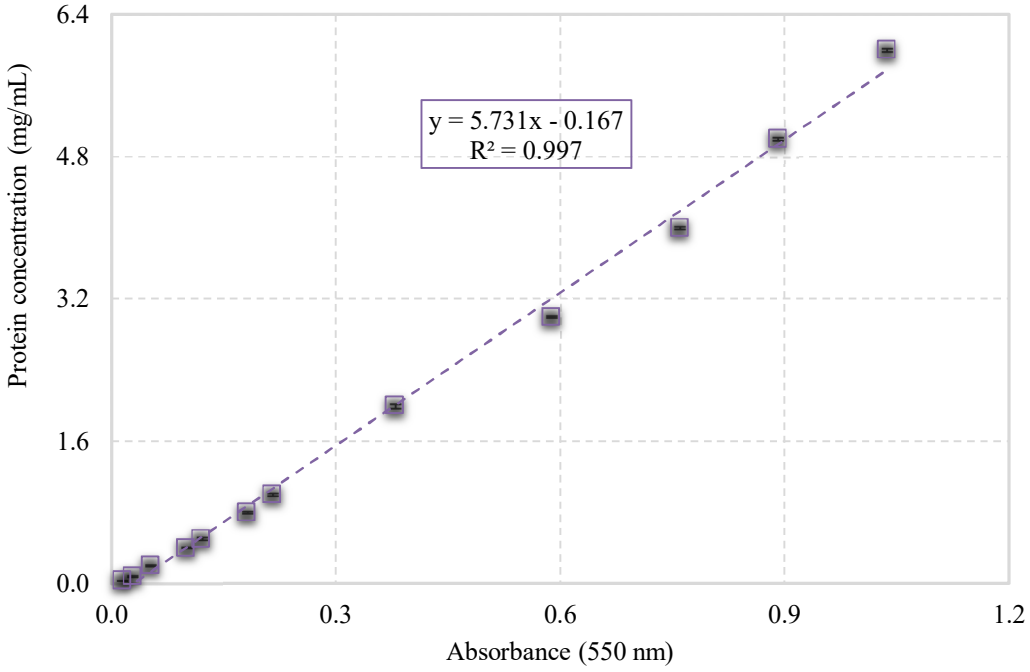
1.1.2. PHB content by the crotonic acid method, a xenon lamp.



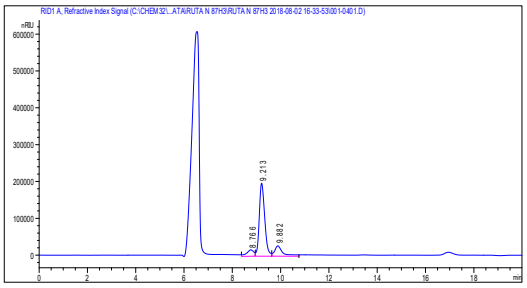
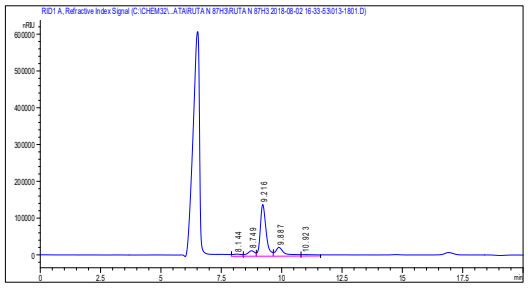
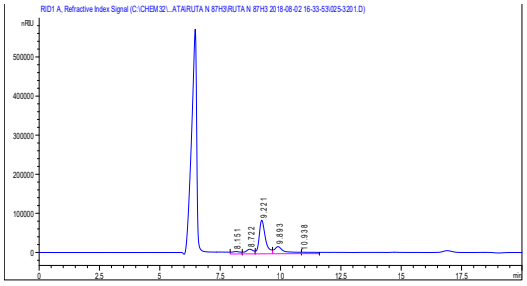
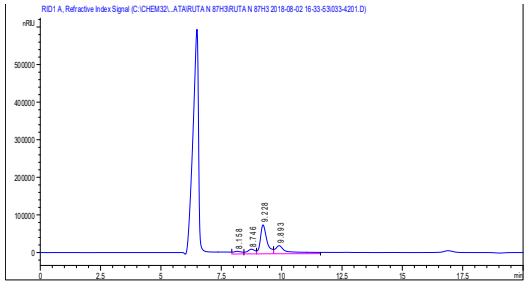
1.1.3. Reducing sugar concentration by DNS method



1.1.4. Protein concentration by Biuret test



1.2. Sugar peaks graphic of samples by HPLC in the evaluation of alternative nitrogen sources

SOLUBLE ISOLATED SOY HYDROLISATE	
0h	24h
	
48h	72h
	

WHEAT GLUTEN HYDROLISATE	
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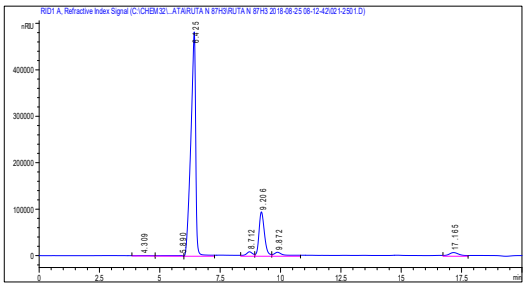	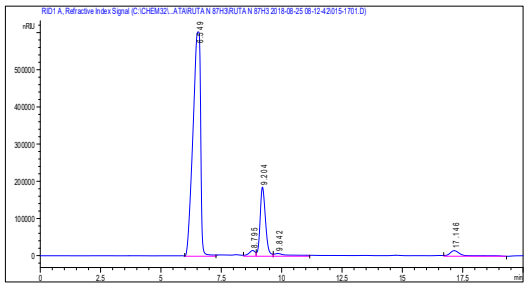

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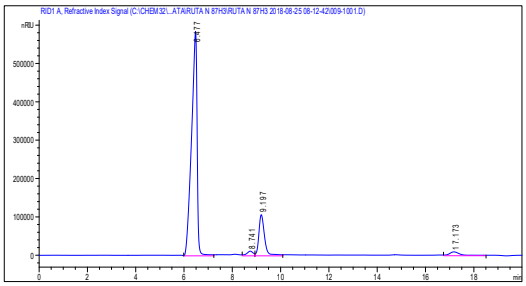	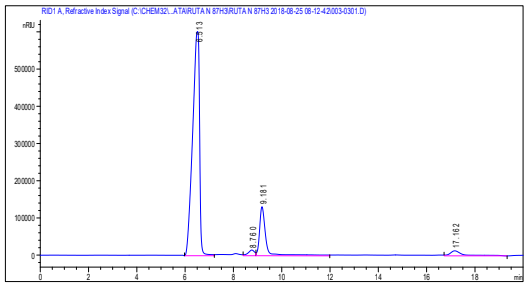

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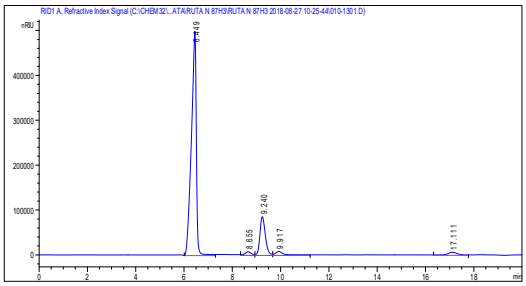	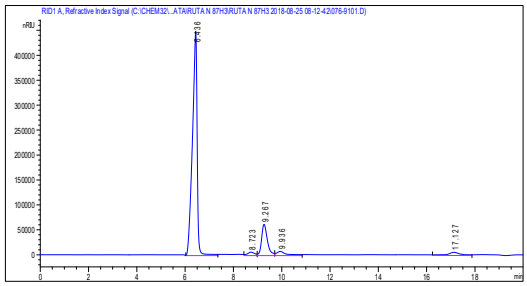
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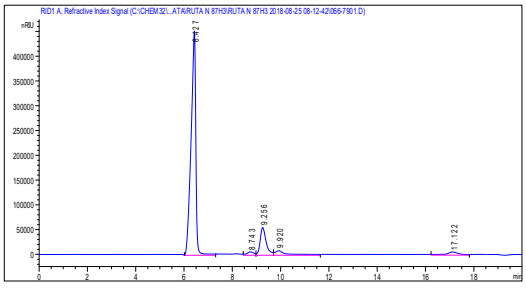	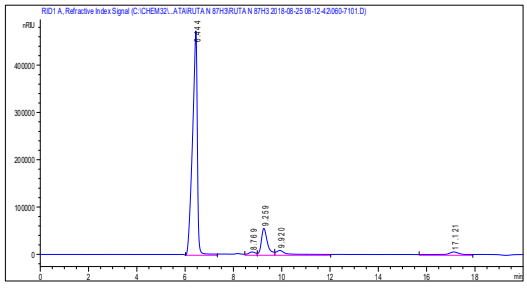

1.3. Statgraphics results chapter 2

1.3.1. ANOVA results

1.3.1.1. ANOVA table for biomass response variable

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	13,40360289	2	6,701801444	84,28	0,0000
Within groups	0,47711	6	0,07951833333		
Total (Corr.)	13,88071289	8			

1.3.1.2. ANOVA table for PHB response variable

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	39,6098	2	19,8049	2253,21	0,0000
Within groups	0,0263689	6	0,00878963		
Total (Corr.)	39,6361	8			

1.3.2. Multiple range Tests results

1.3.2.1. Tukey HSD for biomass response variable

Method: 95.0 percentage Tukey HSD

	Cases	*Mean*	*Homogeneous Groups*	*Contrast*	*Sig.*	*Difference*
Whey	3	1,39633	X	Soy - Wheat	*	-2,45967
Soy	3	1,63767	X	Soy - Whey		0,241333
Wheat	3	4,09733	X	Wheat - Whey	*	2,701

* indicates a significate difference.

1.3.2.2. Tukey HSD for PHB response variable

Method: 95.0 percentage Tukey HSD

	Cases	*Mean*	*Homogeneous Groups*	*Contrast*	*Sig.*	*Difference*
Soy	2	0,012185	X	Soy - Wheat	*	-0,0056113
Whey	2	0,012523	X	Soy - Whey		-0,0003375
Wheat	2	0,017796	X	Wheat - Whey	*	0,0052738

* indicates a significate difference.

1.4.Statgraphics results chapter 3

1.4.1. ANOVA results

1.4.1.1. ANOVA table for biomass response variable

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	82,7682	2	41,3841	1041,69	0,0000
Within groups	0,238368	6	0,039728		
Total (Corr.)	83,0066	8			

1.4.1.2.ANOVA table for PHB response variable

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Between groups	10,5377	2	5,26885	911,09	0,0000
Within groups	0,034698	6	0,005783		
Total (Corr.)	10,5724	8			

1.4.2. Multiple range Tests results

1.4.2.1. Tukey HSD for biomass response variable

Method: 95.0 percentage Tukey HSD

	Cases	*Mean*	*Homogeneous Groups*	*Contrast*	*Sig.*	*Difference*
k_La 2	3	7,047	X	k_La 1 - k_La 2	*	2,305
k_La 1	3	9,352	X	k_La 1 - k_La 3	*	-4,963
k_La 3	3	14,315	X	k_La 2 - k_La 3	*	-7,268

* indicates a significate difference.

1.4.2.2.Tukey HSD for PHB response variable

Method: 95.0 percentage Tukey HSD

	Cases	*Mean*	*Homogeneous Groups*	*Contrast*	*Sig.*	*Difference*
k_La 2	3	2,00333	X	k_La 1 - k_La 2	*	0,459333
k_La 1	3	2,46267	X	k_La 1 - k_La 3	*	-2,031
k_La 3	3	4,49367	X	k_La 2 - k_La 3	*	-2,49033

* indicates a significate difference.

1.4.2.3.Description of kinetic parameters of the unstructured model.

Symbols	Definition
μ (1/h)	Specific growth rate
μm (1/h)	Specific speed of maximum growth
α (g/g)	Constant related to product formation
β (g/g)	Constant related to product formation
K_{SN} (g/L)	Affinity constant to nitrogen source
K_{SO} (g/L)	Affinity constant to oxygen source
$Y_{x/SG}$ (g/L)	Yield biomass product by glucose source
$Y_{x/SN}$ (g/g)	Yield biomass product by nitrogen source
m_N (g/g.h)	Maintenance by nitrogen source
m_G (g/g.h)	Maintenance by glucose source