UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: KINETICAL MODELS FOR FACULTATIVE BACTERIA *CUPRIAVIDUS NECATOR* BEHAVIOR ON OIL DEGRADATION FROM COMESTIBLE OIL WASTEWATER INDUSTRY

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniero en Polímeros

Autor:

Romero López Henry Bryan

Tutor:

PhD. Sommer Márquez Alicia

Co - Tutor:

PhD. Terencio Thibault

Urcuquí, mayo 2021

Urcuquí, 21 de julio de 2021

SECRETARÍA GENERAL
(Vicerrectorado Académico/Cancillería)
ESCUELA DE CIENCIAS QUÍMICAS E INGENIERÍA
CARRERA DE POLÍMEROS
ACTA DE DEFENSA No. UITEY-CHE-2021-00027-AD

A los 21 días del mes de julio de 2021, a las 14:00 horas, de manera virtual mediante videoconferencia, y ante el Tribunal Calificador, integrado por los docentes:

Presidente Tribunal de Defensa	Dr. AVILA SOSA, EDWARD EBNER , Ph.D.
Miembro No Tutor	Dra. HIDALGO BONILLA, SANDRA PATRICIA , Ph.D.
Tutor	Dr. SOMMER MARQUEZ, ALICIA ESTELA , Ph.D.

El(la) señor(ita) estudiante **ROMERO LOPEZ, HENRY BRYAN**, con cédula de identidad No. **2350324402**, de la **ESCUELA DE CIENCIAS QUÍMICAS E INGENIERÍA**, de la Carrera de **POLÍMEROS**, aprobada por el Consejo de Educación Superior (CES), mediante Resolución **RPC-SO-41-No.476-2014**, realiza a través de videoconferencia, la sustentación de su trabajo de titulación denominado: **Kinetical models for facultative bacteria Cupriavidus Necator behavior on oil degradation from comestible oil wastewater industry**, previa a la obtención del título de **INGENIERO/A DE POLÍMEROS**.

El citado trabajo de titulación, fue debidamente aprobado por el(los) docente(s):

Tutor	Dr. SOMMER MARQUEZ, ALICIA ESTELA , Ph.D.

Y recibió las observaciones de los otros miembros del Tribunal Calificador, las mismas que han sido incorporadas por el(la) estudiante.

Previamente cumplidos los requisitos legales y reglamentarios, el trabajo de titulación fue sustentado por el(la) estudiante y examinado por los miembros del Tribunal Calificador. Escuchada la sustentación del trabajo de titulación a través de videoconferencia, que integró la exposición de el(la) estudiante sobre el contenido de la misma y las preguntas formuladas por los miembros del Tribunal, se califica la sustentación del trabajo de titulación con las siguientes calificaciones:

Tipo	Docente	Calificación
Tutor	Dr. SOMMER MARQUEZ, ALICIA ESTELA , Ph.D.	10,0
Miembro Tribunal De Defensa	Dra. HIDALGO BONILLA, SANDRA PATRICIA , Ph.D.	10,0
Presidente Tribunal De Defensa	Dr. AVILA SOSA, EDWARD EBNER , Ph.D.	10,0

Lo que da un promedio de: **10 (Diez punto Cero)**, sobre 10 (diez), equivalente a: **APROBADO**

Para constancia de lo actuado, firman los miembros del Tribunal Calificador, el/la estudiante y el/la secretario ad-hoc.

Certifico que *en cumplimiento del Decreto Ejecutivo 1017 de 16 de marzo de 2020, la defensa de trabajo de titulación (o examen de grado modalidad teórico práctica) se realizó vía virtual, por lo que las firmas de los miembros del Tribunal de Defensa de Grado, constan en forma digital.*

ROMERO LOPEZ, HENRY BRYAN
Estudiante

EDWARD EBNER AVILA SOSA
Digitally signed by EDWARD EBNER AVILA SOSA
Date: 2021.07.30 16:46:18 -05'00'

Dr. AVILA SOSA, EDWARD EBNER , Ph.D.
Presidente Tribunal de Defensa

ALICIA ESTELA SOMMER MARQUEZ
Firmado digitalmente por ALICIA ESTELA SOMMER MARQUEZ
Fecha: 2021.07.30 03:35:56 -05'00'

Dr. SOMMER MARQUEZ, ALICIA ESTELA , Ph.D.
Tutor

SANDRA PATRICIA HIDALGO BONILLA
Firmado digitalmente por SANDRA PATRICIA HIDALGO BONILLA
Fecha: 2021.07.26 22:20:15 -05'00'

Dra. HIDALGO BONILLA, SANDRA PATRICIA , Ph.D.
Miembro No Tutor

CARLA SOFIA YASELGA NARANJO
Digitally signed by CARLA SOFIA YASELGA NARANJO
Date: 2021.07.26 17:09:50 -05'00'

YASELGA NARANJO, CARLA
Secretario Ad-hoc

AUTORÍA

Yo, **HENRY BRYAN ROMERO LÓPEZ**, con cédula de identidad 2350324402, declaro que las ideas, juicios, valoraciones, interpretaciones, consultas bibliográficas, definiciones y conceptualizaciones expuestas en el presente trabajo; así cómo, los procedimientos y herramientas utilizadas en la investigación, son de absoluta responsabilidad de el/la autora (a) del trabajo de integración curricular. Así mismo, me acojo a los reglamentos internos de la Universidad de Investigación de Tecnología Experimental Yachay.

Urcuquí, mayo 2021

Henry Bryan Romero López

CI: 2350324402

AUTORIZACIÓN DE PUBLICACIÓN

Yo, **HENRY BRYAN ROMERO LÓPEZ**, con cédula de identidad 2350324402, cedo a la Universidad de Tecnología Experimental Yachay, los derechos de publicación de la presente obra, sin que deba haber un reconocimiento económico por este concepto. Declaro además que el texto del presente trabajo de titulación no podrá ser cedido a ninguna empresa editorial para su publicación u otros fines, sin contar previamente con la autorización escrita de la Universidad.

Asimismo, autorizo a la Universidad que realice la digitalización y publicación de este trabajo de integración curricular en el repositorio virtual, de conformidad a los dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior.

Urcuquí, mayo 2021

Henry Bryan Romero López

CI: 2350324402

ACKNOWLEDGEMENTS

I want to express my gratitude to Yachay Tech University for allowing me to obtain a high-quality education to improve my academic and professional formation.

I want to express my gratitude to my beloved family, especially to my mother and father, for the constant effort during my personal and professional formation and for encouraging me to continue with my academic life. Thank you for all the sacrifices that you have made for me and your teachings.

I express my gratitude to my aunt Katy López for supporting me during my academic life. You have been like a second mother; thank you for your unconditional support.

I thank my thesis advisor Alicia Sommer for being an essential guide in my professional formation, such as developing this work. Thank you for the dedicated time and patience.

Thank my thesis co-tutor, Thibault Terencio, for helping me develop this work, especially in software development. Thank you for the dedicated time and patience.

I thank Carlos Pazmiño for assisting in the development of this work. Thank you so much for always being available to discuss, share ideas, and help me develop this work.

I want to express my acknowledgments to all my professors for dedicating their time and effort to my personal and professional development.

I want to thank all my friends and classmates for being a fundamental part of my entire academic life.

Henry Bryan Romero López

ABSTRACT

For this thesis, kinetic models were proposed to study the degradation of oils and fats from wastewater from edible oil industries and the production of polyhydroxybutyrate (PHB) through the use of the *Cupriavidus necator* bacteria; some of these models for growth are Monod and a modified Monod model. For the development of kinetic models, experimental data previously reported in Mexico's wastewater industry was used. The wastewater conditions for the production of PHB must be at a temperature of 30 ºC, at a neutral pH, and free of toxic substances. These conditions make it possible to obtain up to 70% PHB in cell dry weight (CDW).

The metabolism of *Cupriavidus necator* under aerobic conditions was reviewed in the literature, with which four biological processes were proposed: heterotrophic growth using fructose as a carbon source, heterotrophic growth using fatty acids as a carbon source, hydrolysis, and heterotrophic lysis. For constructing a mathematical model that describes the kinetical models, the matrix notation was used, which describes a stoichiometric matrix and a matrix of conservative components, which were nitrogen, phosphorus, and organic matter measured as chemical oxygen demand (COD). A kinetic for each process was considered, allowing the development of a mass balance for each component. Finally, software was proposed to simulate PHB production and consumption of fatty acids using *Cupriavidus necator*. The software required continuity equations, material balance, and mass balance. This software, which contains the kinetic models, is a proposal for wastewater from edible oil industries.

Key-words: *Cupriavidus necator*, kinetic models, PHA, PHB, simulation, wastewater.

RESUMEN

Para esta tesis se propuso modelos cinéticos para estudiar la degradación de los aceites y grasas de aguas residuales de industrias de aceite comestible, y la producción de polihidroxibutirato (PHB) mediante el uso de la bacteria *Cupriavidus necator*, algunos de estos modelos para el crecimiento son Monod y un modelo modificado de Monod. Para el desarrollo de modelos cinéticos se usaron datos experimentales reportados previamente en una industria de aguas residuales en México. Las condiciones del agua residual para la producción de PHB deben ser a temperatura de 30 ºC, a pH neutro y libre de sustancias tóxicas. Estas condiciones permiten obtener hasta un 70% de PHB en peso seco celular (PSC).

Se revisó en la literatura el metabolismo de *Cupriavidus necator* en condiciones aerobias, con el cual se propusieron cuatro procesos biológicos. El primer proceso es el crecimiento heterótrofo usando fructosa como fuente de carbono. El segundo proceso es el crecimiento heterótrofo usando ácidos grasos como fuente de carbono. El tercero es la hidrólisis y el cuarto es la lisis heterótrofa. Para la construcción de un modelo matemático se usó la notación matricial, la cual describe un matriz de estequiometria y una matriz de componentes conservativos con el uso de componentes como el nitrógeno, el fósforo y la materia orgánica medida como demanda química de oxígeno (DQO). Se consideró una cinética para cada proceso, lo que permitió desarrollar un balance de masas para cada componente. Finalmente se propuso un software para simular la producción de PHB y consumo de ácidos de grasos usando la bacteria *Cupriavidus necator*, esto se lo realizó mediante ecuaciones de continuidad, balance de materia y balance de masas. Este software que contiene los modelos cinéticos, es una propuesta para las aguas residuales de industrias de aceites comestibles.

Palabras claves: *Cupriavidus necator*, modelos cinéticos, PHA, PHB, simulación, aguas residuales.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ASM	Active sludge model
ASP	Activated Sludge Process
BOD	Biological oxygen demand
CDM	Cell dry mass
COD	Chemical oxygen demand
CSTR	Continuous stirred-tank reactor
DAF	Dissolved air flotation
DO	Dissolved oxygen
DCW	Dry cell weight
GEM	Gas Energy Mixing
g	grams
h	hour
IFAS	Integrated fixed-film activated sludge
kPa	kilopascal
L	liter
m^3	Cubic meter
MBBR	Moving bed biofilm reactor
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PST	Primary sedimentation tank
S	Soluble component
SBR	Sequencing batch reactor
SST	Secondary Settling Tank
UAF	Up-flow Anaerobic Filter
UASB	Up-flow Anaerobic Sludge Blanket
X	Particulate component
WTP	Water treatment plant
WWTP	Wastewater treatment plant

LIST OF SYMBOLS

%	Percentage	
ºC	Degree Celsius	
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
μ_{Fmax}	Maximum specific growth rate using Fructose	h^{-1}
μ_{FAmax}	Maximum specific growth rate using Fatty acids	h^{-1}
μ_{F}	Growth rate equation on Fructose	h^{-1}
μ_{FA}	Growth rate equation on Fatty acids	h^{-1}
μ_{HY}	Hydrolysis process rate equation	h^{-1}
μ_{LY}	Lysis process rate equation	h^{-1}
b_H	Heterotrophic decay rate	h^{-1}
f_{SI}	Fraction of inert COD in particulate substrate	gCOD / gCOD
f_{XI}	Fraction of inert COD generated in biomass lysis	gCOD / gCOD
k_i	Kinetic inhibition	g/L
k_h	Maximum specific hydrolysis rate	g/L
k_X	Half saturation coefficient for hydrolysis of Xs	g/L
k_F	Half saturation coefficient for growth using Fructose	g/L
k_{FA}	Half saturation coefficient for growth using Fatty acids	g/L
k_{NH4}	Half saturation coefficient for growth using Ammonium	g/L
k_O	Half saturation coefficient for growth using Oxygen	g/L
k_{PO4}	Half saturation coefficient for growth using Phosphorus	g/L
Qi	Initial flowrate	g/h
Q_f	Final flowrate	g/h
F	Fructose concentration	g/L
FA	Fatty acid concentration	g/L
GLY	Glycerol concentration	g/L
N	Nitrogen concentration	g/L
O	Oxygen concentration	g/L
P	PHB production	g/L
S_S	Substrate concentration (Fructose)	g/L
S_O	Dissolved oxygen	g/L
S_I	Inert soluble organic material	g/L

S_{NH4}	Ammonium plus ammonia nitrogen	g/L
S_{PO4}	Inorganic soluble phosphorus	g/L
S_{FA}	Substrate concentration (Fatty acids)	g/L
V	Bioreactor working volume	L
X_R	Residual biomass concentration	g/L
X_H	Heterotrophic biomass concentration	g/L
$X_{H,o}$	Initial heterotrophic biomass concentration	g/L
X_I	Particulate inert organic substrate	g/L
X_{NB}	Nitrogen in biomass	g/L
X_{PHB}	Fermentation product (PHB)	g/L
X_S	Slowly biodegradable particulate substrate	g/L
$Y_{PHB/S}$	PHB yield over substrate	gPHB/gSubstrate
$Y_{PHB/X}$	PHB yield over heterotrophic biomass	gPHB/gBiomass
$Y_{X/S}$	Biomass yield over substrate	gcell/gSubstrate
$Y_{X/PHB}$	Biomass yield over PHB production	gcell /gPHB
$Y_{PHB/necator}$	PHB yield over microorganism mass	gPHB/gMicroorganims

CHAPTER I

1. INTRODUCTION

"Wastewater should be considered a sustainable source of water, energy, nutrients and other recoverable by-products, rather than a burden".[1]

The increase in world population has consequences as the scarcity of water resources. According to the World Health Organization (WHO), if one do not control the lack of water resources in a short time, almost half of the world population may have been affected by the consumption of impure water or by water scarcity.[2] Through the National Plan for Good Living and the National Strategy for the Eradication of Poverty (ENIEP by its acronym in Spanish), the government of Ecuador has among its priorities increasing access to drinking water and sanitation. The Ministerio del Ambiente y Agua has presented the National Strategy for Drinking Water and Sanitation (ENAS by its acronym in Spanish), whose objective for the next ten years is to provide universal access to drinking water and sanitation services. With this in mind, the Ministerio del Ambiente y Agua aligned with the Sustainable Development Goals (SDG) of the United Nations (UN), must prioritize access, quality, economical, and environmental sustainability of services.[3] Therefore, Ecuador must comply with the UN objectives, including the sixth objective: "It must carry out projects focused on water resources preservation as soon as possible". Unfortunately, as the number of industries and the population in Ecuador increases, water contamination due to its use in industrial processes also increases; this can generate a problem to access drinking water.

One of the sources of wastewater is due to industrial use. Some pollutants such as oils and fats in wastewater could be found in companies dedicated to producing vegetable oils and similar oil-based products. Ecuador has four prominent companies of vegetable oils: La Fabril S.A., Danec S.A., Ales C.A.,[4] and one developing Uyamafarms Avocado Oil. These companies should present wastewater with a high content of fats and oils, additionally, wastewater treatment processes that one will see later. The wastewater could be used for crop irrigation or as a source of energy through the anaerobic conversion of wastewater's organic content into methane (CH_4) gas, a useful biofuel.[5] Also, there are alternatives for the recovery of residues from wastewater, where microorganisms participate in the decomposition of organic compounds through aerobic conversion.[6] For example, the consumption of oils and fats by

Cupriavidus necator bacteria, for the production of Polyhydroxyalkanoates (PHA) such as PHB.

PHA are polyesters of hydroxyalkanoic acids with a common structure shown in Figure 1. Additionally, PHA are composed of R-ß-hydroxy fatty acids, where the group R in Figure 1 varies from methyl (C_1) to tridecyl (C_{13}).[7] If the R group is a methyl the PHA are P(3HB) or PHB, if R group is an ethyl the PHA are polyhydroxyvalerates (PHV). PHB was one of the first PHA to be widely studied because it can accumulate as a membrane within bacteria up to 80% of the dry cell weight (DCW).[8]

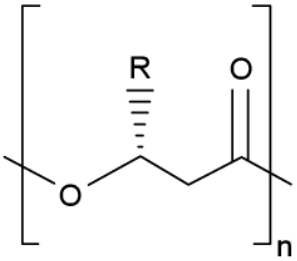

Figure 1. General chemical structure of polyhydroxyalkanoates (PHAs).

Cupriavidus necator is a gram-negative beta proteobacterium for PHA biosynthesis. In recent studies, it has been reported their capability to store Poly(3-hydroxybutyrate), also known as P(3HB) or PHB, up to 90% of its cell dry weight (CDW). *C. necator* strain H16 produces PHA that contains 3-5 carbon length alkanoic acid monomers such as 3HB and 3-hydroxy valerate (3HV), known as short-chain-length PHA (SCL-PHA).[9] In recent years, *C. necator* has been a topic of interest for industrial purposes because it could live in aerobic or anaerobic conditions. Thus, it can produce P(3HB) autotrophically or heterotrophically using carbon sources. Also, the costs of PHA production are very high compared to current traditional plastics; however, if cheap carbon sources are used, PHA production can become profitable.

1.1 Problem Statement

Wastewater treatment in Ecuador is still a challenge for industries. Most wastewater treatment plants meet the minimum requirements for the treated water for domestic consumption or irrigation. The recovery of wastewater from the comestible oil industries and the use of wastewater to produce a higher value-added product is a response against water pollution. Commonly, the production of PHB is expensive compared to polymers of petrochemical origin. However, it is possible to lower PHB production costs, an alternative using cheap carbon

sources such as oils and fats from wastewater, and finally using kinetic models for PHB production. Thus, this thesis seeks to propose kinetic models for the secondary treatment of wastewater from comestible oil industries through *C. necator* bacteria's behavior. *C. Necator*, through its metabolism, can create high value-added products such as PHAs, including PHB; this polymer is an alternative to conventional polymers from the petroleum industry.

1.2 Objectives

1.2.1 General objective

To develop kinetic models for *C. necator* growing and PHB production using fructose, oils, and fats as carbon substrates for companies with wastewater with high content avocado oil waste.

1.2.2 Specific objectives

- To propose components and biological processes according to the metabolism of the bacterium *C. necator* to develop kinetical models.
- To get continuity equations for the calculation of stoichiometric coefficients.
- To adjust the kinetic parameters to optimal conditions for the kinetic model.
- To get a mass balance to simulate the variation of mass in the time.
- To develop simulation software to evaluate the kinetic models.

CHAPTER II

2. BACKGROUND AND LITERATURE REVIEW

2.1 Wastewater Definition

Wastewater is used water that commonly comes from batching, toilet flushing, laundry, dishwashing, etc. Also, wastewater could come from non-domestic sources, such as industrial processes. The wastewater could contain hazardous materials such as heavy metals, in which the use of specific treatments is necessary. About the composition of wastewater, it is 99.94% water by weight. The remaining 0.06% is material dissolved or suspended, which could include nutrients like phosphorous and nitrogen, or carbon sources like fats, oils, grease, or gases like carbon dioxide, nitrogen, oxygen, hydrogen sulfide, and methane.[10,11] In short, wastewater is water contaminated mainly from domestic use or non-domestic use. The organic compounds in wastewater can be characterized by the chemical oxygen demand (COD) test, biochemical oxygen demand (BOD) test, and total organic carbon (TOC) test.[12] The wastewater could contain some characteristics and can be classified as shown below in Figure 2.

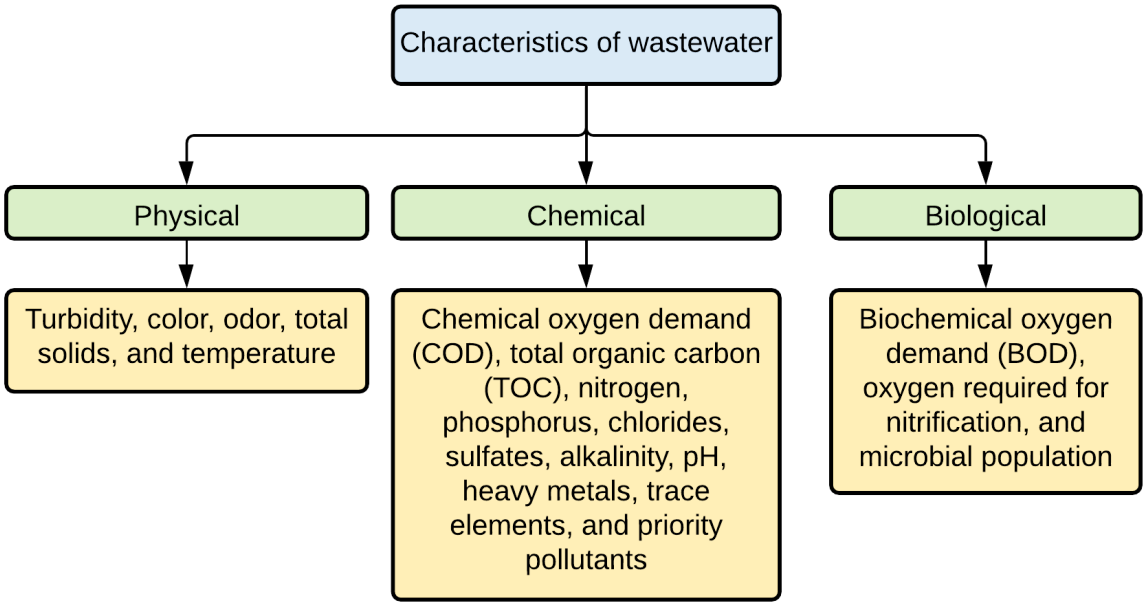

Figure 2. Basic characteristics of wastewater. Source: School of PE.[13]

2.2 Types of wastewater and common treatments

The type of wastewater regarding the source could be classified and described as in Figure 3.

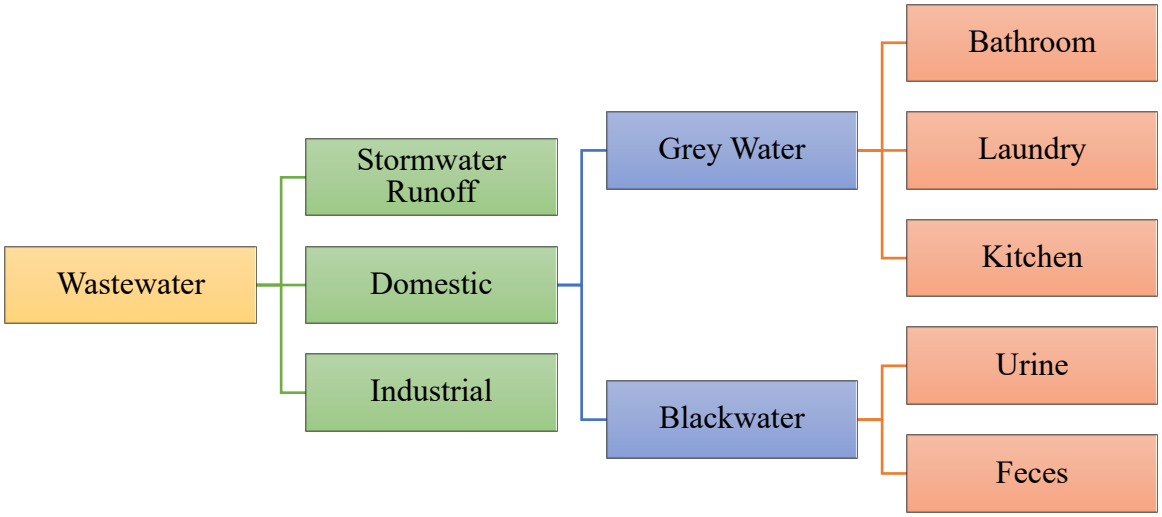

Figure 3. Types of wastewaters. Source: Amoatey and Bani.[14]

2.2.1 Stormwater Runoff

Stormwater runoff promotes contamination in streams, rivers, and lakes because it contains pollutants from many different sources. However, cooperation from residents, businesses, and municipalities can minimize stormwater runoff. In urban areas, the stormwater runoff has precipitation larger than in non-urban areas because surfaces as streets, parking lots, and rooftops increase the stormwater runoff.[15] The total suspended solids is commonly higher in stormwater than a typical municipal wastewater, additionally stormwater may contain amounts of pesticides, halogenated aliphatics, phenols, monocyclic aromatics and phthalate esters.[6]

2.2.2 Domestic Wastewater

Domestic wastewater includes water from commercial and business buildings, institutions, and sometimes water from the storms. The sources of domestic wastewater could be from sanitary facilities, bathing, laundry, and cooking. Domestic wastewater is divided into two categories, depending upon the source: gray water and black water. Gray water is from showers, baths,

whirlpool tubs, washing machines, and dishwater. On the other hand, black water is water from toilets and kitchen sinks.[10]

2.2.3 Industrial Wastewater

Industrial wastewater is water from the manufacturing process, can be complicated to treat due to the variety of pollutants at an industry-based level. The sources of industrial wastewater could be oils, pharmaceuticals, pesticides, silt, chemicals, etc. Generally, industrial wastewater is highly acid or alkaline, and it could contain suspended, colloidal, dissolved solids, inert, organic, or toxic materials, and possibly pathogenic bacteria.[16] However, industrial wastewater may contain recoverable, valuable, and reusable products such as metals or organic material.[6]

The common treatments to all the previous type of known wastewater are described below.

2.3 Types of wastewater treatment

One of the main objectives of wastewater treatment is to restore contaminated water to desirable water quality. There are treatments with physical, chemical, and biological processes or a combination of these processes.[17] The above processes are part of various water treatment systems. Figure 4 shows an example of the types of treatment of municipal wastewater. Wastewater screening will remove solids grades that can damage mechanical equipment. Grit removal separates heavy inorganic solids like sand. A preliminary or primary treatment prepares the water for secondary treatment or/and tertiary treatment.[18]

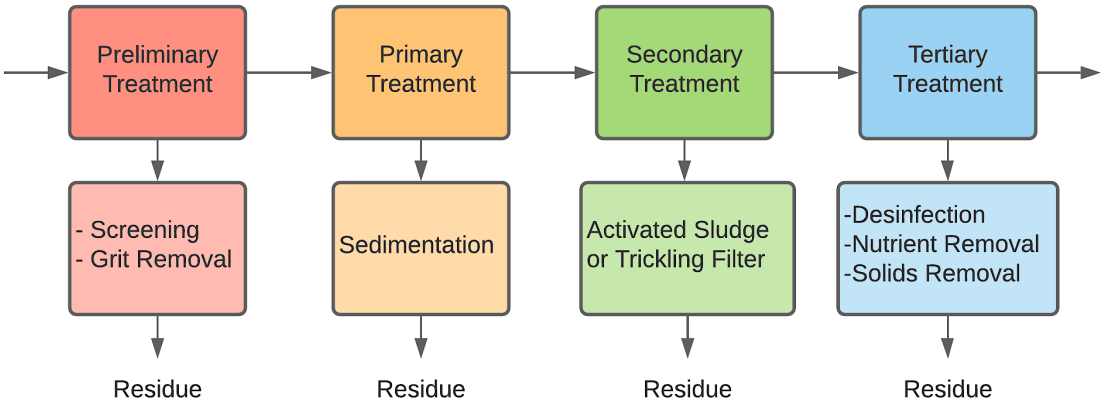

Figure 4. Typical treatments in municipal wastewater.[18]

2.3.1 Preliminary wastewater treatment

The preliminary treatment is useful to remove floating materials and inorganic particles that could cause operational problems in primary or secondary treatment units. The preliminary treatment can contain:[19]

- A sump and pump unit: to collect the wastewater and pump to the first treatment unit.
- An approach channel: to convey the flow of wastewater.
- A screen chamber: to remove large size floating materials.
- A grit chamber: to remove up to 20 mm size suspended solids.
- Oil and grease traps: to remove excessive oil and grease.

2.3.2 Primary wastewater treatment

The primary treatment system includes all the units of preliminary wastewater treatment and a primary sedimentation tank (PST), also known as a primary clarifier. The primary clarifier can reduce settleable suspended solids between 60% - 70%, which can contain between 30% - 32% organic suspended solids.[19] Soluble or colloidal organic matter should be removed in a secondary treatment system.

2.3.3 Secondary wastewater treatment

The secondary treatment system, also called biological treatment, is usually used after the PST to remove soluble organic matter and colloids. Biological processes are used for this treatment. Commonly they use an Activated Sludge Process (ASP) or Trickling filter and a Secondary Settling Tank (SST) also known as a secondary clarifier. Other treatment units used are: Waste Stabilization Ponds, Oxidation Lagoon, Oxidation Ditches, Rotating Biological Contactor (RBC), Up-flow Anaerobic Filter (UAF), and Up-flow Anaerobic Sludge Blanket (UASB).[19] In biological treatment, bacteria can oxidize dissolved and particulate organic matter, also called substrate;[20] for example, *C. necator* can oxidize fatty oils.

Biological treatment can be classified into two types of treatment: aerobic and anaerobic. For this thesis, aerobic treatment will be of interest because it produces greater biomass than an anaerobic process. Therefore, the aerobic process is more effective in the re-absorption of oils and fats in the wastewater.[21] Table 1 shows a comparison between aerobic and anaerobic treatment in wastewater.

Table 1. Advantages and disadvantages of aerobic and anaerobic treatment (Adapted from Shete).[21]

Factors	Aerobic process	Anaerobic Process
Reactors	Aerated lagoons, oxidation ditches, Stabilization ponds, Trickling filters and biological discs.	UASB, Anaerobic filter, Upflow packed bed reactor, CSTR, Down flow fixed- film reactor, Buoyant Filter Bioreactor.
Reactor size	Aerated lagoons, oxidation ditches, Stabilization ponds, trickling filters and biological discs requires larger land area but SBR needs comparatively lower area.	Smaller reactor size is required.
Effluent Quality	Excellent effluent quality in terms of COD, BOD and nutrient removal is achieved.	Effluent quality in terms of COD is fair but further treatment is required. Nutrient removal is very poor.
Energy	High energy is required.	Produce energy in the form of methane.
Biomass yield	In comparison to anaerobic process, 6- 8 times greater biomass is produced.	Lower biomass is produced.
Oil and grease removal	These do not cause serious problems in aerobic processes.	Fats in wastewater shows the inhibitory action during anaerobic treatment of dairy wastewaters.
Shock loading	Excellent performance.	Anaerobic processes showed not good responses to this shock loading.
Alkalinity addition	No need.	There is need for alkalinity addition to maintain the pH because pH changes during the digestion of lactose.

In addition to substrate, in an aerobic process, oxygen and nutrients must complete the secondary treatment process. In equation 1 is shown a general reaction of an aerobic biological process.

$$\nu_1(S) + \nu_2 O_2 + \nu_3 NH_3 + \nu_4 PO_4^{3-} \xrightarrow{microorganisms} \nu_5(new\ cell) + \nu_6 CO_2 + \nu_7 H_2O \quad (1)$$

Where ν_1 to ν_7 are the stoichiometric coefficient, S is the substrate concentration, O_2 is the oxygen concentration, NH_3 is the ammonia concentration, PO_4^{3-} is the phosphate concentration, CO_2 is the carbon dioxide concentration, H_2O is the water produced and new cell is the biomass concentration.[20]

2.3.4 Tertiary or Advanced Treatment System

The tertiary treatment system is used to remove impurities from the effluent that comes from secondary treatment. It is commonly used to meet the minimum requirements or a better quality of water for irrigation, also to remove a specific pollutant or residual nutrients such as nitrogen and phosphorus for reuse of wastewater. A disadvantage of this type of treatment is that it is often expensive to apply. Some techniques for its application are granular-media filtration, biological nitrification/denitrification, ion exchange, air stripping, reverse osmosis, electro dialysis, chemical precipitation, and adsorption, air stripping, reverse osmosis, electro dialysis, chemical precipitation and adsorption.[19]

Generally, all over the world the previous treatments are used to treat wastewater. Ecuador has also a tendency to request wastewater treatment from private companies, like Aguagroup. This company has provided wastewater treatment services through processes of DAF, IFAS, trickling filter, aeration and percolation, to industries such as INAEXPO, Aveguayas - Pronaca, Adelca, Oriental Industries, Juris, Toni Industries, Santana Brewery, among others.[22] Other private companies in Ecuador for wastewater treatment are Sanitron, ISA, Atlas Copco, Andean Water Treatment, Aqualai, among others.

2.4 Common industrial wastewater in Ecuador

The variety of uses of the water produces wastewater with various pollutants, in Ecuador, there is mostly water pollution due to the discharge of water from mining activities, activities in urban areas, artisan activities, hydrocarbons, agriculture, and industrial uses in general.[23] Communities near industrial zones have greater problems of water quality for consumption at a rural or urban level. Among cities with industrial activities that present a problem due to the irrigation of wastewater into rivers are Quito, Guayaquil, Cuenca, Riobamba, Ambato,

Latacunga, Esmeraldas and Santo Domingo.[24] For this thesis, the food oil industries such as Danec, La Fabril, Ales, among others, will be of interest, because one look for cheap carbon sources and this could be achieved given the high content of oils and fats in their wastewater as it will be presented below.

2.4.1 Wastewater from the snack production industry

Carlisnacks Cía. Ltda is a company located in Ecuador that stands out from other companies in the preparation of snacks. Carlisnacks has its location in Quito city and has branch offices in the cities of Guayaquil and Cuenca. Since 1999 it began to market its products, and today is one of the leading snack suppliers in Ecuador.[25] About the management and treatment of its wastewater, Carlisnacks works with the company AFH Services' support. AFH Services has an environmental laboratory for water quality control and works for Carli Snacks, Hospital de Los Valles, the Wyndham Hotel, and the Siegfried industry Guayaquil.[26] Regarding the content of the Carli Snacks wastewater, there is currently no public information. However, if one considers another snack processor, usually there is a discharge of wastewater with a high content of the soil, shells, starch oils, and fats.[27]

2.4.2 Wastewater from milk derivatives production industry

Ecuador has several industries dedicated to the production of dairy products, of which three brands stand out. The first is Chiveria, working since 1970. Among its products are mainly Yogurt and Greek Yogurt.[28] Another company with 30 years of experience is the Pasteurizadora El Ranchito. Among its products are sausages, dairy products, and ice cream.[29] And finally, one has the company El Ordeño S.A. It is an industry that works with more than 6000 small and medium milk producers, with an approximate monthly production of 20 million units.[30] Concerning the management and treatment of their wastewater, it is an information reserved for the organizations in charge of evaluating their activity and its environmental impact. However, the treatments commonly used for the water treatment of the dairy industry are the removal of COD and the removal of oils and fats,[31] the latter is organic matter that can be used as a source of carbon in secondary treatments with microorganisms, such as *C. necator* for PHB production.

2.4.3 Wastewater from comestible oil production industry

According to the United States Department of Agriculture, Ecuador ranks second on a regional scale with 615 million tons of palm oil, after Colombia, which produces 1.67 billion tons per year.[32] One of the most prominent companies to use palm oil to produce edible oils, shortenings, margarine, and soaps in Ecuador is Danec S.A.,[33] this company presents a wide variety of products based on palm oil. Also, they produce canned tuna and sardines, chocolates, cookies, and hygiene products.

In a recent study of wastewater by Acosta M.[34] in his thesis entitled *"Propuesta de optimización del sistema de tratamiento de aguas residuales de la empresa Danec S.A.*" she characterized the effluents from the company Danec S.A with a high content of oils and fats, localized in the province of Pichincha, Rumiñahui canton, Sangolquí parish. Danec S.A. generates liquid waste from tank washing of production processes, approximately 300 gallons of water per load.[34] There is pretreatment of wastewater with grease traps, where manually the grease is removed to incorporate it into the manufacture of detergents and soaps. Table 2 summarizes the characterization of the wastewater in the company Danec S.A., where one can see that in this type of industry, the wastewater has a basic pH, and high content of oils and fats.

Table 2. Results of wastewater characterization in Danec S.A.

Parameters	Concentration
Oils and fats	10447.8 mg/L
BOD_5	1600 mg/L
COD	28900 mg/L
Turbidity	1091.5 NTU
pH	8.7

Source: Adapted from Acosta.[34]

Ales C.A. is another Ecuadorian industry that also produces palm-oil-based products. It works with raw materials extraction from the palm, such as palm olein, palm oil, fatty acids, palm stearin, soap, and materials necessary for oils, butter, soaps, and detergents.[35] Ales C.A. in 2018 presented a new WWTP in the city of Manta, with a capacity to treat 499 m^3/day of the water that comes from its manufacturing processes.[36] Then, a portion of the water treated is reused for the industry's cauldrons, and another part of the water treated is discharged to a near city. About its WWTP, the installed treatment system consists of a primary Gas Energy Mixing

(GEM) system, an MBBR aerobic reactor (secondary treatment), and a sludge treatment system (sludge press).[37]

La Fabril S.A. is an Ecuadorian comestible oil industry. Among its main products are comestible oils, soaps, and detergents. La Fabril S.A., since March 2019, has project respect the treatment of its wastewater with Dow Ultrafiltration Technology, where it consists of reverse osmosis process with a capacity of 10368 m^3/day. The entire system is DOW and PENTAIR WATER brand.[38] The engineer Javier Santana carried out the thesis "*Evaluación técnica de aguas residuales de la empresa Fabril S.A. vertidas al río Muerto, sector Los Ángeles, cantón Montecristi, período 2013*". Santana presented the physicochemical characteristics of wastewater of La Fabril, among them that the daily effluent is 80 m^3/day. Figure 5 shows an illustration of Santana's diagram of the WWTP in the company Fabril S.A..[39]

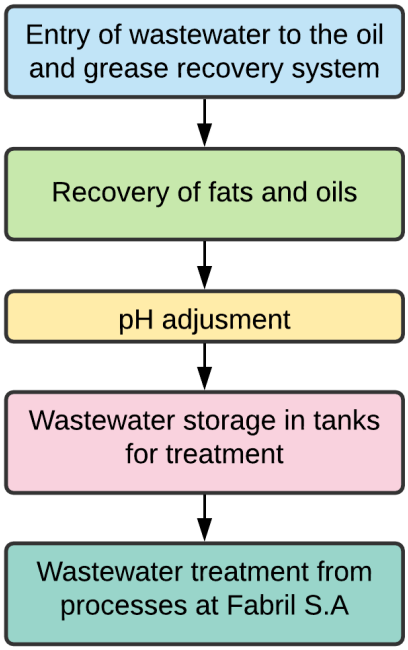

Figure 5. WWTP flowchart at La Fabril S.A (Adapted from Santana).[39]

Another important oil production industry is UYAMA FARMS S.A., which produces oil from avocado source. However, during the purification of avocado oil through a centrifuge, wastewater with avocado oil residues is discarded.[40] Therefore, it will be of interest in this work to propose a mathematical model that uses its wastewater with residual avocado oils as raw material for the production of PHAs such as PHB.

The company works with Avocado Hass variety which composition can be seen in Tables 3 and 4.

Table 3. Avocado composition. Adapted from Teófilo Luna Ochoa.[41]

Component	Composition (%)
Water	70
Proteins	2
Lipids	22
Hydrates of carbon	6

Table 4. Fatty acid composition of the oil of avocado (Persea Americana). Adapted from Ikhuoria and Maliki.[42]

Component	Avocado - Persea americana (% fatty acid)
Myristic	3.99
Palmitic	7.22
Stearic	12.86
Oleic	22.00
Linoleic	24.79
Linolenic	29.14

As seen in Table 4, the wastewater from an avocado industry will have lipids as its main component.

One has mentioned the most important types of wastewaters dealing with oil and fats presence which are coming from industries in Ecuador. All those industries have the responsibility to treat their waste water before disposing it in sweet bodies or other type of allowed discharge bodies. In Ecuador as well as in many different countries exist a regulatory law that indicates the allowed concentration of different pollutant types to be discharged. These regulatory laws are mentioned below.

2.5 Water quality standard in Ecuador

There are technical standards that allow the quality of water supplies and wastewater treatment to be verified and regulated. In Ecuador is recommended to use the book TULSMA and the

book: Standard for environmental quality and effluent discharge: water resource.[43] The book is a guide to regulate the disposal of water-based on safe criteria for the population, the procedures to study the presence of water pollutants, in addition to the permissible limits, among other aspects. Another widely used standards in Ecuador is the *Norma Técnica Ecuatoriana 1108: Agua Potable. Requisitos.*[44] Through international standards, this rule aims to establish the requirements for drinking water.

One agency that regulates water quality in Ecuador is the Ministerio del Ambiente y Agua, which points out that drinking water's physical quality must contain a maximum color value of 300 color units. Furthermore, an acceptable chemical quality control considers compounds that affect drinkability, such as metals, nitrates, arsenic, and pollution indicator compounds such as biochemical oxygen demand (BOD), fats, and oils. Finally, regarding of fats and oils concentration, the Ministerio del Ambiente y Agua recommends a maximum concentration of 0.01 mg/L.[45] Appendix A contains some Ecuadorian standards summarized according to Ministerio del Ambiente y Agua.

2.5.1 Chemical and physical standard parameters for wastewater with fat and oil content

According to the *Norma de Calidad Ambiental y descarga de Efluentes: Recurso Agua*, the maximum permissible limit of oils and fats into water intended for human consumption and domestic is 0.3 mg/L.[43] That parameter applies for freshwaters, for marine and estuary waters, for agricultural use, for recreational purposes, for discharge to a freshwater body, and discharge to seawater.

In Ecuador, according to a study in the company Danec S.A., among the physical parameters for the treatment of wastewater with oil and fats content are pH and turbidity. The turbidity measure consists of determining the scattered and transmitted light by infrared LED with a light source of 850 nm. Some chemical parameters are the calculation of chemical oxygen demand (COD) using the photometric determination method. Respect the determination of oil and fats is made by gravimetric partition.[34] Other techniques to calculate some parameters are in the book Standard Methods for the Examination of Water and Wastewater recommended by Ministerio del Ambiente y Agua.[46]

2.6 Wastewater characterization techniques

The wastewater characterization depends on the composition of the water. Some parameters to take into account are color, pH, electrical conductivity (EC), COD, BOD, hardness, oil and grease, chloride, phenol, total dissolved solids (TDS), total alkalinity, fluoride, sulfate, phosphate, silica, sodium, heavy metals, etc. One is interested in the parameters taken for wastewater coming from oil processes industries, considering water with fat and oil content, mainly. As seen in Table 2, the main parameters are oil, pH, turbidity, BOD_5, nitrogen, phosphorus, and COD, these parameters used to treat wastewater containing oils and fats are summarized below.[46]

2.6.1 pH

The objective is to measure the activity of hydrogen ions by potentiometer measurement using a standard sensing electrode and a reference electrode. The standard electrode is a platinum electrode where hydrogen gas is bubbled at 101 kPa. The reference electrode provides constant electrode potential, commonly are used calomel and silver. The pH is the measure of acid-base equilibrium, where this parameter affects the rate of microbial growth and affects the function of metabolic enzymes. Most microorganisms do well within a pH range of 6.5 to 8.5. The principle of the pH is the potential difference "E" established by the Nernst equation (Equation 2):

$$E = E^o - \left(\frac{RT}{nF}\right)\log\left(a_i\right) \qquad (2)$$

In the Nernst equation, E is the half-cell potential, E^o is the standard potential, R the gas constant, T is the absolute temperature, n the number of electrons exchange, F is the Faraday constant, a_i is the activity of ions, for this work, $\log\left(a_i\right)$ is the pH. Before the pH measurement, two electrodes are into a solution with a known pH, this allows the standardization of electrode and pH meter.[46]

2.6.2 Chemical Oxygen Demand (COD)

COD means the amount of oxygen while oxidizing the organic matter content of a sample under acidic conditions. The oxidation of organic matter releases CO_2 and H_2O. Also, the

values of COD are greater than BOD values. Measuring contamination in industrial wastewater using COD requires 3 hours, compared to the five days using BOD determination. The main limitation is the volatile organic compounds because they are present in the vapor space and not with the oxidizing agent. However, silver sulfate (Ag_2SO_4) for oxidizing those straight chains aliphatic compounds reduce that limitation.[46] In this study, it is of interest to know the COD because it allows us to determine the amount of organic matter susceptible to being oxidized, in our case it would be the concentration of oils or fats in the wastewater.[47] Therefore, a high COD would indicate a high quantity of matter susceptible to being oxidized that fixes a greater quantity of oxygen, generating bacteria in an environment with a deficiency of oxygen due to oxidation. On the other hand, a low COD would represent the opposite, little contamination of the water, in our case, it would be a low number of oils and fats.

2.6.3 Biochemical oxygen demand (BOD_5)

BOD is the amount of oxygen oxidized by bacteria, while decomposable organic matter under aerobic conditions. The term decomposable means that the organic matter can serve as food for bacteria and energy derived from its oxidation.[46] The measurement of BOD is important because it allows knowing the amount of oxygen that microorganisms need in an aerobic process to remove organic matter.[48] In this thesis, one proposes to measure the BOD because it would help to calculate the amount of oxygen, that *C. necator* requires to remove oils and fats, or other readily biodegradable organic matter such as fructose. The technique to measure BOD is called BOD_5, that means the measure of dissolved oxygen (DO) caused by microorganism in a stoppered bottle, before and after incubation during 5 days in the dark at 20 ºC.[46]

2.6.4 Nitrogen and Phosphorus

The types and content of nitrogen and phosphorus vary according to the type of wastewater. Also, all biological material contains nitrogen (N) and phosphorus (P). Therefore, it is crucial to know the amount of these nutrients; because in a biological reactor, nitrogen and phosphorus are part of the nutrients for the growth of microorganisms in wastewater treatment. Also, N and P are part of the residual organic material and microorganisms' biomass, such as *C. necator*. Given the importance, one of the methods to study N and P in wastewater is total Kjeldhal nitrogen (TKN).[49]

2.6.5 Oil and grease

The fats and oil fraction are the hydrocarbons, lipids, fatty acids, soaps, fats and waxes, and oil. The principle consists that fats and oil and similar compounds are dissolved in a suitable solvent and extracted from the aqueous phase. Then, the solvent layer requires evaporation, and the residue is weighted to known their concentration according to equation 3.[50]

$$Fats\ and\ oil\ \left(\frac{mg}{L}\right) = \frac{[(W_2 - W_1) \times 1000]}{Volume\ of\ sample\ taken} \quad (3)$$

Where, W_1 is the weight of the empty evaporation dish, and W_2 is the weight of a sample collected intro evaporation dish. Various processes for their quantification could be used in Ecuador according to the book VI Annex 1 of the Environmental Quality Standard and effluent discharge: water resource. Additionally, this book mentions to comply with the requirements of treated wastewater, follow the manual "Standard Methods for The Examination of Water and Wastewater" from the American Public Health Association. This manual in the 23rd edition presents four techniques to determine the amount of fats and oil: the liquid/liquid partition-gravimetric method, the partition-infrared method, the Soxhlet method, and the solid-phase, partition-gravimetric method. Commonly the liquid/liquid partition-gravimetric technique is used.[46]

The parameters mentioned are essential for this work because they allow to characterize the wastewater from the edible oil industry. For the model proposal of this thesis, it is necessary to consider parameters such as temperature, pH, COD, BOD, the amount of phosphorus and nitrogen, and the number of fats and oil since it affects the growth of *C. necator*. A primary water treatment becomes helpful in reach optimal conditions for using biological treatment. Below are some wastewater treatments with a high content of fats and oil.

2.7 Main water treatments in Ecuador to remove fat and oil

According to the reference *Libro VI Anexo 1 de la Norma de Calidad Ambiental y de Descarga de Efluentes: Recurso Agua* is recommended to apply methods established in the manual *Standard Methods for the Examination of Water and Wastewater.*[43] The most effective primary treatments for solids, fats, and oil removal, is a dissolved air flotation method (DAF). DAF achieves a floating layer that divides the solid from the liquid. This process consists in

the adhesion of the residual water particles by constant bubbling. DAF allows a decrease in the apparent density. Moreover, DAF requires a saturated water stream at pressures between 172 to 630 kPa of gauge pressure. The solubility of air increases with pressure. In the inlet stream to the float chamber the pressure decreases and finally, the generation of small bubbles occurs by the depressurization, and those bubbles allow the residual particles to float.[51]

Another treatment for the removal of fats and oil is the electroflotation (EF). EF is an ascending process of organic matter or other pollutants, where water electrolysis generates hydrogen and oxygen bubbles by specific electrodes.[52] This process efficiency depends on the size of the bubbles generated, where small bubbles are preferable of the better contact surface for the adsorption of the particles to be removed, 90% of the bubbles in the EF can have a size between 15 to 45 μm whereas in dissolved air flotation (DAF) oscillates between 50 to 70 μm.[53] That means that the EF process would be more advantageous than DAF.

Some of the disadvantages of the DAF methods are that compared to simple sedimentation tanks, the DAF is relatively complex and requires more energy due to the recirculation of the water stream.[54] On the other hand, the costs of applying EF depend on the anode and cathode, the cathode can be selected according to the efficiency that is required, and they are generally easily accessible materials like stainless steel, but the anode, if one wants to achieve a high Efficiency, it will need to be Platinum, which can be costly if applied industrially.[55] The following section shows new alternatives for the removal of oils and fats from wastewater using bacteria.

2.8 New tendency for fat and oil removal with bacteria worldwide

In the last decade, several researches have been done on the use of bacteria to remove oil and fats in water. In 2017, a study of three bacteria that took advantage of the lipase enzyme production to remove oils and fats in wastewater was published. These bacteria are *Bacillus subtilis, Staphylococcus Epidermidis, and Pseudomonas Aeruginosa*.[56] In that latter work, it was found a greater enzymatic activity and consequently a greater efficiency in reducing fats and oils in the wastewater. Additionally, it was found that a continuous process shows more efficiency in enzyme activity than a batch process. In another similar study, they use these previously mentioned bacteria and recommend applying biostimulation to improve the removal of oils and fats.[57] Furthermore, they mention that there is greater degradation by mixed culture of the bacteria mentioned above in a fixed-bed bioreactor. Finally, for the bacteria to achieve

maximum performance, it is recommended to use biostimulation techniques in a bioreactor packed with polyethylene (PE).

In 2011, in a study made by the Department of Chemical Engineering of Amirkabir University of Technology in Iran, where they were much interested in using the *Ralstonia Eutropha* bacteria, now known *as C. necator*, to promote biological wastewater treatment with olive oil. This study recommends to adapt the bacteria with phenol before aerobic treatment and use a packed-bed reactor.[58]

In more recent studies, in the year 2020, it has been published that the use of the bacterium *Rhodococcus sp*. AQ5-07, native from Antarctica, has a high degradation activity of canola oil in wastewater from food industries; its efficiency increases by optimizing the environment conditions and controlling a maximum amount of oil that prevents bacteria growth decrease and oil degradation.[59] Another research study also published in 2020, showed that natural strains show greater degradation of oils and fats in wastewater, among which are: *Acinetobacter, Aeromonas, Bacillus, Pseudomonas*, and *Staphylococcus*.[60] Therefore, it is recommended to study the bacteria mentioned above to propose the most optimal for removing oils and fats in wastewater.

However, the interest in using *C. necator* is because it can synthesize and store PHAs, including PHBs up to 80% of its cell dry weight (CDW), with a polydispersity index of approximately 2 M_w/M_n, where M_w is the mass-average molar mass and M_n is the number-average molar mass, the polydispersity index close to unity, means that the polymer has better properties due to a homogeneous distribution of molecular weights of the polymer, for example, the polymer would have high mechanical resistance by acting as a plasticizer.[61] PHB has thermoplastic, elastomeric, biodegradable, non-toxic, thermal, and UV resistance properties, thus it can be an alternative to propylene.[61,62] One can see some properties of PHB concerning other similar polymers in Appendix B. *C. necator* is versatile regarding its living conditions; its metabolism can also be autotrophic and heterotrophic.[63] *C. necator* makes it attractive in the industry because it can produce PHBs at low cost using readily accessible carbon sources, such as oils and fats, and by limiting nutrients. This type of PHA is an excellent alternative to non-biodegradable petroleum-based plastic.[64] In Appendix C, one can see references of researches using *C. necator*, which can use different carbon sources to produce PHB.

CHAPTER III

3. LIPOPHILIC BACTERIA USED FOR PHA AND PHB PRODUCTION IN WASTEWATER TREATMENT

A WWTP can use microorganisms as an alternative to treat the wastewater with fats and oils as it was already explained before. Some microorganisms can produce lipase enzymes to promote the degradation or hydrolysis of oils and fats.[65] A wide variety of microorganisms can degrade oils and fats. There are thermophilic bacteria that can decompose vegetable oils in conditions with a wide temperature range, such as *Bacillus Thermoleovorans* IHI-9.[66] Also, a mixed bacterial culture can degrade oils and fats from wastewater, for example, fifteen bacteria from fatty wastewater samples can degrade oils and fats from plant and animal origins, at pH ranging from 4.5 to 9.5.[67] In the last decade, it was discovered some bacteria that can degrade oils and fats in wastewater can then produce biopolymers such as PHAs like PHB (Figure 6). PHAs have properties similar to polyethylene. Some of these bacteria are *C. necator, Pseudomonas*, and *Bacillus*.[68]

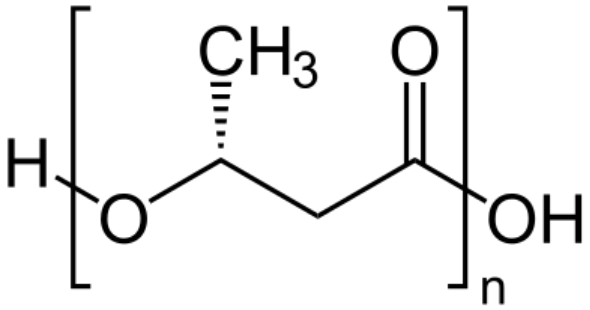

Figure 6. Chemical structure of Poly-3-hydroxybutyrate (PHB).

3.1 *Cupriavidus necator*

C. necator is also known as *Ralstonia eutropha* and previously known as *Hydrogenomonas eutrophus* and *Alcaligenes eutrophus*. *C. necator* is a Gram-negative bacterium belongs to the *ß-Proteobacteria* widely studied for the production of biodegradable plastics. It is a versatile bacterium because it can grow in autotrophic or heterotrophic conditions. Also, it naturally produces some biopolymers like PHB.[69,70] One of the characteristics that make *C. necator*

attractive for industrial use is it contains lipase enzymes. Lipase enzyme allows hydrolysis processes of difficultly degradable compounds like oils in wastewater. Finally, oils are decomposed by hydrolysis into fatty acids, which are carbon sources for the growth of *C. necator*. A study reports the release of extracellular lipase, which indicates the activity of hydrolysis processes in palm oil for the production of PHAs from the growth of *C. necator.*[71] In the Figure 7 is shown the metabolism of *C. necator* using carbon sources such as oils and fats.[72] Also, it shows the mechanism for the production of PHAs, including PHB.

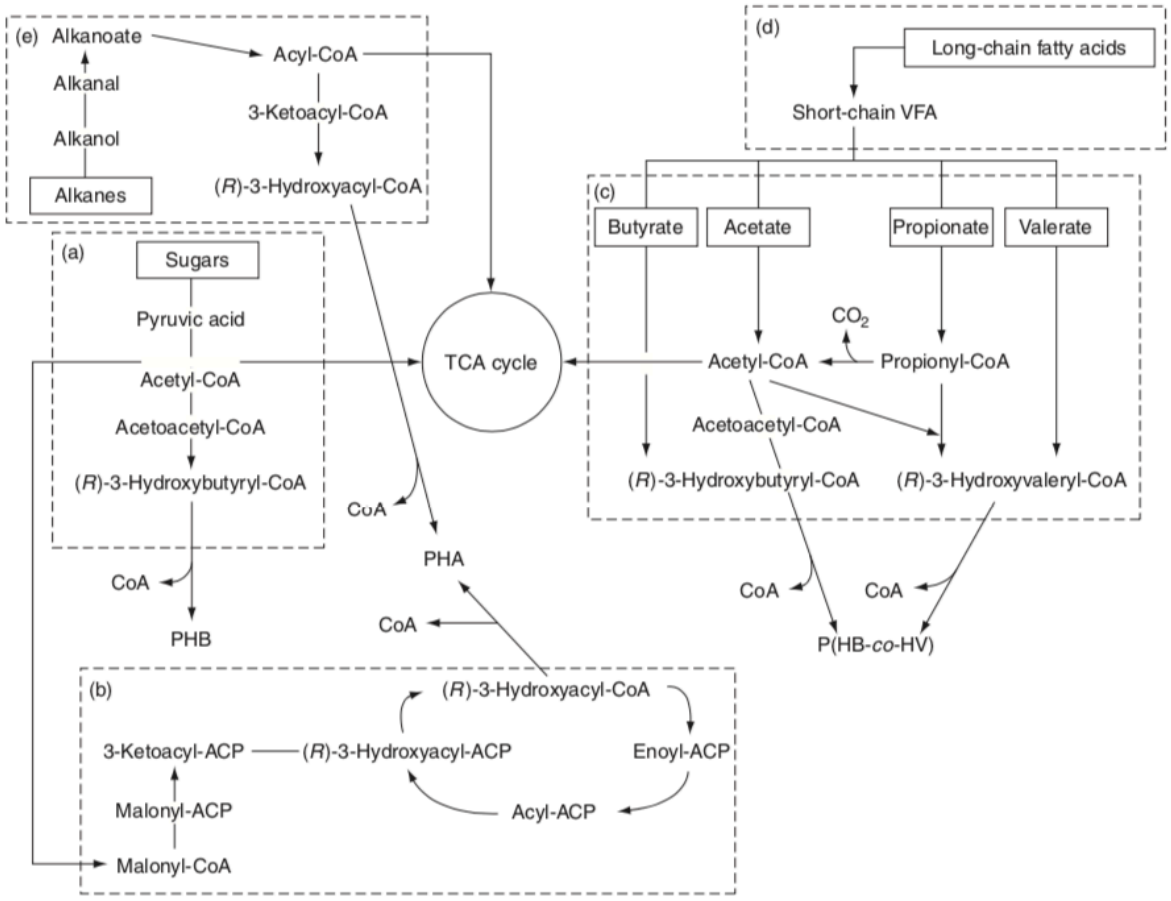

Figure 7. Metabolic pathways for PHAs synthesis from sugars and long chain fatty acids. Through glycolysis (a). Through fatty acid biosynthesis (b). From fatty acids, directly (c). Through fatty acids β oxidation (d). From alkanes (e). Taken from Reis *et al.*[72]

3.2 *Cupriavidus necator* growing conditions

In several reports, the growth conditions of *C. necator* are similar. Among the optimal conditions, one can find the following: pH 7, mineral medium per liter of water with 10 g of fructose for 24 hours, at 30 ºC and 300 rpm; 1.57 g of NH_4SO_4 and other reported nutrients.[73]

C. necator growth can accumulate PHAs at the end of the growth phase. PHB is known to be one of the most abundant PHAs of bacterial origin. The high PHB production requires an optimal carbon concentration, a limitation of nutrients such as nitrogen, and constant pH. According to the literature, the optimal values are C = 20 g/L, N = 1.5 g/L, P = 8.75 g/L, to get a maximum PHB production of 4.6 g/L. In the cytoplasm of *C. Necator*, can be found PHB in the form of granules. For the growth of *C. Necator*, fructose is useful as a carbon source and ammonium as a nitrogen source as well.[74]

Using various sources of substrate encourages the production of copolymers. For example, the use of substrates such as fructose and avocado oil for the growth of *C. necator* allows the production of P(3HB-*co*-3HV) copolymers. However, the yield decreases concerning the use of fructose as the only substrate.[73] On the other hand, the limitation of nutrients such as nitrogen becomes relevant for the maximum production of PHB.

All these mechanisms are very important to study and, in that sense, one can propose some kinetical models to evaluate the *C. necator* behavior and predict the best conditions of growth and PHB production by mathematical modelling which is the purpose of this research work.

CHAPTER IV

4. KINETICAL MODELS AND MATHEMATICAL MODELS

4.1 Kinetical models to follow bacteria growing

Bacteria growth can be described in 4 phases: lag phase, exponential phase, stationary phase, and death phase (Figure 8). In the lag phase, bacteria do not grow in the bioreactor; but are adapting themselves to environmental growth conditions. These conditions can be temperature, pH, and substrate concentration. In the exponential growth phase, the bacteria grow at a high rate. In the stationary phase, bacteria do not growth because of the depletion of nutrients or the presence of toxic substrate toxic. In the death phase, the nutrients required for bacterial growth were totally used.[20]

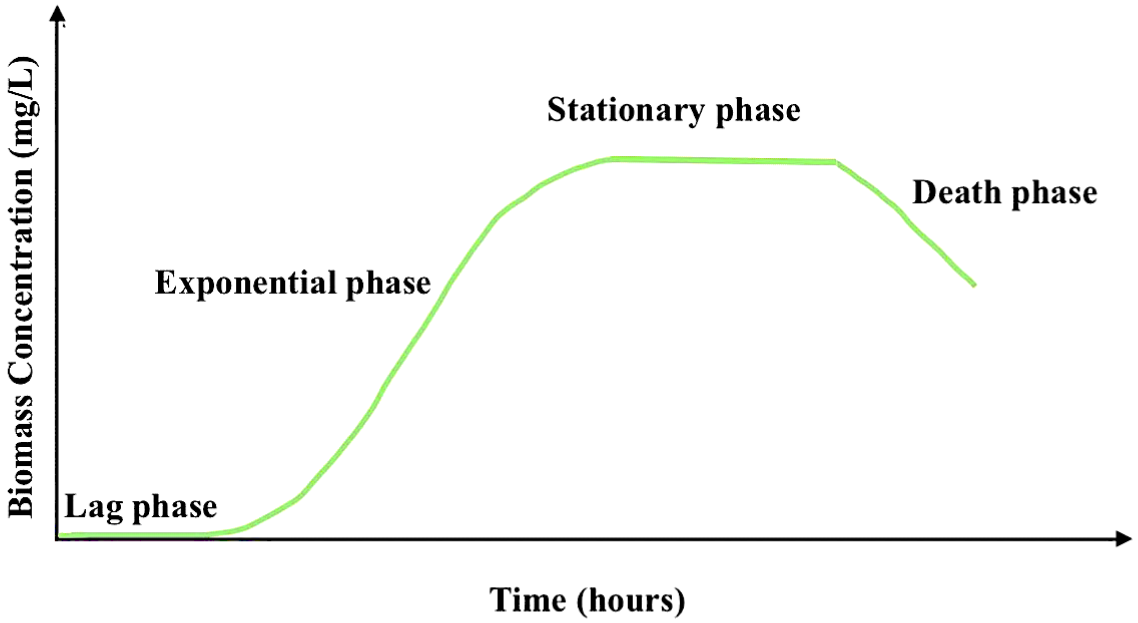

Figure 8. Bacteria growth rate curve.

Kinetic models of bacterial growth are useful for designing and controlling biological processes. The kinetic models can be divided mainly into two types. The first represents the inhibition of substrate, and the second the non-inhibition of the substrate.[20] Appendix D summarizes some kinetic models found in the literature to study the growth of bacteria. One of the most studied models has been Monod (which can also be used to represent the growth of *C. necator*) represented in equation 4.

$$\mu = \frac{\mu_{max} X\, S}{K_s + S} \tag{4}$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, S is the substrate concentration, X is the biomass concentration and K_s is the half saturation constant. However, the Monod model has five limitations.[20].

- The 1st limitation occurs at high concentration substrate, in that case, the maximum specific rate is independent on the substrate concentration.
- The 2nd limitation occurs at low concentration substrate, in that case, the growth rate is dependent on the substrate concentration.
- The 3rd limitation is when a substrate presents inhibition, for example, a toxic substrate then the Monod model cannot be used.
- The 4th limitation is that the Monod model do not consider the ability of bacteria to use substrate during the death phase.
- Finally, the 5th limitation is that Monod model do not take into account the lag phase and the death phase during the growth phase.

However, small modifications are usually made to Monod Model, for example, adding a double substrate relationship or inhibition constants.[75] The equation 5 shows an example of the specific growth rate of biomass, with the concentration of substrates such as glycerol and nitrogen by means of Monod's Model.

$$\mu_1 = \mu_{max} \cdot \frac{GLY}{GLY + k_S} \cdot \frac{N}{N + k_N} \tag{5}$$

However, it is possible to optimize the equation 5. For example, when a substrate such as glycerol is in excess, the Monod kinetic model needs to be modified again, considering the glycerol substrate inhibition.[75] The equation 6 represents this example:

$$\mu_2 = \mu_{max} \frac{GLY}{GLY\left(1 + \left(\frac{GLY}{k_{i,GLY}}\right)^2\right) + k_S} \cdot \frac{N}{N + k_N} \tag{6}$$

Equations 5 and 6 represent Monod models using two substrates: glycerol (GLY) measured in gCOD/L and Nitrogen (N) measured in gN/L, $k_{i,GLY}$ is an inhibition constant for glycerol, k_S and k_N are the saturation constants for glycerol and Nitrogen, respectively. Both growth rates μ_1 and μ_2 have shown similar results. The use of one or the other kinetic model depends on the purpose of the study. For practical purposes and reduction of mathematical calculations, equation 5 is useful in this work.

4.2 Mathematical models for bacteria growing optimization

In general, there are three types of models: physical, conceptual, and mathematical ones. Physical models represent the scale of the system to be modeled. Conceptual models qualitatively describe the modeling system through observation or flow charts. Mathematical models describe the system to be modeled quantitatively through conversion rates and stoichiometric relationships. Mathematical models consider time, scale, environmental conditions that are used to describe relevant processes. Concerning time mathematical models can be classified in a dynamic state, a static state, and a steady-state. Dynamic models represent various states and variables as a function of time. Static models describe processes that may vary as a function of time, and at a scale of interest, looks that it does not change. And finally, steady-state processes consider processes that are so fast, also are called equilibrium processes. In a wastewater treatment system is common to use steady-state models and dynamic models. Finally, a mathematical model could come from empirical models or mechanistic models. Empirical models depend on the identification of essential parameters through observation. On the other hand, a mechanistic model depends on concepts of the chemical, physical, and biological processes that occur within a system and the interaction between chemical compounds.[49]

4.3 Basis for developing a mathematical model

One of the tools to analyze and predict a biological system's behavior is the construction and subsequent simulation of a mathematical model. If a biological phenomenon is known in a reactor, for example, through the metabolism of a bacterium, it is possible to know equations that describe this phenomenon, and therefore a mathematical model can be built.[76] Figure 9 shows a flow chart of the steps to follow for the construction of a mathematical model.

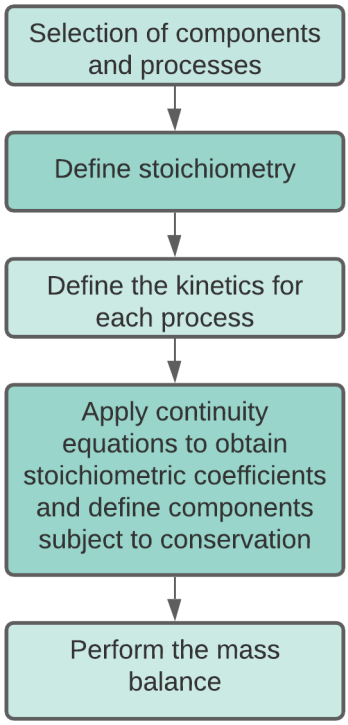

Figure 9. Flowchart for the development of a mathematical model.

For the construction of a mathematical model, one must consider the stoichiometry, kinetics, and processes, all that provide a quantitative description of the system.[49] Later it will be necessary to verify the mathematical model through experiments; if the model fits the experiments, it can be usefully used. Below one can see a description of the factors for developing a mathematical model used.

4.4 Mathematical model

4.4.1 Components and process

The development of a mathematical model begins with selecting the components and processes that are of interest. The components are the species whose concentration one is interested in knowing, and those are defined by the compound name and the units of measurement. The processes are the reactions between the components, and those are defined by stoichiometry. The processes are defined by kinetics, the reactants that are consumed, and the products that are formed.[77] For example, for this work will be of interest to consider processes such as bacterial growth, lysis, and hydrolysis, and compounds such as O, N, P, and organic matter (COD).

4.4.2 Stoichiometry

The stoichiometric of a reaction is a set of stoichiometric coefficients that relate each of the reactants' quantities with the products; in other words, the stoichiometric represents the equality of masses and charges between the reactants and products of a chemical reaction, of that stoichiometry complies with the law of conservation of masses.[78] To understand stoichiometry, one must remember the standard way to write a reaction, as shown in equation 7.

$$a_1A_1 + a_2A_2 + \ldots + a_kA_k \rightarrow a_{k+1}A_{k+1} + a_{k+2}A_{k+2} + \ldots + a_nA_n \quad (7)$$

In equation 7, a_i are the stoichiometric coefficients, and A_i are the reaction components. The stoichiometric coefficients of the reactants have negative values because they express the component's consumption, and the stoichiometric coefficients of the products have positive values because they express the conversion to products. Generally, the material balance of equation 7 is presented in a normalized way; for example, if one takes a reactant as a reference to normalize it, it will take the value -1. The same happens if one wants to normalize towards a product, it will have a value of +1. For example, in a reaction, if a coefficient of a component of interest like heterotrophic biomass (X_H) is selected, a value of 1 will be assigned. [49]

Some stoichiometric coefficients can be calculated using yields, conversion factors, and continuity equations. The components selected for the mathematical model developed in this work do not necessarily have to participate in all reactions. Through continuity equations, it is possible to calculate the lately stoichiometric coefficients unknowns. In addition to considering the relevant processes and compounds, one has to consider conservative mass balances that indicate the number of atoms that a compound enters must be equal to what leaves. Some components of a conservative type are Nitrogen, Phosphorus, BOD, and COD. [49]

As mentioned above, some stoichiometric coefficients can be obtained from yields or conversion factors. The yields are the percentage ratio between two components. Generally, between a produced component and a consumed component. The widely known yield is the yield of heterotrophic organisms Y_H, its expression can be seen in equation 8. On the other hand, a conversion factor is the ratio between the fraction of a component generated and the biomass generated, for example, the fraction of nitrogen in biomass f_N, equation 9.[49]

$$Y_H = \frac{gCOD_{Biomass}}{gCOD_{Sustrate}} \quad (8)$$

$$f_N = \frac{gN}{gCOD_{Biomass}} \quad (9)$$

4.4.3 Kinetics

Kinetics represents the rate in which a process takes place. In a mathematical model, each process will be represented by a kinetic equation. A kinetic equation relates the conversion rate of a compound, and in some cases, it will depend on conversion yields. As it was mentioned before, one of the widely used kinetic models is Monod, which is composed of two main parameters. The first is the maximum specific growth rate (μ_{max},) and the second is the saturation constant (k). k is defined as the concentration at half of the maximum rate. Besides, the saturation term $S/(k_s + S)$ can have a value between 0 to 1. In some cases, the saturation constant can be very small, for example, with ammonia. Therefore, the ammonia's constant saturation would serve to ensure that there is no further growth once the ammonia is depleted.[49] Equation 10 represents a Monod type kinetic with its saturation parameters for various components.

$$\mu = \mu_{max} \frac{S}{k_s + S} \times \frac{S_O}{k_O + S_O} \times \frac{S_N}{k_N + S_N} \dots \quad (10)$$

On the other hand, if one wants to describe inhibition kinetics, the constant will be called the inhibition constant, which will be part of an inhibition term in Monod, which can have a value in a range of 0 and 1. The inhibition constant is defined as the concentration in which the reaction rate of the process is halved. The inhibition parameter commonly used in kinetical models is shown in equation 11.[49]

$$1 - \frac{S}{k_s + S} = \frac{k_s}{k_s + S} \quad (11)$$

4.4.4 Matrix notation

Matrix notation is a way to represent kinetic models. It also facilitates the creation of a software using matrix notation. The first step to construct the matrix for a kinetic model is to identify the components of interest for the model. These will be located in the first row of the matrix and it will also include the units of measurement of each component. It is then necessary to identify the biological processes involved in the transformation processes of the components. Between the biological processes and the components, the stoichiometric coefficients will be located; these will have positive signed values if they are compounds that are being produced and negative values if they are compounds that are consumed. For example, oxygen has a negative COD because in oxidation, it is consumed by accepting electrons from the substrate, and later, water is formed.[49] In appendix E one can see an example with negative values for the stoichiometry of oxygen and compounds where there is consumption.

Suppose one wants to calculate the stoichiometric coefficients of the matrix. It should be considered to apply the continuity law to the sum for each row of the stoichiometric coefficients, resulting in a value equal to zero. Finally, for convenience, the inclusion of a composition matrix such as COD, nitrogen, phosphorus, and the charge may be considered. An example of a stoichiometric matrix together with a composition matrix can be seen in the appendix E. Also, the multiplication of the two matrices must be equal to zero, allowing the conservation of mass. Additionally, equation 12, is an equation to apply to the matrices' above mentioned, where $\nu_{j,i}$ are the stoichiometric coefficients and $i_{c,i}$ are the quantity of material to conservation in a unit of component.[49] Applying the equation 12 will allow to obtain continuity equations necessary for calculating some unknown stoichiometric coefficients of the matrix.

$$\sum_{i} \nu_{j,i} \cdot \ i_{c,i} = 0 \qquad (12)$$

The balance equations are the basis of any model because they describe the change in concentration through chemical and biological conversions as a function of time. However, the concentration change of steady-state models as a function of the time is equal to zero. Biological processes depend on the concentration of the reactor and not on chemical and biological conversions. Also, microorganisms do not distinguish the type of reactor.

Consequently, this facilitates scaling from laboratory to industrial level through the models such as activated sludge model (ASM).[49]

4.5 ASM Models

The ASM models are the most used for WWTP and processes at low temperatures between 5 to 20 ºC. The AMS1 model was the first model published in 1985 in IAWPRC Scientific and Technical Report Series No. 1. This model used for the first time the matrix notation and considers the nitrogen removal. Then, the ASM2 model was required because it includes the removal of biological phosphorus. Consequently, the ASM2D model was necessary to consider phosphorus-accumulating organisms (PAOs) for denitrifying PAOs. The ASM3 model reconsiders the internal storage compounds from the metabolism of microorganisms.[79] All the previously mentioned models share concepts, notations, and terminology. Appendix F presents a summary of various activated sludge models, as well as the number of processes, the maximum of reactions, and the maximum of state variables that each model can consider.

Among the ASM models, the best models that could fit this thesis proposal are ASM1, ASM2, and ASM2D. One wants to propose the kinetic model with components such as COD, nitrogen, and phosphorus; those components are essential nutrients for *C. necator* and PHB accumulation growth. The compounds that are part of the nutrients for the growth of microorganisms are nitrogen and phosphorus. For this work, a model that takes into account both compounds will be considered. The phosphorus (P) component is only part of the ASM2D and not of the ASM1 and the ASM2. The ASM2D is a mathematical model that considers some components and processes of interest, such as aerobic heterotrophic growth, aerobic hydrolysis, and lysis of heterotrophic organisms.[79]

CHAPTER V

5. METHODOLOGY

The development of this thesis has a sequence of activities and parameters, from the literature review until the evaluation of this thesis objectives. The mechanisms to achieve the stablished goals are below.

5.1 Data recompilation for the matrix and model construction (Step 1)

The collection of experimental data is an important step in the construction of a mathematical model. For this reason and in order to make a model that allows predicting the growth of *C. necator* in the wastewater of oil industry, with the production of PHB, was considered the Flores report as reference data.[73] The data was obtained from an avocado industry because the kinetical model could help avocado oil industries like UYAMA FARMS. Data such as the growth medium allows knowing the initial concentrations used as input data for the simulation of the model to be proposed. Table 5 are summarized the components of the growth medium of *C. necator* in a bioreactor.

Table 5. Content of the growth media and the seed culture of *C. necator.*

Medium	Content
Growth medium	10 g/L Fructose 1.57 g/L $(NH_4)SO_4$ 5.66 g/L $Na_2HPO_4 \cdot 12H_2O$ 1.50 g/L KH_2PO_4 0.20 g/L $MgSO_4 \cdot 7H_2O$ 10 mg/L $CaCl_2 \cdot 2H_2O$ 20 mg/L $FeSO_4 \cdot 7H_2O$ 1 ml trace solution

5.1.1 Data recompilation of the growth phases

Table 6 analyzes the growth of *C. necator* in three stages. In summary, *C. necator* is fed from two substrates: fructose (S_S) and fatty acids of avocado oil (S_{FA}). The biomass produced (X_H) will be composed of residual biomass (X_R) and the accumulation of PHB (X_{PHB}). An initial condition is the biomass concentration ($X_{H,o}$). Some yields are biomass produced over substrate consumed ($Y_{X/S}$) and PHB produced over biomass produced ($Y_{PHB/X}$).[42]

Table 6. Stages for *C. necator* growth and PHB accumulation.[73]

Stage 1: Batch cultivation	**Time range:** 0 to 15 hours S_S: 6.70 g/L of Fructose $X_{H,o}$: 0.13 g of initial biomass X_H: 1.30 g of Heterotrophic biomass $Y_{X/S}$: 0.19 g X_H / g Ss
Stage 2: Fed-batch cultivation	**Time range:** 15 to 30 hours S_S: 5.63 g/L of Fructose X_H: 2.35 g/L of Heterotrophic biomass X_{PHB}: 1.14 g/L of PHB X_R: 2.65 g/L of Residual biomass $Y_{X/S}$: 0.42 g X_H / g Ss $Y_{PHB/X}$: 0.48 g X_{PHB} / g X_H
Stage 3: Synthesis of PHB	**Time range:** 30 to 50 hours S_{FA}: 20% V/V avocado oil X_H: 4.91 g/L of Heterotrophic biomass X_{PHB}: 3.48 g/L of PHB X_R: 1.43 g/L of Residual biomass S_S: 6.80 g/L of Fructose $Y_{PHB/X}$: 0.02 g X_{PHB} / g X_H

After considering an experimental reference for data collection. It must be built a mathematical model from the reference data to study the kinetics of *C. necator.* It will be necessary to establish parameters including stoichiometric coefficients, kinetic constants, components, and processes, which will be presented below.

5.2 Mathematical model (Step 2)

5.2.1 Model proposal

For this thesis, one wants to propose an aerobic model for wastewater regulated with neutral pH at a temperature of 30 ºC because data of the growing of *C. necator* with an optimal production of 70% of PHB in CDW (Appendix G). For the development of the model, it will be of interest to simulate the accumulation of PHB in a transitory state; this means that one is interested in modeling PHB production as a function of time.

Some kinetic parameters can be adjusted using data from other models, such as activated sludge models (ASM). As seen in section 4.5, one of the best models that is similar to the model proposed for this thesis is the activated sludge model No. 2d (ASM2D). It includes phosphorus as a component. However, it has the limitation that the temperature to apply is between 10 ºC and 20 ºC. Therefore, the kinetic constants, must be adjusted to the required temperature (30 ºC) using the Arrhenius equation. The proposed initial concentrations were taken considering the Flores report, however for unreported compounds of interest, data from Henze and Katie were taken into account.[73,79,80] All these initial concentrations will serve to simulate the kinetic models of *C. necator* growth and PHB production and are summarized in the table 7:

Table 7. Initial concentrations for simulation of kinetical models.

Initial concentrations	
S_O	0.002 g/L
S_I	0.03 g/L
S_S	10 g/L
S_{FA}	35.42 g/L
S_{NH4}	0.4 g/L
S_{PO4}	0.0036 g/L
X_{PHB}	0 g/L
X_H	0.13 g/L
X_S	0 g/L
X_I	0.025 g/L

5.2.2 Components

To choose the components and processes, one first sought to understand the *C. necator* bacteria's general metabolism (Figure 7). The components selected for this model are classified as soluble (S) and particulate (X) and are presented in the Table 8.

Table 8. Biological components for the mathematical model.

Component	Description	Units
S_O	Dissolved oxygen	$gCOD/m^3$
S_I	Inert soluble organic matter	$gCOD/m^3$
S_S	Readily biodegradable substrate - Fructose	$gCOD/m^3$
S_{FA}	Readily biodegradable substrate - Vegetable Oil Fatty Acids	$gCOD/m^3$
S_{NH4}	Total ammonium nitrogen	gN/m^3
S_{PO4}	Phosphate	gP/m^3
X_H	Active heterotrophic biomass	$gCOD/m^3$
X_{PHB}	PHB accumulated	$gCOD/m^3$
X_I	Inert particulate organic matter	$gCOD/m^3$
X_S	Slowly biodegradable substrate	$gCOD/m^3$

The above components were part of the stoichiometry matrix for creating simulation software. Some components such as nitrogen (N), phosphorus (P), and COD will be part of the composition matrix, as suggested in section 4.3.3.

5.2.3 Processes

Regarding the processes, the study of a dynamic process was considered. In general terms one can say that more variables and parameters a model has, the closer it is to reality. However, this is not always the case because it could generate a more complicated model to understand and with more significant calculation and greater data processing.[49] On the other hand, the fewer processes are considered, simpler and easier the model will be to calculate. However, for this thesis, one proposes four fundamental processes for the growth of *C. necator*, considering the work of Flores,[73] which used two sources of carbon such as fructose and fatty acids from avocado oils to study the synthesis and accumulation of PHB in *C. necator*. The *C. necator* bacteria's metabolism was considered as shown in Figure 7; consequently, it is summarized in Figure 10.

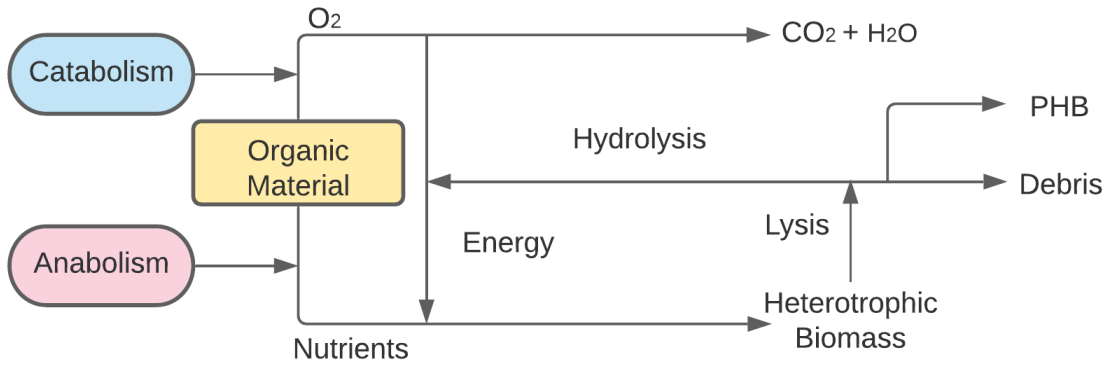

Figure 10. Simplified metabolism for *C. necator*.

In Figure 10, the organic material will be fructose and fatty acids from wastewater of avocado oil manufacturing, the nutrients will be phosphorus (P) and nitrogen (N). The lysis produces PHB and residual cell debris consisting of organic matter such as lipids, proteins, polysaccharides, nucleic acids, and cell wall fragments.[81] From the metabolism of *C. necator* previously presented, the following processes were proposed:

- Heterotrophic aerobic growth through fructose
- Heterotrophic aerobic growth through fatty acids
- Aerobic hydrolysis
- Lysis of heterotrophic organisms

These four processes are part of the construction of the stoichiometry matrix and will be located in the rows of the stoichiometric matrix; and it will also be necessary to consider the kinetics associated with each process.

5.2.4 Stoichiometry

Stoichiometric coefficients must be calculated as mentioned above in section 4.4.2. For example, to calculate the yield of the biomass produced, equation 8 is used, and it would be estimated as described below in eq. 13.

$$Y_{X/S} = \frac{X - X_o}{S_o - S} \tag{13}$$

In the equation 13, X is the amount of biomass produced, X_o is the amount of initial biomass, S the substrate consumed, and S_o is the substrate or carbon source at the beginning of the study stage. Some yields can be obtained from experimental results (Table 6). It will be necessary to calculate yields for the production of PHB as well. This can be done by using eq. 10, where the substrate considered in this work was fructose or fatty acids according to the corresponding stage. The variable X was changed by a variable PHB that represents the PHB production, as shown in equation 14.

$$Y_{P/S} = \frac{PHB - PHB_o}{S_o - S} \tag{14}$$

Where PHB is the final concentration of PHB, PHB_o is the initial concentration of PHB, S_o is the initial concentration of substrate, and S is the final concentration of substrate, like fructose or fats and oil. Other stoichiometric coefficients were obtained from component fractions as mentioned in section 4.4.2 and according to equation 7. However, it is not necessary to calculate all of them because some values are already reported. For example, for the hydrolysis process, the fraction of inert matter in the soluble substrate f_{SI} has a reference value for the ASM2D models equal to zero. In contrast, for the lysis processes, the fraction of inert matter f_{XI} has a value of 0.1.[79]

Henze recommends to use $-1/(Y_{X/S,F})$, to calculate the stoichiometric coefficient between the heterotrophic growth process with fructose $S_{S,F}$. Similarly, the stoichiometric coefficient for the heterotrophic growth process with fatty acids S_{FA} is calculated where this value is equivalent to $-1/(Y_{P/S,A})$. For the stoichiometric coefficients of heterotrophic growth for the production of the PHB polymer it is obtained that, for fructose, it is equivalent to $Y_{P/X}$. For fatty acids, it is equal to $Y_{P/S,A}$. For the stoichiometric coefficient of X_{PHB} in the lysis process, the value was taken from Flores' results, which reported a PHB obtaining of 70.8% CDW. Other stoichiometric coefficients were taken from Henze's ASM2D activated sludge model; these values are shown below, for example, the coefficients of material conservative are taken from Henze. The calculations of the kinetic parameters can be seen in annex H.

5.2.1 Matrix notation

The mathematical model proposed will be based on the matrix notation, as seen in section 4.4.4. It will be necessary to build a stoichiometric matrix, wherein the rows will be the processes together with associated kinetics, and in the columns will be the components. It should also have a composition matrix where the rows will be the components to be conserved. The composition matrix's conversion factors are summarized in Table 9.

Table 9. Typical conversion factors for ASM2D model. [79]

Component	Description	Value	Units
Nitrogen			
i_{NSI}	N content of inert soluble S_I	0.01	gN/gCOD
i_{NBM}	N content of biomass X_H	0.07	gN/gCOD
i_{NXI}	N content of inert particulate X_I	0.02	gN/gCOD
i_{NXS}	N content of slowly biodegradable substrate X_S	0.04	gN/gCOD
Phosphorus			
i_{PSI}	P content of inert soluble S_I	0.00	gP/gCOD
i_{PBM}	P content of biomass X_H	0.02	gP/gCOD
i_{PXI}	P content of inert particulate X_I	0.01	gP/gCOD
i_{PXS}	P content of slowly biodegradable substrate X_S	0.01	gP/gCOD

The stoichiometric matrix and composition matrix are represented in the Table 10, where $a, b, c, d, e, f, g, h, i\ j, k$ and l are the unknown stoichiometric coefficients.

Table 10. Stoichiometric matrix and composition matrix proposed.

Stoichiometric Matrix M(COD)L^{-1})										
Components	S_O	S_I	S_S	S_{NH4}	S_{PO4}	S_{FA}	S_{PHB}	X_H	X_I	X_S
Process	gO2	gCOD	gCOD	gN	gP	gCOD	gCOD	gCOD	gCOD	gCOD
Aerobic heterotrophic Growth in Fructose	a	0	-1/Y_{XS}	b	c	0	$Y_{PHB/XH}$	1	0	0
Aerobic heterotrophic Growth in fatty acids	d	0	0	e	f	-1/$Y_{PHB/FA}$	$Y_{PHB/FA}$	1	0	0
Hydrolysis	0	f_{SI}	g	h	i	0	0	0	0	-1
Lysis of heterotrophic	0	0	0	j	k	0	$Y_{PHB/necator}$	-1	f_{XI}	l
Material Conservation Matrix (conservative components) gCOD										
Conservative components	S_O	S_I	S_S	S_{NH4}	S_{PO4}	S_{FA}	S_{PHB}	X_H	X_I	X_S
COD (g)	-1	1	1	0	0	1	1	1	1	1
N (gN/gCOD)	0	i_{NSI}	0	1	0	0	0	i_{NBM}	i_{NXI}	i_{NXS}
P (gP/gCOD)	0	i_{PSI}	0	0	1	0	0	i_{PBM}	i_{PXI}	i_{PXS}

5.2.2 Continuity equations

The continuity equations are the mathematical equivalent to the mass balance equations. First, to define the continuity equations, the materials subject to conservation must be defined, as mentioned above are nitrogen (N), phosphorus (P), and organic matter measured as chemical oxygen demand (COD). Also, it will be necessary to calculate the unknown stoichiometric coefficients of the stoichiometric matrix expressed in Table 10. The objective is to apply equation 12 for each stoichiometric matrix process using the material conservation matrix mentioned above. Below in Table 11 are presented the expressions of the continuity equations for each process.

Table 11. System of continuity equations for each process.

Conservative components	Continuity equations for growth using fructose
COD	$-a - 2.40 + 0.48 + 1 = 0$
N	$b + 0.07 = 0$
P	$c + 0.02 = 0$
Conservative components	Continuity equations for growth using fatty acids
COD	$-d - 50.89 + u + 1 = 0$
N	$e + 0.07 = 0$
P	$f + 0.02 = 0$
Conservative components	Continuity equations for hydrolysis
COD	$f_{SI} + g - 1 = 0$
N	$0.01 \cdot f_{SI} + h - 0.04 = 0$
P	$j - 0.01 = 0$
Conservative components	Continuity equations for lysis heterotrophic
COD	$0.708 - 1 + f_{XI} + l = 0$
N	$j - 0.07 + 0.02 \cdot f_{XI} + 0.04 \cdot l = 0$
P	$k - 0.02 + 0.01 \cdot f_{XI} + 0.01 \cdot l = 0$

Where in the previous table f_{XI} and f_{SI} are known, a system of equations can be obtained from each process; each equation considers a component to be conserved and is equal to zero to comply with the mass balance.

5.2.3 Kinetics

The kinetics are associated with each process represented in the row of the stoichiometric matrix. For the heterotrophic growth process of *C. necator* using fructose, according to the study by Sharifzadeh.[82] The Monod kinetic model is the one with the highest maximum specific growth rate for fructose. Compared to other kinetic models such as Contois, Westerhoff, Herbert, Moser, and Tessier, Monod has the best fit in kinetic model simulations. Therefore, the kinetics proposed for this thesis is shown below.

$$\mu_F = \mu_{F,max} \cdot \frac{F}{F + k_F} \cdot \frac{N}{N + k_N} \cdot \frac{O}{O + k_O} \cdot \frac{P}{P + k_P} \cdot X_H \qquad (15)$$

Where $\mu_{F,max}$ is the maximum specific growth rate, the substrate is fructose concentration F with its half saturation constant k_F, the nutrients are nitrogen N and phosphorus P with the half saturation constants k_N and k_P respectively.

For the second process of heterotrophic growth through fatty acids, the study by Vrana[75] was considered since no reports for the study with fatty acids were found. He proposes the growth and formation of PHB from glucose and glycerol. However, fatty acids from oil need to be taken into account, thus one proposes a Monod model considering substrate inhibition. Here one has considered the model made for glycerol as a basis (Vrana[75]), given that glycerol is a similar organic molecule. Therefore, the kinetic model associated with growth by fatty acids that one has proposed, is represented in equation 16.

$$\mu_{FA} = \mu_{FA,max} \cdot \frac{FA}{FA + k_{FA}} \cdot \frac{N}{N + k_N} \cdot \frac{k_i}{k_i + F} \cdot \frac{O}{O + k_O} \cdot \frac{P}{P + k_P} \cdot X_H \qquad (16)$$

Where $u_{FA,max}$ is the maximum specific growth rate using fatty acids, k_{FA} is the half saturation coefficient using fatty acids and k_i is the Kinetic inhibition for fructose. For the hydrolysis

process were considered the kinetics for the heterotrophic models of activated sludge proposed by Henze,[79] described in equation 17.

$$\mu_{HY} = k_h \times \frac{O}{k_O + O} \times \frac{{}^{X_S}/_{X_H}}{k_X + {}^{X_S}/_{X_H}} * X_H \quad (17)$$

Where k_h is the maximum specific hydrolysis rate, k_O is the half saturation coefficient using oxygen, k_X is half saturation coefficient for slowly biodegradable particulate substrate X_S. Finally, for the heterotrophic lysis process, a general kinetic equation by Henze,[79] was used, equation 18.

$$\mu_{LY} = b_H \times X_H \quad (18)$$

Where, b_H is the heterotrophic decay rate and X_H is the heterotrophic biomass. The kinetics mentioned above must have an adjustment in their kinetic constants. These kinetic constants are considered to work at neutral pH, but they vary with temperature, so the modified Arrhenius equation was used (eq. 19). Also, kinetics will be an essential parameter for the development of the kinetic model. Therefore, the kinetic constants' values should be adjusted using the modified Arrhenius equation, as shown in equation 19.

$$P(T) = P(20°C)\ \theta_p^{T-20} \quad (19)$$

$P(T)$ is the nominal value of the parameter at 20 ºC and θ_p the temperature correction factor for each parameter.[83] According to Ramirez's study, the kinetic parameter is constant at a higher temperature, for example, if a fixed parameter is taken at a temperature between 31 and 39 ºC.[84] Above 39 ºC at high temperature, a kinetic parameter takes a constant value. For this thesis, the temperature of 30 ºC and pH 7 was considered because as already said most of the data were taken from Flores.[73] The kinetic parameters are summarized Table 12.

Table 12. List of kinetic parameters.

Symbol	Units	Value	θ_P	Reference
$\mu_{F,max}$	h^{-1}	0.125	none	85
$\mu_{FA,max}$	h^{-1}	0.01	none	86
k_F	g/L	30	none	85
k_{NH4}	g/L	0.24	none	86
k_O	g/L	0.0002	none	79
k_{PO4}	g/L	0.00001	none	79
k_{FA}	g/L	11	none	86
$k_{N,F}$	g/L	0.24	none	86
k_i	g/L	$4.28x10^{-4}$	none	86
k_h	h^{-1}	0.187	0.0405	79
k_X	gX_S/gX_H	0.1	none	79
b_H	h^{-1}	0.0333	0.0693	79

5.2.4 Mass balance

To obtain the mass balance equations for the kinetic model that one has proposed, it was applied the principle of conservation of mass, where the general equation is:

$$Accumulation = Input - Output \pm Generation \quad (20)$$

The previous equation has dimensions of mass as a function of time. The input and output are the amounts of matter entering or leaving a system. The accumulation is the variation of the material as a function of time; it will have a positive value when the concentration of the material increases and a negative value when the amount of material decreases. The generation is the amount of material that appears or disappears due to chemical reactions; it will have a positive value when the material is produced by chemical reactions and a negative value when

chemical reactions consume the material. Equation 21 is the equation developed from Equation 20 and will serve to obtain mass balance equations.

$$V_R \times \frac{\partial C}{\partial t} = C_i Q_i - C_f Q_f \pm \upsilon_{reaction} \times V_R \quad (21)$$

The term V_R is the volume of the reactor, C_i and C_f are the initial and final concentration respectively, Q is the component's flow rate, and $\upsilon_{reaction}$ is the rate which a component is produced or consumed. $\upsilon_{reaction}$, is the kinetic rate in which the component participates, that involves the use of the kinetics constants and stoichiometric coefficients.

Since the experimental data were taken in a batch-type reactor for simulation in software, equation 21 can be simplified since there is no inflow or outflow; therefore, the accumulation will be equal to what reacts (Equation 22).

$$V_R \times \frac{\partial C}{\partial t} = \pm \upsilon_{reaction} \times V_R \quad (22)$$

However, for the software development, the balance equations developed for all types of reactors will be taken into account. Below are the mass balances for all the components considered for the development of kinetical models.

$$S_{O,f} = \frac{\frac{Q_i S_{O,o}}{V}(t_f - t_i) + (a\,\mu_F + d\,\mu_{FA})(t_f - t_i) + S_{O,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (23)$$

$$S_{I,f} = \frac{\frac{Q_i S_{I,o}}{V}(t_f - t_i) + (f_{SI}\,\mu_{HY})(t_f - t_i) + S_{I,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (24)$$

$$S_{F,f} = \frac{\frac{Q_i S_{F,o}}{V}(t_f - t_i) + ((-1/Y_{XS})\,\mu_F + g\,\mu_{HY})(t_f - t_i) + S_{F,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (25)$$

$$S_{NH4,f} = \frac{\frac{Q_i S_{NH4,o}}{V}(t_f - t_i) + (b\,\mu_F + e\,\mu_{FA} + h\,\mu_{HY} + j\,\mu_{LY})(t_f - t_i) + S_{NH4,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (26)$$

$$S_{PO4,f} = \frac{\frac{Q_i S_{PO4,o}}{V}(t_f - t_i) + (c\,\mu_F + f\,\mu_{FA} + i\,\mu_{HY} + k\,\mu_{LY})(t_f - t_i) + S_{PO4,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (27)$$

$$S_{FA,f} = \frac{\frac{Q_i S_{FA,o}}{V}(t_f - t_i) + \left((-1/Y_{PHB/FA})\mu_{FA}\right)(t_f - t_i) + S_{FA,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (28)$$

$$X_{PHB,f} = \frac{\frac{Q_i X_{PHB,o}}{V}(t_f - t_i) + \left(Y_{PHB/XH}\,\mu_F + Y_{PHB/FA}\,\mu_{FA} + Y_{PHB/necator}\,\mu_{LY}\right)(t_f - t_i) + X_{PHB,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (29)$$

$$X_{H,f} = \frac{\frac{Q_i X_{H,o}}{V}(t_f - t_i) + (\mu_F + \mu_{FA} - \mu_{LY})(t_f - t_i) + X_{H,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (30)$$

$$X_{I,f} = \frac{\frac{Q_i X_{I,o}}{V}(t_f - t_i) + (f_{XI}\,\mu_{LY})(t_f - t_i) + X_{I,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (31)$$

$$X_{S,f} = \frac{\frac{Q_i X_{S,o}}{V}(t_f - t_i) + (-\mu_{HY} + l\,\mu_{LY})(t_f - t_i) + X_{S,o}}{1 + \frac{Q_f}{V}(t_f - t_i)} \quad (32)$$

Where, the components with the term S_o or X_o is an initial value, the components with the term S_f or X_f represents the final concentration, Q_i and Q_f are the initial and final flowrate, respectively, and t_f and t_i is the final and initial time. Finally, to calculate the mass balance equations, it is possible to use iterative methods such as using short time intervals using values of initial conditions and calculating the next value with the previous result. The time interval is an arbitrary value for solving mass balance equations; the smaller the value, the more accurate the results; this work will consider a time interval of 0.001 hours during 50 hours.

5.3 Software development for the simulation of kinetic models (Step 3)

For the development of the software, it was considered to do it using the Python programming language because it is open-source, easy to use, easy, and able to work with object-oriented programming. The construction of the software is shown in Figure 11, which begins with the import of the Python libraries. They have the functions of creating the software's graphical interface, importing Excel files that contain a stoichiometric or composition matrix, and solving variables. Once the libraries are imported, the programming code is written, which has the following characteristics:

- Graphical interface with drop-down menus to execute different tasks.
- Menu with the option to import the stoichiometric matrix and the composition matrix from files in Excel format (.csv).
- Window to save and display the data of the stoichiometric matrix and the composition matrix.
- Submenu to add or delete rows and columns as considered by the end-user.
- Submenu that verifies that the system of continuity equations from the stoichiometry matrix and the composition matrix have a solution.
- Submenu to obtain the continuity equations.
- Submenu to find the solutions to the stoichiometric coefficients of the continuity equations.
- Menu with the option to import kinetics and kinetic factors in text format (.txt).
- Window to save the kinetics and kinetic factors of each of the biological processes.
- Submenu to obtain the mass balance equations.
- Entries of initial system conditions.
- Submenu to calculate the mass balance equations.
- Option to save the results of mass balance equations.

.

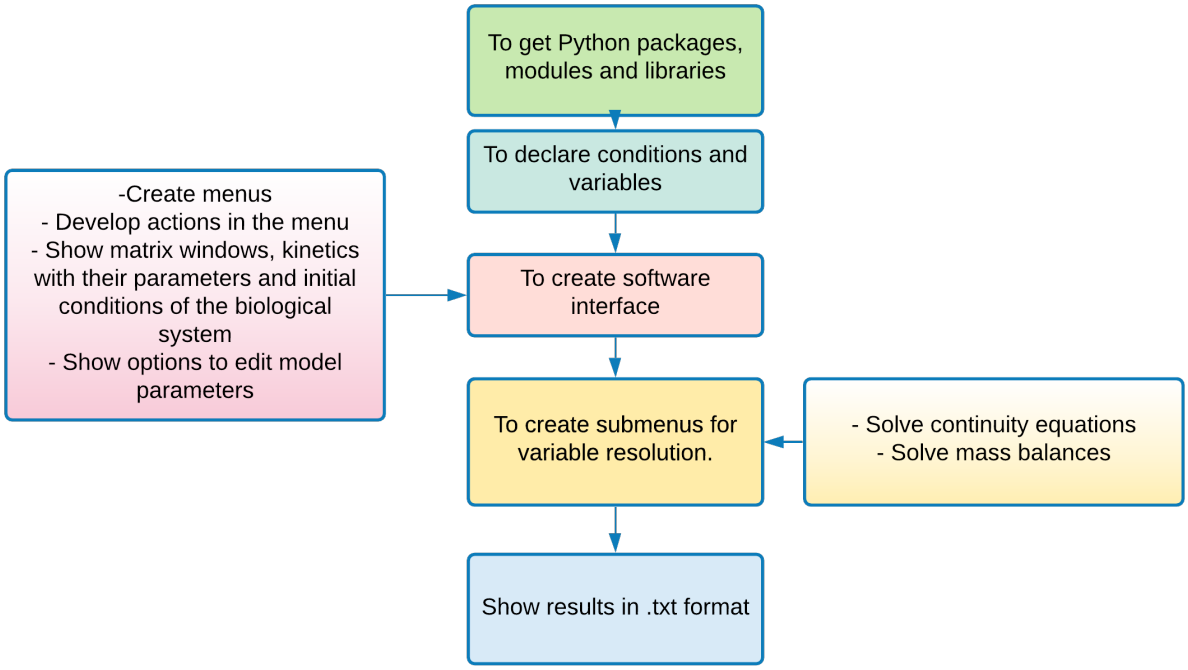

Figure 11. Flow chart of software construction for kinetic models.

As shown in Figure 11, the general steps for constructing the kinetic models for the biological system of this thesis are shown. The details of the programming code can be seen in Annex I.

CHAPTER VI

6. RESULTS AND DISCUSSION

6.1 Data processing for stoichiometric matrix and composition matrix

This study presents the results of using a mathematical model for the construction of kinetic models using matrix notation. Subsequent, in Table 13, a summary of the stoichiometric parameters for developing the stoichiometric matrix is shown.

Table 13. Calculations of stoichiometric parameters.

Stoichiometric parameters
$Y_{PHB/XH} = 0.484$
$Y_{X/S} = 0.417$
$Y_{PHB/FA} = 0.020$
$Y_{PHB/necator} = 0.708$

Table 14 shows the results obtained using the continuity equations previously shown in the methodology, which show values consistent with the literature; for example, for oxygen (S_O), negative values are shown that would indicate that oxygen should be consumed in heterotrophic aerobic growth processes using fructose and fatty acids. In inert soluble matter. In inert soluble matter (S_I) neither consumption nor production is seen according to the f_{SI} value used from the literature. However, for this model, it was taken into account because this component can be considered in future works. The respective negative value for the substrate or fructose (S_S) is seen for consumption in the aerobic growth process.

In the same way, negative values are seen, which indicates the tendency to consume the nutrients with nitrogen (S_{NH4}) and phosphorus (S_{PO4}) or the heterotrophic growth processes, and positive values in the hydrolysis and lysis processes that indicate their formation from slowly biodegradable (X_S). Finally, a negative value for fatty acids (S_{FA}) is also seen, which indicates the consumption for aerobic growth through this component.

Table 14. Stoichiometric coefficients obtained by solving the continuity equations.

Stoichiometric Matrix										
Components	S_O	S_I	S_S	S_{NH4}	S_{PO4}	S_{FA}	S_{PHB}	X_H	X_I	X_S
Process	gO2	gCOD	gCOD	gN	gP	gCOD	gCOD	gCOD	gCOD	gCOD
Aerobic heterotrophic Growth in Fructose	-0.912	0	-2.396	-0.07	-0.02	0	0.484	1	0	0
Aerobic heterotrophic Growth in fatty acids	-49.865	0	0	-0.07	-0.02	-50.885	0.020	1	0	0
Hydrolysis	0	0	1	0.04	0.01	0	0	0	0	-1
Lysis of heterotrophic	0	0	0	0.06	0.02	0	0.708	-1	0.1	0.19
Material Conservation Matrix (conservative components)										
Conservative components	S_O	S_I	S_S	S_{NH4}	S_{PO4}	S_{FA}	S_{PHB}	X_H	X_I	X_S
COD (g)	-1	1	1	0	0	1	1	1	1	1
N (gN/gCOD)	0	0.01	0	1	0	0	0	0.07	0.02	0.04
P (gP/gCOD)	0	0	0	0	1	0	0	0.02	0.01	0.01

For the non-soluble components, it is seen that in the production of PHB (X_{PHB}) there are positive values for the aerobic growth processes through fructose and fatty acids, which is consistent with the literature that proposes the use of both substrates in the same way. A positive value is seen for the lysis process because, in cell death, PHB can be produced or released from biomass. The biomass (X_H) values are positive normalized values that indicate PHB production and compound of interest for the development of the models. In the inert insoluble matter (X_I), a positive referential value of f_{XI} indicates its production in lysis. It becomes part of the sludge that remains floating in the residual water. Finally, the slowly biodegradable substrate (X_S) has a negative normalized value for hydrolysis, which indicates consumption for conversion to readily biodegradable substrates, and a positive lysis value is consistent with the literature.

6.2 Simulation of components behavior using mass balance

As mentioned in the literature, the mass balance equations can be simplified, such that for an aerobic batch process, the inflow and outflow are equal to zero. Therefore, the balance equations and their calculations are simplified. The results of the equations for our model are shown below. In Figure 12, we can see that the behavior of PHB production increases with time; it is expected behavior because the formation of PHB must occur from the consumption of substrates: fructose and fatty acids, as shown in Figures 13 and 14.

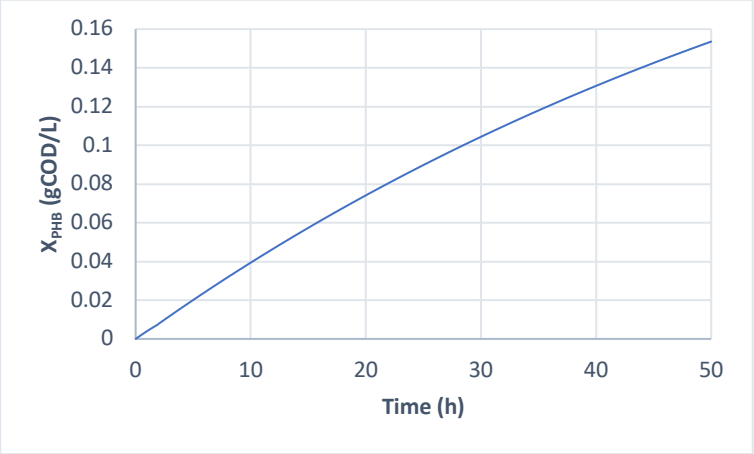

Figure 12. Results of simulation for PHB.

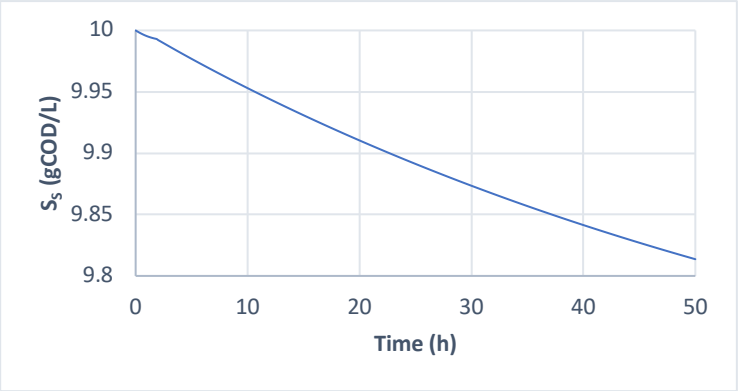

Figure 13. Results of simulation for Fructose.

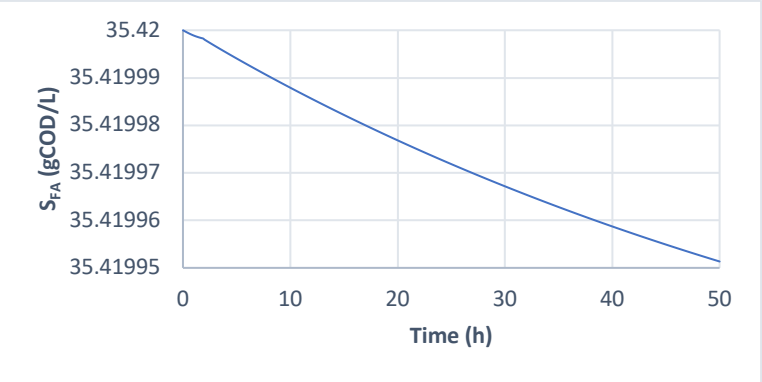

Figure 14. Results of simulation for Fatty acids.

As can be seen in Figures 13 and 14, they present an expected behavior because the substrates must be consumed to produce PHB. In Figure 15, the behavior of the slowly biodegradable substrate (X_S), is shown, which is seen that in the first two hours it presents a maximum concentration; this behavior makes sense because the slowly biodegradable matter can be generated, from the initial growth of microorganisms and lysis respectively, since the amount of initial biomass is low, the maximum peak it reaches is small, then we see that it is consumed due to the hydrolysis process, such that X_S is transformed into easily biodegradable components such as fructose (S_S).

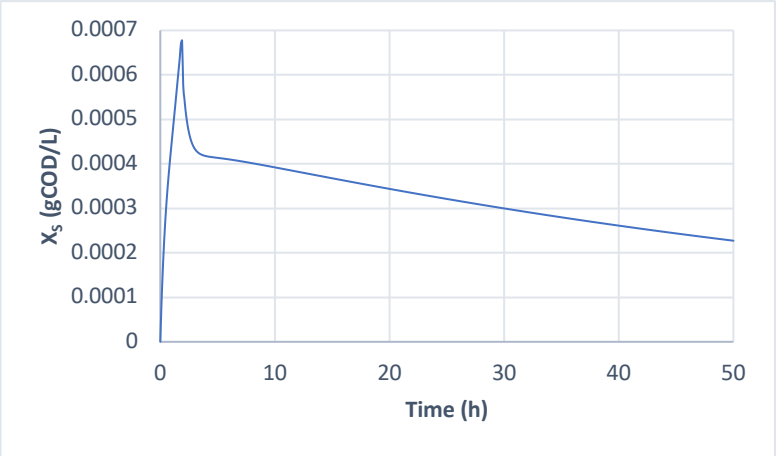

Figure 15. Results of simulation for slowly biodegradable substrate.

Figure 16 shows the oxygen simulation; the values make sense because it is considered a batch-type process that required constant aeration. It can be seen that in a specific time, the oxygen will be exhausted. These results are related to Figure 15; as mentioned in the beginning, oxygen is consumed for growth and generates X_S.

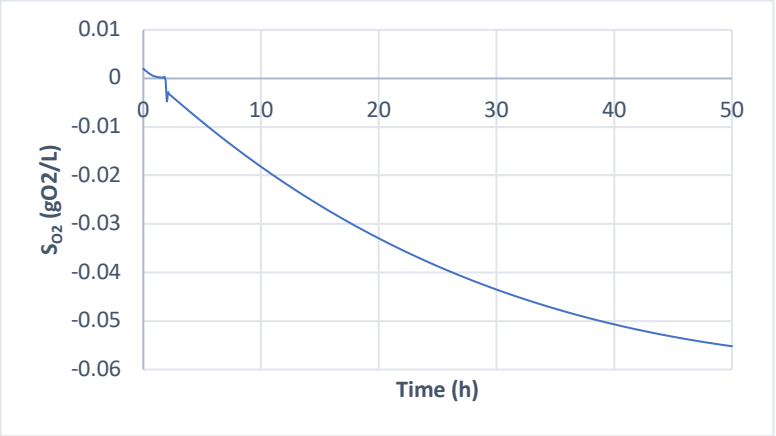

Figure 16. Results of simulation for Oxygen.

Figure 17 shows the behavior of the non-soluble inert matter X_I; an expected behavior is seen, which increases due to the lysis processes.

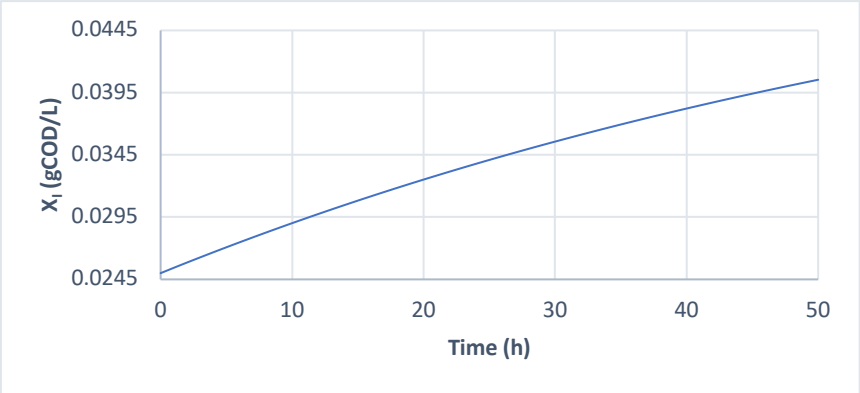

Figure 17. Results of simulation for inert material X_I.

Finally, figures 18 and 19 show a nutrient production behavior, which indicates that the microorganisms cannot consume the nutrients that are produced. This is related to the lysis process in which it releases nutrients from cell death.

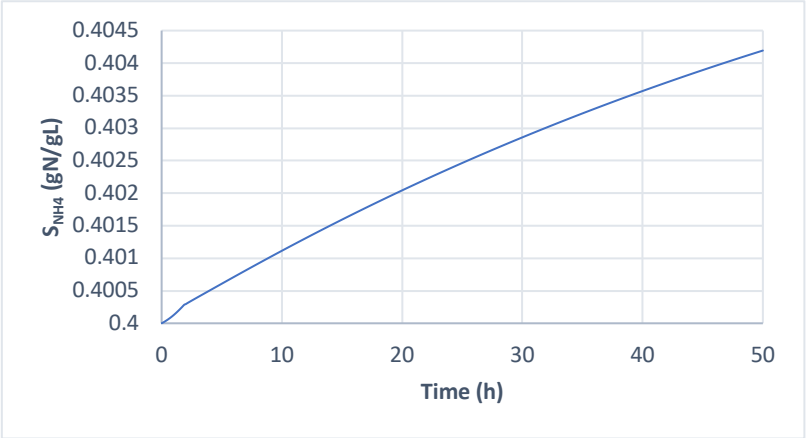

Figure 18. Results of simulation for NH_4.

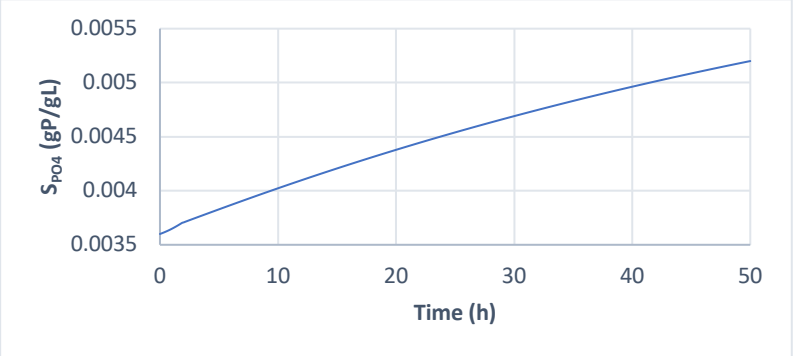

Figure 19. Results of simulation for PO_4.

Figure 20 is consistent with the initial data, which indicates that the microorganisms that tend to consume fructose. Suppose the fructose concentration decreases, the consumption of fatty acids increases. If it is related to the kinetics, the kinetic constant of the Fatty acid growth is very low compared to the lysis constant, so cell death occurs faster. Also, there are not enough microorganisms that consume the nutrients as seen previously. Therefore, the lysis process obscures the growth of *C. necator*, although the PHB continues to increase due to the lysis of the few remaining organisms.

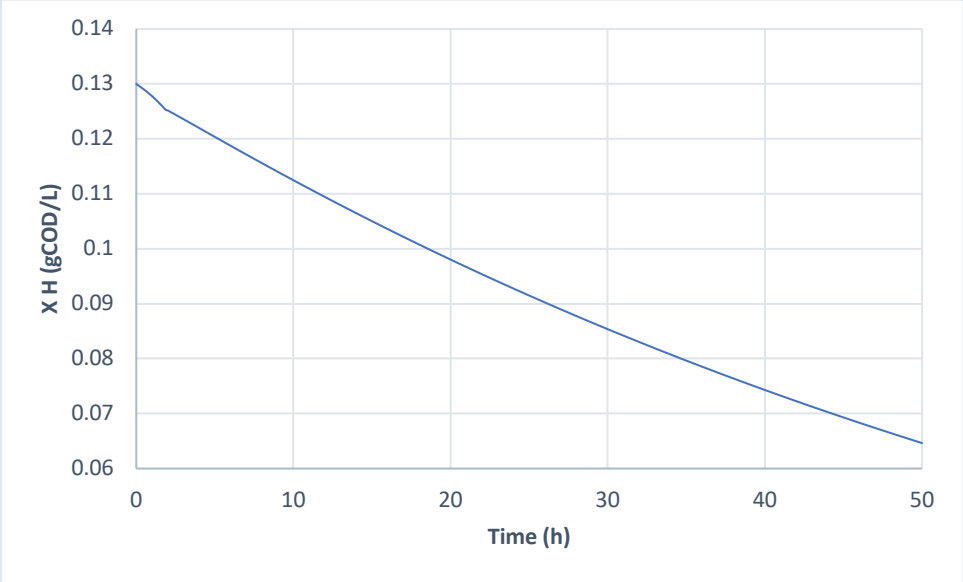

Figure 20. Results of simulation for X_H.

Finally, the soluble inert compounds S_I will have a constant initial value. In summary, these changes in behavior can be explained because referential values of kinetic parameters and initial conditions were taken for which there was no control. However, the stoichiometry and composition matrices were constructed in a referential manner with experimental values according to the metabolism of *C. necator*.

6.3 Software simulation

Figure 21 shows the main window, which contains the respective menus to execute different commands to help the simulation. Figure 22 shows the window with the stoichiometric matrix loaded from a .csv file, with options to add or delete rows and columns, with the ability to print the table and manually edit the coefficient values.

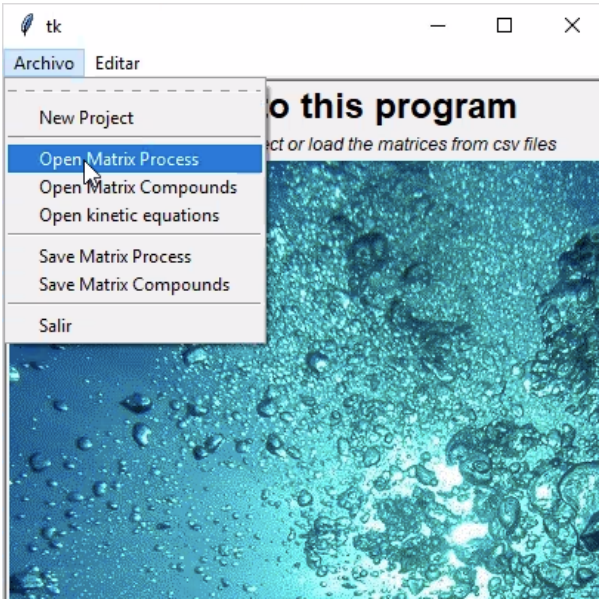

Figure 21. Initial software window.

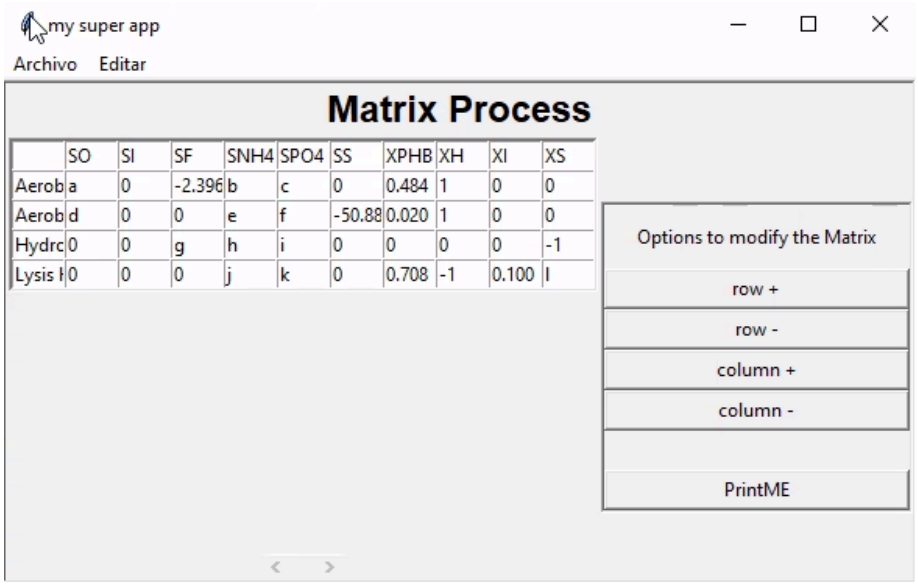

Figure 22. Window with stoichiometric matrix.

Figure 23 shows the stoichiometry and composition matrices added to the simulator to later obtain the resolution of the continuity equations. Figure 24 shows the generation of the continuity equations to be calculated. Figure 25 shows the results of the stoichiometric coefficients using the continuity equations. Figure 26 shows the windows of the kinetic equations and initial conditions. Figure 27 shows the material balance equations developed, to later in figure 28 simulate the behavior of the concentrations of the compounds as a function of time.

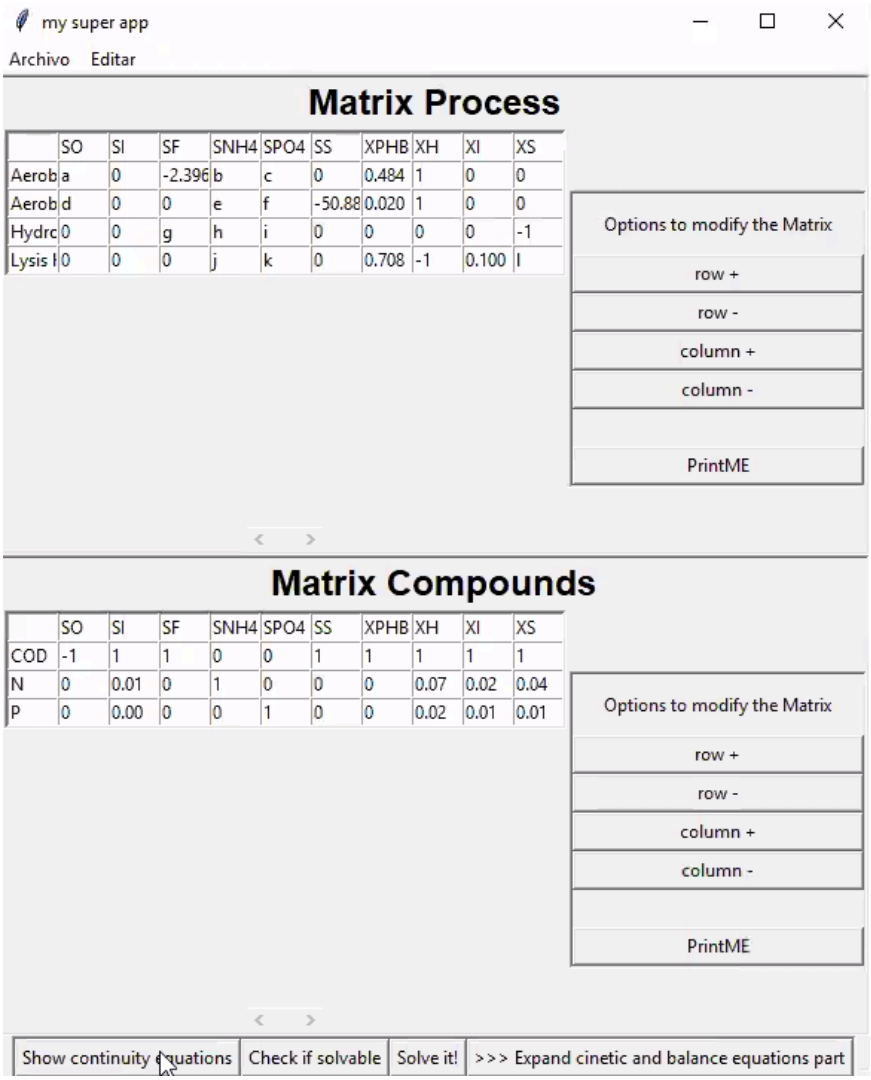

Figure 23. Window with stoichiometric and composition matrix.

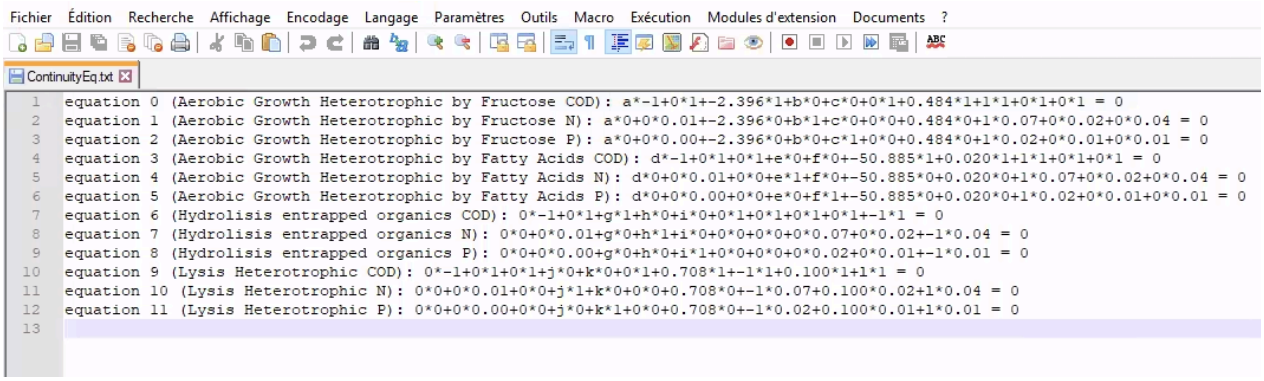

Figure 24. Presentation of the calculated continuity equations.

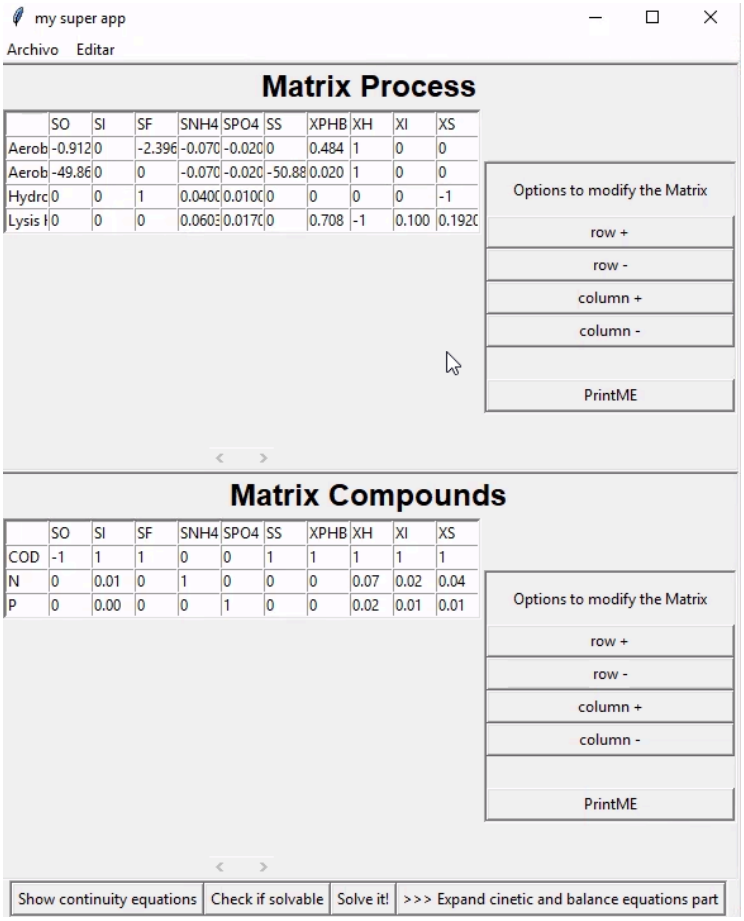

Figure 25. Results of stoichiometric coefficients using the continuity equations.

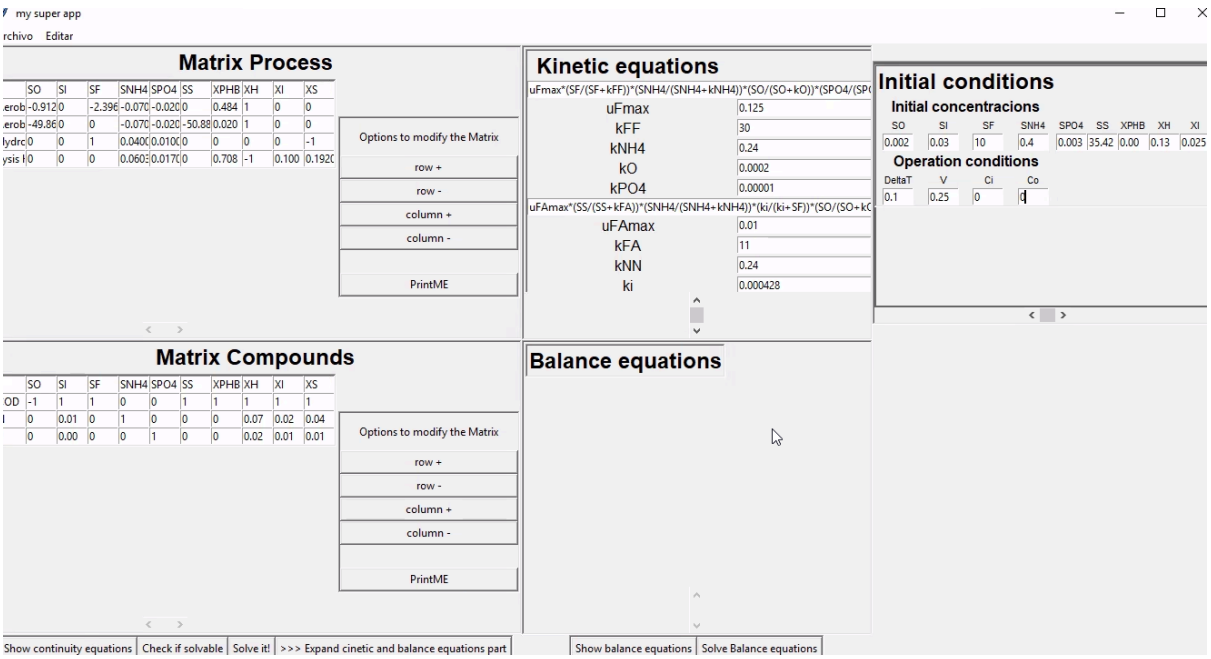

Figure 26. Windows for stoichiometric equations and initial conditions.

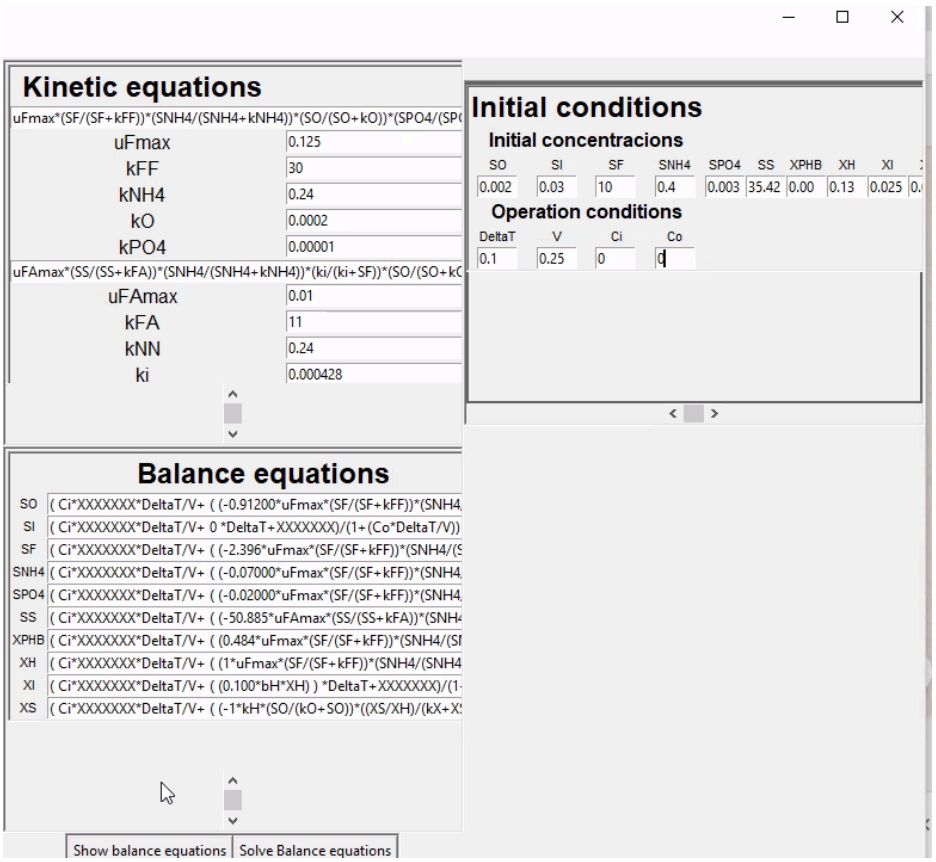

Figure 27. Presentation of mass balances.

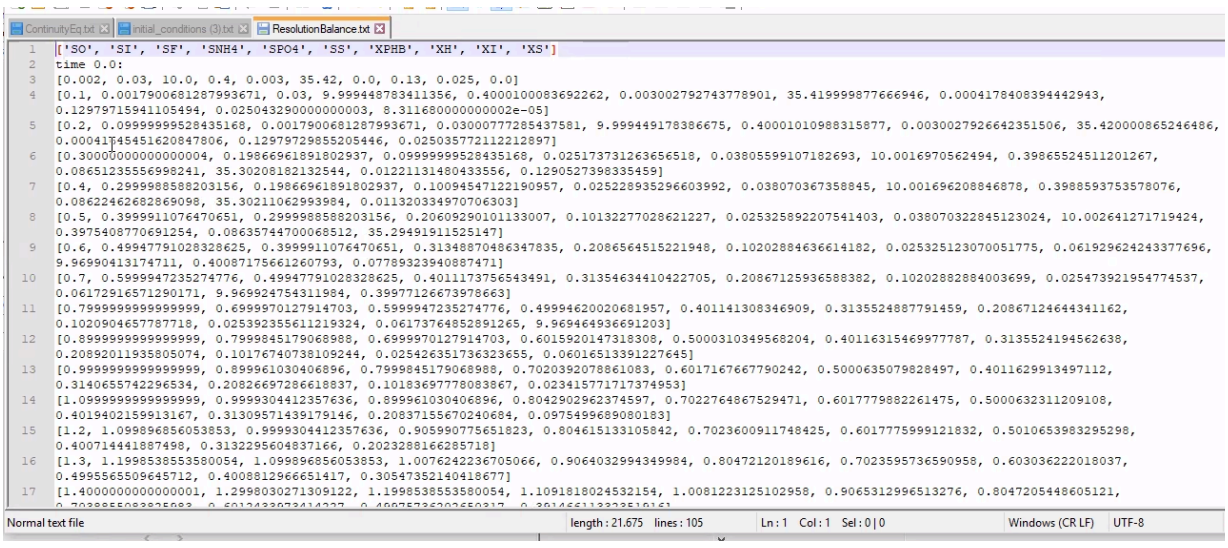

Figure 28. Mass balance simulation results.

The software simulation results make sense with the creation of the graphs of the variation of concentration of soluble and non-soluble components as a function of time seen above.

CHAPTER VII

7. CONCLUSIONS AND RECOMENDATIONS

7.1 Conclusions

- A kinetic model was developed from the conceptualization of *C. necator* metabolism for biological processes such as hydrolysis of heterotrophic microorganisms, lysis of heterotrophic microorganisms, aerobic growth of *C. necator* using fructose as substrate, and aerobic growth of *C. necator* using fatty acids as substrate, to describe PHB production in wastewater from an avocado oil industry.
- The stoichiometric coefficients of *C. necator* metabolism were obtained by using stoichiometric matrices and continuity equations.
- The kinetic parameters of bacterial metabolism were adjusted using the Arrhenius equation.
- Mass balance equations were obtained that allowed describing the biological system of *C. necator* for the production of PHB.
- Python programming software was developed to describe *C. necator* metabolic processes in wastewater for a biological wastewater reactor with avocado oils.
- Some biological processes for the production of PHB in a batch-type reactor were simulated through the proposed software, which may be of interest to the industry with wastewater with avocado oils.
- It was established that the proposed model for the production of PHB depends significantly on the initial concentrations of the components of the model and the kinetic parameters taken from the literature.

7.2 Recommendations

- To validate this model, it is recommended in the future to carry out experimental tests to obtain kinetic constants, control parameters such as pH, aeration, temperature, initial concentrations, among others, so this model is a good reference for future validation work.

- It is recommended to carry out a sensitivity analysis of the proposed model to optimize its performance concerning the calculation process and the parameters considered for its development.
- It is recommended that future works carry out a cost and profit analysis for the production of PHB with the help of kinetic models applied in a treatment plant in operation.

REFERENCES

(1) WHO. *Progress of Wastewater Treatment Piloting the Monitoring Methodology and Initial Findings for SDG6.3.1*; 2018.

(2) WHO. *Sanitation Safety Planning: Manual for Safe Use and Disposal of Wastewater, Greywater and Excreta*; 2015.

(3) Pozo, M.; Serrano, J. C.; Castillo, R. Construcción Metodológica de Los Indicadores ODS de Agua, Saneamiento e Higiene. *Inec* **2017**, 1–67.

(4) Nacional, C. F. *Ficha Sectorial: Elaboración de Aceites Crudos Vegetales*; 2017.

(5) McCarty, P. L.; Bae, J.; Kim, J. Domestic Wastewater Treatment as a Net Energy Producer-Can This Be Achieved? *Environ. Sci. Technol.* **2011**, *45* (17), 7100–7106. https://doi.org/10.1021/es2014264.

(6) Ahuja, S. *Comprehensive Water Quality and Purification*; Elsevier, 2013; Vol. 1–2.

(7) Madison, L. L.; Huisman, G. W. Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic. *Microbiol. Mol. Biol. Rev.* **1999**, *63* (1), 21–53.

(8) Khanna, S.; Srivastava, A. K. Recent Advances in Microbial Polyhydroxyalkanoates. *Process Biochem.* **2005**, *40* (2), 607–619. https://doi.org/10.1016/j.procbio.2004.01.053.

(9) Purama, R. K.; Al-Sabahi, J. N.; Sudesh, K. Evaluation of Date Seed Oil and Date Molasses as Novel Carbon Sources for the Production of Poly(3Hydroxybutyrate-Co-3Hydroxyhexanoate) by Cupriavidus Necator H16 Re 2058/PCB113. *Ind. Crops Prod.* **2018**, *119*, 83–92. https://doi.org/10.1016/j.indcrop.2018.04.013.

(10) UNL. Wastewater - What Is It? | UNL Water https://water.unl.edu/article/wastewater/wastewater-what-it (accessed Sep 15, 2020).

(11) Lin, Shun Dar; Lee, C. *Water and Wastewater Calculations Manual*, 2nd Editio.; McGraw-Hill Education: New York, 2007.

(12) Zheng, C.; Zhao, L.; Zhou, X.; Fu, Z.; Li, A. Treatment Technologies for Organic

Wastewater. In *Water Treatment*; InTech, 2013. https://doi.org/10.5772/52665.

(13) School of PE. Basic Characteristics of Wastewater https://www.schoolofpe.com/blog/2018/03/basic-characteristics-of-wastewater.html.

(14) Amoatey, P.; Bani, R. Wastewater Management. In *Waste Water - Evaluation and Management*; InTech, 2011; p 380. https://doi.org/10.5772/16158.

(15) Rasmussen, T. J.; Schmidt, H. C. Stormwater Runoff : What It Is and Why It Is Important in Johnson County , Kansas. *Sci. a Chang. World* **2009**, 1–2.

(16) Alturkmani, A. Industrial Wastewater. *Enviromental Eng. Website Manag.* **2013**.

(17) Englande, A. J.; Krenkel, P.; Shamas, J. Wastewater Treatment &Water Reclamation. In *Reference Module in Earth Systems and Environmental Sciences*; Elsevier, 2015. https://doi.org/10.1016/B978-0-12-409548-9.09508-7.

(18) National Research Council. *Use of Reclaimed Water and Sludge in Food Crop Production*; National Academies Press: Washington, D.C., 1996. https://doi.org/10.17226/5175.

(19) Karia, G. L.; Christian, R. A. *Wastewater Treatment: Concepts and Design Approach*, 2nd ed.; PHI Learning, 2013.

(20) Muloiwa, M.; Nyende-Byakika, S.; Dinka, M. Comparison of Unstructured Kinetic Bacterial Growth Models. *South African J. Chem. Eng.* **2020**, *33*, 141–150. https://doi.org/10.1016/j.sajce.2020.07.006.

(21) Shete, M. B. S. Comparative Study of Various Treatments For Dairy Industry Wastewater. *IOSR J. Eng.* **2013**, *03* (08), 42–47. https://doi.org/10.9790/3021-03844247.

(22) Aquagroup Cia. Ltda. Tratamientos de aguas residuales en Quito - Ecuador https://www.aquagroup.ec (accessed Mar 1, 2021).

(23) Foro de Recursos Hídricos. *La Gestión Comunitaria de Agua Para Consumo Humano y El Saneamiento En El Ecuador: Diagnóstico y Propuestas*; Quito, 2013.

(24) MAGAP. *Plan Nacional de Riego y Drenaje*; Quito, 2013.

(25) Carli Snacks. Carli Snacks http://www.carlisnacks.com/WebCarli/Pagina_Web/index.php (accessed Sep 15, 2020).

(26) AFH. Plantas de Aguas Residuales Industriales https://afh.com.ec/plantas-de-aguas-resdiduales-industriales/ (accessed Sep 24, 2020).

(27) Espinosa, K. Producción Industrial y Contaminación Ambiental Del Agua En Los Procesos En La Planta de Producción de Snacks de La Asociación Nueva Esperanza de Mulalillo Año 2013 Diseño de Un Plan de Intervención, Universidad Técnica de Cotopaxi, 2014.

(28) Chiveria. Chiveria http://www.chiveria.com.ec/ (accessed Sep 24, 2020).

(29) Ranchito, E. Pasteurizadora El Ranchito http://elranchito.com.ec/ (accessed Sep 24, 2020).

(30) Ordeño, E. Sociedad Industrial Ganadera El Ordeño S.A. https://www.elordeno.com/ (accessed Sep 24, 2020).

(31) Arango, Á.; Garcés, L. Tratamiento de Aguas Residuales de La Industria Láctea. *Prod. más Limpia* **2007**, *2*, 28–29.

(32) USDA. Oil, Palm Explorer https://ipad.fas.usda.gov/cropexplorer/cropview/commodityView.aspx?cropid=4243000&sel_year=2020 (accessed Sep 19, 2020).

(33) Danec. Danec – Home https://www.danec.com/ (accessed Sep 19, 2021).

(34) Acosta Yépez, M. P. *Propuesta de Optimización Del Sistema de Tratamiento de Aguas Residuales de La Empresa DANEC S. A.*; Quito: Universidad de las Américas, 2017: Quito, 2017.

(35) Industrias Ales. Industrias Ales http://www.ales.com.ec/ (accessed Sep 20, 2020).

(36) Mendoza, M. Industrias Ales Reusará 60% de Sus Aguas. **2018**, No. I, 2018.

(37) Sanitron. Industrias Ales Manta. March 7, 2019, pp 1–1.

(38) Sanitron. La Fabril Manta. March 7, 2019, pp 1–1.

(39) Santana, J. *Evaluación Técnica de Aguas Residuales de La Empresa Fabril S.A. Vertidas Al Río Muerto, Sector Los Ángeles, Cantón Montecristi, Periodo 2013.*; Manta, 2014.

(40) Cabrera, J. Estudio de Factibilidad Para La Producción de Aceite de Aguacate En El Cantón Mira y Su Comercialización En La Ciudad de Quito, Universidad Politécnica Estatal Del Carchi, 2015.

(41) Ochoa, T. *Las Frutas Su Poder Curativo*, 2nd ed.; Lima, 2002.

(42) Ikhuoria, E. U.; Maliki, M. Characterization of Avocado Pear (Persea Americana) and African Pear (Dacryodes Edulis) Extracts. *African J. Biotechnol.* **2007**, *6* (7), 946–948. https://doi.org/10.5897/AJB2007.000-2115.

(43) Ministerio del Ambiente. *Texto Unificado de La Legislación Secundaria de Medio Ambiente*; 2017.

(44) INEN. *NORMA TÉCNICA ECUATORIANA NTE INEN 1 108 : 2011 Primera Edición*; 2011.

(45) Secretaría del Agua. *Norma de Diseño Para Sistemas de Abastecimiento de Agua Potable, Disposición de Excretas y Residuos Líquidos En El Área Rural*; 2014.

(46) Baird, R. B.; Eaton, A. D.; Rice, E. W. *Standard Methods for the Examination of Water and Wastewater, American Public Health Association*, 23rd editi.; 2017.

(47) iagua. La importancia de la separación de aceites y grasas en el tratamiento del agua residual urbana https://www.iagua.es/noticias/teqma/importancia-separacion-aceites-y-grasas-tratamiento-agua-residual-urbana.

(48) USGS. Biological Oxygen Demand (BOD) and Water https://www.usgs.gov/special-topic/water-science-school/science/biological-oxygen-demand-bod-and-water?qt-science_center_objects=0#qt-science_center_objects.

(49) López Vázquez, C. M.; Buitrón Méndez, G.; García, H. A.; Cervantes Carrillo, F. J. Tratamiento Biológico de Aguas Residuales: Principios, Modelación y Diseño. *Water Intell. Online* **2017**, *16*, 405–427. https://doi.org/10.2166/9781780409146.

(50) Patel, H.; Vashi, R. T. *Characterization and Treatment of Textile Wastewater*; Elsevier Inc., 2015. https://doi.org/10.1016/c2014-0-02395-7.

(51) Diaz, J. M. *Ecuaciones y Cálculos Para El Tratamiento de Aguas*; Madrid, 2018.

(52) Castells, X. E. *Diccionario de Términos Ambientales: Reciclaje de Residuos Industriales*; Ediciones Díaz de Santos: Madrid, 2012.

(53) Suárez, J.; Jácome, A.; Ures, P. *Electrocoagulación Electroflotación (FT-AVA-002)*; 2015.

(54) Moran, S. Clean Water Unit Operation Design. In *An Applied Guide to Water and Effluent Treatment Plant Design*; Elsevier, 2018; pp 69–100. https://doi.org/10.1016/B978-0-12-811309-7.00007-2.

(55) University of Massachusetts Amherst. Electroflotation http://www.ecs.umass.edu/cee/reckhow/courses/Etreat/slides/597tl09p.pdf.

(56) Lauprasert, P.; Chansirirattana, J.; Paengjan, J. Effect of Selected Bacteria as Bioremediation on the Degradation of Fats Oils and Greases in Wastewater from Cafeteria Grease Traps. *Eur. J. Sustain. Dev.* **2017**, *6* (2). https://doi.org/10.14207/ejsd.2017.v6n2p181.

(57) El-Borai, A.; Eltayeb, K.; Mostafa, A.; El-Assar, S. Biodegradation of Industrial Oil-Polluted Wastewater in Egypt by Bacterial Consortium Immobilized in Different Types of Carriers. *Polish J. Environ. Stud.* **2016**, *25* (5). https://doi.org/10.15244/pjoes/62301.

(58) Jalilnejad, E.; Mogharei, A.; Vahabzadeh, F. Aerobic Pretreatment of Olive Oil Mill Wastewater Using Ralstonia Eutropha. *Environ. Technol.* **2011**, *32* (10), 1085–1093. https://doi.org/10.1080/09593330.2010.528040.

(59) Ibrahim, S.; Zahri, K. N. M.; Convey, P.; Khalil, K. A.; Gomez-Fuentes, C.; Zulkarnain, A.; Alias, S. A.; González-Rocha, G.; Ahmad, S. A. Optimisation of Biodegradation Conditions for Waste Canola Oil by Cold-Adapted Rhodococcus Sp. AQ5-07 from Antarctica. *Electron. J. Biotechnol.* **2020**, *48*, 1–12. https://doi.org/10.1016/j.ejbt.2020.07.005.

(60) Teixeira, P. D.; Silva, V. S.; Tenreiro, R. Integrated Selection and Identification of

Bacteria from Polluted Sites for Biodegradation of Lipids. *Int. Microbiol.* **2020**, *23* (3), 367–380. https://doi.org/10.1007/s10123-019-00109-w.

(61) Fiorese, M. L.; Freitas, F.; Pais, J.; Ramos, A. M.; de AragÃ£o, G. M. F.; Reis, M. A. M. Recovery of Polyhydroxybutyrate (PHB) from Cupriavidus Necator Biomass by Solvent Extraction with 1,2-Propylene Carbonate. *Eng. Life Sci.* **2009**, *9* (6), 454–461. https://doi.org/10.1002/elsc.200900034.

(62) Passanha, P.; Kedia, G.; Dinsdale, R. M.; Guwy, A. J.; Esteves, S. R. The Use of NaCl Addition for the Improvement of Polyhydroxyalkanoate Production by Cupriavidus Necator. *Bioresour. Technol.* **2014**, *163*, 287–294. https://doi.org/10.1016/j.biortech.2014.04.068.

(63) Grousseau, E.; Blanchet, E.; Déléris, S.; Albuquerque, M. G. E.; Paul, E.; Uribelarrea, J.-L. Impact of Sustaining a Controlled Residual Growth on Polyhydroxybutyrate Yield and Production Kinetics in Cupriavidus Necator. *Bioresour. Technol.* **2013**, *148*, 30–38. https://doi.org/10.1016/j.biortech.2013.08.120.

(64) Carpine, R.; Olivieri, G.; Hellingwerf, K. J.; Pollio, A.; Marzocchella, A. Industrial Production of Poly-β-Hydroxybutyrate from CO2: Can Cyanobacteria Meet This Challenge? *Processes* **2020**, *8* (3), 323. https://doi.org/10.3390/pr8030323.

(65) Shabtai, Y.; Wang, D. I. C. Production of Emulsan in a Fermentation Process Using Soybean Oil (SBO) in a Carbon-Nitrogen Coordinated Feed. *Biotechnol. Bioeng.* **1990**, *35* (8), 753–765. https://doi.org/10.1002/bit.260350802.

(66) Markossian, S.; Becker, P.; Märkl, H.; Antranikian, G. Isolation and Characterization of Lipid-Degrading Bacillus Thermoleovorans IHI-91 from an Icelandic Hot Spring. *Extremophiles* **2000**, *4* (6), 365–371. https://doi.org/10.1007/s007920070006.

(67) Tano-Debrah, K.; Fukuyama, S.; Otonari, N.; Taniguchi, F.; Ogura, M. An Inoculum for the Aerobic Treatment of Wastewaters with High Concentrations of Fats and Oils. *Bioresour. Technol.* **1999**, *69* (2), 133–139. https://doi.org/10.1016/S0960-8524(98)00181-3.

(68) González García, Y.; Meza Contreras, J. C.; González Reynoso, O.; Córdova López, J. A. Synthesis and Degradation of Polyhydroxyalkanoates: Plastic Microbial. *Revista*

Internacional de Contaminacion Ambiental. 2013, pp 77–115.

(69) Sydow, A.; Pannek, A.; Krieg, T.; Huth, I.; Guillouet, S. E.; Holtmann, D. Expanding the Genetic Tool Box for Cupriavidus Necator by a Stabilized L-Rhamnose Inducible Plasmid System. *J. Biotechnol.* **2017**, *263*, 1–10. https://doi.org/10.1016/j.jbiotec.2017.10.002.

(70) Lykidis, A.; Pérez-Pantoja, D.; Ledger, T.; Mavromatis, K.; Anderson, I. J.; Ivanova, N. N.; Hooper, S. D.; Lapidus, A.; Lucas, S.; González, B.; Kyrpides, N. C. The Complete Multipartite Genome Sequence of Cupriavidus Necator JMP134, a Versatile Pollutant Degrader. *PLoS One* **2010**, *5* (3), e9729. https://doi.org/10.1371/journal.pone.0009729.

(71) Rao, U.; Sridhar, R.; Sehgal, P. K. Biosynthesis and Biocompatibility of Poly(3-Hydroxybutyrate-Co-4-Hydroxybutyrate) Produced by Cupriavidus Necator from Spent Palm Oil. *Biochem. Eng. J.* **2010**, *49* (1), 13–20. https://doi.org/10.1016/j.bej.2009.11.005.

(72) Reis, M.; Albuquerque, M.; Villano, M.; Majone, M. Mixed Culture Processes for Polyhydroxyalkanoate Production from Agro-Industrial Surplus/Wastes as Feedstocks. In *Comprehensive Biotechnology*; Elsevier, 2011; pp 669–683. https://doi.org/10.1016/B978-0-08-088504-9.00464-5.

(73) Flores-Sánchez, A.; López-Cuellar, M. del R.; Pérez-Guevara, F.; Figueroa López, U.; Martín-Bufájer, J. M.; Vergara-Porras, B. Synthesis of Poly-(R-Hydroxyalkanoates) by Cupriavidus Necator ATCC 17699 Using Mexican Avocado (Persea Americana) Oil as a Carbon Source. *Int. J. Polym. Sci.* **2017**, *2017*, 1–10. https://doi.org/10.1155/2017/6942950.

(74) Nygaard, D.; Yashchuk, O.; Hermida, É. B. Evaluation of Culture Medium on Poly(3-Hydroxybutyrate) Production by Cupriavidus Necator ATCC 17697: Application of the Response Surface Methodology. *Heliyon* **2019**, *5* (3), e01374. https://doi.org/10.1016/j.heliyon.2019.e01374.

(75) Špoljarić, I. V.; Lopar, M.; Koller, M.; Muhr, A.; Salerno, A.; Reiterer, A.; Horvat, P. In Silico Optimization and Low Structured Kinetic Model of Poly[(R)-3-Hydroxybutyrate] Synthesis by Cupriavidus Necator DSM 545 by Fed-Batch

Cultivation on Glycerol. *J. Biotechnol.* **2013**, *168* (4), 625–635. https://doi.org/10.1016/j.jbiotec.2013.08.019.

(76) Universidad de Jaén. Tema 1: Modelos Matemáticos http://matema.ujaen.es/jnavas/web_modelos/pdf_mmb08_09/introduccion.pdf (accessed Dec 7, 2020).

(77) Serralta, J.; Barat, R. Desarrollo de un modelo matemático: Selección de componentes y procesos http://hdl.handle.net/10251/121528.

(78) Bottani, E.; Odetti, H.; Pliego, O.; Villarreal, E. *Química General*; Universidad Nacional del Litoral, 2001.

(79) Henze, M.; Gujer, W.; Mino, T.; van Loosedrecht, M. *Activated Sludge Models ASM1, ASM2, ASM2d and ASM3*; IWA Publishing: London, 2000. https://doi.org/10.2166/9781780402369.

(80) Third, K. A.; Newland, M.; Cord-Ruwisch, R. The Effect of Dissolved Oxygen on PHB Accumulation in Activated Sludge Cultures. *Biotechnol. Bioeng.* **2003**, *82* (2), 238–250. https://doi.org/10.1002/bit.10564.

(81) Yadav, B.; Chavan, S.; Atmakuri, A.; Tyagi, R. D.; Drogui, P. A Review on Recovery of Proteins from Industrial Wastewaters with Special Emphasis on PHA Production Process: Sustainable Circular Bioeconomy Process Development. *Bioresour. Technol.* **2020**, *317*, 124006. https://doi.org/10.1016/j.biortech.2020.124006.

(82) Fereidouni, M.; Younesi, H.; Daneshi, A.; Sharifzadeh, M. The Effect of Carbon Source Supplementation on the Production of Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) by Cupriavidus Necator. *Biotechnol. Appl. Biochem.* **2011**, *58* (3), 203–211. https://doi.org/10.1002/bab.29.

(83) Caravaca, J. Design of Simulation Models with Automatic Control of Wastewater Treatment Plants, Universidad de Zaragoza, 2018.

(84) Ramírez, E. Fundamentos teóricos de lodos activados y aereación extendida http://documentacion.ideam.gov.co/openbiblio/bvirtual/018834/MEMORIAS2004/CapituloII/1Fundamentosdelprocesodelodosactivados.pdf.

(85) Baei, S.; Najafpour, G. D.; Younesi, H.; Tabandeh, F.; Issazadeh, H.; Khodabandeh, M. Growth Kinetic Parameters and Biosynthesis of Polyhydroxybutyrate in Cupriavidus Necator DSMZ 545 on Selected Substrates. *Chem. Ind. Chem. Eng. Q.* **2011**, *17* (1), 1–8. https://doi.org/10.2298/CICEQ100216043B.

(86) Vrana Špoljarić, I.; Lopar, M.; Koller, M.; Muhr, A.; Salerno, A.; Reiterer, A.; Malli, K.; Angerer, H.; Strohmeier, K.; Schober, S.; Mittelbach, M.; Horvat, P. Mathematical Modeling of Poly[(R)-3-Hydroxyalkanoate] Synthesis by Cupriavidus Necator DSM 545 on Substrates Stemming from Biodiesel Production. *Bioresour. Technol.* **2013**, *133*, 482–494. https://doi.org/10.1016/j.biortech.2013.01.126.

ANNEXES

Appendix A. Standards for treatment of wastewater in Ecuador.

Standard	Description
INEN 910	Color determination
INEN 971	Determination of turbidity
INEN 972	Determination of total dry residue
INEN 973	Determination of pH
INEN 975	Nitrate Nitrogen Determination
INEN 976	Determination of chlorides
INEN 977	Determination of residual chlorine
INEN 979	Determination of iron
INEN 980	Determination of arsenic
INEN 982	Determination of cadmium
INEN 983	Determination of hexavalent chromium
INEN 984	Determination of copper
INEN 985	Determination of fluoride
INEN 1102	Determination of lead
INEN 1103	Determination of magnesium
INEN 1104	Determination of total manganese
INEN 1105	Sampling for microbiological examination
INEN 1106	Determination of dissolved oxygen
INEN 1107	Determination of calcium
INEN 1108	Drinking water. Requirements
INEN 1202	Determination of biochemical oxygen demand
INEN 1203	Determination of chemical oxygen demand
INEN 1205	Determination of the total number of bacteria

Appendix B. Physical properties of different types of PHA and their comparison with those observed for plastics derived from petroleum (Babel and Steinbüchel, 2001).

Polymer	Melting temperature (ºC)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation (%)	Transition temperature (ºC)
P(3HB)	179	3,5	40	5	4
P(3HB-*co*-3HV) **3 mol % 3HV** **14 mol % 3HV** **25 mol % 3HV**	170 150 137	2,9 1,5 0,7	38 35 30	* * *	* * *
P(3HB-*co*-4HV) **3 mol % 4HV** **10 mol % 4HV** **64 mol % 4HV**	166 159 50	* * 30	28 24 17	45 242 591	* * *
P(4HB)	53	149	104	1000	*
P(3HHx-*co*-3HO)	61	*	10	300	*
P(3HB-*co*-3HHx)	52	*	20	850	-4
Polypropylene	170	1,7	34,5	400	45
Polyethylene terephthalate	262	2,2	56	7300	3400
Polystyrene	110	3,1	50	*	21
Nylon- 6.6	265	2,8	83	60	*

* No data found

Appendix C. Tables of comparison of PHB production by *C. necator* as an organism in a fed-batch fermentation process.

Substrate	Strain and mutant	Feeding of organic substrate	CDM concentration (g/L)	PHB Production (g/L)	PHB content (%)	PHB Productivity (g/L/h)	Reference
Heterotrophic-heterotrophic process							
Corn syrup	*C. necator* DSM 545	-	16.57	10.75	65	0.22	Daneshi et al., 2010
Waste glycerol	*C. necator* DSM 545	Pulse addition	30.19	10.9	36.1	0.17	Cavalheiro et al., 2012
Fructose	*R. eutropha* B-5786	Continuous	18	15.5	86	0.22	Volova and Kalacheva, 2005
Waste glycerol	*C. necator* DSM 545	-	76.2	38.1	50	1.1	Cavalheiro et al., 2009
Pure glycerol	*C. necator* DSM 545	-	82.6	51.2	62	1.52	Cavalheiro et al., 2009
Pure glycerol	*C. necator* JMP 134	-	102	57.1	56	1.31	Posada et al., 2011
Glucose	*C. necator* DSM 545	Fixed rate	81	63	78	1.85	Atlic et al., 2011
Soybean oil	*C. necator* DSM 545	Pulse addition	83	67	80	2.5	Pradella, 2012
Waste potato starch	*R. eutrophus* NCIMB 11599	-	179	94	53	1.31	Haas et al., 2008
Waste frying	*C. necator* H16	-	138	105	76	1.46	Obruca et al., 2013

oil+propanol							
Glucose	*A. eutrophus* NCIMB 11599	CO_2 evolution rate	164	121	74	2.42	Kim et al., 1994
Autotrophic-autotrophic process							
H_2:O_2:CO_2= 60:20:10 vol%	*C. eutrophus* B 10646	-	30	22	75	0.314	Volova and Voinov, 2003
H_2:O_2:CO_2= 70:20:10 vol%	*C. eutrophus* B 10646	-	48	40.8	85	0.583	Volova et al., 2013a
H_2:O_2:CO_2= 85:5:10 vol%	*C. necator* ATCC 17697	-	69.3	56.4	81.4	0.61	Taga et al., 1997
H_2:O_2:CO_2= 85.2:6.3:8.3 vol%	*C. necator* ATCC 17697	-	91.3	61.9	68	1.55	Tanaka et al., 1995
Heterotrophic-autotrophic process							
Acetic acid+ H_2:O_2:CO_2= 86.5:4.9:9.8 vol%	*C. necator* ATCC 17697	-	22.9	12.6	55	0.224	Sugimoto et al., 1999
Fructose+ H_2:O_2:CO_2= 86.5:4.9:9.8 vol%	*C. necator* ATCC 17697	-	26.3	21.6	82.1	0.556	Tanaka and Ishizaki, 1994

Appendix D. Unstructured Kinetic growth models.

No	Models	References	Description
1	Monod: $\mu = \frac{\mu_{max} S}{K_s + S}$, $\mu = \frac{\mu_{max} XS}{K_s + S}$	Monod, J., 1949. The growth of bacterial cultures. *Annual Reviews in Microbiology, 3*(1), pp.371–394. https://doi.org/10.1146/annurev.mi.03.100149.002103	μ = Specific growth rate μ_{max} = Maximum Specific growth rate S = Substrate concentration X = Biomass concentration K_s = Half saturation constant K_i = Inhibition constant n = Dynamic behaviour in the bioreactor a and b = are constants S_m = Inhibitor concentration n and m = are constants X_m = Maximum biomass concentration Powell (m) is the cell maintenance parameter
2	Blackman: $\mu = \frac{\mu_{max} S}{K_S}$	Blackman, F.F, 1905. Optima and limiting factors. Annals of botany. 19(74), pp.281–295. https://doi.org/10.1093/oxfordjournals.aob.a089000	
3	Haldane: $\mu = \frac{\mu_{max} S}{K_s + S + \frac{s^2}{K_i}}$	Haldane, J. B. S., Enzymes, MIT Press, Cambridge, 1965, pp 84	
4	Tesseir: $\mu = \mu_{max} (1 - e^{K_i S})$	Teissier, G., 1942. Growth of bacterial populations and the available substrate concentration. Rev Sci Instrum, 3208(3208), pp.209–214	
5	Moser: $\mu = \frac{\mu_{max} S^n}{K_s + S^n}$	Moser, H., 1958. The dynamics of bacterial populations maintained in the chemostat. The dynamics of bacterial populations maintained in the chemostat. pp.136. 19591604707. Carnegie Institution of Washington.	
6	Contois: $\mu = \frac{\mu_{max} S}{K_s X + S}$	Contois, D.E., 1959. Kinetics of bacterial growth: relationship between population density and specific growth rate of continuous cultures. Microbiology. 21(1), pp.40–50. https://doi.org/10.1099/00221287–21–1–40.	
7	Logarithmic: $\mu = a + b\ ln(s)$	Westerhoff, H.V., Lolkema, J.S., Otto, R., Hellingwerf, K.J., 1982. Thermodynamics of growth. Non-equilibrium thermodynamics of bacterial growth. The phenomenological and the mosaic approach. Biochimica et biophysica acta. 683(3–4), pp.181–220. https://doi.org/10.1016/0304–4173(82)90001–5	
8	Aiba-Edwards: $\mu = \mu_{max} \frac{S}{K_s + S} \exp(-\frac{S}{K_I})$	Aiba, S., Shoda, M, Nagalani, M, Kinetics of product inhibition in alcohol fermentation. Biotechnol. Bioeng. 10 (1968) 845. doi: http://dx.d0i.0rg/l0.1002/bit.260100610.	
9	Han and Levenspiel: $\mu = \mu_{max}[1 - (\frac{S}{S_m})]^n [\frac{S}{S + K_S(1 - \frac{S}{S_m})^m}]$	Han, K., Levenspiel, O., 1988. Extended Monod kinetics for substrate, product, and cell inhibition. Biotechnology and bioengineering. 32(4), pp.430–447. https://doi.org/10.1002/bit.260320404.	
10	Powell: $\mu = \frac{(\mu_{max} + m)s}{K_s + S} - m$	Powell, E.O., 1967. The growth rate of microorganisms as a function of substrate concentration. Microbial physiology and continuous culture. 3.	
11	Verhulst: $\mu = \mu_{max}[1 - \frac{X}{X_m}]$	Verhulst, P.F., 1838. Notice sur la loi que la population suit dans son accroissement. Corresp. Math. Phys., 10, pp.113–126.	
12	Luong: $\mu = \mu_{max} \frac{S}{K_S + S} (1 - \frac{S}{S_m})^n$	Luong, J. H. T., Generalization of Monod kinetics for analysis of growth data with substrate inhibition. Biotechnol. Bioeng. 29 (1987) 242. http://dx.doi.Org/l0.1002/bit.260290215.	
13	Webb: $\mu = \frac{\mu_{max} S (1 + \frac{S}{K_i})}{S + K_s + (\frac{S^2}{K_i})}$	Webb, J. L., Enzyme and Metabolic Inhibitors, Academic Press, Boston, USA, 1963. doi: http://dx.doi.org/10.5962/bhl.title.7320	
14	Yano and Koga: $\mu = \frac{\mu_{max}}{K_s + S + \frac{S^2}{K_1} + \frac{S^3}{K_2^2}}$	Yano, T., Koga, S., 1969. Dynamic behaviour of the chemostat subject to substrate inhibition. Biotechnology and Bioengineering, 11(2), pp.139–153. https://doi.org/10.1002/bit.260110204.	

Appendix E. Example of a stoichiometric matrix for the modeling of activated sludge (adapted from Gujer and Larsen, 1995).

Component	Oxygen	Inert	Substrate	Ammonia	Alkalinity	Biomass	Inert	Substrate	TSS	Rate
Symbol	S_O	S_I	S_S	S_{NH}	S_{HCO}	X_H	X_I	X_S	X_{TSS}	
Unit	gO_2	gCOD	gCOD	gN	mole	gCOD	gCOD	gCOD	gTSS	
Process	STOCIHIOMETRY MATRIX									
Hydrolysis			1					-1	-0.75	r_1
Aerobic growth	-0.5		-1.5	-0.08	-0.005714	1			0.9	r_2
Lysis				0.07	0.005	-1	0.2	0.8	-0.12	r_3
Conservatives	COMPOSITION MATRIX									
ThOD-COD	-1	1	1	0		1	1	1		
N		0.02		1		0.08	0.05	0		
Charge				0.071429	-1					
Observables										
TSS						0.9	0.9	0.75		

Appendix F. List of activated sludge models and their size in terms of number of processes, state variables and parameters.

Models	Refs.	Substrates	# of processes	# of state variables	# of interacting processes versus variables	# of parameters												
									Stoichiometry					Kinetic				
						Total	Composition matrix parameters	Temperature adjustment	Hydrolysis	OHO	ANO	PAO	Biomass general	Hydrolysis	OHO	ANO	PAO	Biomass general
ASM1	Henze et al. (2000a)[a]	CN	8	13	31	26	2	7	—	1	1	—	1	3	6	5	—	—
Barker & Dold	Barker and Dold (1997)	CNP	36	19	153	81	16	18	2	5	2	8	—	4	9	5	11	1
ASM2d	Henze et al. (2000b)[b]	CNP	21	19	136	74	13	12	1	1	1	3	1	6	12	6	18	—
ASM3	Gujer et al. (2000)[c]	CN	12	13	72	46	8	10	1	4	1	—	1	2	13	6	—	—
ASM3 + BioP	Rieger et al. (2001)	CNP	23	17	148	83	15	13	1	4	1	5	1	2	13	7	21	—
UCTPHO+	Hu et al. (2007)	CNP	35	16	169	66	12	10	—	3	2	7	—	—	13	4	14	1
ASM2d + TUD	Meijer (2004)	CNP	22	18	154	98	16	15	1	2	2	12	—	6	12	6	26	—

Chemical P precipitation not considered. Biomass general refers to common parameters to OHOs, ANOs, and PAOs.
[a]First published in Henze et al. (1987).
[b]First published in Henze et al. (1999).
[c]First published in Gujer et al. (1999).

Appendix G. Comparison of studies reporting PHAs (PHB) production in *C. necator*.

Strain	Substrate	Scale	Control strategy	PHA[1] produced	Biomass ($g\,L^{-1}$)	PHA ($g\,L^{-1}$)	PHA (%)	Productivity ($g\,L^{-1}\,h^{-1}$)	Reference
C. necator	Plant oils[2]	Flask	Batch	P(3HB)	3.6–4.3	2.9–3.4	79–81	0.04–0.05	Fukui and Doi [17]
C. necator (recombinant, *Aeromonas caviae*)	Plant oils[2]			P(3HB-co-3HHX)	3.5–3.6	2.7–2.9	76–81	0.04	
C. necator DSM 541	Fructose	Flask	Batch	P(3HB)	3.4	n.a	55	n.a	Dennis et al. [18]
	Palmitate			P(3HB-co-3HV-co-3HHX)	0.51	n.a	58	n.a	
	Oleate			P(3HB-co-3HHX)	1.44	n.a	57	n.a	
C. necator	Centrifuged fermented organic waste	Flask	Batch	P(3HB-co-3HV)	2.77	1.13	40.0	0.025	Ganzeveld et al., [19]
C. necator	Bagasse hydrolysate	Flask	Batch	n.a	6	3.9	65	0.08	Yu and Stahl [12]
C. necator	Palm oil	Flask	Batch	P(3HB-co-3HV)	3.6	2.66	74	n.a	Liu et al. [20]
C. necator	Fructose[3]	Flask	Fed batch	P(3PHB)	5,25	4.04	76.87	0.081	This study
	Fructose, avocado oil			P(3HB-co-3HV)	4.45–4.91	2.64–3.48	59–70	0.053–0.07	

[1]3PHB, 3 hydroxybutyrate; 3HV, 3 hydroxyvalerate; 3HHX, 3-hydroxyhexanoate. [2]Olive oil, corn oil and palm oil tested once at a time. [3]Control experiments. n.a: Not available.

Appendix H. Calculations to obtain the stoichiometric coefficients.

$$Y_{X/F} = \frac{2.350\ gX_H}{5.630\ gS_F} = 0.417\ gX_H/gS_F$$

$$Y_{PHB/XH} = \frac{1.137\ gX_{PHB}}{2.350\ gX_H} = 0.484\ gX_{PHB}/gX_H$$

$$Y_{PHB/FA} = \frac{0.696\ gX_{PHB}}{35.416\ gS_{FA}} = 0.020\ gX_{PHB}/gS_{FA}$$

$$Y_{PHB/necator} = 0.708\ gX_{PHB}/g\ microorganism$$

Appendix I. Python code used for kinetic model simulation.

```
##
## It is a program for making cinetic model and follow the evolution of different components in time
## It solves the continuity equations and make the calculation of balance equation
## What you need to provide: matrix (for process and compounds), kinetic equations and kinetic constants in a file
## And finally fill all the initial condition, initial concentrations, delta T, Volume, Caudal
#########################################################################################################################
## v0.1 - Modekine
## by Thibault Terencio, Henry Romero, Carlos Pazmino, Alicia Sommer
#########################################################################################################################

import re as reg
from tkinter import *
from tkinter.filedialog import askopenfilename
from tkinter import ttk
import csv

from sympy import *
from sympy.solvers.solveset import linsolve

root = Tk()

##########################################################################
##### Creation of a class named myapp which will contain all the elements of the graphical interface  #############
##########################################################################

inputP=""
inputC=""
outputP=""
outputC=""
ncolFrame=0


class MainWindow:
    def __init__(self,maestro):
        ## Menu definition
        menubar = Menu(root)
        root.config(menu=menubar)
        root.ncolFrame=0
        filemenu = Menu(menubar)
        #lambda used for no executing the command directly
        filemenu.add_command(label="New Project", command=(lambda:self.NewProject()))
        filemenu.add_separator()
        filemenu.add_command(label="Open Matrix Process", command=(lambda:self.OpenFile("Process",entriesP)))
        filemenu.add_command(label="Open Matrix Compounds", command=(lambda:self.OpenFile("Compounds",entriesC)))
        filemenu.add_command(label="Open kinetic equations", command=(lambda:self.OpenKinec("Kinetics","")))
        filemenu.add_separator()
        filemenu.add_command(label="Save Matrix Process", command=(lambda:self.SaveFile(outputP)))
        filemenu.add_command(label="Save Matrix Compounds", command=(lambda:self.SaveFile(outputC)))
        filemenu.add_separator()
        filemenu.add_command(label="Salir", command=root.quit)

        editmenu = Menu(menubar)
```

```
        menubar.add_cascade(label="Archivo", menu=filemenu)
        menubar.add_cascade(label="Editar", menu=editmenu)

        ##############################################################################
        ### Define the welcome part (just when opening the program                 ###
    ##############################################################################

        self.welcome_frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=450,width=350)
        self.welcome = ttk.Label(self.welcome_frame, text="Welcome to this program", font=('Helvetica', 18, 'bold'))
        self.welcome.pack(side='top')
        self.welcome2 = ttk.Label(self.welcome_frame, text="Please choose a new project or load the matrices from csv files", font=('Helvetica', 9, 'italic'))
        self.welcome2.pack(side='top')

        canvaso = Canvas(self.welcome_frame, width = 400, height = 300, background="black")

        self.img = PhotoImage(file='bubbles.gif')
        canvaso.create_image(2,2, anchor=NW, image=self.img)
        canvaso.pack()
        self.welcome_frame.pack()

        ncolFrame=0
        self.WidK=[]
        self.WidB=[]


    def NewProject(self):
        ##############################################################################
        ### Part which generate the two matrix but empty                           ###
    ##############################################################################
        self.welcome_frame.destroy()
        print("-----------------------------------")
        self.Unaapp = myapp(root,"Process",2,2,entriesP,inputP)
        self.otraapp = myapp(root,"Compounds",2,2,entriesC,inputC)
        self.DownPart()

    def DownPart(self):
    ##############################################################################
        ### Part at the bottom of the graphical interface                          ###
    ##############################################################################
        self.next_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=50,width=500)
        self.shooow = Button(self.next_Frame, text="Show continuity equations",command=self.Show)
        self.shooow.pack(side=LEFT)
        self.check = Button(self.next_Frame, text="Check if solvable",command=self.Check)
        self.check.pack(side=LEFT)
        self.solveq = Button(self.next_Frame, text="Solve it!",command=self.SolvEq)
        self.solveq.pack(side=LEFT)
        self.solveq = Button(self.next_Frame, text=">>> Expand cinetic and balance equations part",command=self.ExpandKin)
        self.solveq.pack(side=LEFT)
        self.next_Frame.grid(row=3, column=0)
```

```
def Show(self):
    ##############################################################################
    ### Part to show the continuity equations and keep it in a file              ###
##############################################################################

    listeq=[]
    self.equations=[]
    self.equationss=[]
    self.variables=[]
    self.varwhereis=[]
    self.varwhereis.append([])
    self.varwhereis.append([])
    l=0

    print("CONTINUITY EQUATIONS ")
    for i in range(1,self.Unaapp.n+1):
        for j in range(1,self.otraapp.n+1):
            listeq.append([])
            self.equation=[]

            listeq[l].append("equation "+str(l)+" ("+self.Unaapp.entryname[i][0].get()+" "+self.otraapp.entryname[j][0].get()+"): ")
            for k in range(1,self.Unaapp.ncol+1):
                listeq[l].append(self.Unaapp.entryname[i][k].get()+"*"+self.otraapp.entryname[j][k].get())
                listeq[l].append("+")
                self.equations.append(self.Unaapp.entryname[i][k].get()+"*"+self.otraapp.entryname[j][k].get())
                self.equations.append("+")
                self.equation.append(self.Unaapp.entryname[i][k].get()+"*"+self.otraapp.entryname[j][k].get())
                self.equation.append("+")

                if(self.Unaapp.entryname[i][k].get().isalpha()):
                    CountnoAdd=0
                    for vava in self.variables:
                        if self.Unaapp.entryname[i][k].get()==vava:
                            CountnoAdd+=1
                    if (CountnoAdd==0):
                        self.variables.append(self.Unaapp.entryname[i][k].get())
                        self.varwhereis[0].append(i)
                        self.varwhereis[1].append(k)
            self.equation.pop()
            self.equations.pop()
            self.equationss.append(''.join(self.equation))
            listeq[l].pop()
            listeq[l].append(" = 0")
            l+=1
    fcontinuity = open("ContinuityEq.txt","w")
    for i in range(l):
        print(*listeq[i],sep='',file=fcontinuity)
    print(self.variables)
    print(self.equations)
```

```
def Check(self):
    print("you have the same amount of unknown and equations good!")


def SolvEq(self):
    ###############################################################################
    ### Part for doing a linear solve of continuity equations                   ###
    ### and put the solutions in a file                                         ###
###############################################################################
    print("*****  solving ->")

    fSolcontin = open("ContinuitySolutions.txt","w")
    var4sym=var(','.join(self.variables))
    SolutionsCont=linsolve(self.equationss, var4sym)
    SolContiList=list(SolutionsCont.args[0])

    for i in range(0,len(self.variables)):
        print(self.variables[i],round(SolContiList[i],5),file=fSolcontin)
        ll=self.varwhereis[0][i]
        cc=self.varwhereis[1][i]
        self.Unaapp.entryname[ll][cc].delete(0,END)
        self.Unaapp.entryname[ll][cc].insert(0,round(SolContiList[i],5))

def ExpandKin(self):
###############################################################################
    ### Part which extend the right panel of the interface                  ###
###############################################################################
    #### Kinetic panel
    self.Kin_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=200,width=600)

    self.Kineq_Frame1 = ScrollableFrameV(self.Kin_Frame)
    self.labelo = ttk.Label(self.Kineq_Frame1.scrollable_frame, text="Kinetic equations", font=('Helvetica', 18, 'bold'))
    self.labelo.grid(row=0,column=0)
    self.Kineq_Frame1.pack(side=LEFT)
    self.Kin_Frame.grid(row=0, column=1)
    self.Kineq_Frame1.pack(side=LEFT)

#### Initial panel
    self.Ini_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=200,width=300)
    self.Ini_Frame1 = ScrollableFrame(self.Ini_Frame)
    self.labeli = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Initial conditions", font=('Helvetica', 18, 'bold'))
    self.labeli.grid(row=0,columnspan=4)

    self.labeli = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Initial concentracions", font=('Helvetica', 12, 'bold'))
    self.labeli.grid(row=1,columnspan=4)

    self.IniCon=[]
    self.IniCon.append([])
    self.IniCon.append([])
    self.IniCon.append([])
    self.IniCon.append([])
```

```
#### Initial panel
    self.Ini_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=200,width=300)
    self.Ini_Frame1 = ScrollableFrame(self.Ini_Frame)
    self.labeli = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Initial conditions", font=('Helvetica', 18, 'bold'))
    self.labeli.grid(row=0,columnspan=4)

    self.labeli = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Initial concentracions", font=('Helvetica', 12, 'bold'))
    self.labeli.grid(row=1,columnspan=4)

    self.IniCon=[]
    self.IniCon.append([])
    self.IniCon.append([])
    self.IniCon.append([])
    self.IniCon.append([])
    for i in range(1,self.Unaapp.ncol+1):
        self.IniCon.append([])
        self.labelIniL = ttk.Label(self.Ini_Frame1.scrollable_frame, text=self.Unaapp.entryname[0][i].get(), font=('Helvetica', 8))
        self.labelIniL.grid(row=2, column=i-1)
        self.IniCon[0].append(self.labelIniL)

        self.entry = Entry(self.Ini_Frame1.scrollable_frame, width=5)
        self.IniCon[1].append(self.entry)
        self.entry.grid(row=3, column=i-1)

    self.labeli = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Operation conditions", font=('Helvetica', 12, 'bold'))
    self.labeli.grid(row=4,columnspan=4)

    self.label = ttk.Label(self.Ini_Frame1.scrollable_frame, text="DeltaT", font=('Helvetica', 8))
    self.label.grid(row=5, column=0)
    self.IniCon[2].append(self.label)

    self.label = ttk.Label(self.Ini_Frame1.scrollable_frame, text="V", font=('Helvetica', 8))
    self.label.grid(row=5, column=1)
    self.IniCon[2].append(self.label)
    self.label = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Ci", font=('Helvetica', 8))
    self.label.grid(row=5, column=2)
    self.IniCon[2].append(self.label)
    self.label = ttk.Label(self.Ini_Frame1.scrollable_frame, text="Co", font=('Helvetica', 8))
    self.label.grid(row=5, column=3)
    self.IniCon[2].append(self.label)
    for i in range(4):
       self.entry = Entry(self.Ini_Frame1.scrollable_frame, width=5)
       self.IniCon[3].append(self.entry)
       self.entry.grid(row=6, column=i)        ;

    self.Ini_Frame.grid(row=0, column=2)
    self.Ini_Frame1.pack(side=LEFT)
```

```
#### Balance panel
    self.Bal_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=200,width=600)
    self.Balan_Frame = ScrollableFrameV(self.Bal_Frame)
    self.labelp = ttk.Label(self.Balan_Frame.scrollable_frame, text="Balance equations", font=('Helvetica', 18, 'bold'))
    self.labelp.grid(row=0,columnspan=2)
    self.Balan_Frame.pack(side=LEFT)

    self.Bal_Frame.grid(row=1, column=1)

    self.downBalKin_Frame = Frame(root,borderwidth = 2, relief=SUNKEN, height=50,width=500)
    self.showBal = Button(self.downBalKin_Frame, text="Show balance equations",command=self.ShowB)
    self.showBal.pack(side=LEFT)
    self.solvBal = Button(self.downBalKin_Frame, text="Solve Balance equations",command=self.SolveB)
    self.solvBal.pack(side=LEFT)
    self.downBalKin_Frame.grid(row=3, column=1)

def ShowB(self):
    ##############################################################################
    ### Part to show the balance equations inside the entries in form of variables ###
##############################################################################
    self.listeqB=[]
    self.equationsB=[]
    self.equationssB=[]
    self.variablesB=[]
    l=0
    print(1,self.Unaapp.nom)
    print("BALANCE EQUATIONS ")
    for i in range(1,self.Unaapp.ncol+1):
        self.listeqB.append([])
        self.listeqB[l].append("(")
        self.listeqB[l].append(self.IniCon[2][2].cget("text")+"*XXXXXXX*"+self.IniCon[2][0].cget("text")+"/"+self.IniCon[2][1].cget("text")+"+")
        self.listeqB[l].append("(")
        caca=0
        for j in range(1,self.Unaapp.n+1):
            self.equation=[]
            if (self.Unaapp.entryname[j][i].get()!="0"):
                self.listeqB[l].append("("+self.Unaapp.entryname[j][i].get()+"*"+self.WidK[j-1][0].get()+")")
                self.listeqB[l].append("+")
                caca=caca+1
        self.listeqB[l].pop()
        if caca == 0:
            self.listeqB[l].append("0")
        else:
            self.listeqB[l].append(")")
        self.listeqB[l].append("*"+self.IniCon[2][0].cget("text")+"+XXXXXXX)/(1+("+self.IniCon[2][3].cget("text")+"*"+self.IniCon[2][0].cget("text")+"/"+self.IniCon[2][1].cget("text")+"))")
        l+=1

    self.WidB.append([])
    self.WidB.append([])
    for i in range(l):
```

```
            for i in range(0,NumComp):
                listcompNAME.append(self.IniCon[0][i].cget("text"))
                listcompCONC.append(float(self.IniCon[1][i].get()))
            print(listcompNAME,file=fsolB)
            print("time "+str(round(time,2))+":",file=fsolB)
            print(listcompCONC,file=fsolB)

            listnewcompCONC=listcompCONC

### at time t (each deltat)
            while time<totaltime:
                time = time+Cini_deltaT

                listcompCONC=listnewcompCONC
                listnewcompCONC=[]
# Here, we take the balance equation generated, replace the text with variables and calculate
                for j in range(1,self.Unaapp.ncol+1):

                    Eqqone=Eqq[j-1]
                    Eqqone=Eqqone.replace('XXXXXXX',str(listcompCONC[j-1]))
                    for k in range(0,NumComp):
                        Eqqone=Eqqone.replace(listcompNAME[k],str(listcompCONC[k]))
                    result=eval(Eqqone)

                    listnewcompCONC.append(result)
                listnewcompCONC.insert(0,time)

# Here we write the different components inside the file
                print(listnewcompCONC,file=fsolB)

    def OpenFile(self,prococomp,entritri):
    ##############################################################################
        ### Part for opening files of matrix                                   ###
    ##############################################################################
        self.welcome_frame.destroy()
 #       self.input=input
        self.input = askopenfilename(initialdir="C:/Users/",
                           filetypes =(("csv file", "*.csv"),("Text File", "*.txt"),("All File
                           title = "Choose a file."
                           )
        print(">>>>>>>>>>>")
        print(self.input)
        if prococomp=="Process":
            self.Unaapp = myapp(root,prococomp,3,3,entritri,self.input)
        if prococomp=="Compounds":
            self.otraapp = myapp(root,prococomp,3,3,entritri,self.input)
            self.DownPart()

    def OpenKinec(self,prococomp,entritri):
        ##############################################################################
        ### Part to open a file containing the kinetic equations               ###
    ##############################################################################
```

```
    ##############################################################################
        self.inKin = askopenfilename(initialdir="C:/Users/",
                           filetypes =(("Text File", "*.txt"),("All Files","*.*")),
                           title = "Choose a file."
                           )
        print(">>>>>>>>>>>")
        print(self.inKin)

       # definition of the regex, in that case it would detect 3 columns, but only 2 of them are defined for the match.group (look for the parenthesis)
        paternConst = reg.compile(r"(\S+)\s+:\s+(\S+)")
        paternEq = reg.compile(r"(\S+)\s+=\s+(\S+)")
       # definition of a list of list named dataIR
        dataIR = []
       # definition of the file we would open to look for data
        f=open(self.inKin)
       # definition of the file where we would write something (output)
        out="Salida.txt"

        k=-1
## We would like inside the file and look for the regex line by line
        lines=f.readlines()
        for line in lines:
            print(line)
            match1=paternEq.search(line)
            match2=paternConst.search(line)
            if match1:
                    dataIR.append([])
                    k=k+1
                    dataIR[k].append(match1.group(2))

            if match2:
                    dataIR[k].append(match2.group(1))
                    dataIR[k].append(match2.group(2))
        f.close

        print(dataIR)
        j=1
        for l in range(k+1):
            self.WidK.append([])

            self.entry = Entry(self.Kineq_Frame1.scrollable_frame, width=65)

            self.WidK[l].append(self.entry)
            self.WidK[l][0].delete(0,END)
            self.WidK[l][0].insert(0,dataIR[l][0])
            self.entry.grid(row=l+j, columnspan=2)
            for i in range(1,len(dataIR[l]),2):
                self.labelCons = ttk.Label(self.Kineq_Frame1.scrollable_frame, text=dataIR[l][i], font=('Helvetica', 12))
                self.labelCons.grid(row=l+j+i, column=0)
                self.WidK[l].append(self.labelCons)
```

```
                    self.entry = Entry(self.Kineq_Frame1.scrollable_frame, width=25)
                    self.WidK[l].append(self.entry)
                    self.WidK[l][i+1].delete(0,END)
                    self.WidK[l][i+1].insert(0,dataIR[l][i+1])
                    self.entry.grid(row=l+j+i, column=1)
                j=j+i


    def SaveFile(self,output):
        self.output=output


class ScrollableFrame(ttk.Frame):
    def __init__(self, container, *args, **kwargs):
        super().__init__(container, *args, **kwargs)
        canvas = Canvas(self)
        scrollbar = ttk.Scrollbar(self, orient="horizontal", command=canvas.xview)
        self.scrollable_frame = ttk.Frame(canvas,borderwidth = 2, relief=SUNKEN, height=50,width=600)
        self.scrollable_frame.bind(
            "<Configure>",
            lambda e: canvas.configure(
                scrollregion=canvas.bbox("all")
            )
        )

        canvas.create_window((0, 0), window=self.scrollable_frame, anchor="nw")

        canvas.configure(xscrollcommand=scrollbar.set)
        scrollbar.pack(side="bottom", fill="y")
        canvas.pack(side="left", fill="both", expand=True)

class ScrollableFrameV(ttk.Frame):
    def __init__(self, container, *args, **kwargs):
        super().__init__(container, *args, **kwargs)
        canvas = Canvas(self)
        scrollbar = ttk.Scrollbar(self, orient="vertical", command=canvas.yview)
        self.scrollable_frame = ttk.Frame(canvas,borderwidth = 2, relief=SUNKEN, height=50,width=600)
        self.scrollable_frame.bind(
            "<Configure>",
            lambda e: canvas.configure(
                scrollregion=canvas.bbox("all")
            )
        )

        canvas.create_window((0, 0), window=self.scrollable_frame, anchor="nw")

        canvas.configure(xscrollcommand=scrollbar.set)
        scrollbar.pack(side="bottom", fill="y")
        canvas.pack(side="left", fill="both", expand=True)
```

```
entries_proc = []
entries_proc.append([])
entriesP=[]
entriesC=[]

####################


class myapp:
        ##############################################################################
        ### Class which define the 2 parts with matrix on the left                 ###
    ##############################################################################


    def __init__(self,master,nom,row,col,entryname,inp):
        self.entryname=entryname
        self.entryname=[]
        self.entryname.append([])
        self.inp=inp
        self.nom=nom
        self.master = master
        master.title("my super app")
        self.entered_number = 0
        self.n_row_process=0
        self.n=row
        self.ncol=col
        self.name=""
        self.Matrix_Frame0 = Frame(master,borderwidth = 2, relief=SUNKEN, height=50,width=500)
        self.Matrix_Frame0.grid(row=master.ncolFrame, column=0)
        master.ncolFrame=master.ncolFrame+1
        self.Matrix_Frame1 = ScrollableFrame(self.Matrix_Frame0)
        self.processtit = ttk.Label(self.Matrix_Frame0, text="Matrix "+self.nom, font=('Helvetica', 18, 'bold'))
        self.processtit.pack(side='top')
        self.Matrix_Frame1.pack(side=LEFT)

        vcmd = master.register(self.validate)
        self.Matrix_Frame2 = Frame(self.Matrix_Frame0,borderwidth = 2, relief=SUNKEN, height=200,width=200)
        self.Matrix_Frame2.pack(side=RIGHT)
        self.Matrix_Frame2.pack_propagate(False)
        self.processsizetit = Label(self.Matrix_Frame2, text="Options to modify the Matrix")
        self.processsizetit.pack(pady=10)

        self.process_sizerowp = Button(self.Matrix_Frame2, text="row +",command=self.addrowentry)
        self.process_sizerowm = Button(self.Matrix_Frame2, text="row -",command=self.removerowentry)
        self.process_sizecolp = Button(self.Matrix_Frame2, text="column +",command=self.addcolentry)
        self.process_sizecolm = Button(self.Matrix_Frame2, text="column -",command=self.removecolentry)
        self.process_print = Button(self.Matrix_Frame2, text="PrintME",command=self.printentries)
```

```
        self.process_sizerowp.pack(fill=X)
        self.process_sizerowm.pack(fill=X)
        self.process_sizecolp.pack(fill=X)
        self.process_sizecolm.pack(fill=X)

        self.process_print.pack(side = BOTTOM,fill=X)


       #Defining the menu and the elements of the menus

        for i in range(self.n+1):
            self.entryname.append([])
            for j in range(self.ncol+1):
                self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
                self.entry.grid(row=i, column=j)
                self.entryname[i].append(self.entry)


        self.Matrix_Frame1.pack()

        if inp != "":
            print("Habemus datum")
            self.importdata()

    def addcolentry(self):
        self.ncol+=1
        for i in range(self.n+1):
            print(i)
            self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
            self.entryname[i].append(self.entry)
            print(self.n,"<><><><><>",self.ncol)
            self.entry.grid(row=i, column=self.ncol)

    def removecolentry(self):
        if self.ncol>0:
            self.ncol-=1
            for i in range(self.n+1):
                self.entryname[i][self.ncol+1].destroy()
            self.Matrix_Frame1.pack()                          #     entries_proc[self.n].pop()

    def addrowentry(self):
        self.entryname.append([])
        self.n+=1
        print(len(self.entryname[0]))
        for i in range(self.ncol+1):
            self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
            self.entryname[self.n].append(self.entry)
#           print(self.n,"<><><><><>",self.ncol)
            self.entry.grid(row=self.n, column=i)
        self.Matrix_Frame1.pack()
```

```
    def addrowentry(self):
        self.entryname.append([])
        self.n+=1
        print(len(self.entryname[0]))
        for i in range(self.ncol+1):
            self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
            self.entryname[self.n].append(self.entry)
#            print(self.n,"<><><><><>",self.ncol)
            self.entry.grid(row=self.n, column=i)
        self.Matrix_Frame1.pack()

    def removerowentry(self):
        self.n-=1
        for i in range(self.ncol+1):
#            print(self.n,"<><><><><>",self.ncol)
            self.entryname[self.n+1][i].destroy()
        self.Matrix_Frame1.pack()

    def printentries(self):
        print(self.nom)
        for i in range(self.n+1):
                for j in range(self.ncol+1):
                    print("i: ",i," >>>>>>  j: ",j,"  ",self.entryname[i][j].get())

    def importdata(self):
        csvfile=open(self.inp, newline='')
        csvreader = csv.reader(csvfile, delimiter=';')
        datata=list(csvreader)

        self.n=len(datata)-1
        self.ncol=len(datata[0])-1

        self.entryname=[]
        for i in range(self.n+1):
            self.entryname.append([])
            for j in range(self.ncol+1):
                self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
                self.entry.grid(row=i, column=j)

                self.entryname[i].append(self.entry)
                self.entryname[i][j].delete(0,END)
                self.entryname[i][j].insert(0,datata[i][j])
        self.Matrix_Frame1.pack()

    def validate(self, new_text):
        if not new_text: # the field is being cleared
            self.entered_number = 0
            return True
        try:
            self.n_row_process=int(new_text)
            self.process4 = Entry(self.Matrix_Frame1, validate="key")
            self.process4.pack(side = LEFT)
```

```
    def printentries(self):
        print(self.nom)
        for i in range(self.n+1):
                for j in range(self.ncol+1):
                    print("i: ",i," >>>>>>  j: ",j,"  ",self.entryname[i][j].get())

    def importdata(self):
        csvfile=open(self.inp, newline='')
        csvreader = csv.reader(csvfile, delimiter=';')
        datata=list(csvreader)

        self.n=len(datata)-1
        self.ncol=len(datata[0])-1

        self.entryname=[]
        for i in range(self.n+1):
            self.entryname.append([])
            for j in range(self.ncol+1):
                self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
                self.entry.grid(row=i, column=j)

                self.entryname[i].append(self.entry)
                self.entryname[i][j].delete(0,END)
                self.entryname[i][j].insert(0,datata[i][j])
        self.Matrix_Frame1.pack()

    def validate(self, new_text):
        if not new_text: # the field is being cleared
            self.entered_number = 0
            return True
        try:
            self.n_row_process=int(new_text)
            self.process4 = Entry(self.Matrix_Frame1, validate="key")
            self.process4.pack(side = LEFT)
            self.entered_number = int(new_text)
            for i in range(1):
                self.entry = Entry(self.Matrix_Frame1.scrollable_frame, width=5)
                self.entry.pack(side = LEFT)
            self.Matrix_Frame1.pack()
            return True
        except ValueError:
            return False


#######################################################
##### end of the definition of the class ##############
#######################################################

TheMain=MainWindow(root)

root.mainloop()
```