Iolanda Maria da Silva Reis

Segregated Modeling and Selection of Populations for Polyhydroxyalkanoate Production by Mixed Microbial Cultures

Dissertação para obtenção do Grau de Mestre em
Engenharia Química e Bioquímica

Orientador: Professor Doutor Rui Manuel Freitas Oliveira

Dezembro de 2008

FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Departamento de Química

Segregated Modeling and Selection of Populations for Polyhydroxyalkanoate Production by Mixed Microbial Cultures

Por: Iolanda Maria da Silva Reis

Dissertação apresentada na Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa para obtenção do grau de Mestre em Engenharia Química e Bioquímica

Sob Orientação: Professor Doutor Rui Manuel Freitas Oliveira

Monte de Caparica
Dezembro de 2008

Copyright Iolanda Maria da Silva Reis, FCT/UNL, UNL

A Faculdade de Ciências e Tecnologia e a Universidade Nova de Lisboa têm o direito, perpétuo e sem limites geográficos, de arquivar e publicar esta dissertação através de exemplares impressos reproduzidos em papel ou de forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado, e de a divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objectivos educacionais ou de investigação, não comerciais, desde que seja dado crédito ao autor e editor.

Agradecimentos

O primeiro agradecimento é dirigido à minha família, em especial aos meus pais, que apesar de todas as dificuldades, conseguiram apoiar-me sempre.

Em segundo lugar, gostaria de agradecer ao Professor Rui Oliveira pela atenção e orientação durante toda a dissertação de mestrado.

Aos meus colegas de curso e de faculdade que tornaram estes últimos anos muito especiais, em especial à Filipa e à Andreia.

Aos meus amigos de sempre, que sempre me procuraram e que nunca se esqueceram de mim.

Por último, quero fazer um agradecimento especial ao João Dias, pela paciência e disponibilidade.

Obrigada a Todos!

Sumário

A presente dissertação de mestrado aborda o tópico de modelação e optimização da produção de Polihidroxibutirato (PHB), a partir de culturas microbianas (MMC). Mais especificamente, aborda o design de ciclos de fome/fartura, de limitação de nutrientes, num reactor semi-batch, onde o objective é melhorar o controlo de selecção de populações microbianas, com um taxa de crescimento específico superior e uma capacidade de armazenagem de PHB maior.

Um modelo de população segregada foi implementada em MATLAB e foi efectuada uma análise crítica aos parâmetros cinéticos do modelo (PHB_{max}, $\mu_{S,max}$, $\mu_{PHB,max}$, q_{PHB}, P/O ratio e q_{EPS}) através do controlo dos graus de liberdade que cada parâmetro confere. Dois níveis de parâmetros cinéticos foram definidos de forma a simular uma população de 64 populações de organismos com diferentes genótipos. As simulações foram efectuadas variando a carga orgânica (concentração de acetato) e da razão Carbono/Azoto (C/N ratio), diferenciando se o permeado ou as lamas se manteve. Através destas simulações foi possível demonstrar que parâmetros são mais sensíveis aos graus de liberdade do funcionamento do reactor. De seguida, com o objectivo de optimizar o processo, uma estratégia de múltiplos-objectivos foi implementada em MATLAB. Após a definição dos parâmetros cinéticos e dos dois níveis, 8 populações com diferentes genótipos foram gerados. As condições “manter as lamas” e “manter o permeado” foram novamente inseridas, demonstrando que condição é mais favorável para a selecção de populações. Os principais objectives era maximizar a taxa de crescimento específica de PHB $\mu_{PHB,max,}$ taxa de crescimento específica de Acetado $\mu_{S,max}$, the biomass mean PHB_{max}, the biomass mean P/O ratio e do consumo de PHB $q_{PHB,max}$, and to minimize the biomass mean $q_{EPS,max}$, do processo de selecção em geral, e a diferença entre a concentração inicial de biomassa e a concentração final.

As principais conclusões a retirar desta dissertação são que o regime de fome e fartura, a optimização da alimentação das culturas são importantes factores para a selecção das populações com elevada capacidade de armazenamento de PHB, produção baixa de EPS (extracellular polymeric substances) e uma taxa elevada de crescimento celular.

Summary

The present M.Sc. dissertation addresses the topics of modeling and optimization of polyhydroxybutyrate (PHB) production by mixed microbial cultures (MMC). More specifically, the design of feast and famine cycles, of nutrients limitation, in a sequencing batch reactor is studied with the goal of improved selection control of microbial populations with higher specific growth rate and higher PHB storage capacity.

A segregated population model was implemented in MATLAB and a sensitivity analysis of critical kinetic parameres (PHB_{max}, $\mu_{S,max}$, $\mu_{PHB,max}$, q_{PHB}, P/O ratio and q_{EPS}) over control degrees of freedom was performed. Two levels of key kinetic parameters were defined to mimic a mixture of 64 populations of organisms with different "genotypes". The simulations were made varying organic load (acetate concentration) and the carbon-to-nitrogen ratio (C/N ratio), differentiating if the sludge or permeate were kept. Through these simulations it was possible to demonstrate which parameters are more sensitive to the reactor operating degrees of freedom. Then, in order to optimize the process operation, a multi-objective optimization strategy was implemented in *MATLAB*. After the definition of the kinetic parameters and the two levels of key kinetic parameters, 8 populations with different "genotypes" where generated. The conditions of keeping sludge or keeping permeate were again introduced, demonstrating which condition is more favorable for the selection of populations. The main goals were to maximize the maximum specific growth rate on PHB $\mu_{PHB,max,}$ the maximum specific growth rate on Acetate $\mu_{S,max}$, the capacity to storage PHB PHB_{max}, o P/O ratio and the storage rate $q_{PHB,max}$, and minimize de EPS $q_{EPS,max}$ production, the overall selection process, and the difference between the initial and the final active biomass concentration.

The main conclusions to be taken from this dissertation are that the feast and famine regime and the optimization of the culture feeding are important factors for the selection of populations with high PHB storage capacity, low extracellular polymeric substances production and high cell growth rate.

Nomenclature

Parameter	*Description:*
f_{PHB}	Intracellular PHB content (C–mol PHB/C–mol X)
$f_{PHB,max}$	Maximum intracellular PHB content (C-mo PHB/C-mol X)
k_d	Kinetic constant for PHB degradation (h^{-1})
k_{lyse}	Kinetic constant for Cell degradation (h^{-1})
K_{NS}, K_{NPHB}	Ammonia half-saturation constant (N-mmol/l)
K_{PHB}	Intracellular PHB content half-saturation constants (C-mmol/l)
K_S	Acetate half-saturation constant (C-mmol/l)
m_{ATP}	Maintenance coefficient on ATP (mol ATP/C-mol.h)
m_{PHB}	Maintenance coefficient on PHB (C-mol/C-mol.h)
m_s, $m_{s,max}$	Maintenance coefficient on acetate (C-mol/C-mol.h)
n	Reaction order of PHB degradation for maintenance (dimensionless)
OUR	Oxygen uptake rate (O_2-mmol/h)
q_{EPS}	Specific EPS production rate (C-mol/C-mol.h)
$q_{EPS,max}$	Maximum specific EPS production rate (C-mol/C-mol.h)
q_{PHB}	Specific PHB storage rate (C-mol/C-mol.h)
$q_{PHB,c}$	Specific PHB consumption rate (C-mol/C-mol.h)
q_S	Specific acetate consumption rate (C-mol/C-mol.h)
$q_{S,max}$	Maximum specific acetate consumption rate (C-mol/C-mol.h)
q_S^{PHB}	Specific acetate consumption rate for PHB formation (C-mol/C-mol.h)
$q_{S,l}^{PHB}$	Specific acetate consumption rate for PHB formation in conditions of ammonia limitation (C-mol/C-mol.h)
$q_{S,max}^{PHB}$	Maximum specific acetate consumption rate for PHB formation (C-mol/C-mol.h)

q_S^X	Specific acetate consumption rate for cell grwth (C-mol/C-mol.h)
$q_{S,max}^X$	Maximum specific acetate consumption rate for cell growth (C-mol/C-mol.h)
q_S^{EPS}	Specific acetate consumption rate for EPS formation (C-mol/C-mol.h)
$q_{S,max}^{EPS}$	Maximum specific consumption rate for EPS formation (C-mol/C-mol.h)
r_{PHB}	Volumetric PHB formation rate (C-mol/l.h)
r_X	Volumetric active biomass formation rate (C-mol/l.h)
t	Culture time (h)
$Y_{EPS/S}$	Yield of EPS on acetate (C-mol/C-mol)
$Y_{N/X}$	Yield of ammonia on biomass rate (N-mol/C-mol)
$Y_{PHB/S}$	Yield of PHB on acetate (C-mol/C-mol)
$Y_{PHB/X}$	Yield of PHB on biomass (C-mol/C-mol)
$Y_{X/S}$	Yield of biomass on acetate (C-mol/C-mol)

Greek symbols

α	PHB production saturation order constant (dimensionless)
δ	Efficiency of oxidative phosphorylation (mol ATP/mol/$NADH_2$)
ΔPHB_c	PHB consumed (C-mmol/l)
ΔPHB_f	PHB formed (C-mmol/l)
ΔPHB_m	PHB consumed for maintenance (C-mmol/l)
ΔS	Total acetate consumed (C-mmol/l)
ΔS_m	Acetate consumed for maintenance (C-mmol/l)
ΔX	Acetate biomass formed (C-mol/l)
γ_S, γ_{PHB}, γ_X	Degreeof reduction of acetate, PHB and biomass respectively (dimensionless)

μ_{PHB}	Specific growth rate on PHB (C-mol/C-mol.h)
$\mu_{PHB,max}$	Maximum specific growth rate on PHB (C-mol/(C-mol.h))
μ_{S}	Specific growth rate on acetate (C-mol/(C-mol.h))
$\mu_{S,max}$	Maximum specific growth rate on acetate (C-mol/(C-mol.h))

Acronyms

ADF	Aerobic Dynamic Feeding
ASM	Activated Sludge Model
C/N	Carbon/Nitrogen ratio (C-mol/N-mol)
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolymeric substances
GC	Gas Chromatography
HB	Poly-β-hydroxyvalerate monomer
ISA	Ionic Strength Adjuster
MSE	Mean Squared Error
P/O	ATP synthesis/oxygen consumption ratio
PHA	Polyhydroxyalkanoates
PHB	Poly-β-hydroxybutyrate
SBR	Sequencing Batch Reactor
TCA	Tricaboxylic Acid Cycle
VSS	Volatile Suspended Solids

Subscripts

N	Ammonia
PHB	Poly-β-hydroxybutyrate
S	Acetate
X	Active biomass

Metabolite accumulation rate vector

[1]. *AC* *Acetate*

[2]. *AcAcCoA* *Acetoacetyl coenzyme A*

[3]. *AcCoA* *Acetyl coenzyme A*

[4]. *α-KG* *A-Ketoglutarate*

[5]. *ATP* *Adenosine 5'-triphosphate*

[6]. *BIOMASS* *Non-PHB Biomass*

[7]. CO_2 *Carbon dioxide*

[8]. *E4P* *Erythrose-4-phosphate*

[9]. *F6P* *Fructose-6-phosphate*

[10]. *G3P* *3- Phosphoglycerate*

[11]. *GAP* *Glyceraldehyde-3-phosphate*

[12]. *G6P* *Glucose-6-phosphate*

[13]. *Glum* *Glutamine*

[14]. *Glut* *Glutamate*

[15]. *Glyox* *Glyoxylate*

[16]. *IsoCit* *Isocitrate*

[17]. *LAC* *Lactate*

[18]. *MAL* *Malate*

[19]. *NADH* *Nicotinamide adenine dinucleotide, reduced*

[20]. *NADPH* *Nicotinamide adenine dinucleotide phosphate, reduced*

[21]. *OAA* *Oxaloacetate*

[22]. *PEP* *Phosphoeolpyruvate*

[23]. *PHB* *Poly-β-hydroxybutyrate*

[24]. *PYR* *Pyruvate*

[25]. *R5P* *Ribose-5-phosphate*

[26]. *SUC* *Succinate*

[27]. *SucCoA* *Succinate coenzyme A*

Table of Contents

Agradecimentos III

Sumário IV

Summary V

Nomenclature VI

Table of Contents XI

List of Figures IX

List of Tables XVII

1.Introduction 1

1.1.Thesis bibliographic revision 1

1.1.1.History of Polymers 1

1.1.2.Conventional Plastics versus Biodegradable plastics 2

1.1.3.Biogical aspects of Polyhydroxyalkanoates (PHA) and Extracellular Substances (EPS) 2

1.1.4.Typical Process Behavior with Aerobic Dynamic Feeding 4

1.1.5.Applications of PHA 5

1.1.6.Recovery Process 6

1.1.7.Factors Affecting PHA Synthesis and its Composition 7

1.1.8.Process Economics 7

1.2.Thesis motivation 8

1.3.Dissertation objectives 9

1.4.Thesis outline 10

2.Metabolic Model 11

2.1.Metabolic Network 12

2.2.Network stoichiometry and energetic 16

2.3.Determination of the Metabolic Model Yields 16

2.4.Kinetics equations 19

2.4.1.Feast Phase 19

2.4.2.Famine Phase 20

3.Process Optimization: problem statement 23

3.1.Process Description 23

3.1.1.Reactor operational strategies and parameters 24

3.2.Macroscopic Bioreactor Model 27

3.3.Multi-objective Optimization Strategy 29

4.Results and Discussion 32

4.1.Comparison of segregated model and experimental data 32

4.2.Sensitivity analysis of culture dynamics 37

4.2.1.Specific growth rate on acetate and PHB 37

4.2.1.1.Keeping Sludge 39

4.2.1.2.Keeping Permeate 43

4.2.2.Specific PHB production rate 47

4.2.2.1.Keeping Sludge 48

4.2.2.2.Keeping Permeate 52

4.2.3.Maximum PHB storage capacity 56

4.2.3.1.Keeping Sludge 57

4.2.3.2.Keeping Permeate 61

4.2.4.P/O ratio 65

4.2.4.1.Keeping Sludge 66

4.2.4.2.Keeping Permeate 70

4.2.5.Specific EPS production rate 74

4.2.5.1.Keeping Sludge 75

4.2.5.2.Keeping Permeate 76

4.3.Multi-objective optimization 77

4.3.1.Keeping Sludge 78

4.3.2.Keeping Permeate 80

5.Conclusions 83

6.References and Notes 85

Annexes I

Annex A: Nomenclature I

Annex B: Metabolites Accumulation Rate Vector II

Table of Figures

[Figure 1.1]. Appearance of a real linear polymer chains, using an atomic force microscope……………………………………………………………………………1

[Figure 1.2]. Cargill's corn-based plastic decomposes in 45 days……………………………2

[Figure 1.3]. General scheme for the metabolic pathways of PHA synthesis from different carbon sources within a bacterial cell……………………………………………………4

[Figure 1.4]. Aerobic Dynamic Feeding…………………………………………………5

[Figure 2.1]. Pathways for extracellular polymeric substances (EPS) and poly-β-hydroxybutyrate (PHB) biosynthesis and their metabolic reactions…………………………11

[Figure 2.2]. Metabolic network for EPS and PHB production and degradation process……………………………………………………………………………13

[Figure3.1]. Geometrical Representation of the Goal Attainment Method……………………31

[Figure 4.1]. Comparison of segregated model and experimental data; (×) Active Biomass, (♦) Ammonia, (▲) Acetate and (■) PHB content. At $Time_{op}$= 8h……………………………………………………………………………33

[Figure 4.2]. Comparison of segregated model and experimental data; (×) Active Biomass, (♦) Ammonia, (▲) Acetate and (■) PHB content. At $Time_{op}$= 12h……………………………………………………………………………34

[Figure 4.3]. Comparison of segregated model and experimental data; (×) Active Biomass, (♦) Ammonia, (▲) Acetate and (■) PHB content. At $Time_{op}$= 24h……………………………………………………………………………35

[Figure 4.4]. Comparison of segregated model and experimental data; (×) Active Biomass, (♦) Ammonia, (▲) Acetate and (■) PHB content. At $Time_{op}$= 8h……………………………………………………………………………36

[Figure 4.5]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………39

[Figure 4.6]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………40

[Figure 4.7]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………41

[Figure 4.8]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………42

[Figure 4.9]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………43

[Figure 4.10]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation………………………………………………………………………………44

[Figure 4.11]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………45

[Figure 4.12]. Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation………………………………………………………………………………46

[Figure 4.13]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation………………………………………………………………………………48

[Figure 4.14]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………49

[Figure 4.15]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………50

[Figure 4.16]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………51

[Figure 4.17]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………52

[Figure 4.18]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………53

[Figure 4.19]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………54

[Figure 4.20]. Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………55

[Figure 4.21]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………57

[Figure 4.22]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………58

[Figure 4.23]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………59

[Figure 4.24]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………60

[Figure 4.25]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………61

[Figure 4.26]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………62

[Figure 4.27]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………63

[Figure 4.28]. Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………64

[Figure 4.29]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………66

[Figure 4.30]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………67

[Figure 4.31]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………68

[Figure 4.32]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………69

[Figure 4.33]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………70

[Figure 4.34]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………………71

[Figure 4.35]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………...72

[Figure 4.36]. Sensitivity analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation..……………………………………………………………………………...73

[Figure 4.37]. Sensitivity analysis of specific EPS production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation……………………………………………………………………………75

[Figure 4.38]. Sensitivity analysis of specific EPS production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation..……………………………………………………………………………...76

[Figure 4.39]. Multi-objective optimization results of ammonia feeding condition. SRT=36h and HRT=24h……………………………………………………………………………...79

[Figure 4.40]. Multi-objective optimization results of acetate and ammonia feeding conditions. SRT=48h and HRT=36h……………………………………………………………..81

[Figure 4.41]. Multi-objective optimization results of acetate and ammonia feeding conditions. SRT=48h and HRT=36h……………………………………………………………..82

List of Tables

[Table 2.1]. Theoretical yield coefficients and kinetic rate expressions…………………………….....22

[Table 3.1]. Kinetic parameters obtained by several authors for different feeding and operational conditions…………………………..……………………………………………………..26

[Table 4.1]. Multi-objective optimization for absolute feeding and Constant Acetate Mode….…………………………………………………………………………………...78

[Table 4.2]. Multi-objective optimization for absolute feeding and Acetate Mode varies in each cycle..………………………………………………..80

[Table 4.3]. Multi-objective optimization for absolute feeding and Acetate Mode varies in each cycle..…………………………………………………………………………….……81

1. Introduction

1.1. Thesis bibliographic revision

1.1.1. History of Polymers

Polymers are materials that can be organic or inorganic, natural or synthetic. They have a macromolecular structure, made of smaller molecules (monomers) that connect each other through covalent chemical bounds. They are amorphous with a disorderly arrangement, where the more complex the chain is, more amorphous (less crystalline), less rigid and less resistant the polymer is.

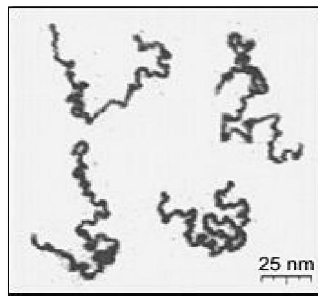


Figure 1.1- Appearance of a real linear polymer chains, using an atomic force microscope

Comparing polymers with metals, the former have lower heat expansion coefficient, good heat and electric resistance, and a low rigidity allowing easy processing. They have a differentiated tension/deformation behavior, where the properties and dimensions change with time: a slight variation in the temperature will lead to a change in the polymer strength and elasticity module.

The structural characteristics are defined by a proportional relation between the melting point, the thickness and resistance that rise with the polymerization degree, and the molecular structure complexity. In agreement with the melting characteristics, the polymers are divided in two groups: thermoplastics and thermo set. The main characteristic of the thermoplastics is the tightening, with the decrease of the temperature and the softening when the temperature is high (riverside process). They are in general, more resistant, less thick, and more ductile. The thermo set are thicker, less resistant and more fragile than thermoplastics.

Nowadays, there are various types of commercial polymers, made of different chemical compounds. Polypropylene, polyesters, polyurethanes, polystyrenes and polyethylene are the most used in the industry depending on the mechanical, physical and electric properties required. The polymers applications are manifold in different areas, like the Textile, Pharmaceutical, Automobile, Agrochemical, and Aerospatiale industry, in design, in decoration, in toys, etc.

1.1.2. Conventional Plastics versus Biodegradable plastics

Conventional plastics are petroleum derivates representing today a big environmental burden because they are not biodegradable thus persisting for a long period of time as contaminants in the environment. They are known to occupy a significant fraction of volume in landfills because of their low density (0.9 g/cm^3) and lack of degradability capacity. The development of new biodegradables plastics with similar properties of conventional plastics, which can substitute them in similar applications and with similar costs, is currently a big challenge for researchers and engineers.

Biodegradable plastics based on biopolymers can answer to some of the environmental problems, because they can be produced from renewable carbon sources, allowing for a sustainable and closed-cycle production process.

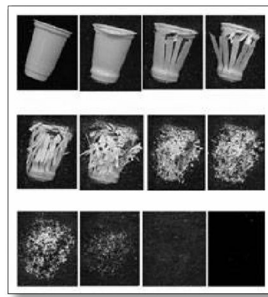


Figure 1.2- Cargill's corn-based plastic decomposes in 45 days

1.1.3. Biological aspects of Polyhydroxyalkanoates (PHA) and Extracellular Substances (EPS)

PHA and EPS are two types of polymers produced by microorganisms under dynamic feeding conditions, inside and outside the cells respectively. An Extracellular polymeric substance, or EPS, is a general designation of different classes of macromolecules such as polysaccharides, proteins, nucleic acids, lipids, and other polymeric compounds, which have been found to occur in the intercellular space of microbial aggregates [86]. They are a complex mixture of high molecular weight polymers (Mw>10,000), and are being currently commercially produced from marine brown algae and by microorganisms, found widely in the nature [78]. The production of EPS from microorganisms, via lysis and hydrolysis, allows them to live continuously at a high-cell density in stable mixed population communities, and is heavily dependent on several factors, such as bacterial type, cultivation time, substrate, and growth state. Its production is considered to confer a competitive advantage to the growth and survival of bacteria in hostile and stress environments [79].

Polyhydroxyalkanoates (PHA) are polyesters of hydroxyalkanoates acid (HAs), from the thermoplastics family. PHA have similar mechanical properties has the conventional plastics, in this

case the polypropylene, with the advantage that it can be produced from renewable carbon sources, such as, sugar and fatty acids. The molecular weight of PHA is in the range of 2×10^5 to 3×10^6, depending on the microorganisms and growth conditions, which are known to be more desirable for industrial applications. The biodegradability is one of the many interesting biological characteristics of PHA materials, in accordance to the different environments. The rate of biodegradability of PHA materials, in natural environments, depends of many factors, such as temperature, moisture level, pH nutrient supply and others related to the PHA physical characteristics, like the composition, the crystalline property, the additives and the surface area [52].

For PHA production, the most used bacteria are: *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinelandii*, *methylotrophus*, *pseudomonads* e *Escherichia coli recombinant.* Currently there are more than 150 different types of hydroxyalkanoate acids constituents of this polymer, where the most known is the Poly (β-hydroxybutyrate) (PHB). PHA production is based mainly on pure cultures, where they grow in a defined nutrient deficient synthetic media and single substrates, such as ammonia and acetate, which can store PHA up to 90% of dry cell weight. PHA production based on pure cultures is currently done by several companies, such as, Biocycle™, with *Burkholderia sacchari*, Biomer™, with *Alcaligenes latus*, Biopol™, with genetically modified chains, like *Escherichia coli,* and Nodax™ with *Wautersia eutropha*. One of the main problems in the production of PHA by pure cultures is the associated production costs, because of the operation control requirements and the auxiliary equipment for the sterilization [87]. The utilization of open mixed microbial cultures is a good way to reduce the high cost of PHA production. Selection of microorganisms occurs naturally on the basis of high capacity for PHA storage, in a sequencing batch reactor with optimized operating conditions. Therefore, there is no need for sterilization and sterile fermentation systems. The value in market increases, because it facilitates the use of complex substrates, such as volatile fatty acids (VFA), since the microbial population can adapt continuously to changes in substrate (industrial waste, agricultural waste), the reactor does not need sterilization and less sophisticated process control is required.

With the aim to decrease the cost of PHA production, it was possible to develop a production process based on mixed cultures, selected based on imposed conditions, where it has been demonstrated that the limitation of their primary metabolism leads to a high intracellular storage capacity.

In the industry the only PHA produced are polymers or copolymers of 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV), and HB and 3-hydroxyhexanoate (HHx). There have been significant advances in molecular biology, more precisely in the understanding of metabolism and genetics of bacteria with PHA synthesis capacity. Due to these studies, it was possible to clone more than 20 genes responsible for the PHA biosynthesis, allowing the construction of several recombinant strains, with the capacity to synthesize polyesters with different monomers units and/or to accumulate higher polymer quantity, e. g., with rapid growth, high cellular density, capacity to use different substrates of low cost and simpler polymer purification.

Through several studies, it has been concluded that the quantity of PHA accumulated and synthesized by several different microorganisms, in response to unbalanced growth and nutrients limitations, increases as the carbon to nitrogen ratio (C/N) increases, which makes it a favoring condition.

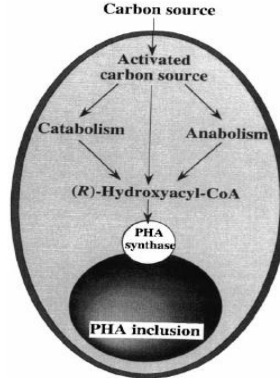


Figure 1.3- General scheme for the metabolic pathways of PHA synthesis from different carbon sources within a bacterial cell [52]

The PHA accumulation occurs when the bacteria is missing supplies necessary for the cell division. Afterwards, stored PHA will be used as energy and carbon source, due to its low solubility characteristic and high molecular weight, during the period of starvation, i.e. the famine phase.

1.1.4. Typical Process Behavior with Aerobic Dynamic Feeding

Through the Figure 1.4 it is possible to demonstrate the typical behavior of an aerobic dynamic feeding and the importance of substrate gradients for the development of filamentous structures in biological flocs of mixed microbial cultures for the production of PHB [21].

In the first part of the diagram, the Famine phase is represented, consisting of a long period of starvation without any external carbon source supply. The second part of the diagram represents the Feast phase, where there is an excess of external carbon substrate, leading to a simultaneously growth and PHA storage. These two phases are repeated in cycles: the feast-and-famine cycles. It is through the repletion of these cycles that the culture selection is induced towards organisms that are able to store high quantities of PHB.

The feast phase begins with carbon source feeding, while the famine phase starts after complete carbon source depletion with the accumulated PHB being metabolized for cell growth and maintenance [5].

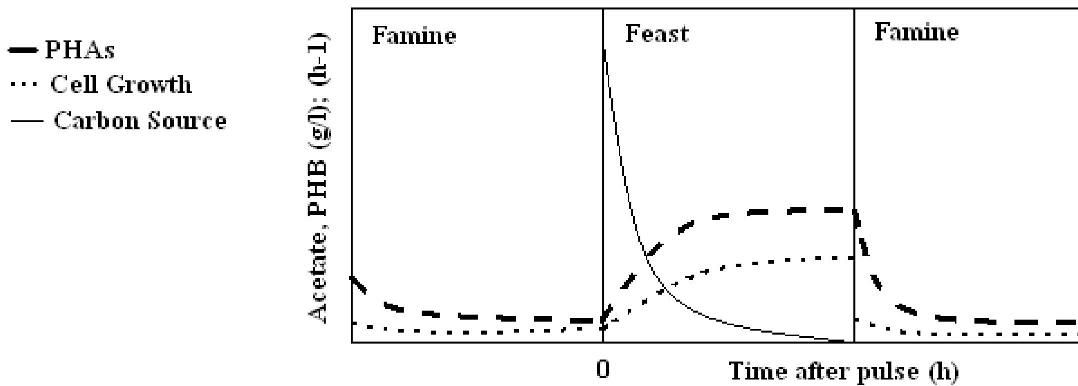


Figure 1.4- Aerobic Dynamic Feeding

1.1.5. Applications of PHA

PHB is applied in a wide range of applications including packaging materials, disposable items, starting material for the synthesis of chiral compound, surgical sutures applications without any toxicity and wound dressing [65]. Currently, the primary applications of PHB are the injection technology, extrusion technology, packaging material (cosmetics, food, pesticides, etc.) and medical applications.

The potential applications of biodegradable plastics produced from PHA are related with their different properties. Such properties lead to three different areas, such as:

I. Agricultural applications*:* PHA is a very promising material in this area, because it can be used as biodegradable carrier for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation, not needing a clean-up after its use.

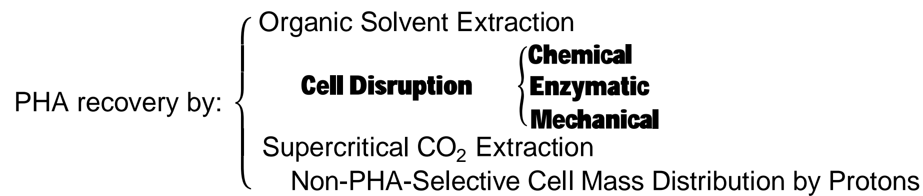
II. Packaging applications: Since the PHAs have similar properties to polystyrene and polyethylene, i.e., flexibility and tensile strength, mentioned before, they can be use in molding and extrusion processes. It is also possible to create new polymers by blending with synthetic polymer (heteropolymers). Some of the products based on PHA are the bags, and containers, packaging films, disposal items such as razors, diapers, utensils, and feminine products.

III. Pharmaceutical and medical applications: Some of the possible applications of PHAs in the pharmaceutical and medical area are: biodegradable carrier for long term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties. One of the major advantage of the use of PHA in medical implantation is that it will not be necessary its removal after surgery, due to its biodegradability.

1.1.6. Recovery Process

Other important aspect of the production of PHA is its recovery process. The recovery aspect contributes significantly to the overall cost of PHA production. Analysis of the entire process for the production and recovery of PHA will allow to design the most efficient method of production, and to evaluate the approximate price of PHA produced on a commercial scale [30] and several methods to isolate and purify PHA have been investigated.

The most used methods are:



In most cases, bacterial biomass is separated from substrate medium by filtration, flocculation or centrifugation. Then, the biomass is lyophilized (freeze dried), where compounds, such as acetone and alcohols, solubilize non-PHA cellular materials whereas PHA granules remain intact. On the other hands, chloroform and other chlorinated hydrocarbons solubilize all PHAs. Therefore, both types of solvents are usually applied during recovery process. Finally, precipitation or evaporation with acetone or alcohol can be used to separate the dissolved polymer from the solvent.

Organic solvent extraction is the most commonly used method for the recovery process. Due to the high viscosities of PHA solutions, a large amount of solvent is used, i.e., 20 parts of solvent to extract 1 part of polymers (Choi et al., 1999). It has been reported that this method uses mainly chloroform, 1,2-propylene carbonate, methylene chloride, 1,2-dichloroethane, tetrahydrofuran methyl cyanide, and ethyl cyanide. The other known method is the cell disruption. This method can be divided into chemical, enzymatic, and mechanical processes, with the purpose to dissolve or destroy the nonpolymeric cell materials (NPCMs).

A method, which was recently developed, is supercritical CO_2 extraction. This method is environmentally friendly and cost – effective, focusing in the removal of lipid impurities, giving a higher purity and maximum efficiency in polymer recovery.

The other environmentally friendly method is the non-PHA-selective cell mass distribution by protons, with PHA crystallization, which is more cost-effective comparing to conventional chemical treatment methods [4].

The choice of a PHA recovery method is based on the evaluation of green strategies involving low-cost processes, with the aim to reduce the polymer degradation, achieving high extraction yield and polymer purity [4].

1.1.7. Factors Affecting PHA Synthesis and Its Composition

The substrate concentration and the strain of microorganisms, according to the type of substrate employed and environmental growth conditions, are the main influences for the PHA synthesis and its composition, affecting the amount of polymer produced. The physical and thermal properties can be controlled by composition and concentration of feeding substrates.

Some studies have showed that factors, such as substrate concentration and temperature, seemed to influence EPS production, where high EPS synthesis was obtained when high substrate concentration was used, and that low temperatures slow down EPS production.

1.1.8. Process Economics

One of the main obstacles, for the replacement of the synthetic plastics by the biodegradable polymers is their production cost (€9/kg for PHA vs. €1/kg for synthetic plastics) [5].

The high PHA production cost is dependent on some factors, such as the downstream process used for polymer extraction efficiency of the polymer cells, the equipment required for aseptic operation, the substrate cost and the PHA yield on substrate. It is known that the operating cost can account for as much as 50% of the raw material, where the carbon source accounts for 70-80 % of the total cost of raw material, becoming very important to the overall economics of PHB production in large scale [65]. The total cost of raw material tends to decrease with the increase of the PHA yield.

An economic evaluation showed that it is possible to reduce the PHA to €4/kg, with the use of low-cost substrates and mixed cultures [5] and doing a complete analysis of the design of the whole processes.

The scale-up of the process leads to an increase of the fraction of the cost of raw materials and contributes negatively to the overall operating cost [30]. The production of PHB on a large scale is limited because of the relative expense of the substrate required, low polymer production, and the cost of maintaining an axenic culture, making it difficult to compete with conventional petroleum-based polymers in the commercial marketplace. In this context it is clear that the ideal biological system for PHA production would be a culture that can store high PHA concentrations while growing on an inexpensive growth substrate [3].

Another factor which influences the PHA production is the recovery process, because of the large amount of solvent used, i.e., 20 parts of solvent to extract 1 part of polymer, due to the high viscosities of PHB solutions [3]. Positive ways to a less expensive recovery are the following methods: Surfactant-hypochlorite digestion, dispersion of chloroform and hypochlorite [3].

1.2. Thesis motivation

The main objective of this thesis is to understand the selection process of populations in mixed microbial cultures, submitted to acetate feast and famine cycles, which are able to store intracellularly high quantities of PHB, through mathematical modeling and model-based process optimization.

Through the knowledge available, it is possible to improve the process control by postulating a segregated population mathematical model, which can then be used to understand the effect of the feast and famine regimen on culture enrichment.

This Thesis attempts to identify new process control strategies with leading to higher PHB productivity by mixed microbial cultures through a better control of culture selection. The new operating strategies suggested in this Thesis could have a positive impact in the process productivity as well as in the overall operating costs. The improvement in the control strategies will optimize substrate consumption, time of operation, and overall expenses.

The other relevant motivation of the present M.Sc. thesis is the fact that the target product is a biological environmental friendly biodegradable polymer, which will allow decreasing the dependency on synthetic petroleum-based non-biodegradable plastics.

1.3. Dissertation objectives

The main objective of this Master's dissertation is to develop a more efficient and competitive process for PHB production by mixed microbial cultures, focusing in the following aspects:

- Select a mixed microbial culture that has, simultaneously, a high specific PHB production and a high specific growth rate;
- To develop a segregated population model and to analyze the effect and efficiency of the feast and famine strategy on the culture enrichment, through the optimization of the acetate and ammonia feeding;
- Sensitivity analysis of the culture dynamics;
- Implementation of a computer program in *MATLAB - The Language of Technical Computing,* of a multi-objective optimization algorithm able to optimize the operation of a SBR for PHB production.

1.4. Thesis outline

This Master's dissertation is constituted by 6 chapters, which are briefly described in the following lines.

Chapter 1 is an introduction. It reviews of the properties and applications of polyhydroxyalkanoates and extracellular polymeric substances, from the agricultural applications to the pharmaceutical and medical applications. It reviews the PHA production process by mixed microbial cultures and the respective recovery process focusing on the factors affecting polyhydroxyalkanoates synthesis and its composition, and the process economics.

Chapter 2 is a detailed description of the metabolic model of PHB production by mixed microbial cultures. A metabolic model was developed, including acetate metabolism, the pathways for extracellular polymeric substances (EPS), biomass and poly-β-hydroxybutyrate (PHB) biosynthesis and their metabolic reactions. The metabolic network for EPS and PHB production and degradation process was studied. The network stoichiometry and energetics were analyzed allowing the determination of the metabolic model yields. Through the metabolic model yields, the kinetic equations, for feast and famine phase, were proposed, based on bibliographic references.

Chapter 3 is a detailed review of the process and its optimization criteria. Process monitoring is described regarding the following variables/techniques: pH, dissolved oxygen (DO), dissolved carbon dioxide, respirometry and tritimetic analysis, of sludge production, acetate consumption and intracellular PHB storage. A macroscopic description is defined as a simplification of a reality, where the average parameters can be used to describe and understand an entire system. The bioreactor dynamics were defined based on transient material balances of the intracellular PHB content, EPS content, acetate, ammonia and biomass. In multiple objective function optimization problems, the objective functions are the maximization of biomass mean $\mu_{PHB,max}$, $\mu_{S,max}$, PHB_{max}, $q_{PHB,max}$, and P/O ratio, and the minimization of biomass mean $q_{EPS,max}$, of the overall selection process, and of the difference between the initial and the final active biomass concentration.

Chapter 4 presents a sensitivity analysis of the culture dynamics. In this chapter the acetate and ammonia feeding will be varied several times and analyzed the effect of feast and famine on culture enrichment supported by the segregated population balance model. This manipulation of the acetate and ammonia feeding will lead to the selection of the populations with the highest PHB production capacity in a SBR operated under the feast and famine regimen.

In the last chapter, **chapter 5,** it is exposed in detail the main conclusion of the present M.Sc. dissertation.

2. Metabolic Model

Figure 2.1 shows the different pathways of EPS and PHB biosynthesis from Acetate in mixed microbial cultures.

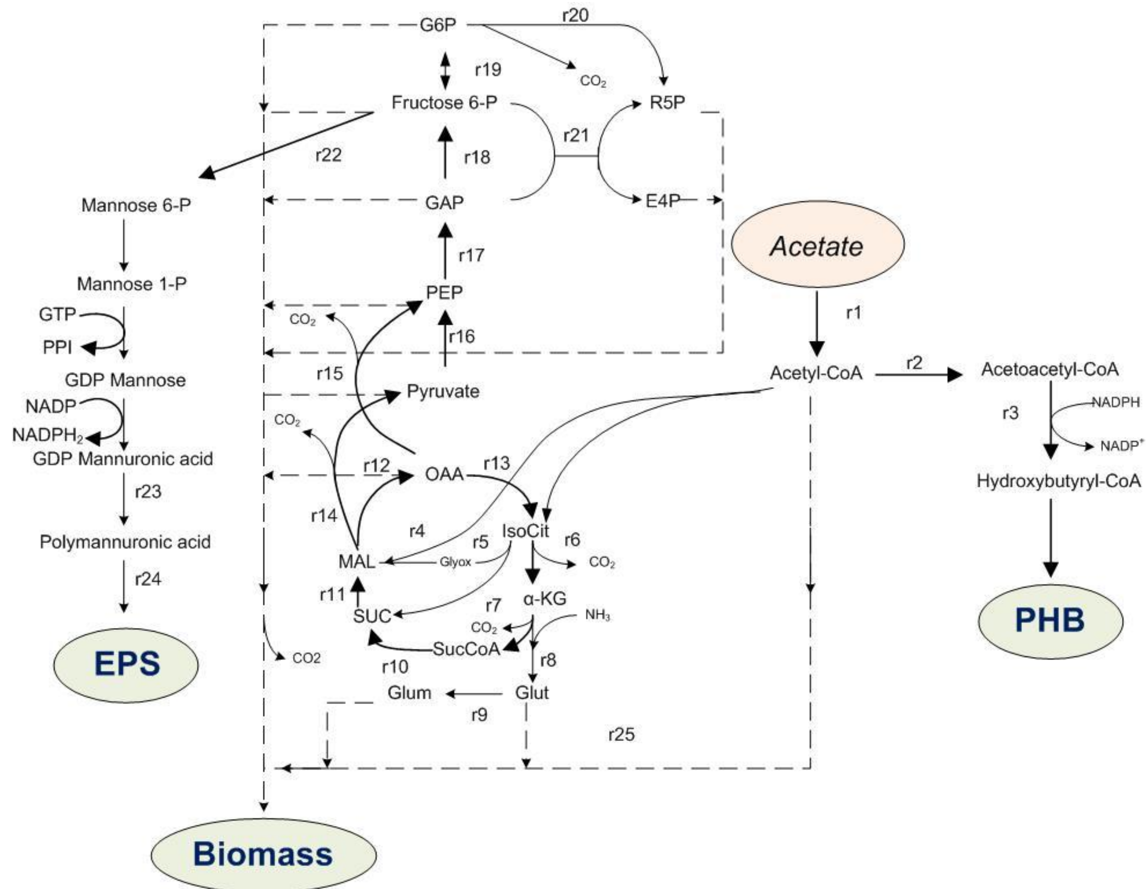


Figure 2.1- Pathways for extracellular polymeric substances (EPS) and poly-β-hydroxybutyrate (PHB) biosynthesis and their metabolic reactions [70], [76], [83], [84], [86].

The main biochemical reactions involved in the metabolism of acetate and formation of EPS and PHB are listed in the section Annex II.

The metabolic network adopted in this study can be briefly summarized as follows. The acetate is condensed to acetyl-CoA. Two moles of acetyl-CoA are converted into acetoacetyl-CoA, which will be then reduced to hydroxybutyrate monomer (HB), and then synthesized to one mole of PHB. PHB metabolism is a cyclic process, where acetyl-CoA is catalyzed by three sequential enzymatic reactions: β-ketothiolase converts two units of acetyl-CoA into acetoacetyl-CoA, acetoacetyl-CoA reductase reduces acetyl-CoA into hydroxybutyrate monomer, and PHB synthase condenses HB units into PHB.

In the synthesis of PHB, one ATP is consumed in activation of one free acid molecule to acetyl-CoA, which implies that to synthesize PHB it is required two units of ATP. The regeneration of ATP is made via TCA cycle, through the oxidation of acetyl-CoA.

Acetyl-CoA is a key metabolite for the energy and carbon skeleton, via the TCA cycle and glyoxylate shunt. For cells grown on acetate, it was assumed that the anaplerotic reaction occurs only through the glyoxylate bypass, which generates malate and thence oxaloacetate that combines with acetyl-CoA for their entering in the TCA cycle, by isocitrate lyase and malate synthetase. In the Gluconeogenesis phase, 2-Methylisocitrate splits into succinate and pyruvate, which is recycled back to oxaloacetate via malate, and the latter is either decarboxylated to acetyl-CoA or converted to P-enolpyruvate (PEP) for gluconeogenesis. This pathway connects to the beginning of the Fructose pathway, where PEP is metabolized into F6P, and transformed through several sequences into EPS [74].

Carbon source is known to be converted to EPS, metabolic waste in respiration as CO_2 or to intracellular storage polymer, PHB, where EPS secretion and PHB accumulation is directly associated to the microbial cyst process in starved cells, making it difficult to direct, preferentially, most of the carbon to the EPS synthesis pathway, rather than separate the synthesis of these two polymers completely [76].

2.1. Metabolic Network

The schematic metabolic model of Figure 2.2 depicts the aerobic metabolism of a bacterium, where the substrates acetate and ammonia are taken up for biomass growth, EPS production and PHA production and storage. The energy required for cell maintenance is produced within the cells, through Oxidative Phosphorylation (r_5).

The Figure 2.2 is representative of the metabolic model for EPS and PHB production and PHB degradation process. It describes the physical and biochemical reaction, the energy and material balances, metabolic fluxes and kinetic reactions.

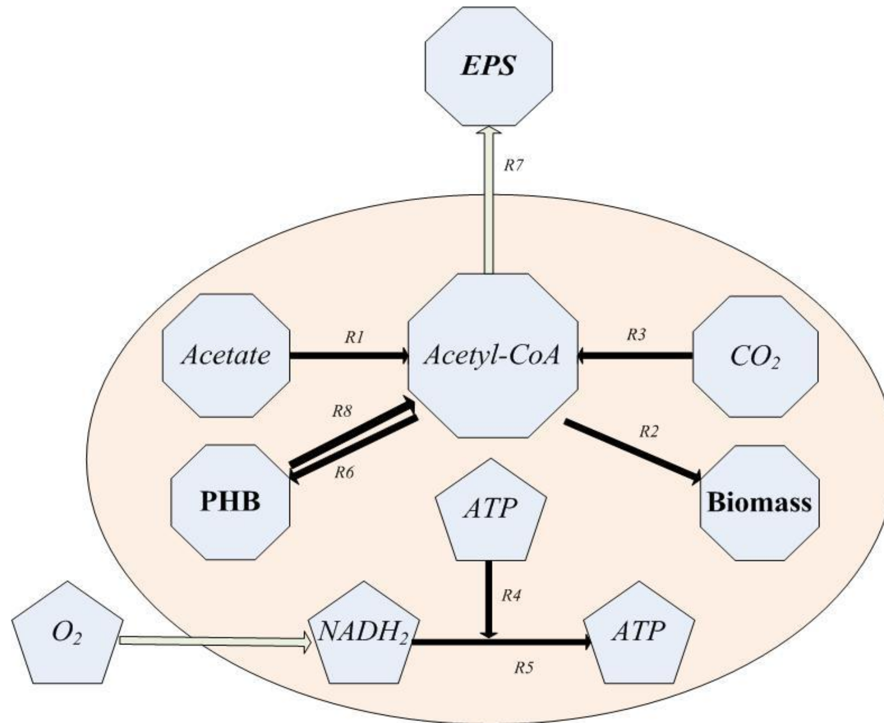


Figure 2.2- Metabolic network for EPS and PHB production and degradation process

The understanding of storage polymers, as an essential intermediate in the overall substrate removal in activated sludge systems was the major motivation behind the modeling of the metabolic network [40] for the selection of an enriched culture with high and stable capacity of PHA production [4].

The biochemical routes and the regulation of PHB synthesis and degradation have been studied extensively by many different authors, as well as the EPS production. It was shown that there are different metabolic pathways to generate these monomers [52], since the microorganisms are capable to produce PHB from different kind of substrates, mainly carbon sources available from inexpensive sources.

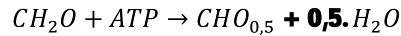
PHA production by MMC has been mainly investigated in SBR operated under feast and famine cycles. In terms of metabolic interpretation, the SBR operation can be classified in four phases with typical metabolic behavior [7]:

i. Cell Growth: promotion of the high cell density, with acetate and ammonia excess, *feast phase*;
ii. Carbon Limitation, *famine phase*;
iii. PHB Production: maximization of the specific PHB productivity, with an excess of acetate and without the introduction of ammonia in this phase.
iv. Biosynthesis of EPS: produced outside the cell, simultaneously with PHB, when they are under starvation conditions.

The biomass empirical formula adopted in this study is $CH_{1,4}N_{0,2}O_{0,4}$. Eight metabolic reactions were adopted to describe the overall metabolism. The most critical unknowns of the model are the specific ATP consumption due to maintenance processes (m_{ATP}) and the amount of ATP produced per mol of $NADH_2$ oxidized, that is, the efficiency of oxidative phosphorylation (δ). The details of this metabolic model are provided below.

R1: synthesis of Acetyl-coA from Acetate

Acetate is taken up by the cell, through means of active transport and activated to form acetyl-P_i. This is further converted into acetyl-coA [109].

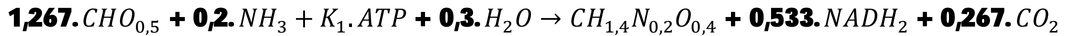


$$CH_2O + ATP \rightarrow CHO_{0,5} + 0{,}5.H_2O$$

Acetyl-CoA is the key compound for the PHB synthesis and degradation pathway, because it can be used for the production of the ATP and $NADH_2$ in the tricarboxylic acid (TCA) cycle, and to form biomass. When growth is unbalanced, due to high concentrations of $NADH_2$, acetyl-CoA is unable to enter the TCA cycle to provide energy for cells.

R2: Synthesis of Biomass from Acetyl-coA

There are two types of synthesis in the anabolism of biomass, which are the synthesis of biomass monomers, followed by polymerization of the biomass precursors into active biomass. It is necessary the amount of K_1 mol ATP to synthesize biomass precursors from acetyl-CoA, where 1 C-mol of biomass precursor is produced with acetate, as the carbon source of 0,267 mol CO_2.



$$1{,}267.CHO_{0,5} + 0{,}2.NH_3 + K_1.ATP + 0{,}3.H_2O \rightarrow CH_{1,4}N_{0,2}O_{0,4} + 0{,}533.NADH_2 + 0{,}267.CO_2$$

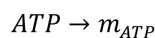
R3: Acetyl-CoA Catabolism

The production of acetyl-CoA is essentially used for energy, growth, and reducing equivalents. The uptake of substrate results in $NADH_2$ formation which is consumed by oxidative phosphorylation, leading to ATP formation [4].

$$CHO_{0,5} + 0{,}667.H_2O \rightarrow CO_2 + 2.NADH_2 + 0{,}5\ ATP$$

R4: Maintenance

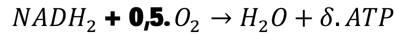
It is possible to determine the specific ATP consumption m_{ATP} from measured specific acetate consumption m_{HAc} due to maintenance processes.



$$ATP \rightarrow m_{ATP}$$

R5: Oxidative Phosphorylation

The uptake of substrate results in $NADH_2$ formation which is consumed by oxidative phosphorylation, leading to ATP production. The ATP is produced by oxidative phosphorylation by the usual pathway for NAD^+, from NADH2 serving as a NADH-overflow mechanism to control the redox state of heterotrophic cells during unbalanced growth conditions [11]. The efficiency of this process, i.e. the amount of ATP produced per molecule of NADH2 oxidized, is expressed by the P/O ratio, or δ.



$$NADH_2 + 0{,}5.O_2 \rightarrow H_2O + \delta.ATP$$

R6: Synthesis of the storage product PHB from Acetyl-coA

The polyhydroxybutyrate synthesis can be considered as a mechanism that regulates the availability of NADH and regulates the redox balance of the cell [13]. PHB acts as an electron and acetate-sink, producing a reduced storage polymer that can be used in times of starvation [11] and is metabolized when no external substrate is available [4]. No decarboxylation occurs during the production of PHB. For the formation of PHB from acetyl-CoA no additional ATP is needed, which results in

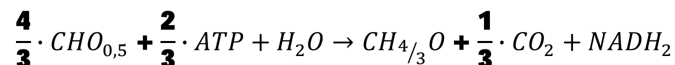
$$CHO_{0,5} + 0{,}25.NADH_2 \rightarrow CH_{15}O_{0,5}$$

R7: Synthesis of EPS from Acetyl-CoA

A small part of substrate is used to produce other extracellular components, such as, EPS, which are not usually taken into account [5] in PHA production models.

The synthesis of EPS from Acetyl-CoA occurs simultaneously with the synthesis of PHB. Alginate does not serve as an overflow metabolite, and rather provides protection against attack and adverse environmental conditions acting as a protective barrier against high metal toxicity, or gives the bacterium with a hydrophilic, negatively charged coating.

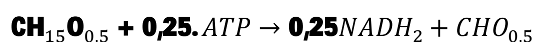
Acetyl groups are always associated with the mannuronic acid residues and they were suggested to protect certain residues from epimerization to guluronic acid. The epimerization provides the cell with a mean to control the composition and hence physical properties of the produced polymer [70].



$$\frac{4}{3} \cdot CHO_{0,5} + \frac{2}{3} \cdot ATP + H_2O \rightarrow CH_{4/3}O + \frac{1}{3} \cdot CO_2 + NADH_2$$

R8: Synthesis of Acetyl-CoA from PHB

Intracellularly accumulated PHB is used by microorganisms as carbon and energy source, in the absence of substrate. The storage polymer is hydrolyzed and converted into acetyl-coA [13].



$$CH_{15}O_{0,5} + 0{,}25.ATP \rightarrow 0{,}25 NADH_2 + CHO_{0,5}$$

One of the steps in the biochemical pathway of the PHB degradation must be the rate limiting one, because, in the famine period, the growth rate of the organisms depends on the PHB degradation rate, but is independent of the type of electron acceptor present in the system and independent of the SRT system [9].

2.2. Network stoichiometry and energetic

For PHB production and degradation process, the maintenance coefficients and theoretical yields are derived based on the mass balance to acetyl-CoA, $NADH_2$ and ATP, presented in the metabolic model. Has described in the model, four macroscopic yields ($Ys/_X$, $Ys/_{EPS}$, $Ys/_{PHB}$, and $Y_X/_{PHB}$) and two macroscopic maintenance coefficients (m_{HAc} and m_{PHB}) are reduced to two unknown parameters which are a microscopic yield, δ, and microscopic maintenance, m_{ATP}, coefficient, showing that there is an advantage in the reduction of the number of free parameters and creating a more consistent description [66]

The specific ATP consumption due to maintenance processes m_{ATP} can be calculated from the measured specific acetate consumption due to maintenance processes ms [13]. The parameter δ can be calculated from OUR (Oxygen uptake rate) measurements and from the stoichiometry of the metabolic reactions of acetate uptake and oxidative phosphorylation. The δ and m_{ATP} coefficients are strongly coupled and cannot easily be estimated independently.

2.3. Determination of the Metabolic Model Yields

One of the advantages of a metabolic model is that the yield and maintenance coefficients depend on the same metabolic parameters [10], and through this metabolic model, a detailed stoichiometry and an energetic analysis, it is possible to derive the expressions for the calculation of the maintenance and theoretical yields coefficients.

Based on this metabolic reactions, material balances of biomass precursors, $NADH_2$, ATP and Ac-CoA and overall degree of reduction balances formulated, it is possible to define $Y_{X/S}$, the yield of biomass on acetate, $Y_{EPS/S}$ the yield of EPS on acetate, $Y_{PHB/S}$ the yield of PHB on acetate, m_S the specific acetate consumption by maintenance processes, in the feast phase, $Y_{EPS/X}$ the yield of EPS on biomass, $Y_{PHB/X}$ the yield of PHB on biomass, m_{PHB} the specific PHB consumption by maintenance processes in the famine phase, δ the efficiency of oxidative phosphorylation and m_{ATP} the specific ATP consumption by maintenance processes [29].

A yield of a process is defined as the quantity of product formed per quantity of substrate utilized for this process [46]. It has been demonstrated that the yield coefficients of storage, direct growth on

substrate and growth on internal storage products respectively are linked to each other through metabolism of the substrate.

DEDUCTION OF YIELS AND MAINTENANCE COEFFICIENTS IN THE FEAST PHASE:

Ac-CoA: $\mathbf{0} = r_1 - \mathbf{1{,}267} \cdot r_2 - r_3 - r_6 - \frac{4}{3} \cdot r_7$ [2.1]

ATP: $\mathbf{0} = -r_1 - K_1 \cdot r_2 + \frac{1}{2} \cdot r_3 - m_{ATP} + \delta \cdot r_5 - \frac{2}{3} \cdot r_7$ [2.2]

NADH₂: $\mathbf{0} = \mathbf{0{,}533} \cdot r_2 + \mathbf{2} \cdot r_3 - r_5 - \frac{1}{4} \cdot r_6 + r_7$ [2.3]

These three equations are then manipulated, leading to one linear equation for the feast phase describing acetate uptake as a function of biomass growth, EPS and PHB production, and maintenance:

A balance of the degree of reduction yields:

$$r_1 = \frac{1{,}266 \cdot r_2 + 4 \cdot \delta \cdot r_2 + 2 \cdot K_1 \cdot r_2 + 4{,}5 \cdot \delta \cdot r_6 + r_6 + 2{,}664 \cdot r_7 + 3{,}33 \cdot \delta \cdot r_7 + 2 \cdot m_{ATP}}{4 \cdot \delta - 1} \quad [2.4]$$

$$r_1 = \left(\frac{1{,}267 + 4 \cdot \delta + 2 \cdot K_1}{4 \cdot \delta - 1}\right) \cdot r_2 + \left(\frac{4{,}5 \cdot \delta + 1}{4 \cdot \delta - 1}\right) \cdot r_6 + \left(\frac{3{,}33 \cdot \delta + 2{,}664}{4 \cdot \delta - 1}\right) \cdot r_7 + \frac{2 \cdot m_{ATP}}{4 \cdot \delta - 1} \quad [2.5]$$

From the reaction stoichiometry, equation **[2.5]**, it results that:

$$r_{HAc} = \frac{1}{Y_{X/HAc}} r_X + \frac{1}{Y_{PHB/HAc}} r_{PHB} + \frac{1}{Y_{EPS/HAc}} r_{EPS} + m_{HAc} \quad [2.6]$$

with $(-r_{HAc}) = r_1$, $r_X = r_2$, $r_{PHB} = r_6$. Based on the balance of the degree of reduction, it is possible to obtain the yield, equations **[2.7]**, **[2.9]**, and **[2.8]**, and maintenance, equation **[2.10]** expressions:

$$Y_{PHB/HAc} = \frac{4 \cdot \delta - 1}{4{,}5 \cdot \delta + 1} \quad [2.7]$$

$$Y_{EPS/HAc} = \frac{4 \cdot \delta - 1}{3{,}33 \cdot \delta + 2{,}664} \quad [2.8]$$

$$Y_{X/HAc} = \frac{4 \cdot \delta - 1}{4 \cdot \delta + 2 \cdot K_1 + 1{,}267} \quad [2.9]$$

$$m_{HAc} = \frac{2 \cdot m_{ATP}}{4 \cdot \delta - 1} \quad [2.10]$$

Linear equation for Acetate Uptake, Growth, and PHB Formation:

Substituting these yield coefficients, determined in the section 3.2, in the following equation **[2.11]**, gives:

$$r_1 = (-r_S) = \frac{1}{Y_{SX}^{max}} \cdot (r_2) + \frac{1}{Y_{SP}^{max}} \cdot (r_6) + m_S \quad [2.11]$$

$$(-q_{HAc})=\frac{1}{Y_{HAc.X}^{max}}\cdot\mu+\frac{1}{Y_{HAc.P}^{max}}\cdot q_{PHB}+m_{HAc} \quad [2.12]$$

Which is a linear equation, equation **[2.13]**, describing substrate uptake, growth, and PHB formation,

$$(-q_{HAc})=q_{HAc}^{X}+q_{HAc}^{PHB}+m_{HAc} \quad [2.13]$$

With q_{HAc}^{X} the flux of acetate for cell growth, q_{HAc}^{PHB} the flux of acetate for PHB formation and m_{HAc} the maintenance flux on acetate [7].

The specific acetate consumption rate could be obtained by dividing the amount of acetate consumed in the feast period by the activate biomass present in the reactor and the duration of the feast period [10].

DEDUCTION OF YIELDS AND MAINTENANCE COEFFICIENTS IN THE FAMINE PHASE:

From steady-state material balances it results

Ac-CoA: $\mathbf{0} = -\mathbf{1{,}267}\cdot r_2 - r_3 + r_8$ [2.14]

ATP: $\mathbf{0} = -K_1\cdot r_2 + \frac{1}{2}\cdot r_3 - m_{ATP} + \delta\cdot r_5 - \frac{1}{4}\cdot r_8$ [2.15]

$NADH_2$: $\mathbf{0} = \mathbf{0{,}533}\cdot r_2 + \mathbf{2}\cdot r_3 - r_5 + \frac{1}{4}\cdot r_7$ [2.16]

These three equations are combined and manipulated leading to one linear equation for the famine period. Furthermore the balance of the degree of reduction yields, equation **[2.17]**:

$$r_2 = \frac{4\cdot m_{ATP} - r_8 - 9\cdot\delta\cdot r_8}{8\cdot\delta + 4\cdot K_1 + 2{,}534} \quad [2.17]$$

$$r_2 = -\left(\frac{9\cdot\delta+1}{8\cdot\delta+4\cdot K_1+2{,}534}\right)\cdot r_8 + \frac{4\cdot m_{ATP}}{8\cdot\delta+4\cdot K_1+2{,}534} \quad [2.18]$$

From the reaction stoichiometry it is clear that:

$$r_X = -\frac{1}{Y_{PHB/X}}\cdot r_{PHB} + m_{PHB} \quad [2.19]$$

with $r_X=r_2$, $r_{PHB}=r_7$. Based on the balance of the degree of reduction, it is possible to obtain the yield, equation **[2.20]**, and maintenance, equation **[2.21]** expressions:

$$Y_{PHB/X} = \frac{8\cdot\delta+4\cdot K_1+2{,}534}{9\cdot\delta+1} \quad [2.20]$$

$$m_{PHB} = \frac{4\cdot m_{ATP}}{8\cdot\delta+4\cdot K_1+2{,}534} \quad [2.21]$$

Linear equation for growth with PHB as Substrate:

An equation for the specific PHB consumption rate can be defined as a function of the observed specific growth rate μ. For this relationship, it is assumed that there is no net production or consumption of biomass precursors, $NADH_2$, ATP, or acetyl-CoA:

$$r_7 = (-r_{PHB}) = \frac{1}{Y_{PX}^{max}} \cdot (r_2) + m_{PHB} \quad [2.22]$$

$$(-q_{PHB}) = \frac{1}{Y_{PX}^{max}} \cdot \mu + m_{PHB} \quad [2.23]$$

The total specific PHB uptake rate $(-q_{PHB})$ accounts for the cell growth term and the maintenance term [7].

The specific PHB production rate could be calculated by dividing the amount of PHB produced in the feast period by active biomass present in the reactor and the duration of the feast period [10].

2.4. Kinetic Equations

The modeling of the storage and growth stages corresponds to two distinct, but complementary, phases: feast and famine. The modeling approaches that have been employed under feast conditions were the traditional ASM and metabolic approaches [40]. Under these dynamic conditions, sludge submitted to consecutive periods of external substrate accessibility and unavailability generates a so-called unbalanced growth [10].

The expressions adopted in this thesis are presented in Table 2.1. They were developed according to the properties of the feast and famine phase. The expressions for specific growth rate, specific PHB production rate, specific EPS production rate and maintenance on acetate were established for the feast phase, and the specific growth rate and maintenance on PHB were defined for the famine phase. It was adopted in the model a new kinetic rate to account for the biomass decay/death process.

All kinetic parameters are presented in Table 2.1. Likewise, parameters for different populations were hypothesized to study the influence of the reactor operation parameters on culture selection.

2.4.1. Feast Phase

Cell Growth:

As stated before, the feast phase initiates with acetate feeding. Cell growth on acetate is expressed by a typical Monod equation with acetate and ammonia limitation.

$$\mu_{HAc} = \mu_{HAc,max} \cdot \frac{HAc}{HAc+K_{HAc}} \cdot \frac{N}{N+K_N} \quad [2.24]$$

Where K_{HAc} is equal to 0.0625 (C-mmol/l) and K_N is equal to 0.5776 (N-mmol/l), taken from Gujer et al., 1999 studies.

EPS Production:

The rate of EPS production is described in equation **[2.25]**, likewise, as a Monod like kinetic rate on acetate concentration:

$$q_{EPS} = q_{EPS,max} \cdot \frac{HAc}{HAc+K_{HAc}} \quad [2.25]$$

PHB Storage:

The rate of PHB synthesis is expressed by a Monod kinetic law on acetate but multiplied by a intracellular PHB concentration saturation term since it is well known that a given organism as an intrinsic maximum storage capacity.

$$q_{PHB} = q_{PHB,max} \cdot \frac{HAc}{HAc+K_{HAc}} \cdot \left[1 - \left(\frac{f_{PHB}}{f_{PHB,max}}\right)^{\alpha}\right] \quad [2.26]$$

where α has the value of 3.83, an empirical parameter that allows increasing the model accuracy in the range of high intracellular PHB contents [7]. Dias et al., 2005, reported a maximum intracellular PHB fraction of $f_{PHB,max}$=2.47 C-mmol/C-mmol in feast and famine experiments.

Maintenance on Acetate:

The acetate consumed for maintenance processes (m_{HAc}) is limited by the acetate concentration. This will prevent negative concentrations.

$$m_{HAc} = m_{HAc,max} \cdot \frac{HAc}{HAc+K_{HAc}} \quad [2.27]$$

According to Beun et al., 2002, the specific ATP consumption for maintenance processes is equal to 0.02 ATP-mol/(C-mol.h) at 20ºC.

2.4.2. Famine Phase

Cell Growth:

The famine phase begins after acetate depletion. Thereafter the stored PHB is metabolized for maintenance processes and active biomass formation. Is this phase the specific growth rate is assumed to be limited by intracellular PHB content ad by the external concentration of ammonia,

$$\mu_{PHB} = \mu_{PHB,max} \cdot \frac{f_{PHB}}{f_{PHB}+K_{PHB}} \cdot \frac{N}{N+K_N} \cdot \frac{K_{HAc}}{HAc+K_{HAc}} \qquad [2.28]$$

Where K_{PHB} is a kinetic parameter estimated individually that equals 0.001 C-mmol/l [7]. The last term expresses the inhibition of acetate on cell growth on PHB.

Maintenance on PHB:

The maintenance coefficient on PHB follows a 'n' order kinetic rate on PHB intracellular content [7],

$$m_{PHB} = k \cdot f_{PHB}^{n} \cdot \frac{K_{HAc}}{HAc+K_{HAc}} \qquad [2.29]$$

The last term expresses the inhibition of acetate on PHB degradation for maintenance.

Cell Decay:

In order to evaluate the need to describe the cell decay rate of PHB, in famine period as function of the PHB content, a general differential equation of chemical reaction was used, which is often applied to microbial mixed culture processes [10].

$$\frac{df_{PHB}}{d_t} = -k \cdot f_{PHB}^{n} \qquad [2.30]$$

In this study, the cell decay rate was defined as follows

$$k_d = 0{,}5 \times b_H \times \left(4 - 2 \cdot max\left(\frac{HAc}{HAc+K_{HAc}}, \frac{f_{PHB}}{f_{PHB,max}+K_{PHB}}\right)\right) \qquad [2.31]$$

The rationale behind this law is that the decay rate is b_h when the cells have access to a carbon source (either acetate in the medium or have stored PHB) and $2 \times b_h$ otherwise.

Cell Lysis:

The lysis parameter is considered null in this study. It represents the solubilisation of biomass, releasing secondary substrates into the liquid. The lysis mechanism is normally caused by enzymes, pH, toxicants or viruses:

$$k_{lysis} = 0 \qquad [2.32]$$

Table 2.1 compiles all the metabolic model equations for aerobic PHB storage, PHB degradation and EPS production.

		Acetate	NH3	X	EPS	PHB	Kinetics
Feast Phase	**Cell growth**	$Y_{X/HAc} = \frac{4\cdot\delta-1}{4\cdot\delta+2\cdot K_1+1{,}267}$	$-Y_{X/N}=5$	1	-	-	$\mu_{HAc}=\mu_{HAc,max}\cdot\frac{HAc}{HAc+K_S}\cdot\frac{N}{N+K_N}$
	PHB Storage	$Y_{PHB/HAc}=\frac{4\cdot\delta-1}{4{,}5\cdot\delta+1}$	-	-	-	1	$q_{PHB}=q_{PHB,max}\cdot\frac{HAc}{HAc+K_S}\cdot\left[1-\left(\frac{f_{PHB}}{f_{PHB,max}}\right)^{\alpha}\right]$
	EPS Production	$Y_{EPS/HAc}=\frac{4\cdot\delta-1}{3{,}33\cdot\delta+2{,}664}$	-	-	1	-	$q_{EPS}=q_{EPS,max}\cdot\frac{HAc}{HAc+K_{HAc}}$
	Maintenance on PHB	-1	-	-	-	-	$m_{HAc}=m_{HAc,max}\cdot\frac{HAc}{HAc+K_S}, m_{HAc,max}=\frac{2\cdot m_{ATP}}{4\cdot\delta-1}$
Famine Phase	**Cell growth**	-	$-Y_{X/N}=5$	1	-	$Y_{PHB/X}=\frac{8\cdot\delta+4\cdot K_1+2{,}534}{9\cdot\delta+0{,}25}$	$\mu_{PHB}=\mu_{PHB,max}\cdot\frac{f_{PHB}}{f_{PHB}+K_{PHB}}\cdot\frac{N}{N+K_N}\cdot\frac{K_S}{HAc+K_S}$
	Maintenance on PHB	-	-	-	-	-1	$m_{PHB}=k\cdot f_{PHB}^{n}\cdot\frac{K_S}{HAc+K_S}$
Cell Decay		-	-	-1		-	$k_d=0{,}5\times b_H\times\left(4-2\cdot max\left(\frac{S}{S+K_S},\frac{f_{PHB}}{f_{PHB,max}+K_{PHB}}\right)\right)$
Constant Parameters		K_1=1.7 mol-ATP/(C-mmol-X)[1], K_S=0.0625 (C-mmol/l)[2], K_N=0.5776(N-mmol/l)[2] α=3.83 (dimensionless)[3], m_{ATP}=0.02 mol-ATP/(C-mol.h)[3], K_{PHB}=0.001 (C-mmol/l)[4] k= 0.067 C-mmol/(C-mmol.h)[4], n=1.94 (dimensionless)[4], k_d=0.062 (C-mmol/(C-mmol.h^{-1})[2]					
Parameter values (-/+) for simulation		δ= 1.0/3.0 (mol-ATP/mol-$NADH_2$), μ_{HAc}= 0.30/1.0 (Cmmol/(Cmmol.h^{-1})), μ_{PHB}= 0.10/0.20 (C-mmol/(C-mmol.h^{-1}) q_{PHB}= 0.30/0.60 (C-mmol/(C-mmol.h^{-1})), $f_{PHB,max}$= 0.35/0.70 (C-mmol/C-mmol), q_{EPS}=0.30/0.60 (C-mmol/(C-mmol.h^{-1}))					
Parameter values for optimization (-/+)		δ= 1.0/3.0 (mol-ATP/mol-NADH2), μ_{HAc}= 0.15/0.30 (Cmmol/(Cmmol.h^{-1})), μ_{PHB}= 0.10/0.20 (C-mmol/(C-mmol.h^{-1}) q_{PHB}= 0.30/0.60 (C-mmol/(C-mmol.h^{-1})), $f_{PHB,max}$= 0.35/0.7.0 (C-mmol/C-mmol), q_{EPS}=0.30/0.60 (C-mmol/(C-mmol.h^{-1}))					

1Gommers et al., (1998), 2Gujer et al., (1999), 3Beun et al., (2002) and 4Dias et al., (2005)

Table 2.1- Theoretical yield coefficients and kinetic rate expressions

3. Process Optimization: Problem Statement

3.1. Process Description

The present M.Sc. thesis follows a previous study by Dias et al., 2006. In the latter work, activated sludge was submitted to aerobic dynamic substrate feeding, ADF, in a SBR, operated for a long time under ammonia limitation, with the purpose to select a culture with a high PHB storage capacity [5]. The volumetric PHB productivity reported was however still not competitive.

The SBR operation consists of a cyclic repetition of a sequence of steps like medium feeding, reaction, settling and medium withdrawing. The reaction phase can be further split into the feast and famine phases [29]. In this kind of cyclic process operation, biomass grows under transient conditions that are imposed by the reactor operating conditions. The reactor operating degrees of freedom are typically the cycle length, organic load rate, the feeding carbon-to-nitrogen (C/N) ratio, the hydraulic retention time (HRT) and the sludge retention time (SRT) thus this type of operation is highly flexible [29] [4].

The control of population dynamics in MMC towards the selection of organisms with the highest and stable PHB storage capacity by manipulating the SBR degrees of freedom is the central goal of the present work. It is well known that the SBR operation as a strong effect on PHB storage capacity [11]. On the other side, EPS synthesis is heavily dependent on cultivation time, substrate, and growth state (attached or suspended). It is known through several studies that the PHB and EPS production can both happen simultaneously and are both able to serve as energy and carbon sources when microorganisms are under starvation conditions [19].

The product, PHB, is formed only during the feast phase. The famine phase starts after complete acetate depletion, causing a cell physiological adaptation to carbon source limitation that will result subsequently in higher PHB formation rates [5]. The famine period should be long enough to guarantee high and stable PHB storage capacities in long term operation, but also as short as possible [7]. Ammonia is consumed while the substrate acetate is taken up, and is responsible for the increase of organism's selectivity with high cell growth rate favoring the microorganisms PHA storage capacity, when working in ADF [5].

In this chapter, the adopted model-based optimization methodology of the SBR operation is described. In the modeling of activated sludge processes, the Activated Sludge Model No. 3 (ASM3) is generally considered as a well established model of the aerobic and anoxic processes [14]. This model explicitly describes the conversion of storage polymers, which is responsible for the substrate removal by the heterotrophic organisms, in a dynamically fed microbial growth system independent of the operating conditions. Subsequently, this stored substrate is assimilated to biomass. This result implies that both biomass growth and EPS production were limited by the carbon source. The growing cells generate EPS in constant proportion to their growth [19]. In the present work we propose a model based on the ASM3 model and the previous model by Dias et al. (2006).

3.1.1. Reactor operational strategies and parameters

The main operation parameters are substrate concentration, carbon to nitrogen ratio (C/N), organic loading rate (OLR), sludge retention time (SRT), pH, oxygen concentration, and temperature. The dynamic feeding of the substrate is monitored based on the oxygen concentration, on-line, e. g., the acetate pulses were implemented with a DO-based feed controller, where its signal was used to detect the DO peaks associated with acetate depletion [5]. This kind of strategy prevented substrate limitation or inhibition. With the feeding of ammonia in the medium, cell growth and PHB formation occurs simultaneously. In *Serafim et al* [5], when 180 C-mmol/l of acetate was supplied in three pulses, a very high percentage of PHB was achieved, 78.5% of cell dry weight, which is similar to the commercial value for PHB pure producer strains. In some cases, 66% to almost 100% of the acetate consumed in steady state systems growing under non-growth-limiting conditions is used for PHB synthesis, and the percentage increases with increasing SRT [10]. In Dias et al., 2005 [7], the intracellular PHB inhibits its own rate of formation, it may not be advantageous to operate close to the maximum intracellular PHB contents $f_{PHB,max}$=2.47 C-mol PHB/C-mol X. The parameters which are known to affect the selection process are described bellow in more detail:

- The length of the total cycle varies from 2 – 12 h, where the substrate feeding period is always shorter [5].
- The sludge retention time, SRT, varies between 2-24 h and is independent of the hydraulic retention time, even though it is an important control parameter and exerting a major influence on the contact time among different reactants and the hydraulic conditions [22]. The selection of the SRT has a huge impact on the PHB content. Studies have shown that low SRT values leads to higher cell growth rate, and less PHB storage. At low SRTs, more biomass is wasted during a cycle resulting in a higher initial substrate: microorganism ratio [11].
- HRT regulates the hydraulic selection pressure to preferentially choose microbial cells that are more hydrophobic and that have better polysaccharides production. Such microbial survival strategies enable the cells to aggregate into granules and avoid hydraulic whashout [22]. The value of SRT varies independently of the HRT, in order to prevent a limitation of medium nutrients at some point of selection In *Serafim et al., 2004* [5], the total cycle had duration of 12 h and the hydraulic retention time (HRT) was 1 day.
- Ammonia feeding is an important factor in the Feast phase for the maximization of the volumetric productivity, because a high concentration of ammonia favors cell growth at the expense of low specific PHB formation rate, while inversely, low concentrations favors the specific PHB formation rate to the detriment of the specific growth rate [5].
- The carbon to nitrogen ratio (C/N) is an important parameter, because the increase of C/N leads to an increase of PHB content and of the PHB on acetate yield. In van Aalst-van Leeuwen et al., 1997 at μ=0.05 h^{-1} and μ=0.10 h^{-1}, initial rates of consumed acetate, is converted to PHB 25% - 30% [13].
- The organic loading rate (OLR) is related indirectly to the famine phase, when the OLR increases, the famine phase length tends to decrease, which favors the microorganisms why high

PHB storage capacity. A high OLR is desirable in biological wastewater treatment of high-strength wastewaters using compact reactors with small footprints [23].

- A comprehensive simulation study showed that oxygen supply rates might favor cell growth at the expense of lower PHB formation rates [4].

- The pH of the operating reactor increases and then stabilizes in the feast phase, between pH 7.5 – 10, because a low pH inhibits the bacteria, diminishing the PHB content. In this case, it is more advantageous to have an uncontrolled pH system, making a less complex and more cost-effective operation. In some cases, the pH increases considerably at the beginning of the cycle, usually between 8.0 – 8.5, rising to 9.4 -10.0 at the end of the feast phase and then slightly decreases at the beginning of the famine phase [5].

- Studies have demonstrated that high oxygen transfer rate results in a surplus of ATP, leading to high cell growth rates, decreasing the PHB yield. The author Third et al., 2003, showed that increased dissolved oxygen concentrations favored the prevalence of ATP and NAD+, whereas low DO concentrations favored the inverse, during the feast phase. The DO was maintained at around 80% of the saturation value, with an air flow rate of 1.0 vvm. In the studies by Serafim et al., 2004, the DO decreases immediately after substrate addition, remaining almost constant during the feast period and rising again after the carbon source exhaustion, leading to the conclusion that the amount of substrate consumed during the feast period was used for PHB storage, cell growth and maintenance processes (oxygen respiration). The reduction of the sludge PHB content was caused by the low DO concentration inside the reactor, which reached values below 10 % of the oxygen saturation during the substrate consumption, resulting in oxygen limitation inside the sludge clusters, and when the oxygen flow rate was changed from 0.4 vvm to 1.0 vvm, the amount of PHB content stored increased (36.9 ± 6.2 %). In another case, increasing dissolved oxygen concentration to ≥ 2.5 mg $O_2.l^{-1}$ in a filamentous sludge led to a rapid improvement of the settleability [21].

- The increase o temperature causes a decrease in the PHB yield on acetate. In this study, the temperature was kept at 22 ºC. Timers controlled the air pump, the stirring, at $\approx \mathbf{250}\, rpm$ and the pumps for medium feed/removal.

Table 3.1 compile the kinetic parameters obtained under varying hydraulic retention time (HRT), and sludge retention time (SRT), SBR cycle length, acetate and ammonia concentrations in the feeding conditions reported in several studies.

HRT (h)	SRT (h)	Cycle length (h)	[HAc]	C/N ratio	q_S	q_{PHB}	PHA content (%)	μ (h^{-1})	References
8	240	4	12.5	6.3	0.39-0.56	0.16-0.28	3.7-4.6	0.05-0.22	Martins et al. (2003)[1]
24-144	24-144	0.75-6	1.1-8.5	-	0.32-0.38	0.06-0.77	-	0.03-0.29	Dionisi et al. (2001)[2]
-	264	6.7	12.2	5.2	0.05-0.59	0.03-0.28	38	0.01-0.16	Third et al. (2004)[1]
8	91-475	4	12.5	6.3	0.37-0.64	0.23-0.27	9.1	0.004-0.065	Beun et al. (2000)[1]
-	96	4	12-96	4.8-19.2	0.85-0.97	0.54-0.66	13.8-44	0.04-0.11	Beun et al. (2002)[1]
1	10	12	15-180	11-258	0.63-1.55	0.15-0.82	65.0	0.01-0.33	Serafim et al. (2004)[1]
Batch			0.077	-		0.023	26.5	0.23	Carucci et al. (2001)[2]
Batch			0.33	20.6	0.94	0.46	-	0.28	Dionisi et al. (2004)[2]
Batch			0.11	12.2	0.027	0.012	16.6	0.004	Dionisi et al. (2002)[2]

[1][HAc] in (C-mmol/l), C/N ratio in (C-mol/N-mol) and q_S, q_{PHB} and μ in [C-mol/(C-mol.h)]

[2][HAc] in (g-COD/l), C/N ratio in (g-COD/g-N), q_S and q_{PHB} and μ in [g-COD/(g-COD.h)]

Table 3.1- Kinetic parameters obtained by several authors for different feeding and operational conditions

3.2. Macroscopic bioreactor model

A macroscopic bioreactor model establishes the link between macroscopic reactor degrees of freedom, which for an SBR are the parameters described in the previous section, and the biological system, which in our case is expressed by the metabolic model of section 2. Since the goal of the model is to understand the impact of macroscopic control on population dynamics, a population segregated model was formulated that considers NPOP populations of different organisms with biomass concentration X_i and intracellular PHB content $f_{PHB,i}$. Each population is thought to mimic a particular phenotype and thus it is characterized by a differentiated set of intrinsic stoichiometric and kinetic parameters.

Bioreactor dynamic operation was formulated based on transient material balances of, biomass of each population, X_i, the population intracellular PHB content, $f_{PHB,i}$, extracellular EPS concentration, *EPS*, extracellular acetate concentration, *HAc* and extracellular ammonia concentration, *N*. The material balance equations assume batch operation, perfect mixing and negligible formation of flocs. These equations describe a dynamical relationship between the most important operating parameters with process performance are thus essential to quantitatively study bioreactor optimization and control. The material balance equations were integrated using a 4th/5th order Runge-Kutta solver (MATLAB's *ode45* function) [4].

According to the conditions postulated above, the material balances of ammonia and acetate can be described as shown below

$$\frac{dHAc}{dt} = -\sum_{i=1}^{NPOP}\left(\frac{\mu_{HAc,i}}{Y^i_{X/HAc}} + \frac{q_{PHB,i}}{Y^i_{PHB/HAc}} + \frac{q_{EPS,i}}{Y^i_{EPS/HAc}} + m_{HAc,i}\right)\cdot X_i \qquad [3.1]$$

$$\frac{dN}{dt} = -\frac{1}{Y_{X/N}}\cdot\sum_{i=1}^{NPOP}\left(\mu_{HAc,i} + \mu_{PHB,i}\right)\cdot X_i \qquad [3.2]$$

The extracellular polymeric substances formed outside the cells, as described in Figure 6, can be described by the following material balance equation:

$$\frac{dEPS}{dt} = \left(q_{EPS,i} + \frac{q_{EPS,i}}{Y^i_{EPS/HAc}}\right)\cdot X_i \qquad [3.3]$$

where $\frac{q_{EPS,i}}{Y^i_{EPS/HAc}} = q_{HAc/EPS}$ is the flux of acetate for EPS production. The material balance of extracellular polymeric substances enables study of the effect of the limiting amounts of acetate in the bioreactor.

The PHB is an intracellular compound. The intracellular PHB content is defined by the ratio of PHB per active biomass ($f_{PHB} = \frac{PHB}{X}$). The material balances of intracellular PHB content is defined for each population is given by:

$$\frac{df_{PHB,i}\cdot X_i}{dt} = \left(q_{PHB,i} + \frac{\mu_{PHB,i}}{Y^i_{X_i/PHB}} - m_{PHB} \right) \cdot X_i \qquad [3.4]$$

By decomposing the derivative the following more convenient form is obtained [7]:

$$\frac{df_{PHB,i}}{dt} = \left(q_{PHB,i} + \frac{\mu_{PHB,i}}{Y^i_{X_i/PHB}} - m_{PHB} \right) - \left(\mu_{Ac,i} + \mu_{PHB,i}\right) \cdot f_{PHB,i} \qquad [3.5]$$

i= 1, …, NPOP

The material balance of biomass for different populations with different biomass concentrations is as follows:

$$\frac{dX_i}{dt} = \left(\mu_{Ac,i} + \mu_{PHB,i} - k_{d,i}\right) \cdot X_i,\ i = 1, \dots, NPOP \qquad [3.6]$$

The SRT theoretically determines mean microbial life-time. In SBR operation the SRT determines the amount of biomass that is removed from the reactor at the end of each cycle. The initial biomass in cycle n+1, $X(0)_{n+1}$, is calculated based on the final biomass concentration of cycle n, $X(Tcycle)_n$, as follows:

$$X(0)_{n+1} = X(T_{cycle})_n \cdot \left(1 - \frac{T_{cycle}}{SRT}\right) \qquad [3.7]$$

In the *(n+1)* cycle, the initial acetate, $S(0)_{n+1}$ and initial ammonia concentrations, $N(0)_{n+1}$, are determined by the respective concentrations at the end of cycle n ($S(T_{cycle})_n$ and $N(T_{cycle})_n$), by the dilution due to replenishment with fresh medium at the end of each cycle (the hydraulic retention time (*HRT*) imposes that a fraction of the medium is replaced with fresh medium at the end of each cycle *n*) and by the amount of acetate and ammonia fed at the beginning of cycle n+1 (ΔS_{n+1} and ΔN_{n+1})

$$\begin{cases} S(0)_{n+1} = S(T_{cycle})_{n,f} \cdot \left(1 - \frac{T_{cycle}}{HRT}\right) + \Delta S_{n+1} \\ N(0)_{n+1} = N(T_{cycle})_{n,f} \cdot \left(1 - \frac{T_{cycle}}{HRT}\right) + \Delta N_{n+1} \end{cases}, \qquad [3.8]$$

3.3. Multi-objective Optimization Strategy

Multiple objective optimization strategy is a method used in different areas, such as economical, technical, environmental, or, in this case, biotechnology, addressing multiple optimization goals, e. g., it is use when there is more than one objective function.

In multi-objective function optimization problems, there are three possible situations: Minimize all the objective functions, maximize all the objective functions and minimize some and maximize others, which is our case. In this study the objectives function are the following:

i. Maximization of population mean specific growth rate on PHB, $\mu_{PHB,max}$;

ii. Maximization of population mean specific growth rate on acetate, $\mu_{S,max}$;

iii. Maximization of population mean maximum intracellular PHB content, PHB_{max};

iv. Maximization of population mean P/O ratio;

v. Minimization of population mean maximum EPS synthesis rate, $q_{EPS,max}$;

vi. Maximization of population mean maximum PHB synthesis rate, $q_{PHB,max}$;

vii. Minimization of the overall selection process;

viii. Minimization of the difference between the initial and the final active biomass concentration.

The mathematical representation of the multiple objective function optimization is as follows:

$$\vec{f}(\vec{x}) = \underset{x \in \Omega}{opt}\{\vec{f}(\vec{x})\} \qquad [3.9]$$

$$\vec{f}: \Omega \rightarrow \mathbb{R}^k \qquad [3.10]$$

$$\Omega = \{x \in \mathbb{R}^n | g(x) \geq 0, h(x) = 0\} \qquad [3.11]$$

$$□□□□□□□□ \quad □(□) = \{□_1(□) \dots □_□(□) \dots □_□(□)\} \qquad [3.12]$$

$$□.□. \quad □ \in \Omega \qquad [3.13]$$

$$□ = \left\{ □ \left| \begin{array}{c} □_□(□) \leq 0 \; □ = 1, \dots, □_1 \\ h_□(□) = 0 \; □ = 1, \dots, □_2 \\ □ = [□_1 \dots □_□]^□ \end{array} \right. \right\} \qquad [3.14]$$

Where:

T – Indicates the transposition of the column vector to the row vector;

x - Solution

$\square_1(\square)$ - Nonlinear objective function

$\square_\square(\square), \square_1$ - Nonlinear inequality and equality constraint functions

$h_\square(\square), \square_2$ – Nonlinear equality constraint functions

Multiple objective function optimization reaches to the components of a vector-valued cost function. Unlike single objective optimization, the solution to this problem is not a single point, but a group of points. Each point in this surface is optimal in the sense that no improvement can be achieved in one cost vector component that does not lead to degradation in at least one of the remaining components [85].

This kind of strategy has conflicts with the final solution, because it gives more than one response, according to the objective functions processed, on the other hand, it can maximize/minimize more than one solution, simultaneously.

Due to the simultaneously maximization/minimization, the *p*-Norm Methods are implemented, which has the purpose of searching the best compromise solution- *Goal Attainment Method*.

The Goal Attainment Method involves a set design goal, the ideal point, $\square^* = [\square_1^*, \square_2^*, \dots, \square_\square^*]$, being associated with a set of objectives functions, $\square(\square) = [\square_1(\square), \square_2(\square), \dots, \square_\square(\square)]$, where x^t is the efficient solution:

$$\square\square\square\square\square\square\square\square\ \square, \quad \square \in \mathbb{R}, \square \in \square \qquad [3.15]$$

$$\square_\square(\square) - \square_\square \cdot \square \leq \square_\square^*, \ \square = 1,2,\dots,\square, \qquad [3.16]$$

where w_i is the vector of weighting coefficients$[\square = \{\square_1, \square_2, \dots, \square_\square\}]$, and it is expressed as a standard optimization problem, and λ is an auxiliary variable. $\square_\square(\square) + \square_\square \cdot \square$ is the desired solution vector, and by fixing the vectors and weights, e. g., the goal values, it is possible to obtain the direction. When $\square_\square = \square_\square(\square)$, every objective function has the same relevance to reach the solution. Until the desired solution vector hits the lower boundary of the objective space, it is necessary to minimize the auxiliary variable.

This method can be represented, for example, geometrically in the two dimensional figure:

$$\underset{\gamma,\, x \in \Omega}{\text{minimize}}\ \gamma \quad \text{subject to} \quad \begin{aligned} F_1(x) - w_1\gamma &\le F_1^* \\ F_2(x) - w_2\gamma &\le F_2^* \end{aligned}$$

Figure 3.1- Geometrical Representation of the Goal Attainment Method

Source: http://www.mathworks.com

The specification of the goals, and , define the goal point, which is in this case P. *λ* is varied, during all the optimization, changing the size of the feasible region. The constraint boundaries converge to the unique solution point F_{1s}, F_{2s}.

Constraints were added to the optimization, to prevent biomass washout or overproduction and the number of cycles was chosen based on the final results of the optimizations. The decision variables are the amount of acetate and ammonia at the beginning of each cycle.

4. Results and Discussion

4.1. Comparison of segregated model and experimental data

The experimental data of a culture submitted for more than two years to feast and famine conditions [5] was used for the validation of the segregated population model.

The setup of model was made with NPOP= 32 populations, where one of two different levels (either higher (+) or lower (-)) for P/O ratio, specific PHB production rate, maximum PHB storage capacity, specific growth rates on acetate and PHB was chosen for each population. A number of populations were defined, $32= 2^5$, so it could be possible to cover all possible combinations between these 5 parameters. The kinetic parameters were previous discussed in section 2 and listed in Table 2.1 – model fitting parameters.

The experimental data was collected with a culture that was submitted to dynamic feeding, with feast and famine conditions, for more than two years in a sequencing batch reactor. In a previous study by Dias et al., 2005 [7], a mathematical model of PHB production by mixed cultures has been developed using this data, which was the basis for the definition and calibration of the population segregated model used in the present work..

The SBR was inoculated with biomass taken from SBRs enriched in PHA-accumulating bacteria. The SBR cycle had varying length depending on the feeding conditions. Acetate (HAc) and ammonia (N) were fed at the beginning of the each cycle through 1 to 4 pulses to control the duration of the feast phase while avoiding possible inhibition of acetate or ammonia. The data shows the typical behavior: during a period of excess of external substrate, carbon uptake is driven to cell growth and PHB storage and, after substrate exhaustion, the stored polymer is used as carbon and energy sources. A longer feast phase with higher acetate feeding leads to an increase of the polymer content, while the excess of ammonia increases the selective pressure of organisms with a high cell growth rate, and, when combined with the feast and famine regimen, it also favors the organisms with a high PHA storage capacity [4].

Acetate concentration decreases linearly while PHB content increases linearly, in the feast phase. In the famine phase it is possible to observe the PHB degradation.

Figure 4.1 – 4.4 compare segregated model simulations with the experimental data. These results demonstrate that there is an agreement between the selected range of parameters and the real process. It should be noted that with the experimental data available, it is only possible to study the production of polyhydroxybutyrate but not the production of extracellular polymeric substances because there are no experimental data about the mixed microbial cultures cultivation for production of PHB and EPS simultaneously.

Comparison of Segregated Model and Experimental Data

Feeding: **NH_3=0.7 N-mmol/l and HAc=60.0 C-mmol/l**

$Time_{op}$= 8h

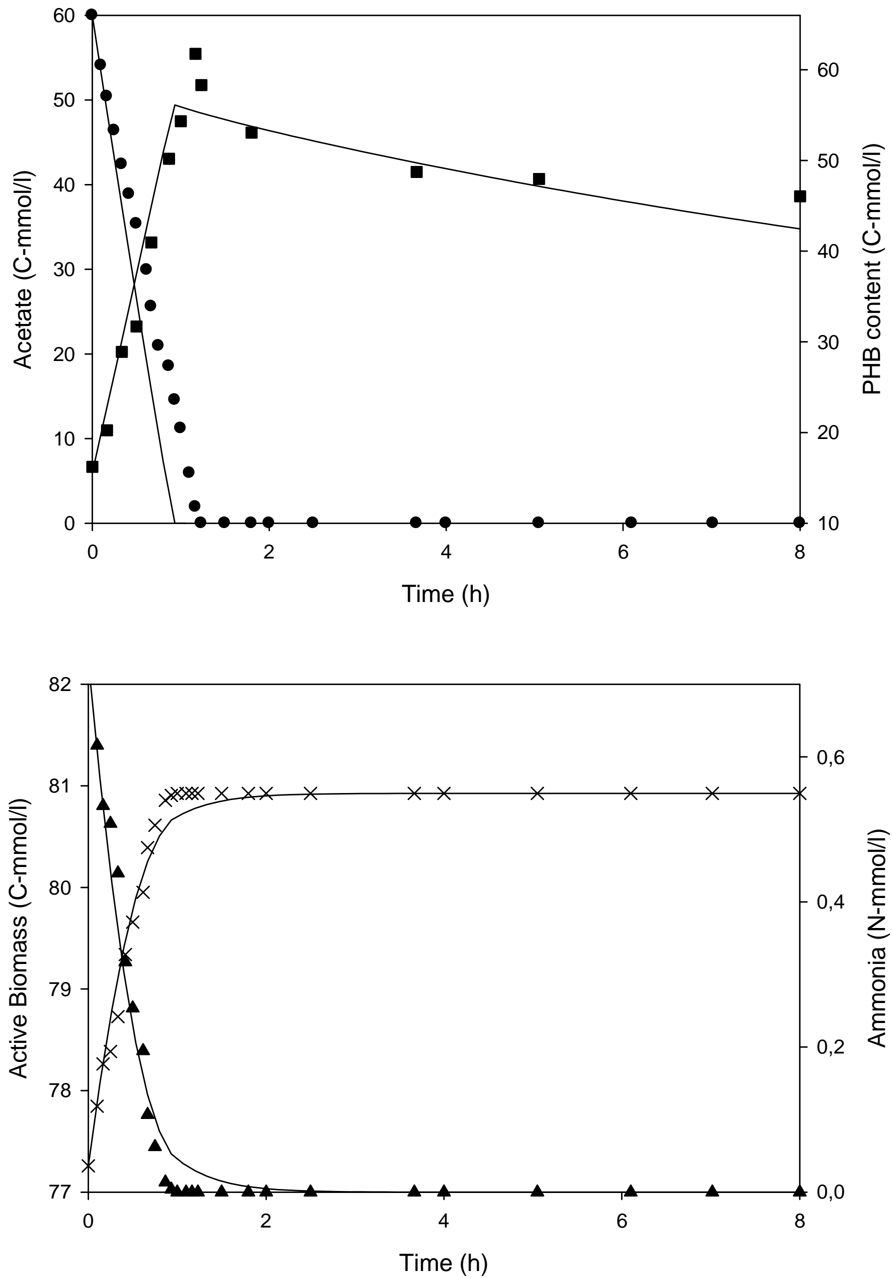


Figure 4.1- Comparison of segregated model and experimental data; (×) Active Biomass, (●) Acetate, (▲)Ammonia and (■) PHB content. At $Time_{op}$= 8h

Comparison of Segregated Model and Experimental Data

Feeding: **NH_3=0.7 N-mmol/l and HAc=30.0 C-mmol/l**

$Time_{op}$= 12h

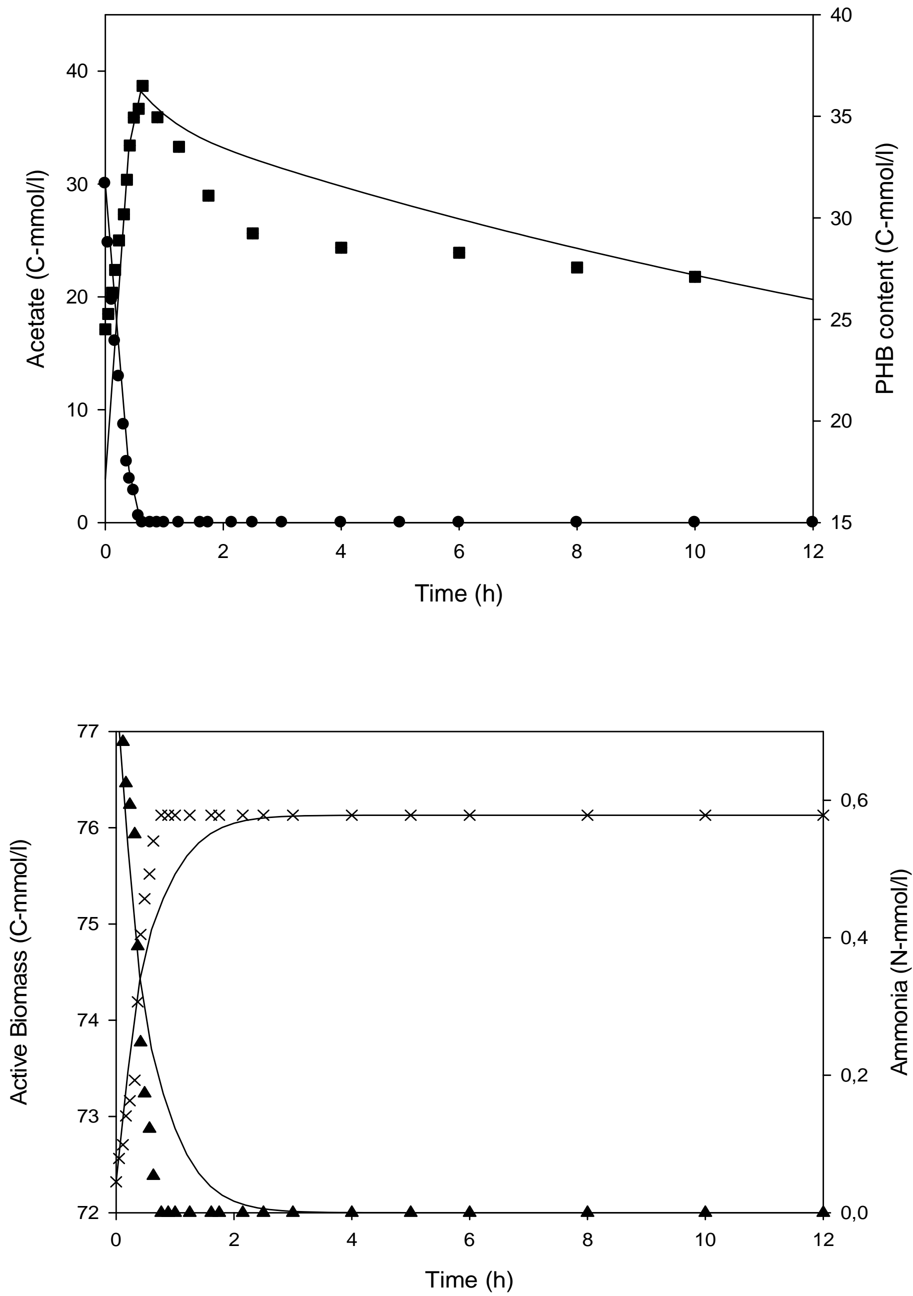
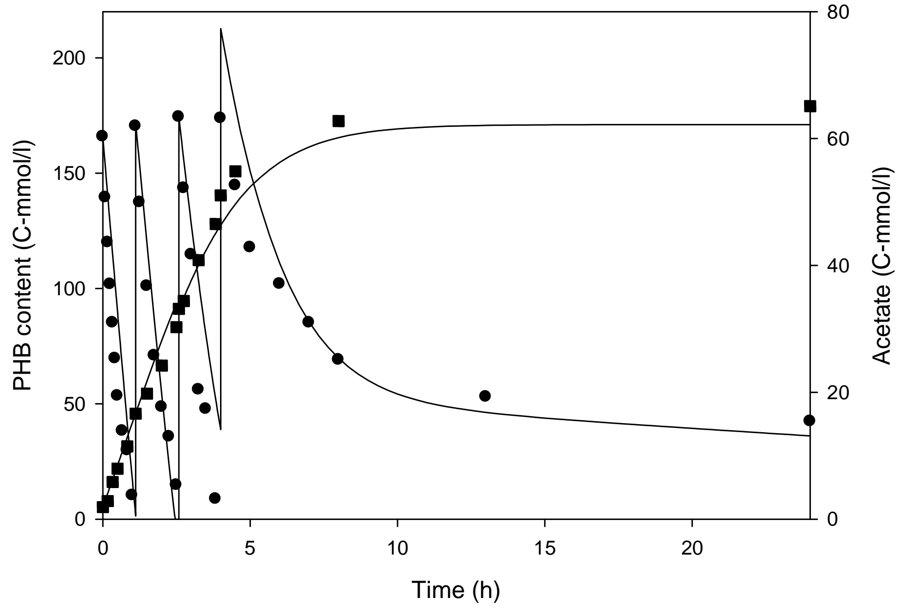


Figure 4.2- Comparison of segregated model and experimental data; (×) Active Biomass, (●) Acetate, (▲) Ammonia and (■) PHB content. At $Time_{op}$= 12h

Comparison of Segregated Model and Experimental Data

Feeding: **NH_3=0.7 N-mmol/l and HAc=60 C-mmol/l**

$Time_{op}$= 24h



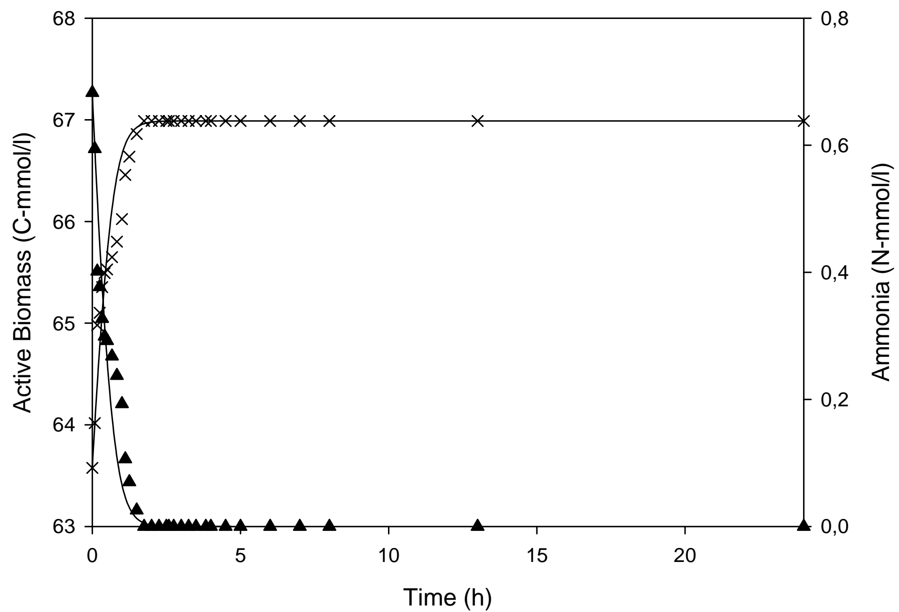


Figure 4.3- Comparison of segregated model and experimental data; (×) Active Biomass, (●) Acetate, (▲) Ammonia and (■) PHB content. At $Time_{op}$= 24h

Comparison of Segregated Model and Experimental Data

***Feeding:* NH_3=1.1 N-mmol/l and HAc=24.0 C-mmol/l**

$Time_{op}$= 8h

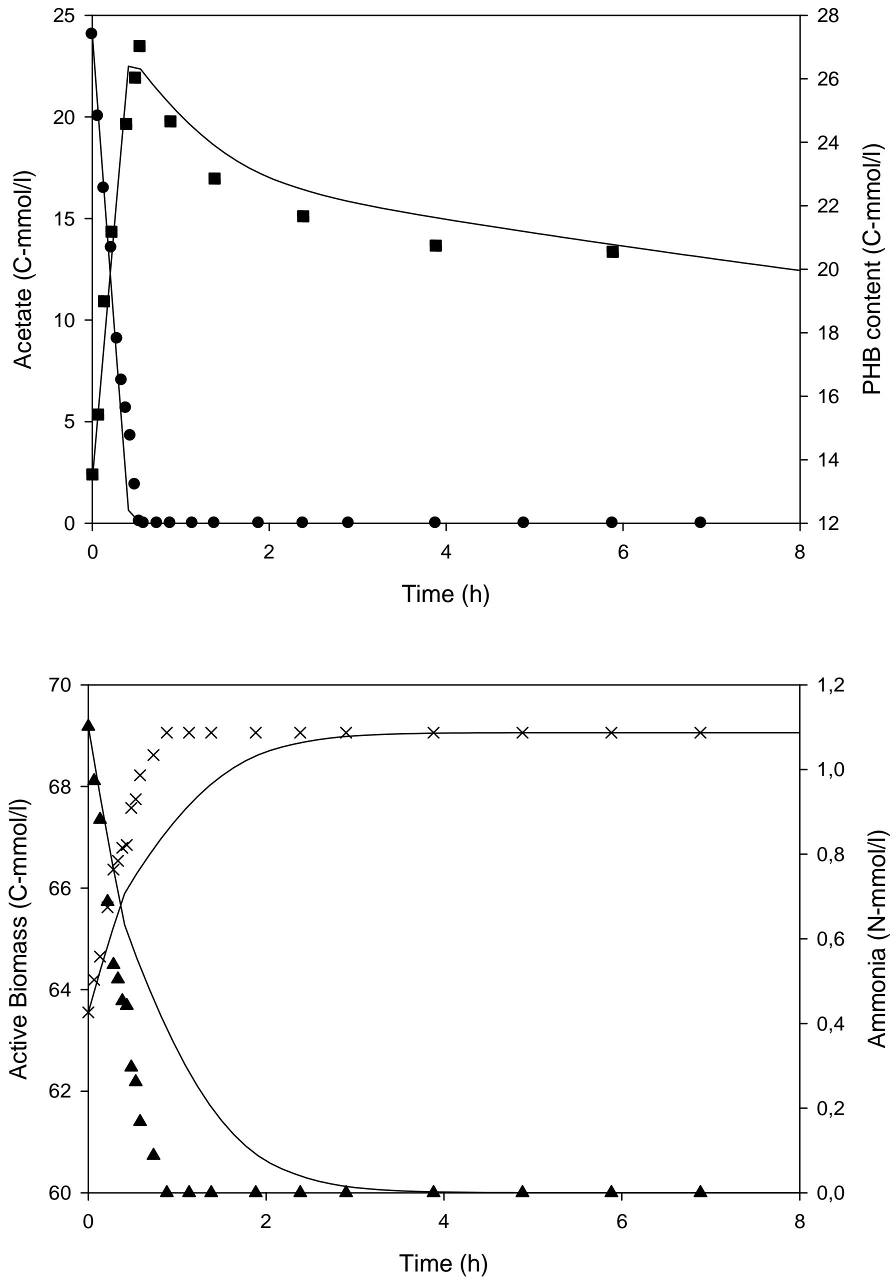


Figure 4.4- Comparison of segregated model and experimental data; (×) Active Biomass, (♦) Ammonia, (▲) Acetate and (■) PHB content. At $Time_{op}$= 8h

4.2. Sensibility analysis of culture dynamics

Through the simulations of the segregated model with NPOP= 64 populations, where one of two different levels (either higher (+) or lower (-)) for P/O ratio, specific PHB production rate, specific EPS production rate, maximum PHB storage capacity, specific growth rates on acetate and PHB was chosen for each population, the effect of conditions on culture selection was evaluated.

The difference between the lower and the upper levels was increased in order to better study the behavior of a culture composed by a population with a diversity of organisms with distinct metabolisms.

Considering an initial active biomass concentration of 200 C-mmol/l, for all combinations of feeding of acetate set by [15, 30, 60, 120 and 240] C-mmol, and ammonia, set by [0.7, 1.4, 2.8, 5.6, 11.2 and 22.4] N-mmol.

It was considered 60 cycles in all simulations. The cycle length was varied to be coincident with the time required for complete PHB depletion in the best population. The cycle length varied in each cycle, but the constraint of 12 hours was imposed. Through the cycle length it was possible to define the HRT and SRT values, which are:

□□□ = *240* □ [49]

□□□ = *24* □ [50]

This means:

□□□ = *10* · □□□ [51]

The main objective at this point is to establish a relationship between the mean and standard deviation of average culture parameters through the variations of the initial acetate and ammonia feeding conditions.

The results show that in the feast and famine strategy, the C/N ratio is a parameter very sensitive to the different variations of the feeding conditions, e. g., is the parameter that more influences the culture selection towards the best performing organisms.

The feeding strategy has proven to have a high influence on the population dynamics.

The sensitivity analysis of the culture dynamics was made for two different conditions: keeping sludge and keeping permeate.

4.2.1. Specific growth rates on acetate and PHB

It was found that unbalanced growth conditions stimulated massive PHB production in activated sludge biomass. The C/N ratio was shown to be an important control parameter of cell growth and PHB formation. Through the figures shown bellow it is possible to deduce that the population average specific growth rate is sensitive to the C/N ratio, and that at low C/N ratios the population specific growth rate on PHB tends to converge to higher values.

When ammonia is present in the medium, the acetate is metabolized simultaneously for cell growth and PHB formation, and the active cell mass increases, but then in the famine phase the specific

growth rate is much lower [7]. The population average specific PHB formation rate is higher for experiments with lower ammonia supply [7]. In the short feast phase, the growth rates are higher than the average growth rate in the famine phase [31].

The SRT seems to be very important for the selection of populations with high specific growth rates, because at low SRT **(Figure 4.5 – 4.12)**, these populations tend o predominate over the remaining populations.

Microbial cultures subjected to changes in substrate concentrations use PHB to efficiently balance the difference between substrate uptake rate and substrate requirements for growth [13].

4.2.1.1. Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=5

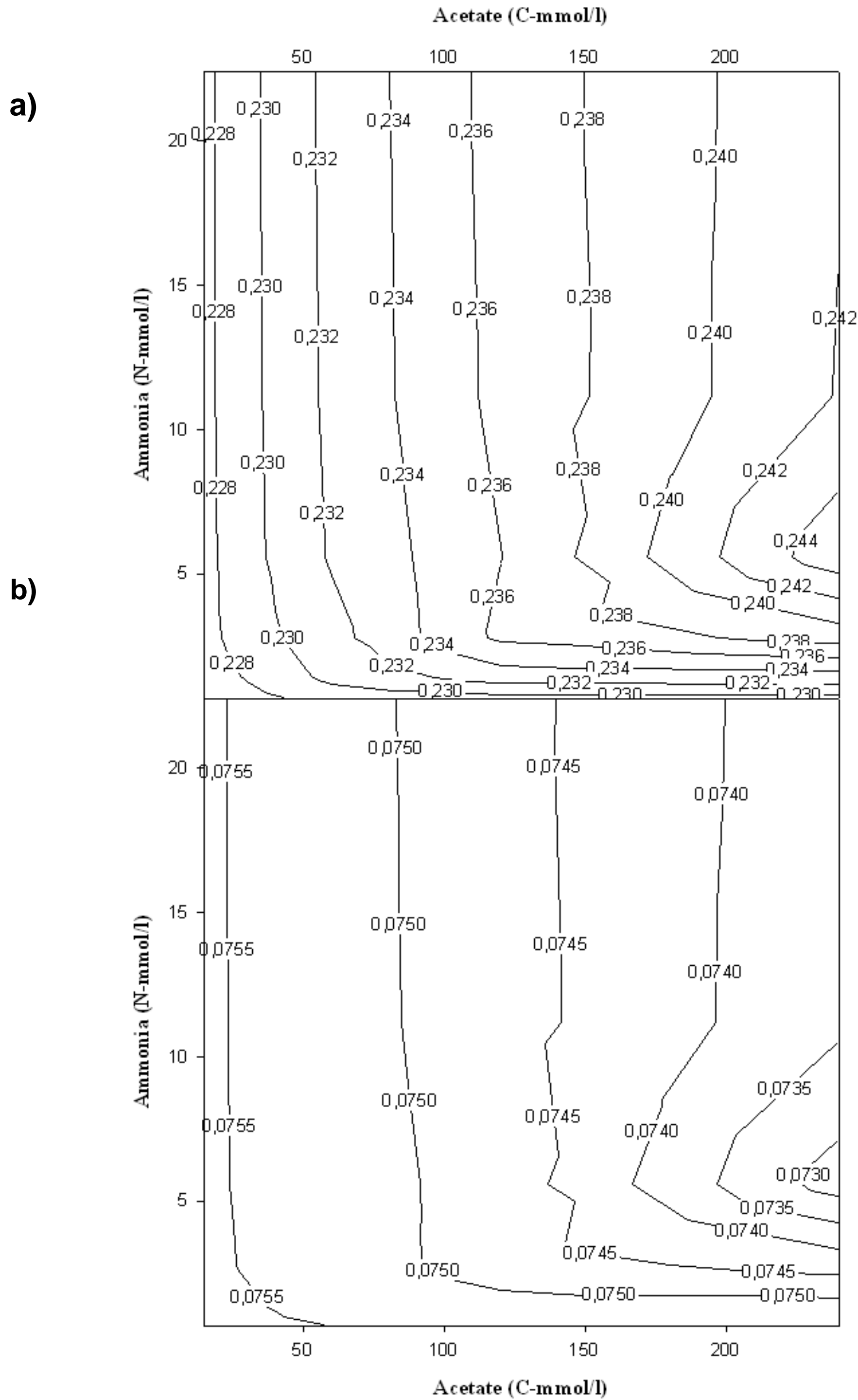


Figure 4.5- Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

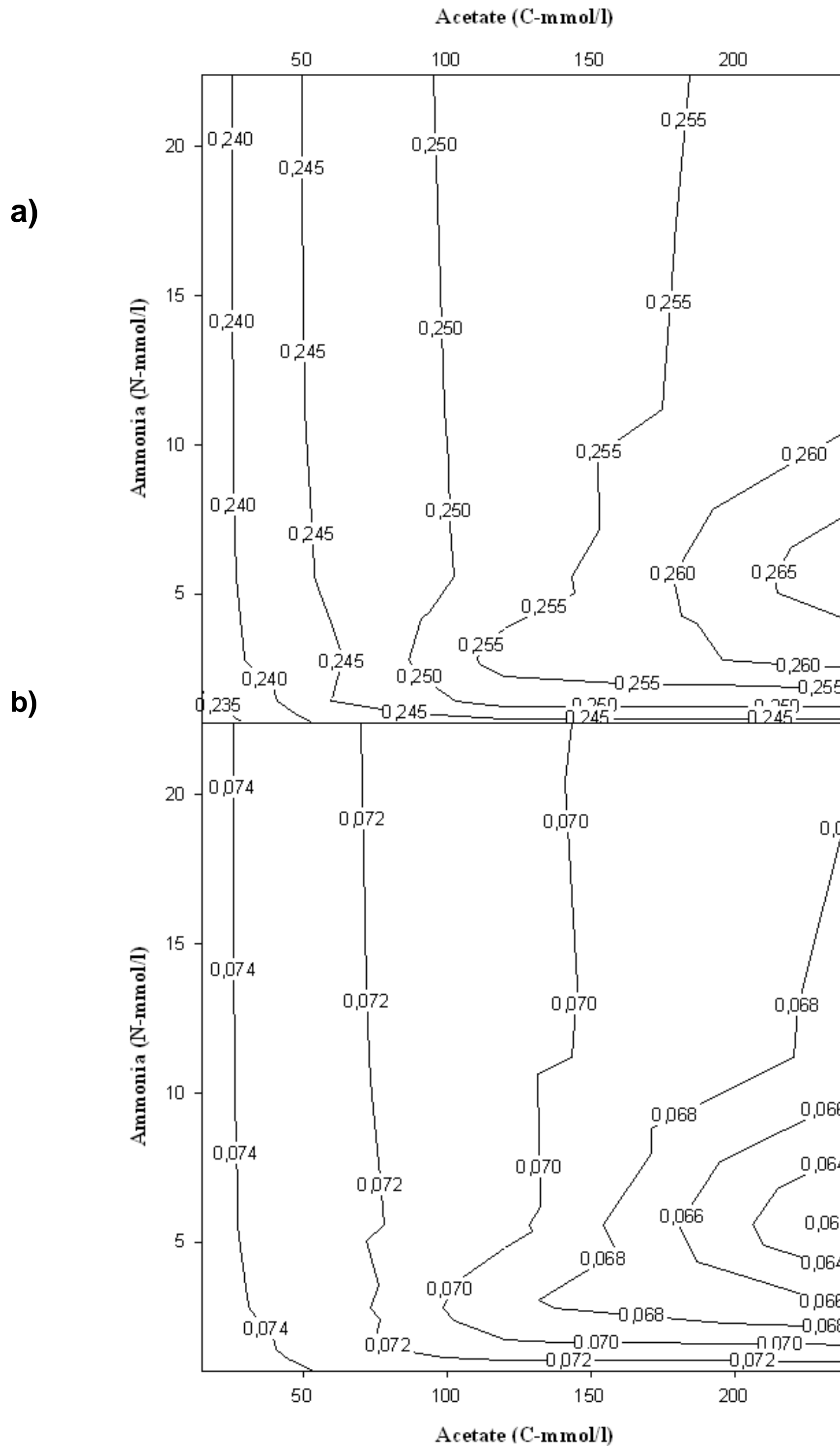


Figure 4.6- Sensitivity analysis of specific growth rates of acetate and PHB on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

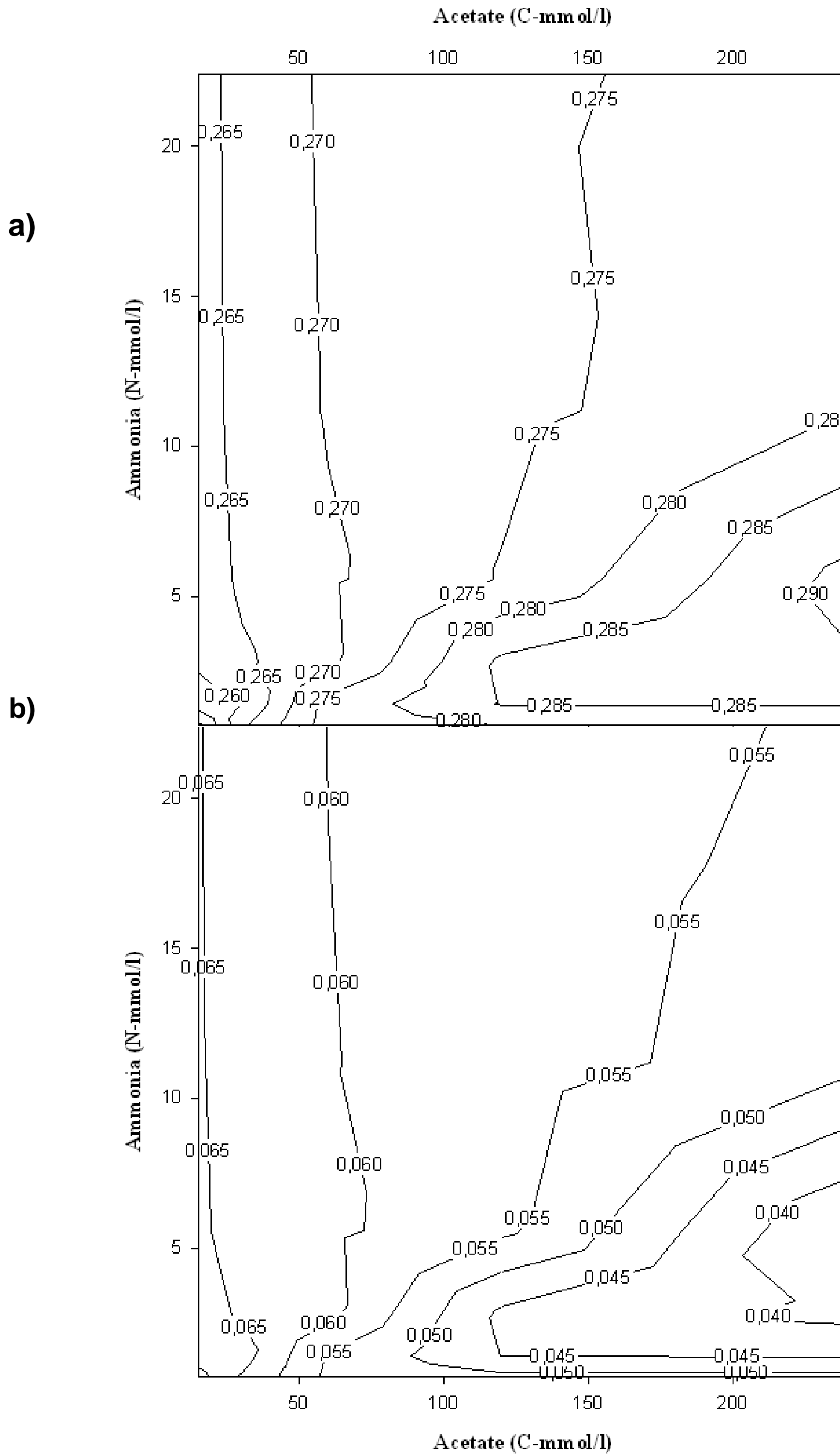


Figure 4.7- Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

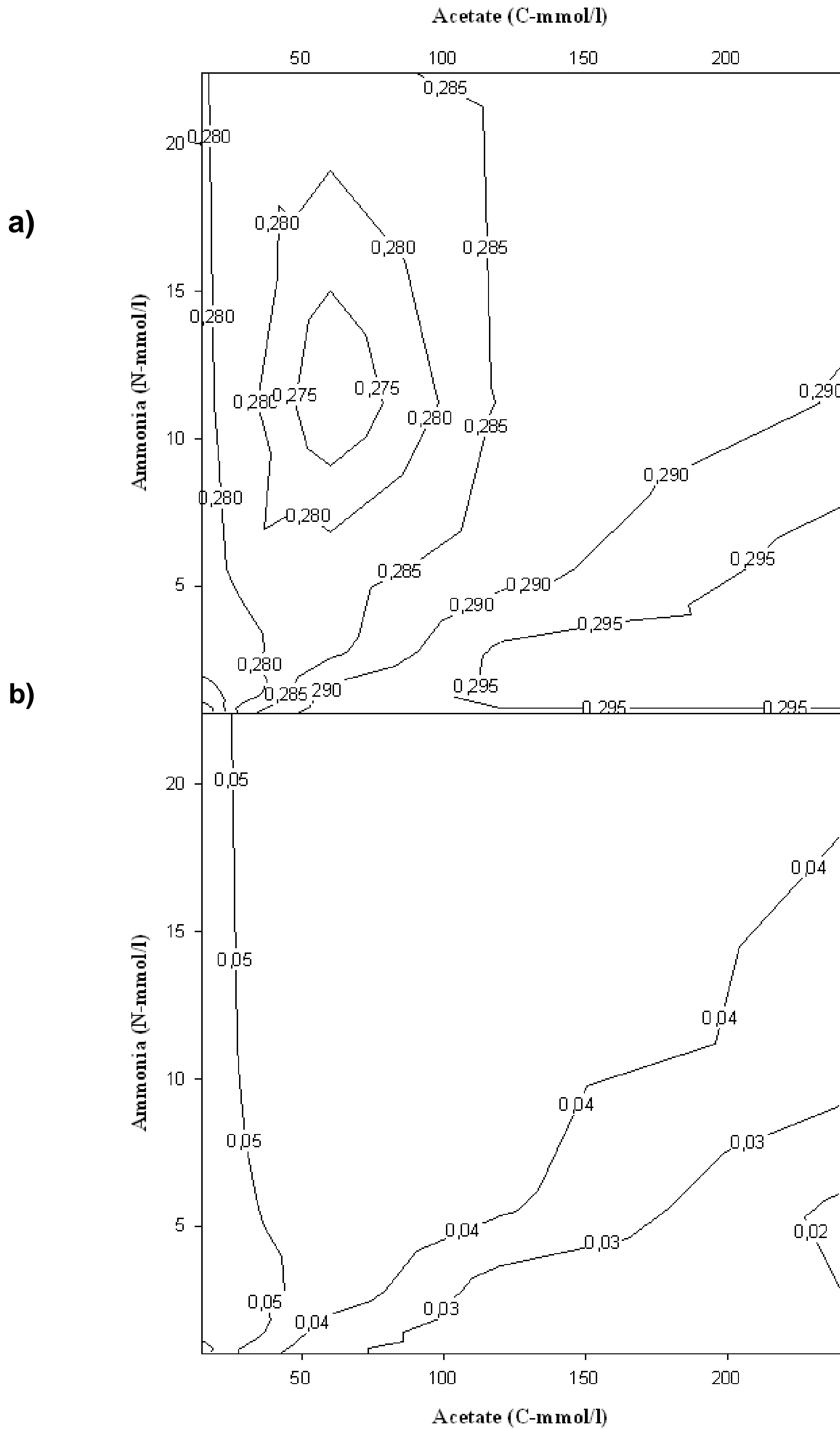


Figure 4.8- Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.1.2. Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=5

a)

b)

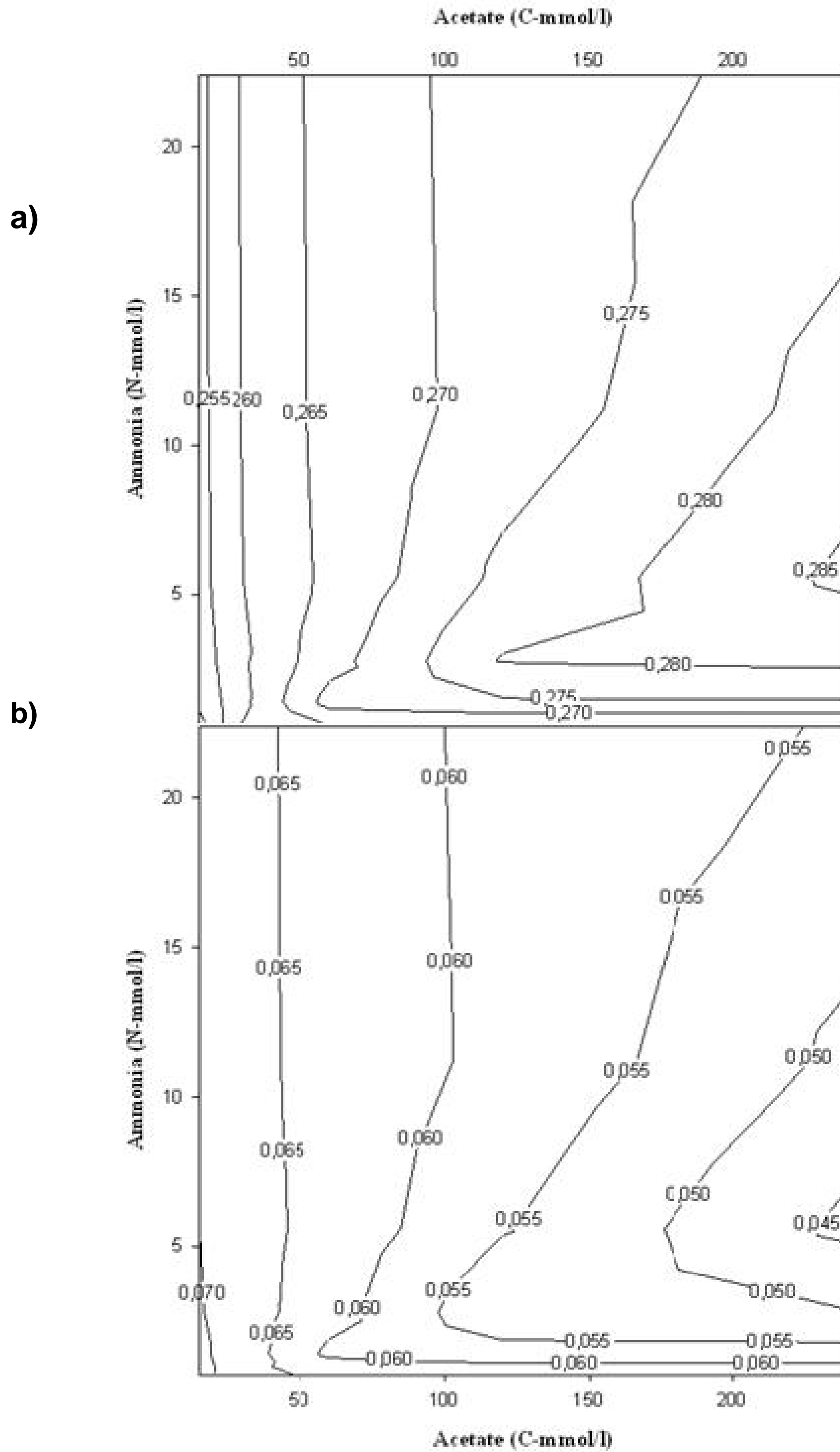


Figure 4.9- Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

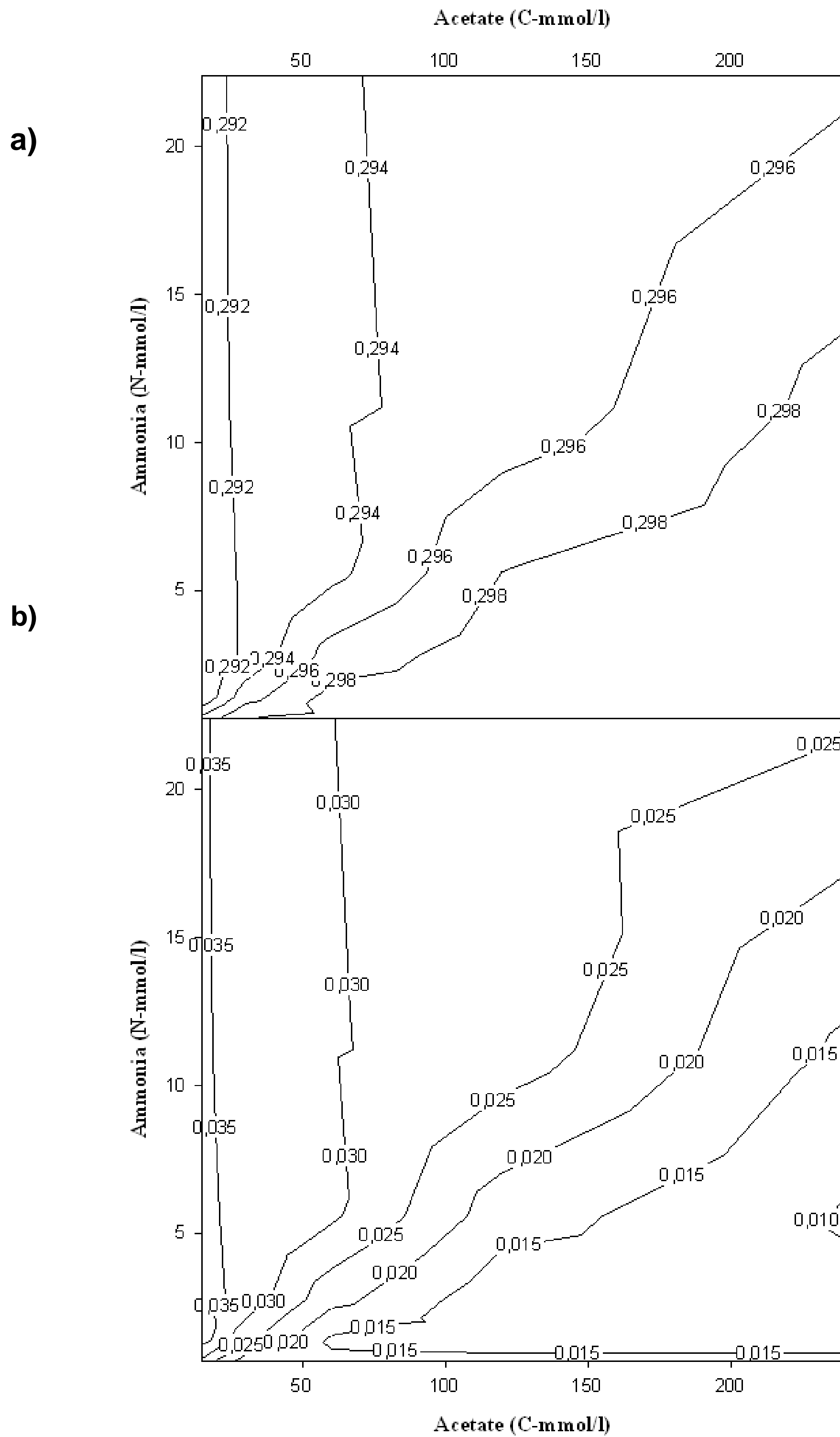


Figure 4.10- Sensitivity analysis of specific growth rates of acetate and PHB on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

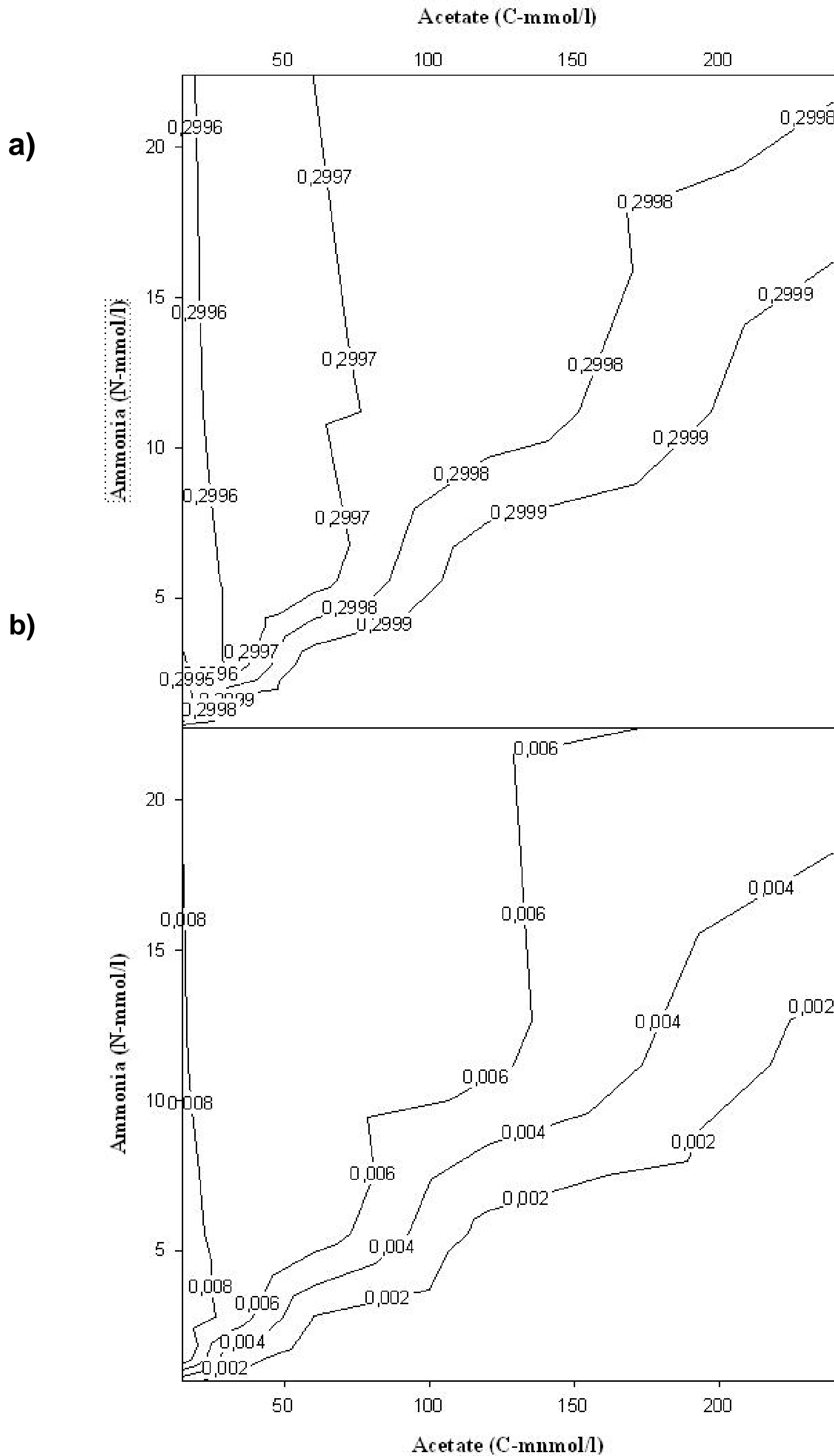


Figure 4.11- Sensitivity analysis of specific growth rates of acetate and PHB on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

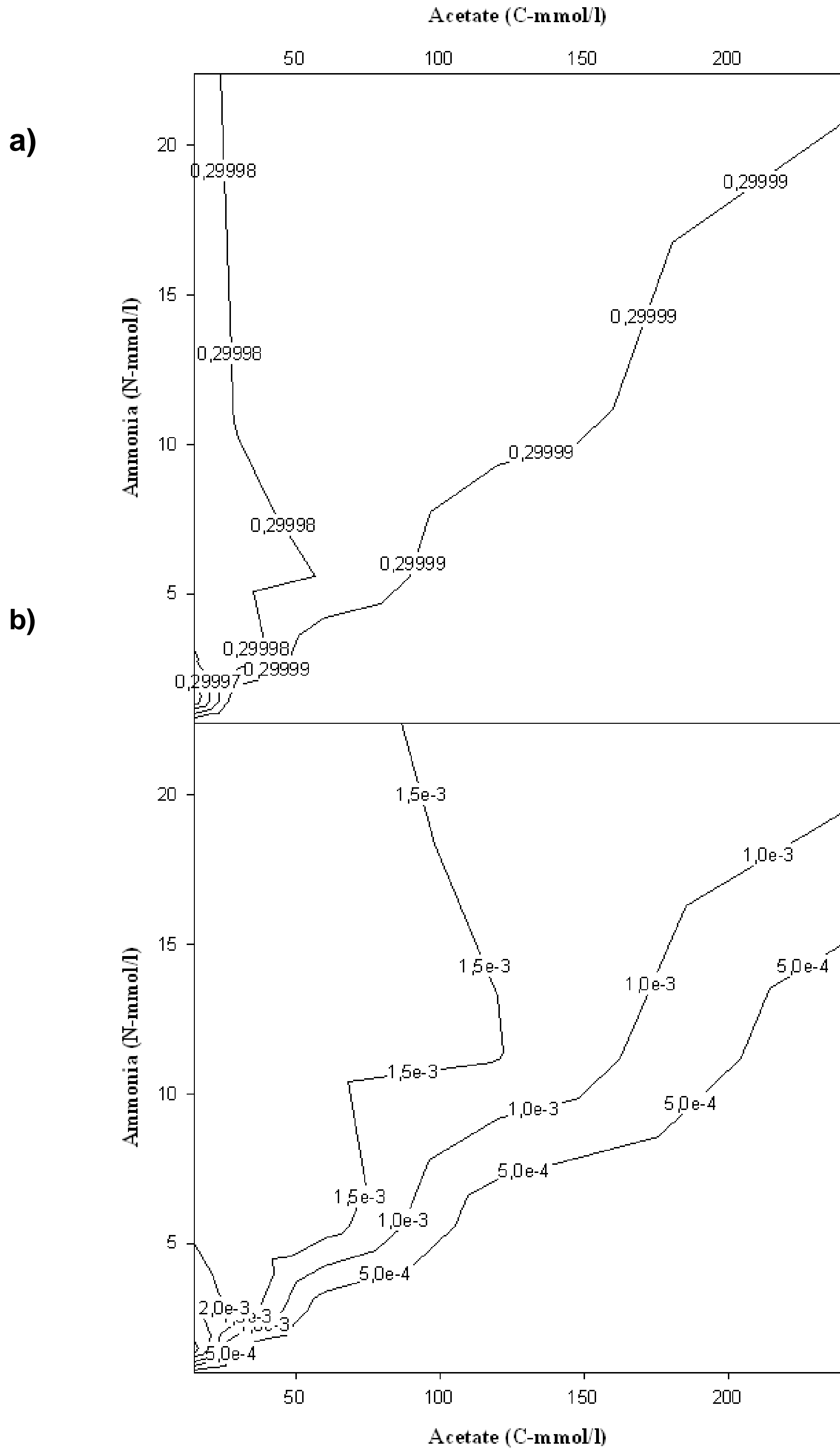


Figure 4.12- Sensitivity analysis of specific growth rates of acetate and PHB on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.2. Specific PHB production rate

This study is one more proof that activated sludge organisms respond to feast and famine regimens by the production of the storage polymer PHB. According to van Aalst-van Leeuwen et al., 1997, the microorganisms capable of utilizing acetate for growth and PHB formation first take up acetate by means of active transport and then activate it to acetyl-CoA, from which biomass monomers and PHB are formed.

The specific PHB formation rate increases considerably when complete ammonia depletion is observed, suggesting that the flux carbon previously used for cell growth was redirected for PHB formation [7]. The specific PHB formation rate is higher at high amounts of acetate, and lower of ammonia. After acetate depletion, the PHB concentration decreased during the famine period to the concentration level al the beginning of the cycle [36].

With a certain relaxation time the specific growth rate will increase and simultaneously the PHB production decreases [13].

This parameter is sensitive to C/N ratio, as it is possible to see in **Figures 4.13 – 4.20.** At high C/N ratio, the population average specific PHB production rate present high values, thus the selection of populations in these conditions and low SRT is not efficient, being selected more efficiently at low C/N ratios.

4.2.2.1. Keeping Sludge

Sensitivity Analysis of Culture Dynamics

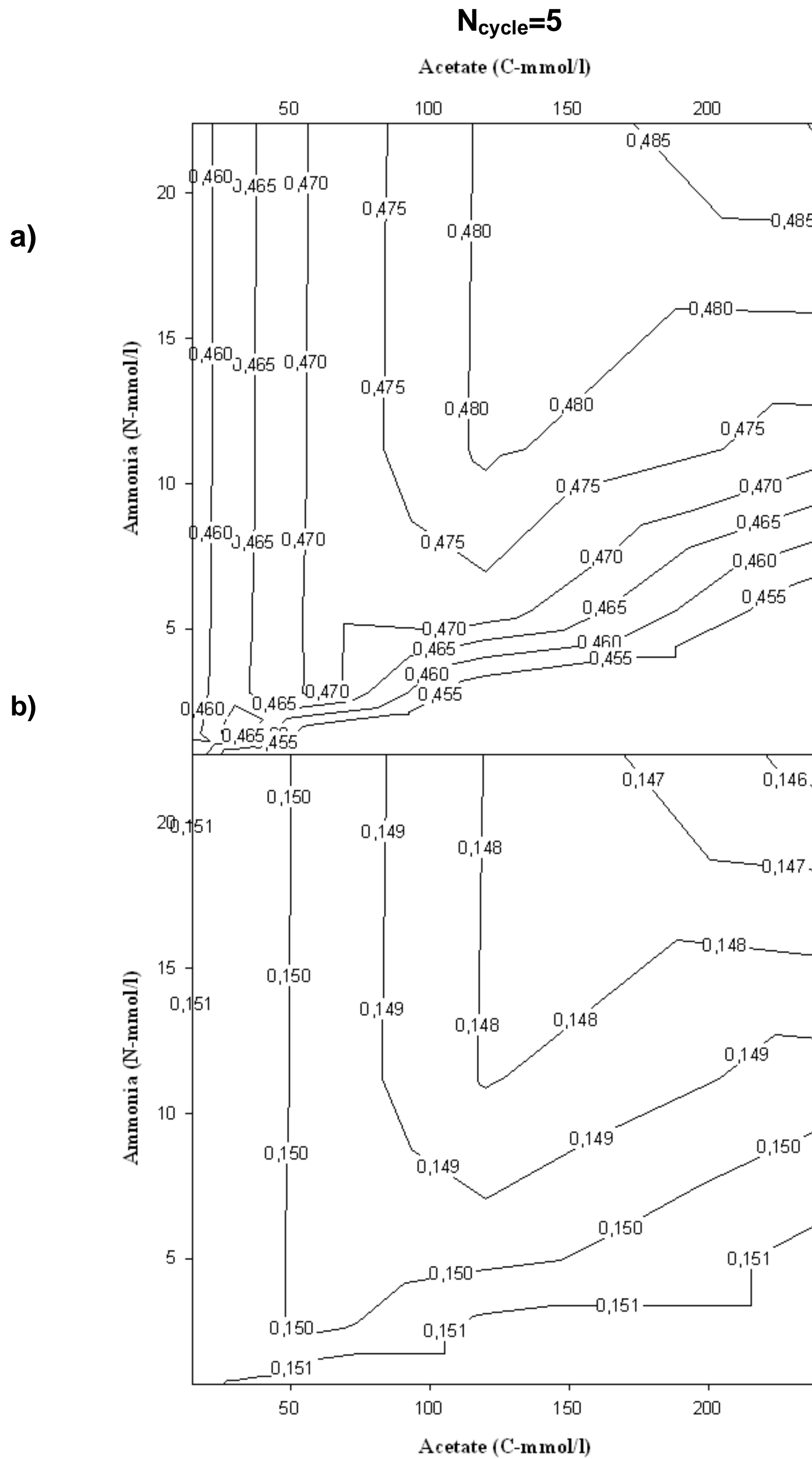


Figure 4.13- Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

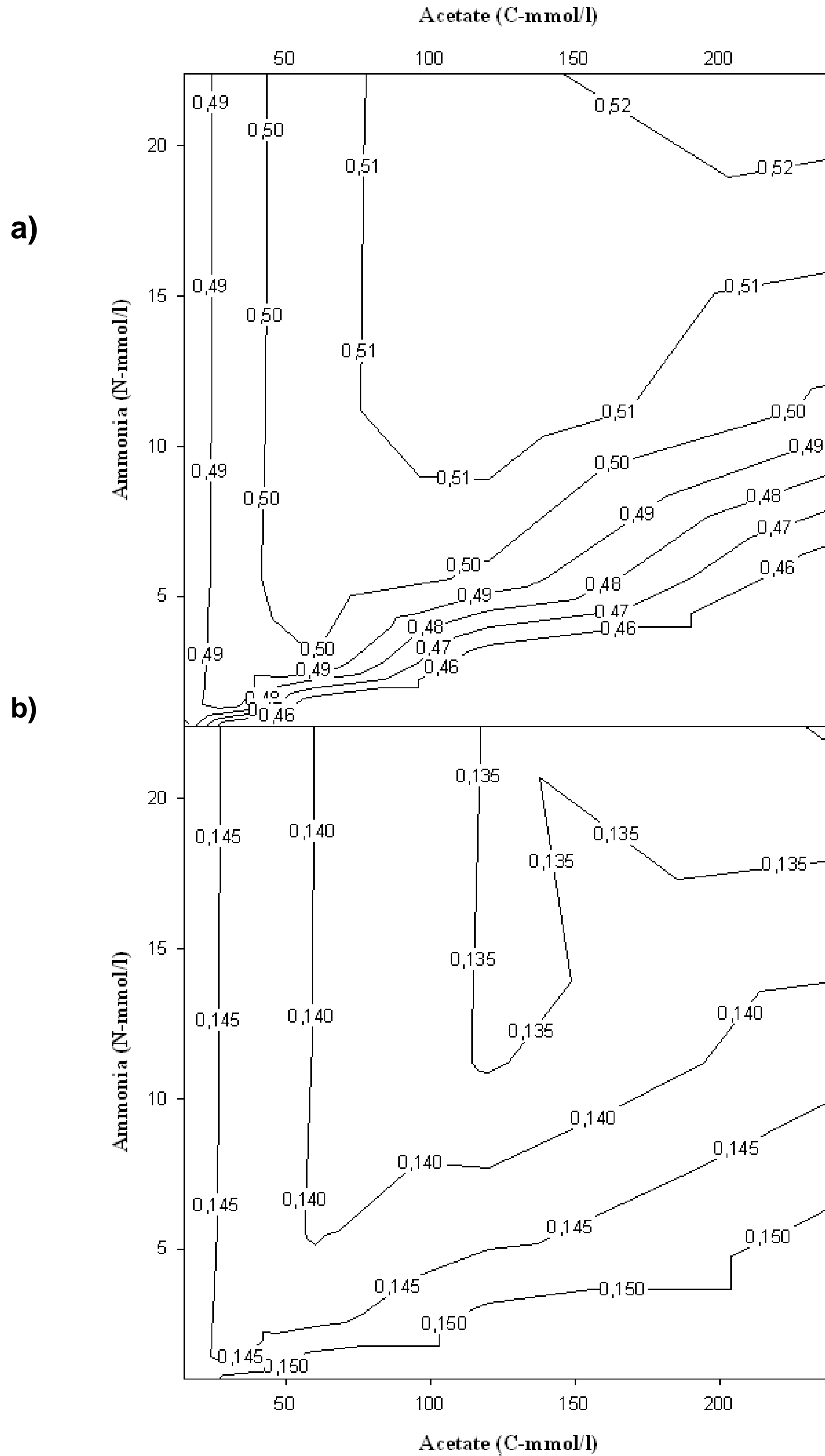


Figure 4.14- Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

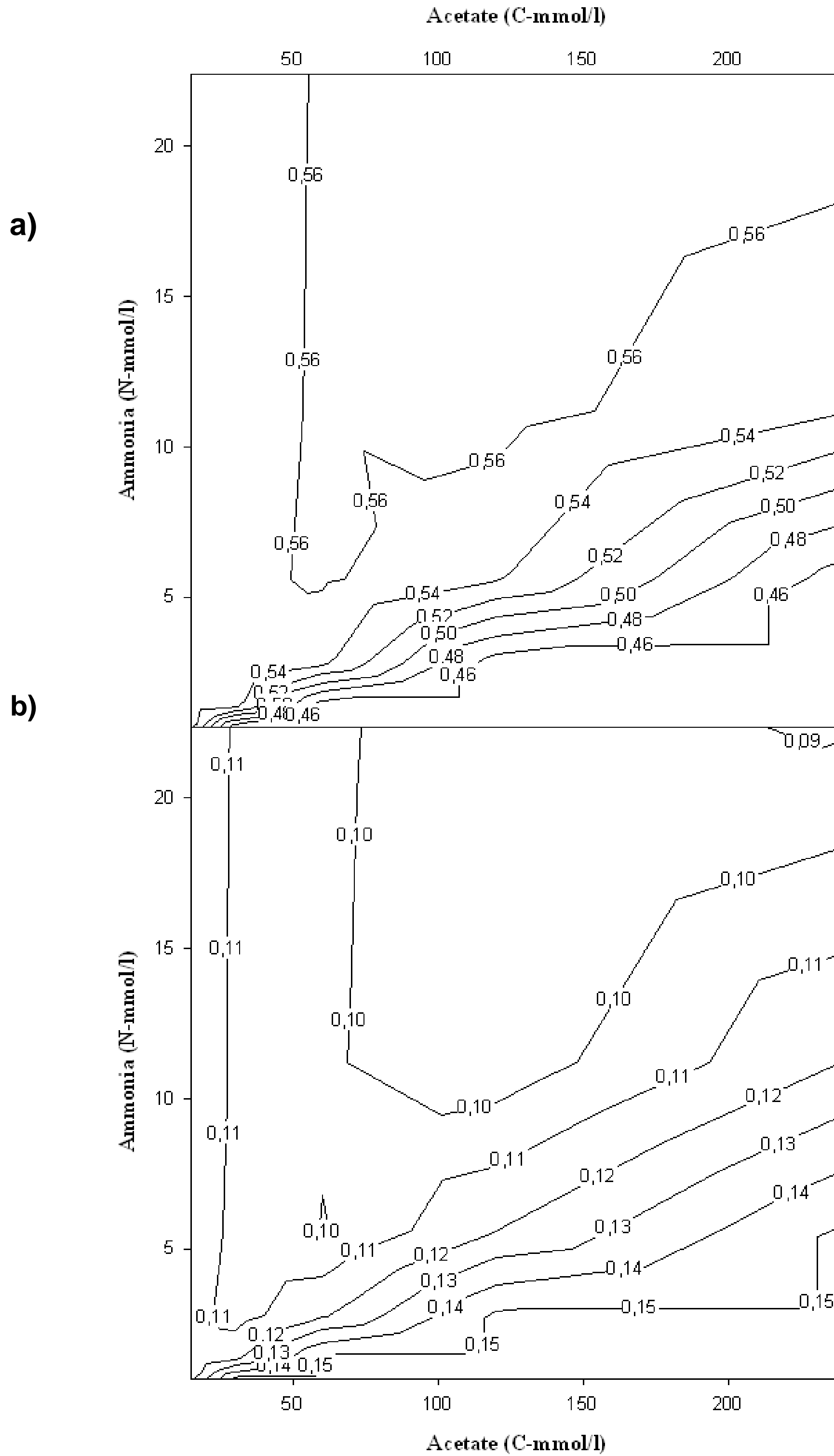


Figure 4.15- Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

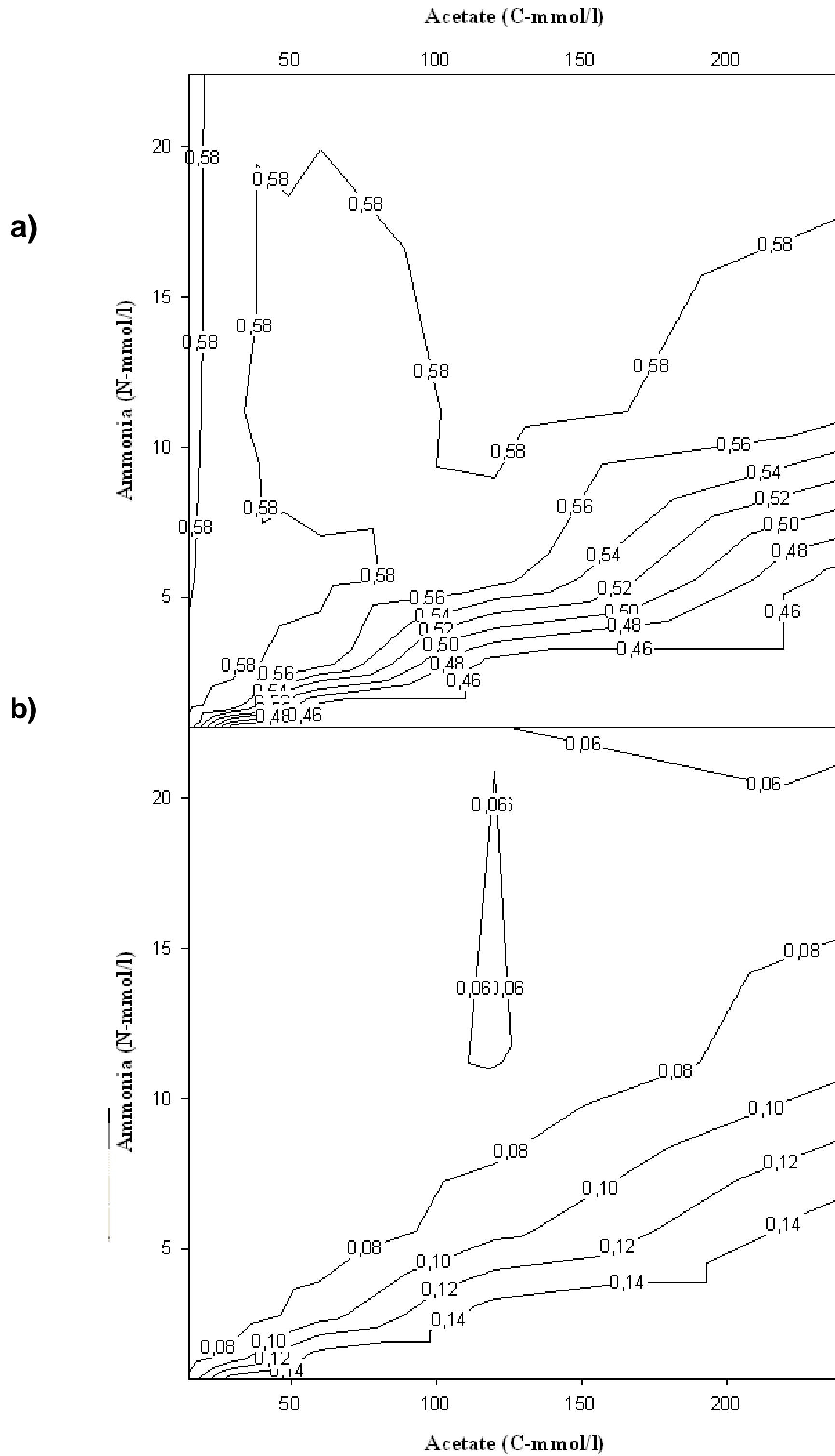


Figure 4.16- Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.2.2. Keeping Permeate

Sensitivity Analysis of Culture Dynamics

N_{cycle}=5

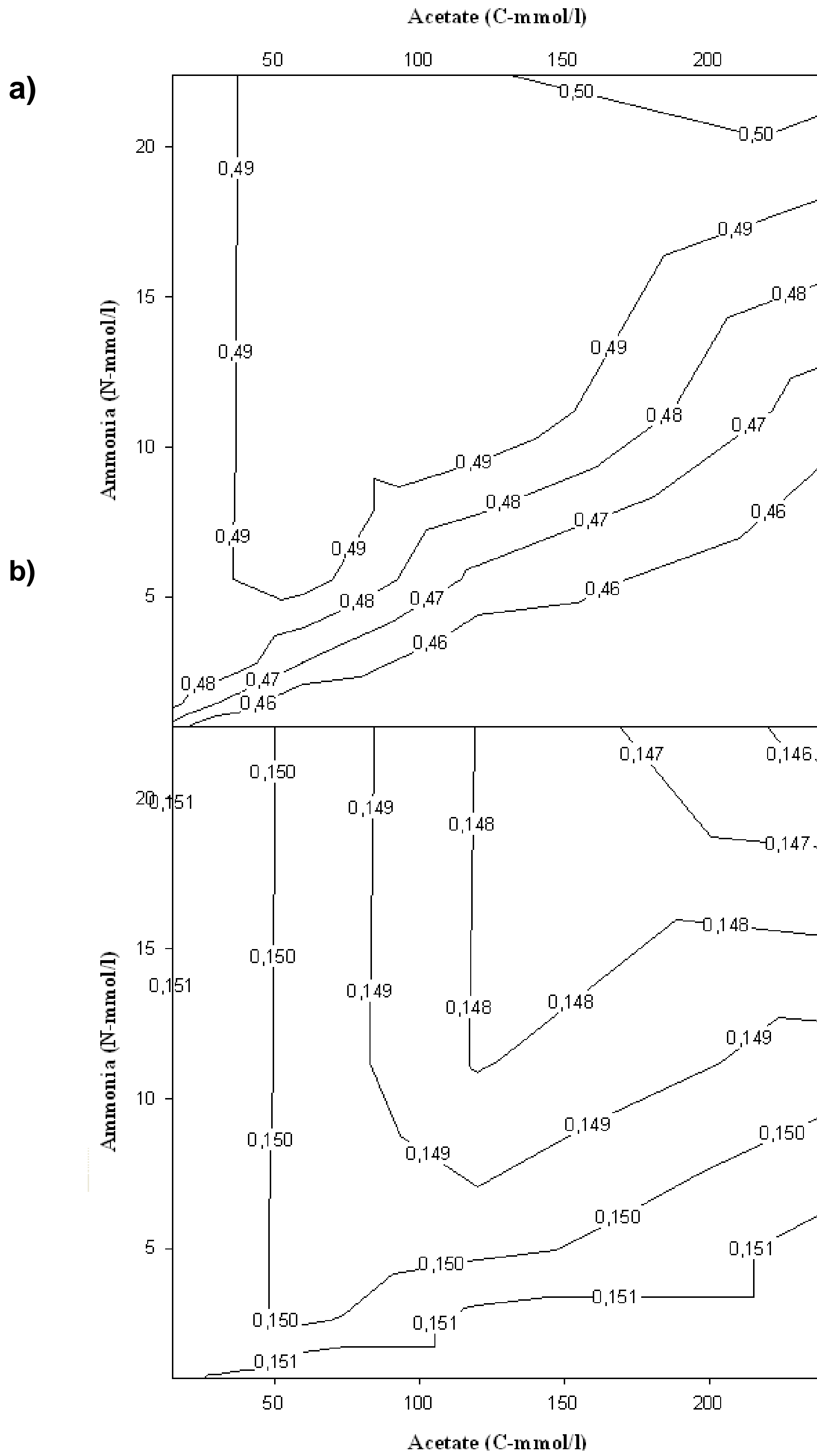


Figure 4.17- Sensitivity analysis of specific PHB production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

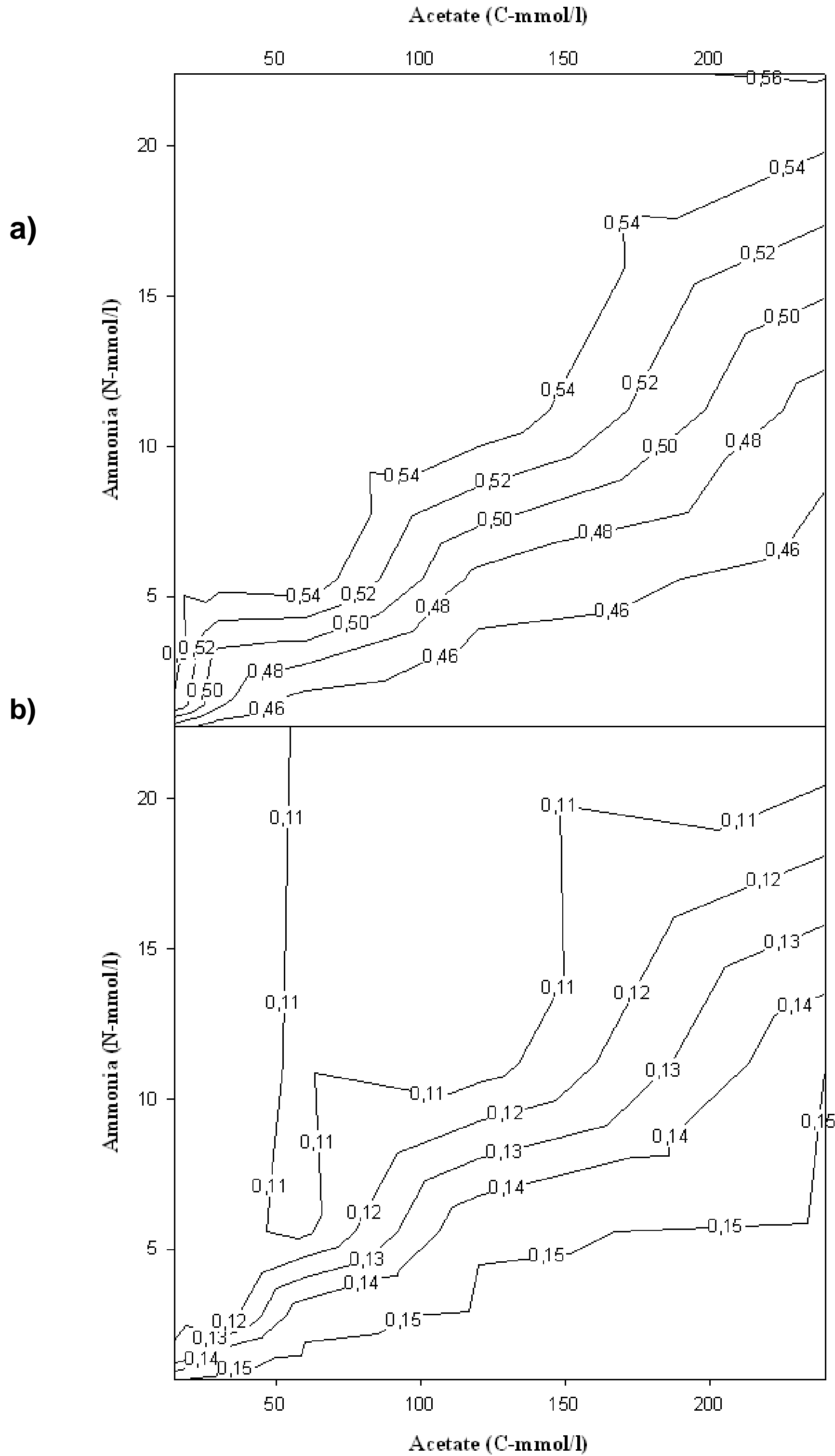


Figure 4.18- Sensitive analysis of specific PHB production rate on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

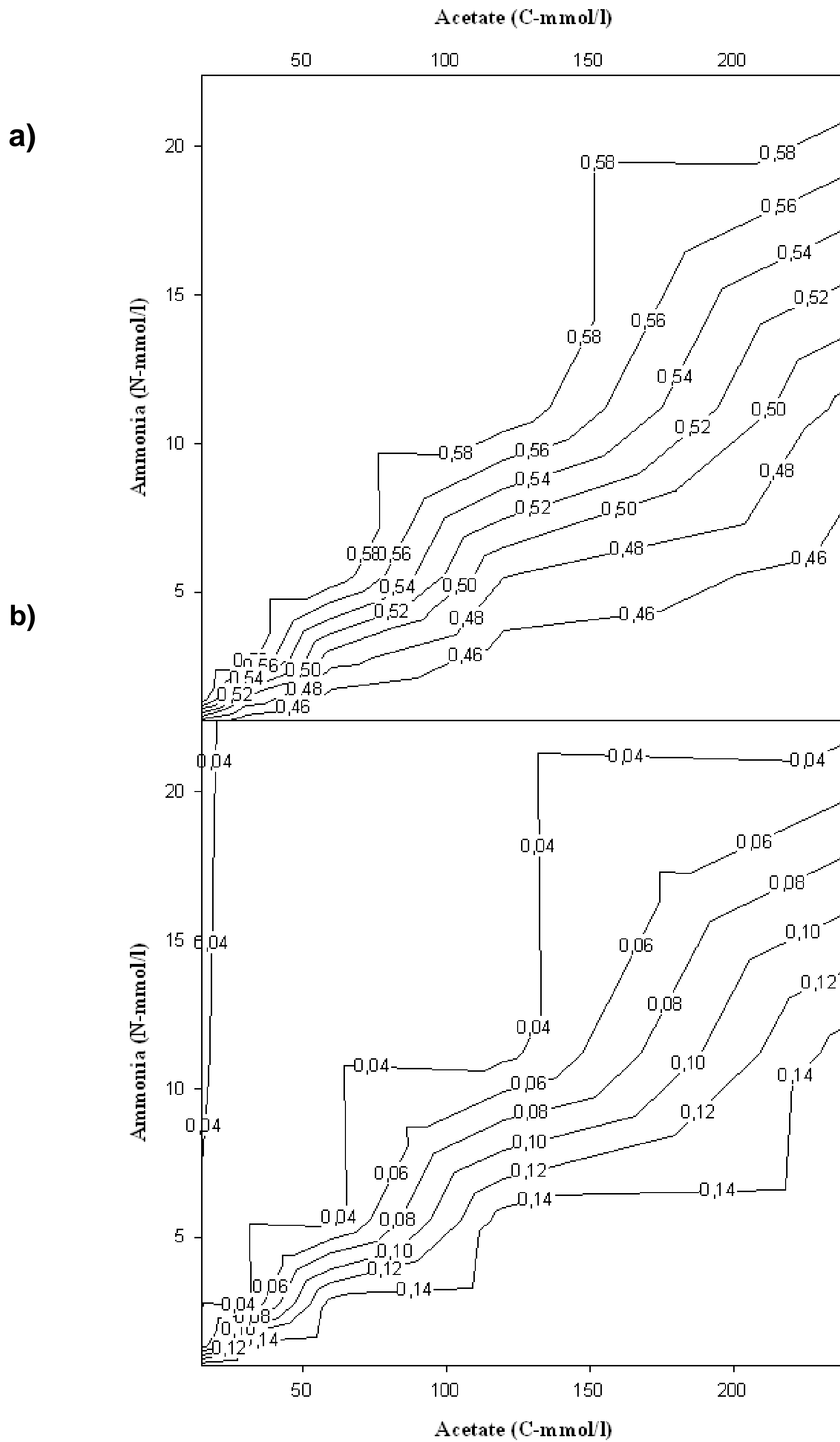


Figure 4.19- Sensitivity analysis of specific PHB production rate on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

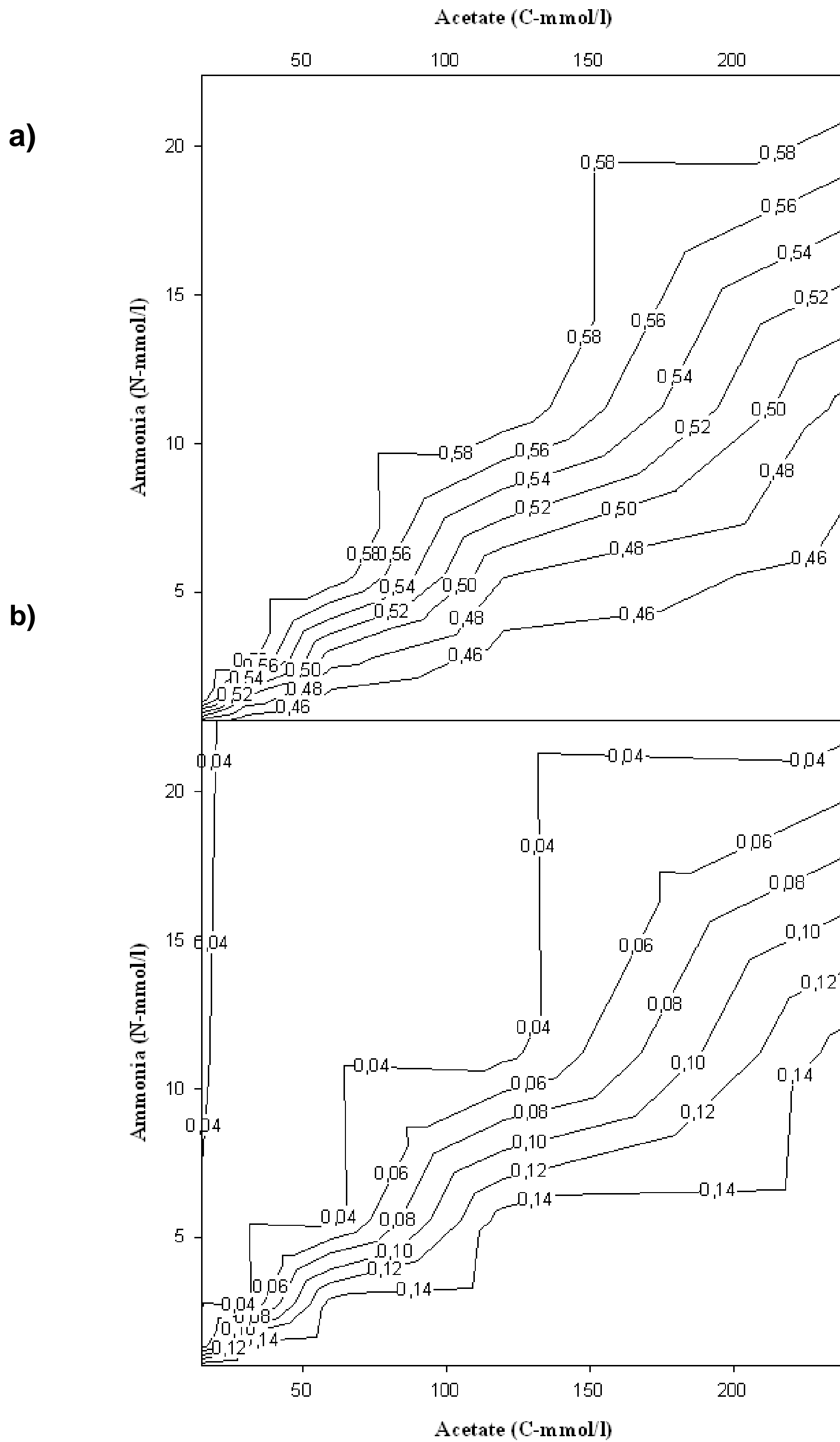


Figure 4.20- Sensitivity analysis of specific PHB production rate on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.3. Maximum PHB storage capacity

Storage of PHB by mixed microbial cultures occurs when they are subjected to transient carbon supply in which a long period of lack of substrate is alternated with a short time of time of excess of substrate [7].

This parameter is very sensitive to different acetate and ammonia feeding conditions. The populations which are able to store more PHB are predominant over the remaining populations, e.g., the selection is based on the populations with the higher PHB storage capacity, at low SRT.

The microbial response to dynamic conditions can be different from several aspects, including the storage. From the modeling point of view it is noteworthy that activated sludge models have evolved from ASM1, which considers only growth, with no any role of storage, to ASM3 (Gujer et al., 1999) [87], which considers storage on external substrates and growth only on internal stored polymers [39]. Current conceptual models of PHA storage assume that storage occurs when some limitation to growth is present [26].

Through the results obtained, it is possible to affirm that at low C/N ratio it is possible to make the selection among the microbial culture of the populations with the higher PHB storage capacity.

4.2.3.1. Keeping Sludge

Sensitivity Analysis of Culture Dynamics

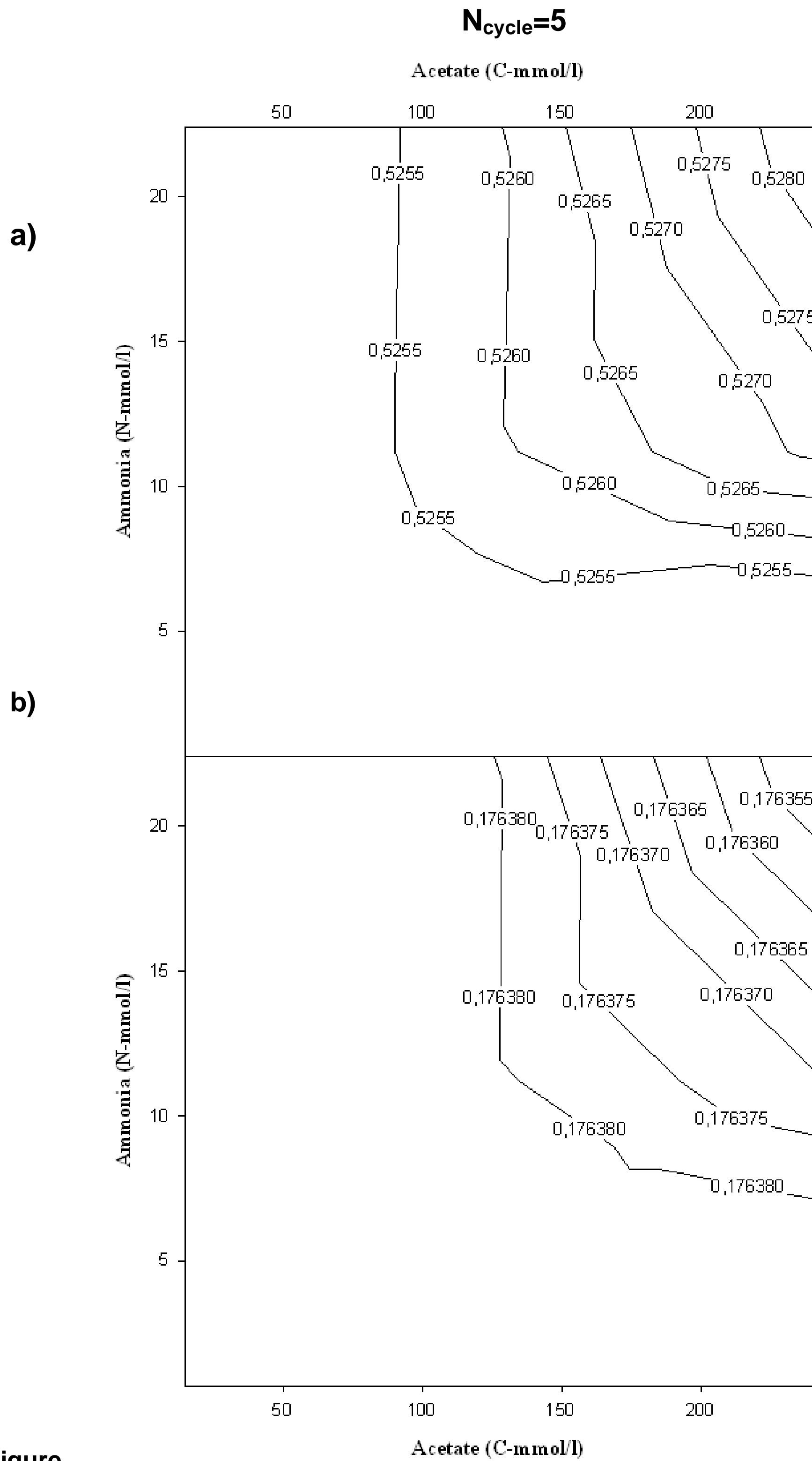


Figure 4.21- Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

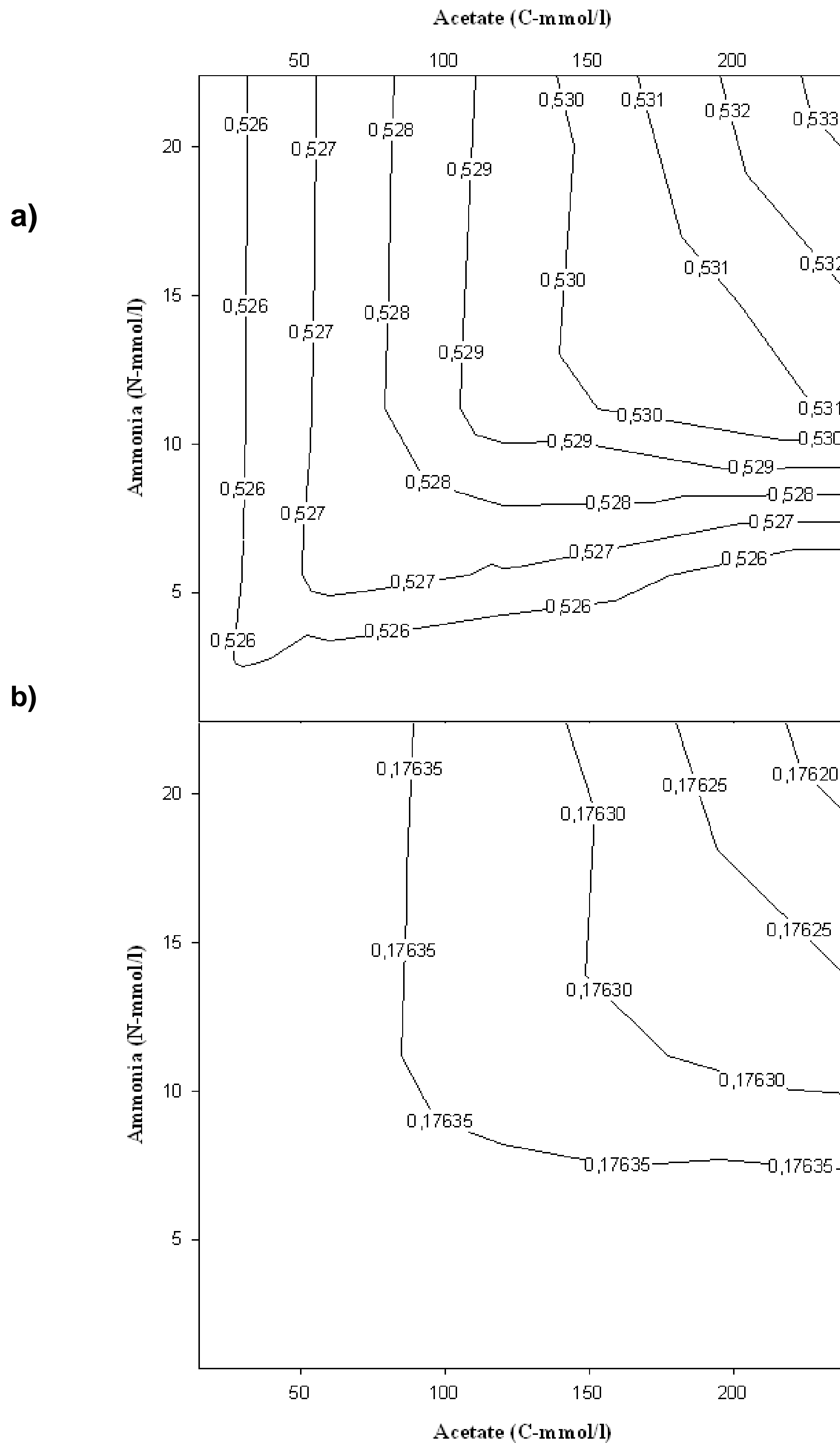


Figure 4.22- Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

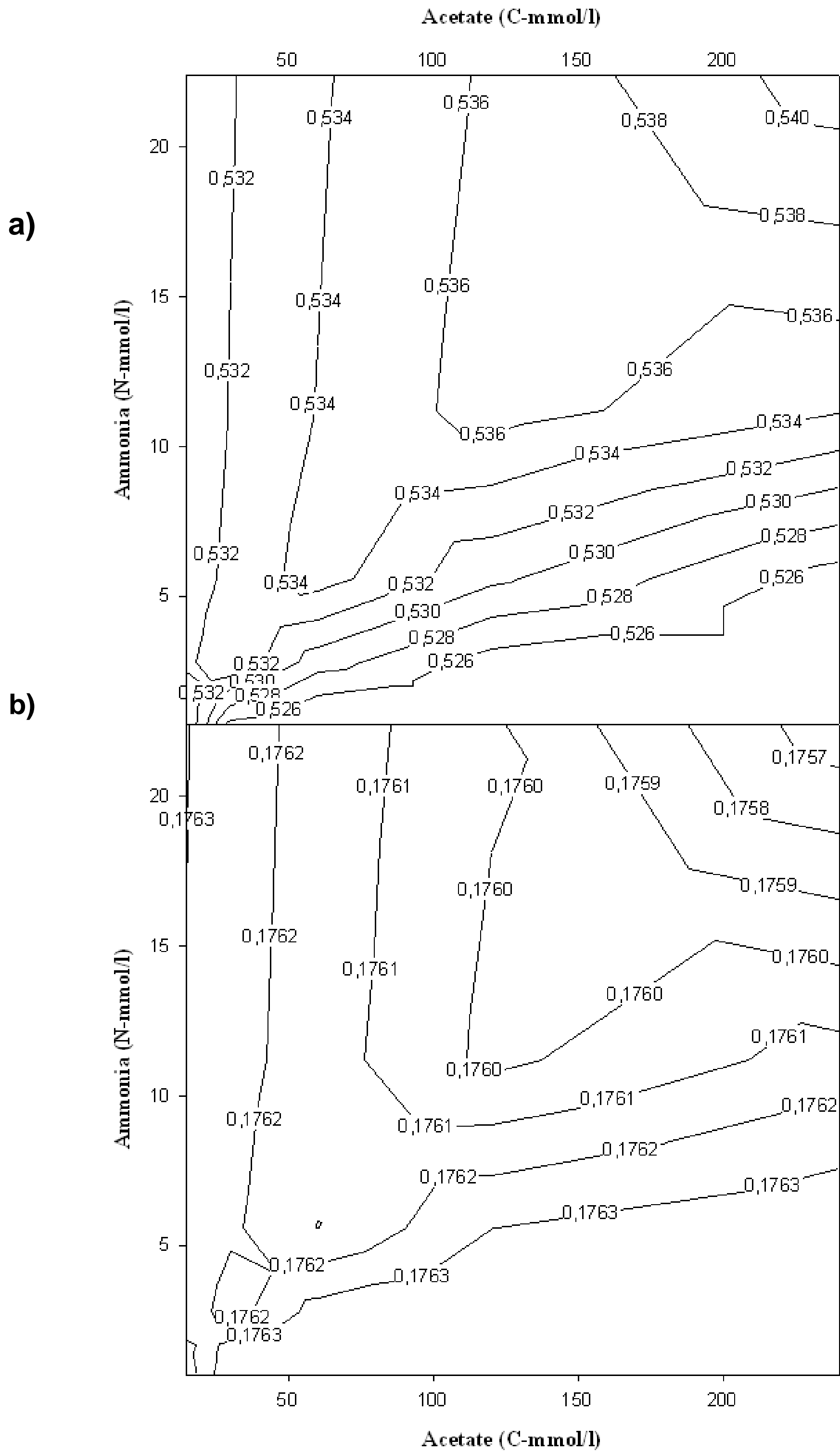


Figure 4.23- Sensitive analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

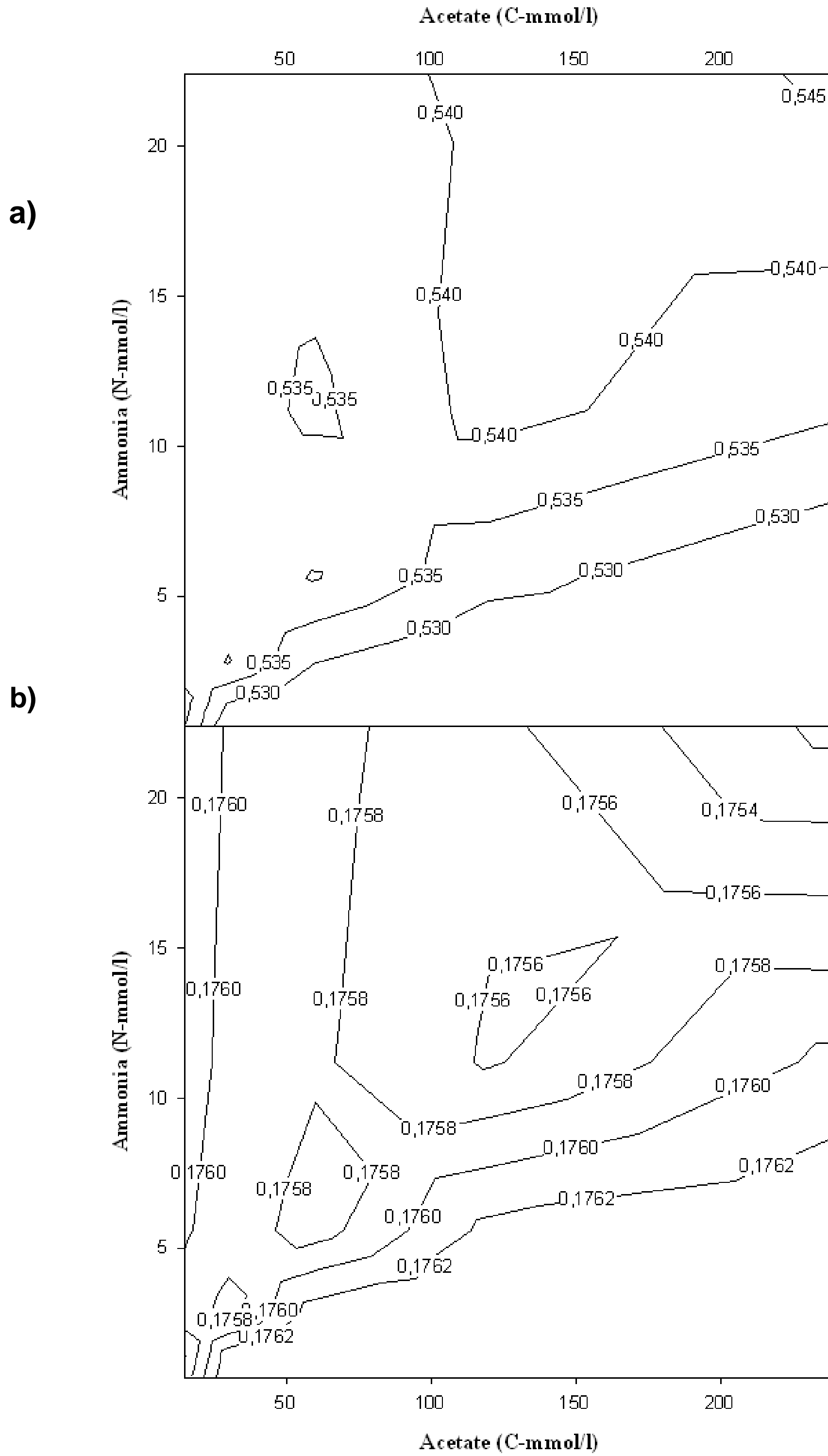


Figure 4.24- Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.3.2. Keeping Permeate

Sensitivity Analysis of Culture Dynamics

N_{cycle}=5

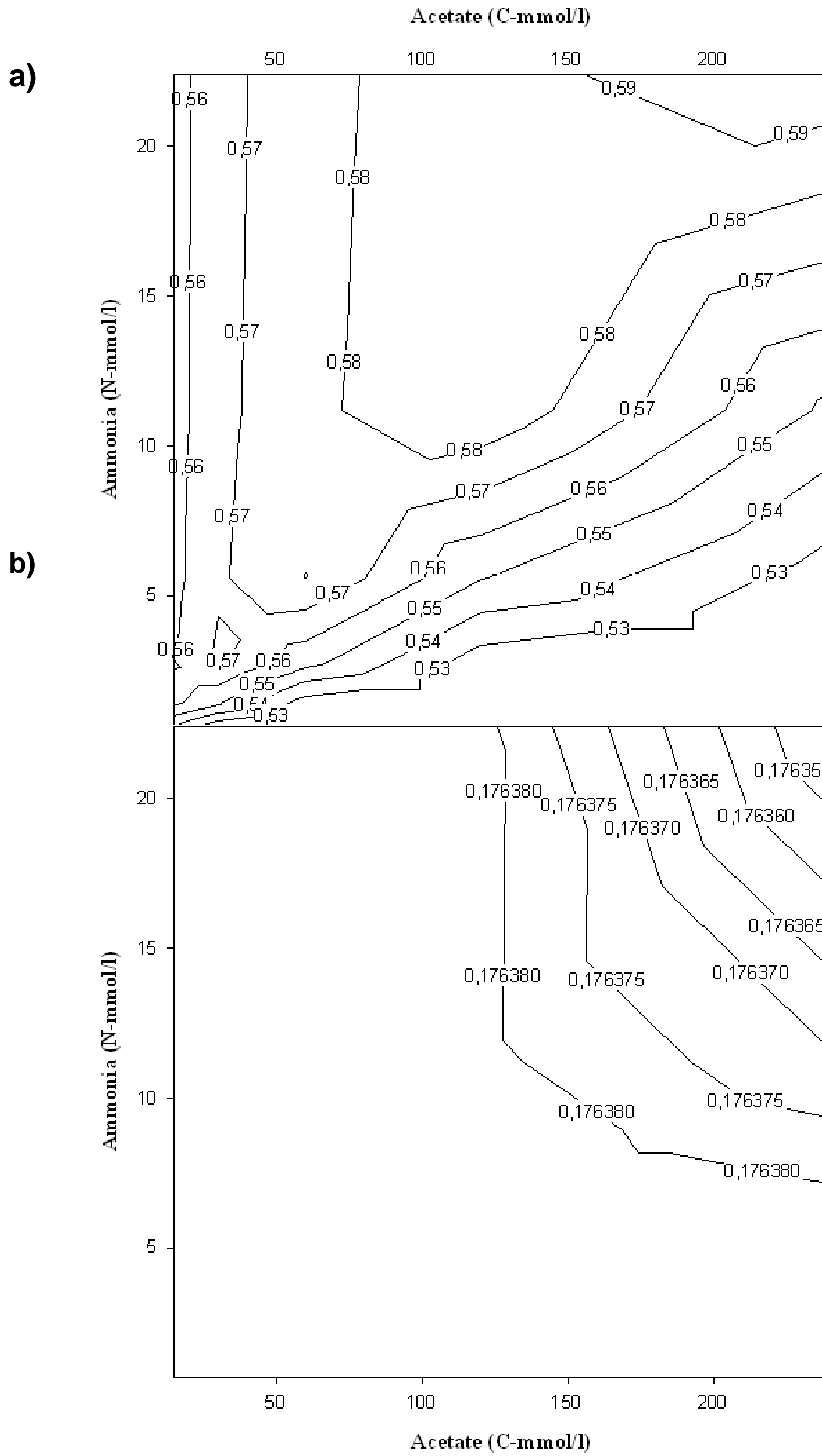


Figure 4.25- Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

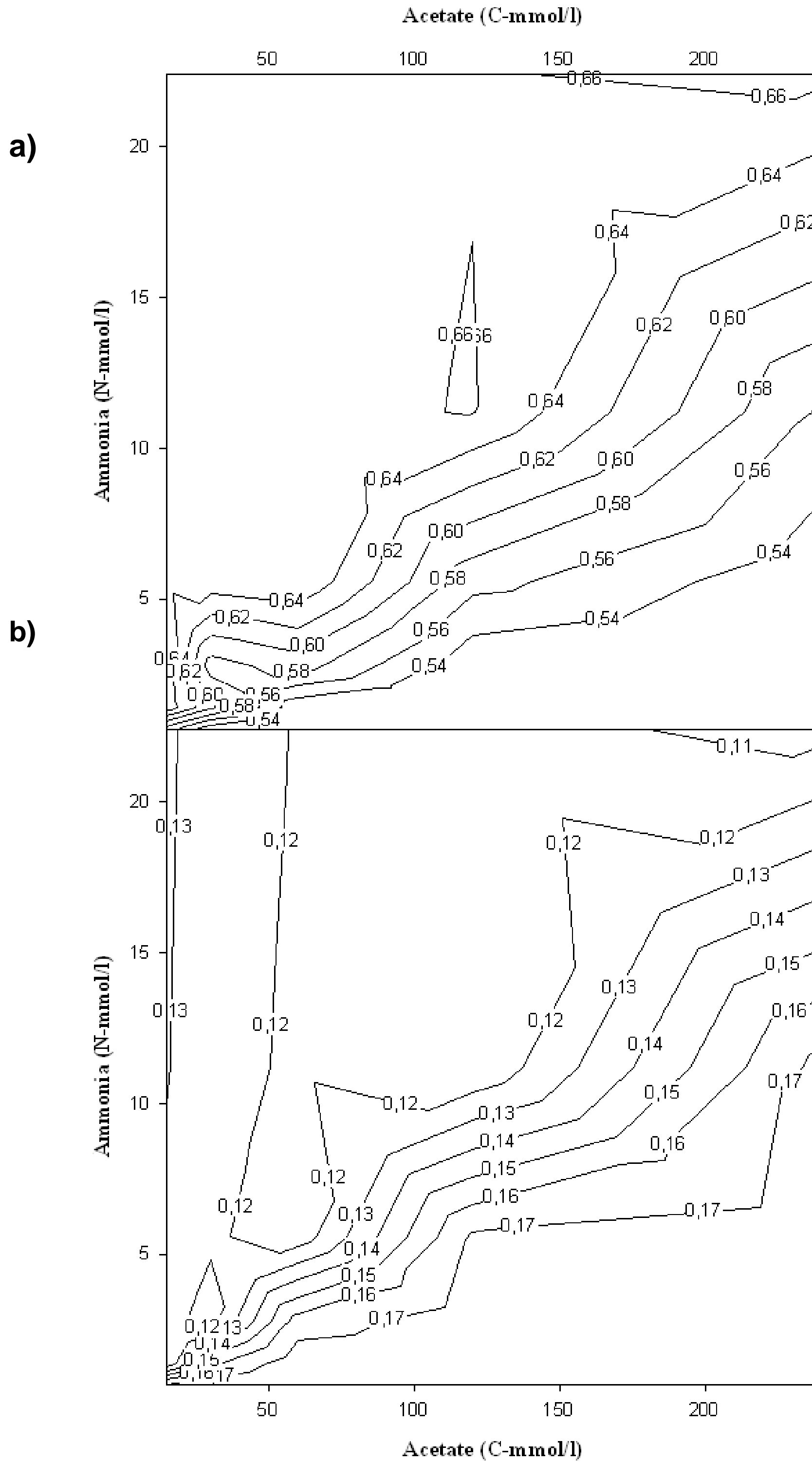


Figure 4.26- Sensitivity analysis of maximum PHB storage capacity on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

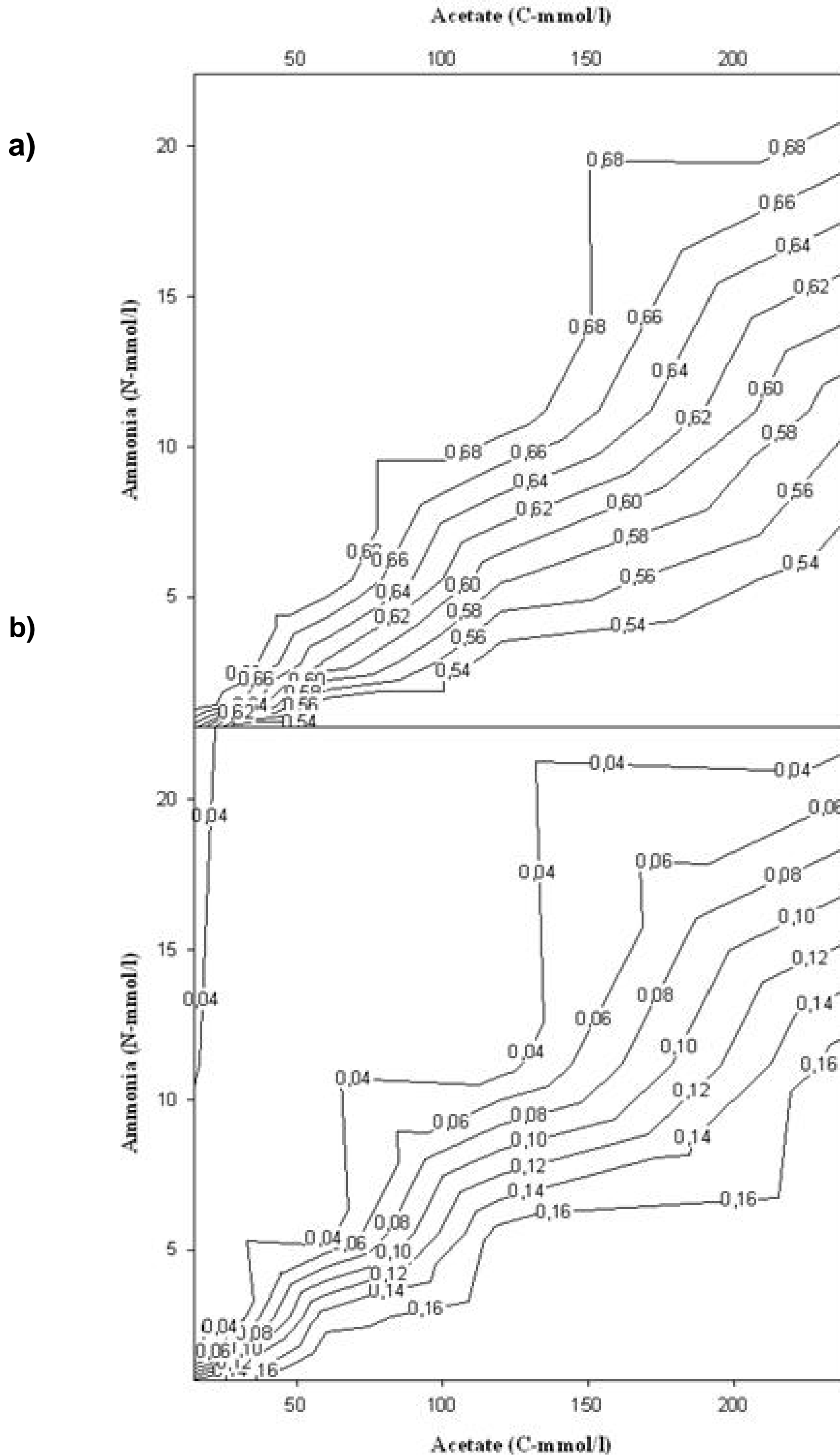


Figure 4.27- Sensitivity analysis of maximum PHB storage capacity on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

a)

b)

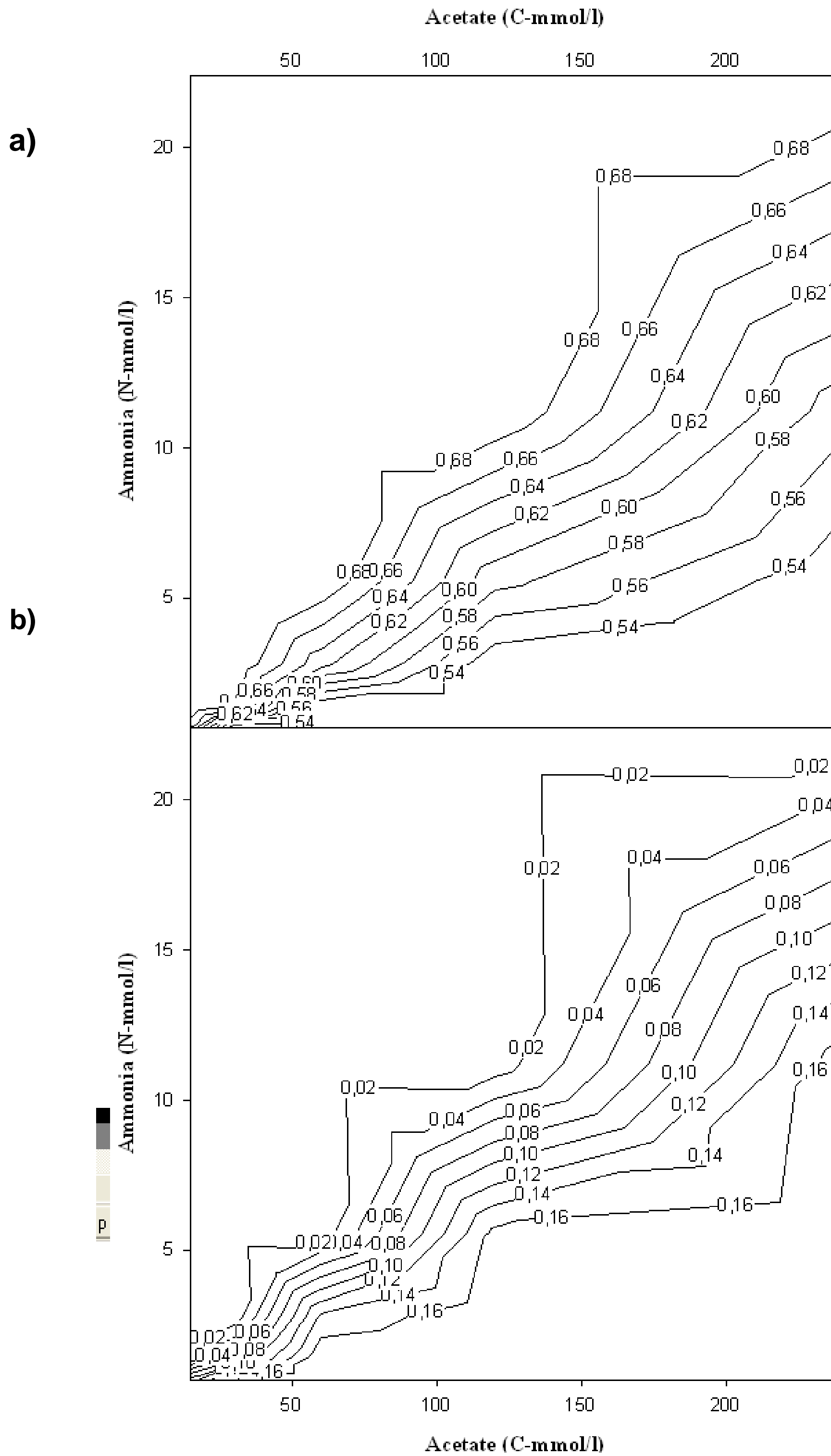


Figure 4.28- Sensitivity analysis of maximum PHB storage capacity on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.4. P/O ratio, or δ

As stated previously, the oxidative phosphorylation efficiency (P/O ratio, or δ) quantifies the amount of ATP produced per molecule of $NADH_2$ oxidized.

According to the results obtained, the selection of the population with the higher P/O ratio is obtained at low C/N ratio. In the table 1 it is represented the metabolic model yields and network stoichiometry, where the higher the P/O ratio, the higher is the yield of PHB on acetate. It is possible to demonstrate also that the high P/O ratio value leads to a low maintenance coefficient. These results are sustained by the experimental data, presented in the study by Dias et al., 2005, where the culture is highly efficient in storing PHB with the maximum theoretical value of P/O ratio.

4.2.4.1. Keeping Sludge

Sensitivity Analysis of Culture Dynamics

N_{cycle}=5

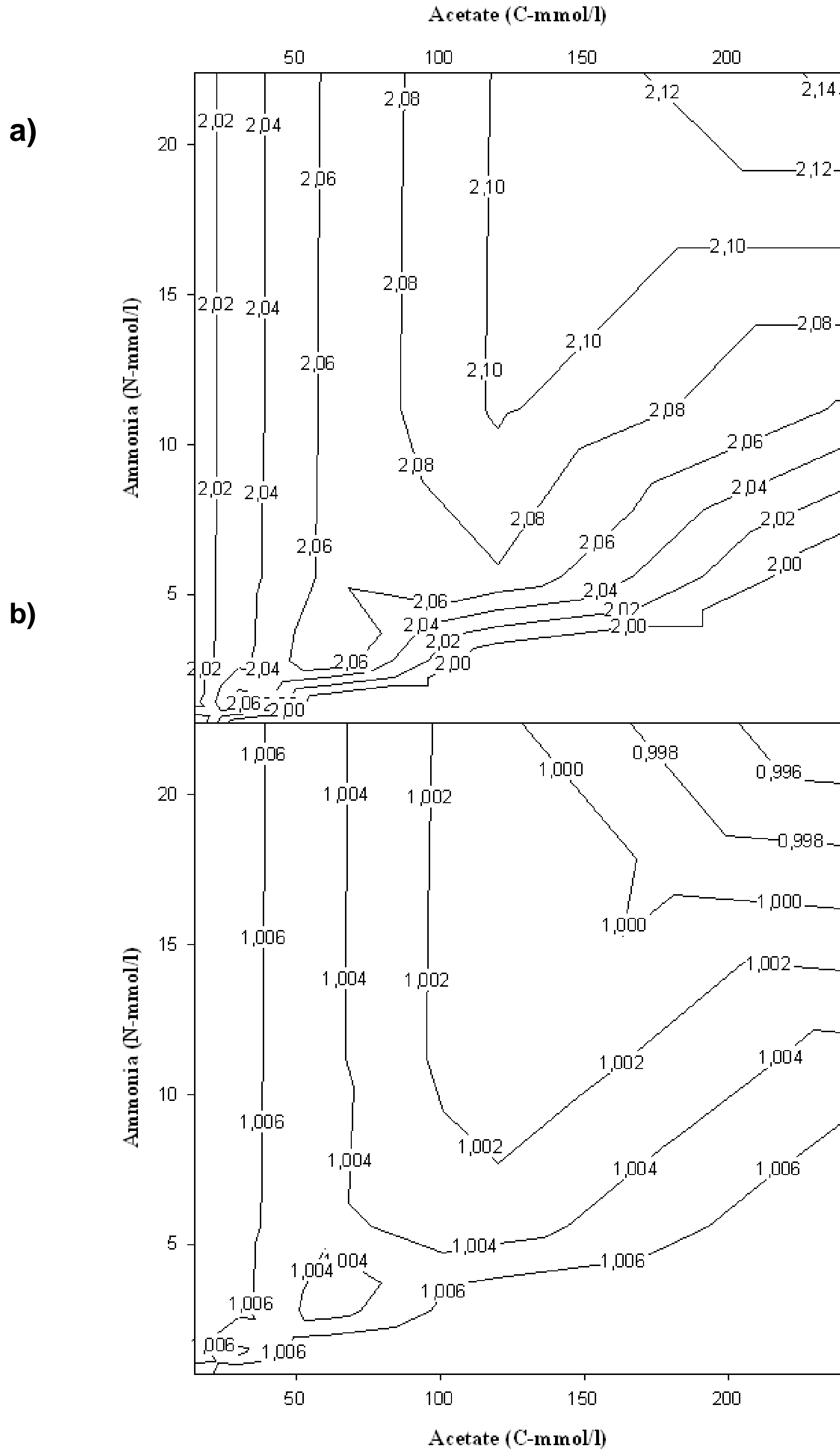


Figure 4.29- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

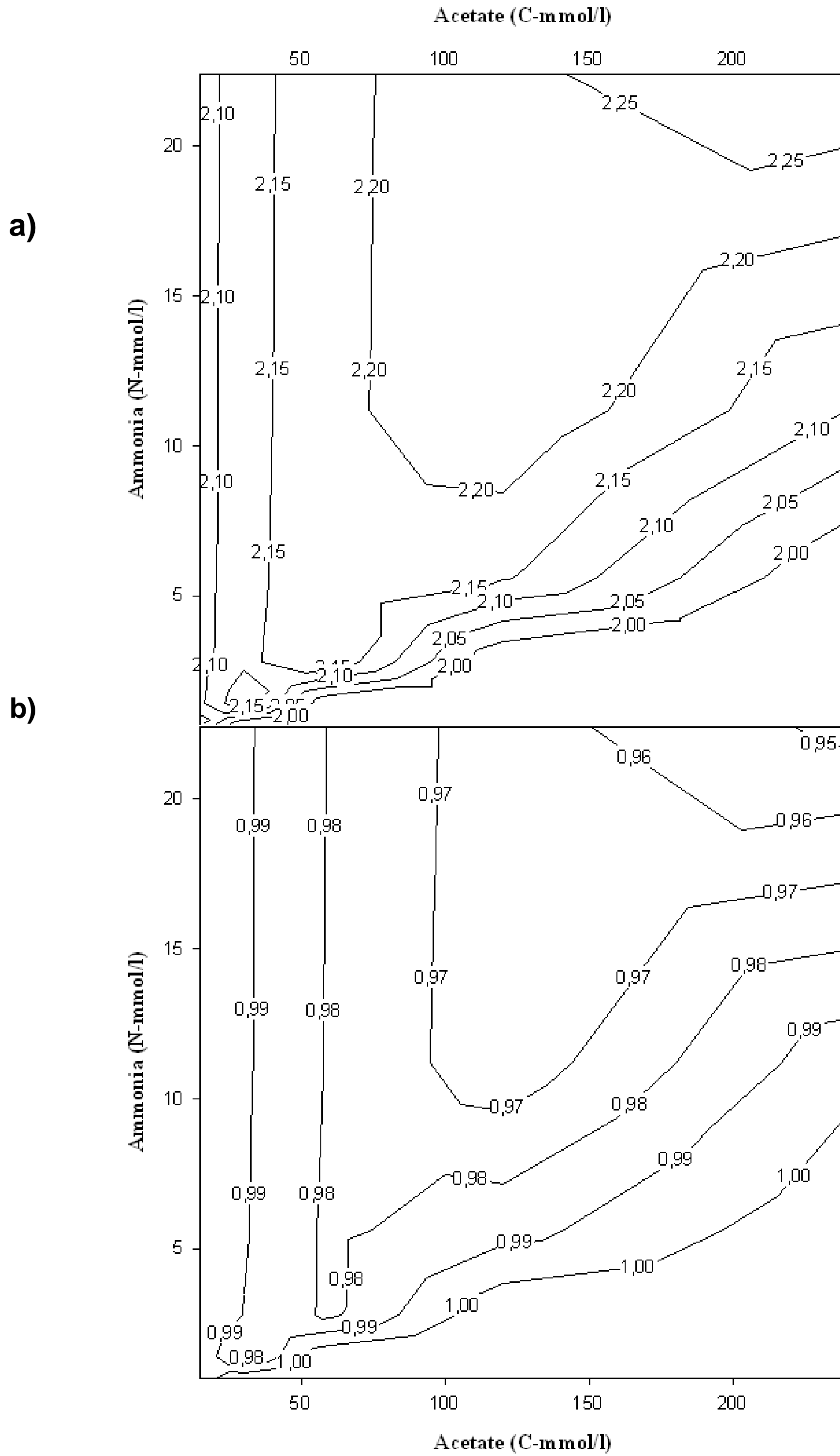


Figure 4.30- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

a)

b)

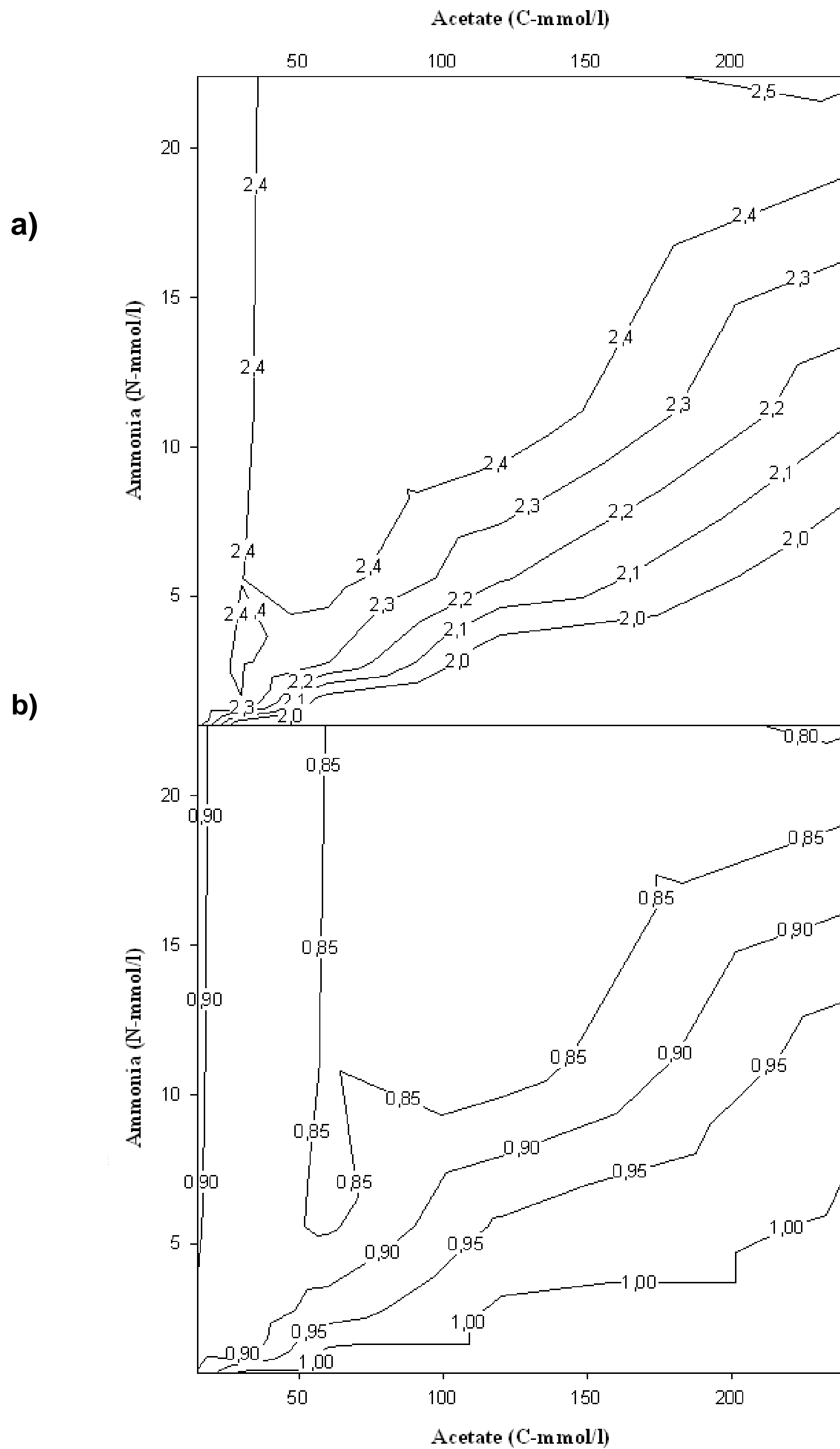


Figure 4.31- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Sludge

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

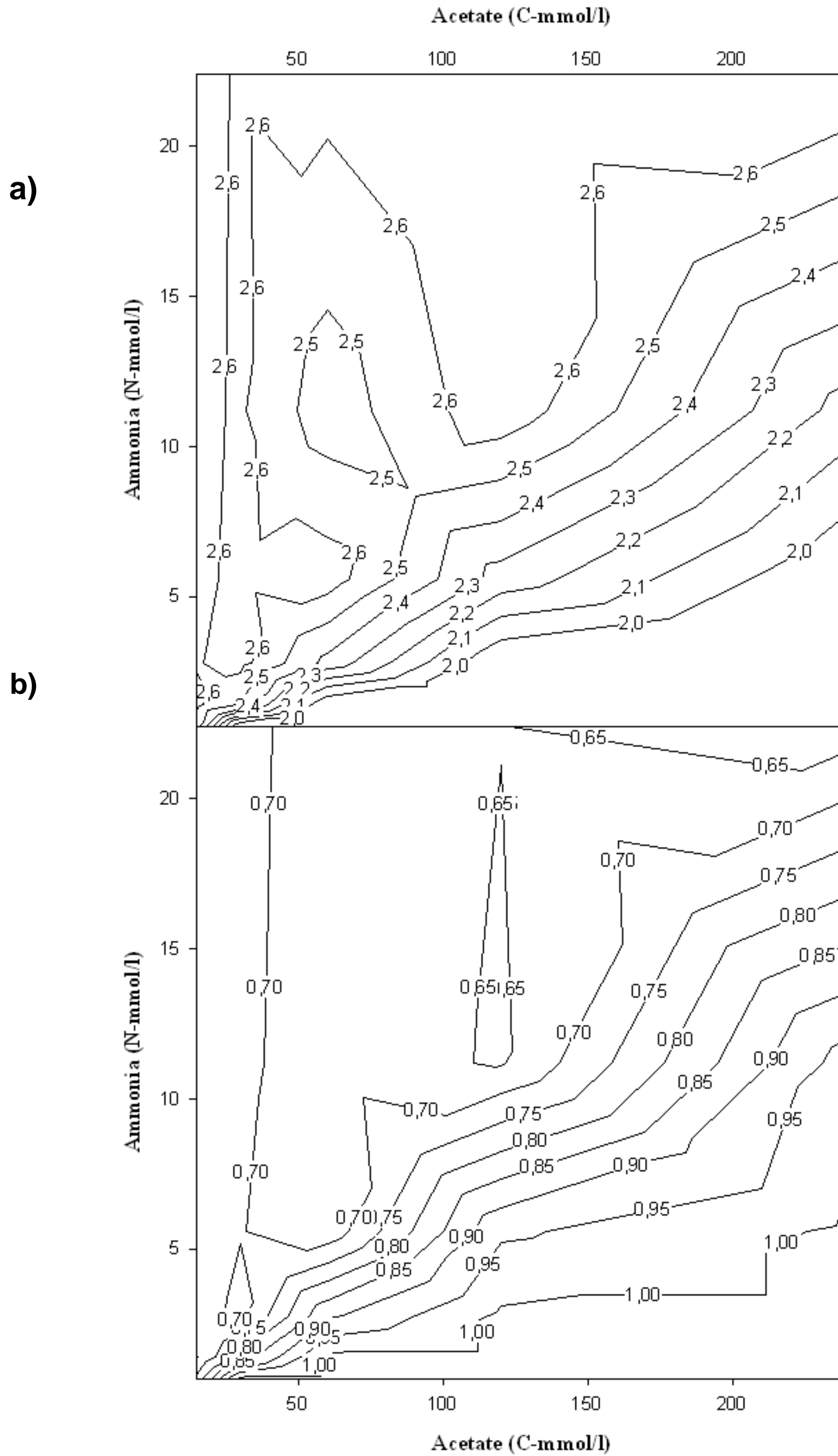


Figure 4.32- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.4.2. Keeping Permeate

Sensitivity Analysis of Culture Dynamics

N_{cycle}=5

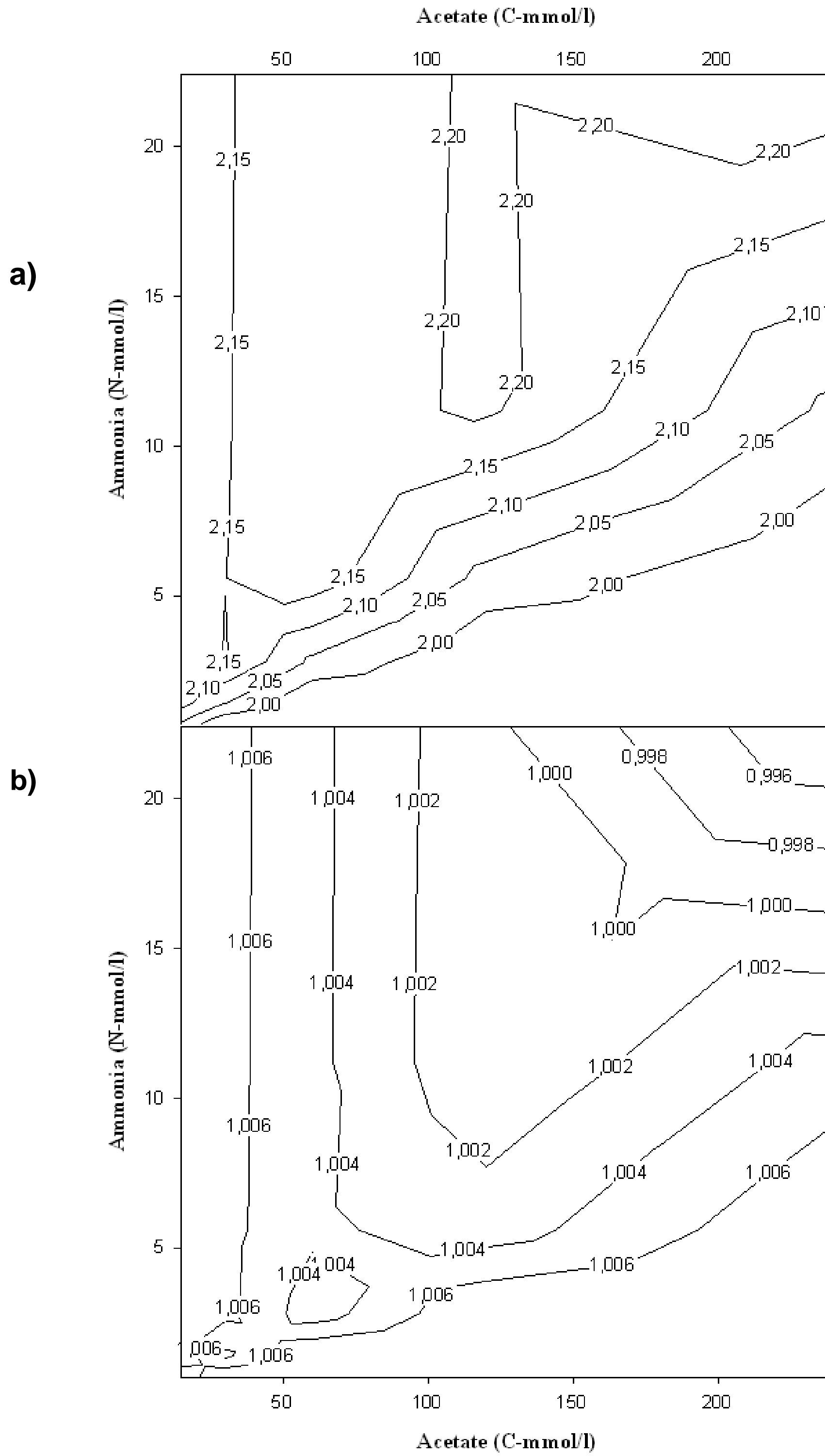


Figure 4.33- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=10

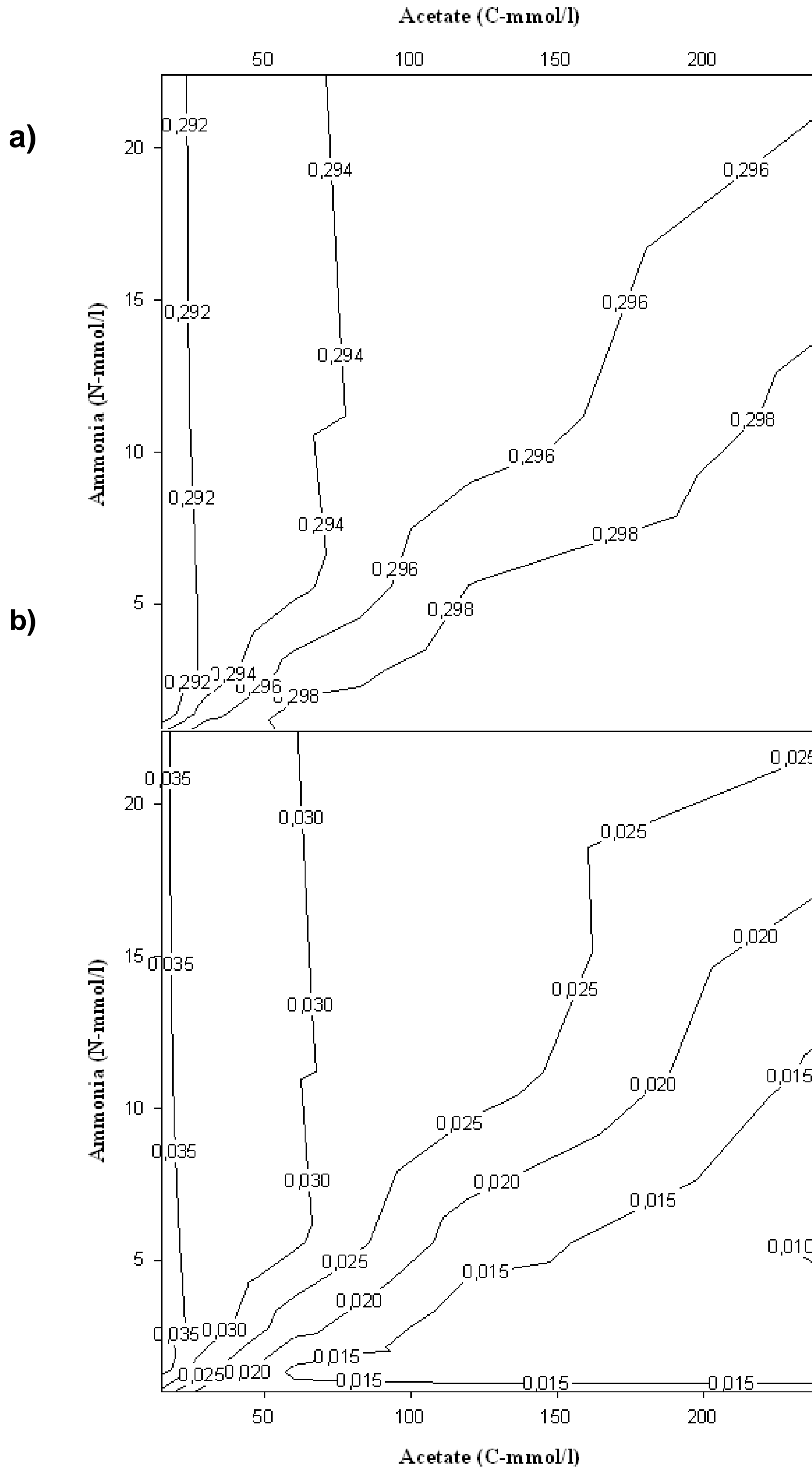


Figure 4.34- Sensitive analysis of P/O ratio on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=20

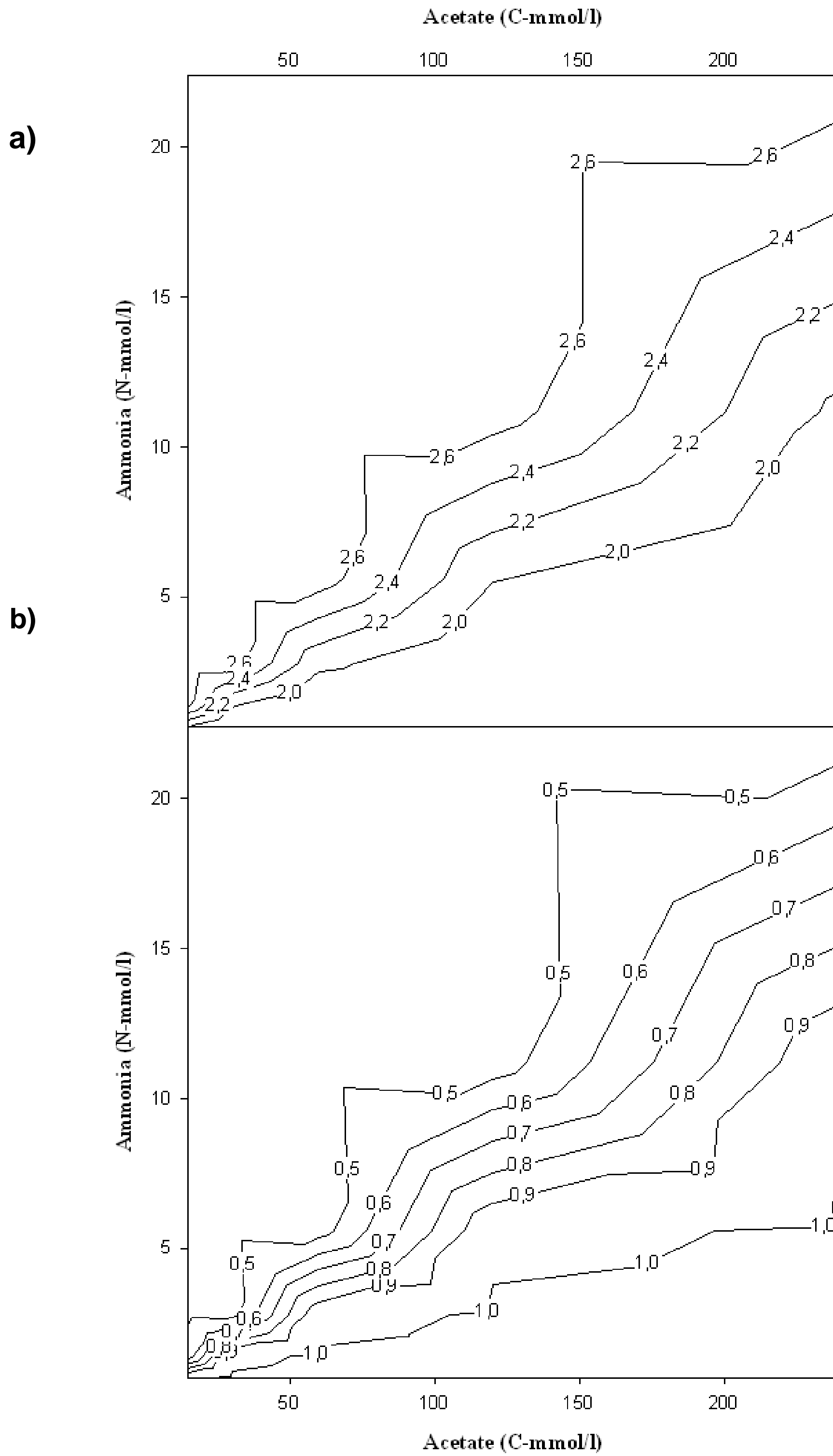


Figure 4.35- Sensitivity analysis of P/O ratio on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

Keeping Permeate

Sensitivity Analysis of Culture Dynamics: N_{cycle}=30

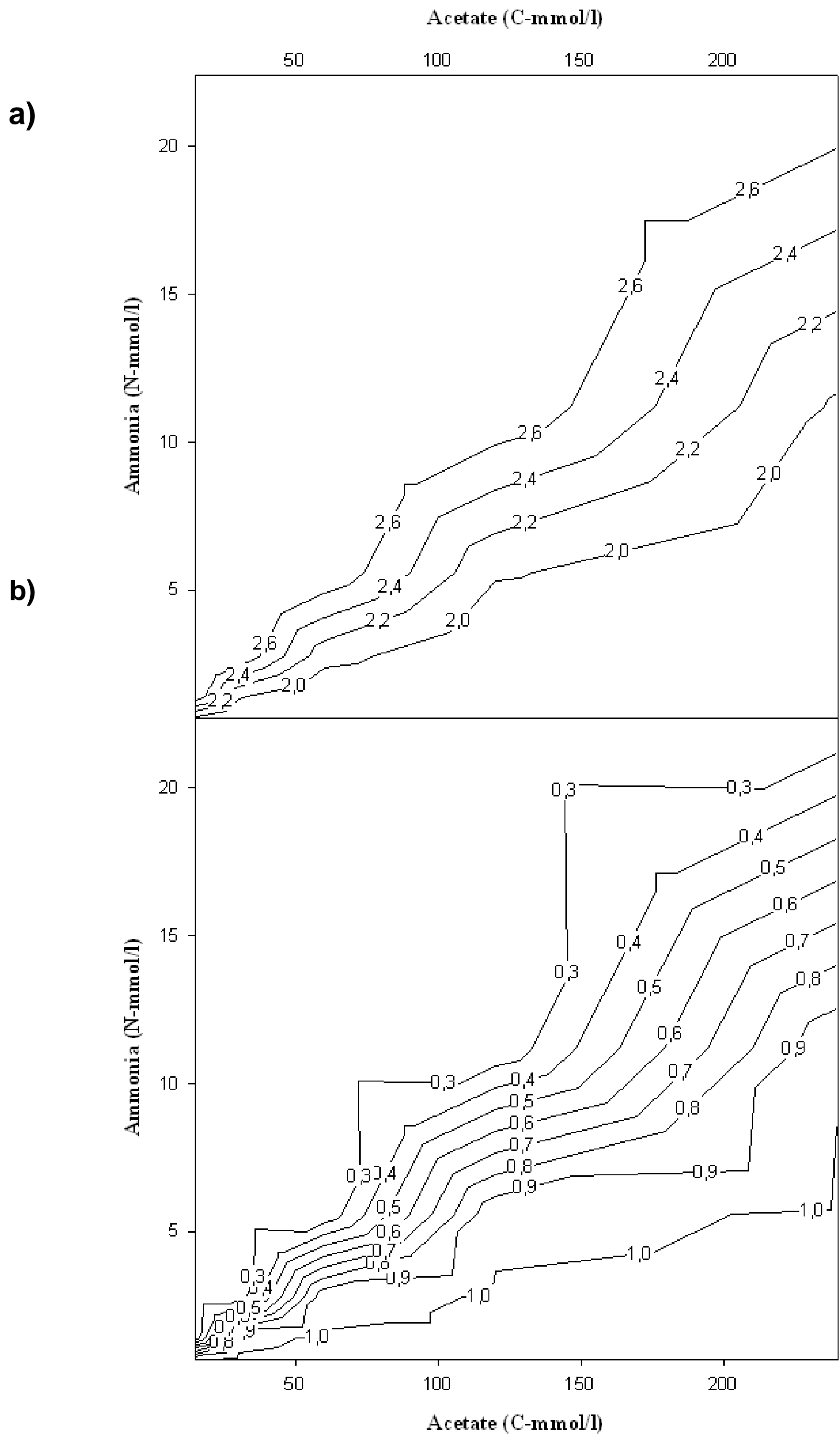


Figure 4.36- Sensitivity analysis of P/O ratio on acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.5. Specific EPS production rate

The average population EPS synthesis rate, $q_{EPS,}$ has shown to be rather insensitive to the different acetate and ammonia feeding conditions, depending only on the number of cycles.

The average EPS synthesis rate tends to increase with increasing number of cycles, achieving the maximum theoretical value imposed, when keeping sludge. In the present study, it is not advantageous that the parameter has high values, because the objective of this work is to minimize this value, and to make sure that it does not affect the amount of PHB produced, because they both compete for the same substrate.

When keeping permeate, the average EPS synthesis rate tends to decrease with increasing number of cycles, achieving the minimal theoretical value imposed. This is the more favorable situation for maximization the volumetric PHB productivity.

Experimental results presented by Wang et al., 2007 support that the specific PHB production rate has an exponential correlation with both specific cell growth rate and EPS production rate, which is strongly influenced by the bacteria growth conditions related to the cultivation time and cell growth [72].

The selection of the populations with low specific EPS production rate will be made by selecting a low SRT. Since it is not sensitive to C/N ratio, it is not possible to determine which ratio is more favorable, depending only on the SRT value.

4.2.5.1. Keeping Sludge

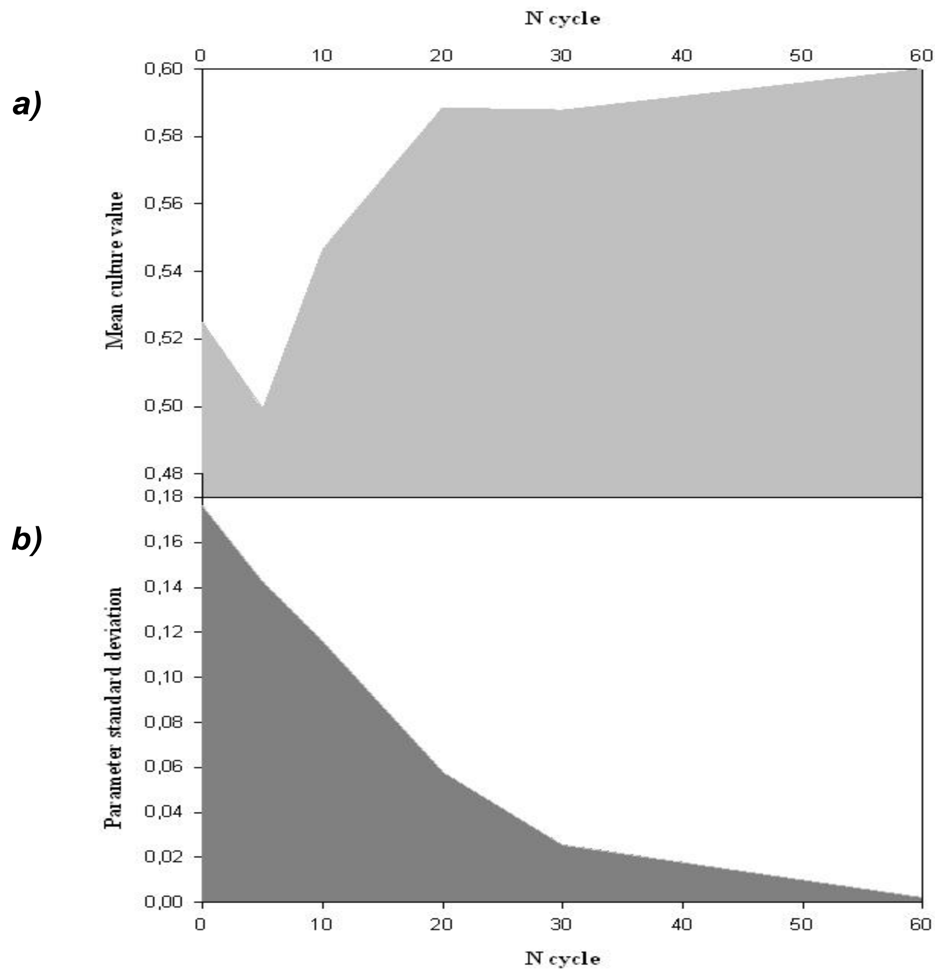


Figure 4.37- Sensitive analysis of specific EPS production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.2.5.2. Keeping Permeate

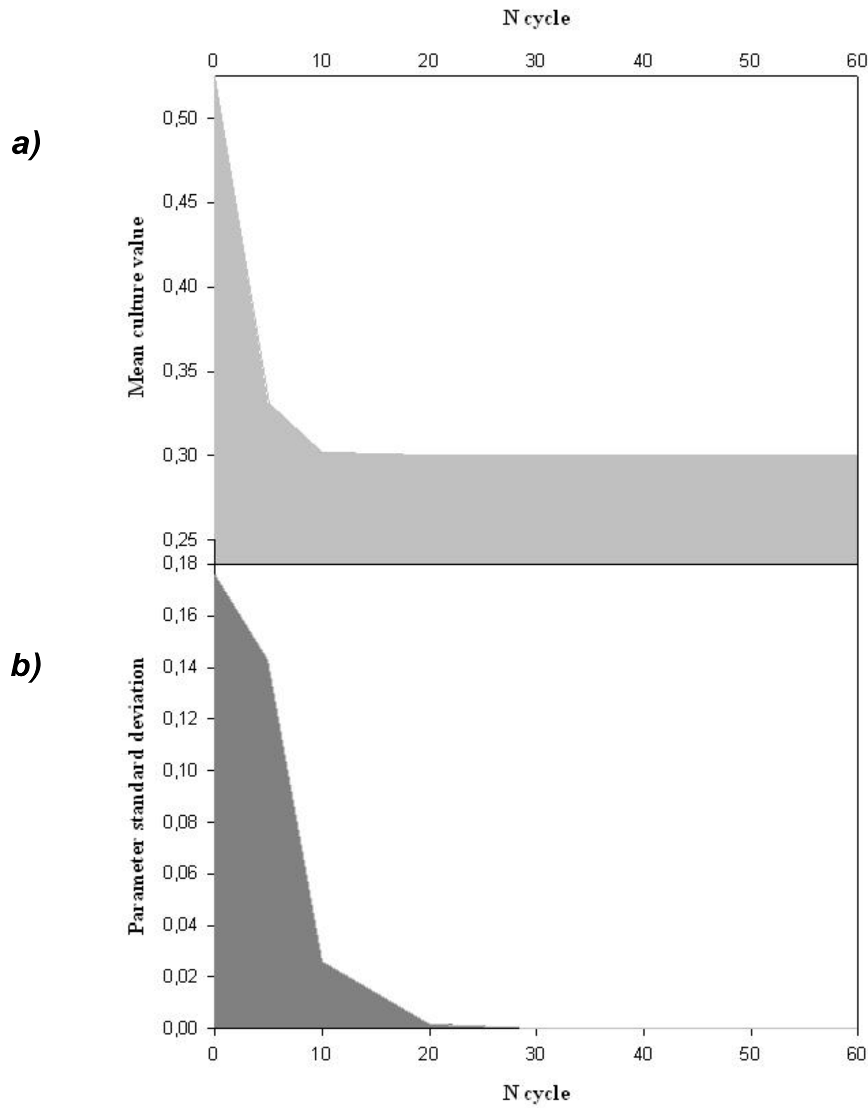


Figure 4.38- Sensitive analysis of specific EPS production rate on SBR acetate and ammonia feeding strategy: a) mean culture value b) parameter standard deviation

4.3. Multi-objective optimization strategy

Through the multiple objective optimization study with NPOP= 8 populations, where one of two different levels (either higher (+) or lower (-)) for, specific PHB production rate, specific EPS production rate, specific growth rate was chosen for each population, it was possible to find the optimal acetate and ammonia feeding strategy corresponding to maximal PHB storage capacity, while maintaining high biomass concentrations at the end of the process. The starting non optimal feeding strategy consisted of constant feedings at the beginning of each cycle of acetate, 120 C-mmol/l, and ammonia, 11.2 N-mmol/l.

The optimization function was constrained by the feeding lower and upper bounds. For the acetate (S) feeding lower and upper is equal to 15 C-mmol/l and 560 C-mmol/l, respectively, while for the ammonia (N) feeding lower and upper bound is equal to 0.7 N-mmol/l and 22.4 N-mmol/l, respectively.

The determination of the amounts of acetate (S) and ammonia (N), at the beginning of each cycle (n), was done indirectly through the yields,

$$\square^{\square}_{\square} = \frac{\square(0)^{\square}}{\square(0)^{\square}} \text{ and } \square^{\square}_{\square} = \frac{\square(0)^{\square}}{\square(0)^{\square}}, \qquad [52]$$

which were the optimization degrees of freedom implemented. This modification proved to facilitate the convergence of the optimizer to the final optimal solution. As shown in the equations, this strategy implies the knowledge of the biomass at the beginning of each cycle.

$$\square^{\square}_{\square} = \frac{\square(0)^{\square}}{\square(0)^{\square}} \text{ and } \square^{\square}_{\square} = \frac{\square(0)^{\square}}{\square(0)^{\square}}, \qquad [53]$$

The multi-objective optimization study had the goal of maximizing the mean of specific growth rate on acetate and on PHB, the mean of maximum PHB storage capacity, the mean P/O ratio, the mean of specific PHB production rate, and the minimization of the specific EPS production rate.

The definitions of the hydraulic retention time and the sludge retention time are the same has in the section 4.2 (sensitivity analysis). The final goal for multi-objective criteria was defined as μ_{max}=0.3, q_{EPS}=0.3, q_{PHB}=0.6, t_{max}=0 and X=200.

Through a run on 30 cycles, with different SRT and HRT values, it was possible to study how keeping the sludge or permeate can affect the optimization of the culture parameters.

With the sensitivity analysis it was possible to confirm that with dynamic feeding conditions, it is possible to select the populations with the highest PHB production capacity. In this section, it is possible to optimize the feeding conditions, in order to achieve the main goals.

The **Tables 4.1 – 4.3** show the results obtained by the optimizations made in *Matlab*. This study was based on absolute feeding mode (i.e. not dependent on biomass concentration), with the amounts of acetate and ammonia fed in each cycle being freely optimized.

In the constant acetate mode (Table and Figure not shown), keeping sludge, there were no meaningful results to analyze.

With an acetate feeding varying from cycle-to-cycle, keeping permeate, the amount of active biomass achieved is not as great as with constant acetate mode, but the parameters do converge to the theoretical optimal values. Through the **Figure 4.41** it is possible to see the optimal cycle-to-cycle acetate and ammonia feeding strategy.

With acetate feeding varying from cycle-to-cycle, keeping sludge, high amounts of active biomass are achieved, but the q_{EPS} parameter is not optimized. In **Figure 4.39**, the acetate and ammonia feeding conditions have the same behavior when keeping permeate.

4.3.1. Keeping Sludge

Multi-objective optimization

Absolute Feeding Mode; Acetate Mode Varies each Cycles

SRT	HRT	n_{pack}	d_{pack}	μ_{max}	q_{EPS}	q_{PHB}	t_{max}	Biomass
36	24	20	1	0,288	0,500	0,545	79,29	40,18
48	36	30	1	0,288	0,520	0,582	115,86	169,19
60	24	30	1	0,291	0,600	0,559	180,32	307,90
60	36	30	1	0,279	0,552	0,580	64,20	127,90

Table 4.1- Multi-objective optimization for absolute feeding and Acetate Mode Varies in each cycle

In these conditions, high specific growth rate, high specific EPS production rate and high specific PHB production rate values are achieved. These results demonstrate that it is difficult to achieve low EPS production rate values.

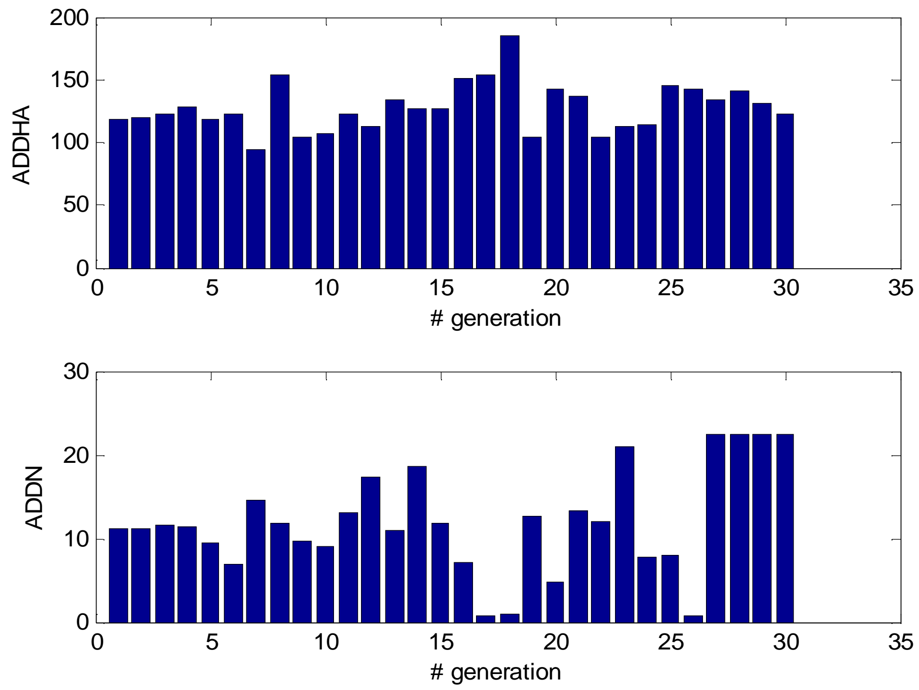


Figure 4.39- Multi-objective optimization results of acetate and ammonia feeding strategy. For SRT= 48 h and HRT= 36 h.

4.3.2. Keeping Permeate

Multi-objective optimization

Absolute Feeding Mode; Constant Acetate Mode

SRT	HRT	n_{pack}	d_{pack}	μ_{max}	q_{EPS}	q_{PHB}	t_{max}	Biomass
36	24	20	1	0,2987	0,3038	0,5904	101,5108	90,1328
36	24	30	1	0,2999	0,3105	0,5975	169,1821	94,1541
48	24	20	1	0,2992	0,3001	0,5831	122,9799	57,4825
48	24	30	1	0,3000	0,3121	0,5990	166,9149	78,5198
48	36	10	1	0,2969	0,3502	0,5333	54,3102	72,4334
60	24	20	1	0,2995	0,3010	0,5929	107,5448	91,6890
60	24	30	1	0,3000	0,3000	0,5966	73,7741	86,4292
60	36	10	1	0,2965	0,3957	0,5557	73,7750	65,0853
60	36	20	1	0,2998	0,3005	0,5931	134,3142	22,6272
60	36	30	1	0,3000	0,3000	0,5989	184,1981	23,1985
60	48	20	1	0,3000	0,4001	0,5941	157,3789	59,4297
60	48	30	1	0,3000	0,3324	0,5983	198,5297	66,0906

Table 4.2- Multi-objective optimization for absolute feeding and Constant Acetate Mode

In these conditions the low EPS production rate, high specific growth rate and high specific PHB production rate values are achieved, in different cycles.

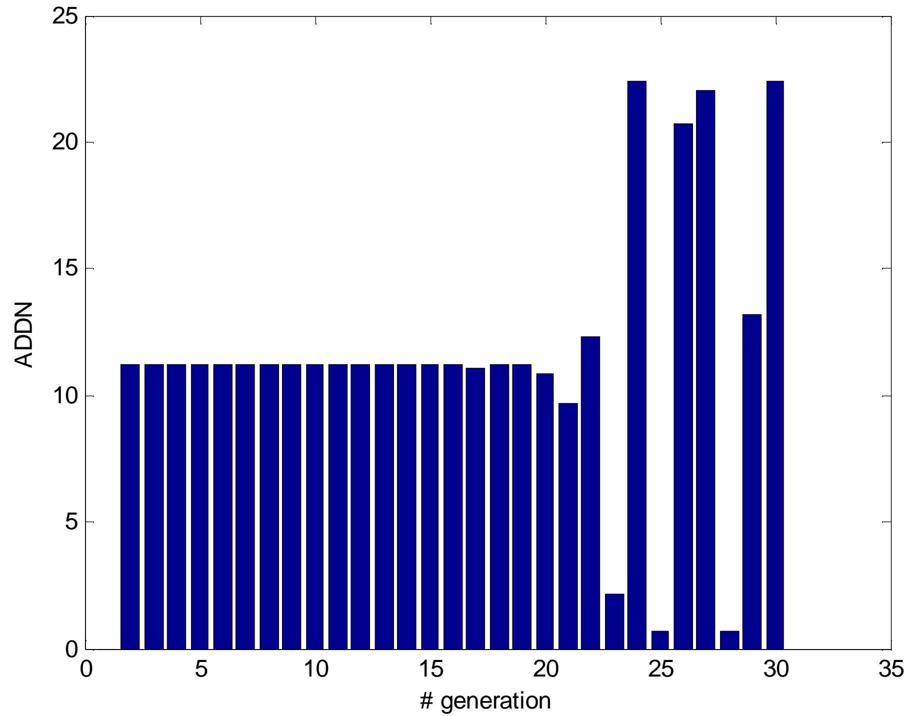


Figure 4.40- Multi-objective optimization results of ammonia feeding strategy. For SRT= 36 h and HRT= 24 h.

Multi-objective optimization

Absolute Feeding Mode; Acetate Mode Varies each Cycles

SRT	HRT	n_{pack}	d_{pack}	μ_{max}	q_{EPS}	q_{PHB}	t_{max}	Biomass
36	24	5	2	0,278	0,334	0,538	36,515	19,533
36	24	10	2	0,299	0,304	0,571	123,800	81,706
48	24	10	2	0,299	0,300	0,590	84,262	96,720
60	24	5	2	0,294	0,319	0,597	60,017	42,469
60	24	10	2	0,300	0,300	0,578	133,715	151,669
48	36	10	2	0,300	0,303	0,584	111,630	22,186
48	36	20	1	0,300	0,310	0,575	126,270	0,7827
60	36	10	2	0,300	0,312	0,591	113,468	59,365
60	48	10	2	0,300	0,360	0,588	125,999	65,483

Table 4.3- Multi-objective optimization for absolute feeding and Acetate Mode Varies in each cycle

In these conditions the low EPS production rate, high specific growth rate and high specific PHB production rate values are achieved. These goals are reached in different cycles.

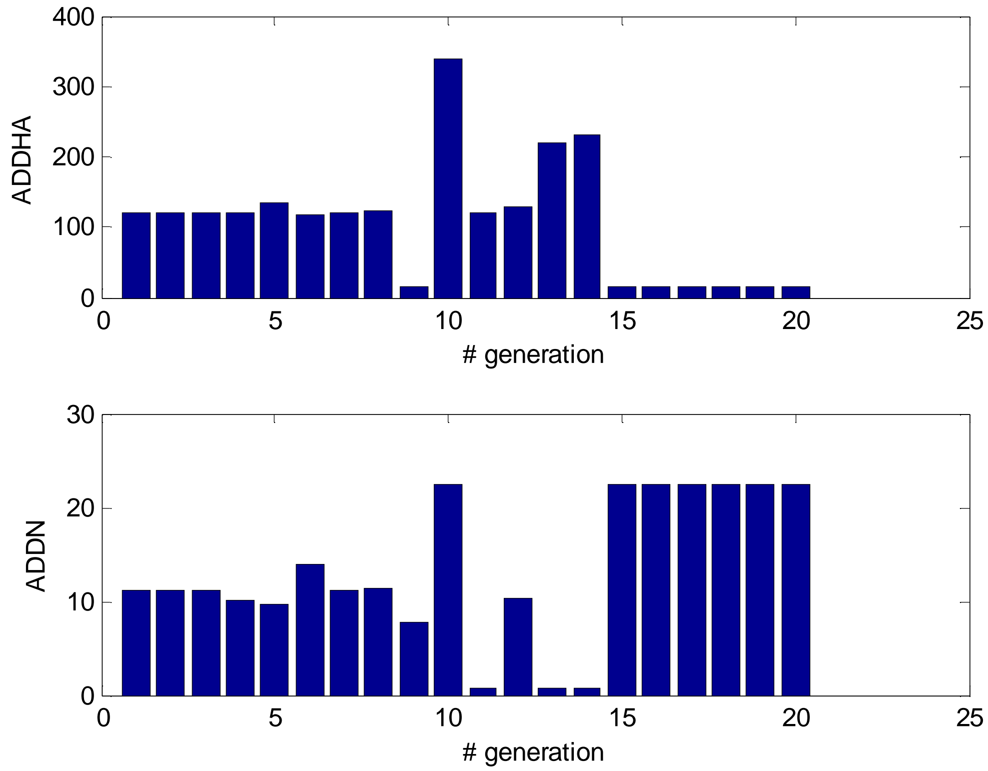


Figure 4.41- Multi-objective optimization results of acetate and ammonia feeding strategy. For SRT= 48 h and HRT= 36 h.

5. Conclusions

The present M.Sc. thesis demonstrates through mathematical modeling and model based optimization studies that in theory the MMC process for PHB synthesis can be highly effective with competitive PHB volumetric productivity if more sophisticated process control schemes of population dynamics are developed and implemented at the macroscopic level. Indirectly this study shows that it is possible to produce PHB by mixed cultures as an alternative to pure cultures that since the beginning were one of the factors that made the PHB production economically inefficient.

The first step of this study was to formulate and implement in a computer program a hypothetical segregated population model based on a previously developed metabolic model. The results showed that the hypothetical segregated model fits well (**Figure 4.1 – 4.4**) with experimental data, which leads to the conclusion that the selected kinetic parameters values are consistent with the real process, showing a consistency with the typical dynamical behavior.

A sensitivity analysis was performed of key population kinetic parameters (PHB_{max}, $\mu_{S,max}$, $\mu_{PHB,max}$, q_{PHB}, P/O ratio and q_{EPS}) over reactor degrees of freedom. This analysis was made based on the sensitivity of these parameters towards the dynamic feeding conditions of acetate and ammonia. The overall results demonstrate clearly that the ammonia and acetate feeding, in each cycle, are key factors for the enrichment process.

The more specific conclusions to be taken from this study are the following:

- The q_{EPS} parameter is very difficult to control and is not sensitive to the manipulation on the acetate and ammonia feeding, showing only sensitivity to different values of SRT. In this case, the selection of populations with low EPS production rate is based on the low values of SRT;

- The P/O ratio is a difficult parameter to analyze, because the sensitivity through the different feeding is not very high. This parameter achieve values close to the theoretical maximum, but only at low SRT, making this factor an important one for the selection of populations with high P/O ratios, thus high values of P/O ratio lead to low maintenance coefficients (equations **[2.10]** and **[2.21]**). This parameter has shown to be more sensitive to the C/N ratio variations when keeping permeate, than when keeping sludge;

- The selection of populations with high specific PHB production rates is more effective under low C/N ratio conditions. C/N ratio is an important factor in the selection of populations based on this parameter. q_{PHB} is directly related to the specific EPS production rate, because the microorganisms convert the substrate into EPS and PHB. The effect of high PHB accumulation lead to a less amount of EPS produced, and the objective is to channel the substrate towards the PHB production and cell growth;

- The specific growth rate is a highly sensitive parameter of the dynamic feeding conditions, e. g., is highly sensitive to C/N ratio, converging to high values when C/N ratio is low, because when the C/N ratio is high, the amount of ammonia supplied depletes before reaching famine phase;

- The maximum PHB intracellular content showed to be sensitive to the C/N ratio and SRT. The population selection is made at low SRT values, because at high values the washout of the competing populations becomes negligible. This kinetic parameter shows to be more sensitive to keeping permeate conditions, than keeping sludge;

- The lower the SRT values are, the faster the population selection is.

The last step of this dissertation was the multi-objective optimization. Through this study it is was possible to optimize the process by maximizing the population mean $\mu_{PHB,max}$, mean $\mu_{S,max}$, mean PHB_{max}, mean P/O ratio and mean $q_{PHB,max}$, and minimizing the overall time of selection and the population mean $q_{EPS,max}$, and also the difference between the initial and the final active biomass concentration. Results show that through dynamic acetate and ammonia feeding conditions, it is possible to achieve the optimal values of the parameters permitting to increase the speed of selection of the best microbial population that has the highest PHB storage capacity and the highest cell growth rate, and it was also possible to see how the SRT and HRT values affect the PHB production. Results also show that is not possible to minimize the specific EPS production rate when keeping sludge, and the best condition to minimize this kinetic parameter is keeping permeate.

Many of these conclusions are supported by experimental data obtained in published studies, but others require the execution of new experiments to confirm results. As final conclusion it can be stated that this M.Sc. thesis provides new guidelines for process design and control with a strong theoretical basis but that were not yet proven experimentally. Some of these ideas could possibly lead to the development of a more competitive PHB production process by MMC.

6. References and Notes

[1]. Doty, L. F., 2005, *"Uma Breve Visão Geral sobre Plásticos Biodegradáveis"*, PhD Thesis, Oxo-biodegradable Plastics Institute, Edmonton AB, Canada;

[2]. Lemos, P. C., Serfim, L., Reis, M. A. M., 2006, *"Synthesis of polyhydroxyalkanoates from diferent short – chain fatty acids by mixed cultures submitted to aerobic dynamic feeding"*, Journal of Biotechnology 122, 226 – 238;

[3]. W. Punrattanasim, 2001, *"The Utilization of Activated Sludge Polyhydroxyalkanoates for the Production of Biodegradable Plastics"*, PhD Thesis, Virginia Polytechnic Institute and State University, Blacksburg, EUA;

[4]. Dias, J. M. L., Lemos, P. C., Serafim, L. S., Oliveira, C., Eiroa, M., Albuquerque, M. G. E., Ramos, A. M., Oliveira, R., Reis, M. A. M., 2006, *"Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product"*, Macromol. Biosci., 6, 885-906;

[5]. Serafim, L. S., Lemos, P. C., Oliveira, R., Reis, M. A. M., 2004, *"Optimization of Polyhydroxybutyrate Production by Mixed Cultures Submitted to Aerobic Dynamic Feeding Conditions"*, Biotechnology and Bioengineering, Vol. 87, No. 2;

[6]. Beccari, M., Dionisi, D., Giuliani, A., Majone, M., Ramadori, R., 2002, *"Effect of different carbon sources on aerobic storage by activated sludge",* Water Science and Technology, Vol 45 No 6 pp 157 – 168;

[7]. Dias, J. M. L., Serafim, L. S., Lemos, P. C., Reis, M. A. M., Oliveira, R., 2005, *"Mathematical Modelling of a Mixed Culture Cultivation Process for the Production of Polyhydroxybutyrate"*, Biotechnology and Bioengineering, Vol. 92, No. 2;

[8]. Dionisi, D., Majone, M., Tandoi, V., Beccari, M., 2001, *"Sequencing Batch Reactor: Influence of Periodic Operation on Performance of Activated Sludges in Biological Wastewater Treatment"*, Ind. Eng. Chem. Res., 40, 5110 – 5119;

[9]. Beun, J. J., Dircks, K., Van Loosdrecht, M. C. M., Heijnen, J. J., 2002, *"Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures"*, Water Research 36, 1167 – 1180;

[10]. Beun, J. J., , Paletta, F., Van Loosdrecht, M. C. M., Heijnen, 2000, J. J., *"Stoichiometry and Kinetics of Poly-β-hydroxybutyrate Metabolism in Aerobic, Slow Growing, Activated Sludge Cultures"*, Biotechnology and Bioengineering, Vol. 67, No. 4;

[11]. Third, K. A., Newland, M., Cord-Ruwisch, R., 2003, *"The Effect of Dissolved Oxygen on PHB Accumulation in Activted Sludge Cultures"*, Biotechnology and Bioengineering, Vol. 82, No. 2;

[12]. Martins, A. M. P., Heijnen, J. J., van Loosdrecht, M. C. M., 2003,*"Effect of feeding pattern and storage on the sludge settle ability under aerobic conditions"*, Water Research 37, 2555 – 2570;

[13]. van Aalst-van Leeuwen, M. A., Pot, M. A., van Loosdrecht, M. C. M., Heijnen, J. J., 1997, *"Kinetic Modeling of Poly(β-hydroxybutyrate) Production and Consumption by Paracoccus*

pantotrophus under Dynamic Substrate Supply", Biotechnology and Bioengineering, Vol. 55, No. 5;

[14]. Balku, S., Berber, R., 2006, *"Dynamics of an activated sludge process with nitrification and denitrification: Start – up simulation and optimization using evolutionary algorithm"*, Computers and Chemical Engineering 30, 490 – 499;

[15]. Banga, J. R., Balsa – Canto, E., Moles, C. G., Alonso, A. A., 2003, *"Dynamic Optimization of Bioreactors: a Review"*, Proc Indian Natn Sci Acad, **69, A,** Nos. 3 & 4, pp. 257 – 265;

[16]. Franco-Lara, E., Wenster-Botz, D., 2005, *"Estimation of optimal feeding strategies for fed-batch bioprocesses"*, Biopercess Biosyst Eng, 27:255 – 262;

[17]. Oehmen, A., Yuan,Z., Blackall, L. L., Keller, J., 2005, *"Comparison of Acetate and Propionate Uptake by Polyphosphate Accumulating Organisms and Glycogen Accumulating Organisms"*, Biotechnology and Bioengineering, Vol. 91, No. 2;

[18]. Liu, Y. Q., Tay, J. H., 2007, *"Characteristics and stability of aerobic granules cultivated with different starvation time"*, Appl Microbiol Biotechnol, 75:205 – 210;

[19]. Wang, X. H., Zhang, H. M., Yang, F. L., Xia, L. P., Gao, M. M., 2007, *"Improved stability and performance of aerobic granules under stepwise increased selection pressure"*, Enzyme and Microbial Tecnology 41, 205 – 211;

[20]. Sheintch, M., Tartakovsky, B., 1997, *"Activated – sludge system design for species selection: analysis of a detailes multispecies model"*, Chemical Engineering Science, Vol. 52, No. 17, pp. 3033 – 3046;

[21]. Martins, A. M., Heijnen, J. J., van Loosdrecht, M. C. M., 2003, *"Effect of dissolved oxygen concentration on sludge settleability"*, Appl Microbiol Biotchnol, 62: 586 – 593;

[22]. Pan, S., Tay, T.-H., He, Y.-X., Tay, S. T.-L., 2004, *"The effect of hydraulic retention time on the stability of aerobically grown microbial granules"*, Letters in Applied Microbiology, 38, 158 e 160;

[23]. Moy, B. Y.-P., Tay J.-H., Toh, S.-K., Liu, Y., Tay, S. T.-L., 2002, *"High organic loading influences the physical characteristics of aerobic sludge granules",* Lettes in Applied Microbiology, 34, 407 – 412;

[24]. Kim, H., Pagilla, K. R., 2000, *"Competitive Growth of Nocardia and Acinetobacter under Anaerobic/Aerobic Batch Operation"*, Water Research. Vol. 34, No. 10, pp. 2667 – 2674;

[25]. Tay, J.-H., Yang, S.-F., Liu, Y., 2002, *"Hydraulic selection pressure-induced nitrifying granulation in sequencing batch reactors"*, Appl Microbiol Biotechnol, 59:332-337;

[26]. Dionisi, D., Majone, M., Miccheli, A., Puccetti, C., Sinisi, C., 2004, *"Glutamic Acid Removal and PHB Storage in the Activated Sludge Process under Dynamic Conditions"*, Biotechnology and Bioengineering, Vol. 86, No. 7;

[27]. Dionisi, D., Beccari, M., Di Gregorio, S., Majone, M., Papini, M. P., Vallini, G., 2005, *"Storage of biodegradable polymers by na enriched microbial community in a sequencing batch reactor operated at high organic load rate"*, J. Chem. Technol. Biotechnol. 80: 1306-1318;

[28]. Levantesi, C., Serafim, L. S., Crocetti, G. R., Lemos, P. C., Rossetti, S., Blackall, L. L., Reis, M. A. M., Tandoi, V., 2002, *"Analysis of the microbial community structure and function of a*

laboratory scale enhanced biological phosphorus removal reactor”, Environmental Microbiology, 4, 559-569;

[29]. Aulenta, F., Dionisi, D., Majone, M., Parisi A., Ramadori, R., Tandoi, V., 2003, *“Effect of periodic feeding in sequencing batch reactor on substrate uptake and storage rates by a pure culture of Amaricoccus kaplicensis”*, Water Research 37, 2764-2772;

[30]. Choi, J., Lee, S. Y., 1999, *“Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation”*, Appl. Microbiol. Biotechnol., 51: 13-21;

[31]. Dircks, K., Beun, J. J., van Loosdrecht, M., Heijnen, J. J., Henze, M., 2001, *“Glycogen Metabolism in Aerobic Mixed Cultures“*, Biotechnology and Bioengineering, Vol. 73, No. 2;

[32]. Karahan, O., van Loosdrecht, M. C. M., Orhon, D., 2006, *“Modeling the Utilization of Starch by Activated Sludge for Simultaneous Substrate Storage and Microbial Growth“*, Biotechnology and Bioengineering, Vol. 94, No. 1;

[33]. Yamane, T., Fukunaga, M., Lee, Y. W., 1996, *“Increased PHB Productivity by High-Cell-Density Fed-Batch Culture of Alcaligenes latus, a Growth-Associated PHB Producer”*, Biotechnology and Bioengineering, Vol. 50, Pp. 197-202;

[34]. Dionisi, D., Majone, M., Papa, V., Beccari, M., 2004, *“Biodegradable Polymers From Organic Acids by Using Activated Sludge Enriched by Aerobic Periodic Feeding”*, Biotechnology and Bioengineering, Vol. 85, No. 6;

[35]. Beccari, M., Majone, M., Massanisso, P., Ramadori, R., 1998, *“A Bulking Slude with High Storage Response Selected under Intermittent Feeding”*, Water Research, Vol. 32, No. 11, pp. 3403-3413;

[36]. Carta, F., Beun, J. J., van Loosdrecht, M. C. M., Heijnen, J. J., 2001, *“Simultaneous Storage and Degradation of PHB and Glycogen in Activated Sludge Cultures“*, Water Research, Vol. 35, No. 11, pp. 2693-2701;

[37]. Dionisi, D., Carucci, G., Papini, M. P., Riccardi, C., Majone, M., Carrasco, F., 2005, *“Olive oil mill effluents as a feedstock for production of biodegradable polymers”*, Water Research 39, 2067-2084;

[38]. Rhu, D. H., Lee, W. H., Kim, J. Y., Choi, E., 2003, *“Polydyhroxyalkanoate (PHA) production from waste”*, Water Science Technology, Vol. 48, pp. 221-228;

[39]. Carucci, A., Dionisi, D., Majone, M., Rolle, E., Smurra, P., 2001, *“Aerobic Storage by Activated Slude on Real Wastewater“*, Water Research Vol. 16, pp. 3833-3844;

[40]. Sin, G., Guisasola, A., De Pauw, D. J. W., Baeza, J. A., Carrera, J., Vanrolleghem, P. A., 2005, *“A New Approach for Modelling Simultaneous Storage and Growth Processes for Activated Sludge Systems Under Aerobic Conditions”*, Biotechnology and Bioengineering, Vol. 92, No. 5;

[41]. Chua, A. S. M., Takabatake, H., Satoh, H., Mino, T., 2003, *“Production of Polyhydroxyalkanoates (PHA) by activated sludge treating municipal wastewater: effect of pH, sludge retention time (SRT), and acetate concentration in influent”*, Water Research 37, 3602-3611;

[42]. Grothe, E., Chisti, Y., 2000, *"Poly(β-hydroxybutyric acid) thermoplastic production by Alcaligenes latus: Behavior of fed-batch cultures"*, Bioprocess Engineering 22, 441-449;

[43]. Ahn, W. S., Park, S. J., Lee, S. Y., 2000, *"Production of Poly(3-Hydroxybutyrate) by Fed-Batch Culture of Recombinant Escherichia coli with a Highly Concentrated Whey Solution"*, Applied and Environmental Microbiology, Vol. 66, No. 8, p. 3624-3627;

[44]. Park, S. J., Ahn, W. S., Green, P. R., Lee, S. Y., 2001, *"Production of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) by Metabolically Engineered Escherichia coli Strains"*, Biomacromolecules, 2, 248-254;

[45]. Pratt, S., Yuan, Z., Gapes, D., Dorigo, M., Zeng, R. J., Keller, J., 2003, *"Development of a Novel Titration and Off-Gas Analysis (TOGA) Sensor for Study of Biological Processes in Wastewater Treatment Systems"*, Biotechnology and Bioengineering, Vol. 81, No. 4;

[46]. Pratt, S., Yuan, Z., Keller, J., 2004, *"Modeling Aerobic Carbon Oxidation and Storage by Integration Respirometric, Titrimetric, and Off-Gas CO_2 Measurements"*, Biotechnology and Bioengineering, Vol. 88, No. 2;

[47]. Pons, M.-N., Le Bonté, S., Potier, O., 2004, *"Spectral analysis and fingerprinting for biomedia characterization"*, Journal of Biotechnology, 113, 211-230;

[48]. Kapritchkoff, F. M., Viotti, A. P., Alli, R. C. P., Zuccolo M., Pradella, J. G. C., Maiorano, A. E., Miranda, E. A., Bonomi, A., 2006, *"Enzymatic recovery and purification of polyhydroxybutyrate produced by Ralstonia eutropha",* Journal of Biotechnology, 122, 453-462;

[49]. Ghatnekar, M. S., Pai, J. S., Ganesh, M., 2002, *"Production and recovery of poly-3-hydroxybutyrate from Methylobacterium sp V49"*, J. Chem. Technol. Biotechnol. , 77: 444-448;

[50]. Hejazi, P., Vasheghani-Farahani, E., Yamini, Y., 2003, *"Supercritical Fluid Disruption of Ralstonia eutropha for Poly (β-hydroxybutyrate) Recovery"*, Biotechnol. Prog., 19, 1519-1523;

[51]. Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S. A., Yamini, Y., 2004, *"Effect of Process Variables on Supercritical Fluid Disruption of Ralstonia eutropha Cells for Poly (R-hydroxybutyrate) Recovery"*, Biotechnol. Prog., 20, 1757-1765;

[52]. Sudesh, K., Abe, H., Doi, Y., 2000, *"Synthesis, structure and properties of Polyhydroxyalkanoates: biological polyesters"*, Prog. Polym. Sci., 25, 1503-1555;

[53]. Carrasco, F., Dionisi, D., Matinelli, A., Majone, M., 2006, *"Thermal Stability of Polyhydroxyalkanoates"*, Journal of Applied Polymer Science, Vol. 100, 2111-2121;

[54]. Huisman, G. W., Wonink, E., Meima, R., Kazemier, B., Terpstra, P., Witholt, B., 1991, *"Metabolism of Poly (3-hydroxyalkanoates) (PHAs) by Pseudomonas oleovorans"*, The Journal of Biological Chemistry, Vol. 266, No. 4, pp. 2191-2198;

[55]. Wang, J., Yu, J., 2001, *"Kinetic analysis on formation of poly (3-hydroxybutyrate) from acetic acid by Ralstonia eutropha under chemically defined conditions"*, Journal of Industrial Microbiology & Biotechnology, 26, 121-126;

[56]. Ryu, H. W., Hahn, S. K., Chang, Y. K., Chang, H. N., 1997, *"Production of Poly (3-hydroxybutyrate) by High Cell Density Fed-Batch Culture of Alcaligenes eutrophus with Phosphate Limitation"*, Biotechnology and Bioengineering, Vol. 55, No. 1;

[57]. Liangqi, Z., Jingfan, X., Tao, F., Haibin, W., 2006, *"Synthesisof poly (3-hydroxybutyrate-co-3-hydroxyoctanoate) by a Sinorhizobium fredii strain"*, Applied Microbiology, 42, 344-349;

[58]. Cenens, C., Smets, I. Y., Van Impe, J. F., 2000, *"Modeling the Competition Between Floc – Forming and Filamentous Bacteria in Activated Sludge Waste Water Treatment Systems – II. A Prototype Mathematical Model Based on Kinetic Selection and Filamentous Backbone Theory"*, Water Research, Vol. 34, No. 9, pp. 2535-2541;

[59]. Blackall, L. L., Crocetti, G. R., Saunders, A. M., Bond, P. L., 2002, *"A review and update of the microbiology of enhanced biological phosphorus removal in wastewater treatment plants"*, Antonie van Leeuwenhoek **81:** 681-61;

[60]. Gernaey, K. V., van Loosdrecht, M. C. M., Henze, M., Lind, M., Jørgensen, S. B., 2004, *"Activated sludge wastewater treatment plant modeling and simulation: state of the art"*, Environmental Modeling & Software 19, 763-783;

[61]. Kharoune, L., Kharoune, M., Lebeault, J. M., 2002, *"Aeobic degradation of 2,4,6-trichlorophenol by a microbial consortium – selection and characterization of microbial consortium"*, Appl Mircrobiol Biotechnol, 59: 112-117;

[62]. Cenens, C., Smets, I. Y., Ryckaert,V. G., Van Impe, J. F., 2000, *"Modeling the Competition Between Floc – Forming and Filamentous Bacteria in Activated Sludge Waste Water Treatment Systems – I. Evaluation of Mathematical Models based on Kinetic Selection Theory"*, Water Research Vol. 34, No. 9, pp. 2525-2534;

[63]. Sötemann, S. W., Musvoto, E. V., Wentzel, M. C., Ekama, G. A., *"Integrated biological, chemical and physical processes kinetic modelling Part 1 – Anoxic-aerobic C and N removal in the activated sludge system"*,Water SA Vol. 31, No. 4;

[64]. Wang, C., Meek, D. J., Panchal, P., Boruvka, N., Archibald, F. S., Driscoll, B. T., Charles, T. C., 2006, *"Isolation of Poly-3-Hydroxybutyrate Metabolism Genes from Complex Microbial Communities by Phenotypic Complementation of Bacterial Mutants"*, Applied and Environmental Microbiology, Vol. 72, No. 1, pp. 384-391;

[65]. Choi, J.-I., Lee, S. Y., 1997, *"Process analysis and economic evaluation for Poly(3-hydroxybutyrate) production by fermentation"*, Bioprocess Engineering 17, 335-342;

[66]. Beun, J. J., Dircks, K., van Loosdrecht, M. C. M., Heijen, J. J., 2002, *„Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures"*, Water Research 36, 1167-1180;

[67]. Flores, X., Bonmatí, A., Poch, M., Rodríguez-Roda, I., 2005, *"Selection of the Activated Sludge Configuration during the Conceptual Design of Activated Sludge Plants Using Multicriteria Analysis"*, Ind. Eng. Chem. Res., 44, 3556-3566;

[68]. Shi, H.,Shiraishe M., Shimizu, K., 1997, *"Metabolic Flux Analysis for Biosynthesis of Poly (β-Hydroxybutyric Acid) in Alcaligenes eutrophus from Various Carbon Sources"*, Journal of Fermentation and Bioengineering Vol. 84, No. 6, 579-587;

[69]. Akar, A., Akkaya, E. U., Yesiladali, S. K., Çelikyilmaz, G., Çokgor, E. U., Tamerler, C., Orhon, D., Çakar, Z. P., 2006, *"Accumulation of Polyhydroxyalkanoates by Microlunatus phosphovorus under various growth conditions"*, J. Ind. Microbiol. Biotechnol., 33: 215-220;

[70]. Dulekgurgen, E., Ovez, S., Artan, N., Orhon, D., 2003, *„Enhanced biological phosphate removal by granular sludge in a sequencing batch reactor"*. Biotechnology Letters **25:** 687-693;

[71]. Lee, N., Nielsen, P. J., Aspegren, H., Henze, M., Schleifer, K.-H., Jansen, J. la C., 2003, *"Long-term Population Dynamics and in situ Physiology in Activated Sludge Systems with Enhanced Biological Phosphorus Removal Operated with and without Nitrogen Removal"*, System. Appl. Microbiol. 26, 211-227;

[72]. Wang, J., Yu, H.-Q., 2007, *"Biosynthesis of plyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by Ralstonia eutropha ATCC 17699 in batch cultures"*, Appl. Microbiol. Biotechnol. 75:871-878;

[73]. Wang, C., Sheng, X., Equi, R. C., Trainer, M. A., Charles, T. C., Sobral, B. W. S., 2007, *"Influence of the Poly-3-Hydroxybutyrate (PHB) Granule-Associated Proteins (PhaP1 and PhaP2) on PHB Accumulation and Symbiotic Nitrogen Fixation in Sinorhizobium meliloti Rm1021"*, Journal of Bacteriology Vol. 189, No. 24, pp. 9050-9056;

[74]. Sabra, W., 1999, *"Microaerophilic production of alginate by Azotobacte vinelandii"*, PhD Thesis, Von der Gemeinsamen Naturwissenschaftlichen Fakultät der Technischen Universität Carolo-Wilhelmina zu Braunschweig, Germany;

[75]. Ramos-Cormenzana, A., Monteliva-Sanchez, M., Lopez, M. J., 1995, *"Bioremediation of Alpechin"*, International Biodeterioration & Biodegradation 249-268;

[76]. Encarnación, S., Vargas, M. del C., Dunn, M. F., Dávalos, A., Mendoza, G., Mora, Y., Mora, J., 2002, "*AniA Regulates Reserve Polymer Accumulation and Global Protein Expression in Rhizobium etli"*, Journal of Bacteriology Vol. 184, No. 8, pp. 2287-2295;

[77]. Povolo, S., Casella, S., 2000, *"A critical role for AniA in energy-carbon flux and symbiotic nitrogen fixation in Sinorhizobium meliloti"*, Arch Microbiol 174: 42-49;

[78]. Arco, Y., Llamas, I., Martínez-Checa, F., Argandoña, M., Quesada, E., del Moral, A., 2005, *"epsABCJ genes are involved in the biosynthesis of the exopolysaccharide mauran produced by Halomonas maura"*, Microbiology, 151, 2841-2851;

[79]. Hart, T. D., Chamberlain, A. H. L., Lynch, J. M., Newling, B., McDonald, P. J., 1999, *"A stray field magnetic resonance study of water diffusion in bacterial exopolysaccharides"*, Enzyme and Microbial Technology 24: 339-347;

[80]. Yu, J., Si,Y., 2004, *"Polyhydroxyalkanoates in Ralstonia eutropha on Short Chain Fatty Acids"*, Biotechnol. Prog. 2004, 20, 1015 – 1024;

[81]. Sabra, W., Zeng, A.-P., Sabry, S., Omar, S., Deckwer, 1999, *"Effect of phosphate and oxygen concentrations on alginate production and stoichiometry of metabolismo f Azotobacter vinelandii under microaerobic conditions"*, Appl. Microbiol. Biotechnol. 52: 773 – 780;Sabra, W., Zeng, A.-P., Deckwer, W.-D., 2001, *"Bacterial alginate: physiology, prduct quality and process aspects"*, Appl. Microbiol. Biotechnol. 56: 315 – 325;

[82]. Liu, H., Fang, H. H. P., 2002, *“Extraction of extracellular polymeric substances (EPS) of sludges“*, Journal of Biotechnology 95, 249 – 256;

[83]. Galindo, E., Peña, C., Núñez, C., Segura, D., Espín, G., 2007, *“Review: Molecular and bioengineering strategies to improve alginate and polyhydroxyalkanoate production by Azotobacter vinelandii”*, Microbiol Cell Factories, 6:7;

[84]. Fonseca, C. M., Fleming, P. J., 1993, *“Genetic Algorithms for Multiobjective Optimization: Formulation, Discussion and Generalization”*, Genetic Algorithms: Proceedings of the Fifth International Conference (S. Forrest, ed.);

[85]. Flemming, H. –C., Wingender, J., 2001, *Relevance of microbial extracellular polymeric substances (EPSs) – Part I; Structural and ecological aspects*, Water Science and Technology Vol 43, No 6 pp 1 – 8;

[86]. Gujer, W., Henze, M., Mino, T., van Loosdrecht, M., 1999, *Activated Sludge Mode No. 3*, Wat. Sci. Tech., Vol. 39, No. 1, pp 183 – 193;

[87]. Van Loosdrecht, M. C. M., Henze, M., 1999, *Maintenance, Endogeneou Respiration, Lysis, Decay and Predation*, Wat. Sci. Tech. Vol. 39, No. 1, pp 107 – 117.

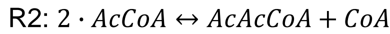
ANNEX

Annex A: PHA and EPS Metabolic Model (C-mol Basis)

Acetate Uptake (Lumping acetate kinase plus phosphate acetyltransferase)

R1: $AC + ATP + CoA \rightarrow AcCoA + ADP$

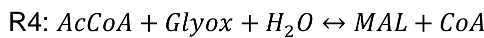
Beta-oxidation

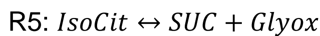

R2: $2 \cdot AcCoA \leftrightarrow AcAcCoA + CoA$

PHB Synthesis

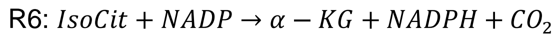
R3: $AcAcCoA + NADPH \leftrightarrow PHB + NADP$

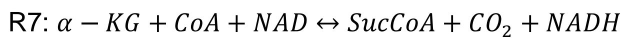
Glyoxylate shunt

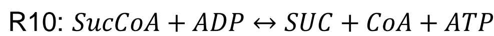

R4: $AcCoA + Glyox + H_2O \leftrightarrow MAL + CoA$


R5: $IsoCit \leftrightarrow SUC + Glyox$

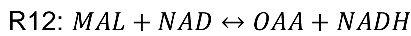
TCA cycle

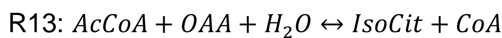

R6: $IsoCit + NADP \rightarrow \alpha - KG + NADPH + CO_2$


R7: $\alpha - KG + CoA + NAD \leftrightarrow SucCoA + CO_2 + NADH$


R10: $SucCoA + ADP \leftrightarrow SUC + CoA + ATP$

R11: $SUC + H_2O + FAD \leftrightarrow MAL + FADH$


R12: $MAL + NAD \leftrightarrow OAA + NADH$

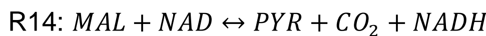

R13: $AcCoA + OAA + H_2O \leftrightarrow IsoCit + CoA$

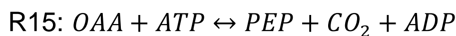
Glutamate, Glutamine Production

R8: $NH_3 + \alpha - KG + NADPH \leftrightarrow Glut + H_2O + NADP$

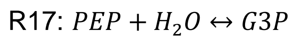
R9: $Glut + NH_3 + ATP \leftrightarrow Glum + ADP$

Gluconeogenesis pathway

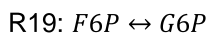

R14: $MAL + NAD \leftrightarrow PYR + CO_2 + NADH$


R15: $OAA + ATP \leftrightarrow PEP + CO_2 + ADP$

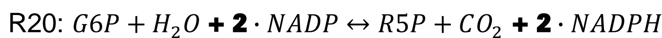
R16: $PYR + ATP \leftrightarrow PEP + ADP$


R17: $PEP + H_2O \leftrightarrow G3P$

R18: $2 \cdot GAP + ADP \leftrightarrow F6P + ATP$


R19: $F6P \leftrightarrow G6P$

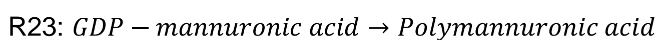
Pentose phosphate pathway


R20: $G6P + H_2O \mathbf{+ 2} \cdot NADP \leftrightarrow R5P + CO_2 \mathbf{+ 2} \cdot NADPH$

R21: $F6P + GAP \leftrightarrow R5P + E4P$

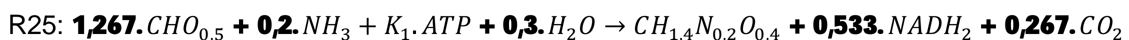
Alginate biosynthesis

R22: *F6P+GTP+NADP*$^+$*→NADPH*$_2$*+GDP-mannuronic acid*


R23: $GDP - mannuronic\ acid \rightarrow Polymannuronic\ acid$

R24: $Polymannuronic\ acid \rightarrow Alginate$

Synthesis of Biomass from Acetyl-CoA


R25: $\mathbf{1{,}267.} CHO_{0,5} \mathbf{+ 0{,}2.} NH_3 + K_1 . ATP \mathbf{+ 0{,}3.} H_2O \rightarrow CH_{1,4}N_{0,2}O_{0,4} \mathbf{+ 0{,}533.} NADH_2 \mathbf{+ 0{,}267.} CO_2$

Annex B: Metabolic model reactions for mixed cultures

	Reactions
Acetate Uptake	$CH_2O + ATP \rightarrow CHO_{0,5} + 0{,}5.H_2O$
Biomass precursors synthesis	$1{,}267.CHO_{0,5} + 0{,}2.NH_3 + K_1.ATP + 0{,}3.H_2O \rightarrow CH_{1,4}N_{0,2}O_{0,4} + 0{,}533.NADH_2 + 0{,}267.CO_2$
Respiration	$CHO_{0,5} + 0{,}667.H_2O \rightarrow CO_2 + 2.NADH_2 + 0{,}5\ ATP$
Maintenance	$ATP \rightarrow m_{ATP}$
Oxydative phosphorylation	$NADH_2 + 0{,}5.O_2 \rightarrow H_2O + \delta.ATP$
Aerobic PHB storage	$CHO_{0,5} + 0{,}25.NADH_2 \rightarrow CH_{15}O_{0,5}$
EPS precursors synthesis	$\frac{4}{3}\cdot CHO_{0,5} + \frac{2}{3}\cdot ATP + H_2O \rightarrow CH_{4/3}O + \frac{1}{3}\cdot CO_2 + NADH_2$
Aerobic PHB consumption	$CH_{15}O_{0,5} + 0{,}25.ATP \rightarrow 0{,}25NADH_2 + CHO_{0,5}$

Table 11 - Metabolic model reactions for microbial mixed cultures (adapted from Dias e tal., 2005)